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Advances in heterologous biosynthesis of plant and fungal natural products by modular co-culture engineering

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Abstract

Heterologous biosynthesis has been long pursued as a viable approach for high efficiency production of natural products with various industrial values. Conventional methods for heterologous biosynthesis use the mono-culture of an engineered microbe for accommodating the whole target biosynthetic pathway to produce the desired product. The emergence of modular co-culture engineering, which divides the pathway between multiple co-culture strains, presents a new perspective to conduct heterologous biosynthesis and improve the bioproduction performance of natural products. This review highlights recent advances in utilizing the modular co-culture engineering approaches to address the challenges of plant and fungal natural product biosynthesis. Potential directions for future research in this promising field are also discussed.

Keywords Bioproduction improvement, Heterologous biosynthesis, Modular co-culture engineering, Natural product, Plants and fungus

Introduction

Plant and fungal natural products have a long history of being used as a major source of nutraceutical and pharmaceutical molecules (Cragg and Newman 2013; Gupta 1994; Hoffmeister and Keller 2007; Lee 2004; Schueffler and Anke 2014). Traditionally, these biological molecules are obtained from plants or fungi through complex extraction and purification processes, which often suffer from low production yield. The relatively slow growth rate of plants and fungi as well as the difficulties associated with their metabolic engineering also largely limit the high-productivity natural product biosynthesis using these native hosts. As such, heterologous biosynthesis using a surrogate host has been developed as an alternative and viable method for generation of the plant and fungal natural products (Ahmadi and Pfeifer 2016; Becker and Wittmann 2016; Li et al. 2018a; Luo et al. 2015; Zhang et al. 2011; Zhang et al. 2008; Zhang et al. 2016). Specifically, a target natural product biosynthetic pathway is introduced into a selected heterologous host which is genetically and metabolically engineered to support the biosynthesis of the pathway products. This approach has been successfully utilized to make tremendous accomplishments in the past decades. Yet, it often relies on the use of a particular microbial host for accommodation of the entire complex biosynthetic pathways for targeted natural products. As such, it encounters major challenges such as imbalanced expression of pathway enzymes, impaired host cell growth due to overwhelming metabolic burden, and lack of flexibility to simultaneously satisfy the need of different heterologous and endogenous enzymes. Recent development of biosynthesis of complex natural products, especially those with highly complicated biosynthetic pathways, calls for more sophisticated methodologies for meeting the need of higher biosynthesis performance.

To this end, modular co-culture engineering has emerged as an alternative approach for biosynthesis of a wide range of natural products (Jones and Wang 2017; Zhang and Wang 2016) . Specifically, individually engineered strains of a designed co-culture system are used to harbor different modules of the target natural product biosynthetic pathway. Owing to the versatile pathway modularization in the context of the co-culture, this methodology offers important advantages for biosynthesis including lowered metabolic stress on each strain, reduced interference between pathway enzymes, flexible balancing between pathway modules and plug-and-play fashion biosynthesis. In fact, most plant and fungal natural products have a long and complex biosynthetic pathway involving a large number of pathway enzymes with various biochemical properties, which present outstanding opportunities to leverage the power of modular co-culture engineering for improving the biosynthesis performance. A wide range of various biochemicals, including simple biofuel molecules, commodity aromatics, and complex natural products, have been successfully produced using engineered microbial co-cultures, which have been summarized in previous reviews (Jones and Wang 2017; Zhang and Wang 2016). In particular, novel engineering approaches, such as employment of multiple species co-cultures (Zhou et al. 2015), simultaneous utilization of different carbon substrates (Zhang et al. 2015), and convergent pathway engineering (Liu et al. 2018a), have been adapted to improve the capability of microbial co-cultures. The focus of this review is placed on recent achievements of plant and fungal natural product biosynthesis using the modular co-culture engineering approach between 2016-2018.

Table 1. Recent achievements of engineering microbial co-cultures for biosynthesis of plant and fungal natural products between 2016-2018

Compound	Co-culture system	substrate	Product Conc.	Improvement over the mono-culture	Ref

Pinene	*E.coli-E.coli*	Sucrose, peptone, yeast extract	64.9 mg/L	1.9-fold	Niu et al. 2018
Monacolin J	*P. pastoris-P. pastoris*	Methanol	593.9 mg/L	13.4%	Liu et al. 2018b
Lovastatin	*P. pastoris-P. pastoris*	Methanol	250.8 mg/L	2.2-fold	Liu et al. 2018b
Caffeoylmalic acid	*E.coli-E.coli*	Glucose	570.1 mg/L	3-fold	Li et al. 2018b
Resveratrol	*E.coli-E.coli*	Glycerol	22.58 mg/L	N/A	Camacho-Zaragoza et al. 2016
Resveratrol glucosides	*E.coli-E.coli*	*p*-coumaric acid	92.3 mg/L	2.9-fold	Thuan et al. 2018b
Apigetrin	*E.coli-E.coli*	*p*-coumaric acid	16.6 mg/L	2.1-fold	Thuan et al. 2018a
Salicylate 2-O-β-D-glucoside	*E.coli-E.coli*	Glucose and glycerol	2.5 g/L	19%	Ahmadi et al. 2016
Salidroside	*E.coli-E.coli*	Glucose and xylose	6.03 g/L	N/A	Liu et al. 2018a
Cadaverine	*E.coli-E.coli*	Glucose and glycerol	28.5 g/L	2.1-fold	Wang et al. 2018
Cadaverine	*C. glutamicum -E. coli*	Starch	6.8 mM	N/A	Sgobba et al. 2018
Pipecolic acid	*C. glutamicum-E. coli*	Starch	3.4 mM	N/A	Sgobba et al. 2018
Naringenin	*S. cerevisiae and E. coli*	Xylose, yeast extract	21.16 mg/L	8-fold	Zhang et al. 2017

70

71 **Co-culture biosynthesis of natural products in recent years**

72 Pinene

73 Pinene is a monoterpene widely found in conifers. As this compound is considered a
74 promising biofuel candidate, its heterologous biosynthesis using renewable feedstocks is of great
75 research interest. Niu et al. developed an *E. coli-E. coli* co-culture system for pinene
76 bioproduction using a rich medium containing sucrose, peptone and yeast extract (Niu et al.
77 2018). The upstream strain in the co-culture was engineered for a heterologous mevalonate
78 pathway to produce the pathway intermediate isopentenyl diphosphate, whereas the downstream

79 strain was dedicated to functional expression of the geranyl diphosphate synthase and pinene
80 synthase, the last two enzymes of the pathway to convert isopentenyl diphosphate to pinene.
81 Importantly, the designated pathway genes were integrated into the chromosome of the
82 corresponding co-culture strains and were evolved for high copy number expression. It should be
83 noted that, isopentenyl diphosphate, in spite of carrying the diphosphate group, was confirmed to
84 be able to travel across the cell membrane for connecting the separate pathway modules in the
85 context of the constructed co-culture. The optimization of the inoculation ratio between the co-
86 culture strains resulted in the production of 64.9 mg/L pinene, which was 1.9-fold higher than the
87 mono-culture control. The biosynthesis improvement should be attributed to the reduced
88 metabolic burden on each strain as well as the coordinated bioconversion capabilities between
89 the co-culture strains through the strain-to-strain ratio manipulation. Interestingly, the cells of the
90 co-culture were also harvested, centrifuged and re-suspended in a phosphate buffer to carry out
91 the whole-cell biocatalysis conversion, which further increased the pinene biosynthesis to 166.5
92 mg/L.

93

94 Monacolin J and lovastatin

95 Lovastatin and its precursor monacolin J are polyketide natural products found in fungi such
96 as *A. terreus* and *P. ostreatus*. Lovastatin has been successfully developed as a commercial
97 cholesterol-reducing drug. Liu et al. developed microbial biosynthetic systems, including *Pichia*
98 *pastoris-Pichia pastoris* co-cultures, for de novo production of monacolin J and lovastatin using
99 methanol as the carbon substrate (Liu et al. 2018b). To this end, the complex lovastatin
100 biosynthesis pathway was first reconstituted in the heterologous host *P. pastoris* by metabolic
101 engineering approaches. Several *P. pastoris-P. pastoris* co-culture strategies with different

102 pathway splitting nodes were employed for biosynthesis improvement. The inoculation ratio
103 between the co-culture strains was also optimized for production improvement. Notably, the
104 variation of both the pathway splitting node and corresponding co-culture strain inoculation ratio
105 allowed for more systematic pathway balancing than using a fixed pathway modularization
106 pattern for biosynthesis optimization. As a result, an engineered co-culture for monacolin J
107 biosynthesis produced 93 mg/L product, and another co-culture harboring the complete
108 lovastatin pathway produced 24.6 mg/L lovastatin. Furthermore, the co-culture biosynthesis was
109 scaled up using a 5 L bioreactor. The optimal inoculation ratios at this scale was investigated and
110 found to be the same with the results of the shake flask. The highest monacolin J and lovastatin
111 production reached 593.9 and 250.8 mg/L in the monacolin J-producing and lovastatin-producing
112 co-cultures, respectively. Compared with the mono-culture, the biosynthesis was improved by
113 13.4% for monacolin J and 2.2-fold for lovastatin. Although the strain-to-strain ratio fluctuation
114 was found during the co-culture biosynthesis, the results of the bioreactor cultivation showed the
115 scalability and promise of the developed co-culture strategies for converting methanol to high
116 value pharmaceuticals.
117
118 <u>Caffeoylmalic acid</u>
119 Caffeoylmalic acid, a hydroxycinnamoyl-malate ester, has been found to be an anti-oxidant and
120 proteolytic inhibition agent and it possesses other activities beneficial for human health. This
121 plant natural product's biosynthesis requires the combination of caffeic acid and malate catalyzed
122 by the hydroxycinnamoyl transferase. Li *et al*. reconstituted the caffeoylmalic acid pathway in *E.*
123 *coli* by over-expression of heterologous enzymes tyrosine ammonia lyase, 4-coumarate-
124 coenzyme A ligase, hydroxycinnamoyl transferase, enabling the de novo production on glucose

125 (Li et al. 2018b). Moreover, an *E. coli-E. coli* co-culture was developed to improve the
126 biosynthesis efficiency. In particular, the co-culture design reduced the formation of the
127 byproduct *p*-coumaroylmalic acid, as the hydroxycinnamoyl transferase with promiscuous
128 activity was put into the downstream co-culture strain and thus was segregated from the
129 byproduct's precursor *p*-coumaric acid in the upstream strain. This strategy highlighted the
130 advantage of modular co-culture engineering for reducing the undesired interference between
131 different pathway modules, which has also been demonstrated in previous studies (Chen et al.
132 2017). This effort, together with the control of the co-culture strain inoculum ratio, resulted in
133 the production of 570.1 mg/L caffeoylmalic acid at the inoculum ratio of 6:1. Notably, the
134 biosynthesis was three times higher than that of the mono-culture.
135
136 <u>Resveratrol</u>
137 Resveratrol is a polyphenol compound with many health-beneficial effects that makes it an
138 attractive chemical both for academic studies and industrial purposes (Kovacic and Somanathan
139 2010). Co-culture biosynthesis of resveratrol precursor naringenin and other flavonoids has been
140 reported in previous studies (Ganesan et al. 2017; Jones et al. 2017; Jones et al. 2016). To further
141 achieve co-culture biosynthesis of resveratrol, Camacho-Zaragoza et al. adapted a co-culture
142 system comprised of two populations of *Escherichia coli* strains, each with a partial and
143 complementary section of the heterologous pathway (Camacho-Zaragoza et al. 2016). The
144 upstream co-culture strain harbored an engineered tyrosine pathway and a heterologous tyrosine

145 ammonia lyase for tyrosine formation and conversion to *p*-coumaric acid. The downstream strain
146 was constructed to produce malonyl-CoA and provide it to the stilbene synthase STS for yielding
147 resveratrol. The co-culture of these strains inoculated at a 1:1 ratio resulted in the production of
148 22.58 mg/L resveratrol from 10 g/L glycerol after 30 h. Also, *p*-coumaric acid was accumulated
149 to a level lower than the mono-culture of the upstream strain, due to better conversion to
150 resveratrol in the co-culture. Interestingly, the growth rates of both strains during co-culture
151 cultivation were found to be similar to those of their mono-cultures, respectively.
152
153 Resveratrol glucosides
154 Similar co-culture design was utilized for biosynthesis of resveratrol glucosides, water-
155 soluble derivatives of resveratrol that has been found to possess the antioxidant, estrogenic and
156 anticancer activities (Thuan et al. 2018b). Specifically, the upstream strain of the engineered co-
157 culture system converted exogenous *p*-coumaric acid to resveratrol through over-expression of 4-
158 coumarate-coenzyme A ligase and stilbene synthase, and the downstream strains were engineered
159 to enhance UPD-glucose formation and catalyze resveratrol glycosylation. Two glucosidated
160 resveratrol products, polydatin and resveratroloside were successfully produced by the co-culture
161 system with high *p*-coumaric acid bioconversion. Notably, the optimization of the upstream and
162 downstream strain inoculum ratio showed that high or low ratio both increased the final
163 polydatin production whereas the lowest production was observed at the ratio of 1:1.
164 Nevertheless, compared to the mono-culture strategy, the co-culture improved the biosynthesis
165 performance by 2.9-fold at the optimal inoculum ratio of 9:1, highlighting the modular co-culture
166 engineering's advances of straightforward balancing between individual pathway modules.

167 Importantly, the co-culture biosynthesis was successfully scaled up using a 3 L bioreactor,
168 leading to the production of 92.3 mg/L resveratrol glucosides.
169
170 Apigetrin
171 Co-culture biosynthesis has been extended for production of other flavonoids such as
172 apigenin and its glycosylated product apigetrin, both of which possess important biological
173 activities. Thuan and coworkers constructed an engineered *E. coli*-*E. coli* co-culture system to
174 accommodate the apigetrin pathway (Thuan et al. 2018a). The upstream strain of the co-culture
175 contained the pathway module for converting exogenous *p*-coumaric acid to apigenin through
176 functional expression of 4-coumarate-coenzyme A ligase, chalcone synthase, chalcone flavanone
177 isomerase, flavone synthase I. The downstream strain was specifically engineered for over-
178 expressing UDP-glucose biosynthesis genes and its attachment to apigenin to generate apigetrin.
179 For biosynthesis optimization, the initial inoculum ratio of the co-culture strains was varied to
180 balance the biosynthesis capabilities of the upstream and downstream pathway modules. As a
181 result, the apigetrin biosynthesis was improved by 2.1-fold, compared to the mono-culture
182 strategy. In addition, the cultivation temperature was also optimized to coordinate the need of the
183 different pathway modules. The optimized co-culture was periodically fed with exogenous *p*-
184 coumaric acid and produced 16.6 mg/L apigetrin.
185
186 Salicylate 2-O-β-D-glucoside
187 Microbial co-cultures have also been utilized for producing other glycosylated natural
188 products, such as a plant-based anti-inflammatory agent salicylate 2-O-β-D-glucoside. Ahmadi et
189 al. engineered the salicylate 2-O-β-D-glucoside biosynthesis pathway in the context of *E. coli*-*E.*

190 *coli* co-cultures (Ahmadi et al. 2016). In one co-culture design, the upstream strain and
191 downstream strain were genetically modified for salicylic acid formation and glycosylation,
192 respectively. Interestingly, it was found that the biosynthesis was not improved by this linear
193 modularization design. To overcome this in another co-culture design, two *E. coli* strains, one
194 containing the whole pathway and the other containing only the downstream glycosylation
195 module, were co-cultivated. This strategy resulted in the production of 2.5 g/L salicylate 2-O-β-
196 D-glucoside, which was higher than the mono-culture control. Moreover, the intermediate
197 salicylic acid accumulation was significantly reduced in the co-culture system. This finding
198 indicated that nonlinear pathway modularization with certain overlap between the modules may
199 be a viable strategy for co-culture biosynthesis. Importantly, the heterologously produced
200 salicylate 2-O-β-D-glucoside was tested and confirmed that it possessed the anti-inflammatory
201 activity without significant mammalian cell toxicity.
202
203 Salidroside
204 Salidroside is a glucoside of tyrosol with demonstrated medicinal values. This natural
205 product biosynthesis requires a convergent pathway that involves tyrosol formation and
206 glycosylation. Liu et al. constructed an *E. coli-E. coli* co-culture to overcome the challenge of
207 salidroside biosynthesis (Liu et al. 2018a). In this system, an aglycone strain was specifically
208 engineered for tyrosol biosynthesis, and a glycoside strain was constructed to overproduce UDP-
209 glucose and attach it to tyrosol for salidroside formation. These two strains were also genetically
210 modified to establish the syntrophic co-culture with a phenylalanine-tyrosine cross-feeding
211 mechanism. In order to reduce the growth competition for carbon source and improve the co-
212 culture stability, the two strains were engineered to grow on glucose and xylose, respectively.

213 Importantly, the glucose/xylose ratio and inoculation ratio of two strains were both adjusted for
214 biosynthesis optimization. The optimized system was able to keep the pathway intermediate
215 tyrosol in a low level during the co-culture cultivation. The adaption of the fed-batch bioreactor
216 technique further improved the salidroside production to 6.03 g/L after 129 h cultivation, which
217 demonstrated the scalability of the constructed syntrophic co-culture system. It should be noted
218 that the modular nature of the co-culture design allowed for flexible swapping of the UDP-
219 glycosyltransferase in the downstream strain, which led to the co-culture production of another
220 glycoside, icariside D2.

221

222 Cadaverine

223 Microbial co-culture biosynthesis of cadaverine, a diamine involved in plant growth, has
224 been reported using an *E. coli-E. coli* co-culture (Wang et al. 2018). In this system, two *E. coli*
225 strains were used for L-lysine production and conversion to cadaverine, respectively. The two *E.*
226 *coli* strains were also engineered to grow on glucose and glycerol, respectively, which reduced
227 their growth competition for the carbon substrates and increased the growth balance. The co-
228 culture cultivation conditions, including inoculation ratio, glucose/glycerol ratio, induction time,
229 temperature, nitrogen source and concentration, C/N ratio were systematically optimized for
230 cadaverine production. The cultivation of the engineered co-culture system in a 7.5 L fermenter
231 led to the production of 28.5g/L cadaverine, which was 2.1-fold higher than the mono-culture
232 system. The biosynthesis improvement can be largely attributed to the fact that the two co-
233 culture strains were rationally modified using metabolic engineering and bioprocess engineering
234 tools to specifically suit the needs of the corresponding biosynthetic tasks. Importantly, the co-

235 culture biosynthesis using the fermentor demonstrated the applicability of the microbial co-
236 cultures at large scales.
237
238 Multi-species co-culture for cadaverine, pipecolic acid and naringenin biosynthesis
239 Multi-species co-cultures have also received increasing research interest, as they combine
240 the biosynthesis powers of different species for better serving the purpose of production
241 optimization. For example, *C. glutamicum* is known for robust lysine biosynthesis capability,
242 which makes it useful for producing lysine-derived natural products. Sogbba et al. established an
243 *E. coli-C. glutamicum* consortia to convert starch to pipecolic acid and cadaverine via L-lysine in
244 one consolidated culture (Sgobba et al. 2018). Specifically, *E. coli* and *C. glutamicum* were co-
245 cultured under the cultivation conditions that was set up to coordinate the growth between the
246 two species. In addition, the two strains were engineered to generate a mutualistic system for
247 improving the co-culture stability. To this end, heterologous α-amylase from *S. griseus* was
248 introduced into starch-negative *E. coli*, allowing it to utilize starch as carbon source. The
249 resulting glucose converted from starch by *E. coli* was used to feed *C. glutamicum*. Meanwhile,
250 *C. glutamicum* provided lysine to lysine auxotrophic *E. coli* to facilitate its growth.
251 Such constructed co-culture was first employed for cadaverine production. It should be
252 noted that, in this co-culture system, the *E. coli* strain was only a starch degrader, and it did not
253 directly participate in the cadaverine biosynthesis via lysine. By deleting gene *snaA* encoding
254 spermi(di)ne N-acetyltransferase and introducing lysine decarboxylase gene *ldcC*, *C.*
255 *glutamicum* was engineered to enhance cadaverine bioproduction *in vivo*. For pipecolic acid
256 production, gene *proC* encoding endogenous pyrroline 5-carboxylic acid reductase and *lysDH*
257 endocing L-lysine-6-dehydrogenase were over-expressed to attract more carbon flow to pipecolic

258 acid pathway in *C. glutamicum*. Based on these efforts, the production of cadaverine and
259 pipecolic acid by the engineered co-cultures reached 6.8 mM and 3.4 mM, with the yield of
260 0.025g/g (starch) and 0.012g/g (starch), respectively.

261 Multi-species co-cultures have also been developed for biosynthesis of complex natural
262 product naringenin. Zhang et al. constructed an *E. coli-S. cerevisiae* co-culture to combined the
263 biosynthetic capabilities of both the prokaryotic and eukaryotic microbes for improving
264 naringenin bioproduction efficiency (Zhang et al. 2017). Specifically, *E. coli* was engineered to
265 enhance the endogenous tyrosine pathway for high level production of amino acid tyrosine, the
266 intermediate of the naringenin pathway; *S. cerevisiae* was engineered to functionally express the
267 downstream naringenin pathway enzymes, including tyrosine ammonia lyase, 4-coumarate-
268 coenzyme A ligase, chalcone synthase, chalcone flavanone isomerase, for naringenin production.
269 Notably, all of downstream pathway enzymes were derived from eukaryotic organisms and thus
270 could be better expressed in *S. cerevisiae*.

271 In addition, the constituent co-culture strains were engineered to generate the commensalism
272 for stabilization of the co-culture population composition, which had been successfully used for
273 co-culture biosynthesis of oxygenated taxanes, tanshinone precursors and functionalized
274 sesquiterpenes in other studies (Zhou et al. 2015). To this end, the *E. coli* strain was utilized to
275 metabolize xylose and produced acetate that could be used by *S. cerevisiae* as the carbon source
276 for growth. This design removed the acetate toxicity towards *E. coli* and also enabled *S.*
277 *cerevisiae* to indirectly utilize xylose for growth. After the commensalism was established in the
278 co-culture, orthogonal experiment design was carried out to optimize the cultivation conditions,
279 including xylose concentration, inorganic salt mixtures, yeast extract and initial yeast inoculation
280 for the co-culture biosynthesis. On top of these efforts, this study also investigated the effect of

281 inoculum size and inoculum ratio of *S. cerevisiae* and *E. coli*. The OD600 ratio of two
282 microorganisms was found to change dramatically after inoculation but stabilized after 48 h.
283 Based these engineering strategies, 21.16 mg/L of naringenin was successfully produced from
284 xylose using the optimized co-culture system.

285

286 **Conclusion and future directions**

287 In recent years, utilization of microbial co-cultures have emerged as a novel methodology for
288 biosynthesis of various biochemicals. Due to its outstanding advantages, rationally designed co-
289 cultures have also been increasingly used for biosynthesis of plant and fungal natural products.
290 To date, there have been many successful studies confirming the applicability and effectiveness
291 of this new approach for improving natural product bioproduction performance, which are
292 summarized in this and previous reviews (Jones and Wang 2017; Zhang and Wang 2016).
293 Therefore, further development of the modular co-culture engineering will provide a robust
294 toolkit for overcoming the challenges of using engineered microorganisms for high-efficiency
295 production of complex plant natural products. In the meantime, the successful application of
296 modular co-culture engineering in plant and fungal natural product biosynthesis will generate
297 important techniques and knowledges for wider utility of this emerging engineering approach in
298 other biochemicals' heterologous biosynthesis.

299 One of the key issues that needs to be addressed in the future is to improve the stability of
300 the co-culture system for bioproduction enhancement. To this end, establishing mutualism or
301 commensalism between co-culture members can be a powerful approach and is expected to
302 receive increasing interest. In addition, selection of appropriate microbial strains or species and
303 controlling their growth rates using metabolic engineering approaches at a similar level is also

304 critical for improving the co-culture stability. At large scales, advanced bioprocess engineering
305 approaches, such as fed-batch bioreactor techniques, can be adapted for coordinating the growth
306 of different co-culture strains for biosynthesis scale-up. Another direction for future development
307 is to more extensively adapt multi-species co-cultures for harnessing the biosynthetic capabilities
308 of different organisms. This is of particular interest for plant and fungal natural product
309 biosynthesis, as the use of eukaryotic organisms in the co-culture is highly beneficial for
310 functional expression of plant and fungal natural product pathway enzymes. Successful
311 employment of multi-species co-cultures will require manipulation of the cultivation conditions
312 to meet the need of all involved species. It also requires in-depth understanding of species-to-
313 species interaction in the context of co-cultures, which can be facilitated by development of
314 sophisticated models simulating the dynamic growth and biosynthetic behaviors of the co-culture
315 strains. Last but not least, the design of the pathway modularization plays a vital role in adapting
316 the modular co-culture engineering approach. The proper design should choose appropriate
317 pathway intermediates that can travel across the cell membrane of different co-culture strains for
318 connection of the pathway modules. Moreover, the pathway modules upstream and downstream
319 of the chosen pathway intermediate should be relatively balanced in terms of biosynthetic labor,
320 which will greatly facilitate the biosynthetic optimization by changing the ratio between the co-
321 culture strains carrying these pathway modules.

322 Moreover, it should be noted that microbial co-cultures have been developed for mimicking
323 complex ecological systems and promoting biosynthesis of a variety of biological molecules
324 including plant and fungal natural products, which are well summarized in recent reviews
325 (Marmann et al. 2014). However, most of these studies focused on the activation of silenced
326 gene expression through manipulated strain-to-strain interaction in the co-cultures, and there was

327 not a high level of pathway modularization and bioengineering components involved in these
328 studies. Yet, the advances in these areas offer another perspective for exploring the biosynthetic
329 power of microbial co-cultures.
330
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334
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