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Studies on the production of branched-chain alcohols in engineered Ralstonia eutropha

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2

3 Title: Studies on the production of branched-chain alcohols in engineered *Ralstonia*

4 *eutropha*

5 Running title: Branched-chain alcohols production in engineered *Ralstonia eutropha*

6

7 Jingnan Lu¹, Christopher J. Brigham², Claudia S. Gai², Anthony J. Sinskey^{2, 3, 4*}

8 Department of Chemistry¹, Department of Biology², Division of Health Sciences and

9 Technology³, Engineering Systems Division⁴, Massachusetts Institute of Technology, 77

10 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

11 * Corresponding author. Mailing address: Bldg. 68-370, Department of Biology,

12 Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, USA.

13 Phone: (617) 253-6721. Fax: (617) 253-8550. E-mail: asinskey@mit.edu

14

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16 butanol, branched-chain amino acid

17 **ABSTRACT**

18 Wild type *Ralstonia eutropha* H16 produces polyhydroxybutyrate (PHB) as an
19 intracellular carbon storage material during nutrient stress in the presence of excess carbon.
20 In this study, the excess carbon was redirected in engineered strains from PHB storage to the
21 production of isobutanol and 3-methyl-1-butanol (branched-chain higher alcohols). These
22 branched-chain higher alcohols can directly substitute for fossil-based fuels and be employed
23 within the current infrastructure. Various mutant strains of *R. eutropha* with isobutyraldehyde
24 dehydrogenase activity, in combination with the overexpression of plasmid-borne, native
25 branched-chain amino acid biosynthesis pathway genes and the overexpression of
26 heterologous ketoisovalerate decarboxylase gene, were employed for the biosynthesis of
27 isobutanol and 3-methyl-1-butanol. Production of these branched-chain alcohols was initiated
28 during nitrogen or phosphorus limitation in the engineered *R. eutropha*. One mutant strain not
29 only produced over 180 mg/L branched-chain alcohols in flask culture, but also was
30 significantly more tolerant of isobutanol toxicity than wild type *R. eutropha*. After
31 elimination of genes encoding three potential carbon sinks (*ilvE*, *bkdAB*, and *aceE*), the
32 production titer improved to 270 mg/L isobutanol and 40 mg/L 3-methyl-1-butanol.
33 Continuous flask cultivation was utilized to minimize the toxicity caused by isobutanol while
34 supplying cells with sufficient nutrients. Under this continuous flask cultivation, the *R.*
35 *eutropha* mutant grew and produced more than 14 g/L branched-chain alcohols over the
36 duration of 50 days. These results demonstrate that *R. eutropha* carbon flux can be redirected
37 from PHB to branched-chain alcohols and that engineered *R. eutropha* can be cultivated over
38 prolonged periods of time for product biosynthesis.

39

40 INTRODUCTION

41 Catabolism of branched-chain amino acids (leucine, valine, and isoleucine) to fusel
42 acids and alcohols was first described in 1904 (Hazelwood et al. 2008). The transfer of an
43 amino group from a branched-chain amino acid via transamination to an α -keto acid results in
44 a branched-chain α -keto acid. Unlike α -ketoglutarate and oxaloacetate, the deamination
45 products of glutamate and aspartate respectively, branched-chain α -keto acid cannot be
46 redirected into central carbon metabolism. Before the branched-chain α -keto acid is excreted
47 into the surrounding medium, microorganisms such as *Saccharomyces* and *Lactococcus* are
48 able to decarboxylate it into its corresponding aldehyde and subsequently reduce it into a fusel
49 alcohol via the Ehrlich pathway (Hazelwood et al. 2008; Larroy et al. 2003). For more than a
50 century, the Ehrlich pathway was mainly studied in the food industry for off-flavor formations
51 in cheese and beer (de Palencia et al. 2006). Current scientific interest in the Ehrlich pathway
52 was initiated by Atsumi et al. who demonstrated the production of branched-chain alcohols
53 from glucose in engineered *Escherchia coli* strains (Atsumi et al. 2008). As depicted in Fig. 1,
54 2-ketoisovalerate and 2-ketoisocaproate, the intermediates of valine and leucine biosynthesis
55 pathway are decarboxylated to form isobutyraldehyde and 3-methyl-1-butyraldehyde and
56 subsequently reduced to branched-chain alcohols isobutanol and 3-methyl-1-butanol,
57 respectively. Previous studies showed that these branched-chain alcohols could be produced
58 by a synthetic pathway using heterologous branched-chain amino acid biosynthesis and
59 Ehrlich pathway enzymes from *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Lactococcus*
60 *lactis* (Atsumi et al. 2008; Atsumi et al. 2009; Blombach et al. 2011; Savrasova et al. 2011;
61 Smith et al. 2010).

62 Branched-chain alcohols, such as isobutanol and 3-mehtyl-1-butanol, have attracted
63 both research and industrial attentions as an alternative biofuel to ethanol and biodiesel.
64 These alcohols have approximately 98 % of the energy content of gasoline, 17 % higher than
65 ethanol, the current gasoline-additive (Sheehan 2009). Unlike ethanol, these branched-chain

66 alcohols have low vapor pressure, hygroscopicity, and water solubility, which make them
67 compatible with the existing pipelines, gasoline pumps, and engines (Atsumi et al. 2008;
68 Atsumi et al. 2009; Blombach et al. 2011; Smith et al. 2010; Yan and Liao 2009). Due to the
69 current concerns of fossil fuel shortage and rising oil prices, use of alternative energies from
70 solar, wind, geothermal and hydroelectric has spread. These energy sources, although
71 effective in stationary power applications, cannot be easily or efficiently employed in current
72 or future transportation systems (Connor and Liao 2009; Connor and Atsumi 2010). Thus
73 alternative biofuels like branched-chain higher alcohols hold promise as a more suitable
74 'mobile' energy in the future. Additionally, isobutanol can be utilized as a precursor for the
75 production of isobutylene, which is used in large quantity by the oil refinery, rubber, and
76 specialty chemical industries (Gogerty and Bobik 2010; Macho et al. 2001).

77 *Ralstonia eutropha* (also known as *Cuprivaidus necator*) is a Gram-negative,
78 facultatively chemolithoautotrophic organism (Pohlmann et al. 2006). It has long been the
79 model organism for the study of polyhydroxybutyrate (PHB) biosynthesis. Under nutrient
80 stress in the presence of excess carbon source, wild type *R. eutropha* can accumulate
81 approximately 80 % of its cell dry weight (CDW) as PHB, an intracellular carbon storage
82 material (Budde et al. 2011; Yang et al. 2010). This intracellular polymer can be isolated
83 from the cell and processed into biodegradable and biocompatible plastic for various
84 applications (Rehm 2003; Brigham et al. 2012). Uniquely, *R. eutropha* also contains two
85 carbon-fixation Calvin-Benson-Bassham cycle operons, two oxygen-tolerant hydrogenases,
86 and several formate dehydrogenases and has been studied extensively for its ability to fix
87 carbon dioxide into complex cellular molecules while obtaining energy from hydrogen or
88 formate oxidation under ambient oxygen concentration conditions (Bowien and Kusian 2002;
89 Ishizaki et al. 2001; Lenz et al.; Pohlmann et al. 2006; Schwartz et al. 2009). Recently, Li et
90 al. constructed a system that couples electrochemical generation of formate with CO₂ fixation
91 and the addition of heterologous genes for the conversion of formate to isobutanol and 3-

92 methyl-1-butanol by *R. eutropha*. Via this electromicrobial conversion, over 140 mg/L
93 branched-chain alcohols were synthesized in engineered *R. eutropha* strains (Li et al. 2012).

94 Steinbüchel et al. examined *R. eutropha* mutant strains that were defective in PHB
95 formation and found that these mutants secreted large amounts of pyruvate into the growth
96 medium when cultivated under nitrogen starvation (Steinbüchel and Schlegel 1989). Such
97 findings suggest that, under stress conditions, the pyruvate dehydrogenase complex becomes
98 less active in these *R. eutropha* strains, leading to the buildup of excess pyruvate.
99 Additionally, it suggested that the excess carbon might be effectively redirected from PHB
100 storage to the production of other molecules such as branched-chain alcohols. In this study,
101 we examined the conditions for the production of branched-chain alcohols in *R. eutropha*
102 using native branched-chain amino acid biosynthesis genes. We also surveyed numerous *R.*
103 *eutropha* mutant strains constitutively expressing alcohol dehydrogenase gene isolated
104 previously (Jendrossek et al. 1990; Steinbüchel and Schlegel 1984; Steinbüchel et al. 1987)
105 for their ability to reduce isobutyraldehyde into isobutanol. In addition, we tested tolerance to
106 isobutanol by wild type and mutant *R. eutropha* strains. Lastly, we demonstrated a prolonged
107 continuous flask cultivation of engineered *R. eutropha* for the production of branched-chain
108 alcohols.

109

110 **MATERIALS AND METHODS**

111 Chemicals, bacterial strains and plasmids

112 Chemicals were purchased from Sigma-Aldrich unless indicated otherwise.
113 Experiments were performed with strains and plasmids listed in Table 1. Primers used in the
114 construction of these strains and plasmids are listed in Online Resource 1. Mutant and
115 engineered strains were all derived from wild type *R. eutropha* H16 (ATCC 17699).

116

117 Growth media and cultivation conditions

118 All *R. eutropha* strains were cultivated aerobically in rich and minimal media at 30°C.
119 Rich medium consisted of 2.75 % (w/v) dextrose-free tryptic soy broth (TSB) (Becton
120 Dickinson, Sparks, MD). Minimal medium used to cultivate *R. eutropha* was formulated as
121 described previously (Lu et al. 2012). Carbon sources used in minimal medium cultivations
122 were 2 % (final w/v) fructose or sodium gluconate. For all *R. eutropha* cultures, 10 µg/mL
123 final concentration of gentamicin was added. In cultivations of *R. eutropha* containing
124 plasmid, kanamycin at 200 µg/mL final concentration was also supplemented.

125 A single colony of *R. eutropha* from a TSB agar plate was used to inoculate 5 mL of
126 TSB medium. The culture was then incubated on a roller drum for 24 h at 30°C before being
127 used to inoculate a flask culture of 100 mL minimal medium, containing carbon sources
128 mentioned above, to an initial OD₆₀₀ of 0.1. The minimal medium culture was continuously
129 shaken in a 30°C incubator at 200 rpm. At intermittent time points, aliquots were removed
130 from the flask culture for analysis, as described below.

131

132 Plasmid and strain construction

133 Standard molecular biology techniques were performed for all DNA manipulations
134 (Chong, 2001). DNA sequence amplification was achieved using Phusion DNA polymerase
135 (New England Biolabs, Ipswich, MA). QIAquick gel extraction kit (Qiagen, Valencia, CA)
136 was used for gel purifications of all DNA products. Plasmid extractions were carried out
137 using QIAprep spin miniprep kit (Qiagen, Valencia, CA). Restriction enzymes used in this
138 study were from New England Biolabs (Ipswich, MA).

139 The plasmids for markerless deletion were constructed by first amplifying
140 approximately 500 base pairs of DNA sequences upstream and downstream of the gene
141 targeted for deletion using primers with identical sequence overlap at the end (Online
142 Resource 1). Overlap PCR using these primers resulted in a DNA fragment of approximately
143 1,000 bp in length that contained both the upstream and downstream region of the target

144 deletion gene. Both the resulting DNA fragment and the parent plasmid, pJV7 (Budde et al.
145 2010) (Table 1), were digested with the restriction enzymes XbaI and SacI or BamHI. The
146 digested plasmid and DNA fragment were then ligated together and transformed into high
147 efficiency *Escherichia coli* DH10-beta competent cells (New England Biolabs, Ipswich, MA)
148 to create the gene deletion plasmid. The gene deletion plasmid was then isolated from *E. coli*
149 DH10-beta cells and transformed into *E. coli* S17-1 (Simon et al. 1983), which was used as a
150 donor for the conjugative transfer of mobilizable plasmids. A standard mating-procedure was
151 performed to introduce the gene deletion plasmid into *R. eutropha* via conjugation (Slater et al.
152 1998). Gene deletions from *R. eutropha* H16 genome were carried out by a standard
153 procedure described previously (Quandt and Hynes 1993; York et al. 2001). Deletion strains
154 were screened by diagnostic PCR with pairs of internal and external primer sets (Online
155 Resource 1).

156 A synthetic isobutanol production operon was constructed by overlap PCR using
157 primers with identical sequence overlap. Each gene was first amplified, purified, and
158 sequenced. Then an artificial ribosome-binding site (5'-AAAGGAGG-3') and a nucleotide
159 linker sequence (5'-ACAACC-3') were incorporated in the beginning of each gene. Finally,
160 all the genes were ligated together in an artificial operon via multiple rounds of overlap PCR.
161 The isobutanol production operon and broad-host-range cloning vector pBBR1MCS-2
162 (Kovach et al. 1995) were digested with BamHI and SacII. The digested vector and operon
163 DNA insert were ligated, transformed, and transferred into *R. eutropha* as described above.

164

165 Polymer quantification

166 The cell dry weight (CDW) and PHB content were measured as described previously
167 (Karr et al. 1983; York et al. 2003).

168

169 Branched-chain alcohols extraction and detection

170 Culture aliquots, taken at various time points, were centrifuged at $4,000 \times g$ to separate
171 the pellet from the supernatant. Isobutanol and 3-methyl-1-butanol were extracted from the
172 supernatant using chloroform in a 1:1 ratio. The concentrations of isobutanol and 3-methyl-1-
173 butanol were determined using gas chromatograph (GC; Agilent Technologies, Santa Clara,
174 CA) with a DB-Wax column (Agilent Technologies, 30 m x 0.32 mm x 0.5 μm) and a flame
175 ionization detector. The split ratio is 20:1 and a 2 μL sample was injected at each run.
176 Hydrogen was used as the carrier gas at a flow rate of 1.1 mL/min. The oven was held at
177 35°C for 5 min, then heated to 230°C at a rate of 12°C/min, and lastly held at 220°C for 5 min.
178 Commercial isobutanol and 3-methyl-1-butanol were analyzed on the GC as described above
179 for standards.

180

181 Carbon, nitrogen, reducing-cofactor analysis

182 Culture supernatants were filtered and injected into HPLC to determine concentrations
183 of fructose or gluconate, and pyruvate. The HPLC was equipped with an ion exchange
184 column, and the detection methods used were previously described (Kurosawa et al. 2010).
185 Ammonium concentrations in the supernatant were measured using an ammonium assay kit
186 (Sigma-Aldrich) following the manufacturer's instructions. The intracellular concentrations
187 of NADH and NADPH were measured using modified assays from previously described
188 works (Leyval et al. 2003; Zhang et al. 2000). The concentrations of NADH and NADPH
189 were normalized per colony forming unit (CFU).

190

191 Product tolerance assay

192 Cultures of *R. eutropha* were grown in minimal medium with 2 % fructose and 0.05 %
193 NH_4Cl . Various concentrations of isobutanol (0%, 0.2%, 0.5%, 0.8%, and 1% v/v) were
194 added to the growth media at 0 h. Growth was monitored by measuring OD_{600} intermittently
195 throughout the 96 h cultivation time. Percent viability was calculated by a modified tolerance

196 assay described by Smith et al. (Smith et al. 2010). Briefly, OD₆₀₀ measurements of remaining
197 viable cells cultured in the presence of isobutanol were normalized with the values obtained
198 from cells cultured in the absence of isobutanol, 24 h after inoculation.

199

200 Enzymatic activity assays

201 *R. eutropha* cultures harvested at different time points were pelleted and stored at -
202 80°C for activity assays of acetohydroxyacid synthase (AHAS), acetohydroxyacid
203 isomeroreductase (AHAIR), dihydroxyacid dehydratase (DHAH), ketoisovalerate
204 decarboxylase (KIVD), and alcohol dehydrogenase (ADH). Cell lysates were prepared by
205 thawing the frozen pellets on ice and resuspending them in phosphate buffered saline.
206 Zirconia/silica beads (0.1 mm; BioSpec Products, Bartlesville, OK) were added to the
207 resuspended cells. These samples were shaken vigorously three times at 5.0 m/s for 30 s, with
208 a 5 min rest between each treatment at 4°C by FastPrep-24 (MP Biomedicals, Solon, OH).
209 Cellular debris and beads were removed by centrifugation for 10 min at 4°C and 6,500 × g.
210 The soluble cell lysates were filtered through 0.2 µm low-protein-binding Supor syringe filters
211 (Pall, NY) and stored on ice for enzymatic assays. Protein concentrations were determined by
212 a modified Bradford assay (Zor and Selinger, 1996) using bovine serum albumin as the
213 protein concentration standard.

214 The AHAS, AHAIR, and DHAD activity assays were based and modified from Leyval,
215 et al. (Leyval et al. 2003). The AHAS activity assay is a discontinuous assay that converts
216 pyruvate first to α-acetolactate and finally to acetoin. The 1 mL assay mixture contained 100
217 mM potassium phosphate buffer at pH 7.0, 50 mM sodium pyruvate, 100 mM MgCl₂, 100 µM
218 thiamine pyrophosphate (TPP), and cell lysate. The reaction was initiated by the addition of
219 sodium pyruvate at 30°C and terminated by acidifying 100 µL aliquots of assay mixture with
220 10 µL 50% H₂SO₄ every 3 min for 30 min total. The acidified assay mixture was then
221 incubated at 37°C for 30 min to allow the formation of acetoin from α-acetolactate. The

222 acetoin formed was quantified by the Voges-Proskauer method (Westerfield, 1945) with a
223 slight adjustment of mixing 35 μL instead of 25 μL of 1-naphthol (5 % w/v in 2.5 M NaOH).
224 The mixture of acetoin, 1-naphthol, and creatine created a pink color and was measured at 535
225 nm with Varioskan Flash Plate Reader (Thermo Scientific, Asheville, NC). Pure acetoin was
226 used as a standard.

227 The AHAIR activity assay mixture (1 mL total volume) contains 100 mM potassium
228 phosphate buffer (pH 7.0), 10 mM α -acetolactate, 3 mM MgCl_2 , 0.1 mM NADPH, and cell
229 lysate. The reaction was initiated by the addition of α -acetolactate at 30°C. A
230 spectrophotometer (Agilent 8453 UV-visible Kinetic Mode) was used to monitor the reaction
231 at 340 nm for the consumption of NADPH. The α -acetolactate was chemically synthesized
232 based on the previously developed method (Leyval et al. 2003). Enzyme activity was
233 calculated in μmol of NADPH oxidized using its molar extinction coefficient of $6220 \text{ M}^{-1}\text{cm}^{-1}$.

234 The DHAD activity assay is also a discontinuous assay. The 1 mL reaction mixture
235 contained 100 mM potassium phosphate buffer (pH 7.0), 5 mM MgCl_2 , 10 mM $\text{DL-}\alpha,\beta$ -
236 dihydroxyisovalerate, and cell lysate. Substrate $\text{DL-}\alpha,\beta$ -dihydroxyisovalerate was added to
237 initiate the reaction. Every 2 min, for a total of 20 min, 100 μL of the reaction was removed
238 and terminated by mixing with 12.5 μL trichloroacetic acid (10 % v/v). Terminated reaction
239 mixtures were mixed with 25 μL saturated 2,4-dinitrophenylhydrazine in 2 M HCl and
240 incubated at room temperature for 20 min and afterwards neutralized with 85 μL of 2 M
241 NaOH for 30 min. The product derivative (α -ketoisovalerate-dinitrophenylhydrazone) was
242 detected at 540 nm using the plate reader. Commercial α -ketoisovaleric acid sodium salt
243 served as standard.

244 The KIVD activity assay was adapted and modified from de la Plaza et al. and de
245 Palencia et al. (de la Plaza et al. 2004; de Palencia et al. 2006). The assay couples the
246 formation of isobutyraldehyde, using aldehyde dehydrogenase from *Saccharomyces*
247 *cerevisiae*, to the formation of isobutyrate. In the 1 mL assay mixture were 100 mM

248 potassium phosphate buffer (pH 7.0), 15 mM pyrazole, 30 mM TPP, 1 mM NAD⁺, 3 μM
249 MgCl₂, 20 mM α-ketoisovaleric acid, 1 mM DTT, 0.5 mg aldehyde dehydrogenase, and cell
250 lysate. The reaction was initiated by the addition of α-ketoisovaleric acid and monitored at
251 340 nm for the reduction of NAD⁺. Enzyme activity was calculated in μmol of NAD⁺
252 reduced, using the molar extinction coefficient of 6220 M⁻¹cm⁻¹.

253 The ADH activity assay was based on Steinbüchel, et al. (Steinbüchel and Schlegel
254 1984). The activity was monitored at 340 nm and the 1 mL enzyme assay mixture consists of
255 95 mM citrate buffer (pH 5.8), 0.1 mM NADPH, 200 mM isobutyraldehyde, and cell lysate.
256 Enzyme activity was calculated in μmol of NADPH oxidized, using the molar extinction
257 coefficient mentioned above.

258 All enzyme activities discussed in this work are a result of triplicate assays reported ±
259 standard deviation. As controls, assays were conducted in the absence of cell lysates and also
260 separately in the absence of substrates. Enzyme unit (U) was defined as 1 μmol product
261 formed per min.

262

263 Continuous flask cultures

264 Triplicate cultures of Re2425/pJL26 (Table 1) were performed in 100 mL minimal
265 medium containing 1 % fructose and 0.05 % NH₄Cl. Every 24 h, the growth media
266 containing branched-chain alcohols were separated from the cells via centrifugation at 4,000 ×
267 g. Isobutanol and 3-methyl-1-butanol were extracted from the growth media and analyzed as
268 described above. Fresh media (without branched-chain alcohols) were then added to the cells
269 in each culture. The cultures continued for another 24 h, until the process was repeated. This
270 cycle was continued for 50 days. At each 24 h cycle, OD₆₀₀ and concentrations of branched-
271 chain alcohols were measured.

272

273 **RESULTS**

274 Redirecting excess carbon and reducing-equivalents

275 Wild type *R. eutropha* produces PHB as an intracellular carbon and energy storage
276 polymer during nutrient stress in the presence of excess carbon (Potter et al. 2004). In order
277 to redirect the excess carbon from PHB, the *phaCAB* operon, which encodes the polymer
278 biosynthesis enzymes β -ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHB
279 synthase (PhaC) (Pohlmann et al. 2006), was eliminated from the *R. eutropha* genome. As
280 shown in Table 2, the wild type (H16) was able to produce more than 80 % of CDW as PHB,
281 but strain Re2061 (H16 Δ *phaCAB*), produced no detectable PHB after 96 h of growth.
282 Deletion of the *phaCAB* operon did not affect cell growth significantly, since the residual
283 CDW were similar in both strains. Compared to H16, Re2061 utilized 0.5 % (w/v) less
284 gluconate during growth. Re2061 also secreted pyruvate into the growth medium, but H16
285 did not secrete any pyruvate throughout the entire growth period. This finding was similar to
286 those reported by Steinbüchel et al. (Steinbüchel and Schlegel 1989). In addition, Re2061
287 cells contained greater concentrations of reducing-cofactors (NADH and NADPH) than H16
288 (Table 2). Since PHB acts as a carbon and energy storage mechanism in *R. eutropha*
289 (Schwartz et al. 2009), Re2061 cells used less carbon source, secreted pyruvate into the
290 extracellular milieu, and contained more reducing-cofactors, likely directly resulting from
291 their inability to produce polymer.

292

293 Assembly of a branched-chain alcohols biosynthesis operon

294 In order for *R. eutropha* to appropriate its branched-chain amino acid pathway,
295 specifically the intermediates α -ketoisovalerate and α -ketoisocaproate, for the production of
296 isobutyraldehyde and 3-methyl-1-butyraldehyde respectively, a heterologous ketoisovalerate
297 decarboxylase (KivD) is needed. The *kivD* gene from *L. lactis* (de la Plaza et al. 2004), which
298 encodes a ketoisovalerate decarboxylase enzyme was expressed and found active towards α -
299 ketoisovalerate when expressed on a plasmid in *R. eutropha* (Data not shown). Subsequently,

300 isobutyraldehyde and 3-methyl-1-butyraldehyde are reduced to their corresponding alcohols
301 by alcohol dehydrogenase with short-chain substrate specificity. In an environment with
302 ambient oxygen concentrations, *R. eutropha* does not exhibit any alcohol dehydrogenase
303 (ADH) activity (Fig. 3A), despite the presence of various putative alcohol dehydrogenase
304 genes on both chromosomes. A search in the *R. eutropha* H16 genome database (NCBI:
305 <http://www.ncbi.nlm.nih.gov/>) revealed putative short-chain substrate *adh* genes, locus tags
306 H16_A0757 (*adh*) and H16_A0861 (*adhA*). The sequence of *adh* is similar to the well-
307 studied *E. coli* Zn-dependent NADPH alcohol dehydrogenase (Jarboe 2011), YqhD (data not
308 shown). Genes encoding *adh*, *adhA*, and *yqhD* were separately inserted into constitutively
309 expressed plasmid pBBR1MCS-2 to create pJL20, pJL21, and pJL22 respectively. The
310 plasmid-borne *adh* genes were introduced into Re2061, and assayed for ADH activity towards
311 isobutyraldehyde. ADH, AdhA, and YqhD were active toward isobutyraldehyde (Fig. 3A)
312 and prefer NADPH as cofactor (data not shown). ADH and YqhD had similar activities of
313 180 mU/mg and 200 mU/mg, respectively, whereas AdhA at 20 mU/mg was less active
314 towards isobutyraldehyde.

315 Expressing *kivD* and *adh* in Re2061 (Re2061/pJL23) allowed the strain to produce 10
316 mg/L isobutanol from fructose (Fig. 2). Such low production suggests that there was
317 insufficient α -ketoisovalerate synthesized from the native branched-chain amino acid
318 biosynthesis pathway to be diverted to isobutanol. To test this hypothesis, 1 % (w/v) pyruvate
319 and 1 % (w/v) α -ketoisovaleric acid were separately supplemented to the growth media.
320 Addition of pyruvate increased isobutanol production to 350 mg/L and production reached 4.5
321 g/L after supplying the growth media with α -ketoisovaleric acid (Fig. 2). Thus, we concluded
322 that indeed insufficient carbon was being shunted through the branched-chain amino acid
323 pathway in Re2061/pJL23 for the production of isobutanol. To address this, the *ilvBHCD*
324 genes from the valine biosynthesis pathway in *R. eutropha* H16 were overexpressed, along
325 with *kivD*, on plasmid pJL26 (Table 1, Fig. 1). Online Resource 2 compares the activities of

326 valine biosynthesis pathway enzymes from wild type *R. eutropha* with engineered strains
327 containing plasmid pJL26 at 24 h. Overexpression of *ilvBHCD* and *kivD* genes from the
328 plasmid pJL26 increased the overall activities of valine biosynthesis pathway enzymes.

329

330 *R. eutropha adh* mutant has isobutyraldehyde dehydrogenase activity

331 Steinbüchel et al. demonstrated that the *adh* gene in wild type *R. eutropha* was only
332 expressed and active when cells were cultivated under anaerobic conditions. It was
333 hypothesized that such gene activation increases the usage of reducing power in the absence
334 of the terminal electron acceptor, oxygen (Steinbüchel and Schlegel 1984; Steinbüchel et al.
335 1987). In previous studies, a number of *R. eutropha* mutant strains constitutively expressing
336 *adh* under aerobic conditions were isolated after continuous growth of H16 on ethanol and/or
337 2,3-butanediol and characterized for their alcohol dehydrogenase activities (Jendrossek et al.
338 1990; Steinbüchel et al. 1987). We demonstrate, as shown in Fig. 3A, that these strains also
339 reduce isobutyraldehyde. In all cases, the cofactor NADPH is preferred over NADH for the
340 reduction of isobutyraldehyde (data not shown). Strain Re2403 (CF17 Δ *phaCAB*) showed
341 similar activity compared to strains that overexpress ADH and YqhD. The strain with the
342 most active isobutyraldehyde dehydrogenase activity (300 mU/mg) was Re2401
343 (DJ21 Δ *phaCAB*) (Fig. 3A). DJ21 was also found to have the highest activity towards the
344 reduction of 2,3-butanediol and ethanol compared to all other *adh* mutant strains (Jendrossek
345 et al. 1990).

346 The engineered isobutanol production plasmid, pJL26, was incorporated into each of
347 the constitutive ADH mutant strains (CF17, CF 101, CF 106, CF 108 CF 303 and DJ21) from
348 which the *phaCAB* operon was previously deleted (Table 1). Growth rates were similar
349 among all these strains (Online Resource 3). With valine biosynthesis pathway genes
350 overexpressed on pJL26, the production of isobutanol and 3-methyl-1-butanol was increased
351 by at least 10 fold (Fig. 3B). Since wild type *R. eutropha* is not able to reduce

352 isobutyraldehyde under ambient culture conditions, no isobutanol or 3-methyl-1-butanol were
353 produced by Re2061/pJL26. Strains Re2403, Re2404, Re2405, Re2406, and Re2407, all
354 containing pJL26, each produced over 100 mg/L isobutanol. Re2401/pJL26, with the highest
355 isobutyraldehyde dehydrogenase activity, also produced the highest amount of isobutanol
356 (200 mg/L). All strains produced similar amount (30 mg/L) of 3-methyl-1-butanol, likely due
357 to low activity of the IPMS enzyme, responsible for the conversion of α -ketovalerate to α -
358 isopropylmalate (Fig. 3B). The amounts of isobutanol produced increased concomitant with
359 higher measured activities of ADH towards isobutyraldehyde and vice versa (Fig. 3A and B).

360 Fig. 4 summarizes the enzymatic activities of isobutanol production pathway enzymes
361 in Re2401/pJL26 over a 96 h cultivation period. All enzymes were active throughout the
362 entire cultivation time. AHAS, AHAI, DHAD, and KIVD exhibited the same trend of
363 decrease in activity over time, since these genes were engineered into an operon
364 (*ilvBHCDkivD*), overexpressed from a single promoter on plasmid pJL26. Alcohol
365 dehydrogenase, on the other hand, was constitutively expressed in our engineered strain, thus
366 its activity was correlated with cell growth. AHAS, being a potentially rate limiting node in
367 the branched-chain biosynthesis pathway, exhibited the lowest activity of all enzymes tested
368 (9 mU/mg at 24 h). KivD also exhibited relatively low activity in *R. eutropha*, as it is a
369 heterologously expressed enzyme from gene originating in the AT-rich bacterium, *L. lactis*
370 (Fig. 4).

371

372 Engineered *R. eutropha* strains exhibit greater isobutanol tolerance than wild type

373 Short chain alcohols like isobutanol are known to cause toxicity to organisms by
374 inserting themselves in the phospholipid membrane, thus causing membrane fluidity and cell
375 death (Atsumi et al. 2010b; Baer et al. 1987; Minty et al. 2011; Vollherbst-Schneck et al.
376 1984). Isobutanol tolerance by *R. eutropha* wild type and engineered strains was evaluated
377 and summarized in Table 3. After 24 h, all strains had grown to the same levels ($OD_{600} \approx 2.3$)

378 in the absence of isobutanol. In the presence of 0.2 % (v/v) isobutanol, only strains Re2403
379 and Re2405 experienced any toxicity. Re2403 was unable to grow, and viability of Re2405
380 was decreased by 70% in the presence of 0.2 % (v/v) isobutanol. In the presence of 0.5 %
381 (v/v) isobutanol concentrations, viability of wild type cells was decreased by half, while
382 Re2406, Re2407 and Re2401 experienced only minimal toxicity effects from this
383 concentration of isobutanol. Concentrations of isobutanol at 0.8 % to 1.0 % (v/v) were
384 extremely toxic to all strains tested, with nearly no cell growth observed (Table 3). Since *R.*
385 *eutropha* mutant strains contained an active ADH enzyme under the conditions studied here,
386 they were likely able to convert the isobutanol to other less toxic molecules, thus experiencing
387 less isobutanol toxicity than wild type at concentrations lower than 0.8 % (v/v).

388

389 Production of branched-chain alcohols in response to nutrient stress

390 As mentioned previously, intracellular PHB is produced when the cells undergo
391 nutrient stress, such as nitrogen or phosphorus limitation (Khanna and Srivastava 2005). In
392 our initial isobutanol production experiments, the growth medium contained 2 % fructose and
393 0.05 % NH₄Cl. Under these conditions, *R. eutropha* cells became nitrogen limited by 24 h,
394 and the production of isobutanol was detected after this time (Online Resource 4). To test if
395 the production of isobutanol was also associated with nitrogen starvation, different
396 concentrations of NH₄Cl (0.07 %, 0.12 %, 0.27 %, and 0.4 % w/v) were used in cultivations
397 of Re2401/pJL26. Results in Fig. 5A demonstrate the amounts of NH₄Cl present in the
398 growth media measured enzymatically (see Materials and Methods) at various time points,
399 while Fig. 5B revealed the amount of isobutanol produced in these cultures. At high NH₄Cl
400 concentrations (0.27 and 0.4 %), Re2401/pJL26 cells were unable to utilize the entire amount
401 of nitrogen source, and the production of isobutanol was extremely low (30 mg/L and 20
402 mg/L respectively). On the other hand, when the NH₄Cl concentrations were lower, at 0.07
403 and 0.12 %, the cells entered nitrogen limitation at approximately 24 h, and isobutanol

404 production initiated after nitrogen depletion and reached ~170 mg/L (Fig. 5). Similar results
405 were seen with phosphorus limitation (data not shown). These results suggest that, under
406 nutrient-limited conditions, engineered *R. eutropha* could convert carbon that would
407 otherwise be used for the secreted pyruvate to other molecules like branched-chain alcohols.

408

409 Production optimization

410 *R. eutropha* strain Re2401/pJL26 was able to produce 150 mg/L isobutanol and 28
411 mg/L 3-methyl-1-butanol in flask cultures using fructose as the main carbon source. In order
412 to improve this production yield, we identified and deleted various carbon sinks from the
413 genome of Re2401. First, the valine-specific transaminase (*ilvE*) gene, the product of which
414 converts 2-ketoisovalerate to valine, was deleted to create strain Re2402. The resulting strain
415 did not become a valine auxotroph (data not shown), since other *ilvE* homologs are present in
416 *R. eutropha*. Re2402/pJL26 was improved in isobutanol production by 33 %, compared to
417 Re2401/pJL26 (Fig. 6). Subsequently, the *bkdAB* operon, which encodes for a branched-
418 chain keto acid dehydrogenase complex for conversion of α -ketoisovalerate to isobutyryl-CoA,
419 was also eliminated from Re2402 to produce strain Re2410. Re2410/pJL26 was boosted in
420 isobutanol production by only an extra 5 % compared to Re2402/pJL26, possibly because
421 isobutyryl-CoA was not the prominent α -ketoisovalerate sink present in *R. eutropha*. Lastly,
422 pyruvate dehydrogenase complex enzyme, encoded by *aceE*, was deleted in Re2410 to
423 produce strain Re2425. These genetic manipulations in Re2425/pJL26 enhanced the
424 production of isobutanol to 270 mg/L, an 80 % increase from the production of Re2401/pJL26
425 (Fig. 6). Elimination of these genes did not affect the overall growth of the engineered *R.*
426 *eutropha* strain (Online Resource 3).

427

428 Continuous flask cultivations

429 Since *R. eutropha* experiences isobutanol toxicity at concentrations above 0.5 % (v/v),
430 the production yield might be adversely affected by the product itself. In order to alleviate
431 this inhibition and determine the longevity of engineered *R. eutropha* in both growth and
432 alcohol production, Re2425/pJL26 was cultivated in 100 mL minimal media with 1 %
433 fructose and 0.05 % NH₄Cl. At the end of every 24 h, all isobutanol and 3-methyl-1-butanol
434 produced were removed with the spent growth media, and fresh minimal media were added to
435 the cultures. Each day, approximately 200 mg/L to 500 mg/L branched-chain alcohols were
436 produced. Re2425/pJL26 was able to continuously utilize fructose as the main carbon source
437 and produce branched-chain alcohols for a duration of greater than 50 days. The total
438 accumulated alcohols produced reached levels of over 14 g/ L (Figure 7).

439

440 **DISCUSSION**

441 A biosynthetic pathway for branched-chain alcohols production, while utilizing several
442 native genes and gene products, is a heterologous pathway in *R. eutropha*, and it could
443 decrease the overall fitness of the cell though unbalanced pathway precursors or product
444 inhibition. In order to achieve optimal production of branched-chain alcohols, balanced
445 carbon and energy flow must be achieved and properly analyzed. As mentioned previously, *R.*
446 *eutropha* is a model organism for carbon storage, due to its ability to redirect carbon flow for
447 the synthesis of large quantities of intracellular polymer (Potter et al. 2004). With PHB
448 biosynthesis enzymes deleted, *R. eutropha* secreted carbon in the form of pyruvate and stored
449 the reducing-energy, originally used by the PhaB enzymes, in the form of NADH (Table 2).
450 Branched-chain alcohols production utilizes pyruvate as an initial pathway precursor into the
451 valine-biosynthesis pathway. Additionally, NADPH is the cofactor required by the additional
452 Ehrlich pathway enzyme ADH. Therefore, it is advantageous to use mutant *R. eutropha*
453 incapable of PHB production for biofuel production.

454 With only the incorporation of the Ehrlich pathway enzymes KivD and ADH, *R.*
455 *eutropha* synthesized low levels of isobutanol (Fig. 2). When isobutanol precursors, pyruvate
456 or α -ketoisovalerate, were supplied extracellularly, the isobutanol production levels
457 dramatically improved to \sim 4.5 g/L (Fig. 2). This result suggested that the production process
458 was limited by low activities of branched-chain amino acid biosynthesis pathway enzymes,
459 specifically the first enzyme of the pathway, AHAS (Online Resource 2). AHAS contains a
460 catalytic large subunit, encoded by the gene *ilvB*, and a small regulatory subunit, encoded by
461 *ilvH* (Vyazmensky et al. 2009). Although there is no crystal structure of the AHAS enzyme to
462 date, ultracentrifugation studies using *E. coli* AHAS revealed that AHAS is a heterotetramer.
463 Catalysis occurs at the tetramer interface and the regulatory subunit binds valine at its
464 homodimer interface as concluded by site-directed mutagenesis and amino acid binding
465 experiments. The binding of valine causes a conformational change at the subunit interface,
466 resulting in a less stable, thus less active complex. AHAS not only was tightly regulated by
467 product inhibition, but also through tRNA repression, substrate specificity, and protein
468 degradation (Chipman et al. 1998; Chipman et al. 2005; Gollop et al. 1990; McCourt and
469 Duggleby 2006). In order to shunt more precursors into the production of isobutanol,
470 duplication of selected native branched-chain amino acid biosynthesis genes was employed in
471 this study (Online Resource 2 and Fig. 4). Additionally, mutagenic techniques can be
472 employed to decrease regulation of AHAS by feedback inhibition and substrate specificity
473 (Engel et al. 2004; Gollop et al. 1990; Mendel et al. 2001; Slutzker et al. 2011). As described
474 previously, the inhibition of *E. coli* AHAS by valine was alleviated by elimination of the
475 valine-binding residues or C-terminal domain of the regulatory subunit (Mendel et al. 2001;
476 Slutzker et al. 2011). Additionally, Engel et al. were able to construct a mutant AHAS from *E.*
477 *coli* with increased substrate specificity towards pyruvate instead of α -ketobutyrate by
478 incorporation of a bulky amino acid residue at the AHAS active site (Engel et al. 2004).
479 These techniques can be utilized to engineer *R. eutropha* AHAS with decreased feedback

480 inhibition and substrate specificity, thus increase AHAS activity for the production of
481 isobutanol.

482 *R. eutropha* does not have endogenous decarboxylase activity towards branched-chain
483 α -keto acids (Pohlmann et al. 2007; Schwartz et al. 2009). The overexpression of the native
484 valine biosynthesis pathway, the addition of heterologous KivD, in addition to the constitutive
485 expression of native ADH under aerobic conditions, resulted in the production of isobutanol
486 and 3-methyl-1-butanol (Fig. 3), thus demonstrating that *R. eutropha* can be engineered for
487 the production of branched-chain higher alcohols. Furthermore, the production of branched-
488 chain alcohols in *R. eutropha* was shown to be triggered by nitrogen limitation (Fig. 5).
489 These observations suggest that such regulation options might be employed for controlled
490 production of isobutanol during fermentation scale ups. Furthermore, controlled production
491 of isobutanol could be achieved by utilization of inducible promoters like propionate
492 (*P*_{lassmeier} et al. 2012) and *P*_{lac} (Fukui et al. 2011).

493 One of the key points in the production of branched-chain higher alcohols was the
494 utilization of broad substrate range ADH for the conversion of branched-chain aldehydes to
495 alcohols. Although various heterologous ADH enzymes from *L. lactis* (AdhA),
496 *Saccharomyces cerevisiae* (Adh2), and *E. coli* (YqhD) were employed for the production of
497 branched-chain alcohols in *E. coli*, *Corynebacterium glutamicum*, and *R. eutropha* (Atsumi et
498 al. 2008; Atsumi et al. 2010a; Blombach et al. 2011; Jarboe, 2011; Li et al. 2012; Smith et al.
499 2010), the use of a native ADH enzyme expressed from *R. eutropha* would be more
500 compatible with the host organism. *R. eutropha* mutant strain DJ21 constitutively expressed
501 ADH and exhibited a higher activity towards the reduction of isobutyraldehyde compared to
502 YqhD (Fig. 3A), which is an enzyme that has been utilized often in heterologous microbial
503 isobutanol production studies (Jarboe, 2011). The DJ21 strain, after deletion of the PHB
504 biosynthesis operon, was also able to produce isobutanol and 3-methyl-1-butanol when native
505 valine-biosynthesis and *kivD* genes were overexpressed (Fig. 3B). The activity of ADH

506 significantly affected the concentration of isobutanol produced, as shown in Fig. 3. This
507 could be explained by the fact that the ADH enzyme is bidirectional (Steinbüchel and
508 Schlegel 1984), thus the production of isobutanol relies on the ability of ADH to catalyze
509 isobutyraldehyde reduction instead of isobutanol oxidation.

510 Production of isobutanol and 3-methyl-1-butanol in engineered *R. eutropha* strains
511 reached a maximum of 270 mg/L and 40 mg/L, respectively, in a $\Delta phaCAB \Delta ilvE \Delta bkdAB$
512 $\Delta aceE$ (Re2425) background at 48 h. The bottleneck still appears to involve low activities of
513 some of the pathway enzymes (Fig. 4) potentially due to product inhibition, poor expression,
514 solubility, oxygen sensitivity, and codon usage. Li, et al. used AlsS from *Bacillus subtilis* as
515 an AHAS for branched-chain alcohols production in *R. eutropha*, because AlsS does not
516 experience product inhibition like AHAS from other organisms (Gollop et al. 1990). In this
517 work, it was also shown that the activity of AlsS was five times higher than the native AHAS
518 (Li et al. 2012). However, after substituting *alsS* into our isobutanol production operon, no
519 significant change in isobutanol production was observed (Online Resource 5). Furthermore,
520 incorporation of valine biosynthesis pathway enzymes from *Corynebacterium glutamicum* for
521 the production of isobutanol in *R. eutropha* also did not improve production (Online Resource
522 5). These results could be due to the differences in codon usage between *R. eutropha* and *B.*
523 *subtilis* or *C. glutamicum*. Synthetic codon-optimized heterologous genes encoding AHAS
524 and KivD could be used to improved protein expression, thus enzyme activity. On the other
525 hand, besides gene duplication, incorporation of promoters at the beginning of each individual
526 pathway genes could also enhance branched-chain alcohol production.

527 Cultivation of *R. eutropha* beyond 48 h resulted in a loss of more than 50 % of the
528 branched-chain alcohols produced (Fig. 6). We anticipated that the cells might have
529 converted isobutanol into less toxic compounds, given the toxic effects of isobutanol (Atsumi
530 et al. 2010b; McGowan 1954; Minty et al. 2011), and the *R. eutropha* mutant strain DJ21 has
531 been shown to utilize isobutanol as its sole carbon source for growth (data not shown). To

532 minimize product consumption, removal of isobutanol from the culture upon formation or
533 deletion of isobutanol utilization pathway genes could be applied.

534 We investigated the ability of *R. eutropha* to tolerate isobutanol toxicity by growing
535 the cells in the presence of different isobutanol concentrations (0 to 1 %, v/v). Despite
536 observing that some mutants were more tolerant to isobutanol than the wild type strain, the *R.*
537 *eutropha* strains tested showed that overall isobutanol tolerance was extremely low. At
538 concentrations as low as 0.8 %, no cell growth was detected (Table 3). Such tolerance is
539 much lower than those reported for *E. coli* (1.5 %) and *C. glutamicum* (>2 %) (Smith et al.
540 2010). Increasing isobutanol tolerance will be crucial for the effective production of
541 isobutanol by *R. eutropha*. Higher tolerance can potentially be achieved by these approaches:
542 overexpression of stress related (heat shock) proteins, directed evolution by challenging cells
543 with increasing concentrations of isobutanol, elimination of transporter genes, or rapid
544 product removal from the growth media (Atsumi et al. 2010b; Baez et al. 2011; Minty et al.
545 2011; Nielsen et al. 2009; Nielsen and Prather 2009). To test the effectiveness of product
546 elimination, we removed all the isobutanol and 3-methyl-1-butanol produced in the media at
547 the end of each 24 h growth period and resupplemented *R. eutropha* with nutrients. The
548 highest rate of branched-chain alcohols produced under such condition was 30 mg/L/h.
549 Overall, more than 14 g/L total branched-chain alcohols were accumulated by *R. eutropha* in
550 the continuous flask culture (Fig. 7). Such prolonged cultivation and branched-chain alcohols
551 production time make *R. eutropha* a favorable candidate for industrial fermentation scale up
552 processes.

553 Although *R. eutropha* is traditionally employed for the production of PHB, a growing
554 amount of attention has now centered on engineering this strain for the production of biofuels.
555 Production of biofuels, such as branched-chain alcohols, in *R. eutropha* can act as an
556 alternative to carbon storage when redirected from PHB biosynthesis. Since *R. eutropha* was
557 able to produce ~60 g/L PHB using CO₂ and H₂ as the sole carbon and energy source,

558 respectively (Ishizaki et al. 2001), engineered *R. eutropha*, with branched-chain alcohols
559 production ability, could be utilized to convert CO₂- and H₂-rich gas streams to
560 transportational biofuel such as branched-chain alcohols. In this study, we examined the
561 ability for *R. eutropha* to redirect its carbon and energy storage system from PHB to the
562 production of isobutanol and 3-methyl-1-butanol via the native branched-chain amino acid
563 biosynthesis pathway, with highlights on the production response to nutrient stress, product
564 tolerance, and continuous flask cultivation.

565

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584 References

- 585
586 Atsumi S, Hanai T, Liao JC (2008) Non-fermentative pathways for synthesis of branched-
587 chain higher alcohols as biofuels. *Nature* 451:86-89
588
589 Atsumi S, Higashide W, Liao JC (2009) Direct photosynthetic recycling of carbon dioxide to
590 isobutyraldehyde. *Nat Biotechnol* 27:1177-1180
591
592 Atsumi S, Wu TY, Eckl EM, Hawkins SD, Buelter T, Liao JC (2010a) Engineering the
593 isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde
594 reductase/alcohol dehydrogenase genes. *Appl Microbiol Biotechnol* 85:651-657
595
596 Atsumi S, Wu TY, Machado IMP, Huang WC, Chen PY, Pellegrini M, Liao JC (2010b)
597 Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*.
598 *Mol Syst Biol* 6:449-450
599
600 Baer SH, Blaschek HP, Smith TL (1987) Effect of butanol challenge and temperature on lipid
601 composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. *Appl*
602 *Environ Microbiol* 53:2854-2861
603
604 Baez A, Cho KM, Liao JC (2011) High-flux isobutanol production using engineered
605 *Escherichia coli*: a bioreactor study with in situ product removal. *Appl Microbiol Biotechnol*
606 90:1681-1690
607
608 Blombach B, Riester T, Wieschalka S, Ziert C, Youn JW, Wendisch VF, Eikmanns BJ, (2011)
609 *Corynebacterium glutamicum* tailored for efficient isobutanol production. *Appl Environ*
610 *Microbiol* 77:3300-3310
611
612 Bowien B, Kusian B (2002) Genetics and control of CO₂ assimilation in the chemoautotroph
613 *Ralstonia eutropha*. *Arch Microbiol* 178:85-93
614
615 Brigham CJ, Sinskey AJ (2012) Applications of polyhydroxyalkanoates in the medical
616 industry. *Int J Biotechnol Wellness Ind.* 1: 53-60.
617
618 Budde CF, Mahan AE, Lu J, Rha C, Sinskey AJ (2010) Roles of multiple acetoacetyl
619 coenzyme A reductases in polyhydroxybutyrate biosynthesis in *Ralstonia eutropha* H16. *J*
620 *Bacteriol* 192:5319-5328
621
622 Budde CF, Riedel SL, Hubner F, Risch S, Popovic MK, Rha C, Sinskey AJ (2011) Growth
623 and polyhydroxybutyrate production by *Ralstonia eutropha* in emulsified plant oil medium.
624 *Appl Microbiol Biotechnol* 89:1611-1619
625
626 Chipman DM, Barak Z, Schloss JV (1998) Biosynthesis of 2-aceto-2-hydroxy acids:
627 acetolactate synthases and acetohydroxyacid synthases. *Biochim Biophys Acta* 1385:401-419
628
629 Chipman DM, Duggleby RG, Tittmann K (2005) Mechanisms of acetohydroxyacid synthases.
630 *Curr Opin Chem Biol* 9:475-481
631
632 Chong L (2001) Molecular cloning - A laboratory manual, 3rd edition. *Science* 292:446-446
633

634 Connor MR, Liao JC (2009) Microbial production of advanced transportation fuels in non-
635 natural hosts. *Curr Opin Biotechnol* 20:307-315.
636
637 Connor MR, Atsumi S (2010) Synthetic biology guides biofuel production. *J Biomed*
638 *Biotechnol* 2010:1-9
639
640 de la Plaza M, Fernandez de Palencia P, Pelaez C, Requena T (2004) Biochemical and
641 molecular characterization of alpha-ketoisovalerate decarboxylase, an enzyme involved in the
642 formation of aldehydes from amino acids by *Lactococcus lactis*. *FEMS Microbiol Lett*
643 238:367-374.
644
645 de Palencia PF, de la Plaza A, Amarita F, Requena T, Pelaez C (2006) Diversity of amino
646 acid converting enzymes in wild lactic acid bacteria. *Enzyme Microb Technol* 38:88-93
647
648 Engel S, Vyazmensky M, Vinogradov M, Berkovich D, Bar-Ilan A, Qimron U, Rosiansky Y,
649 Barak Z, Chipman DM (2004) Role of a conserved arginine in the mechanism of
650 acetohydroxyacid synthase: catalysis of condensation with a specific ketoacid substrate. *J Biol*
651 *Chem* 279:24803-24812
652
653 Fukui T, Ohsawa K, Mifune J, Orita I, Nakamura S (2011) Evaluation of promoters for gene
654 expression in polyhydroxyalkanoate-producing *Cupriavidus necator* H16. *Appl Microbiol*
655 *Biotechnol* 89:1527-1536
656
657 Gogerty DS, Bobik TA (2010) Formation of isobutene from 3-hydroxy-3-methylbutyrate by
658 diphosphomevalonate decarboxylase. *Appl Environ Microbiol* 76:8004-8010
659
660 Gollop N, Damri B, Chipman DM, Barak Z (1990) Physiological implications of the substrate
661 specificities of acetohydroxy acid synthases from varied organisms. *J Bacteriol* 172:3444-
662 3449
663
664 Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR (2008) The Ehrlich
665 pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae*
666 metabolism. *Appl Environ Microbiol* 74:2259-2266
667
668 Ishizaki A, Tanaka K, Taga N (2001) Microbial production of poly-D-3-hydroxybutyrate
669 from CO₂. *Appl Microbiol Biotechnol* 57:6-12
670
671 Jarboe LR (2011) YqhD: a broad-substrate range aldehyde reductase with various applications
672 in production of biorenewable fuels and chemicals. *Appl Microbiol Biotechnol* 89:249-257
673
674 Jendrossek D, Kruger N, Steinbüchel A (1990) Characterization of alcohol dehydrogenase
675 genes of derepressible wild-type *Alcaligenes eutrophus* H16 and constitutive mutants. *J*
676 *Bacteriol* 172:4844-4851
677
678 Karr DB, Waters JK, Emerich DW (1983) Analysis of Poly-β-Hydroxybutyrate in *Rhizobium*
679 *japonicum* Bacteroids by Ion-Exclusion High-Pressure Liquid Chromatography and UV
680 Detection. *Appl Environ Microbiol* 46:1339-1344
681
682 Khanna S, Srivastava AK (2005) Statistical media optimization studies for growth and PHB
683 production by *Ralstonia eutropha*. *Process Biochem* 40:2173-2182
684

685 Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Peterson KM (1995)
686 Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different
687 antibiotic-resistance cassettes. *Gene* 166:175-176
688
689 Kurosawa K, Boccazzi P, de Almeida NM, Sinskey AJ (2010) High-cell-density batch
690 fermentation of *Rhodococcus opacus* PD630 using a high glucose concentration for
691 triacylglycerol production. *J Biotechnol* 147:212-218
692
693 Larroy C, Rosario Fernandez M, Gonzalez E, Pares X, Biosca JA (2003) Properties and
694 functional significance of *Saccharomyces cerevisiae* ADHVI. *Chem Biol Interact* 143-
695 144:229-238
696
697 Lenz O, Ludwig M, Schubert T, Burstel I, Ganskow S, Goris T, Schwarze A, Friedrich B
698 (2010) H₂ conversion in the presence of O₂ as performed by the membrane-bound [NiFe]-
699 hydrogenase of *Ralstonia eutropha*. *Chemphyschem* 11:1107-1119
700
701 Leyval D, Uy D, Delaunay S, Goergen JL, Engasser JM (2003) Characterisation of the
702 enzyme activities involved in the valine biosynthetic pathway in a valine-producing strain of
703 *Corynebacterium glutamicum*. *J Biotechnol* 104:241-252
704
705 Li H, Opgenorth PH, Wernick DG, Rogers S, Wu RY, Higashide W, Malati P, Huo YX, Cho
706 KM, Liao JC (2012). Integrated electromicrobial conversion of CO₂ to higher alcohols.
707 *Science* 335:1596
708
709 Lu J, Brigham CJ, Rha C, Sinskey AJ (2012) Characterization of an extracellular lipase and
710 its chaperone from *Ralstonia eutropha* H16. *Appl Microbiol Biotechnol*. DOI:
711 10.1007/s00253-012-4115-z
712
713 Macho V, Kralik M, Jurecekova E, Hudec J, Jurecek L (2001) Dehydration of C-4 alkanols
714 conjugated with a positional and skeletal isomerisation of the formed C-4 alkenes. *Appl Catal*
715 *A Gen* 214:251-257
716
717 McCourt JA, Duggleby RG (2006) Acetohydroxyacid synthase and its role in the biosynthetic
718 pathway for branched-chain amino acids. *Amino Acids* 31:173-210
719
720 McGowan JC (1954) The physical toxicity of chemicals IV. solubilities, partition coefficients
721 and physical toxicities. *J Appl Chem* 4:41-47
722
723 Mendel S, Elkayam T, Sella C, Vinogradov V, Vyazmensky M, Chipman DM, Barak Z (2001)
724 Acetohydroxyacid synthase: a proposed structure for regulatory subunits supported by
725 evidence from mutagenesis. *J Mol Biol* 307: 465-477
726
727 Minty JJ, Lesnefsky AA, Lin F, Chen Y, Zaroff TA, Veloso AB, Xie B, McConnell CA,
728 Ward RJ, Schwartz DR, Rouillard JM, Gao Y, Gulari E, Lin XN (2011) Evolution combined
729 with genomic study elucidates genetic bases of isobutanol tolerance in *Escherichia coli*.
730 *Microb Cell Fact* 10:18-56
731
732 Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Prather KL (2009) Engineering
733 alternative butanol production platforms in heterologous bacteria. *Metab Eng* 11:262-273
734

735 Nielsen DR, Prather KJ (2009) In situ product recovery of n-butanol using polymeric resins.
736 Biotechnol Bioeng 102:811-821
737
738 Plassmeier J, Persicke M, Puhler A, Sterthoff C, Ruckert C, Kalinowski J (2012) Molecular
739 characterization of PrpR, the transcriptional activator of propionate catabolism in
740 *Corynebacterium glutamicum*. J Biotechnol 159:1-11
741
742 Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering
743 C, Potter M, Schwartz E, Strittmatter A, Voß I, Gottschalk G, Stinbüchel A, Friedrich B,
744 Bowien B (2006). Genome sequence of the bioplastic-producing "Knallgas" bacterium
745 *Ralstonia eutropha* H16. Nat Biotechnol 24:1257-1262
746
747
748 Potter M, Muller H, Reinecke F, Wiczorek R, Fricke F, Bowien B, Friedrich B, Steinbüchel
749 A (2004) The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous
750 and paralogous phasins occur in *Ralstonia eutropha*. Microbiology 150:2301-2311
751
752 Quandt J, Hynes MF (1993) Versatile suicide vectors which allow direct selection for gene
753 replacement in gram-negative bacteria. Gene 127:15-21
754
755 Rehm BH (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15-33
756
757 Savrasova EA, Kivero AD, Shakulov RS, Stoyanova NV (2011) Use of the valine biosynthetic
758 pathway to convert glucose into isobutanol. J Ind Microbiol Biotechnol 38:1287-1294
759
760 Schwartz E, Voigt B, Zuhlke D, Pohlmann A, Lenz O, Albrecht D, Schwarze A, Kohlmann Y,
761 Krause C, Hecker M, Friedrich B (2009) A proteomic view of the facultatively
762 chemolithoautotrophic lifestyle of *Ralstonia eutropha* H16. Proteomics 9:5132-5142
763
764 Sheehan J (2009) Engineering direct conversion of CO₂ to biofuel. Nat Biotechnol 27:1128-
765 1129
766
767 Simon R, Priefer U, Puhler A (1983) A broad host range mobilization system for in vivo
768 genetic-engineering - transposon mutagenesis in gram-negative bacteria. Bio-Technology
769 1:784-791
770
771 Slater S, Houmiel KL, Tran M, Mitsky TA, Taylor NB, Padgett SR, Gruys KJ (1998)
772 Multiple β -ketothiolases mediate poly(beta-hydroxyalkanoate) copolymer synthesis in
773 *Ralstonia eutropha*. J Bacteriol 180:1979-1987
774
775 Slutzker A, Vyazmensky M, Chipman DM, Barak Z (2011) Role of the C-terminal domain of
776 the regulatory subunit of AHAS isozyme III: use of random mutagenesis with in vivo
777 reconstitution (REM-ivrs). Biochim Biophys Acta 1814:449-455
778
779 Smith KM, Cho KM, Liao JC (2010) Engineering *Corynebacterium glutamicum* for
780 isobutanol production. Appl Microbiol Biotechnol 87:1045-1055
781
782 Steinbüchel A, Schlegel HG (1984) A multifunctional fermentative alcohol dehydrogenase
783 from the strict aerobic *Alcaligenes eutrophus*: purification and properties. Eur J Biochem
784 141:555-564
785

786 Steinbüchel A, Frund C, Jendrossek D, Schlegel HG (1987) Isolation of mutants of
787 *Alcaligenes eutrophus* unable to derepress the fermentative alcohol-dehydrogenase. Arch
788 Microbiol 148:178-186
789
790 Steinbüchel A, Schlegel HG (1989) Excretion of pyruvate by mutants of *Alcaligenes*
791 *eutrophus*, which are impaired in the accumulation of poly(β -hydroxybutyric acid) (PHB),
792 under conditions permitting synthesis of PHB. Appl Microbiol Biotechnol 31:168-175
793
794 Vollherbst-Schneck K, Sands JA, Montenecourt BS (1984) Effect of butanol on lipid
795 composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol
796 47:193-194
797
798 Vyazmensky M, Zherdev Y, Slutzker A, Belenky I, Kryukov O, Barak Z, Chipman DM (2009)
799 Interactions between large and small subunits of different acetohydroxyacid synthase
800 isozymes of *Escherichia coli*. Biochemistry 48:8731-8737
801
802 Westerfield WW (1945) A colorimetric determination of blood acetoin. J Biol Chem 161:495-
803 502
804
805 Yan Y, Liao JC (2009) Engineering metabolic systems for production of advanced fuels. J Ind
806 Microbiol Biotechnol 36:471-479
807
808 Yang YH, Brigham CJ, Budde CF, Boccazzi P, Willis LB, Hassan MA, Yusof ZA, Rha C,
809 Sinskey AJ (2010) Optimization of growth media components for polyhydroxyalkanoate
810 (PHA) production from organic acids by *Ralstonia eutropha*. Appl Microbiol Biotechnol
811 87:2037-2045
812
813 York GM, Stubbe J, Sinskey AJ (2001) New insight into the role of the PhaP phasin of
814 *Ralstonia eutropha* in promoting synthesis of polyhydroxybutyrate. J Bacteriol 183:2394-
815 2397
816
817 York GM, Lupberger J, Tian J, Lawrence AG, Stubbe J, Sinskey AJ (2003) *Ralstonia*
818 *eutropha* H16 encodes two and possibly three intracellular poly[D-3-hydroxybutyrate]
819 depolymerase genes. J Bacteriol 185:3788-3794
820
821 Zhang ZQ, Yu J, Stanton RC (2000) A method for determination of pyridine nucleotides
822 using a single extract. Anal Biochem 285:163-167
823
824 Zor T, Selinger Z (1996) Linearization of the Bradford protein assay increases its sensitivity:
825 theoretical and experimental studies. Anal Biochem 236: 302-308
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833 **FIGURE LEGENDS**

834

835 Fig. 1

836 Schematic of isobutanol and 3-methyl-1-butanol production pathways. The production of
837 isobutanol (top right, boxed) uses precursors diverted from the valine biosynthesis pathway
838 via enzymes acetohydroxyacid synthase (AHAS), acetohydroxyacid isomeroreductase
839 (AHAIR), dihydroxyacid dehydratase (DHAD), ketoisovalerate decarboxylase (KIVD), and
840 alcohol dehydrogenase (ADH). Redirection of 2-ketoisocaproate from leucine biosynthesis
841 pathway via isopropylmalate synthase (IPMS), isopropylmalate dehydratase (IPMD),
842 isopropylmalate dehydrogenase (IPMDH), KIVD, and ADH leads to the production of 3-
843 methyl-1-butanol (lower left, boxed). In wild type cells, transaminase (TA) converts 2-
844 ketoisovalerate and 2-ketoisocaproate to valine and leucine respectively. The isobutanol
845 production operon (pJL26) consists of the following genes *ilvBH*, *ilvC*, *ilvD*, and *kivD*
846 encodes for the following enzymes respectively AHAS, AHAIR, DHAD, and KIVD.

847

848 Fig. 2

849 Production of isobutanol in strain Re2061/pJL23. *R. eutropha* Re2061/pJL23 (see Table 1)
850 was cultivated in minimal media with 0.05 % NH₄Cl with the following carbon sources: 2 %
851 w/v Fructose, 2 % w/v Fructose with 1 % w/v Pyruvate, or 2 % w/v Fructose with 1 % w/v 2-
852 ketoisovaleric acid. Each value represents the mean ± standard error (error bars) of n = 3.

853

854 Fig. 3

855 Isobutyraldehyde dehydrogenase activity (A) and production of branched-chain alcohols (B)
856 by *R. eutropha* wild type and ADH mutant strains harboring isobutanol production plasmids
857 (see Table 1). 1: Re2061 (H16 Δ *phaCAB*); 2: Re2061/pJL21 (H16 Δ *phaCAB/padhA*); 3:
858 Re2061/pJL20 (H16 Δ *phaCAB/padh*); 4: Re2061/pJL22 (H16 Δ *phaCAB/pyqhD*); 5:
859 Re2061/pBBR1MCS-2; 6: Re2403/pJL26 (CF17 Δ *phaCAB/pilvBHCDkivD*); 7:
860 Re2404/pJL26 (CF101 Δ *phaCAB/pilvBHCDkivD*); 8: Re2405/pJL26 (CF106

861 $\Delta phaCAB/pilvBHCDkivD$); 9: Re2406/pJL26 (CF108 $\Delta phaCAB/pilvBHCDkivD$); 10:
862 Re2407/pJL26 (CF303 $\Delta phaCAB/pilvBHCDkivD$); 11: Re2401/pJL26 (DJ21
863 $\Delta phaCAB/pilvBHCDkivD$). Data points represent the mean values of $n = 3 \pm$ standard
864 deviation (error bars).

865

866 Fig. 4

867 Activities of isobutanol production pathway enzymes: AHAS-acetohydroxyacid synthase,
868 AHAIR-acetohydroxyacid isomeroreductase, DHAD-dihydroxyacid dehydratase, KIVD-
869 ketoisovalerate decarboxylase, and ADH-alcohol dehydrogenase over the course of a 96 h
870 culture of Re2401/pJL26. Each enzymatic unit (U) is defined as 1 μ mol product formed per
871 min. Average values from three experiments were plotted with error bars representing the
872 standard deviation.

873

874 Fig. 5

875 Effect of nitrogen concentrations, in the form of NH_4Cl , on the production of isobutanol by
876 Re2401/pJL26 (DJ21 $\Delta phaCAB/pilvBHCDkivD$). (A) Nitrogen concentrations at various
877 growth time points; (B) Isobutanol produced and collected from the growth media.

878

879 Fig. 6

880 Improvement of branched-chain alcohols yield by elimination of carbon sinks. (A) Isobutanol
881 production curve; (B) 3-methyl-1-butanol production curve. DJ 21: Constitutive ADH mutant
882 *R. eutropha* strain; Re2401: (DJ21 $\Delta phaCAB$); Re2402: (DJ21 $\Delta phaCAB\Delta ilvE$); Re2410
883 (DJ21 $\Delta phaCAB\Delta ilvE\Delta bkdAB$); Re2425 (DJ21 $\Delta phaCAB\Delta ilvE\Delta bkdAB\Delta aceE$). Values were
884 average from three replicates with standard deviation values represented as error bars.

885 Fig. 7

886 Isobutanol and 3-methyl-1-butanol production by Re2425/pJL26 in a continuous flask culture,
887 over the duration of 50 days. Fresh minimal media with 1 % fructose and 0.05 % NH₄Cl was
888 used each day. The concentration values of isobutanol and 3-methyl-1-butanol were added
889 together to get the daily total branched-chain alcohols concentration. The data shown are the
890 average of three replicated experiments with error bars representing the standard deviation.

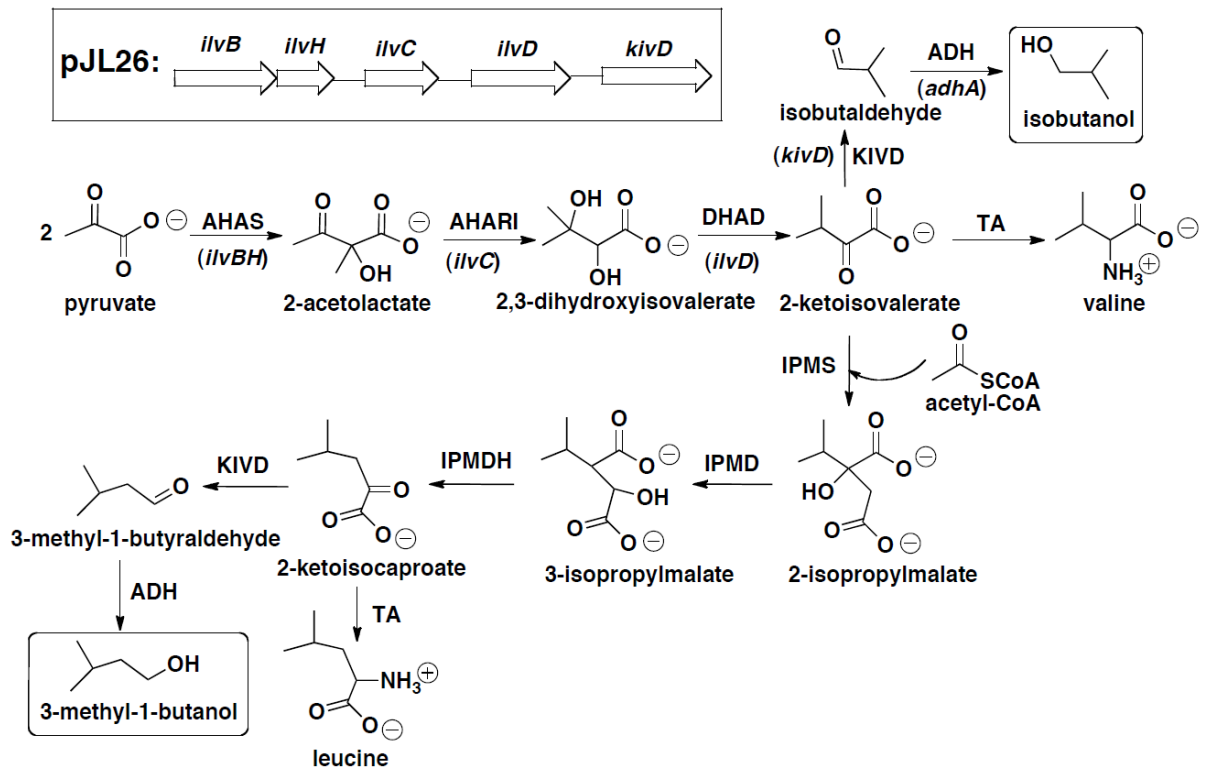
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1 **FIGURES**

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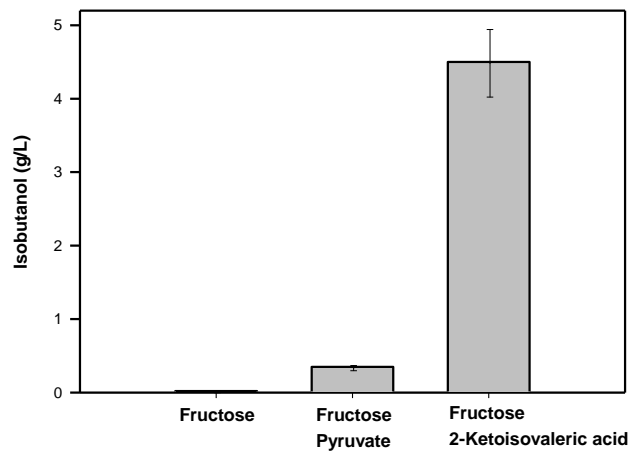
3 **Fig. 1**

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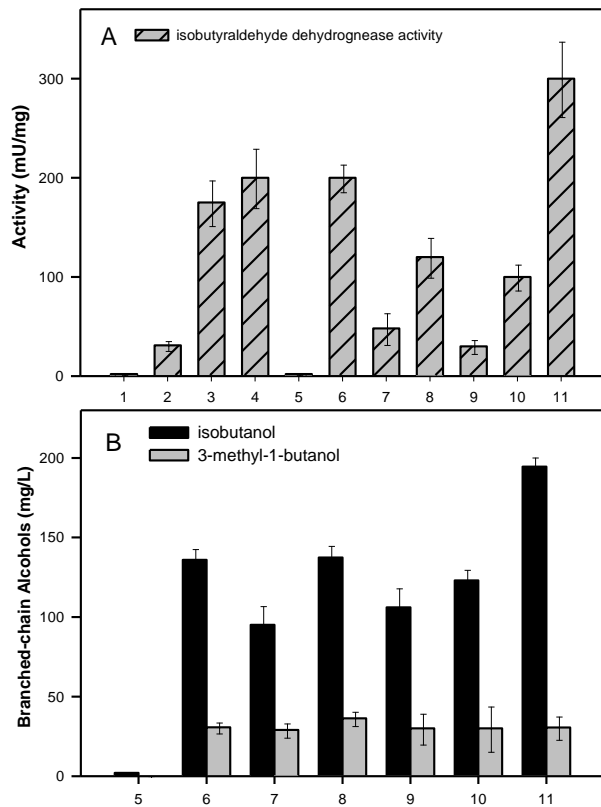
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6 **Fig. 2**



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8 **Fig. 3**



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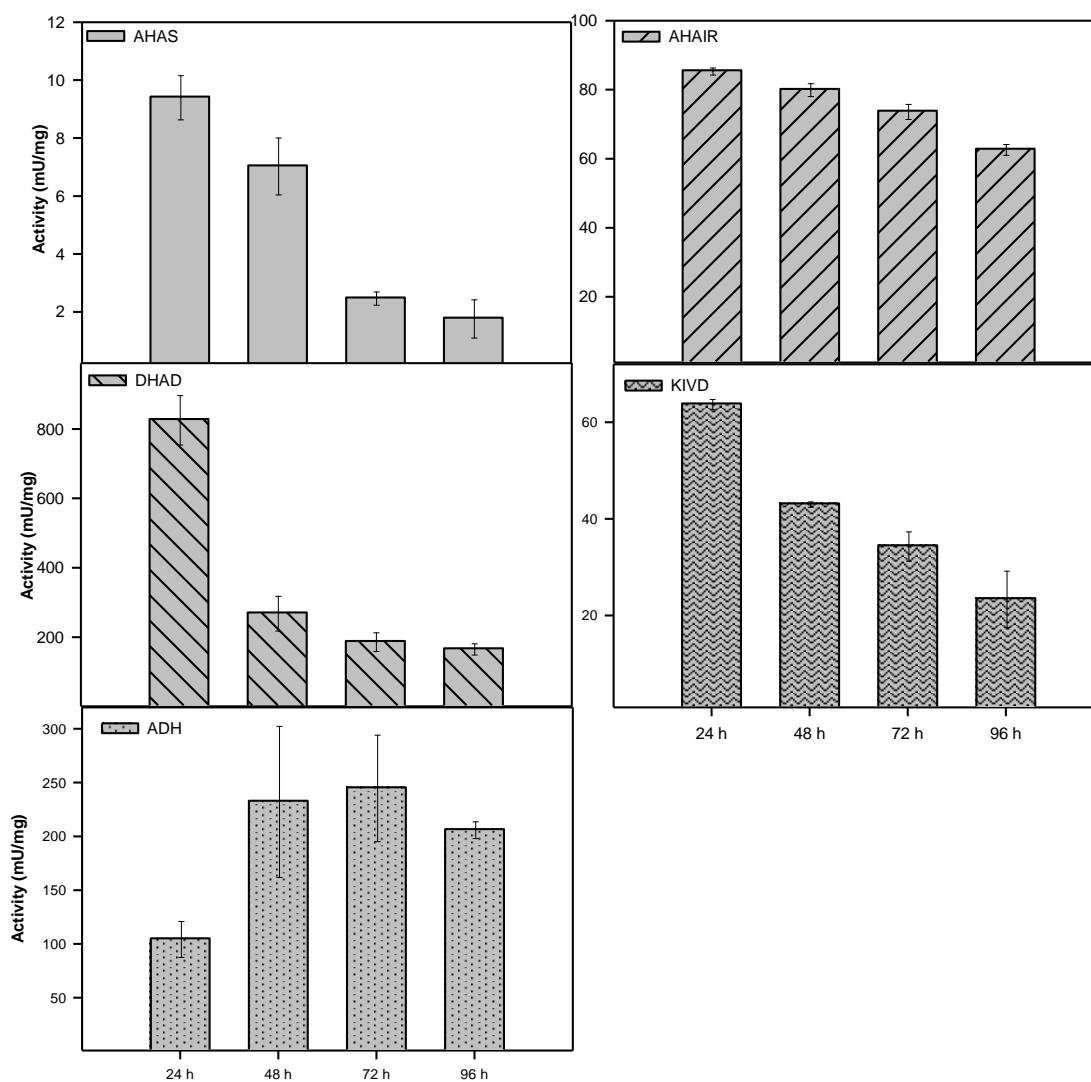
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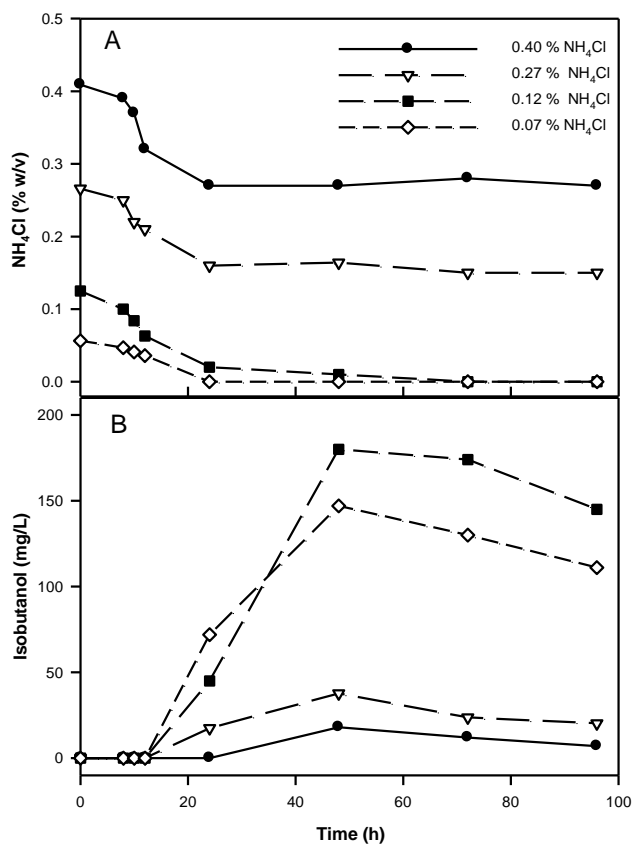
15 **Fig. 4**



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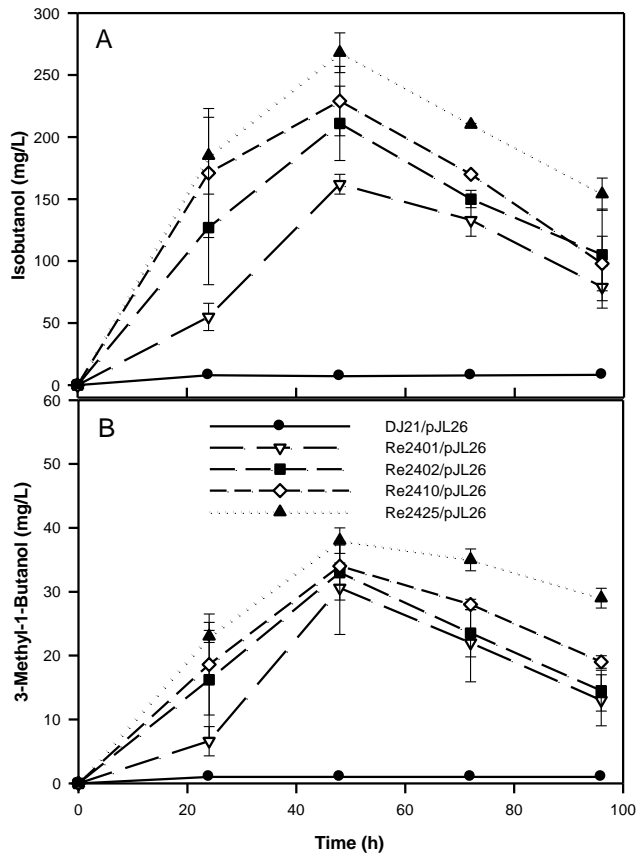
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18 Fig. 5



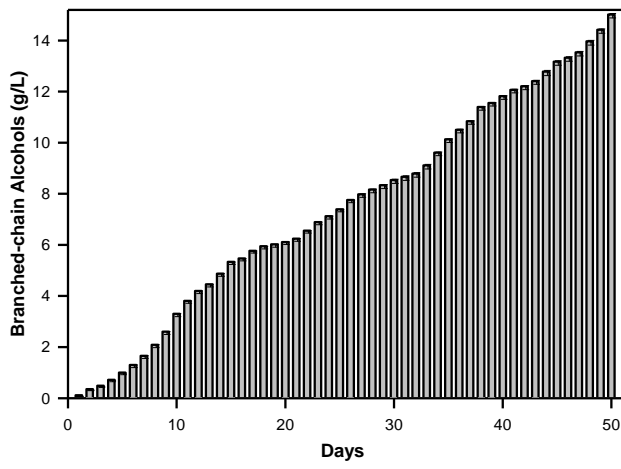
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20 **Fig. 6**



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22 **Fig. 7**



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57 TABLES

58 Table 1

59 Strains and plasmids used in this work.

| Strains or plasmid | Genotype | Reference |
|--------------------|--|----------------------------------|
| Strains | | |
| <i>R. eutropha</i> | | |
| H16 | Wild-type, gentamicin resistant (Gen ^r) | ATCC17699 |
| Re2061 | H16 Δ <i>phaCAB</i> Gen ^r | This work |
| CF17 | H16 <i>adh</i> (Con) ethanol ⁺ 2,3-butanediol ⁺ Gen ^r | (Steinbüchel <i>et al.</i> 1987) |
| CF101 | H16 <i>adh</i> (Con) ethanol ⁺ 2,3-butanediol ⁺ Gen ^r | (Steinbüchel <i>et al.</i> 1987) |
| CF106 | H16 <i>adh</i> (Con) ethanol ⁺ 2,3-butanediol ⁺ Gen ^r | (Jendrossek <i>et al.</i> 1990) |
| CF108 | H16 <i>adh</i> (Con) ethanol ⁺ 2,3-butanediol ⁺ Gen ^r | (Jendrossek <i>et al.</i> 1990) |
| CF303 | H16 <i>adh</i> (Con) ethanol ⁺ 2,3-butanediol ⁺ Gen ^r | (Steinbüchel <i>et al.</i> 1987) |
| DJ21 | H16 <i>adh</i> (Con) ethanol ⁺ 2,3-butanediol ⁺ Gen ^r | (Jendrossek <i>et al.</i> 1990) |
| Re2403 | CF17 Δ <i>phaCAB</i> Gen ^r | This work |
| Re2404 | CF101 Δ <i>phaCAB</i> Gen ^r | This work |
| Re2405 | CF106 Δ <i>phaCAB</i> Gen ^r | This work |
| Re2406 | CF108 Δ <i>phaCAB</i> Gen ^r | This work |
| Re2407 | CF303 Δ <i>phaCAB</i> Gen ^r | This work |
| Re2401 | DJ21 Δ <i>phaCAB</i> Gen ^r | This work |
| Re2402 | DJ21 Δ <i>phaCAB, ilvE</i> Gen ^r | This work |
| Re2410 | DJ21 Δ <i>phaCAB, ilvE, bkdAB</i> Gen ^r | This work |
| Re2425 | DJ21 Δ <i>phaCAB, ilvE, bkdAB, aceE</i> Gen ^r | This work |
| <i>E. coli</i> | | |
| S17-1 | Conjugation strain for transfer of plasmids into <i>R. eutropha</i> | (Simon <i>et al.</i> , 1983) |
| Plasmids | | |
| pJV7 | pJQ200Kan with Δ <i>phaC1</i> allele inserted into BamHI restriction site, confers kanamycin resistance (Kan ^r) | (Budde <i>et al.</i> , 2011a) |
| pJL33 | pJV7 with Δ <i>phaC1</i> allele removed by XbaI and SacI digestion and replace with <i>phaCAB</i> allele (Kan ^r) | This work |
| pCJB6 | pJV7 with Δ <i>phaC1</i> allele removed by XbaI and SacI digestion and replace with Δ <i>ilvE</i> allele (Kan ^r) | This work |
| pCJB7 | pJV7 with Δ <i>phaC1</i> allele removed by XbaI and SacI digestion and replace with Δ <i>bkdAB</i> allele (Kan ^r) | This work |
| pJL32 | pJV7 with Δ <i>phaC1</i> allele removed by XbaI and SacI digestion and replace with Δ <i>aceE</i> allele (Kan ^r) | This work |
| pBBR1MCS-2 | Broad-host-range cloning vector (Kan ^r) | (Kovach <i>et al.</i> , 1996) |
| pJL23 | pBBR1MCS-2 with <i>L. lactis kivD</i> gene and <i>R. eutropha adh</i> (H16_A0757) alcohol dehydrogenase gene inserted into the multiple cloning site (Kan ^r) | This work |
| pJL21 | pBBR1MCS-2 with <i>R. eutropha</i> H16_A0861 alcohol | This work |

| | | |
|-------|---|-----------|
| | dehydrogenase gene inserted into the multiple cloning site (Kan ^r) | |
| pJL20 | pBBR1MCS-2 with <i>R. eutroha</i> H16_A0757 alcohol dehydrogenase gene inserted into the multiple cloning site (Kan ^r) | This work |
| pJL22 | pBBR1MCS-2 with <i>E. coli yqhD</i> alcohol dehydrogenase gene inserted into the multiple cloning site (Kan ^r) | This work |
| pJL26 | pBBR1MCS-2 with branched-chain alcohol production operon (<i>ilvBHCDkivd</i>) inserted into the multiple cloning site (Kan ^r) | This work |

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75 **Table 2**

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77 Physiologic differences between wild type *R. eutropha* (H16) and Re2061 (H16 Δ *phaCAB*)^a.

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| Strains | Residual CDW | [Gluc] (% w/v) | [Pyr] (% w/v) | [PHB] (% CDW) | [NADH] (pmol/CFU) | [NADPH] (pmol/CFU) |
|---------|---------------|----------------|----------------|---------------|-------------------|---------------------|
| H16 | 1.1 \pm 0.1 | 1.2 \pm 0.03 | 0 | 83 \pm 2.9 | 0.01 \pm 0 | 7.5E-5 \pm 1E-5 |
| Re2061 | 1.3 \pm 0.2 | 0.7 \pm 0.04 | 0.6 \pm 0.01 | 0 | 0.04 \pm 0.002 | 8.2E-5 \pm 0.7E-5 |

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80 ^aStrains are grown in minimal media with 2 % gluconate and 0.05 % NH₄Cl. The values

81 presented were measured at the end of 96 h growth period. Concentrations of gluconate (Gluc)

82 consumed, pyruvate (Pyr) and PHB produced were detected by HPLC as discussed in

83 Materials and Methods. Intracellular reducing equivalents NADH and NADPH were

84 measured using a cofactor cycling assay (Ref) and normalized per each colony forming unit

85 (CFU). Each value represents the mean \pm standard error on n = 3.

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106 **Table 3**

107 Isobutanol tolerance of *R. eutropha* wild type and mutant strains^a.

| Strains | OD ₆₀₀ (0% IBT) | % viable (0.2% IBT) | % viable (0.5% IBT) | % viable (0.8% IBT) | % viable (1% IBT) |
|---------|-------------------------------|------------------------|------------------------|------------------------|----------------------|
| Re2061 | 2.3 | 100 | 48 | 4 | 3 |
| Re2403 | 2.2 | 3 | 3 | 3 | 3 |
| Re2404 | 2.2 | 100 | 3 | 3 | 3 |
| Re2405 | 2.3 | 29 | 5 | 3 | 3 |
| Re2406 | 2.3 | 100 | 83 | 12 | 4 |
| Re2407 | 2.3 | 100 | 98 | 6 | 2 |
| Re2401 | 2.3 | 100 | 94 | 10 | 4 |

108
 109 ^aIsobutanol at 0, 0.2, 0.5, 0.8, and 1 % were added to minimal media. Growth of *R. eutropha*
 110 wild type Re2061 and $\Delta phaCAB$ of ADH mutant strains (Table 1) were monitored.
 111 Calculation of % viable was based on the ratio between cells grown in isobutanol to cells
 112 grown without isobutanol at 24 h. Each value represents n = 1.

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Supplementary Material:

Online Resource 1: List of primers used in this study.

| Name | Sequence ^a |
|-----------------------------|--|
| <i>ΔphaCAB</i> upstream F | GAATGGATCCGTGCTCGGTGATCGCCATCAT |
| <i>ΔphaCAB</i> upstream R | GACTGGTTGAACCAGGCCGGCAGGTCACTCGAGCATATGCATGATTTG ATTGTCTCTCTG |
| <i>ΔphaCAB</i> downstream F | CAGAGAGACAATCAAATCATGCATATGCTCGAGTGACCTGCCGGCCTG GTTCAACCAGTC |
| <i>ΔphaCAB</i> downstream R | GAATGGATCCCAGGGTGTAGGTGCTGGT |
| <i>ΔphaCAB</i> digF | CGACGCCACCAACCTGCCGGG |
| <i>ΔphaCAB</i> digR | GTCCACTCCTTGATTGGCTTCG |
| <i>ΔilvE</i> upstream F | ATAAGGATCCTGCTCGAGCGGCTCGATCGT |
| <i>ΔilvE</i> upstream R | GGCTGGCAGCCGGTGTCTCACATGTCTGTTCTCCCTGCG |
| <i>ΔilvE</i> downstream F | CGCAGGGAGAACAGACATGTGAGCACCGGCTGCCAGCC |
| <i>ΔilvE</i> downstream R | AACTGGATCCCCTTGAGCAGCGCAAAGAGC |
| <i>ΔilvE</i> digF | ATGTGGGGTCAAAGGCAC |
| <i>ΔilvE</i> digR | TAGACGGTGCCGCAGTAC |
| <i>ΔbkdAB</i> upstream F | TGAAGAGCTCCTTCGTCAACGGCAACTATG |
| <i>ΔbkdAB</i> upstream R | CCTGTTGTCTTCGACCGCTACATGGCAGGTCTCTCGATGC |
| <i>ΔbkdAB</i> downstream F | GCATCGAGAGACCTGCCATGTAGCGGTCTGAAGACAACAGG |
| <i>ΔbkdAB</i> downstream R | CTTGTCTAGAACTACGTGGATTTCGCTGGC |
| <i>ΔbkdAB</i> digF | TCAAGGACATCCGAGAGGCC |
| <i>ΔbkdAB</i> digR | CGCTGAGTCACTTCTTCTGC |
| <i>ΔaceE</i> upstream F | GCCGGGATCCGAAGCCTTGCTGGCTTCATCC |
| <i>ΔaceE</i> upstream R | TGCCCGATGGCCGATCGTTCACACGGCAAGTCTCCGTTAAGG |
| <i>ΔaceE</i> downstream F | CCTTAACGGAGACTTGCCGTGTGAACGATGGGCCATCGGGCA |
| <i>ΔaceE</i> downstream R | GCATGGATCCGCTGGCAAAACGCTGAGCATTGAG |
| <i>ΔaceE</i> digF | GTGATCCTGGCCAAGACCATC |
| <i>ΔaceE</i> digR | GGCATCCTGCGGGGTGTAGCG |
| <i>kivD</i> F | GCGGTCTAGAAGGAGAATGCGATGTA |
| <i>kivD</i> R | GCACGAGCTCTGAATTATTTTGTTC |
| <i>adh</i> F | GAATGAGCTCGCGGGCCGGCAACGTC |
| <i>adh</i> R | GCCGTCTAGAACTAGTTCAGTGCGGCTTGATGGCG |

| | |
|--|---|
| <i>adhA</i> F | GAAT <u>GAATTC</u> GTGCGCGGAGACCGGCA |
| <i>adhA</i> R | GCCGGGATCCTTACATCGCTGCAGCGAA |
| <i>qyhD</i> F | GCCGGAATTCATGAACAACCTTAATCTGCACACCCC |
| <i>qyhD</i> R | GAATGGATCCTTAGCGGGCGGCTTCGTATATAC |
| <i>ilvBH</i> F | GCATACTAGTATGCCAGCGCGGAATTCTC |
| <i>ilvBH</i> R | CTTGTCGTAAAACACTTTCATGGTTGTCCTCCTTTCTAGAGAGCTTTCGTT TTCATG |
| <i>ilvC</i> F | GCCGCATATGAAAGTGTTTTACGACAAGGACGCG |
| <i>ilvC</i> R | GCCGACTAGTTTAGTTCTTCGACTGGTCGACC |
| <i>ilvD</i> F | GTCGACCAGTGAAGAATAAAAAGGAGGACGACCATGGCATTCAACA AACGCTCGCAG |
| <i>ilvD</i> R | GGTCGTCCTCCTTTTTAGTCCGTCCTGCCCCCTTG |
| <i>kivD</i> F | AAGGGGGCAGTGACGGACTGAAAAGGAGGACGACCATGTATACAGTAG GAGATTACC |
| <i>kivD</i> R | GCAGTCTAGAGGTCGTCCTCCTTTTTATGATTTATTTGTTTCAGC |
| <i>ilvBHCDkivD</i> digF1 | GCCAACATGAACTATTCGATC |
| <i>ilvBHCDkivD</i> digR1 | GCCCTCGGTGCCCATCGACATG |
| <i>ilvBHCDkivD</i> digF2 | CTGTTGCTGCAGCTGAACGTC |
| <i>ilvBHCDkivD</i> digR2 | GCCAAAGCTAATTATTTTCATG |
| <i>alsS</i> F (<i>B. subtilis</i>) | GCATACTAGTGCCGCTCGAGATGACAAAAGCAACAAAAGAA |
| <i>alsS</i> R | GCATGGATCCCTAGAGAGCTTTCGTTTTTCATG |
| <i>ilvBNe</i> F (<i>C. glutamicum</i>) | GCCGTCTAGAGTGAATGTGGCAGCTTCTCAAC |
| <i>ilvBNe</i> R | GCCGTCTAGATTAAGCGGTTTCTGCGCGAGC |
| <i>ilvCe</i> F | GCATACTAGTTTAGTCGACCTGACGGACTGC |
| <i>ilvCe</i> R | CTTTTGAACGAAGTGGGATCATGGTTGTCCTCCTTTTTAAGCGGTTTCTGC GCGAGC |
| <i>ilvDc</i> F | GCTCGCGCAGAAACCGCTTAAAAGGAGGACAACCATGATCCCCTTC GTTCAAAAG |
| <i>ilvDc</i> R | GCAGTCTAGATTAGTCGACCTGACGGACTGC |

^a Restriction sites are underlined

Online Resource 2:

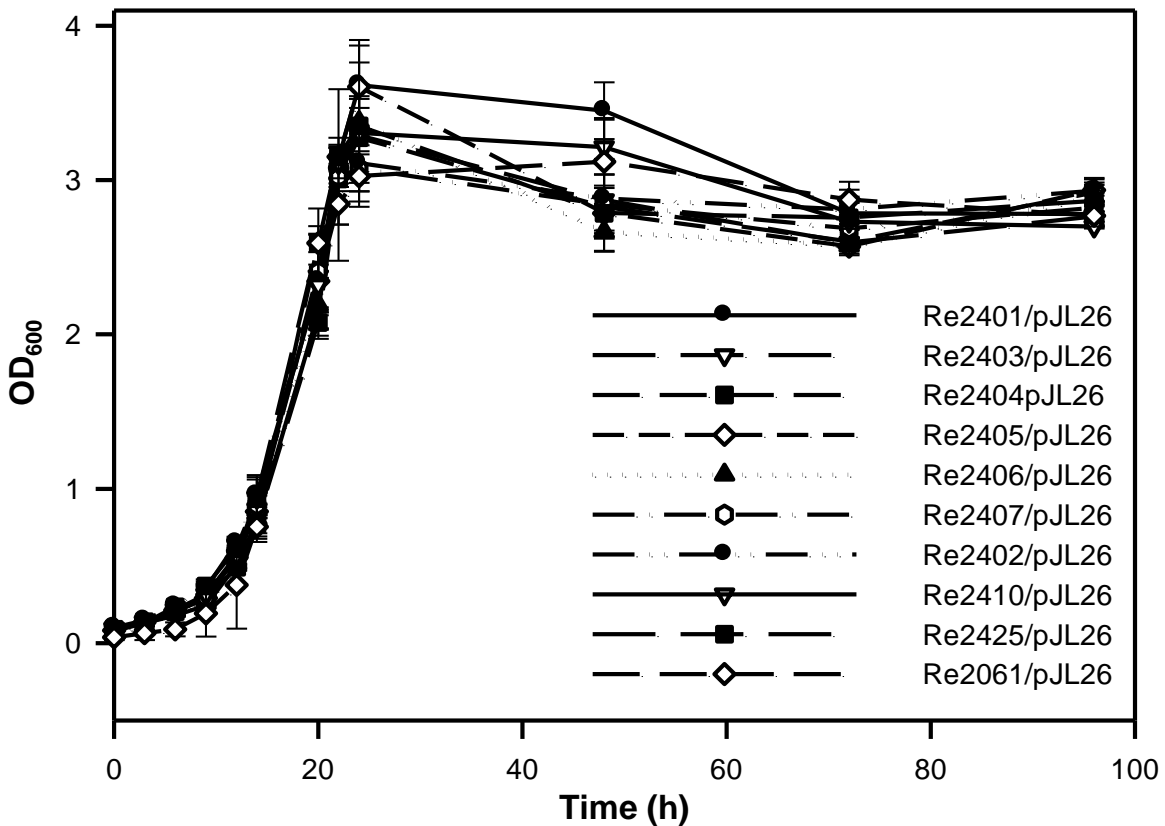
Activities of valine biosynthesis pathway enzymes in *R. eutropha* with empty vector (Re2061/pBBR1MCS-2) and overexpression plasmid (Re2061/pJL26). Cells were grown in minimal media with 2 % fructose and 0.05 % NH₄Cl and harvested for enzymatic activity at 24 h.

| Enzymes | Re2061/pBBR1MCS-2 | Re2061/pJL26 |
|---------------|-------------------|--------------|
| AHAS (mU/mg) | 5 | 10 |
| AHAIR (mU/mg) | 16 | 86 |
| DHAD (mU/mg) | 32 | 780 |

Online Resource 3:

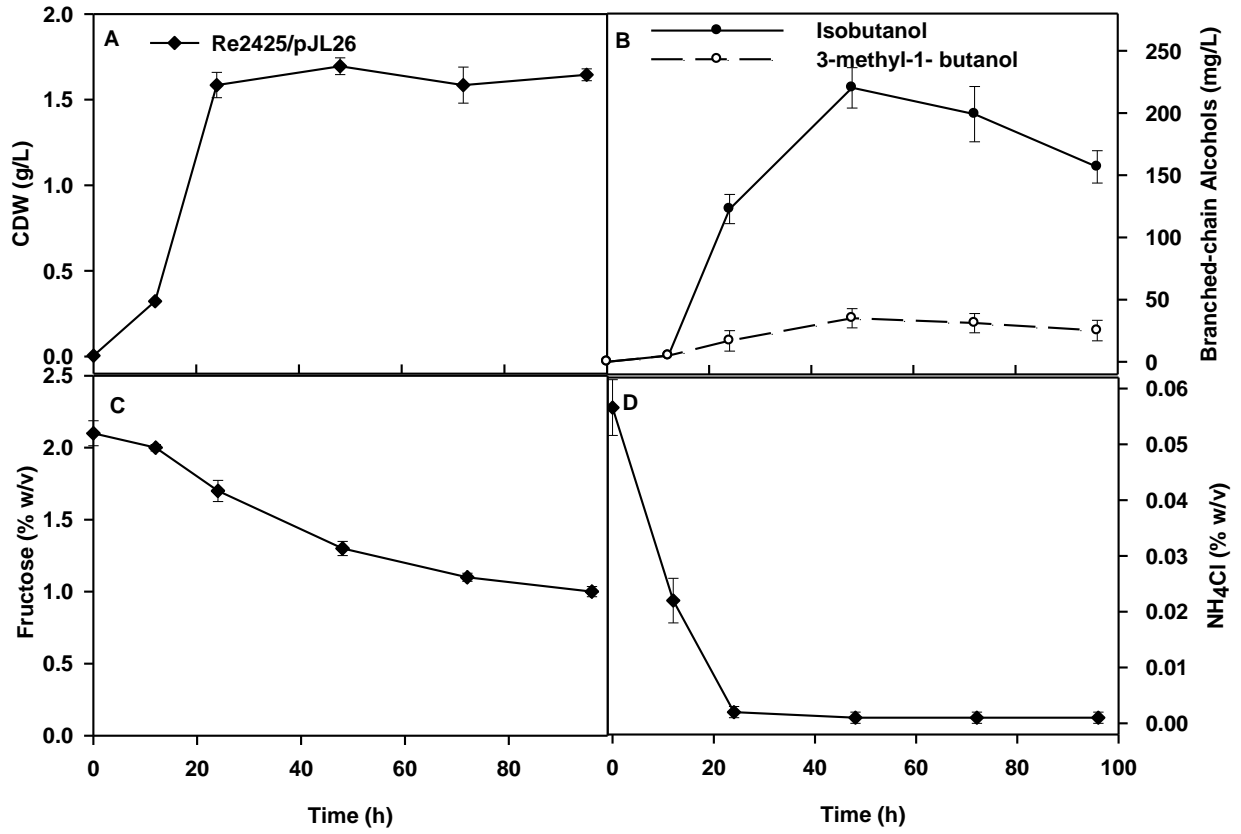
Growth of *R. eutropha* deletion strains and mutant strains constitutively expressing ADH containing the isobutanol production plasmid, pJL26. See Table 1 of text for more information regarding strains and plasmids used in this experiment.

Re2061 ($H16\Delta phaCAB$), Re2401 ($DJ21\Delta phaCAB$), Re2403 ($CF17\Delta phaCAB$), Re2404 ($CF101\Delta phaCAB$), Re2405 ($CF106\Delta phaCAB$), Re2406 ($CF108\Delta phaCAB$), Re2407 ($CF303\Delta phaCAB$). Re2402 ($DJ21\Delta phaCAB \Delta ilvE$), Re2410 ($DJ21\Delta phaCAB \Delta ilvE \Delta bkdAB$), Re2425 ($DJ21\Delta phaCAB \Delta ilvE \Delta bkdAB \Delta aceE$).



Online Resource 4:

Growth (A), branched-chain alcohols (isobutanol and 3-methyl-1-butanol) production (B), fructose utilization (C), and ammonia utilization (D) profile of Re2425/pJL26 over 96 h in minimal media with 2 % fructose and 0.05 % NH_4Cl .



Online Resource 5:

Production of isobutyraldehyde and isobutanol by strain Re2425 with plasmid pJL26 (*ilvBHCDkivD*), pJL27 (*alsS^ailvCDkivD*), pJL29 (*ilvBNCD_c^bkivD*) in minimal media with 2 % fructose and 0.05 % NH₄Cl at 48 h.

| Strain | Isobutyraldehyde (mg/L) | Isobutanol (mg/L) |
|--------------|-------------------------|-------------------|
| Re2425/pJL26 | 150 | 20 |
| Re2425/pJL27 | 30 | 60 |
| Re2425/pJL29 | 80 | 10 |

^a*alsS*-gene encoding AHAS from *Bacillus subtilis*

^b*ilvBNCD_c*-*Corynebacterium glutamicum* valine biosynthesis pathway genes encoding AHAS, AHAIR, and DHAD