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

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

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

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ABSTRACT

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

INTRODUCTION

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

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

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

Ralstonia eutropha (also known as *Cuprivaidus necator*) is a Gram-negative, facultatively chemolithoautotrophic organism (Pohlmann et al. 2006). It has long been the model organism for the study of polyhydroxybutyrate (PHB) biosynthesis. Under nutrient stress in the presence of excess carbon source, wild type *R. eutropha* can accumulate approximately 80 % of its cell dry weight (CDW) as PHB, an intracellular carbon storage material (Budde et al. 2011; Yang et al. 2010). This intracellular polymer can be isolated from the cell and processed into biodegradable and biocompatible plastic for various applications (Rehm 2003; Brigham et al. 2012). Uniquely, *R. eutropha* also contains two carbon-fixation Calvin-Benson-Bassham cycle operons, two oxygen-tolerant hydrogenases, and several formate dehydrogenases and has been studied extensively for its ability to fix carbon dioxide into complex cellular molecules while obtaining energy from hydrogen or formate oxidation under ambient oxygen concentration conditions (Bowien and Kusian 2002; Ishizaki et al. 2001; Lenz et al.; Pohlmann et al. 2006; Schwartz et al. 2009). Recently, Li et al. constructed a system that couples electrochemical generation of formate with CO_2 fixation and the addition of heterologous genes for the conversion of formate to isobutanol and 3-

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

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

MATERIALS AND METHODS

Chemicals, bacterial strains and plasmids

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

Growth media and cultivation conditions

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

A single colony of *R. eutropha* from a TSB agar plate was used to inoculate 5 mL of TSB medium. The culture was then incubated on a roller drum for 24 h at 30°C before being used to inoculate a flask culture of 100 mL minimal medium, containing carbon sources mentioned above, to an initial OD_{600} of 0.1. The minimal medium culture was continuously shaken in a 30°C incubator at 200 rpm. At intermittent time points, aliquots were removed from the flask culture for analysis, as described below.

Plasmid and strain construction

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

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

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

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

Polymer quantification

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

Branched-chain alcohols extraction and detection

Culture aliquots, taken at various time points, were centrifuged at 4,000 × *g* to separate the pellet from the supernatant. Isobutanol and 3-methyl-1-butanol were extracted from the supernatant using chloroform in a 1:1 ratio. The concentrations of isobutanol and 3-methyl-1-butanol were determined using gas chromatograph (GC; Agilent Technologies, Santa Clara, CA) with a DB-Wax column (Agilent Technologies, 30 m x 0.32 mm x 0.5 μm) and a flame ionization detector. The split ratio is 20:1 and a 2 μL sample was injected at each run. Hydrogen was used as the carrier gas at a flow rate of 1.1 mL/min. The oven was held at 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. Commercial isobutanol and 3-methyl-1-butanol were analyzed on the GC as described above for standards.

Carbon, nitrogen, reducing-cofactor analysis

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

Product tolerance assay

Cultures of *R. eutropha* were grown in minimal medium with 2 % fructose and 0.05 % NH_4Cl. Various concentrations of isobutanol (0%, 0.2%, 0.5%, 0.8%, and 1% v/v) were added to the growth media at 0 h. Growth was monitored by measuring OD_{600} intermittently 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, OD_{600} 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 $MgCl_2$, 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% H_2SO_4 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

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

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

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

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

potassium phosphate buffer (pH 7.0), 15 mM pyrazole, 30 mM TPP, 1 mM NAD^+, 3 μM $MgCl_2$, 20 mM α-ketoisovaleric acid, 1 mM DTT, 0.5 mg aldehyde dehydrogenase, and cell lysate. The reaction was initiated by the addition of α-ketoisovaleric acid and monitored at 340 nm for the reduction of NAD^+. Enzyme activity was calculated in μmol of NAD^+ reduced, using the molar extinction coefficient of 6220 $M^{-1}cm^{-1}$.

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

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

Continuous flask cultures

Triplicate cultures of Re2425/pJL26 (Table 1) were performed in 100 mL minimal medium containing 1 % fructose and 0.05 % NH_4Cl. Every 24 h, the growth media containing branched-chain alcohols were separated from the cells via centrifugation at 4,000 × *g*. Isobutanol and 3-methyl-1-butanol were extracted from the growth media and analyzed as described above. Fresh media (without branched-chain alcohols) were then added to the cells in each culture. The cultures continued for another 24 h, until the process was repeated. This cycle was continued for 50 days. At each 24 h cycle, OD_{600} and concentrations of branched-chain alcohols were measured.

RESULTS

Redirecting excess carbon and reducing-equivalents

Wild type *R. eutropha* produces PHB as an intracellular carbon and energy storage polymer during nutrient stress in the presence of excess carbon (Potter et al. 2004). In order to redirect the excess carbon from PHB, the *phaCAB* operon, which encodes the polymer biosynthesis enzymes β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHB synthase (PhaC) (Pohlmann et al. 2006), was eliminated from the *R. eutropha* genome. As shown in Table 2, the wild type (H16) was able to produce more than 80 % of CDW as PHB, but strain Re2061 (H16Δ*phaCAB*), produced no detectable PHB after 96 h of growth. Deletion of the *phaCAB* operon did not affect cell growth significantly, since the residual CDW were similar in both strains. Compared to H16, Re2061 utilized 0.5 % (w/v) less gluconate during growth. Re2061 also secreted pyruvate into the growth medium, but H16 did not secrete any pyruvate throughout the entire growth period. This finding was similar to those reported by Steinbüchel et al. (Steinbüchel and Schlegel 1989). In addition, Re2061 cells contained greater concentrations of reducing-cofactors (NADH and NADPH) than H16 (Table 2). Since PHB acts as a carbon and energy storage mechanism in *R. eutropha* (Schwartz et al. 2009), Re2061 cells used less carbon source, secreted pyruvate into the extracellular milieu, and contained more reducing-cofactors, likely directly resulting from their inability to produce polymer.

Assembly of a branched-chain alcohols biosynthesis operon

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

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

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

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

R. eutropha adh mutant has isobutyraldehyde dehydrogenase activity

Steinbüchel et al. demonstrated that the *adh* gene in wild type *R. eutropha* was only expressed and active when cells were cultivated under anaerobic conditions. It was hypothesized that such gene activation increases the usage of reducing power in the absence of the terminal electron acceptor, oxygen (Steinbüchel and Schlegel 1984; Steinbüchel et al. 1987). In previous studies, a number of *R. eutropha* mutant strains constitutively expressing *adh* under aerobic conditions were isolated after continuous growth of H16 on ethanol and/or 2,3-butanediol and characterized for their alcohol dehydrogenase activities (Jendrossek et al. 1990; Steinbüchel et al. 1987). We demonstrate, as shown in Fig. 3A, that these strains also reduce isobutyraldehyde. In all cases, the cofactor NADPH is preferred over NADH for the reduction of isobutyraldehyde (data not shown). Strain Re2403 (CF17Δ*phaCAB*) showed similar activity compared to strains that overexpress ADH and YqhD. The strain with the most active isobutyraldehyde dehydrogenase activity (300 mU/mg) was Re2401 (DJ21Δ*phaCAB*) (Fig. 3A). DJ21 was also found to have the highest activity towards the reduction of 2,3-butanediol and ethanol compared to all other *adh* mutant strains (Jendrossek et al. 1990).

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

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

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

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

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

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

Production of branched-chain alcohols in response to nutrient stress

As mentioned previously, intracellular PHB is produced when the cells undergo nutrient stress, such as nitrogen or phosphorus limitation (Khanna and Srivastava 2005). In our initial isobutanol production experiments, the growth medium contained 2 % fructose and 0.05 % NH_4Cl. Under these conditions, *R. eutropha* cells became nitrogen limited by 24 h, and the production of isobutanol was detected after this time (Online Resource 4). To test if the production of isobutanol was also associated with nitrogen starvation, different concentrations of NH_4Cl (0.07 %, 0.12 %, 0.27 %, and 0.4 % w/v) were used in cultivations of Re2401/pJL26. Results in Fig. 5A demonstrate the amounts of NH_4Cl present in the growth media measured enzymatically (see Materials and Methods) at various time points, while Fig. 5B revealed the amount of isobutanol produced in these cultures. At high NH_4Cl concentrations (0.27 and 0.4 %), Re2401/pJL26 cells were unable to utilize the entire amount of nitrogen source, and the production of isobutanol was extremely low (30 mg/L and 20 mg/L respectively). On the other hand, when the NH_4Cl concentrations were lower, at 0.07 and 0.12 %, the cells entered nitrogen limitation at approximately 24 h, and isobutanol

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

Production optimization

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

Continuous flask cultivations

Since *R. eutropha* experiences isobutanol toxicity at concentrations above 0.5 % (v/v), the production yield might be adversely affected by the product itself. In order to alleviate this inhibition and determine the longevity of engineered *R. eutropha* in both growth and alcohol production, Re2425/pJL26 was cultivated in 100 mL minimal media with 1 % fructose and 0.05 % NH_4Cl. At the end of every 24 h, all isobutanol and 3-methyl-1-butanol produced were removed with the spent growth media, and fresh minimal media were added to the cultures. Each day, approximately 200 mg/L to 500 mg/L branched-chain alcohols were produced. Re2425/pJL26 was able to continuously utilize fructose as the main carbon source and produce branched-chain alcohols for a duration of greater than 50 days. The total accumulated alcohols produced reached levels of over 14 g/ L (Figure 7).

DISCUSSION

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

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

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

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

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

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

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

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

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

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

Although *R. eutropha* is traditionally employed for the production of PHB, a growing amount of attention has now centered on engineering this strain for the production of biofuels. Production of biofuels, such as branched-chain alcohols, in *R. eutropha* can act as an alternative to carbon storage when redirected from PHB biosynthesis. Since *R. eutropha* was able to produce ~60 g/L PHB using CO_2 and H_2 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 CO_2- and H_2-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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FIGURE LEGENDS

Fig. 1

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

Fig. 2

Production of isobutanol in strain Re2061/pJL23. *R. eutropha* Re2061/pJL23 (see Table 1) was cultivated in minimal media with 0.05 % NH_4Cl with the following carbon sources: 2 % w/v Fructose, 2 % w/v Fructose with 1 % w/v Pyruvate, or 2 % w/v Fructose with 1 % w/v 2-ketoisovaleric acid. Each value represents the mean ± standard error (error bars) of n = 3.

Fig. 3

Isobutyraldehyde dehydrogenase activity (A) and production of branched-chain alcohols (B) by *R. eutropha* wild type and ADH mutant strains harboring isobutanol production plasmids (see Table 1). 1: Re2061 (H16 Δ*phaCAB*); 2: Re2061/pJL21 (H16 Δ*phaCAB*/p*adhA*); 3: Re2061/pJL20 (H16 Δ*phaCAB*/p*adh*); 4: Re2061/pJL22 (H16 Δ*phaCAB*/p*yqhD*); 5: Re2061/pBBR1MCS-2; 6: Re2403/pJL26 (CF17Δ *phaCAB*/p*ilvBHCDkivD*); 7: Re2404/pJL26 (CF101 Δ*phaCAB*/p*ilvBHCDkivD*); 8: Re2405/pJL26 (CF106

861 Δ*phaCAB*/p*ilvBHCDkivD*); 9: Re2406/pJL26 (CF108 Δ*phaCAB*/p*ilvBHCDkivD*); 10:
862 Re2407/pJL26 (CF303 Δ*phaCAB*/p*ilvBHCDkivD*); 11: Re2401/pJL26 (DJ21
863 Δ*phaCAB*/p*ilvBHCDkivD*). Data points represent the mean values of n = 3 ±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 μmol 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 Δ*phaCAB*/p*ilvBHCDkivD*). (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: (DJ21Δ*phaCAB*); Re2402: (DJ21Δ*phaCAB*Δ*ilvE*)*;* Re2410
883 (DJ21Δ*phaCAB*Δ*ilvE*Δ*bkdAB*)*;* Re2425 (DJ21Δ*phaCAB*Δ*ilvE*Δ*bkdAB*Δ*aceE*). Values were
884 average from three replicates with standard deviation values represented as error bars.

Fig. 7

Isobutanol and 3-methyl-1-butanol production by Re2425/pJL26 in a continuous flask culture, over the duration of 50 days. Fresh minimal media with 1 % fructose and 0.05 % NH_4Cl was used each day. The concentration values of isobutanol and 3-methyl-1-butanol were added together to get the daily total branched-chain alcohols concentration. The data shown are the average of three replicated experiments with error bars representing the standard deviation.

FIGURES

Fig. 1

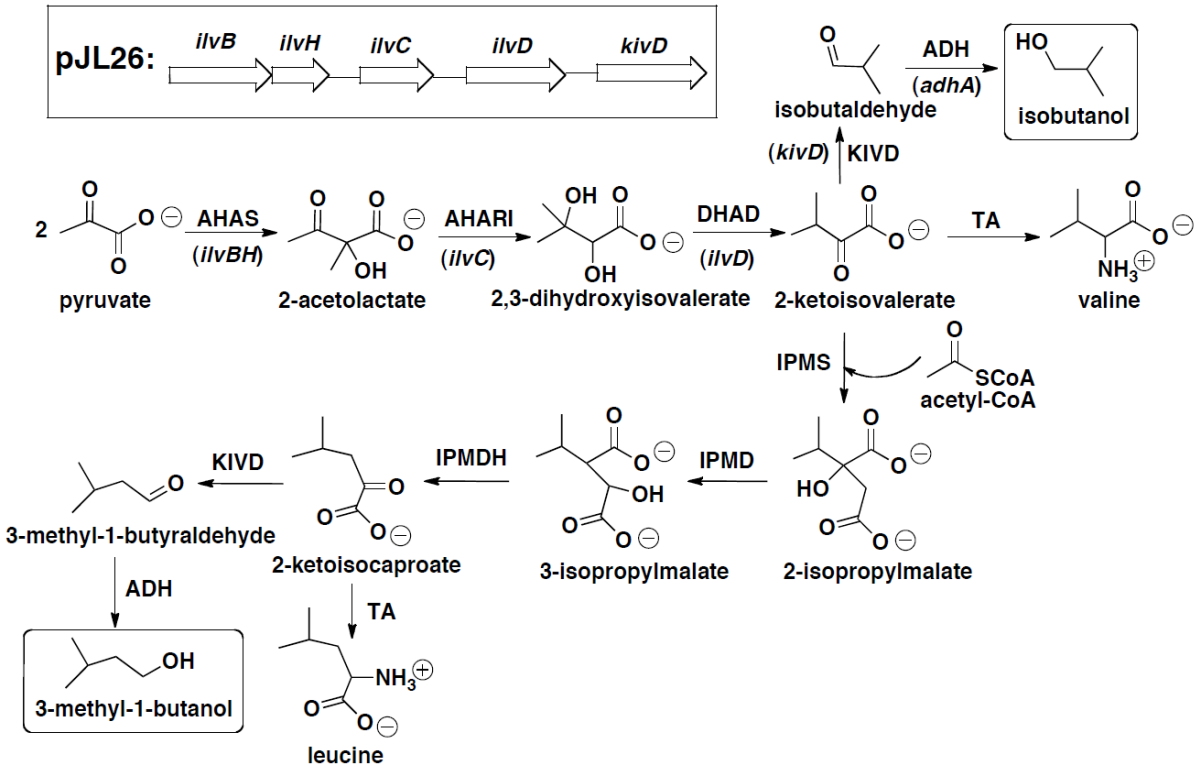


Fig. 2

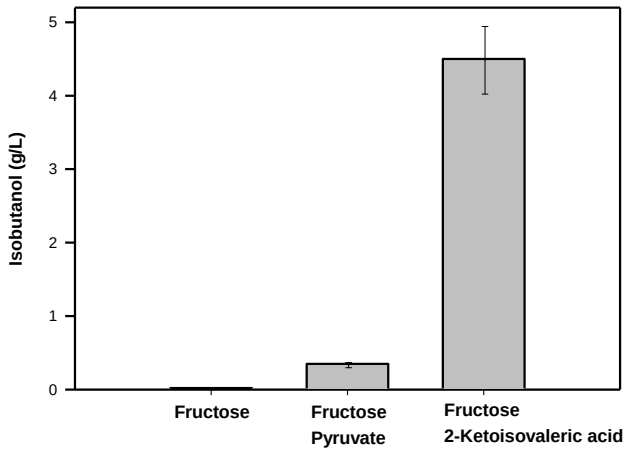
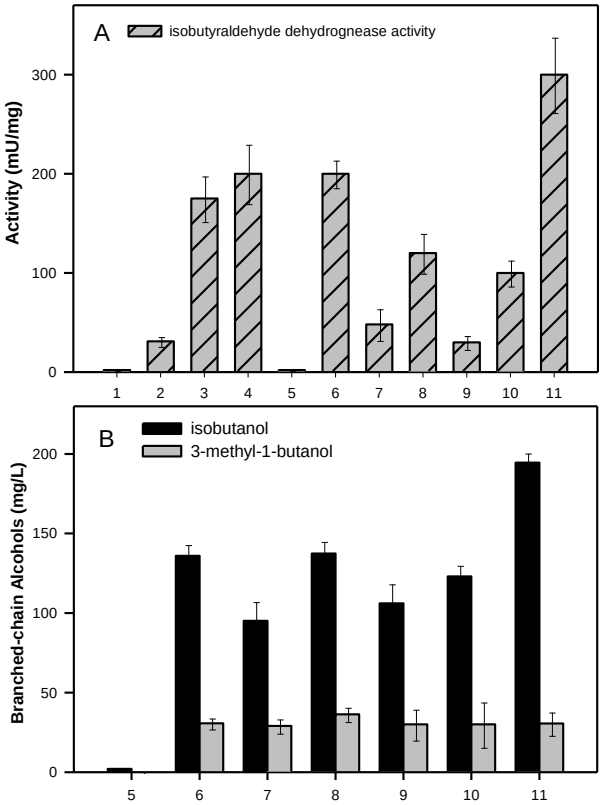
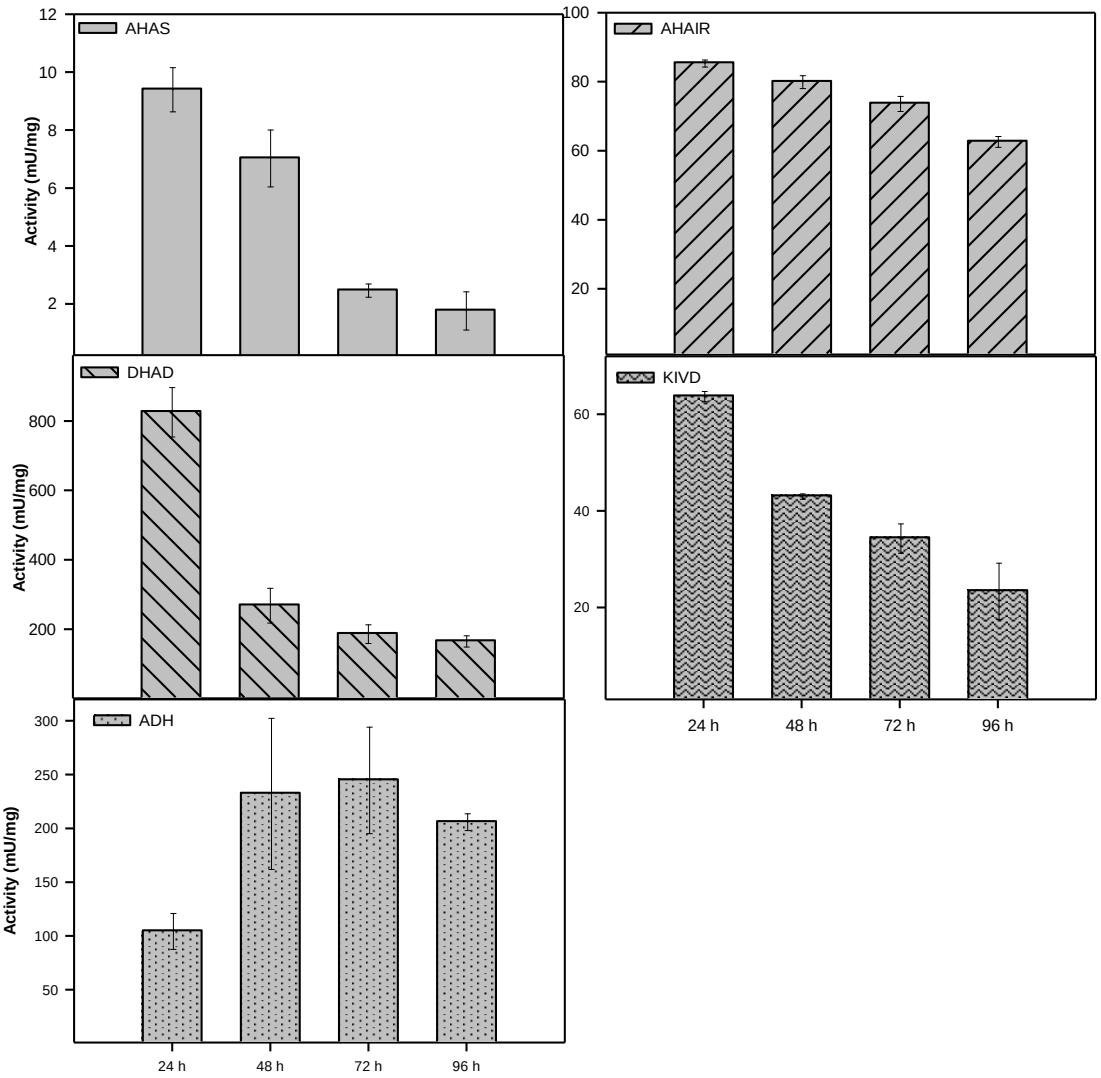

Isobutanol (g/L)
0
1
2
3
4
5
Fructose
Fructose
Pyruvate
Fructose
2-Ketoisovaleric acid

Fig. 3


A
isobutyraldehyde dehydrognease activity
Activity (mU/mg)
0
100
200
300
1
2
3
4
5
6
7
8
9
10
11
B
isobutanol
3-methyl-1-butanol
Branched-chain Alcohols (mg/L)
0
50
100
150
200
5
6
7
8
9
10
11

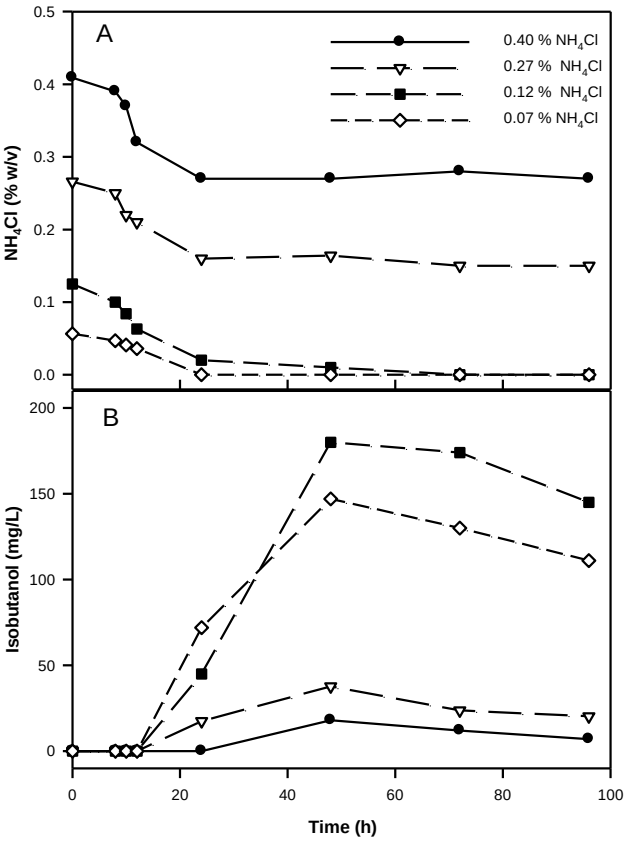
15 **Fig. 4**



16

17

18 **Fig. 5**


A
B
0.40 % NH4Cl
0.27 % NH4Cl
0.12 % NH4Cl
0.07 % NH4Cl
NH4Cl (% w/v)
Isobutanol (mg/L)
Time (h)
0.5
0.4
0.3
0.2
0.1
0.0
200
150
100
50
0
0
20
40
60
80
100

19

Fig. 6

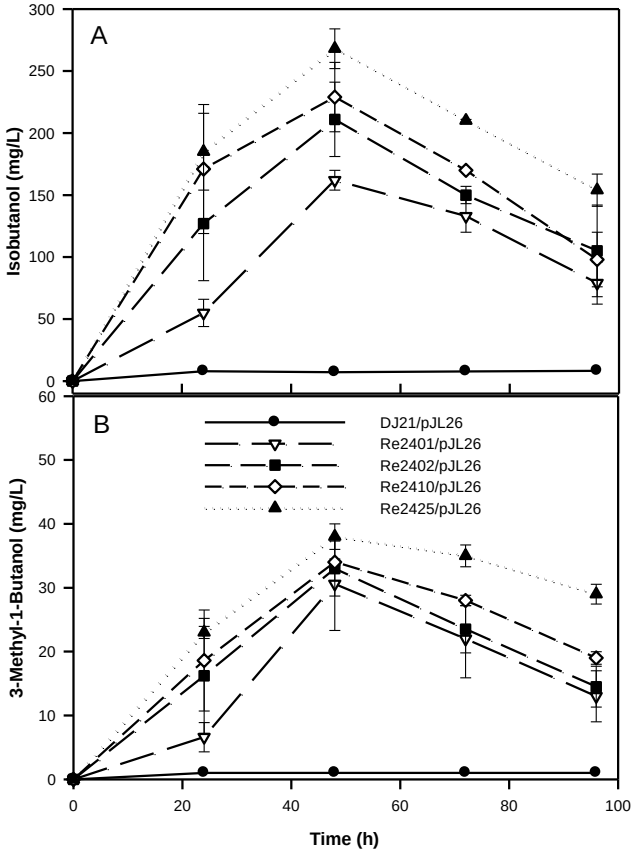
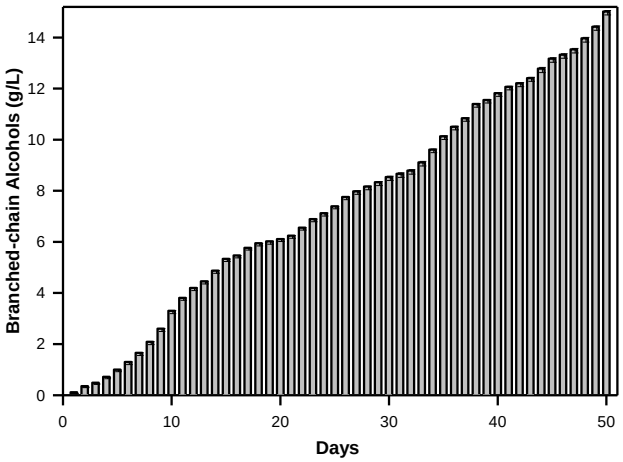

A
B
Isobutanol (mg/L)
3-Methyl-1-Butanol (mg/L)
Time (h)
DJ21/pJL26
Re2401/pJL26
Re2402/pJL26
Re2410/pJL26
Re2425/pJL26

Fig. 7



TABLES

Table 1

Strains and plasmids used in this work.

Strains or plasmid	Genotype	Reference
Strains		
R. eutropha		
H16	Wild-type, gentamicin resistant (Gen^r)	ATCC17699
Re2061	H16Δ*phaCAB* Gen^r	This work
CF17	H16 *adh*(Con) $ethanol^+$ 2,3-$butanediol^+$ Gen^r	(Steinbüchel *et al.* 1987)
CF101	H16 *adh*(Con) $ethanol^+$ 2,3-$butanediol^+$ Gen^r	(Steinbüchel *et al.* 1987)
CF106	H16 *adh*(Con) $ethanol^+$ 2,3-$butanediol^+$ Gen^r	(Jendrossek *et al.* 1990)
CF108	H16 *adh*(Con) $ethanol^+$ 2,3-$butanediol^+$ Gen^r	(Jendrossek *et al.* 1990)
CF303	H16 *adh*(Con) $ethanol^+$ 2,3-$butanediol^+$ Gen^r	(Steinbüchel *et al.* 1987)
DJ21	H16 *adh*(Con) $ethanol^+$ 2,3-$butanediol^+$ Gen^r	(Jendrossek *et al.* 1990)
Re2403	CF17Δ*phaCAB* Gen^r	This work
Re2404	CF101Δ*phaCAB* Gen^r	This work
Re2405	CF106Δ*phaCAB* Gen^r	This work
Re2406	CF108Δ*phaCAB* Gen^r	This work
Re2407	CF303Δ*phaCAB* Gen^r	This work
Re2401	DJ21Δ*phaCAB* Gen^r	This work
Re2402	DJ21Δ*phaCAB, ilvE* Gen^r	This work
Re2410	DJ21Δ*phaCAB, ilvE, bkdAB* Gen^r	This work
Re2425	DJ21Δ*phaCAB, ilvE, bkdAB, aceE* Gen^r	This work
E. coli		
S17-1	Conjugation strain for transfer of plasmids into *R. eutropha*	(Simon *et al.*, 1983)
Plasmids		
pJV7	pJQ200Kan with Δ*phaC1* allele inserted into BamHI restriction site, confers kanamycin resistance (Kan^r)	(Budde *et al.*, 2011a)
pJL33	pJV7 with Δ*phaC1* allele removed by XbaI and SacI digestion and replace with Δ*phaCAB* allele (Kan^r)	This work
pCJB6	pJV7 with Δ*phaC1* allele removed by XbaI and SacI digestion and replace with Δ*ilvE* allele (Kan^r)	This work
pCJB7	pJV7 with Δ*phaC1* allele removed by XbaI and SacI digestion and replace with Δ*bkdAB* allele (Kan^r)	This work
pJL32	pJV7 with Δ*phaC1* allele removed by XbaI and SacI digestion and replace with Δ*aceE* allele (Kan^r)	This work
pBBR1MCS-2	Broad-host-range cloning vector (Kan^r)	(Kovach *et al.*, 1996)
pJL23	pBBR1MCS-2 with *L. lactis kivD* gene and *R. eutropha adh* (H16_A0757) alcohol dehydrogenase gene inserted into the multiple cloning site (Kan^r)	This work
pJL21	pBBR1MCS-2 with *R. eutroha* H16_A0861 alcohol	This work

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

Table 2

Physiologic differences between wild type *R. eutropha* (H16) and Re2061 (H16Δ*phaCAB*)[a].

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

[a]Strains are grown in minimal media with 2 % gluconate and 0.05 % NH_4Cl. The values presented were measured at the end of 96 h growth period. Concentrations of gluconate (Gluc) consumed, pyruvate (Pyr) and PHB produced were detected by HPLC as discussed in Materials and Methods. Intracellular reducing equivalents NADH and NADPH were measured using a cofactor cycling assay (Ref) and normalized per each colony forming unit (CFU). Each value represents the mean ± standard error on n = 3.

Table 3

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

[a]Isobutanol at 0, 0.2, 0.5, 0.8, and 1 % were added to minimal media. Growth of *R. eutropha* wild type Re2061 and Δ*phaCAB* of ADH mutant strains (Table 1) were monitored. Calculation of % viable was based on the ratio between cells grown in isobutanol to cells grown without isobutanol at 24 h. Each value represents n = 1.

Supplementary Material:

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

Name	Sequence[a]
Δ*phaCAB* upstream F	GAATGGATCCGTGCTCGGTGATCGCCATCAT
Δ*phaCAB* upstream R	GACTGGTTGAACCAGGCCGGCAGGTCACTCGAGCATATGCATGATTTG ATTGTCTCTCTG
Δ*phaCAB* downstream F	CAGAGAGACAATCAAATCATGCATATGCTCGAGTGACCTGCCGGCCTG GTTCAACCAGTC
Δ*phaCAB* downstream R	GAATGGATCCCAGGGTGATGTAGGTGCTGGT
Δ*phaCAB* digF	CGACGCCACCAACCTGCCGGG
Δ*phaCAB* digR	GTCCACTCCTTGATTGGCTTCG
Δ*ilvE* upstream F	ATAAGGATCCTGCTCGAGCGGCTCGATCGT
Δ*ilvE* upstream R	GGCTGGCAGCCGGTGCTCACATGTCTGTTCTCCCTGCG
Δ*ilvE* downstream F	CGCAGGGAGAACAGACATGTGAGCACCGGCTGCCAGCC
Δ*ilvE* downstream R	AACTGGATCCCCTTGAGCAGCGCAAAGAGC
Δ*ilvE* digF	ATGTGGGGTCAAAGGCAC
Δ*ilvE* digR	TAGACGGTGCCGCAGTAC
Δ*bkdAB* upstream F	TGAAGAGCTCCTTCGTCAACGGCAACTATG
Δ*bkdAB* upstream R	CCTGTTGTCTTCGACCGCTACATGGCAGGTCTCTCGATGC
Δ*bkdAB* downstream F	GCATCGAGAGACCTGCCATGTAGCGGTCGAAGACAACAGG
Δ*bkdAB* downstream R	CTTGTCTAGAAACTACGTGGATTCGCTGGC
Δ*bkdAB* digF	TCAAGGACATCCGAGAGGCC
Δ*bkdAB* digR	CGCTGAGTCACTTCTTCTGC
Δ*aceE* upstream F	GCCGGGATCCGAAGCCTTGCTGGCTTCATCC
Δ*aceE* upstream R	TGCCCGATGGCCGATCGTTCACACGGCAAGTCTCCGTTAAGG
Δ*aceE* downstream F	CCTTAACGGAGACTTGCCGTGTGAACGATGGGCCATCGGGCA
Δ*aceE* downstream R	GCATGGATCCGCTGGCAAAACGCTGAGCATTGAG
Δ*aceE* digF	GTGATCCTGGCCAAGACCATC
Δ*aceE* digR	GGCATCCTGCGGGGTGTAGCG
kivD F	GCGGTCTAGAAGGAGAATGCGATGTA
kivD R	GCACGAGCTCTGAATTATTTTGTTCA
adh F	GAATGAGCTCGCGGGCCGGCAACGTC
adh R	GCCGTCTAGAACTAGTTCAGTGCGGCTTGATGGCG

adhA F	GAATGAATTCGTGCGCGCGAGACCGGCA
adhA R	GCCGGGATCCTTACATCGCTGCAGCGAA
q*yhD* F	GCCGGAATTCATGAACAACTTTAATCTGCACACCCC
q*yhD* R	GAATGGATCCTTAGCGGGCGGCTTCGTATATAC
ilvBH F	GCATACTAGTATGCCCAGCGCGGAATTCTC
ilvBH R	CTTGTCGTAAAACACTTTCATGGTTGTCCTCCTTTCTAGAGAGCTTTCGTTTTCATG
ilvC F	GCCGCATATGAAAGTGTTTTACGACAAGGACGCG
ilvC R	GCCGACTAGTTTAGTTCTTCGACTGGTCGACC
ilvD F	GTCGACCAGTCGAAGAACTAAAAAGGAGGACGACCATGGCATTCAACAAACGCTCGCAG
ilvD R	GGTCGTCCTCCTTTTCAGTCCGTCACTGCCCCCTTG
kivD F	AAGGGGGCAGTGACGGACTGAAAAGGAGGACGACCATGTATACAGTAGGAGATTACC
kivD R	GCAGTCTAGAGGTCGTCCTCCTTTTTATGATTTATTTTGTTCAGC
ilvBHCDkivD digF1	GCCAACATGAACTATTCGATC
ilvBHCDkivD digR1	GCCCTCGGTGCCCATCGACATG
ilvBHCDkivD digF2	CTGTTGCTGCAGCTGAACGTC
ilvBHCDkivD digR2	GCCAAAGCTAATTATTTCATG
alsS F (*B. subtilis*)	GCATACTAGTGCCGCTCGAGATGACAAAAGCAACAAAAGAA
alsS R	GCATGGATCCCTAGAGAGCTTTCGTTTTCATG
ilvBNc F (*C. glutamicum*)	GCCGTCTAGAGTGAATGTGGCAGCTTCTCAAC
ilvBNc R	GCCGTCTAGATTAAGCGGTTTCTGCGCGAGC
ilvCc F	GCATACTAGTTTAGTCGACCTGACGGACTGC
ilvCc R	CTTTTGAACGAAGTGGGATCATGGTTGTCCTCCTTTTTAAGCGGTTTCTGCGCGAGC
ilvDc F	GCTCGCGCAGAAACCGCTTAAAAAGGAGGACAACCATGATCCCACTTCGTTCAAAAG
ilvDc R	GCAGTCTAGATTAGTCGACCTGACGGACTGC

[a] Restriction sites are underlined

Online Resource 2:

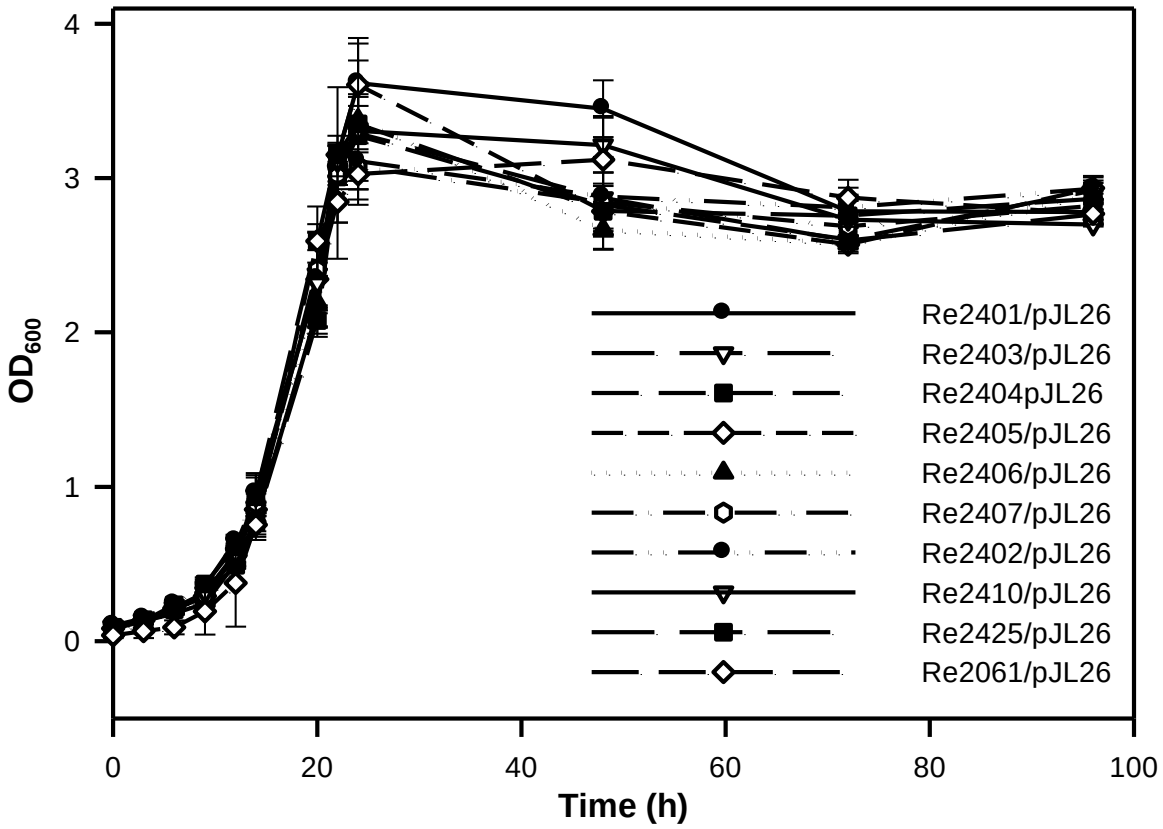
Activities of valine biosynthesis pathway enzymes in *R. eutropha* with empty vector (Re2061/pBBR1MCS-2) and overexpression plasmid (Re2061/pJL26). Cells were grown in minimal media with 2 % fructose and 0.05 % NH_4Cl and harvested for enzymatic activity at 24 h.

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

Online Resource 3:

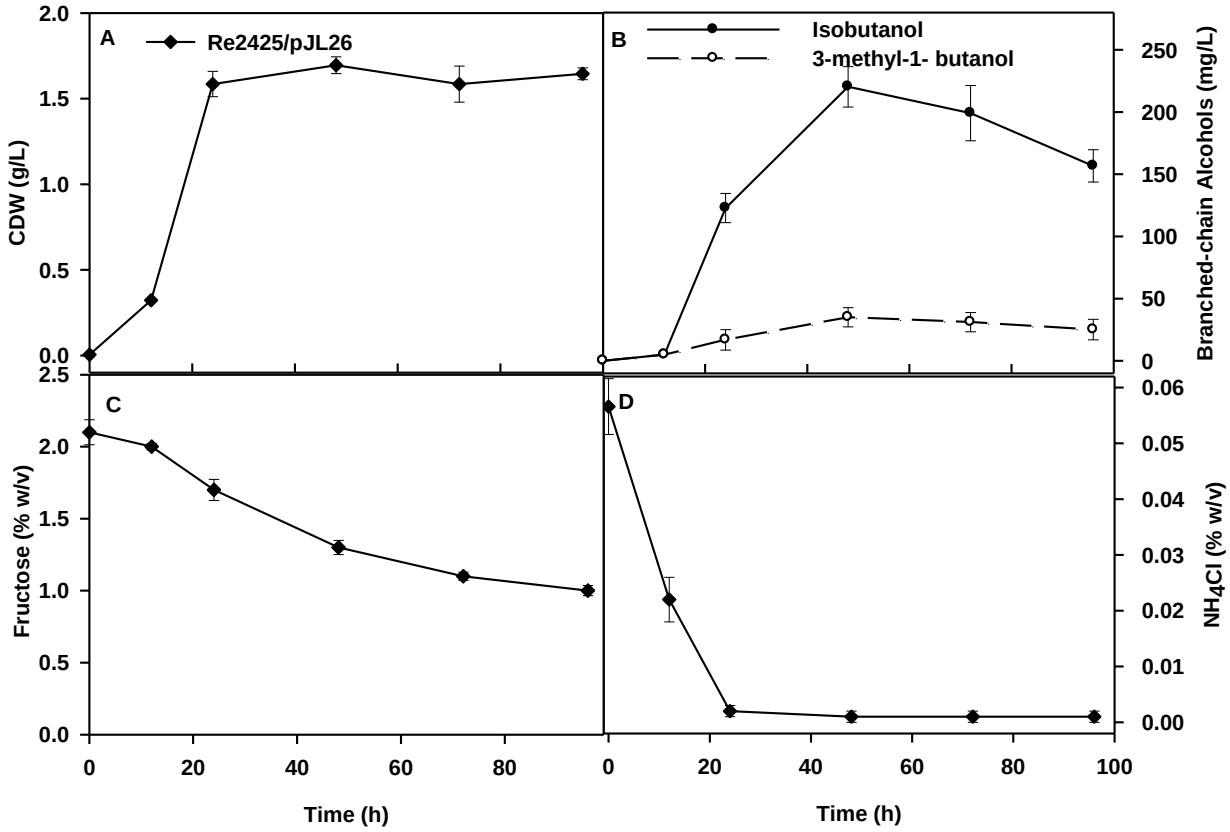
Growth of *R. eutropha* deletion strains and mutant strains constitutively expressing ADH containing the isobutanol production plasmid, pJL26. See Table 1 of text for more information regarding strains and plasmids used in this experiment.

Re2061 (H16Δ*phaCAB*), Re2401 (DJ21Δ*phaCAB*), Re2403 (CF17Δ*phaCAB*), Re2404 (CF101Δ*phaCAB*), Re2405 (CF106Δ*phaCAB*), Re2406 (CF108Δ*phaCAB*), Re2407 (CF303Δ*phaCAB*). Re2402 (DJ21Δ*phaCAB* Δ*ilvE*), Re2410 (DJ21Δ*phaCAB* Δ*ilvE* Δ*bkdAB*), Re2425 (DJ21Δ*phaCAB* Δ*ilvE* Δ*bkdAB* Δ*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*[a]*ilvCDkivD*), pJL29 (*ilvBNCD*$_c$[b]*kivD*) in minimal media with 2 % fructose and 0.05 % NH_4Cl 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 *Bacillis subtilis*
[b]*ilvBNCD*$_c$-*Corynebacterium glutamicum* valine biosynthesis pathway genes encoding AHAS, AHAIR, and DHAD