1 **Evaluating the feasibility of medium chain oleochemical synthesis using microbial**

2 **chain elongation**

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14 **One Sentence Summary:** In this work, the authors use constraint-based metabolic

15 modelling and enzyme cost minimization to assess the feasibility of using metabolic

16 engineering to expand the product spectrum of anaerobic chain elongating bacteria.

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18 **Keywords:** chain elongation, anaerobic bioproduction, metabolic engineering,

19 metabolic modelling, resource allocation, circular bioeconomy

20   **Abstract**

21   Chain elongating bacteria are a unique guild of strictly anaerobic bacteria that have

22   garnered interest for sustainable chemical manufacturing from carbon-rich wet and

23   gaseous waste streams. They produce C_6-C_8 medium-chain fatty acids which are

24   valuable platform chemicals that can be used directly, or derivatized to service a wide

25   range of chemical industries. However, the application of chain elongating bacteria for

26   synthesizing products beyond C_6-C_8 medium-chain fatty acids has not been evaluated.

27   In this study, we assess the feasibility of expanding the product spectrum of chain

28   elongating bacteria to C_9-C_{12} fatty acids, along with the synthesis of C_6 fatty alcohols,

29   dicarboxylic acids, diols, and methyl ketones. We propose several metabolic

30   engineering strategies to accomplish these conversions in chain elongating bacteria

31   and utilize constraint-based metabolic modelling to predict pathway stoichiometries,

32   assess thermodynamic feasibility, and estimate ATP and product yields. We also

33   evaluate how producing alternative products impacts the growth rate of chain

34   elongating bacteria via resource allocation modelling, revealing a trade-off between

35   product carbon length and class versus cell growth rate. Together, these results

36   highlight the potential for using chain elongating bacteria as a platform for diverse

37   oleochemical biomanufacturing and offer a starting point for guiding future metabolic

38   engineering efforts aimed at expanding their product range.
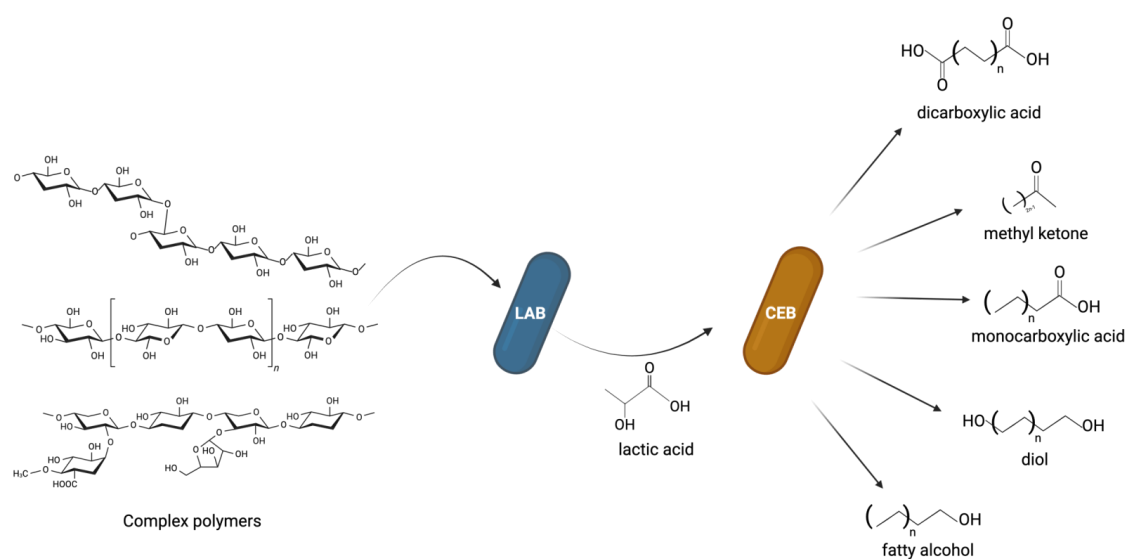
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40   Graphical Abstract: In this work, the authors use constraint-based metabolic modelling

41   and enzyme cost minimization to assess the feasibility of using metabolic engineering

42   to expand the product spectrum of anaerobic chain elongating bacteria.

43 **Introduction**

44

45 Figure 1 – (A) Anaerobic conversion of organic waste to oleochemicals using

46 synthetic co-cultures inspired by natural systems. Complex polymers are degraded by

47 lactic acid bacteria (LAB), which in turn provide lactic acid to chain-elongating bacteria

48 for medium-chain fatty acid synthesis. (B) The reverse beta-oxidation pathway in chain

49 elongating bacteria and proposed product spectrum expansion. EMP: Embden-

50 Meyerhof-Parnas glycolysis; LDH: lactate dehydrogenase; ADH: alcohol

51 dehydrogenase; ADA: acetaldehyde dehydrogenase; PFOR: pyruvate:ferredoxin

52 oxidoreductase; PFL: pyruvate formate lyase; PTA: phosphate acetyltransferase;

53 ACK: acetate kinase; ACACT: acetyl-CoA C acetyltransferase; HACD: 3-hydroxyacyl-

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54   CoA dehydrogenase; ECOAH: enoyl-CoA hydratase; EBACD: electron bifurcating

55   acyl-CoA dehydrogenase; CoAT: CoA transferase; Fd: ferredoxin; RNF: proton

56   translocating ferredoxin:NAD$^+$ oxidoreductase complex; HYD: Fe-Fe hydrogenase.

57   Created with BioRender.com.

58   Circular economies require the recycling of societal "wastes" into new

59   bioproducts to support sustainable human activity. Anaerobic fermentation processes

60   can upcycle carbon from agricultural residues, food waste, and industrial off-gases

61   into useful fuels, chemicals, and materials. One promising process is microbial chain

62   elongation, which uses anaerobic microbiomes in open-culture systems to synthesize

63   medium-chain fatty acids (MCFAs) from complex organic and gaseous waste streams

64   (Angenent et al., 2016; Holtzapple et al., 2022; Scarborough et al., 2022). The process

65   works by producing key intermediates, such as lactate and/or ethanol, through either

66   organic waste fermentation (breaking down) or gas fermentation (building up), which

67   subsequently undergo a secondary fermentation to produce MCFAs via chain

68   elongation. Several studies have demonstrated stable MCFA production from organic

69   waste (Grootscholten et al., 2014; Stamatopoulou et al., 2020) and gaseous

70   feedstocks (Bäumler et al., 2022; Diender et al., 2016; Fernández-Blanco et al., 2022)

71   via chain elongation at the bench- and pilot-scale; and more recently a demonstration

72   plant has been built in the Netherlands by the Dutch company, ChainCraft

73   (https://www.chaincraft.nl/).

74   At the heart of the process is a functional guild of obligate anaerobes called

75   "chain elongating bacteria" that use a native reverse beta-oxidation (RBO) pathway

76   (see Figure 1B) to ferment lactate, ethanol, and other electron-rich organic substrates

77   (e.g., sugars, glycerol) into C_4-C_8 carboxylates (i.e., butyrate, hexanoate, and

78   octanoate) as part of their growth. The pathway is unique because it allows redox

79　balancing, while also conserving energy through a novel flavin-based electron

80　bifurcation (FBEB) mechanism (Buckel & Thauer, 2018; Li et al., 2008). Almost all

81　known chain elongators belong to the phylum *Bacillota* (previously *Firmicutes*) and are

82　a phylogenetically and physiologically diverse group. While most chain elongators

83　remain uncultivated, over 15 strains have been isolated and sequenced to date, with

84　most new isolates being reported in the last 10 years (Candry & Ganigué, 2021).

85　These isolates, particularly *Clostridium kluyveri,* have been used to establish synthetic

86　co-cultures to convert gaseous or sugar-based feedstocks to MCFAs and their

87　corresponding alcohols (Bäumler et al., 2022; Diender et al., 2016; Haas et al., 2018;

88　Otten et al., 2022; R. Lynd et al., 2022) which could be expanded to more complex

89　feedstocks by selecting lactate and/or ethanol-producing partners with improved

90　hydrolytic capabilities.

91　　　　The development of synthetic co-cultures, inspired by mixed culture chain

92　elongation processes, could represent a platform for improving titres, rates, and yields

93　of MCFA production (Figure 1A). Moreover, there is growing interest in genetically

94　modifying the native RBO cycle in chain elongators to expand the product spectrum

95　beyond C_4-C_8 monocarboxylates, including alcohols, diols, dicarboxylates, and other

96　bulk chemicals (Agena et al., 2023; Guss & Riley, 2021; Strik et al., 2022). This would

97　enable the anaerobic synthesis of diverse medium-chain oleochemicals from wet and

98　gaseous waste streams, which is expected to have improved economic feasibility

99　compared to sugar-based aerobic fermentations (Holtzapple et al., 2022). However,

100　the technical feasibility of making products other than C_4-C_8 monocarboxylates via

101　metabolic engineering of chain elongators remains unexplored. As tools to genetically

102　modify chain elongators emerge (Agena et al., 2023; Cheng et al., 2019; Guss & Riley,

103　2021), methodologies to intelligently design these new biocatalysts are needed.

6

104   In this study, we use constraint-based metabolic modelling along with

105   thermodynamic analyses to evaluate the feasibility of synthesizing diverse medium-

106   chain oleochemicals (C_6-C_{12} fatty acids, primary alcohols, dicarboxylates, diols, and

107   methyl ketones) from key intermediate substrates (lactate, ethanol, sugars, glycerol)

108   using anaerobic chain elongating bacteria (Figure 1). We first analyze MCFA

109   production scenarios beyond natural C_8 production up to C_{12}, highlighting trade-offs

110   between ATP yield and expected growth rate using a combination of metabolic

111   modelling and enzyme cost minimization analyses. Subsequently, we propose several

112   modifications to the RBO cycle to synthesize target medium chain oleochemical

113   products and use constraint-based metabolic modelling to determine overall pathway

114   stoichiometry, thermodynamic feasibility, and theoretical product yields. Our results

115   indicate that the metabolic engineering of chain elongating bacteria could enable the

116   anaerobic synthesis of diverse medium-chain oleochemicals at industrially relevant

117   yields. Moreover, we identify potential challenges with engineering the RBO pathway

118   in chain elongators and offer potential solutions to overcome them via metabolic

119   engineering. We anticipate these results will provide a useful starting point for

120   engineering microbial chain elongation to serve as a platform for sustainable chemical

121   manufacturing.

122   **Results and Discussion**

123   **Impact of MCFA Chain Length on Growth Rate and ATP Yield**

124   ***Modelling Chain Elongation beyond Octanoic Acid***

125   Currently, the products of chain elongation are restricted to butyric and hexanoic acids

126   (C_4-C_6), with limited synthesis of octanoic acid (C_8) (Nelson et al., 2017; Zhu et al.,

127   2017). Genetically modifying chain elongating bacteria to improve selectivity and

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128   increase MCFA chain length could improve product yields, while also expanding the

129   process to produce MCFAs with larger markets (C_8-C_{12}).

130   To assess the feasibility of MCFA production beyond C_8, we predicted overall

131   pathway stoichiometry and redox balance, free energy change, and theoretical ATP

132   and product yields of C_4-C_{12} fatty acids (FAs) via parsimonious enzyme usage flux

133   balance analysis (pFBA) with ATP yield set as the objective function using a simplified

134   metabolic model describing core chain elongation metabolism (iFermCell193, see

135   Methods) (Table 1). We focused on lactate utilization by chain elongating bacteria

136   because this is their primary substrate in mixed culture processes converting organic

137   wastes (Figure 1) (Contreras-Dávila et al., 2020). Model simulations with different

138   electron donors, including ethanol, glucose, xylose, and glycerol FAs were also

139   evaluated (see Supplemental Material) to demonstrate process feasibility for a range

140   of other possible operating scenarios.

141   The model predicted that ATP yield and overall reaction-free energy increases

142   with MCFA chain length from C_4-C_{12} (Table 1), indicating MCFA synthesis beyond C_8

143   should be theoretically possible. Moreover, the production of C_4-C_{12} chain lengths from

144   lactate released ≥ 50 kJ/mol indicating these reactions produce sufficient energy for

145   ATP synthesis. C_9-C_{12} carboxylate production with other electron donors, including

146   ethanol, glucose, xylose, and glycerol, were also found to be feasible (see

147   Supplemental Material). This observation agrees with past modelling results that

148   suggested hexanoic and octanoic acid production should improve ATP yields from

149   lactate compared to butyric acid production (Scarborough et al., n.d.).

150   The predicted increase in net ATP production is attributed to a greater flux

151   through the proton translocating ferredoxin:NAD$^+$ oxidoreductase complex (RNF) for

152   each turn of the RBO cycle, which leads to greater ATP production via the Ion Motive

153   Force (IMF). In the RBO cycle, each successive elongation generates reduced

154   ferredoxin from the electron-bifurcating acyl-CoA dehydrogenase (EBACD). This

155   ferredoxin goes on to drive RNF to recover some NADH and/or is used by a soluble

156   ferredoxin hydrogenase (HYD1) to evolve H_2 as a terminal electron sink. As confirmed

157   in the predicted flux distributions, elongation to longer chain lengths increases the total

158   NADH demand required to reduce the acyl chains (HACD and EBACD in Figure 1).

159   Thus, with increasing chain length, a greater proportion of the reduced ferredoxin

160   produced by the EBACD is used to regenerate NADH via RNF, rather than driving

161   HYD1. This ultimately leads to increased ATP production through the IMF along with

162   decreased H_2 evolution for increasing chain length. These results also indicate that

163   past a certain chain length ($>C_{12}$), H_2 evolution will cease as all electron equivalents

164   will be required solely for chain elongation.

165   Table 1. Predicted stoichiometry for the synthesis of C_4-C_{12} FAs from lactate.

Chain Length	Overall Equation	ATP Yield (mol ATP / mol Lactate)	$\Delta G_r^{o'}$ (kJ/mol)	$\Delta G_r^{o'}$ per ATP (kJ/mol)	Yield (g FA / g Lactate)
C_4	Lactate + 1/2 H^+ → 1/2 Butyrate + CO_2 + H_2	0.250	-28.98	-160.11	0.49
C_6	Lactate + 2/3 H^+ → 1/3 Hexanoate + CO_2 + 2/3 H_2 + 1/3 H_2O	0.333	-48.53	-255.34	0.43
C_8	Lactate + 3/4 H^+ → 1/4 Octanoate + CO_2 + 1/2 H_2 + 1/2 H_2O	0.375	-48.53	-170.22	0.40
C_{10}	Lactate + 4/5 H^+ → 1/5 Decanoate + CO_2 + 2/5 H_2 + 3/5 H_2O	0.400	-231.56	-804.43	0.38
C_{12}	Lactate + 5/6 H^+ → 1/6 Dodecanoate + CO_2 + 1/3 H_2 + 2/3 H_2O	0.417	-195.82	-483.10	0.37

166

Intracellular Thermodynamic Landscape and Resource Allocation

168   Our initial modelling analysis suggested that the improved ATP yield with longer chain

169   lengths should naturally select for MCFAs beyond C_8. However, all pure and mixed

170   culture chain elongation studies have only observed C_4-C_8 MCFA products. This points

171   to a potential trade-off between growth yield (or ATP yield) and growth rate (or ATP

172   production rate), resulting from optimal resource allocation. In a model based on

173   resource allocation theory (Supplementary Material; inspired by a similar model

174   developed A. I. Flamholz et al., 2024), where catabolic ATP production and anabolic

175   ATP consumption rates are linearly related to the biomass fractions allocated to either

176   function, RBO enzyme cost per unit ATP production flux and chain elongating bacteria

177   growth rate are inversely correlated (Figure 6). This simplified model captures the fact

178   that a higher growth rate is expected to necessitate both a larger pool of anabolic

179   enzyme and ribosomes (Basan, 2018) and a higher ATP production flux, requiring that

180   catabolic machinery produces ATP faster with less enzyme.

181   To evaluate the enzyme investment per ATP flux required to produce

182   carboxylates of different chain lengths, we performed Enzyme Cost Minimization

183   (ECM) analysis. ECM places a lower bound on a pathway's enzyme demand per unit

184   flux (g/(mol/h)) by accounting for the pathway's length and stoichiometry, reaction

185   thermodynamics, and saturation effects following from the optimal set of metabolite

186   concentrations, given appropriate bounds on those concentrations and

187   parametrization of enzyme kinetics (A. Flamholz et al., 2013). This analysis coupled

188   with ATP yield results from our model showed that minimal enzyme cost per unit ATP

189   flux increases with chain length (Figure 2). This suggests that chain elongating

190   bacteria making longer length products invest more of their proteome into high yield

191   catabolism, at the trade-off of having less catalytic capacity for growth.

192   According to the optimized free energy changes predicted by ECM, thiolase

193   reactions (ACACT) are the least exergonic in RBO (Supplementary Figure 1). Since

194   these reactions operate near equilibrium, they have a smaller forward to reverse flux

195   ratio (Noor et al., 2014) and therefore require a larger enzyme pool to sustain a given

196   net flux through the pathway. The set of metabolite concentrations which minimizes

197   pathway enzyme cost fixes the thiolase products (oxoacyl-CoAs) at low concentrations

198   to drive these reactions. This heightens the demand for the proceeding enzymes in

199   the pathway, 3-hydroxyacyl-CoA dehydrogenases (HACD) since they are far from

200   saturation (Figure 2). Similarly, enoyl-CoA hydratase reactions (ECOAH) are

201   thermodynamically constrained, meaning their reactant and product concentrations

202   are kept high and low, respectively. This further increases the protein burden of HACD

203   as well as the proceeding electron bifurcating acyl-CoA dehydrogenase (EBACD)

204   (Figure 2). At longer chain lengths, ECOAH reactions are predicted to be more

205   favourable (Supplementary Figure 1), causing the rate at which enzyme cost increases

206   with chain length to decrease.

207   Interestingly, when oxoacyl-CoA concentrations are constrained by a lower

208   bound of 1 µM, as is the case for all other metabolites, products beyond C_4 are deemed

209   infeasible due to endergonic subsequent thiolase reactions. To reflect observations of

210   chain elongation beyond C_4, oxoacyl-CoAs can fall to sub-micromolar levels in this

211   model. An alternative solution, as previously suggested in a similar instance with

212   malonyl dehydrogenase (Noor et al., 2014), is to posit channelling between ACACT

213   and HACD, effectively merging the two into one favourable reaction. Indeed,

214   channelling between ACACT, HACD and ECOAH has been described with crystal

215   structures of bacterial and human beta-oxidation complexes (Ishikawa et al., 2004; Xia

216   et al., 2019). So long as a similar mechanism is active from C_4 to C_{12}, the trend of

217   enzyme cost per ATP flux increasing with chain length is expected to hold. This has

218   major implications for engineering chain elongating bacteria to produce MCFAs

219   beyond C8, as longer chain carboxylates would exhibit higher yields, but at the cost of

220   a lower growth rate. As a result, compensatory efforts to bolster growth rate, for

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221   example through adaptive laboratory evolution (Sandberg et al., 2019), may be

222   needed to achieve higher productivity.
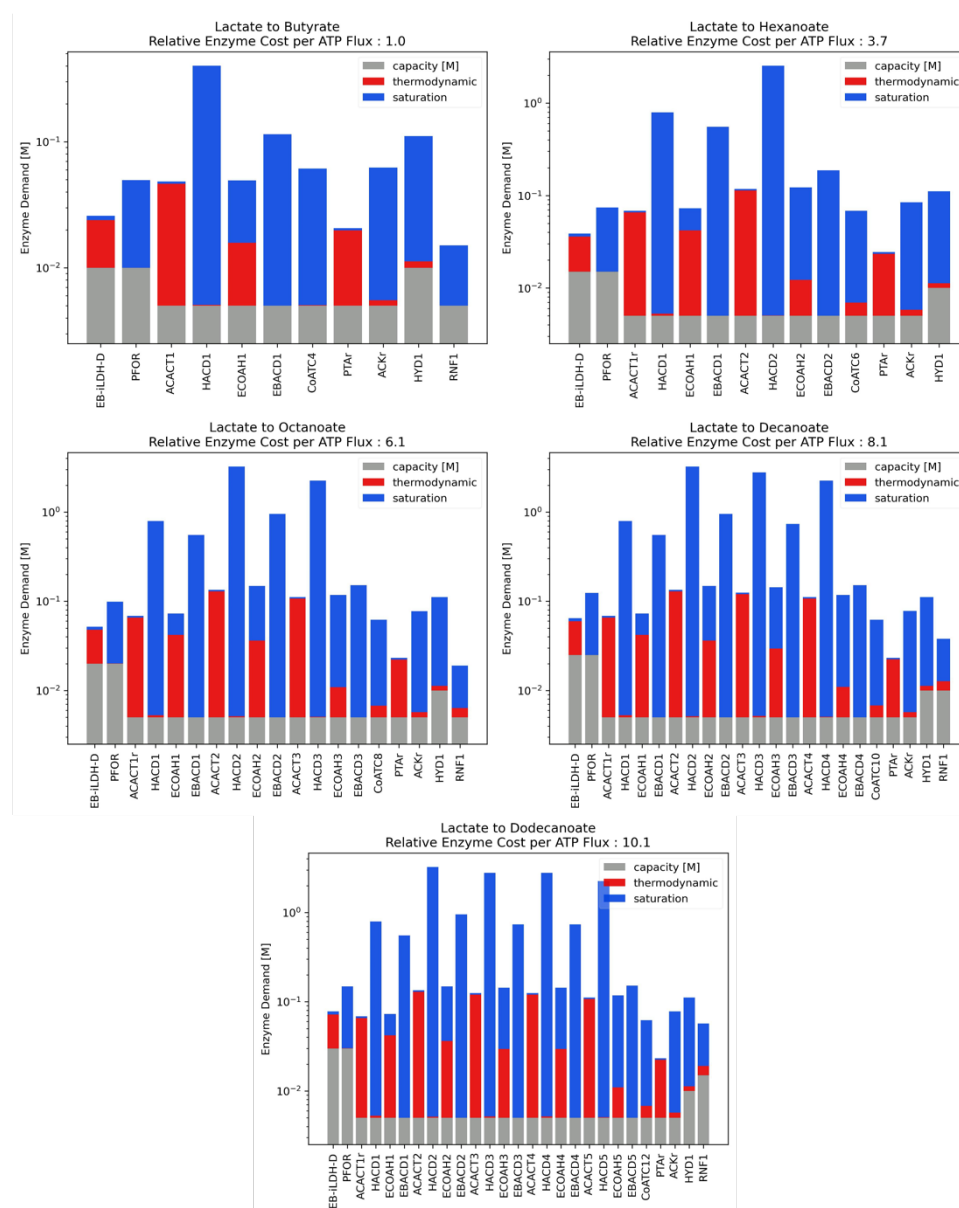

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224   Figure 2. ECM results of RBO for production of carboxylates from lactate.

225   Enzyme cost per unit ATP flux is reported as a multiple of butyrate production's cost.

226   Each reaction carries a cost which accounts for the pathway's stoichiometry (grey),

227   increased cost incurred due to a reaction's proximity to equilibrium (red), and the cost

228   due to sub-saturation reactant concentrations (blue).

229

Expanding and Controlling Chain Elongation Products with Metabolic Engineering

Metabolic engineering strategies for the expansion of RBO-derived products have been reviewed for non-chain elongating, model organisms with established genetic tools, such as *Escherichia coli*, *Saccharomyces cerevisiae*, and select acetogenic *Clostridium* species (Tarasava et al., 2022). The approach for these strains relies on the engineered reversal of β-oxidation accomplished through extensive strain engineering such as knockout or repression of native β-oxidation regulators and overexpression of genes from chain elongating bacteria and other oleaginous hosts (Tarasava et al., 2022). Most of these hosts do not natively rely on the RBO cycle for redox balancing or energy conservation which makes the RBO cycle growth-coupled in the chain elongating bacteria. Further, installing orthogonal systems for the engineered reversal of β-oxidation imposes an increased metabolic burden on non-chain elongating hosts as high expression is likely needed to ensure sufficient flux through the pathway (Liu et al., 2018). This can lead to redox limitations or inefficient substrate use for chain elongation in these non-chain elongating hosts and approaches like two-phase production are likely needed to maximize product yields (Lange et al., 2016). Production with non-chain elongating hosts will likely require further strain engineering to improve product yields and selectivity.

As an alternative to engineering model organisms, tools to genetically modify chain elongating bacteria are beginning to emerge (Agena et al., 2023; Cheng et al., 2019; Guss & Riley, 2021), which will open the door to expanded opportunities to produce oleochemicals via anaerobic fermentation. Chain elongating bacteria are ideal hosts for RBO-based bioproduction as the RBO cycle is innately growth-coupled in these strains (no reconstruction needed) and they can utilize solid and gaseous

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255   waste feedstocks rather than relying on refined substrates (e.g., sugars). Further,

256   product yields in anaerobes are typically much greater as they do not synthesize as

257   much biomass compared to aerobic systems and most electrons end up in products

258   (Cueto-Rojas et al., 2015). However, the feasibility of pathways for the production of

259   other oleochemicals including, fatty alcohols, dicarboxylates, diols, methyl ketones,

260   has not been explored in anaerobic chain elongating bacteria. Here, we propose

261   potential production pathways and enzymes required to generate a wide range of

262   oleochemicals (Figure 3) and use pFBA and ECM to assess the feasibility and

263   potential effects on ATP yield and growth rate.

264   iFermCell193 was further modified to capture the reactions required for

265   hexanoic acid conversion to other products (iFermCell356). Table 2 summarizes the

266   potential pathway stoichiometries for the conversion of hexanoic acid (C_6) to the

267   corresponding fatty alcohol, dicarboxylate, and diol using lactate as an electron donor,

268   along with the net ATP fluxes and overall Gibbs free energy change of reaction for

269   each substrate-product pair as predicted by our model. All predicted pathway

270   stoichiometries have overall favourable standard Gibbs free energies of reaction and

271   release ≥ 50 kJ/mol per ATP required for ATP production. The model also predicts that

272   net ATP yield is not negatively impacted by conversion into these other bioproducts

273   which indicates that their production can be growth-coupled, similar to MCFAs. In the

274   following sections, we describe potential metabolic engineering strategies to

275   accomplish these conversions in chain elongating bacteria and discuss specific shifts

276   in redox requirements that occur predicted by the model, along with the impact on the

277   metabolic cost of the additional enzymes estimated by ECM.

278

279 Table 2. Predicted pathway stoichiometry for different C_6 oleochemicals produced

280 from lactate.

Product; Pathway	Overall Equation	ATP Yield (mol ATP / mol Lactate)	$\Delta G_r^{\circ'}$	$\Delta G_r^{\circ'}$ per ATP	Yield (g P/g S)
Carboxylate; RBO	Lactate + 2/3 H^+ → 1/3 Hexanoate + CO_2 + 2/3 H_2 + 1/3 H_2O	0.333	-26.75	-160.11	0.43
Alcohol; RBO + AdhE	Lactate + H^+ → 1/3 Hexanol +. CO_2 + 2/3 H_2O	0.333	-45.18	-255.34	0.38
Alcohol; RBO + AOR	Lactate + H^+ → 1/3 Hexanol + CO_2 + 2/3 H2O	0.500	-45.18	-170.22	0.57
Dicarboxylate; RBO + AlkBGT	Lactate + H^+ + 2/3 NO_2^- → 1/3 Adipate + CO_2 + 2/3 H_2 + H_2O + 1/3 N_2	0.333	-228.21	-804.43	0.54
Diol; RBO + AlkBGT	Lactate + 5/4 H+ + 1/2 NO_2^- → 1/4 1,6-Hexanediol + 1/4 Acetate + CO_2 + H_2O + 1/4 N_2	0.5	-191.63	-483.10	0.66

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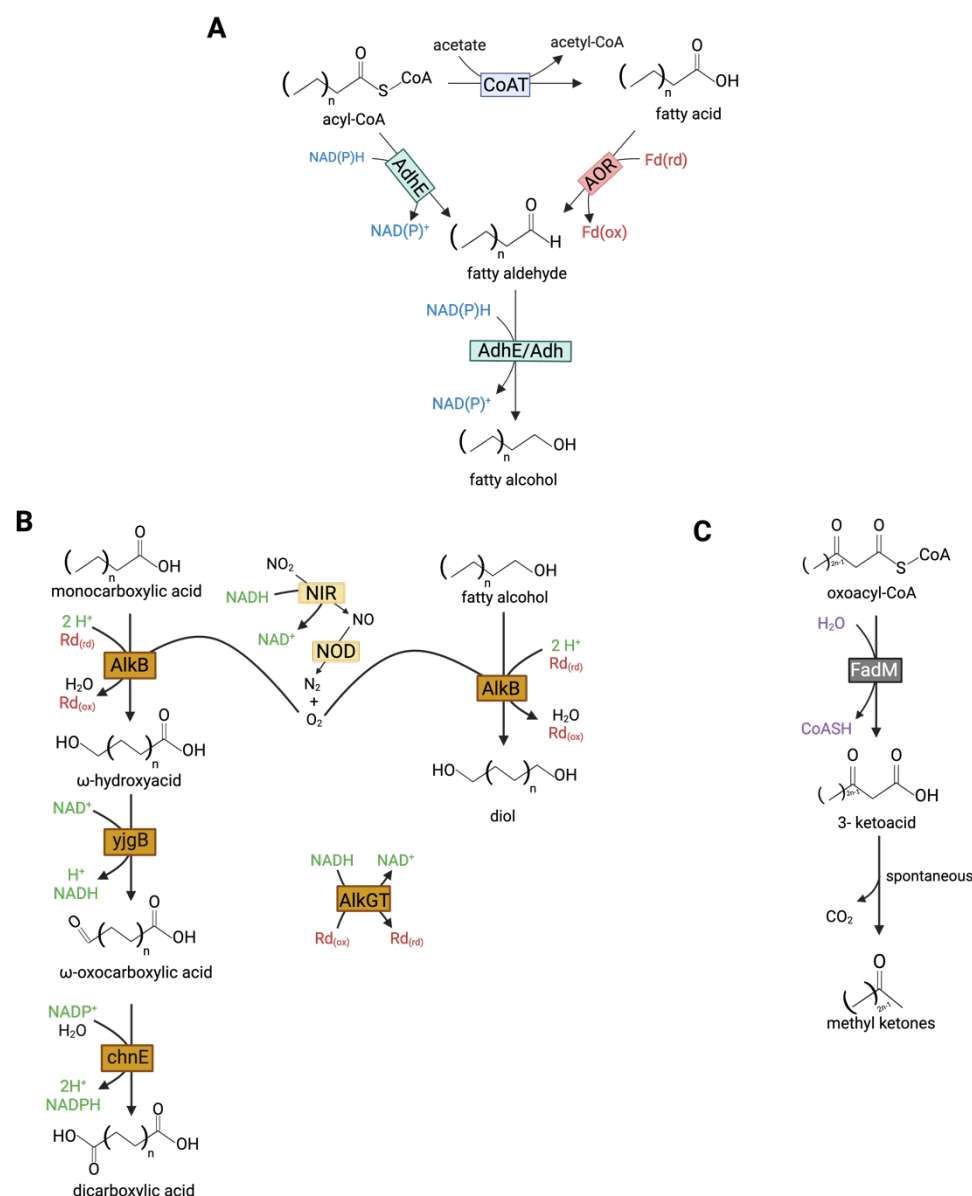
Figure 3. Proposed pathways for chain elongating bacteria product spectrum expansion. A: Fatty alcohols pathway. AdhE: bifunctional aldehyde-alcohol dehydrogenase; AOR: aldehyde:ferredoxin oxidoreductase; FadM: acyl-CoA thioesterase. B. Diol and dicarboxylic acids pathways. NIR: nitrite reductase; NOD: nitric oxide dismutase; AlkBGT: alkane 1-monooxygenase complex; yjgB: cinnamyl alcohol dehydrogenase; chnE: 6-oxohexanoate dehydrogenase; Rd: rubredoxin. C: Methyl ketones pathway. FadM: thioesterase. Created with Biorender.com.

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290   ***Fatty Alcohol Production***

291   Medium-chain fatty alcohols (FAOHs) could be produced using MCFAs as building

292   blocks. Even though FAOH production pathways (Figure 3A) exist in anaerobic

293   bacteria, such as acetogens and butanol-producing clostridia (Moon et al., 2016), this

294   has yet to be observed in most chain elongating bacteria. In nature, acetogenic

295   bacteria use aldehyde:ferredoxin oxidoreductase (AOR) to convert FAs into fatty

296   aldehydes in monoculture or when they are co-cultured with chain elongating bacteria

297   (Benito-Vaquerizo et al., 2020; Diender et al., 2016). In another instance, aldehyde-

298   alcohol dehydrogenase (AdhE) can convert acyl-CoAs into aldehydes (Mehrer et al.,

299   2018). In these two scenarios, specific electron carriers (1 reduced ferredoxin and 1

300   NAD(P)H in the former and 2 NAD(P)H in the latter), are necessary to obtain the final

301   product. Final conversion relies on the alcohol dehydrogenase (Adh) activity or the

302   bifunctionality of AdhE (Liew et al., 2017). Both AOR- and AdhE-based FAOH

303   production would result in redox balance shifts if implemented in the chain elongating

304   bacteria as a net redox demand is needed to conduct the reductions.

305   We incorporated both AOR and AdhE FAOH synthesis pathways in our model

306   and assessed their effects on FAOH production, redox balances, and ATP yield. In

307   chain elongation to FAs, H_2 is evolved as an electron sink to maintain redox balance.

308   We hypothesized that instead of producing H_2, these electron equivalents could be

309   used to reduce the carboxylate to the alcohol. Consistent with this, the model predicted

310   that hexanol (C_6 FAOH) production does not result in H_2 production (Table 2), and

311   instead, flux is redirected from HYD1 to RNF to produce NADH required by the and

312   AOR and AdhE pathways (Supplementary Material). However, in the AOR pathway,

313   the model predicted a net increase in ATP flux yield through the combined action of

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314   IMF and substrate-level phosphorylation as compared to hexanoic acid production

315   (Supplementary Material).

316   ECM results show that hexanol production via AOR carries an enzyme cost per

317   ATP flux of 2.9 times that of butyrate, whereas hexanoic acid production requires 3.7

318   times more than butyrate (Figure 4). This reflects the increased ATP yield from FAOH

319   production with AOR and the fact that the RBO cycle reactions are more

320   thermodynamically hindered than redox reactions for FAOH formation from FAs; the

321   additional protein needed to carry flux through the latter represents a small fraction of

322   the pathway's protein demand. In the AdhE case, ECM results show that hexanoic

323   acid and hexanol production require approximately equal enzyme investments of 3.7

324   and 3.5 times the cost of butyrate production, due to bypassing the use of PTA and

325   ACK for alcohol production. In both AOR and AdhE cases, it can be expected that

326   chain elongating bacteria engineered for FAOH production will not experience

327   significant growth rate penalties compared to production of MCFA of the same chain

328   length, and in fact, may grow faster if the AOR pathway is implemented.

329

330   ***Dicarboxylate and Diol Production: Pathways Requiring Oxygen.***

331   The anaerobic oxidation of MCFAs into diols and dicarboxylic acids poses a significant

332   metabolic engineering challenge in chain elongators, as the lack of oxygen as an

333   electron acceptor results in a considerable decrease in reaction energetics. Methane

334   oxidation is a clear example, where the anaerobic pathway needs to be coupled to

335   nitrite or sulphate reduction (Knittel & Boetius, 2009). Alkane-degrading microbes can

336   anaerobically oxidize C-H bonds by fixing CO_2 and converting alkanes into FAs. It is

337   hypothesized that this mechanism is initiated by an ethylbenzene dehydrogenase and

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338 the whole pathway happens at the great expense of 6 ATP equivalents, suggesting

339 that an unexplored energy-coupling mechanism may take place in these organisms

340 given the low ATP yields in anaerobes (Supplementary Figure 2) (Heider et al., 2016;

341 Shou et al., 2021). Alternatively, alkane monooxygenase complexes like AlkBGT have

342 been used in the hydroxylation of terminal ω-carbons of different compounds,

343 including FAs (Clomburg et al., 2015). However, implementing AlkBGT in anaerobes

344 is currently infeasible due to its requirement for O_2.

345   To solve these problems, we envision making use of another hypothesized

346 hydroxylation mechanism in anaerobes: the intra-aerobic pathway (Ettwig et al., 2010).

347 Achieving such conditions requires "oxygen donors", such as nitrite, hydrogen

348 peroxide, or perchlorate, whose decomposition provides the required oxygen for

349 AlkBGT. Since oxygen presence can be problematic in this scenario, it is important to

350 maintain its intracellular concentrations low, which could be achieved by tuning

351 NIR/NOD and AlkBGT expression or even improving AlkBGT rates through protein

352 engineering. If oxygen levels are still high enough to disrupt cell function, a metabolic

353 strategy that could be explored would be to confine the oxygen generation reaction

354 within microcompartments, pseudo-organelles common in various organisms,

355 including anaerobes, which have been used in several applications, such as protecting

356 cells from toxic intermediates and oxygen (*Bacterial Microcompartments |*

357 *SpringerLink*, n.d.; Kennedy et al., 2021).

358   In our proposed pathway, the first module comprises oxygen generation, where

359 nitrite is reduced to nitric oxide by nitrite reductase (NIR) (Wang et al., 2019), followed

360 by the action of nitric oxide dismutase (NOD) to yield N_2 and O_2. The second module

361 encompasses the oxidation reactions, where AlkB would be used to oxidize FAs and

362 FAOHs to ω-hydroxyacids and diols, respectively, using rubredoxin as electron carrier

19

363   (Figure 3B). The remaining portion of the AlkBGT complex is responsible for

364   regenerating reduced rubredoxin by consuming NADH. Further oxidation of ω-

365   hydroxyacids to dicarboxylic acids, however, would require additional enzymes, like

366   cinnamyl alcohol dehydrogenase (yjgB) and 6-oxohexanoate dehydrogenase (chnE)

367   (Clomburg et al., 2015), even though it is reported that AlkBGT complex can further

368   oxidize hydroxyls to carboxyls and even ester groups, a phenomenon called

369   overoxidation (van Nuland et al., 2017). Protein discovery and engineering will play a

370   key role in bringing these pathways to life in the chain elongating bacteria. For example,

371   understanding anaerobic carboxylation mechanisms and discovering other related

372   pathways would circumvent the need of using oxygen and microcompartments.

373   Additionally, to the best of our knowledge, no FAOHs have been used as substrates

374   for AlkBGT, which would require protein characterization and engineering to achieve

375   diol production if intra-aerobic conditions are necessary.

376   The reactions described above were incorporated into our model and the effect

377   on redox balance and ATP yield was assessed for the production of adipic acid and

378   1,6-hexanediol from hexanoic acid. NO_2^- was supplied in our simulations as a

379   substrate to source O_2 for the hydroxylation step. Nitrite reduction via NIR requires an

380   investment electron equivalents from NADH. However, the model predicted that the

381   production of adipic acid does not require additional electrons equivalents from H_2 nor

382   lactate as H_2 evolution and molar yield of adipic acid from lactate was the same as the

383   hexanoic acid case. For adipic acid, the ω-hydroxyacid intermediate that is produced

384   by AlkBGT is subsequently oxidized, releasing an NADH that is required for the

385   reduction of NO_2^- and balancing redox. However, in the case of 1,6-hexanediol, an

386   additional electron source is required for the initial reduction of hexanoic acid to

387   hexanol. Thus, diol production is not as carbon efficient because additional electron

388   equivalents from lactate are required to reduce both terminal ends into alcohols,

389   leading to the co-production of acetate to balance carbon, yielding a net increase in

390   ATP yield due to substrate-level phosphorylation through PTA/ACK. ECM found that

391   1,6-hexanediol carries an enzyme cost marginally lower than that of hexanol produced

392   via AOR, 2.5 times the cost of butyrate. Cost decreases since both pathways share

393   an ATP yield of 0.5, while diol production does away with the CoAT reaction. This

394   reaction is itself enzymatically expensive due to its low driving force, and it increases

395   the cost of PTA and ACK reactions as CoAT requires a higher acetate concentration.

396   Adipic acid production requires 7.5 times more enzyme investment per ATP flux than

397   butyrate (Figure 4). This suggests that it may be more challenging to achieve high

398   adipic acid productivity due to a reduction in growth rate. On the other hand, 1,6-

399   hexanediol production could even increase growth rate vs. hexanoic acid, similarly to
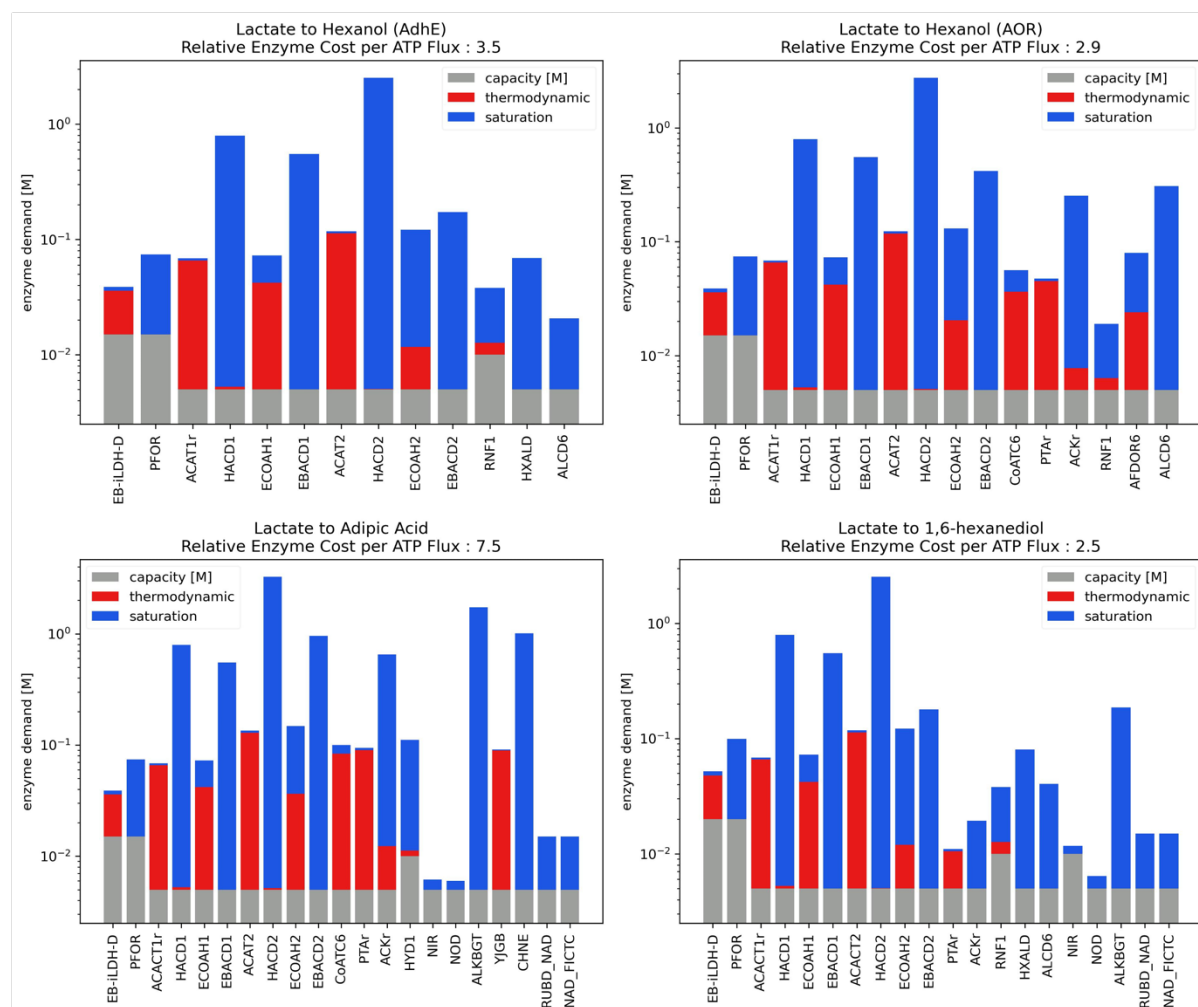
400   hexanol production.

Figure 4. ECM results of RBO for production of novel chain elongation products from lactate. Enzyme cost per unit ATP flux is reported as a multiple of butyrate production's cost.

Methyl ketone Production

Methyl ketones (MKs) are another chemical class that can be produced using MCFAs as precursors. Currently, in the engineered RBO cycle, methyl ketones are obtained from the hydrolysis of oxoacyl-CoA molecules by the thioesterase FadM. This reaction initially generates ketoacids, which in turn spontaneously decarboxylate to result in the final MK (Figure 3C) (Yan et al., 2020). In addition to FadM introduction, other

412 metabolic engineering strategies are necessary to achieve methyl ketone production

413 in chain elongating bacteria. The first strategy would be to substitute the native CoATs

414 by FadM, translocating termination from acyl-CoA to oxoacyl-CoA. Since the RBO

415 cycle is essential to redox balance and ATP production in chain elongating bacteria,

416 sole acetone and 2-butanone production would not be possible, as FadM would

417 terminate before one complete turn of the RBO cycle. Therefore, to optimize MK

418 production, it is necessary to allow MCFA with MK co-production.

419   FadM was incorporated into our model to assess the potential to produce 2-

420 pentanone from 6-oxohexanoyl-CoA. The model predicted that 2-pentanone

421 production is not optimal for ATP yield which aligns with the early termination initiated

422 by FadM at 6-oxohexanoyl-CoA, where the ATP from the complete elongation to

423 hexanoic acid is not produced. To assess the effect of 2-pentanone production on ATP

424 yield, we fixed 2-pentanone production at various values and determined the overall

425 pathway stoichiometry at each point using pFBA with ATP yield set as the objective

426 function (Table 3). It was found that 2-pentanone is negatively coupled with butyrate

427 and ATP production (Figure 5). Further, 2-pentanone production is thermodynamically

428 feasible only for MK production yields less than 0.15 mol 2-pentanone per mol lactate.

429 Since 2-pentanone production is feasible in *Escherichia coli* (Lan et al., 2013), it

430 suggests that the concentrations for standard conditions are likely incorrect to estimate

431 the pathway driving force. CO_2 production also increases with increasing 2-pentanone

432 production due to the spontaneous decarboxylation of the ketoacid. In isolation, this

433 loss of CO_2 severely impacts the carbon efficiency of this production pathway.

434 However, it could be alleviated if methyl ketone production was conducted in the

435 context of a microbial community, where other functional guilds could recycle the CO_2

436 (Baleeiro et al., 2023).

Table 3. Selected predicted pathway stoichiometries for 2-pentanone production.

Product; Pathway	Overall Equation	ATP Yield (mol ATP / mol Lactate)	$\Delta G_r^{\circ\prime}$	$\Delta G_r^{\circ\prime}$ per ATP
	Lactate + 0.5 H^+ → 0.5 Butyrate + CO_2 + H_2	0.750	-14.99	-19.99
	Lactate + 0.62 H^+ → 0.08 2-Pentanone + 0.38 Butyrate + 1.08 CO_2 + 1.08 H_2	0.610	-7.37	-12.08
Methyl ketone; FadM	Lactate + 0.65 H^+ → 0.1 2-Pentanone + 0.35 Butyrate + 1.1 CO_2 + 1.1 H_2	0.575	-5.46	-9.50
	Lactate + 0.89 H^+ → 0.26 2-Pentanone + 0.11 Butyrate + 1.26 CO_2 + 1.26 H_2	0.295	9.78	33.14
	Lactate + H^+ → 0.34 2-Pentanone + 1.3 CO_2 + 1.24 H_2 + 0.06 H_2O	0.030	12.86	428.68
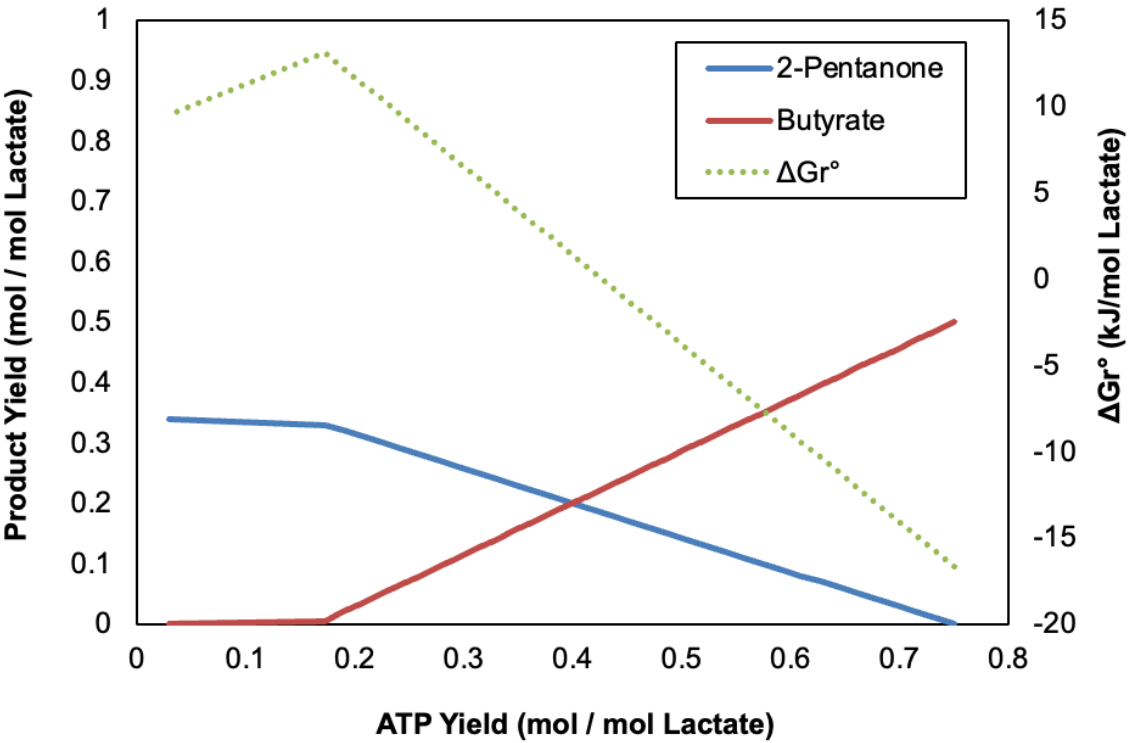
Figure 5. Production profiles for the co-production of 2-pentanone and butyrate from lactate and standard Gibbs free energy of reaction. Molar yields of ATP are negatively related to increasing production of 2-pentanone. Co-production of butyrate is required with increasing 2-pentanone production.
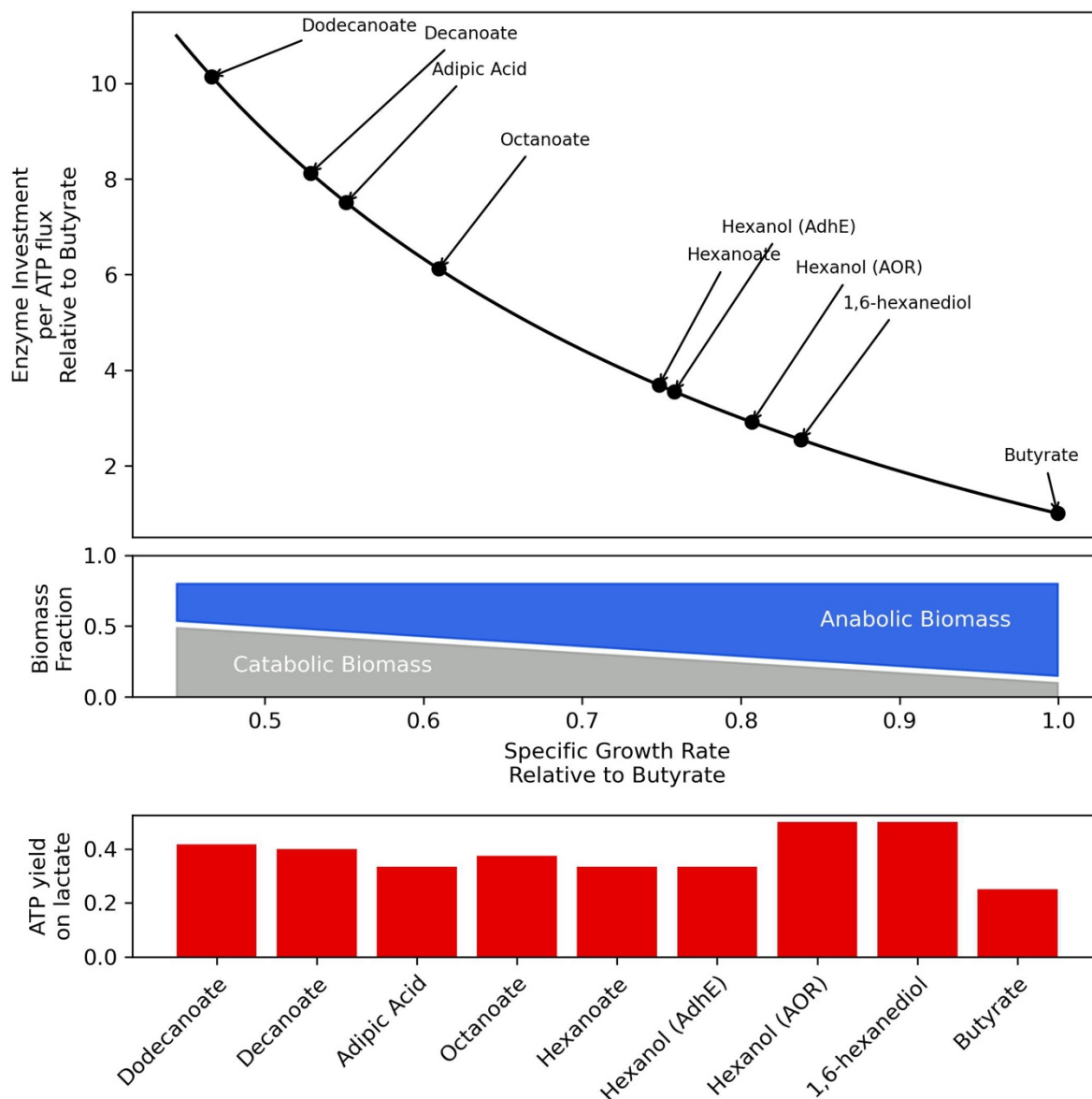
444

445

Figure 6. Top: Enzyme cost per ATP flux relative to butyrate vs. specific growth rate relative to butyrate, as derived from a simplified resource allocation model (Supplementary text). Middle: Anabolic fraction of biomass corresponding to specific growth rate (blue) and catabolic fraction of biomass remaining to supply ATP at a given growth rate. Bottom: ATP yield on lactate for different chain elongation simulated by pFBA.

Conclusion

Developing chain elongating bacteria as a bioproduction platform for oleochemical synthesis could play a crucial role in sustainable manufacturing from carbon-rich wastes streams. Our analyses demonstrate that the synthesis of C_9-C_{12} fatty acids, as well as conversion to their corresponding fatty alcohols, dicarboxylic acids, diols, and methyl ketones are thermodynamically feasible and generate sufficient ATP production for growth coupling. In the case of C_9-C_{12} fatty acids, enzyme cost analyses point towards a trade-off between growth rate and ATP yield with increasing chain length which may explain why chain elongation in nature terminates at chain lengths of C_8.

Other aspects that need to be investigated to understand limitations in chain length include the effect of product toxicity and enzyme-substrate affinity for longer-chain fatty acids. This includes strategies chain elongators employ to protect against MCFA toxicity and evaluation of the substrate range that can be accommodated by native RBO cycle enzymes. To address these problems, genetic engineering tools need to be developed, including genetic cargo delivery systems, the construction of regulatory element libraries and gene knock-in/knock-out methods to delete native chain elongating bacteria genes and to enable heterologous gene expression. These genetic modifications will allow the production of a broad range of oleochemicals through chain elongation using the pathways proposed in this work. The production of longer chain length fatty acids and their subsequent conversion into alcohols are ideal targets for initial demonstrations of oleochemical production using engineered chain elongating bacteria as these products are not redox limited in our analyses. However, the functionalization of the aliphatic end of the fatty acids required for dicarboxylate and diol production will be a significant challenge due to the anaerobic requirements

478   of chain elongating bacteria. While we present an avenue to accomplish this through

479   nitrite reduction and the use of microcompartments, screening of other enzymes that

480   can functionalize fatty acids without oxygen will be important to chain elongating

481   bacteria metabolic engineering efforts. Additionally, the production of methyl ketones

482   was found to be feasible only for select pathway stoichiometries and not strongly

483   growth coupled as it requires carboxylate co-production for ATP production. Given

484   previous demonstrations of methyl ketone production in *Escherichia coli* under

485   microaerobic conditions (Lan et al., 2013), other conditions and production pathways

486   should be investigated to improve yields in chain elongators.

487   Overall, our analyses indicate that chain elongating bacteria are promising

488   biomanufacturing chassis for accessing several oleochemical product classes.

489   Developing genetic tools for chain elongating bacteria will be critical for understanding

490   the fundamental physiology of this functional guild and will be required to expand their

491   product spectrum, in isolation as pure cultures, and in the context of self-assembled

492   and synthetic microbiomes.

493

494   **Methods**

495   ***Predicting Pathway Stoichiometry with Parsimonious Flux Balance Analysis***

496   Python3 and CobraPy were used for the metabolic modelling. Previously, the

497   iFermCell215 model was created to represent a generalized chain elongation

498   microbiome with several functional guilds capable of utilizing different electron donors

499   for elongation up to C8 (Scarborough et al., 2020). iFermCell215 was simplified by

500   removing some inapplicable reactions and further modified with 26 metabolites and 30

501   reactions required for the production of C_9-C_{12} carboxylates to produce iFermCell193.

502   iFermCell193 was further constrained to capture the metabolism of typical CEB, in

503   alignment with the proposed stoichiometric models previously described to obtain a

504   generalized core model for chain elongating bacteria (Angenent et al., 2016;

505   Scarborough et al., n.d.). Using iFermCell193, the production of fatty acids from lactate

506   (main text), ethanol, glucose, xylose, and glycerol (Supplementary Material) was

507   modelled with parsimonious enzyme usage flux balance analysis (pFBA) (Lewis et al.,

508   2010) where net ATP yield was set as the objective function, used here as a proxy for

509   growth.

510   iFermCell193 was further modified with 70 metabolites and 163 reactions to

511   produce iFermCell356 based on the proposed production pathways for expanding the

512   chain elongating bacteria product spectrum to produce fatty alcohols, dicarboxylates,

513   diols, methyl ketones. The production of these bioproducts from different electron

514   donors was modelled with pFBA where net ATP yield was set as the objective function,

515   except methyl ketone production.

516   Python package equilibrator-api (https://gitlab.com/equilibrator) was used to

517   predict the Gibbs free energy of formation for metabolites at physiological conditions

518   of T = 25 °C, pH = 7, pMg = 0, Ionic Strength = 0 M. To calculate overall Gibbs free

519   energy change of reaction for the production of the different products from lactate, the

520   Gibbs free energy change of formation and the overall pathway reaction stoichiometry

521   predicted by the model were used. The models, configuration files, and final flux

522   solutions are provided in the Supplementary Material.

523   ***Enzyme Cost Minimization***

524   ECM was developed by Flamholz et al. and performed here with the Python

525   packages equilibrator-api and equilibrator-pathway (https://gitlab.com/equilibrator). A

526   reversible Michaelis Menten kinetic expression is substituted with a flux-force

527   efficiency term, $\frac{J_+}{J_-} = e^{\Delta G'/RT}$, to yield an expression for the enzyme concentration $[E]$

528   required to sustain a given flux J through a pathway reaction:

529
$$[E] = \frac{J}{k_{cat}} \frac{1 + \prod_i \left(\frac{K_{M,i}}{[C_i]}\right)^{m_i}}{1 - e^{\frac{\Delta G'}{RT}}}$$

530   Where k_{cat} is the enzyme's rate constant, $[C_i]$ is the concentration of reactant

531   i, $K_{M,i}$ is the enzyme's saturation constant for reactant i, m_i is the stoichiometric

532   coefficient of reactant i, and $\Delta G'$ is the Gibbs free energy change, estimated via the

533   component contribution method at pH 6 (Noor et al., 2013). This expression can be

534   decomposed into 3 factors to facilitate interpretation: $\frac{J}{k_{cat}}$ represents the stoichiometric

535   enzyme demand; $1 + \prod_i \left(\frac{K_{M,i}}{[C_i]}\right)^{m_i}$ encapsulates the cost incurred when the enzyme is

536   not saturated; $1 - e^{\frac{\Delta G'}{RT}}$ reflects the increased enzyme requirement to sustain a net flux

537   when a reaction is near equilibrium. The total enzyme cost per unit pathway flux (gram

538   protein/mole product/hour) is calculated by summing reaction costs multiplied by both

539   their stoichiometry in the pathway n_i and their enzyme's molar weight M_i, then dividing

540   by the arbitrary pathway flux J in units of moles per hour. Total enzyme cost per unit

541   flux is minimized by gradient descent given bounds on metabolite concentrations and

542   the requirement for all reactions to be exergonic:

543
$$Minimize \; \Lambda = \frac{1}{J} \sum_i M_i \, n_i [E_i]$$

544 $$\text{Such that } C_{i,min} < C_i < C_{i,max} \; \forall i$$

545 $$\text{And } \Delta G'_i = \Delta G^{\circ \prime}_i + RT\boldsymbol{S}_i^T \cdot \ln(\boldsymbol{C}) < 0 \; \forall i$$

546 Where $\boldsymbol{S}_i^T$ is the ith row of the stoichiometric matrix's transpose, containing the

547 stoichiometric coefficients for each metabolite in the ith reaction, and $\ln(\boldsymbol{C})$ is the

548 vector of logarithms of each metabolite's concentration. As a first approximation, all

549 enzymes are assumed to share rate and saturation constants and molecular weight

550 (k_{cat} = 200 s^{-1}; K_m = 0.2 uM, MW = 40 kDa). Pathway specific metabolites are bounded

551 between 1 uM and 10 mM, apart from oxoacyl-CoAs which can drop to 0.4

552 uM. Universal metabolites such as ATP and NADH are set to concentrations derived

553 from literature (Bennet et al. 2009). The reduction potential of ferredoxin is set to -400

554 mV. Dissolved oxygen concentration was set to 6.6 uM (0.005 atm at equilibrium),

555 which is expected to be sub-lethal to obligate anaerobes (Loesche, 1969). Dissolved

556 hydrogen concentration was 1.4 mM (0.2 atm) and dissolved CO_2 concentration was

557 10 uM. Ionic strength was set to 250 mM. A proton motive force of 200 mV was used

558 for butyrate production, whereas 100 mV was used in all other cases, due to the

559 reversal of RNF activity required for butyrate production without an electron acceptor.

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