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1 **Biotechnological production of acetoin, a bio-based platform**
2 **chemical, from lignocellulosic resource by metabolically**
3 **engineered *Enterobacter cloacae***

4

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21 **Abstract**

22 Acetoin (AC) is regarded as one of top potential sugar-derived chemical building
23 blocks that can be used as food additives, precursors in chemical synthesis, and plant
24 growth promoting molecules. In this study, a low-cost lignocellulosic resource of
25 pretreated corn stover was used as a carbon source to produce AC. After redirecting
26 the metabolic flux, fine tuning reducing power, and eliminating carbon catabolite
27 repression in *Enterobacter cloacae* SDM, a systematically engineered strain SDM 53
28 was constructed, which is able to utilize glucose and xylose efficiently and
29 simultaneously. Using fed-batch fermentation of SDM 53, 45.6 g L^{-1} AC was
30 produced at a rate of 1.52 g L^{-1} h^{-1} using the lignocellulosic hydrolysate.
31 Biotechnological synthesis of AC has various advantages such as being sustainable
32 and environment-friendly. With its desirable properties, the engineered strain SDM 53
33 may be a potential choice for the industrial production of AC.

34

35 **Introduction**

36 Acetoin (3-hydroxy-2-butanone or acetyl methyl carbinol, AC) is a volatile compound
37 that is widely used in foods, plant growth promoters, and biological pest controls.[1] In
38 addition, AC can also be used as a precursor for a variety of chemical compounds,
39 such as diacetyl and alkyl pyrazines, including 2,3,5,6-tetramethylpyrazine, a famous
40 herb in Chinese herbology.[1] Its versatile usage and potential for bulk industrial
41 production make AC one of the 30 platform chemicals that are given priority in
42 development and utilization by the United States Department of Energy.[2]

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43 Currently, commercially available AC is primarily obtained from chemical
44 synthesis of fossil feedstock. The chemical process involves radical reactions and may
45 have negative effects on the environment. Moreover, AC produced from chemical
46 synthesis is unsuitable and unsafe for use in some applications, especially in food
47 additives. In fact, some efforts have been made towards producing AC naturally by
48 biotechnological methods, which are alternatives for the green production of AC.
49 However, a number of bottlenecks, such as the increasing cost of raw materials and
50 low productivity, hinder the development of biotechnological AC production.[3]

51 Biomass is the most abundant renewable material and is more widespread than
52 fossil fuels.[4] The energy in biomass resource is so huge that it is calculated to be five
53 times of the world's energy consumption approximately.[5] In fact, the production of
54 myriad of products from biomass resources, such as biofuels (e.g. ethanol,[6]
55 isobutanol,[7] 1-butanol[8], and hydrogen[4]), biomaterials, and food/feed,[9] have received
56 more and more attention. The biomass-based sustainability revolution has thus arisen,
57 replacing the industrial revolution, and is regarded as the defining challenge in
58 meeting the increasing and simultaneous needs of energy, food, and environment.[10]

59 The dominant biomass sugars are glucose (C6) and xylose (C5). Although xylose
60 can be used by majority of the microbes, its consumption is always delayed due to
61 carbon catabolite repression (CCR), resulting in low volumetric productivity.[11] As a
62 bio-based platform chemical that has commercial uses, AC can be converted into
63 further value-added derivative compounds for the market requirement.[1] The
64 biorefinery manufacturing process not only has the potential to utilize bioresources,

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65 but may also have benefits in lowering the productive cost of AC and finally promote
66 its practical application.[12] Thus, it is important to construct an AC producer that could
67 efficiently produce AC from the carbohydrate of lignocellulosic resource without
68 CCR.

69 In microorganisms, AC is an intermediate product of the 2,3-butanediol (BD)
70 fermentation-pathway. Three enzymes involved in the BD biosynthesis from pyruvate
71 include α-acetolactate synthase (ALS, encoded by *budB*), α-acetolactate
72 decarboxylase (ALDC, encoded by *budA*), and acetoin reductases (ARs, also called
73 DRs or BDHs, encoded by *budC* and *gdh*) (Fig. 1).[13-15] *Enterobacter cloacae* subsp.
74 *dissolvens* strain SDM is theoretically regarded as an efficient producer of AC, as it is
75 able to grow rapidly in simple medium and efficiently metabolize the major
76 lignocellulose-derived sugars glucose and xylose into BD.[15] In this study, an
77 engineered strain SDM 53 was constructed after systematical genetic modification
78 and tight cofactor manipulation in *E. cloacae* SDM. Considering its desirable
79 characteristics, this systematical engineered strain may be a promising alternative for
80 the AC production using the lignocellulosic resource.

81

82 **Materials and methods**

83 **Enzymes and chemicals**

84 Racemic AC and BD were purchased from Sigma. NADH was purchased from Roche
85 (USA). Restriction enzymes were purchased from ThermoFisher (USA). Polymerase
86 chain reaction (PCR) primers were provided by Sangon (Shanghai, China). FastPfu

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87 DNA polymerase and T_4 DNA ligase were purchased from Transgen Biotech (China)
88 and ThermoFisher (USA), respectively. Lignocellulosic hydrolysate from the
89 pretreated corn stover, which contained glucose of 411.0 g L^{-1}, xylose of 140.8 g L^{-1},
90 cellobiose of 39.3 g L^{-1} and arabinose of 5.0 g L^{-1}, was kindly provided by Changchun
91 Dacheng Group Co. Ltd. (China). The hydrolysate also contained some other
92 constituents, including 5-hydroxymethylfurfural of 2.1 g L^{-1}, furfural of 1.8 g L^{-1},
93 acetate of 1.8 g L^{-1} and formate of 22.7 g L^{-1}. All other chemicals were of analytical
94 grade and commercially available.
95
96 **Bacterial strains and plasmids**
97 *E. coli* DH5α was used for general cloning procedures. The genomes of *Lactobacillus*
98 *brevis* CICC 6004 and *Klebsiella pneumoniae* LZ were used as templates for the
99 amplification of NADH oxidase (NOX) and NADH dehydrogenase (NDH),
100 respectively. The pKR6K was used for gene knock-out in *E. cloacae* strain SDM.[16] *E.*
101 *coli* S17-1, which is able to host pKR6K and its derivatives, was used for conjugation
102 with *E. cloacae* SDM.[17] The pET28a (+) and $pETP_C$ were used for overexpression of
103 genes in *E. cloacae* (Table 1).
104 Lysogenic broth (LB) medium was used for the culture of *E. coli* and *E. cloacae*.
105 The selection medium in the conjugation experiments was M9 minimal medium[18]
106 supplemented with 1% sodium citrate as the sole carbon source. Solid LB medium
107 supplemented with 12% sucrose was used to select plasmid excision from the
108 chromosome during the gene allelic exchange experiments. The LBE medium, i.e.,

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109 LB supplemented with 0.7 mM EDTA, was used for the preparation of
110 electrocompetent cells of *E. cloacae*.
111 M9 minimal medium[18] supplemented with 5 g L^{-1} yeast extract was used for the
112 comparison of the capability of AC production between engineered strains. The batch
113 and fed-batch fermentation media was consisted of (g L^{-1}): beef extract 5, corn steep
114 liquor 6, urea 2, and adjusted to pH 7.0 before sterilized. Then sterilized glucose
115 solution or mixed sugars or enriched lignocellulosic hydrolysate was added before
116 fermentation. Kanamycin was used at a concentration of 50 μg mL^{-1}.
117

118 **Gene knock-out in *E. cloacae* SDM**

119 The primers used in this study are listed in Table S1. Vector isolation, restriction
120 enzyme digestion, agarose gel electrophoresis, and other DNA manipulations were
121 carried out by using standard protocols.[19] Knock-out mutants of *E. cloacae* strain
122 SDM were generated via allele exchange using the suicide plasmid pKR6K. *E. coli*
123 S17-1, which is able to host pKR6K and its derivatives, was used for conjugation with
124 *E. cloacae* SDM. The construction of mutant alleles and the methods of gene
125 knock-out were determined by the methods described in the previous reports.[20]
126

127 **Gene cloning and expression in engineered strain**s

128 The genes *nox* (GenBank: 22653410) from *L. brevis* CICC 6004 and *ndh* from *K.*
129 *pneumoniae* LZ were amplified through PCR with the primer pairs
130 NOX-f(*NdeI*)/NOX-r(*SalI*) and NDH-f(*NdeI*)/NDH-r(*SalI*), respectively. (The

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131 sequence of *ndh* in *K. pneumoniae* LZ is consistent with this gene in *K. pneumoniae*
132 MGH 78578. GenBank: 150954509). The gene *vhb* (GenBank: 311024) was
133 synthesized and sequenced by BGI (China) and then amplified through PCR with the
134 primer pairs VHb-f(*NdeI*)/VHb-r(*SalI*). All the three genes were digested with *NdeI*
135 and *SalI*, and then introduced into the *NdeI*/*SalI* site of expression vector $pETP_C$ to
136 construct the vectors designated $pETP_C$-*nox,* $pETP_C$-*ndh*, and $pETP_C$-*vhb*, respectively.
137 These vectors were then transformed into *E. cloacae* SDM or its derivatives by
138 electroporation to produce the respective engineered strains.

139 To construct the vector with *nox* and *galP* co-expression, we amplified the gene
140 fragment P_5-*nox* through PCR by promoter pairs P_5-*nox*-f1(*BglII*)/P_5-*nox*-r2, with
141 plasmid $pETP_5$-*nox* as template. And the gene fragment P_b-*galP* was amplified
142 through PCR by promoter pairs P_b-*galP*-f3/P_b-*galP*-r4(*SacI*), with plasmid
143 $pETP_b$-Bpbdh-P_b-*galP* as template. The DNA fragments P_5-*nox* and P_b-*galP* were
144 then ligated through gene splicing by overlap-extension, and inserted into the
145 *BglII*/*SacI* site of pET28a (+). The vector $pETP_5$-*nox*-P_b-*galP* was then transformed
146 into SDM 46 by electroporation to produce the engineered strain SDM 53.

147 Methods of electrocompetent cells preparation and
148 electroporation-transformation were cited from Wei et al[21] with slight modification:
149 The electrocompetent cells of *E. cloacae* SDM or its derivatives were prepared from
150 culture grown in LBE medium at 37°C. After the cell density reached to OD_{600nm} of
151 0.5–0.7, culture was immediately moved onto ice for 30 min. Then, the cells were
152 washed twice with cold ultrapure water, and resuspended with sterile water to a final

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153 OD_{600nm} of 50. Bio-Rad MicroPulser and 2-mm electroporation cuvette were needed
154 in electroporation-transformation. The instrument was set to 2.0 kV, 200 Ω, and 25 uF.
155 The transformants of *E. cloacae* were then screened from the LB plate supplemented
156 with kanamycin at 37°C.
157

158 **Promoter library construction**

159 Five promoters of varying strength, which were cited from Alper et al.,[22] were
160 synthesized and sequenced by BGI (China). The sequences of promoter were listed at
161 Table S2. All the promoters were amplified through PCR with the primer pairs
162 Promoter-f(*BglII*)/Promoter-r(*XbaI*), and digested with *BglII* and *XbaI*, then
163 introduced into the *BglII*/*XbaI* site of expression vector $pETP_C$-*nox* to replace the P_C
164 promoter.
165

166 **Enzyme activity assays**

167 Cells of these engineered strains were grown for 8 h, then centrifuged at 13,000 × g
168 for 5 min, and washed twice with 67 mM phosphate buffer (pH 7.4). Cells were
169 finally resuspended to an OD_{600nm} of 30 with 67 mM phosphate buffer (pH 7.4), and
170 disrupted with an ultrasonic cell breaking apparatus (Xinzhi, Ningbo, China). Cell
171 debris was removed through centrifugation at 13,000 × g for 30 min. Enzyme
172 activities were assayed in the resulting supernatant.[23]

173 The activity of acetoin reductase (AR) was assayed spectrophotometrically by
174 measuring the change in absorbance at 340 nm corresponding to the oxidation of

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175 NADH (ε_{340} = 6,220 M^{-1} cm^{-1}) using a UV/visible spectrophotometer (Ultrospec 2100
176 pro, Amersham Biosciences, USA). The reaction solution contained 5 mM of acetoin
177 and 0.2 mM of NADH in 67 mM phosphate buffer (pH 7.4). One unit of activity was
178 defined as the amount of enzyme that consumed 1 μmol of NADH per min. The
179 protein concentration was measured by the Lowry method, with bovine serum
180 albumin as the standard.[24] The assay of NADH oxidase (NOX) activity was similar
181 with AR, with some modification in reaction solution. The reaction solution of NOX
182 contained 0.2 mM of NADH in 67 mM phosphate buffer (pH 7.4).
183
184 **Analytical methods**
185 Samples were withdrawn periodically and centrifuged at 13,000 × *g* for 10 min. The
186 cell density was determined by monitoring the absorbance at 600 nm using a
187 spectrophotometer (LENGGUANG-721, China) after an appropriate dilution. The
188 concentration of glucose was measured enzymatically by a bio-analyzer (SBA-40D,
189 Shandong Academy of Sciences, China) after an appropriate dilution with ultrapure
190 water. Concentrations of BD and AC were analyzed by GC as described in Ma et al.[25]
191 Concentrations of by-products were analyzed by the high-performance liquid
192 chromatography system (HPLC) as described in Li et al.[20]
193
194 **Batch and fed-batch fermentations**
195 Batch fermentation was conducted in 500-mL shake flasks containing 100 mL of
196 medium. Cultivation was carried out at 37°C and 180 rpm. The initial pH was

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197 adjusted to 7.0. Samples were withdrawn periodically to determine the cell density,
198 concentrations of glucose, AC, BD and by-products.
199 Fed-batch fermentation was carried out in a 7.5-L bioreactor (CelliG 310, NBS,
200 USA) with an initial glucose concentration of 80 g L^{-1} and broth volume of 4 L. Both
201 cultivations were performed at 37°C with an aeration rate of 1 vvm and agitation
202 speed of 500 rpm. The dissolved oxygen concentration was controlled no less than 5%
203 during fermentation. The pH was maintained at 6.5 by automatic addition of 6 M
204 H_3PO_4 or 6 M NaOH using computer-coupled peristaltic pump. The fed-batch
205 fermentations were conducted by feeding glucose solution or lignocellulosic
206 hydrolysate when the residual glucose in the fermentation broth decreased to
207 approximately 20 g L^{-1}.
208

209 **Results and discussion**

210 **Enhanced AC production by blocking the main pathway from AC to BD**

211 *E. cloacae* SDM has an extraordinary ability of utilizing biomass for BD production,
212 during which the intermediary AC is formed. As shown in Wang et al.,[26] *E. cloacae*
213 SDM produced 93.9 g L^{-1} BD during 47 h of fermentation using cassava powder as
214 substrate, but produced only 5.3 g L^{-1} of AC. This result suggests that acetoin
215 reductases (ARs, also called DRs or BDHs) in this strain direct the main AC reduced
216 to BD. Two enzymes, AR-I and AR-II that are, respectively, encoded by *gdh* and *budC*
217 were found to be capable of catalyzing the reduction of AC to BD. Then the *gdh* and
218 *budC* genes were knocked out individually or in combination. As shown in Fig. 2 and

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219 Table 2, inactivation of *gdh* or/and *budC* reduced the enzyme activity of AR, and had
220 a significant improvement on AC production. After glucose was consumed nearly
221 completely, AC concentrations of 27.6 g L^{-1} and 29.8 g L^{-1} were obtained from strains
222 SDM 12 and SDM 13, respectively, compared to AC concentration of 2.7 g L^{-1}
223 obtained from *E. cloacae* SDM (Table 2). Moreover, when the enzyme activity of AR
224 decreased, the ratio of AC to BD improved (Fig. 2). Both results demonstrate that the
225 inactivation of ARs has positive effects on AC production. However, enhanced
226 accumulation of some reduction by-products was observed, such as lactate and
227 ethanol, after ARs inactivation (Table 3). This result suggests that the inactivation of
228 ARs limits the regeneration of NAD^+ in engineered strains. Thus, an enzyme that can
229 regenerate NAD^+ *in vivo* is needed in our successive investigation.

230 In addition, as shown in Table 2, although the *gdh* and *budC* double knock-out
231 strain SDM 13 accumulates slightly higher concentrations of AC than the *budC* single
232 knock-out strain SDM 12, SDM 12 shows higher AC productivity in comparison to
233 strain SDM 13. Moreover, higher cell density and glucose consumption rate were
234 reached by strain SDM 12 (Table 2). Therefore, *E. cloacae* SDM 12 was chosen for
235 further investigation for AC production eventually.

236

237 **NADH oxidase is chosen for NAD^+ regeneration in SDM 12**

238 NADH oxidase (NOX), NADH dehydrogenase (NDH), and *Vitreoscilla* hemoglobin
239 (VHb) were reported to have the capability to improve the NAD^+/NADH ratio *in*
240 *vivo*.[27-29] In this study, the genes encoding these three enzymes were cloned in plasmid

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241 pETP$_C$, a modified pET28a (+) vector with the constitutive promoter P_C, to produce
242 pETP$_C$-*nox*, pETP$_C$-*ndh*, and pETP$_C$-*vhb*, respectively. These expression constructs
243 were subsequently transformed into SDM 12 to produce strains SDM 21, SDM 22,
244 and SDM 23, respectively. As shown in Fig. 3A, compared with the control strain
245 SDM 24, all three of the modified strains increased the ratios of NAD^+ to NADH,
246 suggesting that they have the capability of decreasing the reducing force *in vivo*.
247 Moreover, the highest ratio of NAD^+ to NADH (improved 1.5 times compared to that
248 exhibited by SDM 24), and the highest titer of AC (25.3 g L^{-1}) was obtained when
249 NOX was overexpressed (Fig. 3B). Thus, NOX was chosen to regenerate the NAD^+ in
250 subsequent experiments.
251

252 **Enhancement of AC yield by fine tuning NAD^+/NADH ratio in vivo**

253 It has been reported that NOX can regenerate NAD^+, and has positive effect on the
254 production of AC.[27,] In our study, however, overexpression of NOX, by using the P_C
255 promoter, only increased the yield of AC from 68.1% to 70.4% (%, AC mol/mol
256 glucose) (Fig. 3). Thus, tightly control of NOX expression may be required for the
257 improvement of AC yield and titer. Two ways of accomplishing this include: (1) using
258 an inducible promoter at varying inducer concentrations (e.g., isopropyl
259 *β*-D-1-thiogalactopyranoside, IPTG); (2) using a constitutive promoter library with
260 varying promoter strengths. Although inducible promoters allow for continuous
261 control of expression, these systems are limited in practical applications by
262 high-priced inducer costs and hypersensitivity to inducer concentration.[22] Thus, in this

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263 study, a constitutive promoter library was the preferred method chosen for tightly
264 control the overexpression of NOX.

265 The P_C promoter in pETP$_C$-*nox* was subsequently replaced by promoters of
266 varying strengths, forming the following five plasmids: pETP$_1$-*nox*, pETP$_2$-*nox*,
267 pETP$_3$-*nox*, pETP$_4$-*nox*, and pETP$_5$-*nox*. These plasmids were transformed into strain
268 SDM 12 by electroporation. As shown in Fig. 4A and Fig. 4B, in seven engineered
269 strains with varying promoter strength, the overexpression of NOX is successfully
270 controlled, with the specific activity of NOX decreasing from 53.4 U mg^{-1} to 7.3 U
271 mg^{-1}. Moreover, the *in vivo* NAD$^+$/NADH ratio is closely reflected by the specific
272 activity of NOX. This indicates that the reducing force *in vivo* can be fine tuned by
273 controlling the overexpression of NOX or other similar enzymes. Finally, as shown in
274 Fig. 4C and Fig. 4D, the SDM 35 strain containing P_5 promoter had the highest titer
275 (29.3 g L^{-1}), yield of AC (3.5% higher compared to control strain SDM 24), and ratio
276 of AC to BD (7.9).

277

278 **Inactivation of by-product pathways**

279 Although fine tuning of reducing power *in vivo* improved the AC yield in strain SDM
280 35, by-products accumulated at the end of fermentation, including ethanol, succinate,
281 and lactate at concentrations of 3.2 g L-1, 2.2 g L-1, and 0.04 g L^{-1}, respectively. In *E.*
282 *cloacae* SDM, the formation of lactate, succinate, and ethanol is catalyzed by *ldhA*
283 (GenBank: 392324837), *frdA* (GenBank: 392323201), and *adhE* (GenBank:
284 392325376), respectively. To achieve higher yield of AC, these genes were knocked

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285 out individually in strain SDM 12. The mutant strains were cultured at 37°C in
286 500-mL shake flasks containing 100 mL M9 medium supplemented with 90.0 g L^{-1}
287 glucose and 5.0 g L^{-1} yeast extract. The initial pH was 7.0, and the fermentation was
288 finished when glucose was consumed nearly completely.
289 As shown in Fig. 5, the *adhE*/*budC* double mutant strain SDM 43 displayed the
290 highest AC yields compared to the other double mutant strains. However, in all
291 engineered strains, SDM 43 showed the lowest productivity of 0.87 g L^{-1} h^{-1}. Since
292 AdhE modulates the level of NADH,[30] its inactivation may result in slow growth and
293 glucose utilization. Given the positive effects on AC yield displayed by the other two
294 double mutant strains (*budC*/*frdA* and *budC*/*ldhA*) (Fig. 5), we subsequently
295 constructed the *budC*/*frdA*/*ldhA* triple mutant strain SDM 44. As shown in Fig. 5, a
296 high AC yield of 76% (%, mol AC/mol glucose) was obtained.
297
298 **Utilization of glucose in fed-batch fermentation**
299 In this study, the *budC*/*frdA*/*ldhA* triple-mutant strain SDM 44 was further modified to
300 overexpress NOX using promoter P_5, generating strain SDM 45. To detect AC
301 production using glucose as a carbon source, fed-batch fermentation was carried out
302 by strain SDM 45 in a 7.5-L fermenter with an operating volume of 4 L.
303 As shown in Fig. 6A, 55.2 g L^{-1} AC and 15.2 g L^{-1} BD were obtained from 148.0
304 g L^{-1} glucose after 20.5 h of fermentation. The highest productivity of AC was 2.69 g
305 L^{-1} h^{-1} that was reported to data, and the yield of AC was 74.6%. As shown in Fig. 6B,
306 the major by-products in this fermentation are acetate and ethanol, which were found

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307 at concentrations of 5.3 g L^{-1} and 3.6 g L^{-1}, respectively. All other by-products,
308 including succinate, lactate and formate, were found at less than 0.4 g L^{-1}.
309

310 **Simultaneous utilization of glucose and xylose in batch fermentation**

311 Lignocellulosic resource, one of the most accessible and renewable carbon source,
312 was reported to generate a solution containing primarily glucose and xylose.[31] In this
313 study, the ratio of these two sugars of the lignocellulosic resource was detected
314 approximately at 3:1 (w/w). In the batch fermentation, the engineered strain SDM 45
315 is capable of utilizing glucose and xylose as carbon sources. However, as shown in
316 Fig. 7A, the consumption of xylose began only when glucose was consumed nearly
317 completely. After 35 h of fermentation, 21.7 g L^{-1} AC was produced while 10.4 g L^{-1}
318 xylose remained in the fermentation broth.

319 To eliminate the carbon catabolite repression (CCR) in strain SDM 45, the gene
320 encoding the major glucose transporter $IICB^{Glc}$ in PTS, *ptsG* (GenBank: 392324502)
321 was knocked out. As shown in Fig. 7B, the resulting strain SDM 51 is capable of
322 co-utilizing glucose and xylose. However, this strain showed decreasing rate of
323 glucose consumption.

324 Although 23.3 g L^{-1} AC accumulated after 35 h of fermentation, 7.0 g L^{-1} glucose
325 remained in the fermentation broth by strain SDM 51. To improve glucose utilization,
326 a galactose permease encoding gene (*galP*, GenBank: 392326539) was
327 over-expressed in strain SDM 51. As shown in Fig. 7C, the resulting strain SDM 53
328 displays a good ability to consume glucose and xylose simultaneously. After 35 h of

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329 fermentation, 27.5 g L^{-1} AC was produced from 55.8 g L^{-1} glucose and 23.5 g L^{-1}
330 xylose, and these sugars were almost completely consumed. Batch-fermentation was
331 also conducted using mixed sugars with different ratios of glucose to xylose (from 3:1
332 to 1:3), which are the major two fermentable sugars in various lignocellulose
333 materials. As shown in Fig. S1, production of AC was similar when the mixed sugars
334 were used, which suggested that the systematically engineered strain SDM 53 may
335 have the potential to utilize various lignocellulose materials from different plant
336 sources to produce AC.
337
338 **Utilization of the lignocellulosic hydrolysate in fed-batch fermentation**
339 Fed-batch fermentation using the lignocellulosic hydrolysate as carbon source by
340 strain SDM 53 was also carried out. As shown in Fig. 8A, the lignocellulosic
341 hydrolysate was fed into the fermentation broth to maintain the glucose concentration
342 at no less than 20.0 g L^{-1}. After 30 h of fermentation, 45.6 g L^{-1} AC was obtained, and
343 the productivity and yield of AC were 1.52 g L^{-1} h^{-1} and 58.4%, respectively. In
344 addition, the major by-products were acetate and formate, which were found at
345 concentrations of 4.0 g L^{-1} and 2.4 g L^{-1}, respectively (Fig. 8B).
346 Many researches have focused on the production of AC via microbial
347 fermentation (Table 4). Sun et al. demonstrated that overexpression of water forming
348 NOX in *Serratia marcescens* could increase the intracellular NAD^+ concentration and
349 NAD^+/NADH ratio, resulting in a high accumulation of 75.2 g L^{-1} AC.[27] Known as
350 GARS (generally regarded as safe), *Bacillus* strains have also shown excellent

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351 performances for the production of AC. For example, after inactivation of acetoin
352 reductase AR and moderate expression of NOX, the recombinant *Bacillus subtilis*
353 BMN accumulated AC of 56.7 g L^{-1} and had a yield of 75.6% from glucose.[32] Another
354 GARS strain, *Bacillus amyloliquefaciens* E-11 accumulated 71.5 g L^{-1} AC at a high
355 yield of 84.5% from glucose after enhancing AC tolerance.[33]

356 However, current supply of commercial AC is primarily from fossil feedstocks
357 via chemical synthesis, and the production cost for AC is still lower than that via
358 microbial fermentation (personal communication, Apple Flavor & Fragrance Group
359 Co., Ltd,China). Thus, more attention should be focused on sustainable production of
360 AC using the renewable, accessible and low-cost carbon sources such as
361 lignocellulose. In this study, four strategies of metabolic engineering were carried out
362 for enhancing AC production: choosing a suitable host bacterium, redirecting the
363 metabolic flux, fine tuning the reducing power, and eliminating the carbon catabolite
364 repression. *E. cloacae* can rapidly grow in a simple medium and displays a wide
365 substrate spectrum.[15] In addition, this bacterium can tolerate high salinity and high
366 osmotic conditions.[34, 35] With these superior characteristics, *E. cloacae* was chosen as
367 the host for the production of AC. Then, gene *budC* was knocked out for reducing the
368 transformation of AC to BD, and the pathways of by-products were blocked for
369 enhancing the precursor pools of AC. After controlling the overexpression of NOX,
370 the reducing power (NAD^+/NADH ratio) was successfully fine tuned. Finally, after
371 gene *ptsG* was inactivated and gene *galP* was overexpressed, the co-utilization of
372 glucose and xylose was realized.

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373 All these strategies have gained positive effect on the accumulation of AC.
374 However, the improvement of AC yield is not so ideal when the reducing power was
375 tuned through regulating the NOX overexpression. This suggests that the reducing
376 power might not so crucial for the accumulation of AC in *E. cloacae*. In contrast, both
377 enhancing the accumulation of precursor and decreasing the further reduction have
378 obvious improvement on AC accumulation (Table 2, Fig. 5). The biological
379 production of many other platform chemicals, for example, ethanol, BD, iso-butanol
380 and n-butanol, has also gained a lot of attentions[6-8, 36]. Until now, BD has gained a
381 higher titer of 152.0 g L^{-1} than other platform chemicals,[36] largely because of its
382 low-toxicity to microorganisms. Thus, as shown of the studies of Luo et al., selecting
383 the AC tolerance strain would be another recommendable method for further
384 enhancing AC production.[33]
385

386 **Conclusions**

387 Efficient producers of AC were successfully redesigned by blocking AC reduction to
388 BD and fine tuning NAD^+/NADH *in vivo* by tightly controlling the overexpression of
389 NOX, a water-forming NADH oxidase. In addition, the by-product pathways of
390 succinate and lactate were blocked in order to redistribute the metabolic flux to AC.
391 By using the engineered strain SDM 45, 55.2 g L^{-1} of AC was produced using glucose
392 as substrate. The productivity was 2.69 g L^{-1} h^{-1}, which is the highest productivity of
393 AC reported thus far. Furthermore, the engineered strain SDM 53 was able to utilize
394 glucose and xylose simultaneously and efficiently after inactivation of *ptsG* and

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395 overexpression of *galP*. Using the lignocellulosic hydrolysate of the pretreated corn
396 stover as carbon source, 45.6 g L^{-1} of AC accumulated with a productivity of 1.52 g
397 L^{-1} h^{-1}.

398

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405

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Table 1 Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strain		
E. coli DH5α	F^-, φ*80 lacZΔM15*, Δ(*lacZYA-argF*)*U169*, *recA1*, *endA1*, *hsdR17*, *phoA*, *supE44*λ^-, thi^{-1}, *gyrA96*, *relA1*	Novagen
E. coli S17-1	*recA*, *pro*, *thi*, conjugative strain able to host λ-pir-dependent plasmids	[17]
E. cloacae SDM	Wild-type	[26]
Lactobacillus brevis CICC 6004	Wild-type	CICC 6004
Klebsiella pneumoniae LZ	Wild-type	[37]
SDM 11	*E. cloacae* SDM Δ*gdh*	This study
SDM 12	*E. cloacae* SDM Δ*budC*	This study
SDM 13	*E. cloacae* SDM Δ*budC*Δ*gdh*	This study
SDM 21	*E. cloacae* SDM Δ*budC*/$pETP_C$-*nox*	This study
SDM 22	*E. cloacae* SDM Δ*budC*/$pETP_C$-*ndh*	This study
SDM 23	*E. cloacae* SDM Δ*budC*/$pETP_C$-*vhb*	This study
SDM 24	*E. cloacae* SDM Δ*budC*/$pETP_C$	This study
SDM 31	*E. cloacae* SDM Δ*budC*/$pETP_1$-*nox*	This study
SDM 32	*E. cloacae* SDM Δ*budC*/$pETP_2$-*nox*	This study

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SDM 33	*E. cloacae* SDM Δ*budC*/pETP$_3$-*nox*	This study
SDM 34	*E. cloacae* SDM Δ*budC*/pETP$_4$-*nox*	This study
SDM 35	*E. cloacae* SDM Δ*budC*/pETP$_5$-*nox*	This study
SDM 41	*E. cloacae* SDM Δ*budC*Δ*ldhA*	This study
SDM 42	*E. cloacae* SDM Δ*budC*Δ*frdA*	This study
SDM 43	*E. cloacae* SDM Δ*budC*Δ*adhE*	This study
SDM 44	*E. cloacae* SDM Δ*budC*Δ*frdA*Δ*ldhA*	This study
SDM 45	*E. cloacae* SDM Δ*budC*Δ*frdA*Δ*ldhA*/pETP$_5$-*nox*	This study
SDM 46	*E. cloacae* SDM Δ*budC*Δ*frdA*Δ*ldhA*Δ*ptsG*	This study
SDM 51	*E. cloacae* SDM Δ*budC*Δ*frdA*Δ*ldhA*Δ*ptsG*/pETP$_5$-*nox*	This study
SDM 53	*E. cloacae* SDM Δ*budC*Δ*frdA*Δ*ldhA*Δ*ptsG*/pETP$_5$-*nox*-P_b-*galP*	This study
Plasmid		
pET28a (+)	Km^r, expression vector, pMB1 replicon	Novagen
pKR6K	Km^r, gene replacement vector derived from plasmid pK18*mobsacB*, R6K origin, Mob$^+$ *sacB*	[16]
pKΔ*budC*	pKR6K derivative, carries a 639 bp deletion of *budC*	[20]
pKΔ*gdh*	pKR6K derivative, carries a 302 bp deletion of *gdh*	[23]

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pKΔ*ldhA*	pKR6K derivative, carries a 420 bp deletion of *ldhA*	20
pKΔ*frdA*	pKR6K derivative, carries a 817 bp deletion of *frdA*	20
pKΔ*adhE*	pKR6K derivative, carries a 1679 bp deletion of *adhE*	This study
pKΔ*ptsG*	pKR6K derivative, carries a 473 bp deletion of *ptsG*	20
pETP$_C$	Km^r, the promoter P_{T7} of pET28a (+) replaced by P_C	38
pETP$_C$-*nox*	pETP$_C$ carrying gene *nox*, originated from *L. brevis* CICC 6004	This study
pETP$_C$-*ndh*	pETP$_C$ carrying gene *ndh*, originated from *K. pneumoniae* LZ	This study
pETP$_C$-*vhb*	pETP$_C$ carrying gene *vhb*, originated from *Vitreoscilla*	This study
pETP$_1$-*nox*	pET28a (+) carrying promoter P_1 and *nox* (*L. brevis*)	This study
pETP$_2$-*nox*	pET28a (+) carrying promoter P_2 and *nox* (*L. brevis*)	This study
pETP$_3$-*nox*	pET28a (+) carrying promoter P_3 and *nox* (*L. brevis*)	This study
pETP$_4$-*nox*	pET28a (+) carrying promoter P_4 and *nox* (*L. brevis*)	This study
pETP$_5$-*nox*	pET28a (+) carrying promoter P_5 and *nox* (*L. brevis*)	This study
pETP$_b$-Bpbdh-P_b-*galP*	pET28a (+) carrying promoter P_b[b], *bdh* originated from *Bacillus pumilus* and *galP* originated from strain SDM	20
pETP$_5$-*nox*-P_b-*galP*	pET28a (+) carrying promoter P_5, *nox* (*L. brevis*), promoter P_b[b] and *galP* from strain SDM	This study

[a]Km^r, kanamycin. [b]P_b, the promoter of BD synthesis cluster from *E. cloacae* SDM.

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Table 2 Fermentation products of the recombinant *E. cloacae* strains with ARs inactivation[a]

Strain	AC (g L^{-1})	BD (g L^{-1})	Cell density (OD_{600nm})	Glucose (g L^{-1} h^{-1}) [b]	Productivity (g L^{-1} h^{-1}) [c]
SDM	2.72 ± 1.13	40.30 ± 0.89	11.16 ± 0.22	3.65 ± 0.11	0.11 ± 0.05
SDM 11	1.63 ± 0.22	39.74 ± 1.33	9.77 ± 0.34	3.42 ± 0.28	0.07 ± 0.01
SDM 12	27.61 ± 0.92	10.42 ± 0.64	10.52 ± 0.65	2.26 ± 0.07	0.84 ± 0.03
SDM 13	29.77 ± 1.50	7.94 ± 0.45	9.16 ± 0.05	2.03 ± 0.07	0.76 ± 0.04

[a]Data are the means ± standard deviations (SDs) from three parallel experiments.

[b]The rate of glucose consumed in this study.

[c]The rate of AC production in engineered strains.

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Table 3 Fermentation by-products of the recombinant *E. cloacae* strains[a]

Strain	Succinate (g L^{-1})	Lactate (g L^{-1})	Formate (g L^{-1})	Acetate (g L^{-1})	Ethanol (g L^{-1})	Ethanol/acetate
SDM	0.70 ± 0.07	0.07 ± 0.07	0.79 ± 0.03	0.34 ± 0.31	2.10 ± 0.08	6.14
SDM 11	0.57 ± 0.03	0.14 ± 0.04	0.89 ± 0.02	0.19 ± 0.04	2.35 ± 0.12	12.40
SDM 12	0.27 ± 0.05	0.03 ± 0.00	0.77 ± 0.05	0.16 ± 0.03	2.79 ± 0.10	17.74
SDM 13	0.22 ± 0.02	0.68 ± 0.10	0.82 ± 0.03	0.19 ± 0.03	3.15 ± 0.21	16.56

Ethanol/acetate: the ratio of ethanol to acetate.

[a]Data are the means ± standard deviations (SDs) from three parallel experiments.

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Table 4 Comparison of AC production by different microorganisms using various carbon sources

Strain	Method	Concentration (g L^{-1})	Yield (%)	Productivity (g L^{-1} h^{-1})	Reference
Glucose as substrate					
Bacillus subtilis	Inactivation of AR, moderate expression of NOX	56.70	75.6	0.68	[32]
Bacillus amyloliquefaciens	Wild-type	51.20	86	1.42	[39]
Candida glabrata	Enhancing CAR pathway, and inactivation of by-product pathways	7.33	14.9	0.11	[40]
B. amyloliquefaciens	Enhancing AC tolerance in mutated strain	71.50	84.5	1.62	[33]
C. glabrata	Over-expression of ALDC and ALS	3.67	6	0.05	[41]
C. glabrata	Acetoin pathway was targeted into the mitochondria	3.26	—	—	[42]
Clostridium acetobutylicum	Co-producing with butanol, over-expression of ALDC, abolished acetone formation	4.3	14.3	0.04	[43]
E. coli[a]	Over-expression of BD operon using BICES	31.00	94	0.43	[44]
B. subtilis	Moderate expression of AlsR	41.50	69.1	0.43	[45]

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B. subtilis	Random mutation	53.90	73.5	0.37	[46]
E. cloacae	Inactivation of AR and by-product pathways, cofactor engineering	55.22	76.3	2.69	This study
Sucrose as substrate					
Serratia marcescens	Over-expression of NOX	75.20	—	1.88	[27]
S. marcescens	Medium optimization and speed control strategy	60.50	—	1.44	[47]
Saccharified cedar as substrate					
E. coli	Over-expression of BD operon using BICES	19.00	91	0.16	[44]
Lignocellulosic hydrolysate as substrate					
E. cloacae	Inactivation of AR and by-product pathways, cofactor engineering, blocking CCR by inactivation of PtsG, and over-expression of GalP	45.6	58	1.52	This study

[a]Using mixed sugars (glucose and xylose as carbon source).

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Figure legends:

Fig. 1 Biotechnological strategies in *Enterobacter cloacae* SDM to produce acetoin. In this work, the AR-II (encoded by *budC*) was knocked out to block the main pathway from acetoin (AC) to 2,3-butanediol (BD). Subsequently the NADH oxidase (NOX, encoded by *nox*) was over-expressed with various constitutive promoters for fine tuning NAD^+/NADH *in vivo*. Lactate dehydrogenase (encoded by *ldhA*), alcohol dehydrogenase (encoded by *adhE*) and fumarate reductase (encoded by *frdA*) were inactivated individually or in combination for improvement of AC yield. In addition, PTS (phosphotransferase system) was blocked and GalP (galactose permease) was over-expressed for co-utilization of glucose and xylose. Other genes and enzymes: XylE: xylose transporter; PPP: pentose phosphate pathway; *glk*: glucose kinase; *budB*: α-acetolactate synthase; *budA*: α-acetolactate decarboxylase; *budC*/*gdh*: acetoin reductases; *pps*: phosphoenolpyruvate synthase; *ppc*: phosphoenolpyruvate carboxylase; *mdh*: malate dehydrogenase; *fumABC*: fumarate hydratase; *pflB*: pyruvate formate lyase; *pta*: phosphate acetyltransferase; *ackA*: acetate kinase. Blue crosses indicated the genes were inactivated in this study; Dotted blue crosses indicated the gene inactivated in this work, but not inactivated in the engineered strain for fermentation eventually.

Fig. 2 The enzyme activity of AR and the ratio of AC to BD in engineered strains. AR: acetoin reductase; AC: acetoin; BD: 2,3-butanediol. +/–: gene existed (+) or deleted (–) in engineered strains. All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by error bars.

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Fig. 3 Effects of different enzymes on the NAD^+/NADH *in vivo* (A) and on the production of AC and BD (B). All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by error bars.

Fig. 4 Effects of different expression levels of NOX (A) on NAD^+/NADH *in vivo* (B), AC production (C) and the ratio of AC to BD (D) in engineered *E. cloacae* SDM. Strains SDM 31, SDM 32, SDM 33, SDM 34, SDM 35 and SDM 21 harbored promoters P_1, P_2, P_3, P_4, P_5, and P_c, respectively. And strain SDM 24 was a control strain with no NOX over-expression. All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by error bars.

Fig. 5 Effects of by-products elimination on the production of AC. The experiments were conducted in 500-ml Erlenmeyer flasks containing 100 ml of medium with pH adjusted to 7.4. The initial glucose concentration used was 90 g L^{-1} approximately. Data was obtained when glucose was consumed nearly completely. All assays were performed by triplicate cultures, standard deviations of the biological replicates were represented by error bars.

Fig. 6 Fed-batch fermentation using glucose as carbon source by strain SDM 45. The experiments were conducted in 7.5-L fermenter containing 4 L of medium with an initial glucose concentration of 80 g L^{-1} approximately. Cultivation was carried out at an initial pH of 6.5 and maintained at 6.5 by automatic addition of 6 M H_3PO_4 or 6 M NaOH using a program-controlled peristaltic pump. Agitation speed was 500 rpm and aeration rate was 1 vvm.

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Fig. 7 Time course of batch fermentation using strains SDM 45 (A), SDM 51 (B) and SDM 53 (C). The experiments were conducted in 500-ml Erlenmeyer flasks containing 100 ml of medium with pH adjusted to 7.0. Mixed sugars (58 g L^{-1} glucose and 25 g L^{-1} xylose, approximately) were added in the medium before fermentation. Samples were withdrawn every 3 h for detection of cell density and concentration of substrates and products.

Fig. 8 Fed-batch fermentation using lignocellulosic hydrolysate as carbon source by SDM 53. The experiments were conducted in 7.5-L fermenter containing 4 L of medium with an initial glucose concentration of 80 g L^{-1} approximately. Cultivation was carried out at an initial pH of 6.5 and maintained at 6.5 by automatic addition of 6 M H_3PO_4 or 6 M NaOH using a program-controlled peristaltic pump. Agitation speed was 500 rpm and aeration rate was 1 vvm.

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Fig. 1 Biotechnological strategies in Enterobacter cloacae SDM to produce acetoin.
106x71mm (300 x 300 DPI)

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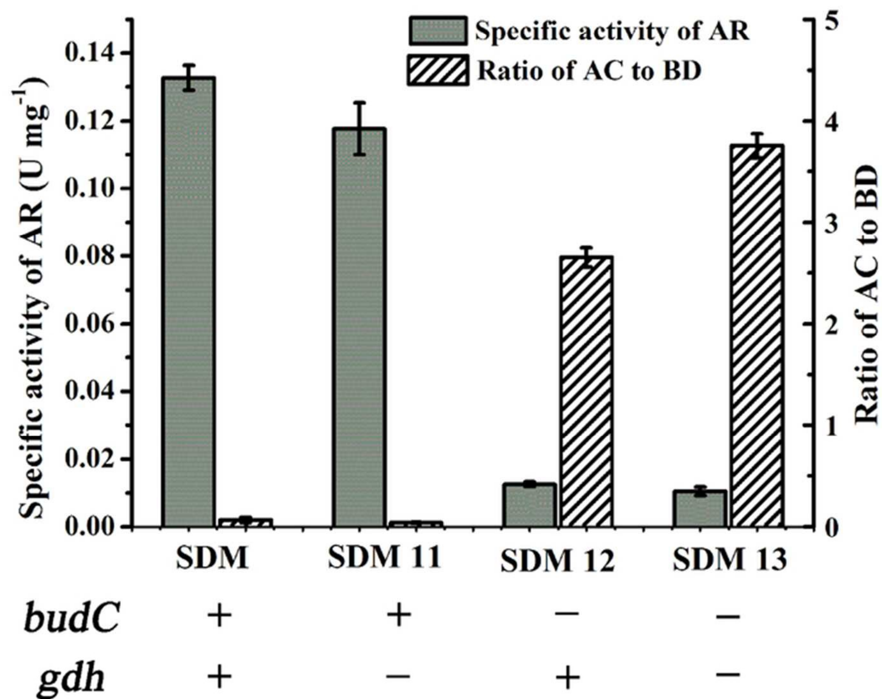

Fig. 2 The enzyme activity of AR and the ratio of AC to BD in engineered strains
66x53mm (300 x 300 DPI)

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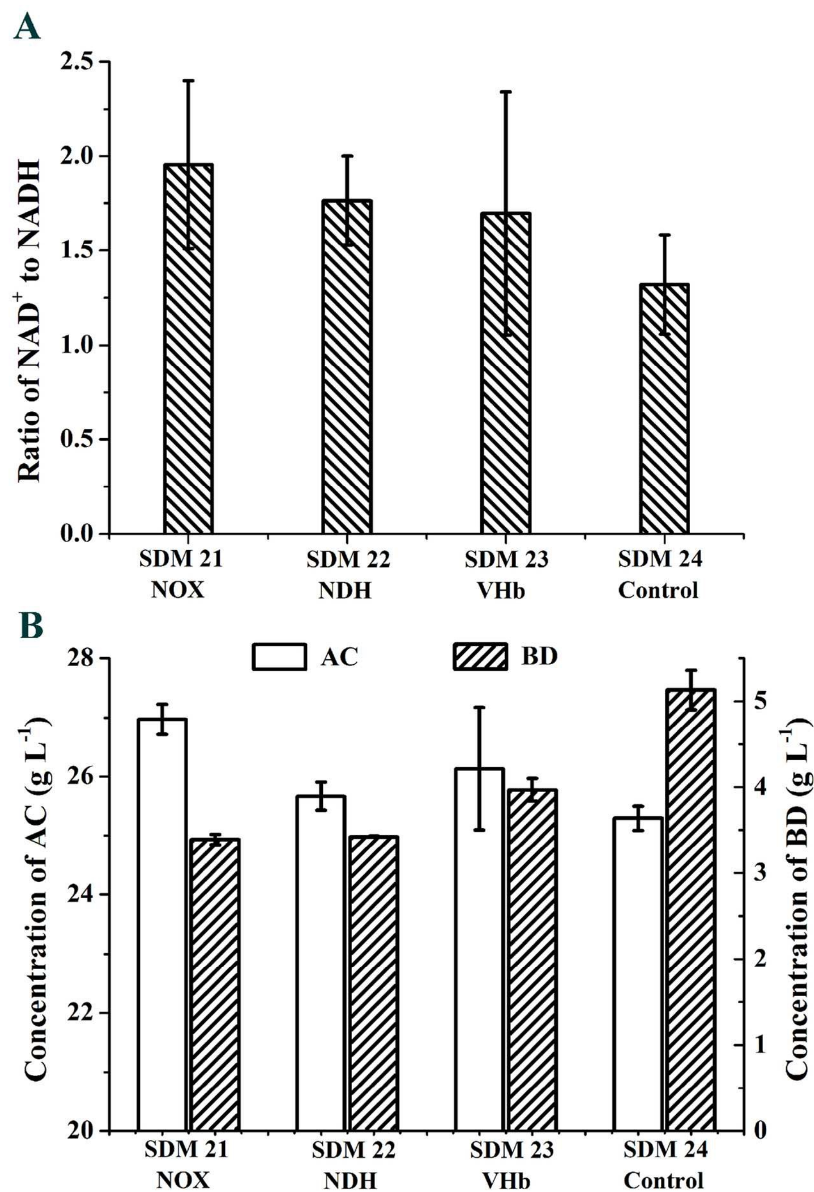

Fig. 3 Effects of different enzymes on the NAD+/NADH in vivo (A) and on the production of AC and BD (B)
115x159mm (300 x 300 DPI)

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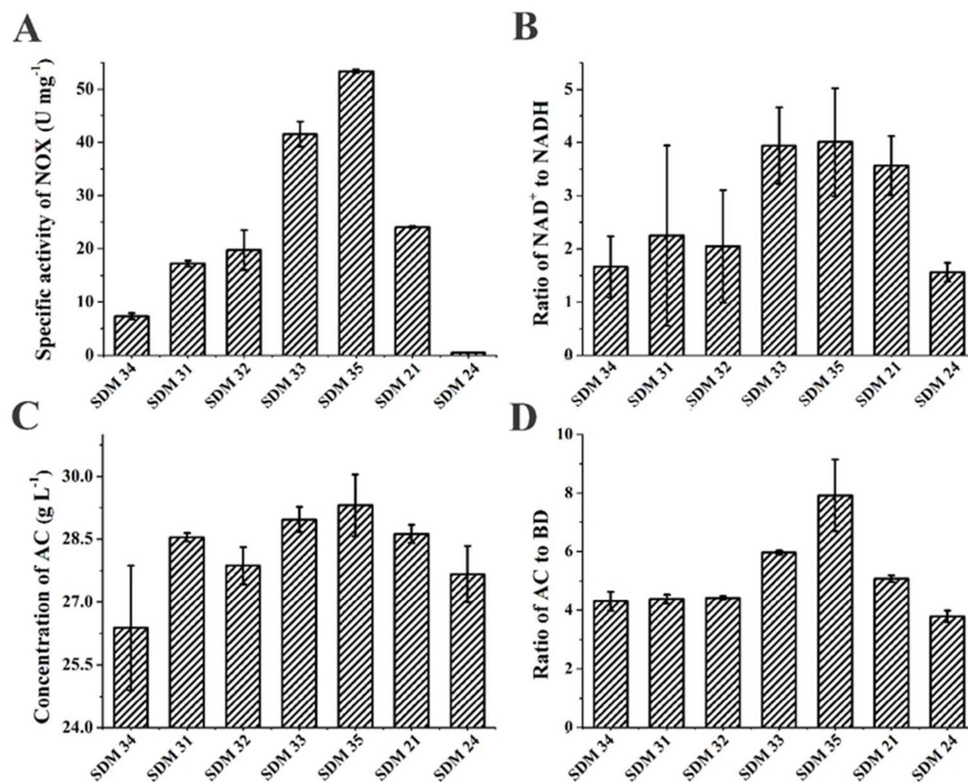

Fig. 4 Effects of different expression levels of NOX (A) on NAD+/NADH in vivo (B), AC production (C) and the ratio of AC to BD (D) in engineered E. cloacae SDM.
66x52mm (300 x 300 DPI)

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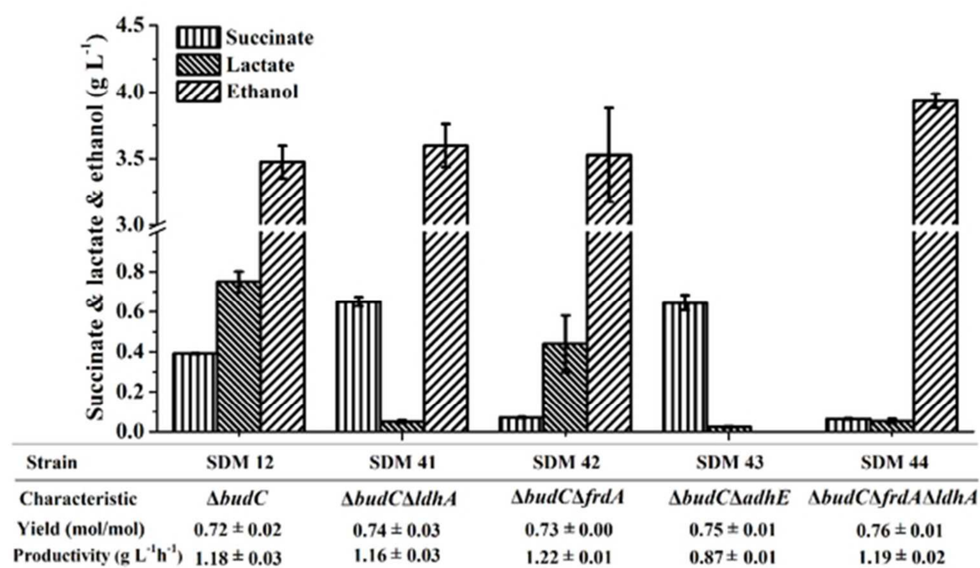

Strain	SDM 12	SDM 41	SDM 42	SDM 43	SDM 44
Characteristic	Δ*budC*	Δ*budC*Δ*ldhA*	Δ*budC*Δ*frdA*	Δ*budC*Δ*adhE*	Δ*budC*Δ*frdA*Δ*ldhA*
Yield (mol/mol)	0.72 ± 0.02	0.74 ± 0.03	0.73 ± 0.00	0.75 ± 0.01	0.76 ± 0.01
Productivity (g $L^{-1}h^{-1}$)	1.18 ± 0.03	1.16 ± 0.03	1.22 ± 0.01	0.87 ± 0.01	1.19 ± 0.02

Fig. 5 Effects of by-products elimination on the production of AC.
51x31mm (300 x 300 DPI)

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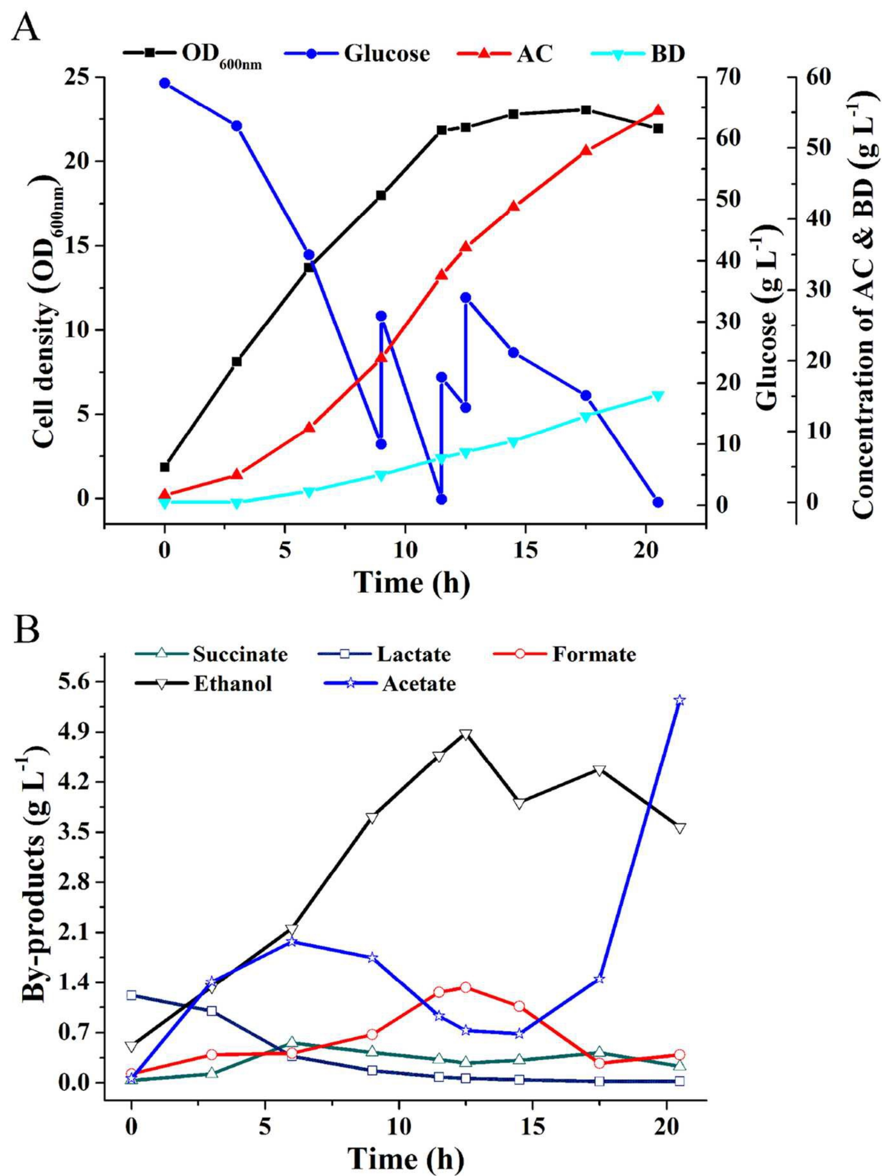

Fig. 6 Fed-batch fermentation using glucose as carbon source by strain SDM 45.
109x145mm (300 x 300 DPI)

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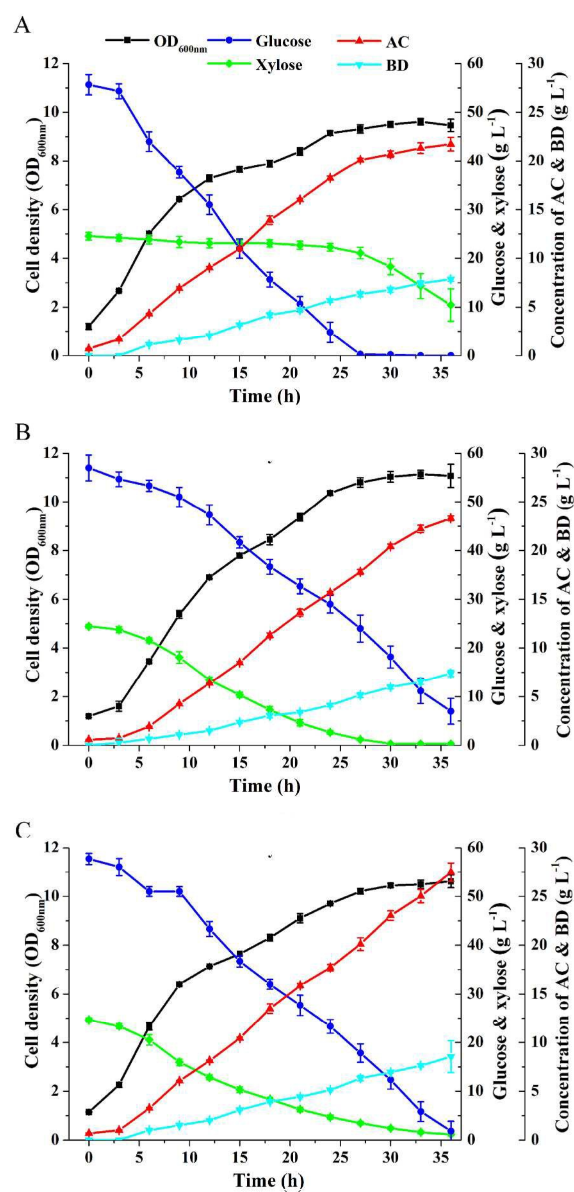

Fig. 7 Time course of batch fermentation using strains SDM 45 (A), SDM 51 (B) and SDM 53 (C).
167x337mm (300 x 300 DPI)

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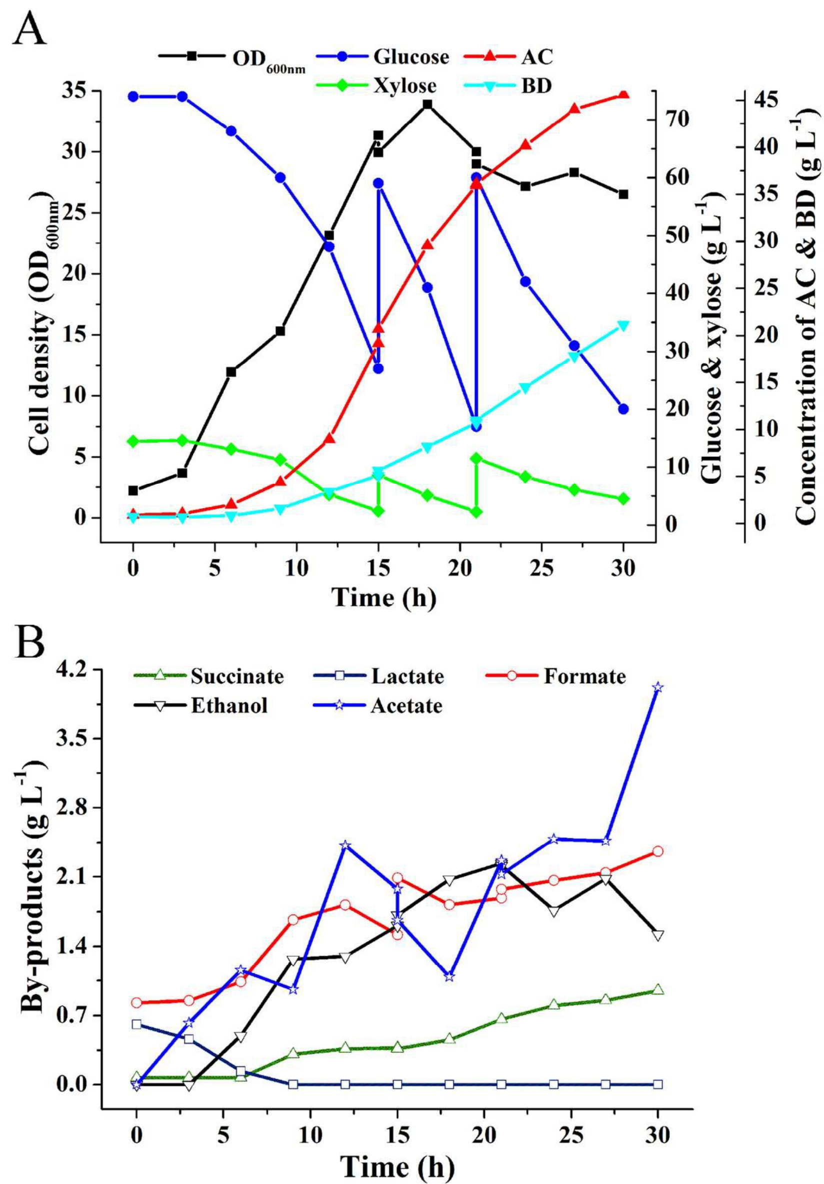

Fig. 8 Fed-batch fermentation using lignocellulosic hydrolysate as carbon source by SDM 53.
120x174mm (300 x 300 DPI)

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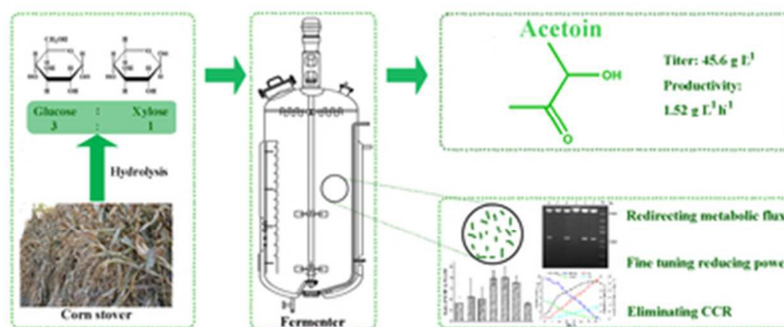

Biotechnological production of acetoin, a bio-based platform chemical, from lignocellulosic resource by metabolically engineered Enterobacter cloacae
33x14mm (300 x 300 DPI)

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