Renewable Fatty Acid Ester Production in *Clostridium*

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Abstract

Endproduct toxicity is a key bottleneck for biofuel/biochemical production. Biosynthesis of high-value derivatives that can easily separate would alleviate such toxicity and thus enhance bioproduction efficiency. As a proof of principle, biosynthesis (along with in-line extractive recovery) of fatty acid esters using clostridia was investigated in this study. We hypothesize that solventogenic clostridia are excellent platforms for ester production, because they co-produce acyl-CoAs (acetyl-CoA and butyryl-CoA), acids (acetate and butyrate), and alcohols (ethanol and butanol). Through rational screening for host strains and genes (encoding alcohol acyl transferases and lipase), systematic metabolic engineering, and elimination of putative prophages, we obtained strains that can produce 20.3 g/L butyl acetate and 1.6 g/L butyl butyrate respectively, which were both historically highest levels in microbial hosts. Our principle for engineering microorganisms to produce high-value and easy-recoverable endproducts is highly applicable to other bioprocesses, and could lead to breakthroughs in biofuel/biochemical production and general bioeconomy.

Keywords: clostridia, biofuels and biochemicals, fatty acid ester, butyl acetate, butyl butyrate, ethyl acetate, CRISPR-Cas9, hydrolysates, prophages

Main

Although tremendous efforts have been invested for biofuel and biochemical research, it is still challenging to generate robust strains that can produce target products at desirable levels[1]. One key bottleneck is the intrinsic toxicity of endproducts to host cells[2]. Therefore, the production of high-value bioproducts which can be easily recovered from fermentation might be a solution to tackle the bottleneck in bioproduction. Fatty acid esters, or mono-alkyl esters, can be used as valuable fuels such as diesel components or specialty chemicals for food flavoring, cosmetic and pharmaceutical industries[3]. It is projected that the US market demand for fatty acid esters could reach $4.99 billion by 2025[4]. In addition, esters, with fatty acid and alcohol moieties, are generally hydrophobic and can easily separate from fermentation; thus the production of ester can help mitigate endproduct toxicity to host cells and efficient bioproduction can be achieved.

Conventionally, esters are produced through Fischer esterification which involves high temperature and inorganic catalysts[5,6]. The reaction consumes a large amount of energy and generates tremendous wastes, and thus is not environmentally friendly[5]. On the other hand, ester production through biological routes is becoming more and more attractive because it is renewable and environmentally benign. There are two primary biological pathways for ester production: one is through esterification of fatty acid and alcohol catalyzed by lipases[7], and the other is based on condensation of acyl-CoA and alcohol catalyzed by alcohol acyl transferases (AATs)[5]. Previously, lipases from bacteria or fungi have been employed for catalyzing esterification for ester production[8]. For instance, lipase from *Candida sp*. has been recruited to

drive production of butyl butyrate (BB) with *Clostridium tyrobutyricum*. *C. tyrobutyricum*, a natural hyper-butyrate producer, could generate 34.7 g/L BB with supplementation of lipase and butanol[9]. However, in such a process, the supplemented enzyme accounts a big cost and meanwhile the operation needs to be carefully managed to achieve the optimum performance of esterification[9]. Therefore, a whole microbial cell factory able of ester production in one pot is highly desired. *Saccharomyces cerevisiae* has been reported to produce various esters with its native AATs, but generally at very low levels (< 1 g/L)[10]. Rodriguez et al. metabolically engineered *E. coli* to produce esters by introducing heterologous AATs[5]. Although the production of some acetate esters can reach decent levels (such as 17.2 g/L isobutyl acetate), the production of most of the esters was rather low, probably due to the unavailability of intrinsic substrates/precursors and limited tolerance of *E. coli* to organic endproducts.

Solventogenic clostridia are well known for solvent production through the Acetone-Butanol-Ethanol (ABE) pathway[11]. During fermentation, acyl-CoAs (acetyl-CoA and butyryl-CoA), fatty acids (acetate and butyrate), and alcohols (ethanol and butanol) are co-produced, either as intermediates or endproducts. Therefore, we hypothesized that clostridia can be excellent platforms to be engineered for ester production by introducing heterologous AATs and/or lipase genes. In this study, strains from four well-known clostridial species were evaluated as hosts for ester production. Through systematic metabolic engineering and elimination of putative prophages, we ultimately obtained two strains which can produce 20.3 g/L butyl acetate (BA) and 1.6 g/L BB respectively in extractive batch fermentations. These production levels were both highest in record. Besides, some of our engineered strains

107 demonstrated great potential for ethyl acetate (EA; 8.4 g/L) production.

108

109 **Results**

110 **Screening of host strains and genes for ester production**

111 We considered clostridia as ideal platforms for ester production thanks to their intrinsic
112 intermediates (fatty acids, acyl-CoAs, and alcohols) serving as precursors for ester biosynthesis
113 (Fig. 1). We hypothesized that different flux levels of these precursors within various clostridial
114 strains would make a big difference for the specific type(s) of ester production. Therefore, we
115 selected five strains (from four representative species) including *C. tyrobutyricum*
116 Δ*cat1::adhE1*[12], *C. tyrobutyricum* Δ*cat1::adhE2*[12], *C. pasteurianum* SD-1[13], *C.*
117 *saccharoperbutylacetonicum* N1-4-C[14] and *C. beijerinckii* NCIMB 8052[15] to evaluate their
118 capabilities for ester production through metabolic engineering (Table S1). We included both
119 *C. tyrobutyricum* Δ*cat1::adhE1* and Δ*cat1::adhE2* here because they produce different levels
120 (and thus ratios) of butanol and ethanol[12,16]. Esters can be synthesized either through
121 esterification of acid and alcohol catalyzed by lipase, or through condensation of acyl-CoA and
122 alcohol catalyzed by AATs (Fig. 1). Previously, lipase B (CALB) from *Candida antarctica* has
123 been employed for efficient ester production through esterification[9,17]. In addition, four AATs
124 including VAAT[18,19], SAAT[18], ATF1[3,10], EHT1[20] have been recruited for ester production in
125 various hosts[5,21-23]. Therefore, here, we evaluated all these genes in our clostridial hosts for
126 ester production.

127 Six plasmids (pMTL-P_{cat}-*vaat*, pMTL-P_{cat}-*saat*, pMTL-P_{cat}-*atf1*, pMTL-P_{cat}-*eht1*, pMTL-

128 P_{cat}-*lipaseB* as well as pMTL82151 as the control) were individually transformed into *C.*
129 *saccharoperbutylacetonicum* N1-4-C, *C. pasteurianum* SD-1, *C. tyrobutyricum cat1::adhE1*
130 and *cat1::adhE2* respectively. While pTJ1-P_{cat}-*vaat*, pTJ1-P_{cat}-*saat*, pTJ1-P_{cat}-*atf1*, pTJ1-P_{cat}-
131 *ehtl* and pTJ1-P_{cat}-*lipaseB* as well as pTJ1 were transformed into *C. beijerinckii* 8052.
132 Fermentations were performed (Fig. 2a), and results were shown in Fig. 2b & Table S3. Four
133 types of esters were detected: EA, BA, ethyl butyrate (EB) and BB. Interestingly, control strains
134 with the empty plasmid (pMTL82151 or pTJ1) also produced noticeable EA, BA and BB. This
135 could be because: 1) the endogenous lipase in clostridia can catalyze ester production[14]; 2) *catP*
136 on pMTL82151 encoding a chloramphenicol acetyltransferase (belonging to the same class of
137 enzymes as AATs) has AAT activities[5,24].

138 Based on the results, it could be concluded that ATF1 is more favorable for BA production.
139 All strains with *atf1* produced higher levels of BA compared to the same strain but with the
140 overexpression of other genes (Fig. 2b & Table S3). While VAAT, SAAT and EHT1 seemed to
141 have better activities for BB production. Among all the strains, *C. saccharoperbutylacetonicum*
142 FJ-004 produced the highest titer of 5.5 g/L BA. This is the highest BA production that has
143 ever been reported through microbial fermentation. *C. acetobutylicum* CaSAAT (with the
144 overexpression of *saat* from *Fragaria xananassa*) was reported to produce 8.37 mg/L BA[23].
145 While this manuscript was prepared, a newly engineered *C. diolis* strain was reported to
146 produce 1.37 g/L BA[25]. The highest BB production of 0.3 g/L was observed in *C. pasteurianum*
147 J-5 with the overexpression of *eht1*. This is also the highest BB production reported so far
148 directly from glucose with engineered microorganisms, which is significantly higher than the

149 recently reported 50.07 mg/L in an engineered *C. acetobutylicum*[23]. Couple of our engineered
150 strains could also produce small amount of EB with the highest of 0.02 g/L been observed in
151 *C. pasteurianum* J-5. With the *lipaseB* overexpression, *C. tyrobutyricum* JZ-6 could generate
152 8.4 g/L EA. This was 48.2% of the highest EA production that has ever been reported (17.35
153 g/L with an engineered *Wickerhamomyces anomalus*[26]). As we reported previously, the mother
154 strain *C. tyrobutyricum* Δ*cat1::adhE1* could produce 20.8 g/L acetate and 5.3 g/L ethanol
155 (precursors for EA synthesis) during a batch fermentation, which might have enabled high-
156 level EA production in *C. tyrobutyricum* JZ-6[12].

157 The production levels of BA and BB achieved above are both significantly higher than the
158 previously reported in microbial hosts. In comparison, the BA level is much higher than BB
159 level, and thus has greater potentials towards economic viability. Therefore, in the following
160 steps, we primarily focused on systematic metabolic engineering of the strain for further
161 enhanced BA production.

162

163 **Deletion of *nuoG* increased BA production**

164 Enhancement of the pool of precursors is one common strategy to improve the production
165 of targeted bioproduct. Butanol and acetyl-CoA are the two precursors for BA production.
166 Therefore, we set out to increase butanol synthesis to improve BA production. The *nuoG* gene
167 encodes the NADH-quinone oxidoreductase subunit G, which is a subunit of the electron
168 transport chain complex I[27]. NADH-quinone oxidoreductase can oxidize NADH to NAD^+ and
169 transfer protons from cytoplasm to periplasm to form a proton gradient between periplasm and

170 cytoplasm, which can then contribute to the energy conversion[27] (Fig. S1). It has been reported
171 that inactivation of *nuoG* could increase both glucose consumption and butanol production in
172 *C. beijerinckii*[28]. In this study, we hypothesized that by deleting *nuoG*, BA production would
173 be boosted because of the potentially increased butanol production. Thus, we deleted *nuoG*
174 (Cspa_c47560) in N1-4-C and generated FJ-100. Further, FJ-101 was constructed based on FJ-
175 100 for BA production. Results demonstrated that, although butanol production in FJ-100 was
176 only slightly improved than N1-4-C, BA production in FJ-101 was remarkably enhanced
177 compared to FJ-004 (7.8 g/L vs. 5.5 g/L) (Table S4). EA production in FJ-101 was also
178 increased to 0.9 g/L (vs. 0.5 g/L in FJ-004). We speculate that deletion of *nuoG* saved NADH,
179 which could be used for enhanced production of alcohols (precursors for BA and EA synthesis),
180 and thus increased production of BA (and EA).
181
182 **Enhancement of acetyl-CoA availability to improve BA production**
183 At the end of fermentation with FJ-101, there was still 7.6 g/L of butanol remained (Table
184 S4). This suggested that limited availability of intracellular acetyl-CoA was likely the
185 bottleneck for the further improvement of BA production. To enhance the availability of acetyl-
186 CoA, we firstly introduced isopropanol synthesis (from acetone) pathway (Fig. 3a). We
187 hypothesized that this could pull flux from acetone to isopropanol, and thus boost the
188 transferring of CoA from acetoacetyl-CoA to acetate driven by the CoA transferase, resulting
189 in increased availability of acetyl-CoA. The *sadh* gene in *C. beijerinckii* B593 encoding a
190 secondary alcohol dehydrogenase can convert acetone into isopropanol[29]. The *hydG* gene in

the same operon as *sadh* encodes a putative electron transfer protein. It has been demonstrated to play important roles for the conversion of acetone into isopropanol[30,31]. In this study, either *sadh* alone or the *sadh-hydG* gene cluster was integrated into the chromosome of FJ-100, generating FJ-200 and FJ-300, respectively. Further, by introducing pMTL-cat-*atf1*, FJ-201 and FJ301 were obtained. As shown in Fig. 3b and Table S4, compared to FJ-101, about 50% of the acetone could be converted into isopropanol in FJ-201, while ~95% of the acetone in FJ-301 could be converted into isopropanol. The total titers of acetone plus isopropanol in FJ-301 and FJ-201 were 6.6 and 5.1 g/L respectively, both of which were higher than 4.8 g/L acetone in FJ-101. More significantly, BA production in FJ-201 and FJ-301 has been remarkably increased compared to FJ-101, and reached 10.1 and 12.9 g/L, respectively, likely due to enhanced 'regeneration' of acetyl-CoA as described above.

Dynamic expression of *atf1* enhanced BA production

In our engineered strain, BA is synthesized through condensation of butanol and acetyl-CoA catalyzed by ATF1. The constitutively high expression of ATF1would not necessarily lead to the high BA production. For example, BA production in FJ-008 in which *atf1* was driven by the constitutive strong promoter P_{thl} from *C. tyrobutyricum* was actually much lower (3.5 g/L vs 5.5 g/L) than in FJ-004 in which *atf1* was expressed under the promoter P_{cat} from *C. tyrobutyricum*. We hypothesized that, in order to obtain more efficient BA production, the synthesis of ATF1 should be dynamically controlled and thus synchronous with the synthesis of precursors (butanol or acetyl-CoA). Therefore, for the next step, we attempted to evaluate

212 various native promoters for *atf1* expression, and identify the one(s) that can enable an
213 appropriately dynamic expression of ATF1 and lead to enhanced BA production.

214 Four promoters associated with the synthesis of acetyl-CoA or alcohols were selected to
215 drive the *atf1* expression, and four strains were constructed correspondingly for BA production
216 (Fig. 3c). Fermentation results were shown in Fig. 3d and Table S5. Indeed, distinct results for
217 BA production were observed in these strains. The BA level was only 1.0 and 1.1 g/L in FJ-
218 305 and FJ-302 with P_{bdh} and P_{pfl} for *atf1 expression*, respectively. P_{ald} is an important promoter
219 in *C. saccharoperbutylacetonicum*, which can sense the acidic state and switch cell metabolism
220 from acidogenesis to solventogenesis[32]. FJ-303 with the *atf1* expression driven by P_{ald}
221 produced 9.9 g/L BA, which was still 20% lower than in FJ-301. Interestingly, FJ-304 in which
222 *atf1* was expressed under P_{adh} produced 13.7 g/L BA, which was about 10.5% higher than in
223 FJ-301. Based on the results, we speculated that the promoter of *adh* (which encodes the key
224 enzyme catalyzing butanol and ethanol production) might have resulted in the more appropriate
225 dynamic expression of *atf1* in line with the flux of the precursors and thus led to enhanced BA
226 production. On the other hand, EA production in FJ-304 was only 0.8 g/L, which was lower
227 than all the other four strains (all > 1.1 g/L). Ethanol production in FJ-304 was also lower than
228 in FJ-301 (0.3 g/L vs 0.6 g/L). The enhanced BA production in FJ-304 consumed more acetyl-
229 CoA, and thus decreased production of ethanol and EA, both of which also needed acetyl-CoA
230 as the precursor.

231

232 **Rational organization of BA-synthesis enzymes to enhance BA production**

233 Rational organization of enzymes associated with the synthesis of target product is an

effective strategy to improve the bioproduction[33]. In this study, we evaluated several such approaches to increase BA production (Fig. 4).

PduA* protein, derived from *Citrobacter freundii* Pdu bacterial microcompartment could form filaments in bacteria like *E. coli*[33]. The CC-Di-A and CC-Di-B are designed parallel heterodimeric coiled coils and two proteins with each of these self-assembling tags could combine and shorten the catalytic distance. The enzymes (from the same metabolic pathway), tagged with one of the coiled coils (CC-Di-A or CC-Di-B) would attach onto the formed intracellular filaments (its PduA* was tagged by the other coiled coil); thus, the organized enzymes on the filaments would improve the catalytic efficiency of the target metabolic pathway. MinD is a membrane-associated protein and the localization of MinD is mediated by an 8-12 residue C-terminal membrane-targeting sequence. The proteins with MinD C-terminal sequence were able to be drawn to the cell membrane[33,34]. Thus, the application of MinD C-tag can facilitate the secretion of target product and enhance its production by mitigating the intracellular toxicity as well as promoting the catalyzing process (Fig. 4a).

To evaluate whether the organization of enzymes could improve BA production in our strain, three strategies were recruited: 1) assembling two of the three enzymes (enzymes associated with BA synthesis: NifJ (related to acetyl-CoA synthesis), BdhA (related to butanol synthesis) and ATF1) with the CC-Di-A and CC-Di-B tags; 2) organizing the three enzymes onto PduA* formed scaffold; or 3) introducing MinD C-tag to draw ATF1 onto the cell membrane.

Firstly, we assembled enzymes for BA synthesis by adding the CC-Di-A tag to ATF1 and the CC-Di-B tag to NifJ and BdhA (Fig. 4b). Fermentation results showed that the addition of CC-Di-A tag to the C-terminus of ATF1 in FJ-306 had significantly negative effects on BA synthesis with only 2.6 g/L BA was produced (Fig. 4e & Table S6). The assembly of ATF1

together with NifJ or BdhA had even more negative effects on BA synthesis and BA production was only 0.06 and 0.03 g/L in FJ-309 and FJ-310, respectively. Further, we organized ATF1, NifJ and BdhA onto the PduA* scaffold. PduA* was tagged with CC-Di-B, while the other three enzymes were tagged with CC-Di-A (Fig. 4c). The generated FJ-311 (harboring the scaffold and ATF1-CC-Di-A) produced 3.1 g/L BA, while FJ-312 (harboring the scaffold, ATF1-CC-Di-A and NifJ-CC-Di-A) and FJ-313 (harboring the scaffold, ATF1-CC-Di-A, NifJ-CC-Di-A and BdhA-CC-Di-A) produced slightly higher amount of BA both at 3.5 g/L. The scaffold seemed to have some positive effects on BA synthesis but didn't work as efficient as it was reported in other studies[33]. Moreover, we evaluated the effect of the introduction of MinD C-tag (to draw ATF1 onto the cell membrane) on BA production (Fig. 4d). Fermentation results showed that the corresponding FJ-308 strain could produce 16.4 g/L BA, which was 20% higher than FJ-304 (Fig. 4e & Table S6). The FJ-307 strain (with CC-Di-A-Atf1-*MinD*) could also produce higher BA of 3.9 g/L compared with FJ-306 (2.6 g/L). All these results indicated that the addition of the cell membrane associated motif to draw ATF1 onto the cell membrane could facilitate the BA excretion and mitigate the intracellular toxicity and therefore enhance BA production. However, the assembly of BA synthetic enzymes or the organization of relevant enzymes onto the scaffold would significantly decrease BA production.

Elimination of prophages increased BA (and BB) production

During our fermentations, we noticed that the ester production of the strains was not stable and could be varied from batch to batch. Our industrial collaborator also observed that *C. saccharoperbutylacetonicum* often had instable performance for ABE production in continuous fermentatio (data not shown). It has been reported that the N1-4 (HMT) strain contains a

281 temperate phage named HM T which could release from the chromosome even without
282 induction[35]. In addition, the N1-4 (HMT) strain can produce a phage-like particle clostocin O
283 with the induction of mitomycin C[36]. We hypothesized that the instability of fermentations with
284 *C. saccharoperbutylacetonicum* might be related to the prophages, and the deletion of
285 prophages would enable more stable and enhanced production of desired endproducts. We
286 identified five prophage-like genomes (referred here as P1-P5 respectively) within the
287 chromosome of N1-4 (HMT) (Fig. 5a)[37]. Based on systematic evaluation through individual
288 and combinatory deletion of the prophages, we demonstrated that P5 is responsible for the
289 clostocin O synthesis (Figs. 5f, h & S4) [36], and further confirmed that P1 was HM T phage
290 genome (Figs. 5g & S6). However, the phage image was different from what was described
291 before[38]. It was more like HM 7 (a head with a long tail), rather than HM 1 (a head with multiple
292 short tails). This is the first time that an image of the HM T phage has been reported. Both
293 ΔP1234 (with the deletion of P1-P4) and ΔP12345 (with the deletion of P1-P5) exhibited
294 improved cell growth and enhanced butanol production (Figs. 5b-5e, Figs. S2, S7 & S8).
295 ΔP12345 should be a more stable platform as there is not cell lysis at any conditions of
296 mitomycin C and norfloxacin induction (Fig. 5i). While ΔP1234 showed similar growth and
297 even slightly higher butanol production compared to ΔP12345 (Figs. 5d & 5e).

298 Thus, in a further step, we used ΔP1234 and ΔP12345 as the platform to be engineered for
299 enhanced and more stable BA production. We deleted *nouG* and integrated *sadh-hydG* cluster
300 in both ΔP1234 and ΔP12345, and obtained FJ-1200 and FJ-1300 correspondingly. The
301 plasmid pMTL-P_{adh}-*atf1-MinD* was transformed into FJ-1200 and FJ-1300, generating FJ-1201

302 and FJ-1301, respectively. Fermentation results showed that FJ-1201 and FJ-1301 produced
303 19.7 g/L and 19.4 g/L BA, respectively, which were both higher than FJ-308 (Fig. 6a & Table
304 S7). BA production in both FJ-1201 and FJ-1301 could be completed within 48 h, resulting in
305 a productivity of ~0.41 g/L/h, which was significantly higher than 0.23 g/L/h in FJ-308. BA
306 yield in FJ-1201 reached 0.26 g/g, which was also higher than FJ-308 (0.24 g/g). We further
307 determined BA concentrations in the fermentation broth as 0.6 g/L and 0.5 g/L respectively for
308 the fermentation with FJ-1201 and FJ-1301. Taken together, total BA production in FJ-1201
309 was 20.3 g/L, which was the highest level that has ever been reported in a microbial host. It is
310 2400-fold higher than the highest level that has been previously reported[23], and also 14.8-fold
311 higher than that by the very recently reported *C. diolis* strain[25].

312 Besides BA production, BB production in FJ-1201 also reached 0.9 g/L, which was
313 significantly higher than in FJ-308 (0.01 g/L) and in *C. pasteurianum* J-5 (0.3 g/L) (Fig. 2b,
314 Fig. 6a, & Table S7). This level (0.9 g/L) was 18.6-fold higher than the highest BB production
315 that has been previously reported (0.05 g/L in *C. acetobutylicum*)[23]. In addition, EA production
316 in FJ-1201 increased to 0.8 g/L as compared to 0.4 g/L in FJ-308. All these results confirmed
317 our hypothesis that the elimination of prophages would make more robust host strain for
318 enhanced and more stable ester production.

319

320 **Expression of SAAT in FJ-1200 further enhanced BB production**

321 As demonstrated above, SAAT and EHT1 were more relevant for BB production (Fig. 2b
322 & Table S3). Therefore, to achieve higher BB production, we expressed *saat* and *eht1* in FJ-

1200 and obtained FJ-1202 and FJ-1203, respectively. Fermentation showed that FJ-1202 and FJ-1203 produced 1.3 and 0.2 g/L BB, respectively (Fig.6d & Table S8). Further, we added MinD C-tag to the SAAT and introduced the recombinant gene into FJ-1200 and obtained FJ-1204 for an attempt to further improve BB production as observed for BA production in FJ-308. However, BB production in FJ-1204 was only 1.0 g/L. Notwithstanding, 1.3 g/L BB obtained in FJ-1202 is 25.8-fold higher than the highest level that has been previously reported[23].

Esters production with biomass hydrolysates as substrate

Fermentations were carried out using biomass hydrolysates as the substrate. In the hydrolysates, besides sugars (57.4 g/L glucose and 27.2 g/L xylose) as carbon source, there were also nutrients converted from biomass (corn stover). Therefore, we tested the effect of organic nitrogen (yeast and tryptone) of various levels on ester production. Interestingly, results showed that the highest BA production of 17.5 g/L was achieved in FJ-1201 (in the extractant phase) without any exogenous nitrogen source supplemented (Table S9). In addition, 0.3 g/L BA was detected in aqueous phase, making total BA production 17.8 g/L in FJ-1201 (Fig. 6a & Table S9). Although this was slightly lower than when glucose was used as substrate (20.3 g/L), fermentation with hydrolysates did not need any supplementation of nutrients, which could significantly reduce production cost. We further performed fermentation in 500-mL bioreactor with pH controlled >5.0. BA production reached 16.0 g/L with hydrolysates and 18.0 g/L with glucose as substrate, both of which were lower than results from fermentation

under the same conditions but with serum bottles (Figs. 6b & 6c). During bioreactor fermentation, we noticed very strong smell of BA around the reactor. We suspected that significant evaporation during fermentation with bioreactor resulted in the lower level of final BA titer as compared to fermentation with serum bottle, which was securely sealed with only minimum outlet for releasing gases (Fig. 2a). An improved bioprocess needs to be carefully designed for larger scale fermentation to minimize BA evaporation and enhance BA production and recovery.

Furthermore, we performed fermentation with FJ-1202 for BB production using hydrolysates in both serum bottle and 500-mL bioreactor. Results demonstrated that BB production in serum bottle from hydrolysates was 0.9 g/L (compared to 1.3 g/L when glucose used as substrate; Fig. 6d). BB production in bioreactor from hydrolysates reached 0.9 g/L compared to 1.6 g/L when glucose was used as substrate (Figs. 6e & 6f). The results were consistent with the case for BA production that lower-level BB was obtained when hydrolysates (compared to glucose) was used as substrate. However, interestingly, larger scale fermentation with bioreactor produced slightly higher-level BB than the fermentation under the same conditions with serum bottle, which was different from the case for BA production. This might be because BB is less evaporative (in bioreactor) than BA.

Discussion

Although tremendous efforts have been invested on biofuel/biochemical research worldwide, very limited success has been achieved. A key bottleneck is that the microbial host

is subject to endproduct toxicity and thus desirable production efficiency cannot be obtained[39]. Our central hypothesis was that metabolically engineering of microorganisms for high-value and easy-recoverable bioproduct production can help alleviate endproduct toxicity and thus high titer and productivity can be achieved, with which economically viable biofuel/biochemical production can be ultimately established. Engineering solventogenic clostridia for ester (high-value and easy-recoverable) production was the example we implemented here to test this hypothesis. Our group have previously established versatile genome engineering tools for clostridia[12,15,40], putting us at a strong position to perform this study.

Based on the systematic screening of host strains and enzymes as well as multiple rounds of rational metabolic engineering: enriching precursors (alcohols and acetyl-CoA) for ester production, dynamically expressing heterologous ester-production pathway, rationally organizing ester-synthesis enzymes, and improving strain robustness by eliminating putative prophages, we ultimately obtained strains for efficient production of esters in both synthetic fermentation medium and biomass hydrolysates. To the best of our knowledge, the production levels of BA and BB we achieved set up the new records. Overall, we demonstrated that clostridia are excellent platforms for valuable biofuel and biochemical production. The general principle that we demonstrated here is highly applicable to other relevant bioprocesses, and may result in breakthroughs in biofuel/biochemical production.

Methods

386 **Microorganisms and cultivation conditions**

387 All the strains and plasmids used in this study are listed in Table S1. *C. pasteurianum* ATCC
388 6013 and *C. saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923) were requested from
389 American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen
390 und Zellkulturen (DSMZ), respectively. *C. beijerinckii* NCIMB 8052 was requested from Dr.
391 Hans P. Blaschek[15]. *C. tyrobutyricum* Δ*cat1*::*adhE1* and *C. tyrobutyricum* Δ*cat1*::*adhE2* are
392 hyper-butanol producing mutants constructed in our lab[12]. All the clostridial strains were grown
393 in an anaerobic chamber (N_2-CO_2-H_2 with a volume ratio of 85:10:5) at 35 ºC. Strains of *C.*
394 *tyrobutyricum*, *C. saccharoperbutylacetonicum* and *C. beijerinckii* were cultivated using
395 tryptone-glucose-yeast extract (TGY) medium[41], while strains of *C. pasteurianum* were
396 cultivated using 2×YTG medium[42]. When required, clarithromycin (Cla) or thiamphenicol
397 (Tm) was supplemented into the medium at a final concentration of 30 μg/mL and 15 μg/mL,
398 respectively. *E. coli* DH5α was used for routine plasmid propagation and maintenance. *E. coli*
399 CA434 was used as the donor strain for plasmid conjugation for *C. tyrobutyricum*. Strains of
400 *E. coli* were grown aerobically at 37 ºC in Luria-Bertani (LB) medium supplemented with 100
401 μg/mL of ampicillin (Amp), 50 μg/mL kanamycin (Kan) or 34 μg/mL chloramphenical (Cm)
402 as needed.

403

404 **Plasmid construction**

405 All the plasmids used in this study are listed in Table S1, and all the primers used in this
406 study are listed in Table S2.

407 The plasmids pMTL82151 and pTJ1 were used as mother vectors for heterogeneous gene
408 expression[43,44]. The promoter of the *cat1* gene (CTK_C06520) (P_{cat}) and the promoter of the
409 *thl* gene (CTK_C01450) (P_{thl}) from *C. tyrobutyricum* ATCC 25755 were amplified and inserted
410 into pMTL82151 at the *Eco*RI site, and the generated plasmids were named as pMTL82151-
411 P_{cat} and pMTL82151-P_{thl}, respectively. Promoters of the following gene, *pflA* (Cspa_c13710)
412 (P_{pfl}), *ald* (Cspa_c56880) (P_{ald}), *adh* (Cspa_c04380) (P_{adh}) and *bdh* (Cspa_c56790) (P_{bdh}), all
413 from *C. saccharoperbutylacetonicum* N1-4 (HMT) were amplified and inserted into
414 pMTL82151 at the *Eco*RI site, and the generated plasmids were named as pMTL82151-P_{pfl},
415 pMTL82151-P_{ald}, pMTL82151-P_{adh} and pMTL82151-P_{bdh}, respectively.
416 The *vaat* gene from *Fragaria vesca*, the *saat* gene from *F. ananassa* and the *atf1* gene from
417 *S. cerevisiae* were amplified from plasmids pDL006, pDL001 and pDL004, respectively[3,22].
418 The *atf1'*(the codon optimized *atf1* gene), *eht1* from *S. cerevisiae*[20], and *lipaseB* from *Candida*
419 *antarctica*[45] were all synthesized by GenScript (Piscataway, NJ, USA). The obtained gene
420 fragments of *vaat*, *saat*, *atf1*, *atf1', eth1*, and *lipaseB* were inserted between the *Btg*ZI and
421 *Eco*RI sites in pMTL82151-P_{cat}, generating pMTL-P_{cat}-*vaat*, pMTL-P_{cat}-*saat*, pMTL-P_{cat}-*atf1*,
422 pMTL-P_{cat}-*atf1'*, pMTL-P_{cat}-*eht1*, and pMTL-P_{cat}-*lipaseB*, respectively. The *atf1* gene was
423 inserted between the *Btg*ZI and *Eco*RI sites in pMTL82151-P_{thl}, generating pMTL-P_{thl}-*atf1*.
424 The P_{cat} promoter and the gene fragments of *vaat*, *saat*, *atf1*, *eth1*, and *lipase* were amplified
425 and ligated into the *Eco*RI site of pTJ1, generating pTJ1-P_{cat}-*vaat*, pTJ1-P_{cat}-*saat*, pTJ1-P_{cat}-
426 *atf1*, pTJ1-P_{cat}-*eht1* and pTJ1-P_{cat}-*lipaseB*, respectively. The *atf1* gene was inserted into the
427 *Eco*RI site of pMTL82151-P_{pfl}, pMTL82151-P_{ald}, pMTL82151-P_{adh} and pMTL82151-P_{bdh},

428 generating pMTL-P_{pfl}-*atf1*, pMTL-P_{ald}-*atf1*, pMTL-P_{adh}-*atf1* and pMTL-P_{bdh}-*aft1*, respectively.

429 DNA sequences of *CC-Di-A*, *CC-Di-B*, *MinD* and *pduA** were synthesized by GenScript

430 (Piscataway, NJ, USA). The MinD-tag was fused to the end of *atf1* by PCR and ligated into the

431 *Eco*RI site of pMTL-P_{adh}, generating pMTL-P_{adh}-*atf1-MinD*. In addition, the MinD-tag was

432 fused to the end of *saat* by PCR and inserted between the *Btg*ZI and *Eco*RI sites of pMTL-P_{cat},

433 generating pMTL-P_{cat}-*saat-MinD*.

434 The synthesized *CC-Di-A* fragment was ligated into the *Eco*RI site of pMTL-P_{adh},

435 generating pMTL-P_{adh}-CC-Di-A. The DNA fragments of *atf1* and *atf1-MinD* were amplified

436 from pMTL-P_{adh}-*atf1* and pMTL-P_{adh}-*atf1-MinD* and then inserted into the *Eco*RI site of

437 pMTL-P_{adh}-CC-Di-A, obtaining pMTL-P_{adh}-A-*atf1* and pMTL-P_{adh}-A-*atf1-MinD*. The *CC-Di-*

438 *B* sequence with the *nifJ* gene and *CC-Di-B* with the *bdhA* gene were subsequently inserted

439 into the *Kpn*I site of pMTL-P_{adh}-A-*atf1*, generating pMTL-P_{adh}-A-*atf1*-B-*nifJ* and pMTL-P_{adh}-

440 A-*atf1*-B-*nifJ*-B-*bdhA*. The DNA fragments of *CC-Di-B-pduA**, *CC-Di-A-nifJ*, *CC-Di-A-bdhA*

441 were inserted into the *Eco*RI site of pTJ1-P_{cat}, generating pTJ1-P_{cat}-B-*pduA**, pTJ1-P_{cat}-B-

442 *pduA**-A-*nifJ* and pTJ1-P_{cat}-B-*pduA**-A-*nifJ*-A-*bdhA*, respectively.

443 For the gene deletion or integration in *C. saccharoperbutylacetonicum*, all the relevant

444 plasmids were constructed based on pYW34, which carries the customized CRISPR-Cas9

445 system for genome editing in *C. saccharoperbutylacetonicum*[15,40]. The promoter P_{J23119} and the

446 gRNA (with 20-nt guide sequence targeting on the specific gene) were amplified by two rounds

447 of PCR with primers N-20nt/YW1342 and YW1339/YW1342 as described previously (N

448 represents the targeted gene)[40]. The obtained fragment was then inserted into pYW34 (digested

with *Btg*ZI and *Not*I) through Gibson Assembly, generating the intermediate vectors. For gene deletion, the fragment containing the two corresponding homology arms (~500-bp for each) for deleting the specific gene through homologous recombination was amplified and inserted into the *Not*I site of the obtained intermediate vector as described above, generating pYW34-ΔN (N represents the targeted gene). For gene integration, the fragment containing the two corresponding homology arms (~1000-bp for each), the promoter and the gene fragment to be integrated, was amplified and inserted into the *Not*I site of the obtained intermediate vector as described above, generating the final plasmid for gene integration purpose.

Fermentation with glucose as the substrate

For the fermentation for ester production, the *C. pasteurianum* strain was cultivated in Biebl medium[46] with 50 g/L glycerol as the carbon source at 35 °C in the anaerobic chamber. When the OD_{600} reached ~0.8, the seed culture was inoculated at a ratio of 10% into 100 mL of the same medium in a 250-mL serum bottle and then cultivated at an agitation of 150 rpm and 30 °C (on a shaker incubator) for 72 h. The *C. beijerinckii* strain was cultivated in TGY medium until the OD_{600} reached ~0.8. Then the seed culture was inoculated at a ratio of 5% into 100 mL P2 medium along with 60 g/L glucose and 1 g/L yeast extract in a 250 mL serum bottle. The fermentation was carried out at an agitation of 150 rpm and 37 °C for 72 h[41]. The *C. tyrobutyricum* strain was cultivated in RCM medium at 35 °C until the OD_{600} reached ~1.5. Then the seed culture was inoculated at a ratio of 5% into 200 mL fermentation medium (containing: 50 g/L glucose, 5 g/L yeast extract, 5 g/L tryptone, 3 g/L $(NH_4)_2SO_4$, 1.5 g/L

K_2HPO_4, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 0.03 g/L $FeSO_4 \cdot 7H_2O$, and 1 g/L L-cysteine) in a 500-mL bioreactor (GS-MFC, Shanghai Gu Xin biological technology Co., Shanghai, China) and the fermentation was carried out at an agitation of 150 rpm and 37 °C for 120 h with pH controlled >6.0[9]. The *C. saccharoperbutylacetonicum* strain was cultivated in TGY medium at 35 °C in the anaerobic chamber until the OD_{600} reached ~0.8. Then the seed culture was inoculated at a ratio of 5% into 100 mL P2 medium along with 60 g/L glucose and 1 g/L yeast extract in a 250 mL serum bottle. The fermentation was carried out at an agitation of 150 rpm and 30 °C for 120 h[41]. For the fermentation at larger scales in bioreactors, it was carried out in a 500-mL fermenter (GS-MFC, Shanghai Gu Xin biological technology Co., Shanghai, China) with a working volume of 250 mL with pH controlled >5.0, at 50 rpm and 30 °C for 120 h. Samples were taken every 24 h for analysis.

For all fermentations in the serum bottle, a needle and hosepipe were connected to the top of bottle for releasing the gases produced during the fermentation (Fig. 2a). For all the fermentations for ester production, the extractant *n*-hexadecane was added into the fermentation with a ratio of 1:1 (volume of the extractant vs. volume of fermentation broth) for *in situ* ester extraction. The reported ester concentrations were the determined values in the extractant phase.

Fermentation with biomass hydrolysates as the substrate

The biomass hydrolysates was kindly provided by Dr. Daniel Schell from National Renewable Energy Laboratory (NREL) which was generated from corn stover through the innovative

'deacetylation and mechanical refining in a disc refiner (DDR)' approach[47]. For the fermentation, the biomass hydrolysate was diluted and supplemented into the P2 medium as the carbon source (with final sugar concentrations of 57.4 g/L glucose and 27.2 g/L xylose). In addition, various concentrations of yeast extract (Y, g/L) and tryptone (T, g/L) were also added as the nitrogen source to evaluate their effects on the fermentation performance: 0Y+0T; 1Y+3T and 2Y+6T. The fermentation was carried out under the same conditions as described above at 100 mL working volume in a 250-mL serum bottle.

Analytical methods

Concentrations of acetone, ethanol, butanol, acetic acid, butyric acid and glucose were measured using a high-performance liquid chromatography (HPLC, Agilent Technologies 1260 Infinity series, Santa Clara, CA) with a refractive index Detector (RID), equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min at 25 °C. The concentration of the ester in the *n*-hexadecane phase was quantified using a gas chromatography-mass spectrometry (GC-MS, Agilent Technologies 6890N, Santa Clara, CA) equipped with an HP-5 column (60m×0.25 mm, 0.25 mm film thickness). Helium was used as the carrier gas. The initial temperature of the oven was set at 30 °C for 2 min, followed by a ramp of 10 °C/min to reach 300 °C, and a ramp of 2 °C/min to reach the final temperature of 320 °C, which was then held for 2 min. The detector was kept at 225 °C[9].

Authors' contributions

J.F., Y.W. and I.B. designed the experiments. J.F., J.Z. (Jie Zhang), P.W., Y.G., and M.C. performed the experiments. J.F. and Y.W. drafted the manuscript. P.J.B., Y.G. and J.Z. (Junping Zhou) contributed to improve the figures. M.C. and Z.S. contributed to the manuscript revision. All authors read and approved the manuscript.

Data availability

The materials and data reported in this study are available upon reasonable request from the corresponding author.

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Competing interests

Auburn University has filed a patent application covering the work described in this article*. The application names Y.W. and J.F. as inventors.

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Supplementary information

Supplementary Information

Supplementary Information includes partial of the Methods, Results, Discussion and Supplementary Figs. 1–8 and Tables 1–11.

Figure Captions

Fig. 1 Engineering of solventogenic clostridia for fatty acid ester production. Top: Five strains out of four representative clostridial species were selected and evaluated as the host to be engineered for ester production in this study. We hypothesized that the different metabolic fluxes within different strains would make a big difference for the desirable ester production. The metabolic pathways of the four different species were represented in four different colors. **Bottom:** Fatty ester esters could be synthesized through two primary biological pathways: one is through the esterification of fatty acid and alcohol catalyzed by lipases, and the other is through the condensation of acyl-CoA and alcohol catalyzed by alcohol acyl transferases (AATs). Key genes in the pathway: *pta*, phosphotransacetylase; *ack*, acetate kinase; *thl*, thiolase; *hbd*, beta-hydroxybutyryl-CoA dehydrogenase; *crt*, crotonase; *bcd*, butyryl-CoA dehydrogenase; *adh*, alcohol dehydrogenase; *adhE*, Aldehyde-alcohol dehydrogenase; *adc*, acetoacetate decarboxylase; *ctfAB*, CoA transferase; *ptb*, phosphotransbutyrylase; *buk*, butyrate kinase; *ald*, aldehyde dehydrogenase.

Fig. 2 Screening strains and enzymes for ester synthesis. (a) Schematic representation of devices and procedures for ester fermentation with serum bottle. (b) Heatmap results showed the ester production in various *Clostridium* strains with the overexpression of different enzymes. Empty plasmid: overexpression of pMTL82151 (for *C. saccharoperbutylacetonicum* N1-4-C, *C. pasteurianum* SD-1, *C. tyrobutyricum cat1::adhE1* and *cat1::adhE2*) or pTJ1 (for *C. beijerinckii* 8052) as the control; EA: ethyl acetate; EB: ethyl butyrate; BA: butyl acetate;

BB: butyl butyrate. Scale bar on the right is in g/L.

Fig. 3 Enhancing BA production through increasing the availability of acetyl-CoA (a & b) and dynamically expressing the *atf1* gene (c & d). (a) Introduction of isopropanol synthesis pathway (in red) and deletion of thiolase genes. The pathways in purple represent the 'regeneration' of actyl-CoA. There are five annotated genes encoding thiolase in *C. saccharoperbutylacetonicum*, only two (in green) of which could be deleted (see Supplementary materials). (b) The fermentation results for BA production with various mutant strains corresponding to the genetic manipulations in (a). The reported value is mean ± SD. (c) Four promoters associated with the biosynthesis of acetyl-CoA or alcohols in the pathway were selected to drive the expression of *atf1*. (d) The fermentation results for BA production with various mutant strains in which different promoters were used to drive the expression of *atf1* as illustrated in (c). The reported value is mean ± SD. Key genes in the pathway: *adh*, alcohol dehydrogenase; *bdh*, butanol dehydrogenase; *ctfAB*, CoA transferase; *sadh*, secondary alcohol dehydrogenase; *hydG*, putative electron transfer protein; *pfl*, pyruvate formate lyase; *ald*, aldehyde dehydrogenase.

Fig. 4 Enhancing BA production through rational organization of the enzymes associated with BA synthesis. (a) Biological components evaluated in this study for rational organization of enzymes: (I) CC-Di-A and CC-Di-B tags, (II) PduA* scaffold, (III) MinD C-tag. (b) Schematic representation of assembling two of the three enzymes for BA synthesis with the

CC-Di-A and CC-Di-B tags. (c) Schematic representation of organizing the three enzymes for BA synthesis onto the PduA* formed scaffold. (d) Schematic representation of using the MinD C-tag to draw ATF1 onto the cell membrane. (e) The fermentation results for BA production with various mutant strains corresponding to the genetic manipulations from (b)-(d). The reported value is mean ± SD.

Fig. 5 The deletion of the prophage genomes. (a) The gene cluster organization of the HM T prophage (P1) and another four putative prophages (P2-P5). (b) Comparison of the cell growth of prophage deleted mutants and the control N1-4-C strain. (c) Comparison of the butanol production in prophage deleted mutants and the control N1-4-C strain. (d) Comparison of the cell growth between ΔP1234 and ΔP12345. (e) Comparison of butanol production between ΔP1234 and ΔP12345; (f) Transmission election microscopy image of clostocin O; (g) Transmission election microscopy image of the HM T prophage particles; (h, i) Cell growth profiles of ΔP1234 and ΔP12345 with the induction (at various OD_{600}) using mitomycin C at 2 or 4 μg/ml. "-" indicates that there was no cell lysis; "+" indicates that most of the cells were lysed. The value at the right side of the cell growth profile figure represents the actual OD_{600} value at which mitomycin C (with the applied concentration included in the parentheses) was added for the induction.

Fig. 6 Fermentation results of the engineered strains for fatty acid ester production. (a) BA production in serum bottles using glucose (FJ-1201 and FJ-1301) or biomass hydrolysates

(FJ-1201(H)) as the substrate. (b) BA fermentation kinetics in FJ-1201 in 500-mL bioreactor using glucose as the substrate. (c) BA fermentation kinetics in FJ-1201 in 500-mL bioreactor using biomass hydrolysates as the substrate. (d) BB production in serum bottles using glucose (FJ-1202, FJ-1203, and FJ-1204) or biomass hydrolysates (FJ-1202(H)) as the substrate. (e) BB fermentation kinetics in FJ-1202 in 500-mL bioreactor using glucose as the substrate. (f) BB fermentation kinetics in FJ-1202 in 500-mL bioreactor using biomass hydrolysates as the substrate.

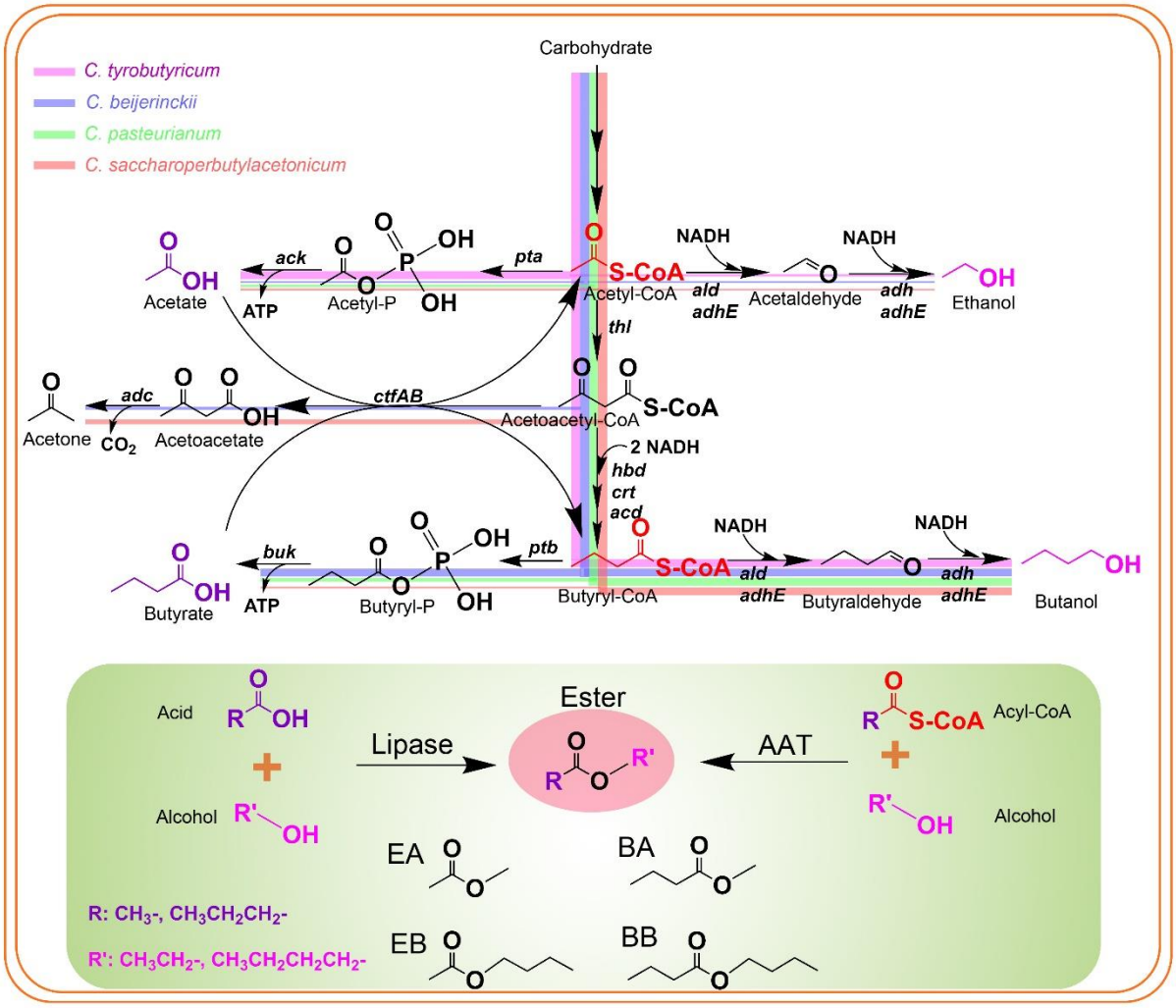

C. tyrobutyricum
C. beijerinckii
C. pasteurianum
C. saccharoperbutylacetonicum
Carbohydrate
Acetate
ack
ATP
Acetyl-P
pta
Acetyl-CoA
S-CoA
NADH
ald
adhE
Acetaldehyde
NADH
adh
adhE
Ethanol
thl
Acetone
adc
CO2
Acetoacetate
ctfAB
Acetoacetyl-CoA
S-CoA
2 NADH
hbd
crt
acd
Butyrate
buk
ATP
Butyryl-P
ptb
Butyryl-CoA
S-CoA
NADH
ald
adhE
Butyraldehyde
NADH
adh
adhE
Butanol
Acid
Alcohol
Lipase
Ester
AAT
Acyl-CoA
Alcohol
EA
BA
EB
BB
R: CH3-, CH3CH2CH2-
R': CH3CH2-, CH3CH2CH2CH2-

Fig. 1

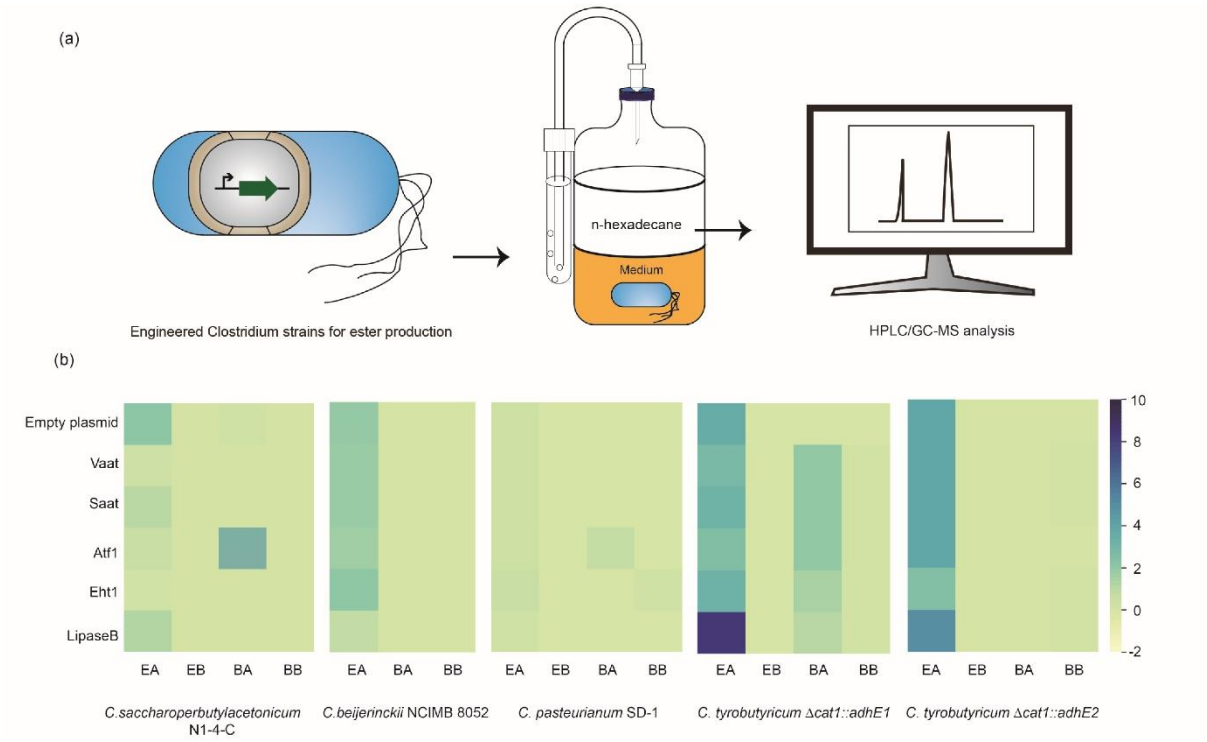

(a)
n-hexadecane
Medium
Engineered Clostridium strains for ester production
HPLC/GC-MS analysis
(b)
Empty plasmid
Vaat
Saat
Atf1
Eht1
LipaseB
EA EB BA BB
C.saccharoperbutylacetonicum N1-4-C
C.beijerinckii NCIMB 8052
C. pasteurianum SD-1
C. tyrobutyricum Δcat1::adhE1
C. tyrobutyricum Δcat1::adhE2
10
8
6
4
2
0
-2

Fig. 2

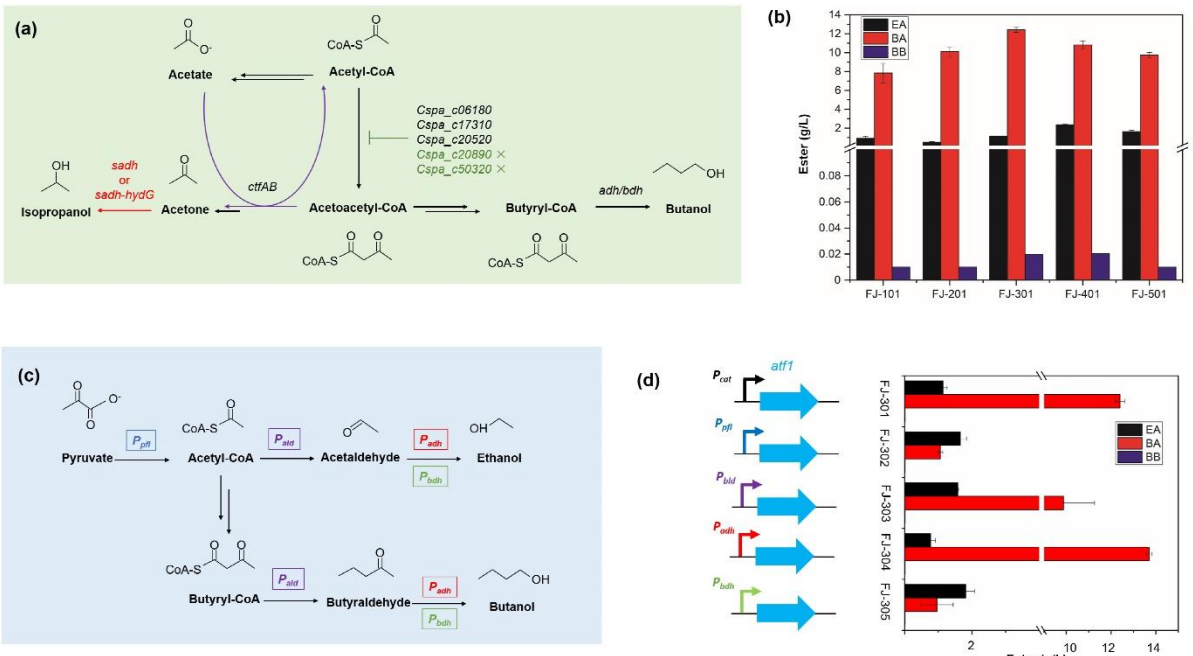

(a)
Acetate
Acetyl-CoA
Cspa_c06180
Cspa_c17310
Cspa_c20520
Cspa_c20890 ×
Cspa_c50320 ×
sadh
or
sadh-hydG
ctfAB
adh/bdh
Isopropanol
Acetone
Acetoacetyl-CoA
Butyryl-CoA
Butanol
(b)
EA
BA
BB
Ester (g/L)
FJ-101
FJ-201
FJ-301
FJ-401
FJ-501
(c)
Pyruvate
Acetyl-CoA
Acetaldehyde
Ethanol
Butyryl-CoA
Butyraldehyde
Butanol
(d)
atf1
FJ-301
FJ-302
FJ-303
FJ-304
FJ-305
Ester (g/L)

Fig. 3

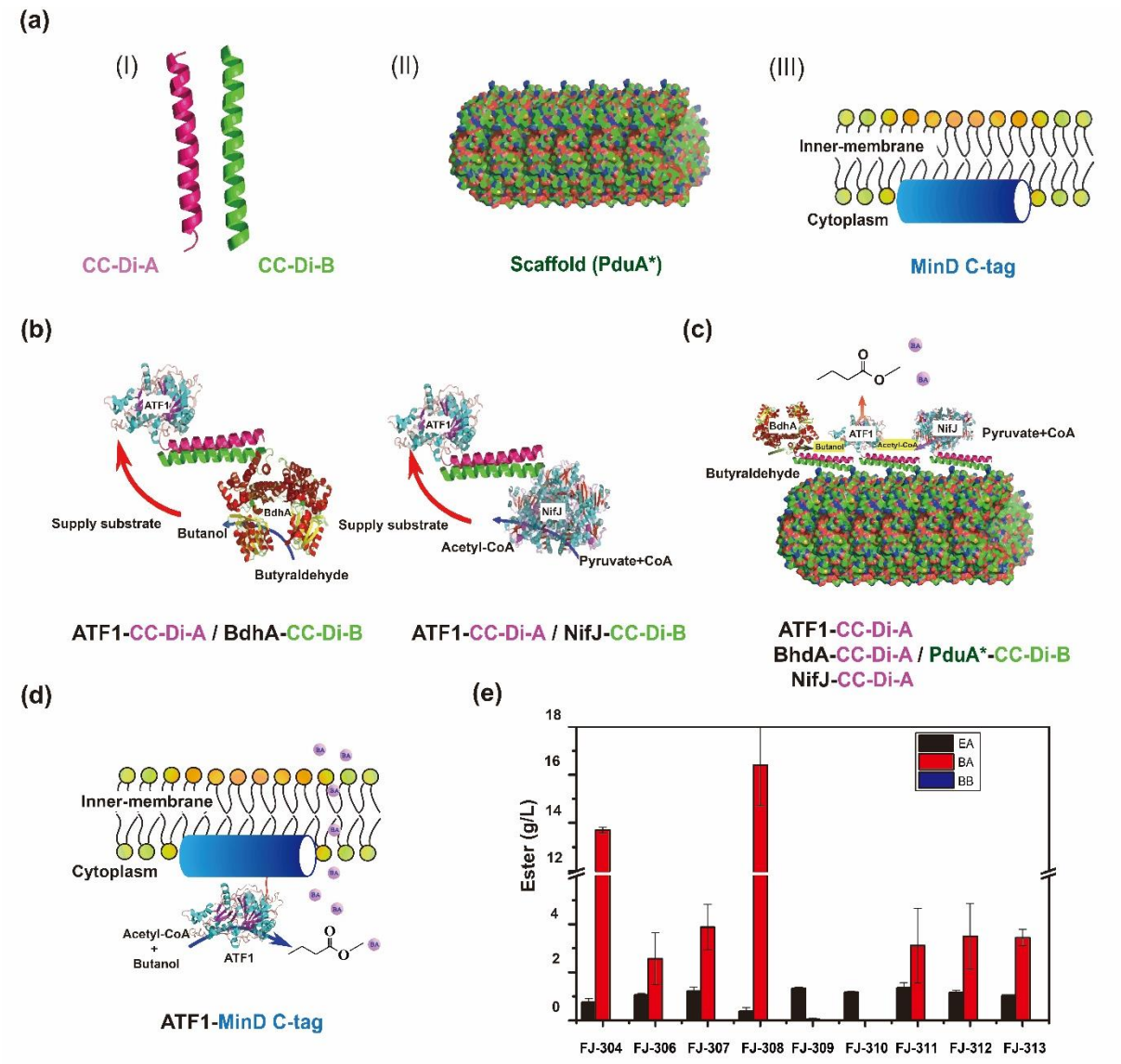
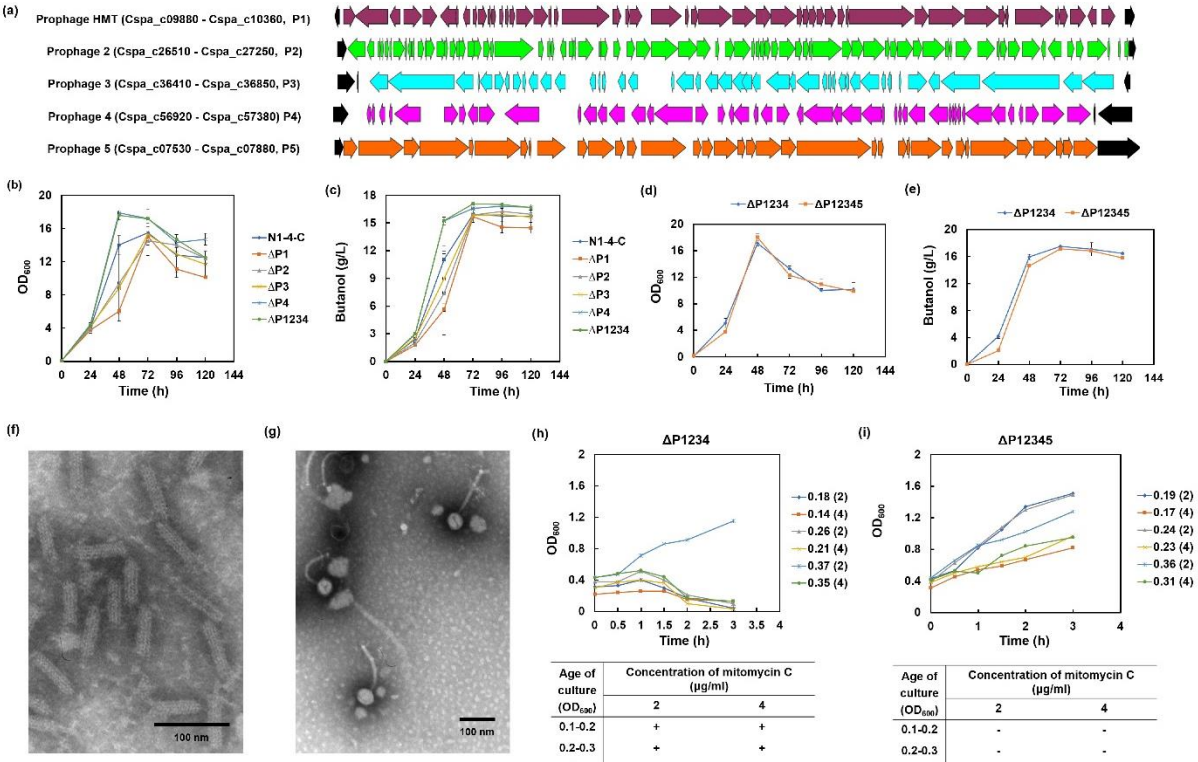

(a)
(I)
(II)
(III)
CC-Di-A
CC-Di-B
Scaffold (PduA*)
Inner-membrane
Cytoplasm
MinD C-tag
(b)
ATF1
BdhA
Supply substrate
Butanol
Butyraldehyde
NifJ
Acetyl-CoA
Pyruvate+CoA
ATF1-CC-Di-A / BdhA-CC-Di-B
ATF1-CC-Di-A / NifJ-CC-Di-B
(c)
Pyruvate+CoA
Butyraldehyde
ATF1-CC-Di-A
BhdA-CC-Di-A / PduA*-CC-Di-B
NifJ-CC-Di-A
(d)
Inner-membrane
Cytoplasm
Acetyl-CoA
+
Butanol
ATF1
ATF1-MinD C-tag
(e)
Ester (g/L)
EA
BA
BB
FJ-304
FJ-306
FJ-307
FJ-308
FJ-309
FJ-310
FJ-311
FJ-312
FJ-313

Fig. 4



Age of culture (OD_{600})	Concentration of mitomycin C (μg/ml)	
	2	4
0.1-0.2	+	+
0.2-0.3	+	+
0.3-0.4	-	+

Age of culture (OD_{600})	Concentration of mitomycin C (μg/ml)	
	2	4
0.1-0.2	-	-
0.2-0.3	-	-
0.3-0.4	-	-

Fig. 5

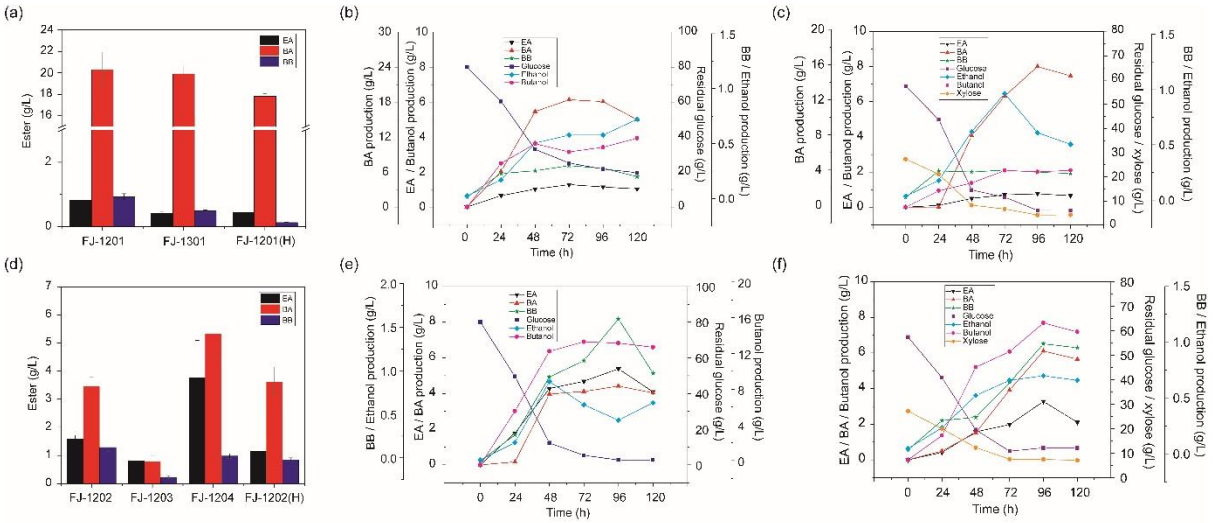

(a)
Ester (g/L)
EA
BA
BB
FJ-1201
FJ-1301
FJ-1201(H)
(b)
BA production (g/L)
EA / Butanol production (g/L)
Residual glucose (g/L)
BB / Ethanol production (g/L)
Time (h)
(c)
BA production (g/L)
EA / Butanol production (g/L)
Residual glucose / xylose (g/L)
BB / Ethanol production (g/L)
Time (h)
(d)
Ester (g/L)
FJ-1202
FJ-1203
FJ-1204
FJ-1202(H)
(e)
BB / Ethanol production (g/L)
EA / BA production (g/L)
Residual glucose (g/L)
Butanol production (g/L)
Time (h)
(f)
EA / BA / Butanol production (g/L)
Residual glucose / xylose (g/L)
BB / Ethanol production (g/L)
Time (h)

Fig. 6