1 **Metabolic pairing of aerobic and anaerobic production in a**

2 **one-pot batch cultivation**

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Abstract

Background

The versatility of microbial metabolic pathways enables their utilization in vast number of applications. However, the electron and carbon recovery rates, essentially constrained by limitations of cell energetics, are often too low in terms of process feasibility. Cocultivation of divergent microbial species in a single process broadens the metabolic landscape and thus, the possibilities for more complete carbon and energy utilization.

Results

In this study, we integrated the metabolisms of two bacteria, an obligate anaerobe *Clostridium butyricum* and an obligate aerobe *Acinetobacter baylyi* ADP1. In the process, a glucose-negative mutant of *A. baylyi* ADP1 first deoxidized the culture allowing *C. butyricum* to grow and produce hydrogen from glucose. In the next phase, ADP1 produced long chain alkyl esters utilizing the by-products of *C. butyricum*, namely acetate and butyrate.

Conclusions

The cocultivation of strictly anaerobic and aerobic bacteria allowed the production of both hydrogen gas and long-chain alkyl esters in a simple one-pot batch process. The study demonstrates the potential of 'metabolic pairing' using designed microbial consortia for optimal electron and carbon recovery.

Keywords

36   Synthetic microbial consortia – metabolic pairing – integrated metabolism –

37   hydrogen production – wax esters

Introduction

39   Almost all known biochemical reactions can be found in microbes, making them the

40   most metabolically diverse class of organisms in the world. The natural versatility of

41   microbes, and on the other hand, the development of cell engineering technologies

42   have enabled their utilization in a great number of applications ranging from

43   bioremediation of xenobiotics to the production of complex drug precursors [1-3].

44   Despite the advancements in cell-based processes enabled by metabolic engineering

45   and synthetic biology, the laws of thermodynamics limit the number and efficiency

46   of different metabolic pathways in a single organism. For example, the anaerobic

47   fermentation of sugars to short-chain alcohols or organic acids is energetically

48   favourable and redox balanced for cells, which makes the process scale-up rather

49   straight forward and economically feasible. As a trade-off, however, in in these

50   catabolic "downhill" pathways the substrate is converted to a product with lower

51   energy and carbon content, causing substantial but inevitable loss of carbon. In

52   contrast, the synthesis of many industrially relevant products with long carbon chain,

53   such as alkanes, fatty acids, and alkyl esters, is energetically expensive for cells, and

54   thus harnessing such thermodynamically "uphill" synthesis pathways for a profitable

55   process is challenging. Recent advances in exploiting microbial electrosynthesis

56   (MES) hold great potential for the energy recovery by external electron transfer, but

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57   further efforts are required to overcome the challenges related to the limitations of

58   metabolic capabilities and systematic strain engineering for greater efficiency [4-5].

59   A potential strategy for combining a broader range of metabolic attributes is to

60   utilize two or more different species or strains in a single process. In recent years,

61   growing attention has been given to rationally designed microbial consortia

62   addressing the issues related to the constraints of a single cell, such as limited

63   substrate range or biosynthetic efficiency. For example, Hanly and Henson (2013)

64   modelled substrate conversion by simultaneous utilization of hexoses and pentoses

65   in a cocultivation by a respiratory deficient *Saccharomyces cerevisiae* and

66   *Scheffersomyces stipitis* [6]. In another example, Minty et al. (2013) demonstrated

67   the production of isobutanol directly from lignocellulose hydrolysate by a coculture

68   of a fungi *Trichoderma reesei* and a bacterium *Escherichia coli*. In the culture, *T.*

69   *reesei* secreted cellulases to create solubilized sugars, which were utilized by *E. coli*

70   and further converted to isobutanol [7]. Park et al. (2012) also used cellulase-

71   excreting fungi in a coculture. In their study, *Acremonium cellulolyticus* was used in a

72   one-pot approach together with *Saccharomyces cerevisiae* to produce ethanol [8].

73   Similarly, Wang et al. (2015) used a bacterium *Clostridium celevecrescens* together

74   with *Clostridium acetobutylicum* ATCC824 to produce butanol [9].Zhang et al. (2015),

75   on the other hand, reported a coculture system, which involved a partial distribution

76   of the metabolic pathway for cis-cis-muconic acid production in two engineered *E.*

77   *coli* strains [10]. As a result of efficient intermediate redirection and alleviated

78   burden to cells, the production of cis-cis-muconic acid was significantly improved.

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79   Other typical issues related to single-cell cultures include excessive byproduct

80   formation and poor tolerance against certain feedstock components and/or the

81   (by)product itself. Organic acids, such as acetate, impose a great challenge in

82   bioprocesses both in terms of toxicity to cells and as a drain for carbon and energy

83   [8]. We have previously demonstrated the benefits of acetate redirection to biomass

84   and product in a consortium of *E. coli* and *Acinetobacter baylyi* ADP1 [12]. In the

85   culture, engineered *A. baylyi* ADP1 consumed the acetate produced by *E. coli*, which

86   improved the biomass production and recombinant protein expression compared to

87   single-cell cultures. More recently, we have successfully combined MES with long-

88   chain ester synthesis by sequentially culturing an acetogenic strain *Sporumusa ovata*

89   and *A. baylyi* ADP1 [13]. In the separated two-stage process, the acetogenic strain

90   utilized carbon dioxide and electricity to produce acetate, which was subsequently

91   fed to ADP1 to produce long-chain alkyl esters. In a somewhat reverse culture set-

92   up, we established a coculture of *A. baylyi* and *Clostridium butyricum* for the

93   production of hydrogen (H_2) gas from simulated and rice straw hydrolysates [14].

94   The glucose-negative *A. baylyi* selectively removed the *C. butyricum* growth

95   inhibitors, namely acetate, formate, 4-hydroxybenzoate, and oxygen from culture,

96   allowing the subsequent growth and H_2 production by *C. butyricum* from hydrolysate

97   sugars.

98   In the present study, we demonstrate the successful integration of anaerobic

99   fermentation (representing the 'downhill' pathway) to aerobic synthesis (the 'uphill'

100   pathway). In the process of two metabolically diverse bacterial species, aerobic and

101   anaerobic phases alternate allowing a complete conversion of carbon and electrons

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102   to biomass and products in a non-optimized simple batch culture. As an ultimate

103   example of a 'carbon wasting' downhill process, glucose is fermented to produce H_2

104   by the obligate anaerobe *C. butyricum*. The byproducts, namely acetate and

105   butyrate, together with naturally occurring oxygen, are converted to highly energy

106   and carbon rich alkyl esters with an average of 34-carbon chains by the strict aerobe

107   *A. baylyi* ADP1. This study demonstrates the potential of combining distinctive

108   bacterial metabolisms for optimal electron and carbon recovery.

109

Results

111   In order to allow efficient distribution and comprehensive consumption of carbon in

112   a cocultivation of *C. butyricum* and *A. baylyi*, a previously engineered knock-out

113   strain of *A. baylyi* deficient in glucose consumption was deployed [14]. The strain *A.*

114   *baylyi* ADP1 Δgcd, designated here as ADP1-g, lacks the gene for glucose

115   dehydrogenase, which is responsible for the first reaction in the modified Entner–

116   Doudoroff pathway of *A. baylyi* sugar catabolism [15]. We hypothesized that in the

117   cocultivation *C. butyricum* consumes the glucose and produces H_2, followed by the

118   production of long chain alkyl esters by ADP1-g from the liquid end-metabolites

119   (acetate and butyrate) of *C. butyricum* (Fig. 1). In the first stage of the process, the

120   growth medium is deoxidized by the metabolic activity of ADP1-g strain, allowing the

121   growth and H_2 production of the obligate aerobe *C. butyricum*. After C. butyricum

122   growth, oxygen is allowed to enter the system, enabling ADP1-g growth and wax

123   ester production from the residual carbon, namely acetate and butyrate.

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Suitability of ADP1-g for the alternating bioprocess conditions

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125   We investigated the capability of the obligate aerobe ADP1-g to survive and

126   subsequently grow in a cultivation with varying oxygen availability. In this

127   experiment, ADP1-g was cultivated with limited oxygen supply in sealed vessels. The

128   cells grew for 30 hours, after which the growth ceased due to oxygen depletion (Fig.

129   2). These anoxic growth conditions were maintained for another 24 hours and then

130   oxygen was released to the vessels. Shortly after, an exponential increase in cell

131   density was observed demonstrating that ADP1-g maintains cell viability after

132   exposure to oxygen-deprived conditions for 24 hours.

133   Another growth test was conducted with ADP1-g to investigate the utilization of

134   acetate and butyrate, which are potential end-products of *C. butyricum* in the

135   cocultivation system. The strain consumed both carbon sources (initial 15 mM

136   butyrate and 25 mM acetate) within 21 hours (Fig 3a.). In addition, wax esters were

137   produced and accumulated up to 18 hours of cultivation (Fig 3b). Thereafter, the

138   amount of WEs decreased due to the carbon depletion, which is a typical

139   phenomenon in carbon limiting conditions [16].

Cocultivation of ADP1-g and *C. butyricum* in batch

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141   In the next stage, ADP1-g and *C. butyricum* were cocultivated in a 300 ml batch

142   culture. In this one-pot approach, aerobic and anaerobic phases alternate allowing

143   both bacteria to grow. Anoxic conditions were produced and maintained by the

144   metabolic activity of the aerobic ADP1-g. The metabolite formation by *C. butyricum*,

145   as well as the growth and end-products of both *C. butyricum* and ADP1-g, were

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146   determined. Twenty millimolar of glucose was used as carbon source for *C.*

147   *Butyricum* growth based on preliminary tests conducted with different substrate

148   concentrations (Additional file 1: Table S1). Ten millimolar acetate supplementation

149   was used for initial growth and deoxygenation by ADP1-g according to ADP1-g

150   acetate utilization trend (Table 1).

151   During the anaerobic phase, *C. butyricum* produced 1.6±0.1 mol H_2/mol glucose with

152   a cumulative H_2 production of 131.5±5.2 ml. A drop in pH associated with *C.*

153   *butyricum* growth occurred (pH 5) and most of the supplemented glucose was

154   consumed (Fig. 4 a-b). Additionally, 11.4±0.3 mM of butyrate and 7.7±0.1 mM of

155   acetate were produced from the *C. butyricum* fermentation (Fig. 4b).

156   After the *C. butyricum* fermentation, oxygen was released to the system enabling

157   ADP1-g growth. Wax esters, the end products of ADP1, accumulated during 24-30 h

158   of cultivations (Fig. 5). During this time, acetate was completely consumed along

159   with most of the butyrate (Fig. 4b). The WEs were solely produced by ADP1-g, as *C.*

160   *butyricum* does not produce WEs (Additional file 2: Fig. S2).

161   **Cocultivation of ADP1-g and *C. butyricum* in a 1-liter bioreactor**

162   For a more detailed analysis of the coculture, a cultivation experiment was

163   conducted in a 1L bioreactor. Similarly to the previous batch cultivation, ADP1-g and

164   *C. butyricum* were simultaneously inoculated in the culture and the cultivation vessel

165   was sealed. Oxygen partial pressure, pH, substrate/metabolite concentrations, and

166   cell growth were monitored during the cultivation. The culture was deoxygenated by

167   ADP1-g within 5 minutes after the inoculation (Fig. 6a) and anoxic conditions were

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168 maintained for the next 24 hours. During this anaerobic phase, *C. butyricum*

169 consumed most of the supplemented glucose (83%) producing 4.8 mM of acetate,

170 8.3 mM of butyrate and 461 ml of H_2 (cumulative). The carbon and electrons from

171 the supplemented glucose were completely recovered in the metabolites and end

172 products of *C. butyricum* (Additional file 3: Table S2). Within 3 hours of the latter

173 aerobic phase, an increase in cell dry weight was observed as ADP1-g strain started

174 to grow. During this aerobic stage, the strain recovered the carbon from *C. butyricum*

175 metabolites for cell growth and storage compounds, namely wax esters (Figures 6a

176 and 6b, Table 2). Acetate was consumed rapidly in 4.5 hours whereas butyrate was

177 consumed more gradually (Fig. 6b). The highest wax ester yield (30 mg/g biomass)

178 was achieved after 8 hours of aerobic cultivation (32 hours of overall cultivation)

179 with 100% of acetate and 61 % of butyrate consumed. The total yields of products

180 recovered from the batch cocultivation were 1.7 mol H_2 /mol glucose$_{consumed}$ and

181 10.8 mg WE/g glucose$_{consumed}$, respectively.

Discussion

183 Efficient recovery of carbon and electrons in the final product is crucial in biological

184 production processes. For cells, however, the optimal balance between carbon

185 utilization and redox state often requires the by-production of unwanted metabolites.

186 Moreover, the product range may be constrained depending on the metabolic

187 capabilities of the cell. To address these issues, we designed and constructed a

188 'metabolic pair' of anaerobic and aerobic metabolisms enabling both fermentative

189 hydrogen production and aerobic lipid synthesis with complete carbon and energy

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190 recovery in a simple one-pot batch cultivation. In the coculture set-up, a strict

191 anaerobe *C. butyricum* was deployed for the production of H_2 gas using glucose as the

192 source of carbon and energy, whereas the strictly aerobic ADP1-g was utilized for

193 maintaining oxygen-free conditions during the anaerobic fermentation, followed by

194 the recovery of any residual carbon in the culture for the aerobic production of wax

195 esters. In the batch process carried out in "one-pot", aerobic and anaerobic phases

196 alternate allowing toggle-like growth of the bacteria.

197 For the coculture system to be functional, the aerobic strain is required to both survive

198 and sustain anaerobic conditions during the H_2 production phase. Previously, ADP1-g

199 has been shown in a proof of concept experiment to be able to deoxygenize growth

200 media for *C. butyricum* [14], albeit the viability of the strain to survive anoxic

201 conditions was not investigated. Thus, in our first experiment, the ability of ADP1-g to

202 restore growth after anoxic phase was studied. According to the growth data, we

203 found out that the strain was able to continue growth after being incubated in oxygen-

204 limited conditions for 24 hours. In the subsequent experiment, we also confirmed that

205 the strain is able to uphold sufficient anaerobic conditions during the oxygen-sensitive

206 *C. butyricum* growth and H_2 production phase. Examples of other approaches for

207 employing oxygen-consuming bacteria as deoxygenizing agents have been previously

208 reported. For example, cocultures for maintaining anoxic conditions (in a culture

209 initially made anaerobic) have been made with E*nterococcus* [17]. Other cocultivation

210 approaches have included systems to remove oxygen for *C. butylicum* growth with the

211 help of *Bacillus subtilis* in order to increase butanol yields [18] [19]. Also, examples of

212 deoxygenation of hydrogen producing cultures involving *C. butyricum* have been

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213   previously reported [17]. However, as the aerobic species do not contribute to product

214   formation in these processes, the substrate utilized for their metabolism is "wasted"

215   and lowers the yield of the overall process. In contrast, in our system, ADP1-g not only

216   consumes the oxygen, but also scavenges and upgrades the otherwise wasted organic

217   acids produced by *C. butyricum*. Notably, ADP1-g does not consume glucose, and

218   thereby does not impair the efficiency of H_2 production from glucose.

219   The incapability of ADP1-g to utilize glucose has been previously demonstrated [14].

220   Wax ester production by *A. baylyi* ADP1 using acetate as a carbon source has been

221   also previously reported [20] whereas WE production from for butyrate has not been

222   recorded to our knowledge. In our study, ADP1-g was shown to grow and produce

223   alkyl esters (WEs) from both acetate and butyrate, which makes it ideal for upgrading

224   the metabolites of *C. butyricum*. It is also notable that the strain was able to continue

225   growth and establish wax ester production directly in the medium 'used' and occupied

226   by *C. butyricum*.

227   *C. butyricum* ferments glucose and other carbohydrates readily to H_2 and volatile fatty

228   acids (VFA), such as acetate and butyrate [21] [22]. In theory, the complete oxidation

229   of glucose yields 12 mol of H_2, though in reality, thermodynamic and metabolic

230   restrictions in biological systems divert the substrate also towards biomass and other

231   metabolites [23]. Thus, the theoretical yields of the NADH dependent H_2 production

232   by *Clostridium* vary between $2 - 4$ mol-H_2/mol-glucose depending on the ratio of

233   produced metabolites butyrate and acetate. As the latter is associated with higher H_2

234   yields - and *butyricum* typically favors butyrate production -the actual yields obtained

235   are closer to the lower range of the theoretical values. In our experiments, the

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236   cocultivation yielded 1.7 ± 0.1 mol H_2/mol glucose$_{consumed}$ with an acetate to butyrate

237   ratio of 0.6:1. As comparison, experiments with pure cultures of *C. butyricum* in

238   optimized production conditions have yielded results such as 2.29 mol H_2/mol glucose

239   [22] and 2.21 mol H_2/mol glucose [21] whereas coculture designs have yielded values

240   such as 1.65 mol H_2/mol glucose [24]. The metabolites (acetate and butyrate)

241   produced in our experiments account up to 49,5% of the carbon and 59.1% of the

242   electrons from the initial glucose supplementation, which are an untapped resource

243   when using pure cultures. In our study, these compounds were scavenged in the

244   second aerobic stage of the one-pot system providing additional value for the process

245   in the form of 10.8 mg WE / g glucose$_{consumed}$.

246   Co-culture systems allow the design of production systems with metabolic capabilities

247   exceeding those of any individual organism. Zhou et al [25] produced oxygenated

248   taxanes by dividing the complex pathway between *E. coli* and *S. cerevisiae*. *E. coli*

249   produced taxadiene, which was oxygenated by *S. cerevisiae*, resulting in the

250   production of complex oxygenated taxane molecules that have not been produced by

251   any single microbe. In another example, Liu et al [26] used a consortium consisting of

252   three different microbes for the division of labor in order to enhance the electricity

253   production from glucose. In the consortium, *E. coli* first converted glucose to lactate,

254   which was oxidized by *Shewanella oneidensis* to acetate. The acetate was further

255   utilized by *E. coli*. *B. subtilis*, in turn, produced riboflavin, which is required as an

256   electron shuttle. These studies exemplify how a single biosynthetic pathway can be

257   divided between different organisms in order to improve the production. In the

258   present study, we combine two very different metabolisms (aerobic and anaerobic,

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259   catabolic and anabolic) that complement each other for the efficient recovery of

260   electrons (H_2 and WE) and carbon (WE).

261   The relevance of the current platform could be further improved by engineering the

262   production efficiency and/or product range. *A. baylyi* ADP1 can be readily engineered

263   for improved production of native or non-native products [20, 27-29], whereas limited

264   number of studies describing successful engineering of *C. butyricum* has been

265   reported. Thus, one of the advantages of metabolically diverse cocultures is the

266   modularity and flexibility of the system; substrate and product range can be

267   individually engineered and tuned for applicable strain(s). Such division of engineering

268   load lowers the metabolic burden in one strain, and on the other hand, reduces the

269   challenges faced with strains for which engineering tools and methods are not readily

270   available.

Conclusions

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272   Cocultivation of organisms with divergent metabolic characteristics can greatly

273   broaden the substrate and product range and facilitates more comprehensive

274   utilization of carbon and energy. We successfully integrated bacterial anaerobic and

275   aerobic metabolisms in a synthetic consortium, and by exploiting the advantageous

276   properties of both metabolisms, demonstrated the production of hydrogen gas and

277   long-chain alkyl esters in a single process. The study demonstrates the power of

278   metabolic pairing in resolving metabolic and thermodynamic limitations of single

279   organisms as well as in developing novel metabolic combinations for more efficient

280   carbon and energy recovery.

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Methods

Strains and cultivation medium

Acinetobacter baylyi ADP1 (DSM 24193) devoid of glucose dehydrogenase gene

ACIAD2983, (referred here as ADP1-g) and *Clostridium butyricum*, isolated from a

hydrogen-producing bioreactor, were used in the study [30]. Unless otherwise

indicated, ADP1-g cells cultivated in low-salt Lysogeny broth (LB) (tryptone, 10

g/liter; yeast extract, 5 g/liter; sodium chloride, 1 g/liter) at 30°C and 300rpm, were

used as pre-inoculums. The growth kinetics of ADP1-g in a three stage bioprocess

was studied in in MA/9 medium (g/L; 5.518 Na2HPO$_4$·2H$_2$O, 3.402 KH$_2$PO$_4$, 1 NH$_4$Cl,

0.008 nitrilotriacetic acid, 0.487 FeCl$_3$, 0.25 MgSO$_4$·7H$_2$O, 0.02 CaCl$_2$·2H$_2$O, 0.2%

casein amino acids and 2ml/L of SL7 trace element solution). ADP1-g acetate and

butyrate utilization experiments were conducted in a minimal medium [24] (referred

as JM medium) with modifications as described in [14]. *C. butyricum* precultivations

were conducted in anaerobic Reinforced Clostridial Medium (RCM, Sigma Aldrich,

US) at 37°C and 150rpm. The co-cultivation experiments, using ADP1-g and C.

butyricum, were conducted in JM medium and the cultures were grown at 30°C and

300rpm.

Experimental procedure

The growth test to investigate ADP1-g viability in the three stage aerobic-anaerobic-

aerobic bioprocess was conducted in tubes containing 10 ml of sterile aerobic MA/9

medium supplemented with casein amino acids. Upon inoculating (initial optical

density 0.02), the cultivation tubes were closed with sterile septum rubber stoppers,

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303   tightened with aluminum crimps and incubated at 30°C and 300rpm. A blank

304   cultivation (devoid of inoculant) was included as control to determine 'false positive'

305   results from contamination and as analytical control. The experiment was conducted

306   in triplicates and the cell growth data measured at specific intervals using a

307   spectrophotometer (Ultrospec 500 pro, Amersham Biosciences, UK) for 120 hours,

308   were averaged.

309   Batch experiments to investigate ADP1-g capacity to utilize organic acids from *C.*

310   *butyricum* fermentation was performed in 120 ml serum bottles with a working

311   volume of 50 ml sterile JM medium. Acetate and butyrate were supplemented at

312   concentrations 20 mM and 15 mM, respectively. The inoculated cells (initial cell

313   density 0.02) were cultured aerobically at 30°C and 300rpm. The cells were also

314   inoculated to a substrate blank, i.e. cultivation medium devoid of acetate and

315   butyrate. Samples to analyze cell growth, organic acid utilization, lipid analysis and

316   medium pH were collected in 3-hour interval for 24 hours.

317   The co-cultivations of ADP1-g and *C. butyricum* were conducted in triplicate 500ml

318   batch bottles containing 300ml of sterile aerobic JM medium. To initiate ADP1-g and

319   *C. butyricum* growth, the medium was supplemented with 10 mM acetate and 20

320   mM glucose, respectively. Two milliliters of precultivated ADP1-g with an optical

321   density at wavelength 600nm (OD$_{600nm}$) of 3.2 was inoculated alone and co-

322   inoculated with 2 ml of *C. butyricum* precultivation (OD$_{600nm}$ 3.1). ADP1-g cultivated

323   in similar medium and a blank medium were used as the controls in this experiment.

324   Following inoculation, the batch bottles were capped (rubber stoppers), tightened

325   (aluminum crimps). Initial samples to determine cell growth, substrate utilization,

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326   liquid metabolites and lipids were collected and the cultures were incubated at 30°C

327   and 200rpm. The gaseous end products were analyzed from the bottle headspace

328   after a 24 hour cultivation period and the rubber stoppers were removed. Opening

329   the bottle caps ensured the end of anaerobic phase and culture samples for analysis

330   were collected. The medium pH, after the anaerobic phase, from the coculture,

331   ADP1-g alone and blank cultivations were measured aseptically and adjusted to pH

332   7.4 (initial pH) with 5 M sterile sodium hydroxide solution. The cultures were divided

333   to sterile 50 ml serum bottles and incubated under aerobic conditions at 30°C and

334   200rpm. The experiment was conducted in triplicates and samples were collected

335   every 3 hours during 20-hour cultivation for analysis.

336   Bioreactor experiment was conducted in a sterile 1-liter vessel (Sartorius Biostat B

337   plus Twin System, Germany) with an online pH monitoring system. To a medium

338   volume of 795 ml, 50ml of ADP1-g precultivated in JM medium supplemented with

339   25 mM acetate (OD$_{600nm}$ 3.9) and 5 ml of *C. butyricum* precultivated in RCM (OD$_{600nm}$

340   2.3) were inoculated, totaling the cultivation volume to 850 ml. To prevent gas

341   leakage, the fermentor outlets were closed and a gas collection bag (Supelco, USA)

342   was connected to the exhaust. After initial sample collection, the cultures were

343   grown at 30°C and 350 rpm for 24 hours. At the end of anaerobic phase, the

344   collection bag was removed, samples were collected for analysis and the medium pH

345   was adjusted 7.3 with sterile 5 M NaOH solution. The aerobic phase was initiated

346   with air supply. The medium pH and partial O2 (pO2) profiles were obtained from

347   the bioreactor. From the bioreactor, 45 ml of culture was removed every hour from

348   which duplicate technical repeats to monitor cell growth and organic acid utilization

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349   were collected and the remaining culture (~40ml) was used for quantitative lipid

350   analysis.

Analytical techniques

352   For qualitative lipid analysis, 4 ml of culture samples were analyzed using thin layer

353   chromatography as described in [16]. For dry cell weight (DCW) calculations in

354   bioreactor experiment, 40 ml of original cultures were pelleted in pre-weighed tubes

355   at 30,000 g (4°C), freeze-dried and weighed. The tube weights were subtracted from

356   the freeze-dried cell weights to determine the DCW (g/L). The lipids extracted from

357   the freeze-dried cells were quantitatively analyzed using NMR as described in [28].

358   Glucose, acetate and butyrate concentrations were analyzed, in triplicates, using

359   High-Performance Liquid Chromatography (HPLC) (LC-20AD, Shimadzu, Japan)

360   equipped with equipped with Shodex SUGAR (SH1011) column (300 × 8 mm),

361   refractive index detector (RID, RID-10A) and 0.01N H_2SO_4 as mobile phase. One

362   milliliter of culture samples were centrifuged at 15,000 g for 7 minutes (4°C), filtered

363   through 0.2μm polycarbonate filter (Chromafil® PET-45/25, Macherey-Nagel,

364   Germany) and diluted tenfold with ultrapure water in 1ml HPLC vials. The injection

365   volume, column temperature and mobile phase flowrate was set to 100μl, 40°C and

366   0.6 ml/min, respectively. Identification and quantification of carbon substrate and

367   liquid fermentation metabolites were based on chromatography using external

368   standards.

369   A gas chromatograph (GC-2014, Shimadzu GC) fitted with a thermal conductivity

370   detector, PORAPAK column (2 m × 2 mm) and N2 as carrier gas was used to analyze

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371   the gaseous end products as described in [31]. In brief, to the GC maintained with

372   column, oven and detector temperatures of 80°C, 80°C and 110°C, respectively,

373   200μl of sample from the collection bag was injected to the sampling port. The

374   overpressure, i.e. gas volume exceeding the headspace volume of the vessel, was

375   analyzed using water displacement method. Hydrogen yields (mol H_2 / mol

376   glucose$_{consumed}$) were calculated by converting the GC data (ml) and overpressure

377   values (ml) to millimolar (mM) values using the ideal gas law equation (for room

378   temperature) and dividing it with the product of cultivation volume (L) and utilized

379   sugar (mM). For batch experiments, each experimental repeats were measured

380   twice and in bioreactor experiment, triplicate technical repeats were included.

381   Carbon balances of *C. butyricum* gaseous and liquid metabolites and utilized

382   substrate were calculated by multiplying the concentrations with the respective

383   number of carbon in the molecular formula. The CO_2 in the liquid phase was ignored

384   during the carbon balance calculations. Electron mass balances were calculated by

385   multiplying the carbon mass of utilized glucose and each metabolite with the

386   corresponding degree of reduction (mol electrons per C-mol). For carbon and

387   electron mass calculations, the chemical formula of *C. butyricum* biomass was

388   assumed to be $CH_{1.624}O_{0.456}N_{0.216}P_{0.033}S_{0.0047}$ [32]. The biomass concentrations (mM)

389   after anaerobic cultivation was calculated by dividing the DCW with the total mass

390   from the formula (g/mol). Both carbon and electron recoveries were calculated by

391   determining the percentage of sum total of carbon and electron mass of liquid and

392   gaseous metabolites divided with the respective masses calculated for the utilized

393   sugar.

Declarations

Authors' contributions

RM and SS designed the system and planned the experimental setup. MS, TL, and RM executed the experimental work. EE and TL carried out the lipid analytics. MS, TL, SS and RM interpreted the data and wrote the manuscript. RM supervised the work. All authors read and approved the final manuscript.

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Availability of data and materials

All the data analysed in this study are included in this manuscript and its additional files.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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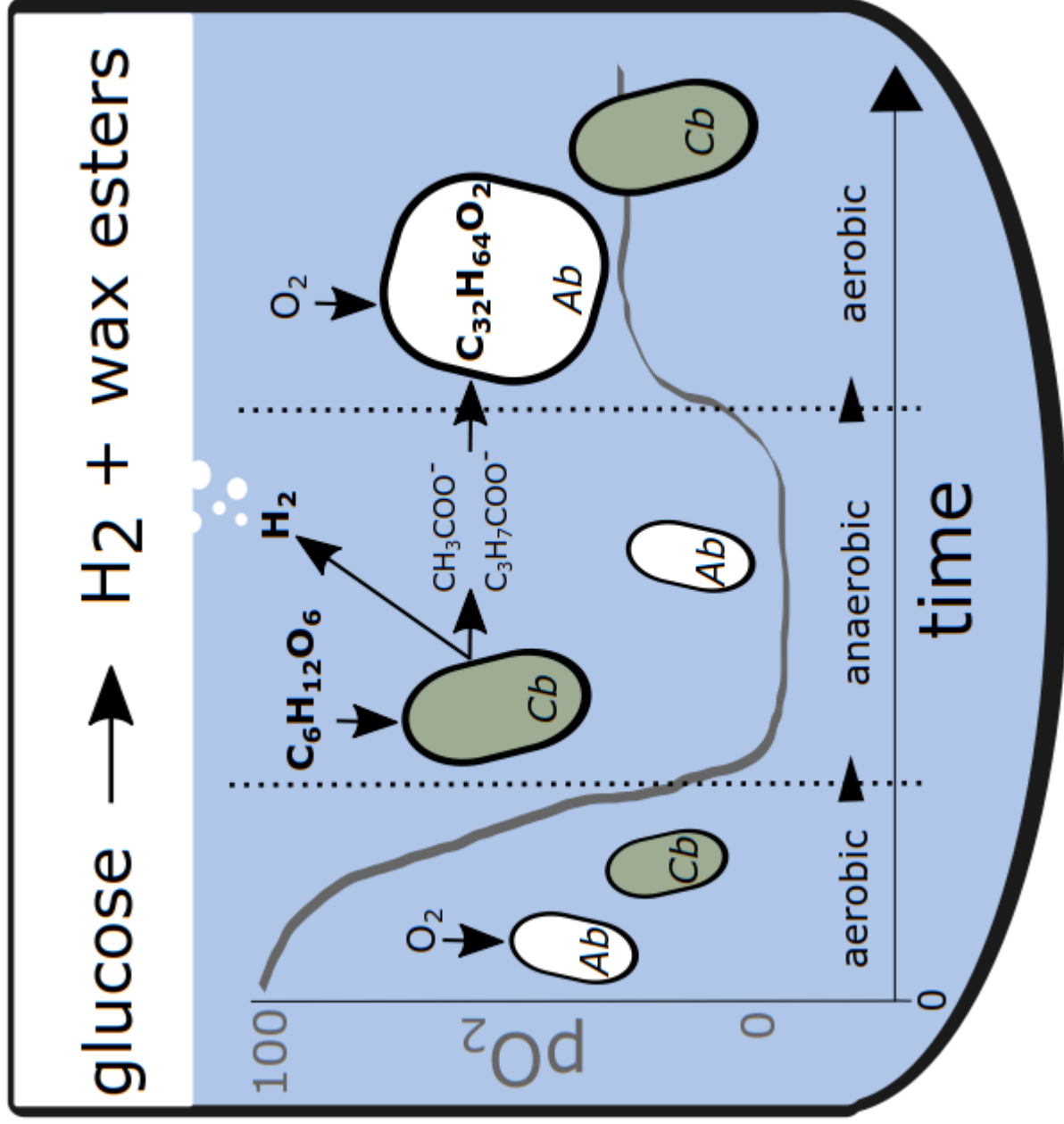
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Figure 1 – Schematic representation of the one-pot batch cultivation of *C. butyricum* and ADP1-g strain.

In the first stage, the metabolic activity of ADP1-g deoxidizes the culture, allowing H_2 production from glucose by *C. butyricum*. Thereafter, oxygen is released to the culture, enabling wax ester production from the residual carbon, namely acetate and butyrate, by ADP1-g.
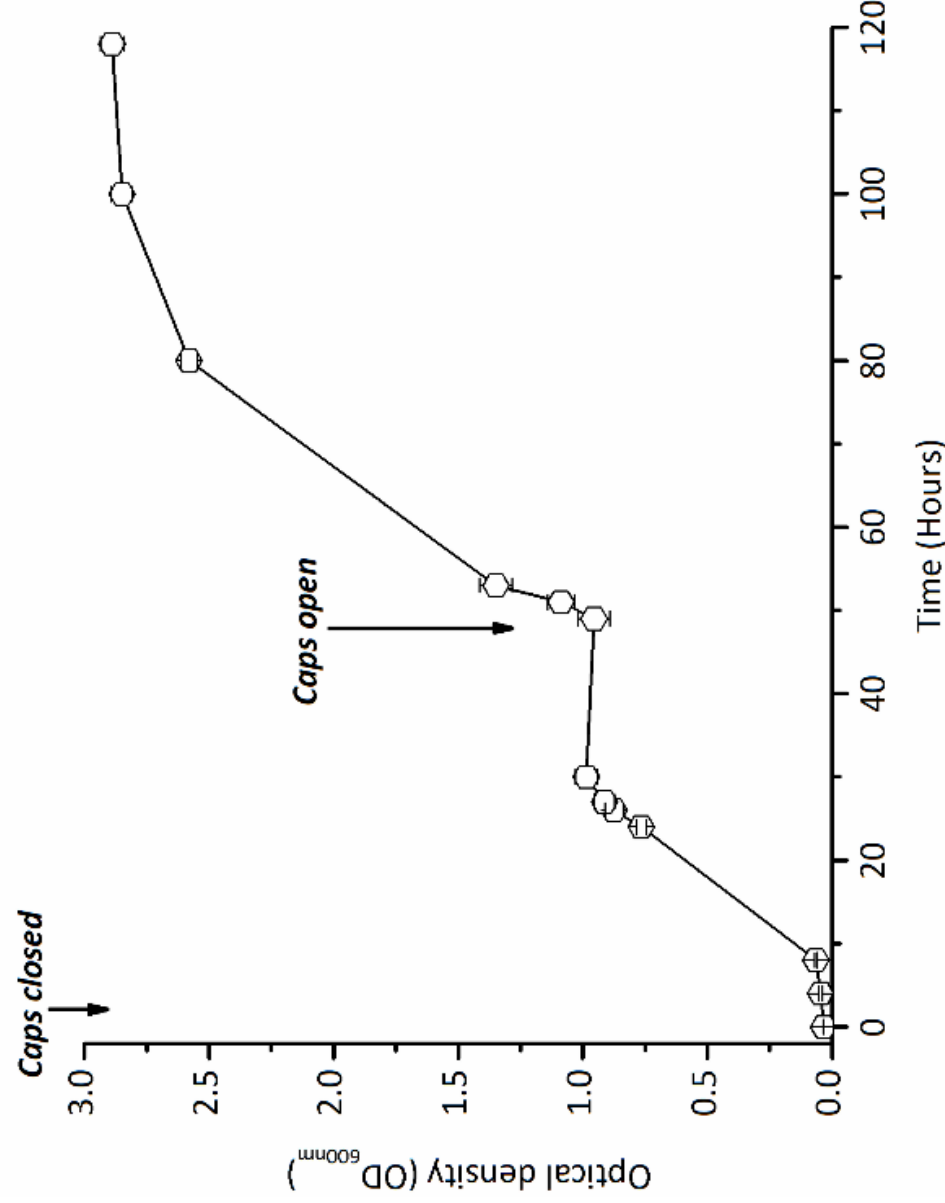
Figure 2 – ADP1-g growth during alternating oxygen availability.

The strain ADP1-g ability to withstand anoxic environment was conducted in 10 ml sterile aerobic MA/9 medium amended with 0.2% casein amino acids. Upon inoculation, the cultivation tubes were closed until growth cessation (30 hours) and opened at 50 hour time-point to resume the growth. The data points and error bars represent mean value and standard deviation from triplicate experimental repeats, respectively. In some cases, the error bars are smaller than the symbol.
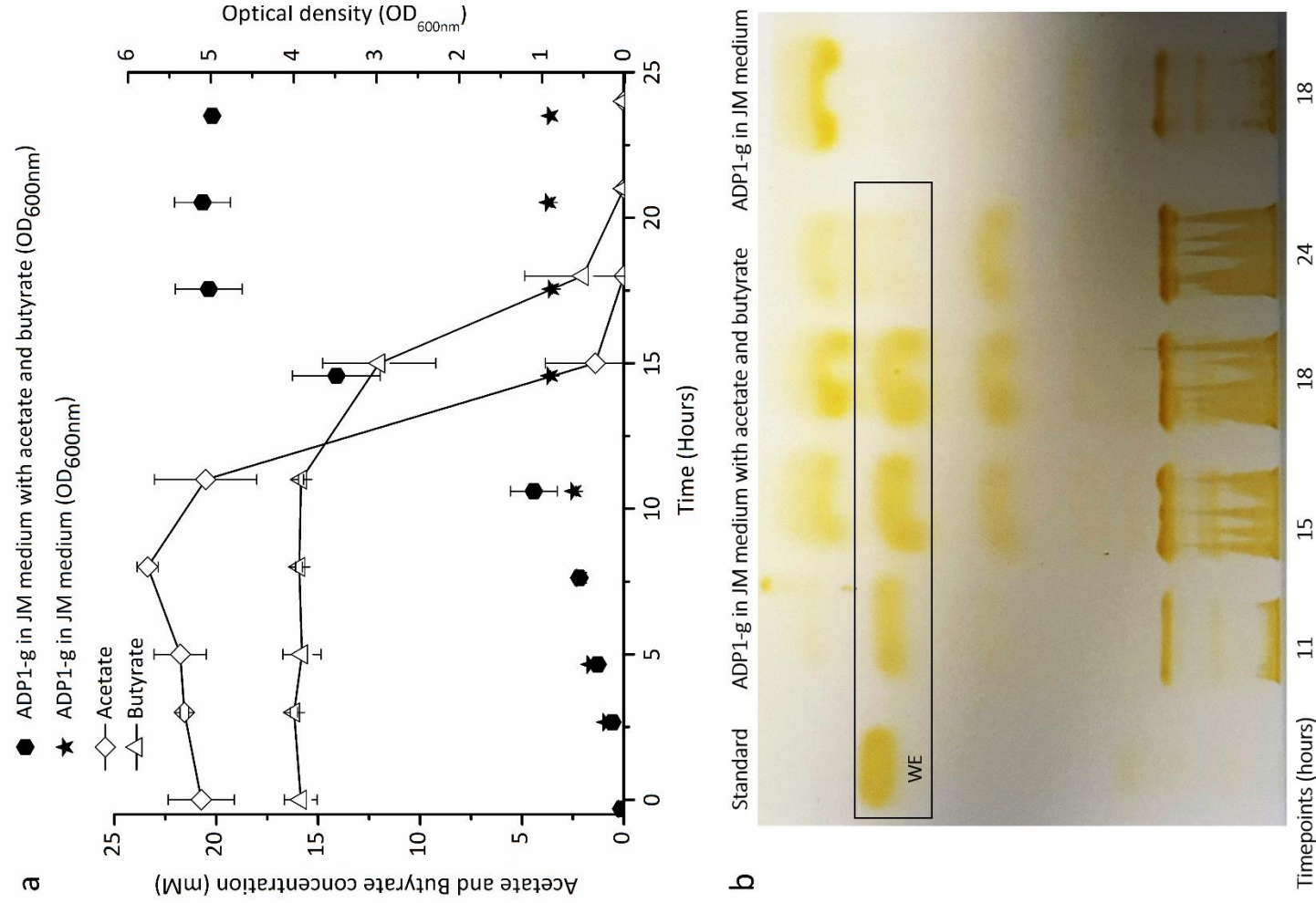
Figure 3 – Growth, substrate utilization and wax ester production of ADP1-g cultivated in JM medium with 20mM acetate and 15mM butyrate.

(a) The growth (closed symbols), determined as optical density at 600 nm wavelength, and organic acid utilization (open symbols). The presented data points are averaged values and error bars indicate standard deviation from triplicate experimental repeats. In some cases, the error bars are smaller than the symbol. (b) Thin layer chromatography analysis of wax ester synthesized by ADP1-g from acetate and butyrate. The time point samples analyzed with TLC correspond to the same presented in (a). In above figures a and b, ADP1-g cells in JM medium represents the growth and wax ester profiles, respectively, of ADP1-g cells when cultivated without acetate and butyrate supplementation (substrate blank).
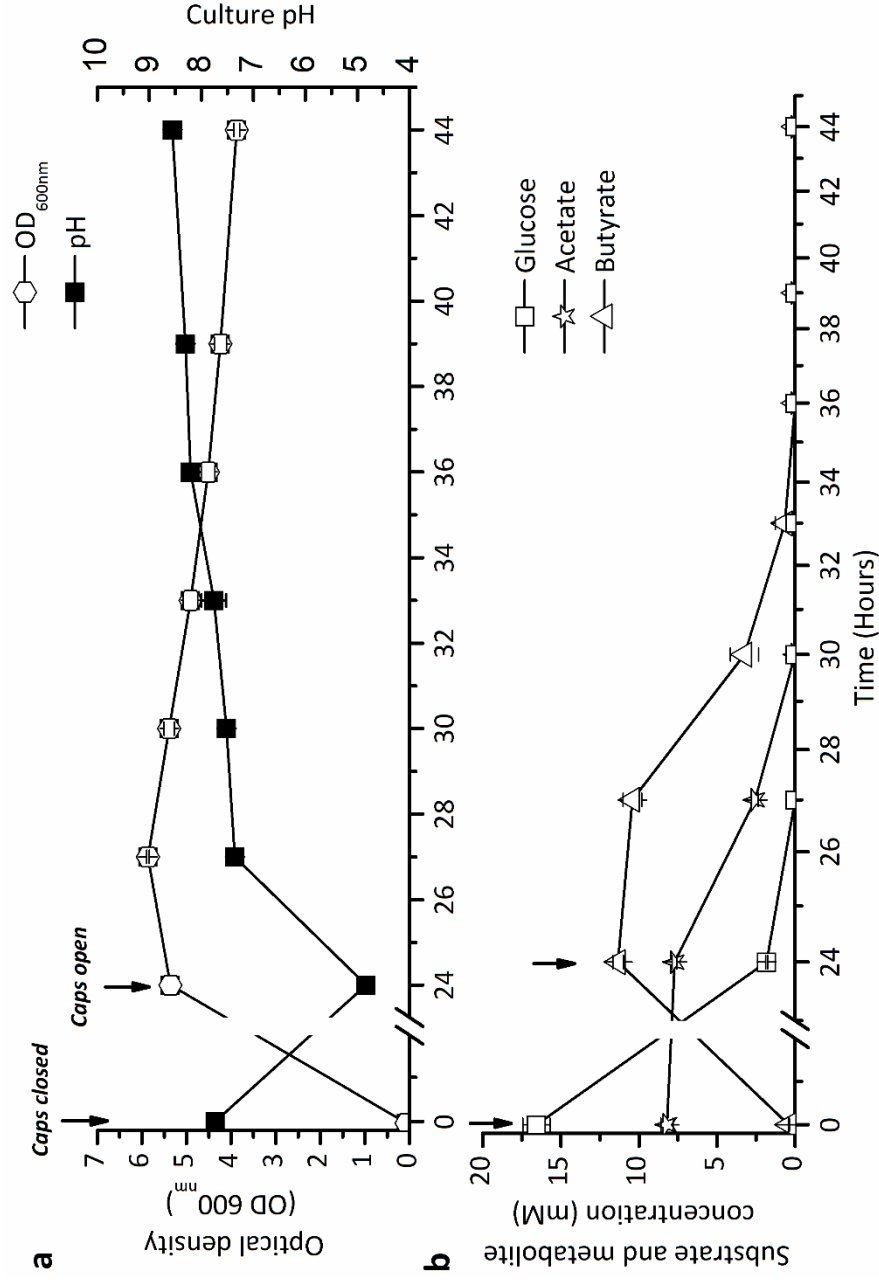
Figure 4 – The growth, pH, and substrate concentrations of ADP1-g – *C. butyricum* cocultivations.

Arrows indicate the time when the growth vessel caps were closed and opened during the experiment. (a) OD_{600nm} and pH trends of one-pot batch ADP1-g – *C. butyricum* cocultivations. (b) Glucose utilization (*C. butyricum*) and acetate-butyrate metabolism (ADP1-g) data from cocultivation experiment. The data points are averaged from triplicate experimental repeats. In some cases, the symbols overlap and the error bars (standard deviation) are smaller than the symbol.
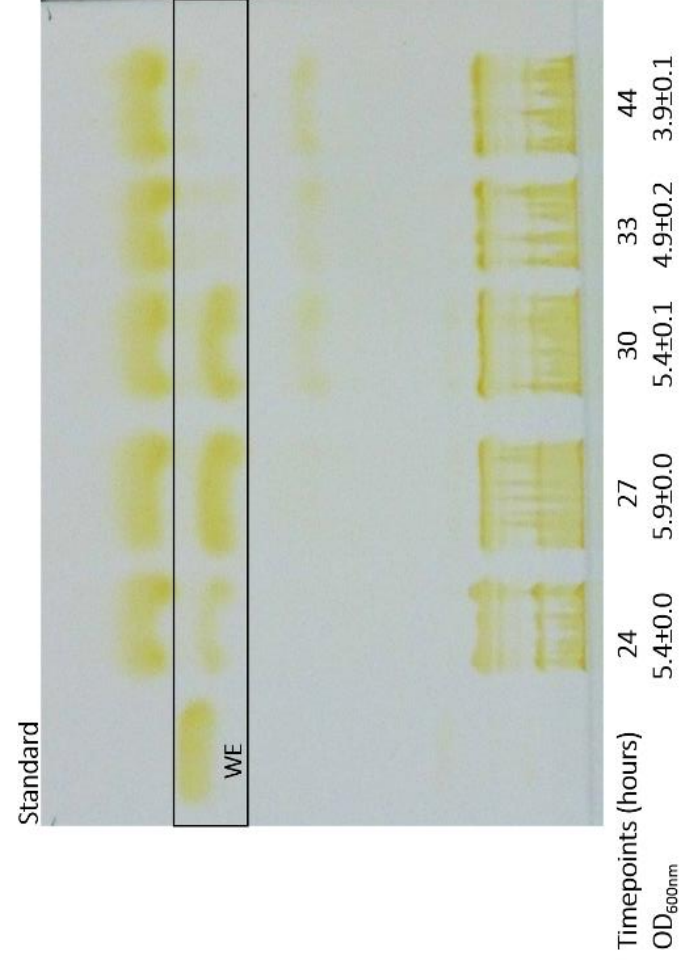
Figure 5 – TLC analyses demonstrating the wax ester production from ADP1-g – *C. butyricum* coculture.

The wax esters were synthesized by ADP1-g strain utilizing the acetate and butyrate generated during *C. butyricum* fermentation. The samples are from the same timepoints shown in Figure 4.
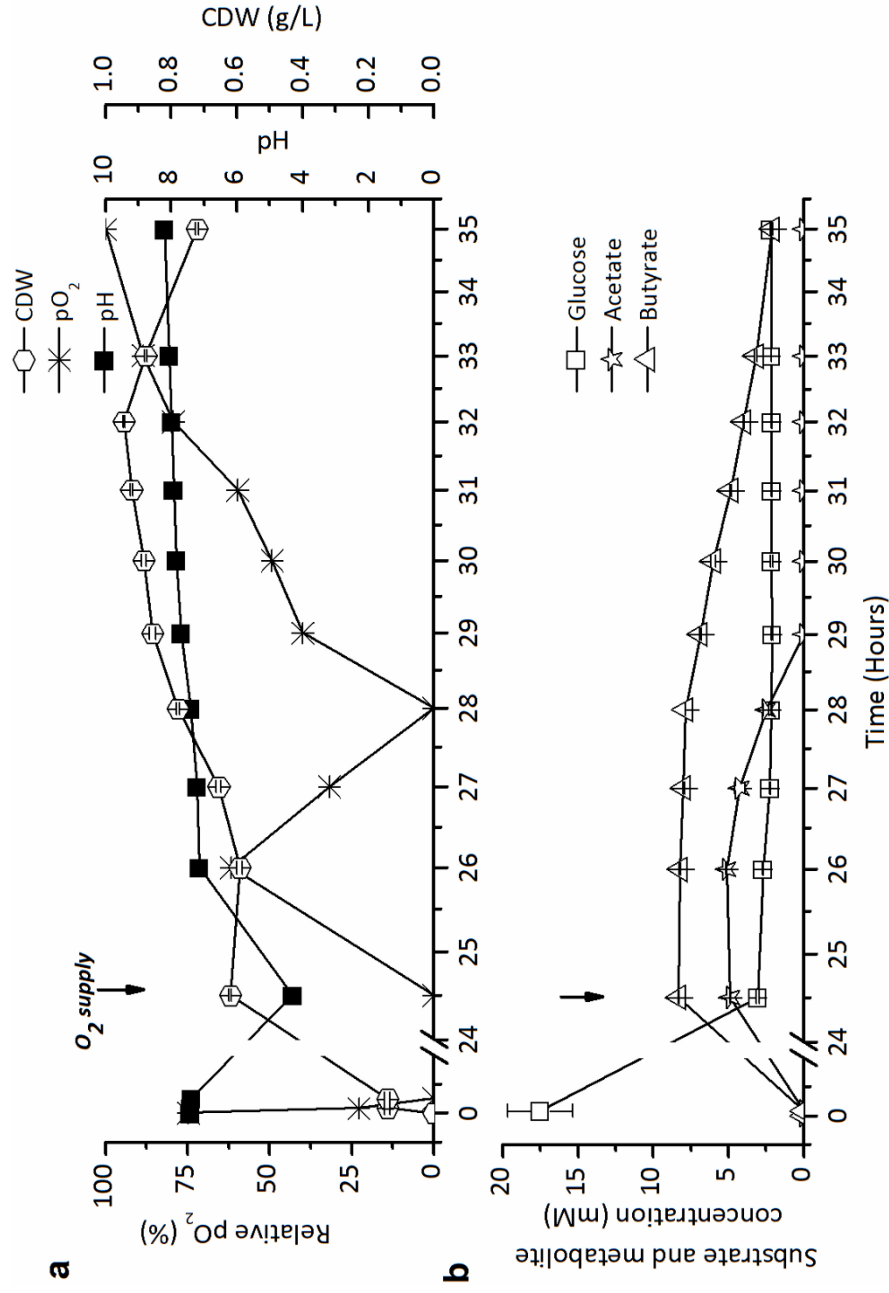
Figure 6 – One-pot batch cultivation of ADP1-g and *C. butyricum* in 1-litre bioreactor.

The cocultivation was carried out in 800 ml JM medium supplemented with 20 mM glucose. The changes in (a) pH, pO_2 and biomass formation (in cell dry weight, CDW), and (b) glucose, acetate and butyrate concentrations are presented. *C. butyricum* consumed glucose producing acetate and butyrate during the first 24 hours, whereas ADP1-g utilized the fermentation end-products during the following 10 hours. Arrows indicate the timepoint at which the oxygen supply was initiated and the culture pH adjusted to 7.3. The data for CDW, glucose, acetate and butyrate are mean values from triplicate technical repeats. The standard deviations (error bars) of each data are plotted and in some cases the symbols overlap the error bars.

Table 1 – Growth, pH and carbon utilization trend of ADP1-g cultivated in JM medium supplemented with glucose and acetate.

Cultivation time (hours) [a]	Growth (OD$_{600nm}$) [b]	Medium pH [b]	Glucose concentration (mM) [b]	Acetate concentration (mM) [b]
0	0.1±0.0	7.8±0.0	17.2±0.2	8.5±0.1
24	0.7±0.0	7.3±0.0	17.1±0.4	BD
27	1.1±0.1	7.9±0.1	17.6±1.4	BD
44	1.0±0.1	8.5±0.1	17.4±0.3	BD

[a] Sampling time similar as indicated in Figure 4

[b] Averaged data from triplicate experimental repeats ± standard deviation

BD, below detection limit

Table 2 – Products generated from glucose in one-pot batch cultivation by *C. butyricum* and ADP1-g.

	Substrate utilized and products generated	Yield	Productivity
Substrate			
Glucose	14.5±2.1 [a]		
Products			
Biomass	0.94 [b]		
Lipids	9.7 [c]		
H$_2$	24.5±0.8 [d]	1.7±0.1 [e]	0.8±0.0 [f]
WE	28 [d]	10.8 [e]	0.9 [f]
		30 [g]	
		31 [h]	