Dynamic decoupling of biomass and lipid biosynthesis by autonomously regulated switch

short title: Dynamic decoupling of biomass and lipid synthesis

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Abstract

For improving the microbial production of fuels and chemicals, gene knock-outs and overexpression are routinely applied to intensify the carbon flow from substrate to product. However, their possibilities in dynamic control of the flux between the biomass and product synthesis are limited, whereas dynamic metabolic switches can be used for optimizing the distribution of carbon and resources. The production of single cell oils is especially challenging, as the synthesis is strongly regulated, competes directly with biomass, and requires defined conditions, such as nitrogen limitation. Here, we engineered a metabolic switch for redirecting carbon flow from biomass to wax ester production in *Acinetobacter baylyi* ADP1 using acetate as a carbon source. Isocitrate lyase, an essential enzyme for growth on acetate, was expressed under an arabinose inducible promoter. The autonomous downregulation of the expression is based on the gradual oxidation of the arabinose inducer by a glucose dehydrogenase *gcd*. The depletion of the inducer, occurring simultaneously to acetate consumption, switches the cells from a biomass mode to a lipid synthesis mode, enabling the efficient channelling of carbon to wax esters in a simple batch culture. In the engineered strain, the yield and titer of wax esters were improved by 3.8 and 3.1 folds, respectively, over the control strain. In addition, the engineered strain accumulated wax esters 19% of cell dry weight, being the highest reported among microbes. The study provides important insights into the dynamic engineering of the biomass-dependent synthesis pathways for the improved production of biocompounds from low-cost, sustainable substrates.

Keywords

Lipid biosynthesis, wax esters, acetate, dynamic control, decoupling, autonomous circuit

Abbreviations

WE – wax esters, AceA – isocitrate lyase

1. Background

Metabolic engineering and synthetic biology provide powerful means for the bio-based production of a variety of chemicals and other commodities by engineered microbes. Compared to conventional chemical synthesis, the superiority of biological production systems lies in the possibility to synthesize both the catalyst (i.e. the cell factory) and the product itself from very simple chemical compounds, such as sugars or organic acids. However, the challenge is to develop a system, which optimally distributes the resources and carbon flux between building up the catalyst and operating the catalyst for the actual production; extensive cell growth takes resources from the product synthesis, whereas too excessive flux towards product synthesis may result in reduced growth, insufficient cofactor regeneration, low enzyme expression levels, and eventually poor titers. To address the challenges related to the optimal distribution of cellular resources, a number of dynamic circuit designs targeting the central pathway nodes have been recently developed (1). While growth-associated genes responsible for central carbon metabolism cannot be directly deleted, various strategies for decoupling growth and product synthesis have been introduced; Soma et al. constructed a metabolic toggle switch for conditional knockout of citrate

62 synthase *gltA,* an enzyme required for functional tricarboxylic acid (TCA) cycle (2). The
63 switch allowed an induced shift of carbon flow from TCA cycle to synthetic isopropanol
64 pathway. More recently, the system was further improved by introducing a sensor-
65 regulator system responsive to a defined cell density (3). Solomon et al. introduced a
66 dynamic approach to controlling the glycolytic flux; antisense RNA technology and an
67 inverting gene circuit were employed for inhibiting the activity of glucokinase (Glk),
68 resulting in a controlled growth rate and a reduced production of acetate (4).
69 Brockman and Prather introduced another example of a dynamic regulation system,
70 where they developed a circuit for dynamic knockdown of phosphofructokinase-1
71 (Pfk-1), the enzyme responsible for the key step in the glycolytic pathway regulating
72 glucose-6-phosphate flux. By the temporal control of Pfk-1 degradation, glucose-6-
73 phosphate could be efficiently directed to heterologous *myo*-inositol synthesis
74 pathway instead of biomass production (5). In a previous work, Doong et al. further
75 improved the system with a *myo*-inositol responsive dynamic sensor that regulated
76 the downstream enzymes of the pathway in converting *myo*-inositol to glucarate. The
77 introduced systems represent highly elegant examples of advanced metabolic control,
78 but the complex circuit designs can be prone to destabilization in prolonged
79 cultivations and function unexpectedly in scaled-up processes (6). Thus, reliable
80 metabolic control systems with simple and robust operation would be also desired.
81
82 Microbial storage compounds, such as polyhydroxyalkanoates (PHA), triacylglycerols
83 (TAG) and wax esters (WE), are industrially relevant and desirable molecules due to
84 their vegetable oil like properties and broad applicability in e.g. fuel, nutritional,

85 cosmetic, and pharmaceutical industries. However, the production of the long carbon
86 chain products derived from fatty acyl-CoA requires significant energy investments
87 from the cell. In addition, the synthesis of storage compounds directly competes with
88 biomass production and is strongly regulated, growth-phase dependent, and requires
89 high amounts of cofactors and excess carbon along with limitation on other nutrients,
90 such as nitrogen (7, 8). While strategies for dynamically regulated production of free
91 fatty acids (FFA) and FA derived products have been introduced (9, 10), means for
92 overcoming the challenges of storage lipid synthesis regulation are still lacking.
93 Therefore, the production processes are conventionally improved by non-specific
94 means, such as bioprocess optimization or in conditions with a defined carbon-
95 nitrogen ratio (11). As an example of a more advanced approach, Xu et al. established
96 a semi-continuous culture system with model-aided bioprocess optimization and cell
97 recycling, which allowed efficient TAG production and high productivities even with
98 dilute acetate feed (12). While also metabolic engineering strategies have been
99 employed for improving TAG (13-16) and WE synthesis (17, 18) in microbes, efficient
100 overproduction of acetyl-CoA coupled with dynamic resource distribution between
101 biomass and storage lipid synthesis remains a challenge.
102
103 When microbes are cultivated on non-glycolytic substrates, such as acetate or fatty
104 acids, complete TCA cycle yielding carbon dioxide cannot be utilized for anabolic
105 processes. In those circumstances, the cells have to rely on an alternative route,
106 namely glyoxylate cycle, which bypasses the two decarboxylation steps of TCA cycle.
107 The key enzyme in the glyoxylate cycle is isocitrate lyase (AceA), responsible for

108 converting isocitrate to glyoxylate and succinate. It was previously demonstrated that
109 a knock-out of the isocitrate lyase in *Pseudomonas putida* improved the production of
110 PHAs when grown on gluconate, apparently for providing surplus acetyl-CoA for the
111 PHA synthesis (19). Some bacteria also exhibit an alternative pathway for glycolysis,
112 such as the modified Entner-Doudoroff pathway of certain *Acinetobacter* strains (20,
113 21). An interesting feature of the glycolysis of *Acinetobacter* is the oxidation of glucose
114 to gluconate prior the transport to cells. In the absence of glucose, the first enzyme of
115 the pathway, glucose dehydrogenase Gcd, can unselectively oxidize other sugar
116 compounds present in the medium, such as pentoses, without the capability to utilize
117 them as a carbon source (22-24). Importantly, this feature does not interfere with the
118 utilization of non-glycolytic carbon sources, such as organic acids, and can be
119 considered being 'orthogonal' to the substrate utilization. Thus, this feature could be
120 exploited in the regulation of metabolic pathways in the cells.
121
122 Here, we construct a dynamic switch for autonomous shifting of cells from the
123 biomass mode to the storage lipid synthesis mode by introducing a circuit for a
124 conditional elimination of the glyoxylate cycle, which is the essential bypass for cells
125 growing on acetate, and the key control node in lipid biosynthesis pathway. We
126 demonstrate the functionality of the switch by the improved (both yield and titer)
127 production of wax esters using *A. baylyi* ADP1 as a host.

2. Material and Methods

Strains and molecular work

A. baylyi ADP1 (DSM 24193, Deutsche Sammlung von Microorganismen und Zellkulturen, Germany) was used in the study. The single gene knock-out strain of *A. baylyi* ADP1Δ*aceA*::*tdk*/*Kan*^{r} (ACIAD1084 deleted) was kindly provided by Veronique de Berardinis (Genoscope, France). In the single gene knock-out mutant, the gene in question is replaced with a gene cassette containing a kanamycin resistance gene (Kan^r) (25). For cloning and plasmid amplification, *Escherichia coli* XL1-Blue (Stratagene, USA) was used. The reagents and primers for molecular work were purchased from ThermoFisher Scientific (USA), unless stated otherwise. The primers used in the study are presented in Table 1. The complementation of *aceA* in the knock-out strain was carried out by constructing a gene cassette with the *aceA* gene under an arabinose inducible promoter AraC-pBAD; a previously described gene cassette (16, 26) was used as the scaffold for the construction. The arabinose promoter and AraC repressor, designated as ara, were amplified from pBAV1C-ara-LuxCDE plasmid (18) using primers VS10_09 and VS10_10 and inserted to the plasmid iluxAB/pIX (26) using restriction sites MfeI and NdeI. The gene *aceA* (ACIAD1084) was amplified from the genome of ADP1 wt with primers SS17_08 and SS17_09 and cloned to the ara-iluxAB/pIX using restriction sites NdeI and XhoI. Transformations of ADP1 were carried out as described previously (16). The transformed cells were selected on LA plates containing 25 µg/ml chloramphenicol. ADP1 strain with a *poxB* deletion, *A. baylyi* ADP1Δ*poxB*::*Cm*^{r}, was used as the reference strain designated as ADP1 wt. All genetic modifications were confirmed with PCR and sequencing.

Cultivations

The components for culture mediums were purchased from Sigma (USA). The strains were cultivated in modified MA/9 minimal salts medium (17) at 25 °C and 300 rpm unless stated otherwise. The medium was supplemented with 25-100 mM Na-acetate or 250 mM glucose, 0.1 % casein amino acids (w/v), and 0-1.0 % L(+)arabinose when appropriate.

The cultivations for optimizing the arabinose concentration for ADP1-ara-aceA growth were carried out using Tecan Spark® (Tecan, Switzerland) microplate reader in 25 °C for 21 hours with two replicate wells for each strain and arabinose concentration. The medium was supplemented with 25 mM Na-acetate and 0.1 % casein amino acids, and arabinose (concentrations 0; 0.05; 0.1; 0.2; 0.5 or 1.0 %) was used for the induction of *aceA*. Mediums without acetate supplementation was used as the control medium to determine the growth on casein amino acids. The strain ADP1 wt was used as the positive control, whereas the knock-out strains ADP1 Δ*aceA*::*Tdk/kan*^{r} and *A. baylyi* ADP1Δ*aceA*::*tdk*/*Kan*^{r} Δ*poxB*::*Cm*^{r} were used as negative controls for growth on acetate. For semi-quantitative determination of the WE production of ADP1-ara-aceA, the cells were cultivated in two parallel 5 ml cultures in the same medium except with 50 mM Na-acetate in 5 ml tubes for 30 hours.

For the quantitative determination of the WEs with NMR, the strain ADP1 wt and ADP1-ara-aceA were cultured in total 600 ml of medium supplemented with 50 mM Na-acetate and 0.5 % (~30 mM) arabinose distributed in 12 Erlenmeyer flasks. HPLC samples were taken every 1-5 hours and two parallel 40 ml samples were taken in five

different time-points to quantitatively determine WE (by NMR) and biomass production (as cell dry weight).

Lipid and end-metabolite analyses

The amount of total lipids and WEs were estimated by TLC or quantified by NMR. For TLC, equal volumes of samples (3 ml) from different cultures were taken and the lipids were extracted using 'miniscale' chloroform-methanol extraction as described previously (17). Thirty µl of the chloroform phase was applied on 20 × 10 cm Silica Gel 60 F_{254} HPTLC glass plates with 2.5 × 10 cm concentrating zone (Merck, USA). Mobile phase used was n-hexane: diethyl ether: acetic acid 90: 15: 1 and iodine was used for visualization. Jojoba oil was used as the standard for WEs. For comparative evaluation of the intensities of the WE bands on TLC, the Gel analysis method of ImageJ software (rsb.info.nih.gov/ij/index.html) was applied as described in the ImageJ documentation.

For NMR analyses, the 40-ml biomass samples were freeze-dried and the cell dry weight (CDW) was determined gravimetrically. The lipid extraction and the quantitative ^{1}H NMR analysis of WEs was carried out as described earlier (26). The amount of total lipids was determined gravimetrically. The areas of the peaks in the NMR spectrum are directly proportional to the molar concentration of each functional group, yielding specific concentration for WEs in total biomass. The concentration of WEs was calculated from the integrated signal at 4.05 ppm which is characteristic for protons of α-alkoxy-methylene group of esters (–CH2-COO-CH2–). For the calculation of the WE titer in grams per liter, an average molar mass of 506 g/mol was used (17).

198

199 The glucose, acetate, and arabinose concentrations were determined by LC-20AC
200 prominence liquid chromatograph (Shimadzu, USA) equipped with RID-10A refractive
201 index detector, DGU-20A5 prominence degasser, CBM-20A prominence
202 communications bus module, SIL-20AC prominence autosampler, and Shodex SUGAR
203 SH1011 (Showa Denko KK, Japan) as described previously (16).

204 # 3. Results

205 In order to investigate the effect of the knock-out of isocitrate lyase AceA on the
206 growth and WE production in *A. baylyi* ADP1, we employed a knock-out mutant strain
207 *A. baylyi* ADP1 Δ*aceA*::Tdk/kanr (25) for preliminary test cultivations. We observed
208 that when grown on glucose, the cells grow more slowly, but produce WE titers
209 comparable to those of the wild type (wt) strain; after 48 hours of cultivation, the wild
210 type had produced 470±150 mg/l WEs compared to 460±40 mg/l WEs produced by
211 the knock-out strain. In opposite to the wt strain, however, the mutant strain did not
212 exhibit growth on minimal medium supplied with acetate as the sole carbon source.
213 This is due to the lack of route for acetyl-CoA to be directed in biosynthetic pathways
214 via malate. Thus, as acetyl-CoA represents the key precursor in both the biomass
215 production through the glyoxylate shunt and the wax ester biosynthesis, we
216 hypothesized that by dynamically regulating the isocitrate lyase, the state of the cells
217 could be switched between biomass and lipid synthesis modes (Fig 1). In order to make
218 the shift dynamic, we introduced an approach for autonomous regulation of the
219 isocitrate lyase AceA; by expressing the enzyme under an arabinose-inducible
220 promoter AraC-pBAD, the induction is gradually repressed due to the depletion of

221 arabinose by the glucose dehydrogenase activity of ADP1. The arabinose inducible
222 promoter has been previously shown to function in *A. baylyi* ADP1 (18, 27). In order
223 to establish a system with maximal linearity and controllability, we constructed a gene
224 cassette for genomic expression of *aceA* (Fig 1c, d). Exploiting the natural
225 transformation machinery of ADP1, the gene cassette was integrated in the genome
226 to replace a gene *poxB* (ACIAD3381), which has been previously shown to be a neutral
227 target site in terms of growth and wax ester production (26, 28). The resulting strain
228 *A. baylyi* ADP1Δ*aceA*::*tdk*/*Kan*^{r} Δ*poxB*::*araC*-pBAD-*aceA*-*Cm*^{r} was designated as ADP1-
229 ara-aceA. The strain *A. baylyi* ADP1Δ*poxB*::*Cm*^{r} was used as the reference strain, from
230 now on designated as the ADP1 wt.
231
232 We investigated the functionality of the complementation in the strain ADP1-ara-aceA
233 in minimal medium supplemented with 50 mM acetate (Figure 2). Arabinose (at
234 concentrations 0; 0.1 and 1.0 %) was added to the cultures, and the cultivations were
235 continued for 68 hours. Cells did not grow or consume acetate in the absence of
236 arabinose, indicating sufficiently tight regulation of the arabinose promoter. In the
237 cultures with small amount of arabinose (0.1 %), the cells stopped growing after
238 reaching an optical density (OD) of 0.3 and consumed only 5 mM acetate, whereas
239 with 1.0 % arabinose the cells reached OD ~3.5 along with complete consumption of
240 acetate. The ADP1 wt grew to slightly lower biomass (OD ~2.4) and consumed all the
241 acetate; arabinose supplementation had no effect on the ADP1 wt growth. After 68
242 hours of cultivation, approximately 85 % of the 1% arabinose had been oxidized. The
243 control knockout strains ADP1Δ*aceA::tdk/Kan*^{r} and ADP1*ΔaceA::tdk/Kan*^{r} *ΔpoxB::Cm*^{r}

244 did not exhibit growth nor acetate consumption with or without the presence of
245 arabinose (OD 0 at 0-68 h). We also confirmed, that the AceA expression is repressed
246 due to arabinose oxidation, i.e. the conversion of arabinose to non-inducive form,
247 arabino-lactone and further to arabonate (Figure S1).
248
249 In order to find the optimal arabinose concentration in terms of both biomass and wax
250 ester production, the strain ADP1-ara-aceA was cultivated in several different
251 arabinose concentrations in minimal salts medium supplemented with acetate (Figure
252 3) for 21 hours. ADP1 wt was cultured as the reference strain. Casein amino acids
253 (0.1%) were added to the culture in order to promote the growth and to prevent
254 nitrogen limitation. As indicated by the previous growth experiment, we found that
255 1% arabinose was sufficient to allow the engineered strain to reach the same biomass
256 as ADP1 wt, albeit the cells grew slower. Within the concentration range 0 – 0.2%, only
257 small differences in growth pattern or biomass production were observed. The slight
258 increase in OD of uninduced cells is due to the utilization of the casein amino acids
259 present in the growth medium; a same amount of biomass is achieved without acetate
260 supplementation with the wild type strain and the knock-out strain
261 ADP1*ΔaceA::tdk/Kan*^{r}*ΔpoxB::Cm*^{r} with both acetate and casein amino acid
262 supplementation (data not shown). For ADP1 wt, all the growth curves were similar
263 regardless of the arabinose concentration used (data not shown).
264
265 Next, we determined which initial arabinose concentration most optimally distributes
266 the carbon between the biomass and the WE production. The strain ADP1-ara-aceA

267 was cultivated in 5 ml minimal salts medium supplemented with 0.1 % casamino acids
268 and 50 mM acetate with different arabinose concentrations (0; 0.1; 0.2; 0.5% and
269 1.0%) for 30 hours, after which biomass and WE production were determined. A clear
270 correlation between the arabinose concentration and biomass production (OD600)
271 was detected (Figure 4). Without arabinose supplementation, the cells grew to an OD
272 of approximately 0.8, which is due to the utilization of casamino acids (the same OD
273 was obtained with ADP1*ΔaceA::tdk/Kan*^{r}*ΔpoxB::Cm*^{r}). A semi-quantitative lipid
274 analyses based on thin layer chromatography (TLC) was carried out to compare the
275 amount of WEs produced (Figure 5B). For all the cultures, the same sample volume
276 was taken for the analysis, thus representing the titer of WEs produced. Based on
277 image analysis, the intensity of the band representing the wax ester titer increases
278 along with the biomass concentration. However, only a slight difference was observed
279 between the bands of the 0.5% and 1.0% cultures, suggesting that in the culture which
280 contains saturating amount of arabinose the growth corresponds to that of the wild-
281 type strain. When the intensities were divided with the optical densities, the highest
282 amounts of WEs (per cell) were produced in the cultures with 0.2% and 0.5%
283 arabinose. Thus, considering both the volumetric titer and the yield of WEs per
284 biomass, the arabinose concentration of 0.5% was found to be optimal in terms of
285 distributing the carbon and cellular resources between biomass and WEs.
286
287 Batch cultivations for ADP1 wt and ADP1-ara-aceA were carried out in minimal
288 medium supplemented with 0.1 % casein amino acids, 50 mM Na-acetate and 0.5 %
289 arabinose. Acetate and arabinose concentrations as well as the WE production (at 5,

290 7, 9 and 10 h time-points) were determined (Figure 5). ADP1 wt consumed all the
291 acetate in approximately 9-10 hours; the amount of biomass increased until the 10-
292 hour time-point, the CDW being 1.3 g/l. The highest amount of WEs was measured at
293 the 9-hour time-point, being 47 mg/g CDW and 60 mg/l. In ADP1 wt, the WEs
294 accounted for 44 % of total lipids. The WE yield was found to be 0.02 g WE/g consumed
295 acetate. The strain ADP1-ara-aceA utilized acetate more steadily and produced less
296 biomass compared to ADP1 wt; the growth ceased after 16 hours along with the
297 arabinose depletion: the biomass remained at 0.6-0.7 g/l CDW between the 16-24
298 hours of cultivation. Thereafter, the WE content of the cells strongly increased, being
299 highest at 38 h time-point, which also increased the amount of total biomass to 1.0
300 g/l. The WE titer was found to be 184 mg/l representing 19 % of CDW, which was 3.8-
301 fold higher compared to the ADP1 wt. In addition, the WEs accounted for 80 % of all
302 cellular lipids in ADP1-ara-aceA. The WE yield was 0.08 g WE/g consumed acetate, also
303 being 4-fold higher over the ADP1 wt.

304 4. Discussion

305 Sugars, mainly glucose, have been the major carbon source for the heterotrophic
306 microbial production of fatty acid derived compounds, such as TAGs and WEs, which
307 can be used for the production of biofuels, biochemicals, and other biocommodities
308 (29, 30). However, in order to increase the feasibility and sustainability of the
309 processes, the possibility to utilize alternative carbon sources is of high interest.
310 Organic acids, such as acetate, serves as a low-cost, abundant carbon source for
311 microbial lipid synthesis. Acetate can be readily derived from the hemicellulose

312 fraction of plant biomass or waste streams, or produced from syngases by microbial
313 fermentation (31). For example, we have previously demonstrated the conversion of
314 carbon dioxide to WEs via acetate intermediate by combining microbial
315 electrosynthesis with aerobic lipid synthesis in a two-stage process (32). Many of the
316 potential acetate streams, however, may have dilute acetate concentrations, thus
317 sustaining conditions that are preferable for biomass production rather than for
318 efficient storage carbon synthesis. On the other hand, highly concentrated acetate
319 feeds can inhibit cell growth (33).
320
321 Nitrogen starvation is a commonly used and efficient means to trigger storage lipid
322 accumulation in microbes (34-36). However, in such conditions the cell biomass
323 typically remains low (37), which can result in lower overall product titres. Thus,
324 genetic reprogramming would serve as a means to bypass the natural regulation for
325 storage lipid synthesis. Mechanisms behind the regulation in microbes are not well
326 understood, and therefore it has been challenging to genetically drive cells to
327 overproduce the storage compounds or other fatty acid derived products. Previous
328 strategies include for example the manipulation of the conserved carbon storage
329 regulator CsrA through the CsrA-CsrB ribonucleoprotein complex, by which alterations
330 in the central carbon metabolism and fatty acid synthesis regulation have led to
331 favourable changes in both native and non-native product synthesis pathways in *E.*
332 *coli* (38). This approach, however, is rather unspecific and potentially difficult to
333 combine with other (targeted) engineering strategies.
334

335 In this study, we developed an autonomously regulated circuit for programmable
336 synthesis of WEs in a native production host, *A. baylyi* ADP1. The circuit allows the
337 cells to shift from the biomass mode to the WE synthesis mode independent from the
338 carbon/nitrogen ratio or the growth phase of the culture. In practice, we replaced the
339 native isocitrate lyase aceA with an arabinose inducible system, which allows a
340 conditional and timed knockdown of the expression of *aceA*. This enzyme is essential
341 for the biomass production when the cells grow on acetate. The timed repression of
342 aceA expression is achieved by gradually eliminating the inducer, namely arabinose;
343 the native enzyme activity of glucose dehydrogenase Gcd of *A. baylyi* oxidizes
344 arabinose to arabino-lactone and further to arabonate, which in turn cannot serve as
345 inducers. Importantly, and in contrast to other auto-induction-based systems,
346 arabinose oxidation does not interfere with the utilization of the carbon source, here
347 acetate, and can be thus considered as an orthogonal system. By adjusting the
348 arabinose concentration, a predefined and optimal amount of biomass can be
349 produced. When the inducer concentration is oxidized below the 'threshold'
350 concentration, the cells shift from the biomass producing mode to the synthesis mode,
351 efficiently directing carbon to product synthesis.
352
353 First, we confirmed that the engineered strain ADP1-ara-aceA with complemented
354 isocitrate lyase was able to grow on acetate as the sole carbon source. We observed
355 that without the presence of arabinose, the cells did not exhibit growth, showing
356 phenotype similar to the knockout strain *A. baylyi* ADP1Δ*aceA*::*tdk*/*Kan*^{r}Δ*poxB*::*Cm*^{r}.

357 We also observed that arabinose concentration 1% is sufficient to allow the growth of
358 ADP1-ara-aceA to reach at least the same biomass as the ADP1 wt.
359
360 Initial arabinose concentrations were in correlation with the obtained biomasses
361 between concentrations 0.2% and 1.0%. The concentration 0.2% was found to be the
362 'threshold' for sufficient biomass production; below this concentration, the cells
363 produced only slightly more biomass compared to the uninduced cells, indicating that
364 arabinose concentrations >0.2% are required for sufficient growth in the studied
365 conditions. This finding was also supported by the reporter induction test; when the
366 cells containing the bacterial luciferase *luxAB* under arabinose promoter were induced
367 with the supernatant from different cultivation time-points (thus having different
368 arabinose concentrations), clear induction of *luxAB* determined as luminescence
369 production was only observed with the sample that contained >0.2% arabinose.
370
371 According to the semi-quantitative WE analyses, the cultures supplemented with 0.2%
372 or 0.5% arabinose produced the highest WE yields (per biomass). The cultures that
373 were induced with 1% arabinose produced nearly two times more biomass compared
374 to that of the 0.5% culture but had lower WE yield, indicating that a significant
375 proportion of the carbon was directed to the biomass when 1% arabinose was used.
376 Interestingly, the cultures with little (0.1%) or no arabinose produced the lowest WE
377 yield, suggesting that at least subtle levels of *aceA* expression are required, not only
378 for biomass production but also to support WE synthesis. The WE titres (WEs/volume)
379 of 0.5% and 1.0% cultures were estimated to be very close to each other in the two

380 cultures, whereas 0.2% culture had clearly lower volumetric WE production due to low
381 biomass production. Thus, we considered 0.5% arabinose as the most effective
382 inducer concentration in terms of optimal distribution of carbon between biomass and
383 products.
384
385 For validation of the system, batch cultures for the ADP1 wt and ADP1-ara-aceA were
386 carried out. It was shown that the engineered strain ADP1-ara-aceA efficiently
387 accumulated WEs in simple batch conditions supplied with relatively low substrate
388 concentration (50 mM acetate) and in non-optimal carbon-nitrogen ratio. The strain
389 ADP1-ara-aceA produced 187 mg/l WEs representing 19 % of the CDW and a yield of
390 0.08 g WE/g consumed acetate. As expected, the strain grew more slowly and
391 produced less biomass than ADP1 wt, but the yield of WEs per biomass and per
392 consumed acetate were 3.8 and 4 folds higher compared to ADP1 wt, respectively. In
393 addition, the WE titer was found to be 3.1 folds higher compared to that of the ADP1
394 wt. Thus, the dynamic regulation not only improved the yield of WEs per biomass and
395 per used carbon, but clearly excelled the volumetric titer of that of the wild type strain.
396 For comparison, in a previous study (32), *A. baylyi* ADP1 produced WEs from acetate
397 with a titer of approximately 90 mg/l (from higher initial acetate concentration, 100
398 mM), with an average yield of 4 % (carbon/carbon), being equal to 0.02 g WE/g
399 consumed acetate. The highest WE titer reported so far has been 450 mg/l, which was
400 obtained when the key enzyme of the pathway (fatty acyl-CoA reductase) was
401 overexpressed and 5 % glucose was used as the substrate (17). However, the yields

402 (0.04g WE/g glucose and 12.5% WEs of CDW) were lower compared to this study. With
403 external alkane supplementation, up to 17% WEs of CDW has been obtained (39).
404
405 Notably, the amount of WEs per cell was nearly constant in ADP1 wt at the analysed
406 time points, being 3.9-4.3% of the CDW. In ADP1-ara-aceA, by contrast, the amount of
407 WEs per cell strongly increased along with the arabinose depletion; the percentage of
408 WEs per cell increased from 5.2% to 19% between the sampling points. The highest
409 increase in the WE content (from 7.5 to 19%) was achieved after the arabinose
410 concentration reached the 'threshold' 0.2% (equivalent to 15 mM arabinose).
411 Although the shift from biomass mode to lipid mode was rather gradual, the arabinose
412 concentration 0.2% seems to be the key turning point in the cellular state. In the batch
413 culture, initial concentrations 50 mM acetate and 0.5% arabinose were used. By the
414 end of the culture, the arabinose was completely oxidized, and only a small amount
415 (5 mM) of acetate remained unutilized. Thus, by adjusting the substrate and inducer
416 concentrations, the system is potentially scalable to a wide range of substrate
417 concentrations. Moreover, coupling this system with other engineering strategies,
418 such as introducing additional knock-outs (16) and/or overexpression of key enzymes
419 of the pathway (17) could further improve the WE production.
420
421 Considering not only the efficient redirection of carbon to product, but also the
422 downstream processing, the purity of the product is important. In ADP1-ara-aceA,
423 WEs constituted 80% of total lipids, indicating high purity of the desired product. In
424 ADP1 wt, only 44% of the total lipids were WEs.

425

426 The results from different experiments indicate that at least low levels of isocitrate
427 lyase are required to maintain WE production from acetate, potentially due to the
428 requirements for cells to generate e.g. NADPH for the synthesis. This hypothesis was
429 also supported by a further observation in an additional experimental set-up where
430 the WE content of the *aceA* knockout strain did not increase after a transfer from a
431 glucose medium to an acetate medium (data not shown). While arabinose
432 concentration <0.2% is not sufficient to promote biomass production, it allows the
433 cells to synthetize and maintain the required cofactor balance, and to efficiently
434 produce WEs.

435

436 Rapid advancements in the CRISPR/Cas9 technologies have broaden the tools
437 available for targeted genome engineering, and especially the employment of the
438 deactivated Cas9 (dCas9) has recently gained interest in the context of targeted gene
439 silencing (40, 41). While the dCas9 –based tools have been shown to be functional and
440 applicable in a wide range of (microbial) hosts, challenges related to unpredictability,
441 cellular burden and off-targeting may limit its use (42, 43). In addition, at least two
442 different constructs are typically required for the fine-tuned expression of dCas9 and
443 the RNA elements, and the system cannot be easily operated 'hands-free' without a
444 timed addition of an inducer. In this context, the system described here provides a
445 straight-forward, readily controllable, and reliable set-up for conditional and timed
446 gene knock-down. In addition, other interesting synthetic biology hosts such as *P.*
447 *putida* (44, 45) exhibit the same glucose dehydrogenase activity and could thus find

448 this strategy applicable. However, the transferability of this system to others hosts
449 such as *E. coli* and *S. cerevisiae* remains to be investigated in the future.
450
451 Our system serves as a simple and scalable method for dynamic, 'hands-free'
452 regulation of growth-essential reactions in the cell, which allows targeted and
453 adjusted biomass and product synthesis. Here, the dynamic regulation system was
454 exploited in the conversion of acetate to carbon-rich storage compounds, namely wax
455 esters, that are otherwise not efficiently accumulated in cells without optimized
456 conditions and high carbon-nitrogen ratio. Moreover, the system could be potentially
457 utilized and generalized for a broad range of synthesis pathways that are dependent
458 on acetyl-CoA (17, 46). In addition, other sustainable carbon sources, such as lignin-
459 derived compounds (47-49) would be compatible with this system.

460 **5. Conclusions**

461 We showed that an autonomously regulated genetic switch allowed the dynamic
462 decoupling of biomass and wax ester production in engineered *A. baylyi* ADP1, which
463 resulted in 3-4 fold improvements in the wax ester yield and titer compared to the
464 wild type strain. Shifting the cells from a biomass mode to a product synthesis mode
465 was achieved by gradually repressing the growth essential gene *aceA* by a simple and
466 robust set-up. The engineered strain produced 19% WEs of its cell dry weight, being
467 the highest reported among microbes. The study demonstrates the possibility to
468 bypass the challenges related to highly regulated storage lipid synthesis, and

strengthens the status of *A. baylyi* ADP1 as a convenient host for metabolic engineering and high-value lipid production from sustainable substrates.

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660 # Figure legends

661

662 **Figure 1.** An autonomously regulated switch for shifting the cells from a biomass to a
663 product synthesis mode. A) Wax ester synthesis strongly competes with biomass
664 production; In the wild type cells, efficient wax ester synthesis is triggered only in
665 defined conditions (i.e. in conditions with nitrogen starvation and excess carbon). In
666 normal growth conditions, most of the carbon is directed to biomass production and
667 cell maintenance. B) The ADP1 wild type strain utilizes acetate for biomass production
668 through a glyoxylate shunt in citric acid cycle. Isocitrate lyase (AceA) is the key enzyme
669 in the glyoxylate shunt and thus essential for growth on acetate. Strain lacking this
670 enzyme is unable to grow on acetate as the sole carbon source. C) The genetic
671 construct for dynamic regulation of *aceA* expression and growth. In the construct,
672 *aceA* is placed under the arabinose-inducible promoter AraC-pBAD. When cultured on
673 acetate, the arabinose used as the inducer is slowly oxidized by the native glucose
674 dehydrogenase Gcd of ADP1, gradually repressing the expression of AceA. Arabinose
675 oxidation does not serve as the carbon source or interfere with the acetate utilization,
676 thus being orthogonal to the circuit function. D) Along with the inducer depletion
677 (arabinose oxidation), the cells gradually shift from the biomass mode to the lipid
678 synthesis mode; the less there is arabinose left in the culture, a higher proportion of
679 the carbon flux is directed to the product. The amount of biomass can be simply
680 regulated by adjusting the initial arabinose concentration.

681

682 **Figure 2.** Growth of ADP1 wt and the engineered strain ADP1-ara-aceA in minimal
683 medium. The cells were cultured for 68 hours at 25 °C in MSM supplemented with 50
684 mM Na-acetate as the sole carbon source. Arabinose concentrations of 0; 0.1 and 1.0%
685 (for ADP1 wt 0 and 1%) were used for the induction. Optical densities representing
686 biomasses are presented as an average of two individual replicates.
687
688 **Figure 3.** Growth of ADP1-ara-aceA and ADP1 wt with different arabinose
689 concentrations. The cells were cultured in MA/9 medium supplemented with 25 mM
690 acetate, 0.1% cas.amino acids, and arabinose (0-1%) at 25 °C in micro well-plates for
691 21 hours. The optical densities are presented as the average of three different
692 replicate wells. For ADP1 wt, only the growth curve of the culture containing 1%
693 arabinose is shown.
694
695 **Figure 4.** Biomass and wax ester production by ADP1-ara-aceA induced with different
696 arabinose concentrations. ADP1-ara-aceA was cultured in MA/9 supplemented with
697 50 mM acetate, 0.1% casam, and arabinose (0, 0.1, 0.2, 0.5, or 1.0%) for 30 hours. A)
698 The amount of the produced biomass (at the end-point) was determined by optical
699 density (600 nm) measurement, presented as an average of two individual replica
700 cultures. B) Wax ester production in the cultures with different arabinose
701 concentrations were determined by thin layer chromatography analysis, for which 3
702 ml samples for taken from each culture. Jojoba oil was used as the standard.
703

704 **Figure 5.** Acetate utilization, arabinose oxidation, and the accumulation of wax esters
705 (WE) in the batch cultures of A) ADP1 wt and B) ADP1-ara-aceA. The cells were
706 cultivated in MA/9 medium supplemented with 50 mM acetate, 0.1% cas. amino acids,
707 and 0.5% arabinose for 12 h (ADP1 wt) or 38 h (ADP1-ara-aceA). For the arabinose and
708 acetate concentrations determined by HPLC, the average and standard deviation for
709 samples from two individual cultures are presented. Similarly, the WEs were
710 quantitatively analyzed by NMR from two individual cultures. The WE production at
711 different time-points is presented as mg/g CDW to demonstrate the different
712 accumulation patterns of the strains.
713
714

715 # Tables

716

717 **Table 1.** List of primers used in the study.

718

Name	***Sequence (5'-> 3')***
VS10_9	CAATGAATTCCGATAAAAGCGGATTCCTGAC
VS10_10	ATCCCATATGTAATTCCTCCTGTTAG
SS17_08	AATACATATGACATATCAATCAGCTCTTGAGC
SS17_09	TAATACAATTGCGAACAGGCTTATGTCAAGACGTC

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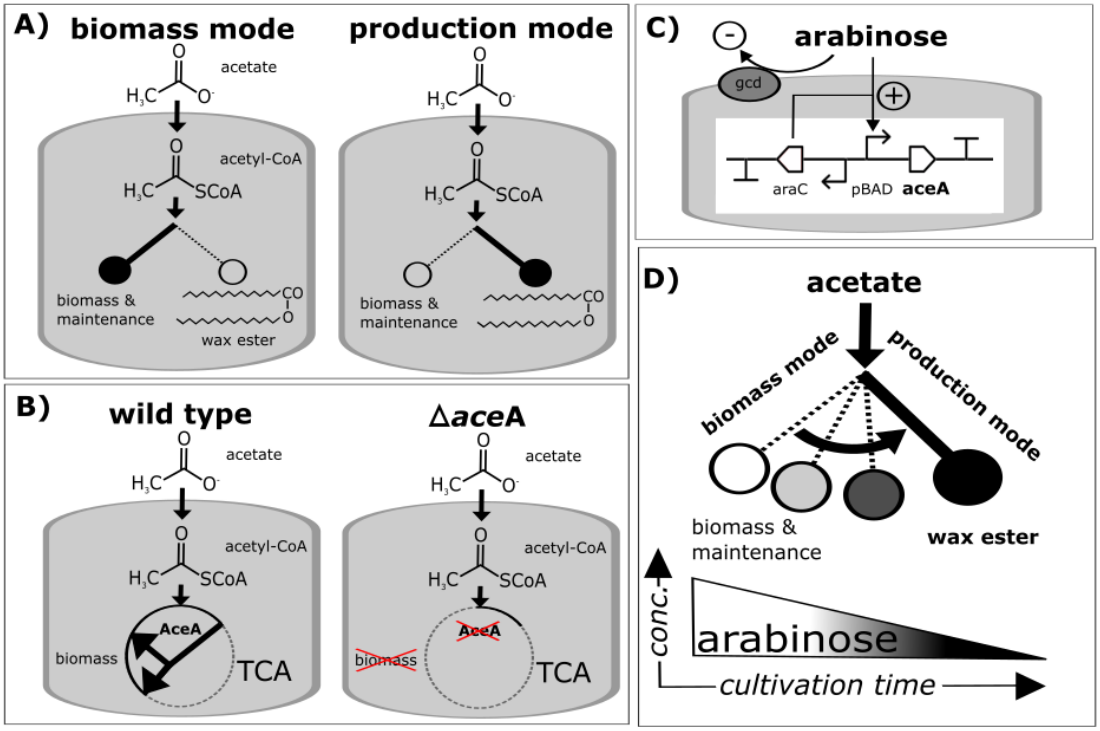
726

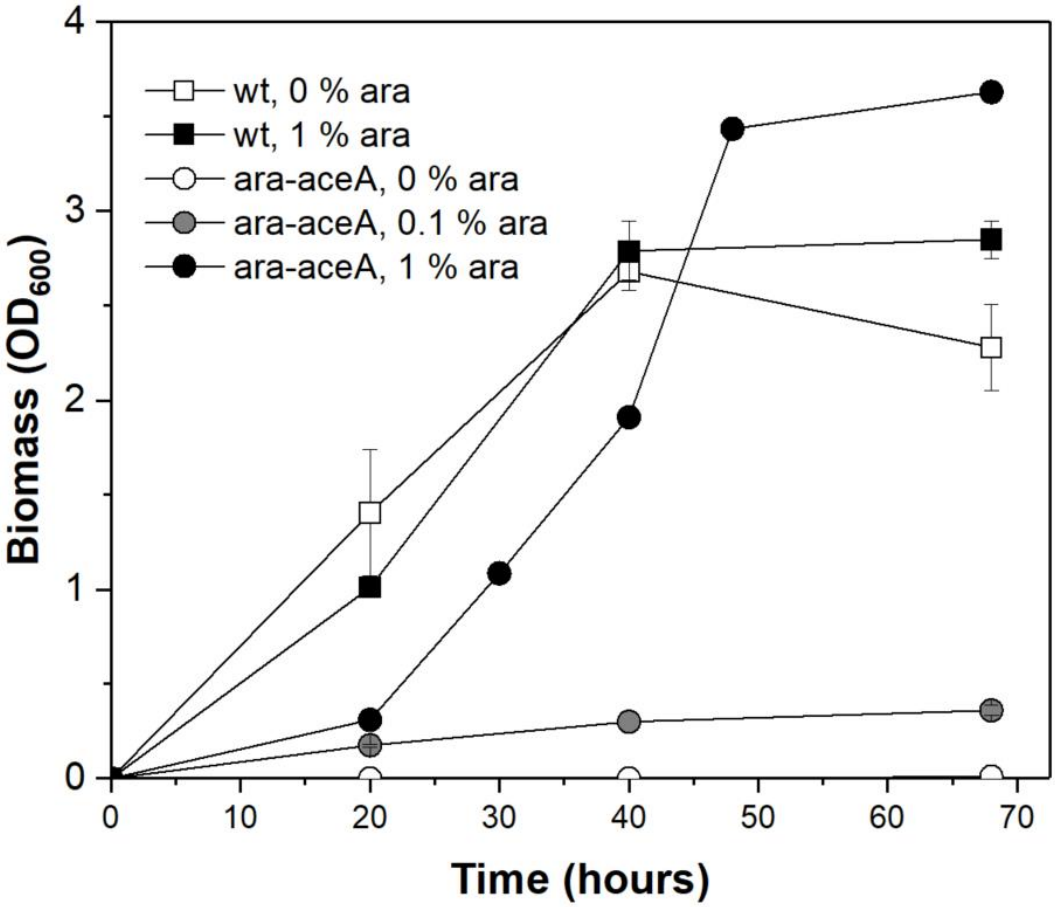
727 Figure 1.



728

729

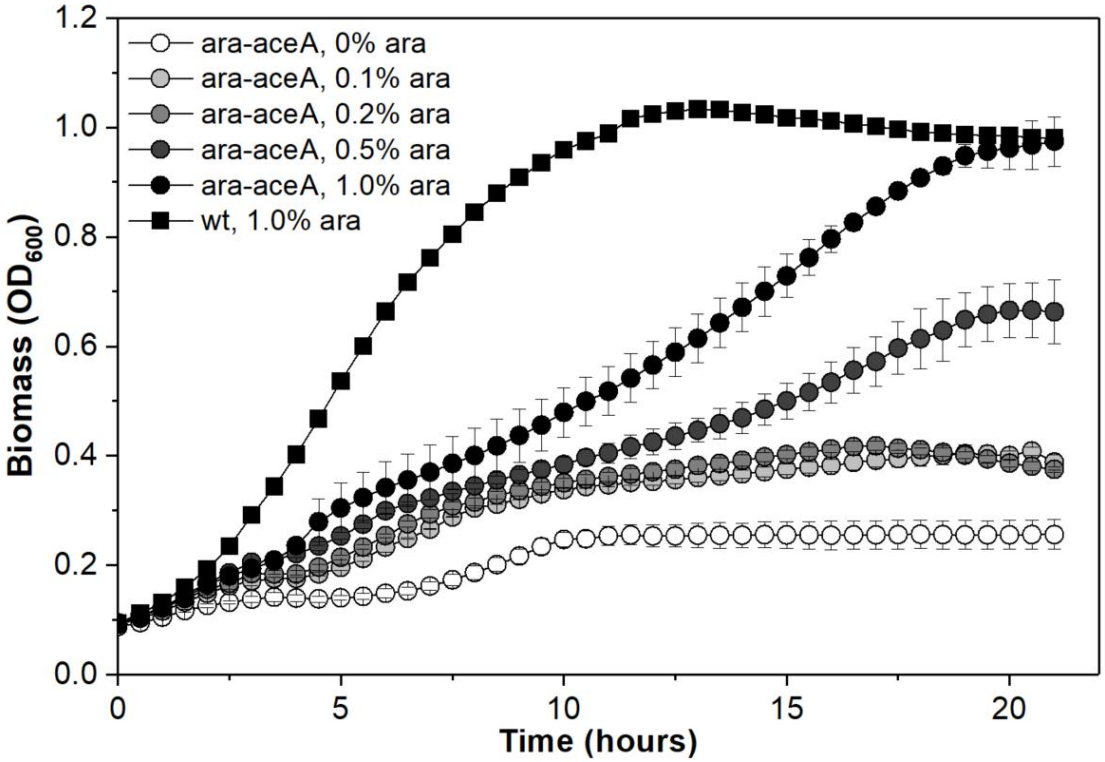
730 Figure 2.



731

732

733 Figure 3.



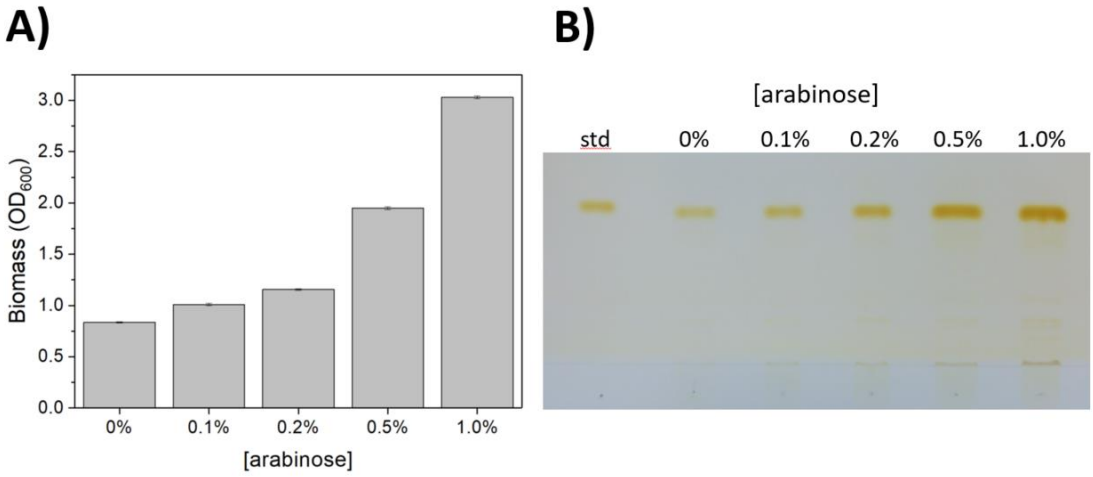
734

735

736

737 Figure 4

738

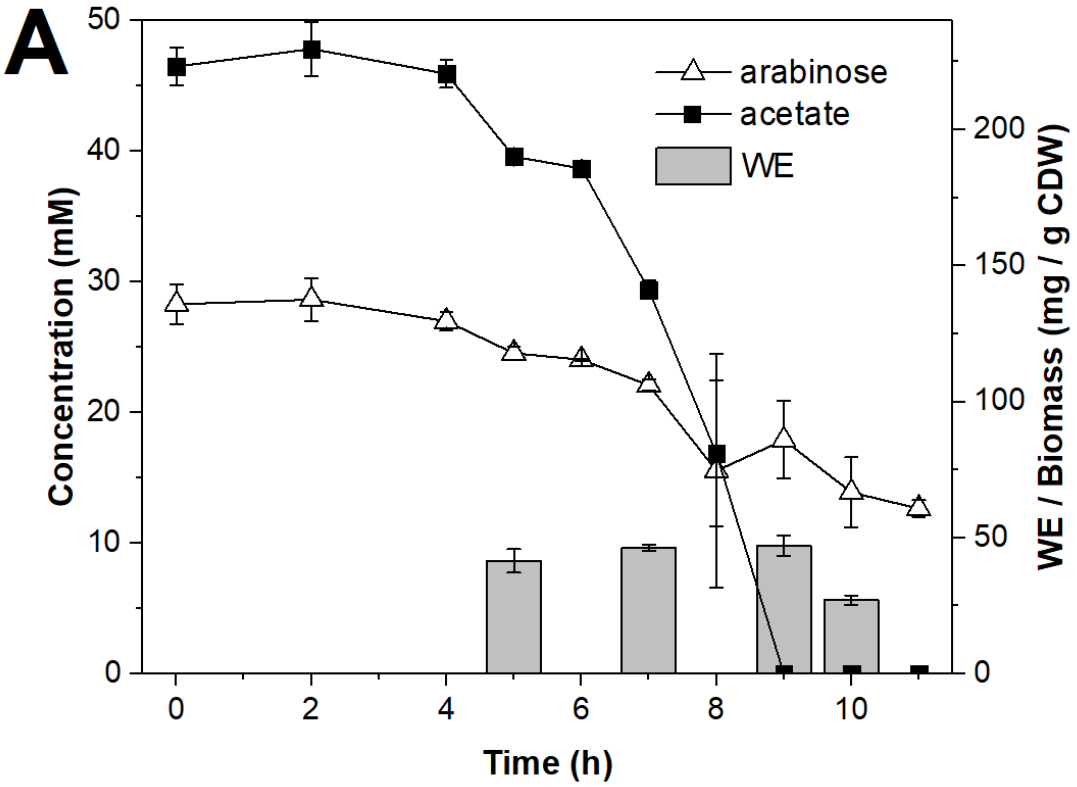


739

740

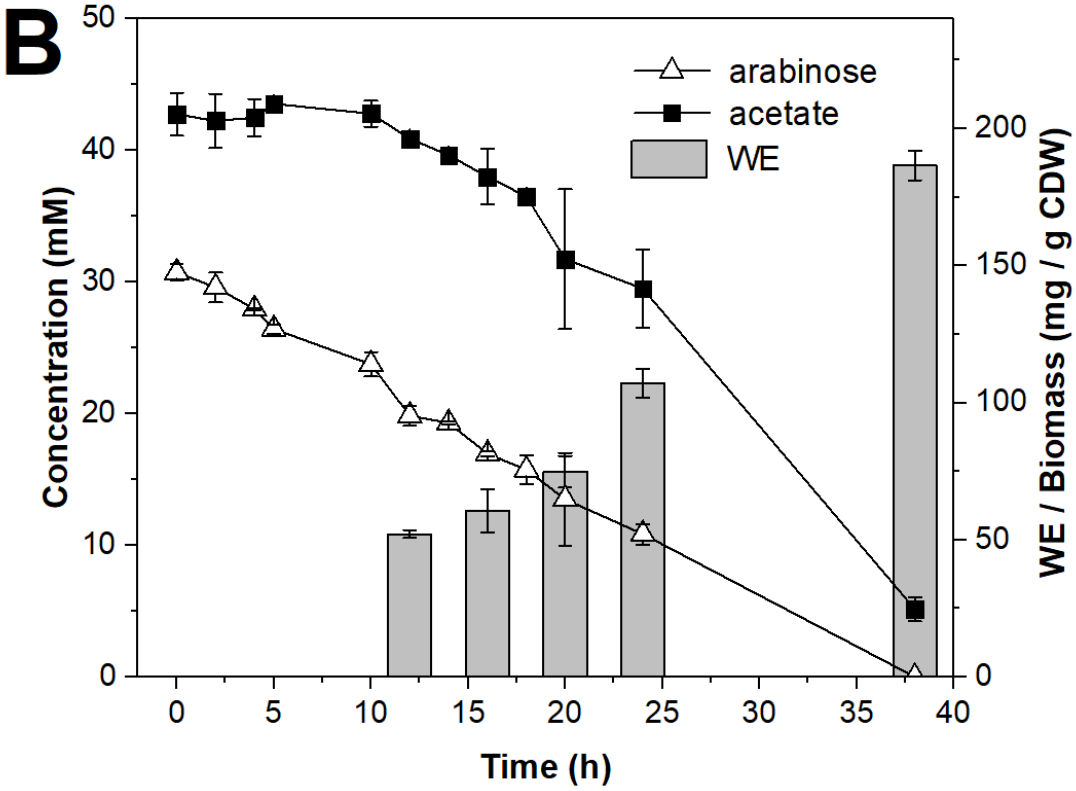
741

742 Figure 5A.



743

744


B
arabinose
acetate
WE
Concentration (mM)
WE / Biomass (mg / g CDW)
Time (h)
0
10
20
30
40
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0
50
100
150
200
0
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