

Isopropanol production with engineered *Cupriavidus necator* as bioproduction platform

Estelle Grousseau · Jingnan Lu · Nathalie Gorret ·
Stéphane E. Guillouet · Anthony J. Sinskey

Received: 6 December 2013 / Revised: 4 February 2014 / Accepted: 4 February 2014 / Published online: 7 March 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract Alleviating our society's dependence on petroleum-based chemicals has been highly emphasized due to fossil fuel shortages and increasing greenhouse gas emissions. Isopropanol is a molecule of high potential to replace some petroleum-based chemicals, which can be produced through biological platforms from renewable waste carbon streams such as carbohydrates, fatty acids, or CO₂. In this study, for the first time, the heterologous expression of

engineered isopropanol pathways were evaluated in a *Cupriavidus necator* strain Re2133, which was incapable of producing poly-3-hydroxybutyrate [P(3HB)]. These synthetic production pathways were rationally designed through codon optimization, gene placement, and gene dosage in order to efficiently divert carbon flow from P(3HB) precursors toward isopropanol. Among the constructed pathways, Re2133/pEG7c overexpressing native *C. necator* genes encoding a β -ketothiolase, a CoA-transferase, and codon-optimized *Clostridium* genes encoding an acetoacetate decarboxylase and an alcohol dehydrogenase produced up to 3.44 g l⁻¹ isopropanol in batch culture, from fructose as a sole carbon source, with only 0.82 g l⁻¹ of biomass. The intrinsic performance of this strain (maximum specific production rate 0.093 g g⁻¹ h⁻¹, yield 0.32 C mole C mole⁻¹) corresponded to more than 60 % of the respective theoretical performance. Moreover, the overall isopropanol production yield (0.24 C mole C mole⁻¹) and the overall specific productivity (0.044 g g⁻¹ h⁻¹) were higher than the values reported in the literature to date for heterologously engineered isopropanol production strains in batch culture. Strain Re2133/pEG7c presents good potential for scale-up production of isopropanol from various substrates in high cell density cultures.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-5591-0) contains supplementary material, which is available to authorized users.

E. Grousseau · N. Gorret · S. E. Guillouet
Université de Toulouse, INSA, UPS, INP; LISBP, 135 Avenue de
Rangueil, F-31077 Toulouse, France

E. Grousseau · N. Gorret · S. E. Guillouet
INRA, UMR792 Ingénierie des Systèmes Biologiques et des
Procédés, F-31400 Toulouse, France

E. Grousseau · N. Gorret · S. E. Guillouet
CNRS, UMR5504, F-31400 Toulouse, France

J. Lu
Department of Chemistry, Massachusetts Institute of Technology, 77
Massachusetts Avenue, Cambridge, MA 02139, USA

A. J. Sinskey (✉)
Department of Biology, Massachusetts Institute of Technology, 77
Massachusetts Avenue, Cambridge, MA 02139, USA
e-mail: asinskey@mit.edu

A. J. Sinskey
Division of Health Sciences and Technology, Massachusetts Institute
of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139,
USA

A. J. Sinskey
Engineering Systems Division, Massachusetts Institute of
Technology, 77 Massachusetts Avenue, Cambridge, MA 02139,
USA

Keywords *Cupriavidus necator* · *Ralstonia eutropha* ·
Isopropanol · Branched-chain alcohols · Biofuel · Metabolic
engineering

Introduction

With the need to reduce consumption of petroleum-based products, diversified alternative fuels and bulk chemicals from renewable carbon sources have to be developed. Current research on fuel substitutes has focused largely on ethanol, even though numerous technical problems associated with

this biofuel are reported. Ethanol is corrosive toward ferrous metals, has lower energy content than gasoline, and degrades elastomers and flexible transfer lines in fuel systems, which makes it challenging to ship via traditional pipelines (Bruno et al. 2009). To overcome some of the challenges associated with the use of ethanol as a fuel, various higher alcohols were evaluated. Higher alcohol molecules, such as isobutanol, n-butanol, isopropanol, 1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and isopentenol, can be blended with gasoline at various ratios and act as drop-in fuels, thus having high potential to be implemented as gasoline replacements (Lee et al. 2008a; Connor and Liao 2009; Bruno et al. 2009). Among these higher alcohols, isopropanol has a very high research octane number (129) and is already used as a gasoline and diesel additive (Peralta-Yahya and Keasling 2010). Currently, isopropanol is mainly used as a solvent in the chemical industry and is blended with many everyday household products such as paints and inks (Pharkya et al. 2011). Isopropanol can also be utilized as a chemical intermediate and be converted to propylene by dehydration and subsequently to “green” polypropylene (Kibby and Hall 1973; Araki et al. 1993). Currently, isopropanol is chemically produced from propylene or acetone (Pharkya et al. 2011), which are petroleum-based products. Isopropanol, bioproduced from renewable carbon sources, is a promising alternative as a green chemical target molecule in the chemical, solvent, and alternative energy industries.

Isopropanol can be naturally produced by *Clostridium* species (Krouwel et al. 1980; Chen and Hiu 1986; Survase et al. 2011; Matsumura et al. 1992) with a reported maximum production level at 5 g l⁻¹ (Matsumura et al. 1992; Survase et al. 2011). Nevertheless, production in *Clostridium* species faces several challenges (Connor and Liao 2009), such as complex physiology and narrow genetic engineering capabilities, even though some engineered *Clostridium* strains have reached titer up to 8.8 g l⁻¹ (Collas et al. 2012). In addition, isopropanol production in *Clostridium* species is still associated with several by-products including lactic acid, acetic acid, butyric acid, butanol, acetone, and ethanol (Dürre 1998; Collas et al. 2012), which impact the carbon yield and recovery processes. In order to overcome the complexity of using *Clostridium* species, heterologous expression of *Clostridium* isopropanol pathway in other microorganisms has been attempted. For instance, heterologous expression of *Clostridium* isopropanol pathway in *Escherichia coli* led to a production of up to 4.9 g l⁻¹ of isopropanol in batch culture (Hanai et al. 2007) and up to 40 g l⁻¹ using a fed-batch strategy (Jojima et al. 2008; Inokuma et al. 2010). An engineered yeast, *Candida utilis*, was capable of isopropanol production at a titer of 9.5 g l⁻¹ and up to 27.2 g l⁻¹ in batch and fed-batch strategies, respectively (Tamakawa et al. 2013). Lately, isopropanol production by a cyanobacterium at a titer of 27 mg l⁻¹ in batch was achieved, although not directly from

CO₂ but hypothesized to be from stored glycogen (Kusakabe et al. 2013).

The facultative chemolithoautotrophic bacterium *Cupriavidus necator* (also known as *Ralstonia eutropha*) is a metabolically versatile bioproduction platform organism. It is metabolically capable of utilizing many simple and complex carbon sources, especially oils (Lee et al. 2008b; Budde et al. 2011), fatty acids (Wilde 1962; Johnson and Stanier 1971; Friedrich et al. 1979; Doi et al. 1989), and CO₂ (Wilde 1962; Repaske and Mayer 1976; Tanaka et al. 1995), which can be derived from agro-industrial waste streams. *C. necator* is a model bacterium for the study of PHAs (polyhydroxyalkanoates) biopolymers, in addition to H₂- and CO₂-based chemolithoautotrophic metabolism for the past few decades (Schlegel 1990; Reinecke and Steinbüchel 2009). *C. necator* is able to divert a significant amount of carbon into poly-3-hydroxybutyrate [P(3HB)] under unfavorable growth conditions of nutrient limitation (oxygen, nitrogen, phosphorus, etc.), with adequate availability of carbon (Koller et al. 2010). This natural ability to store excess carbon is very appealing since isopropanol and P(3HB) share the same production pathway precursors (Fig. 1), which indicates that few genetic modifications are required to divert P(3HB) precursors to the production of isopropanol. As depicted in Fig. 1, expression of two genes encoding for an acetoacetate decarboxylase (ADC) and an alcohol dehydrogenase (ADH) is necessary to redirect carbon flow to isopropanol. Moreover, *C. necator* can be easily engineered, because its genome has been fully sequenced (Schwartz et al. 2003; Pohlmann et al. 2006) and basic genetic tools are available to manipulate the microorganism since the end of the 1980s (Jendrossek et al. 1988; Peoples and Sinskey 1989; Park et al. 1995).

The key features for engineering a microorganism for industrial metabolite production are the following: the selected microorganism must be (i) robust toward industrial process conditions, (ii) able to grow with minimal nutrient supplement for cost effective issues, (iii) able to grow on cheap substrates, (iv) accept heterologous genes; the expression of multiple genes must be coordinated to channel the carbon flow; and growth and product formation must be uncoupled especially for toxic molecules such as isopropanol. *C. necator* meets these requirements and was selected for isopropanol production in this study.

Although *C. necator* seems to be a good host for isopropanol production, it has never been engineered and tested for the production of isopropanol. In this study, we engineered *C. necator* for isopropanol production. A rational design of production pathways was employed and the production of isopropanol by each engineered pathway was evaluated in the strains. The pathway gene-coding sequences, codon usages, gene copy numbers, distance of specific gene from the promoter, and various promoter systems were investigated to optimize the production of isopropanol in terms of titer, yield, and specific rate.

Materials and methods

Strain

C. necator Re2133 (Budde et al. 2011; Fig. 2) was used as the parent strain for isopropanol production since genes encoding for acetoacetyl-CoA reductases (*phaB1B2B3*) and for the PHA synthase (*phaC*) were deleted (Fig. 1) from the wild-type strain *C. necator* H16 (ATCC17699, Gen^l).

Medium and cultivation conditions

Rich medium used for the precultures consisted of 27.5 g l⁻¹ dextrose-free tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA) with addition of 10 mg l⁻¹ gentamycin and 200 mg l⁻¹ kanamycin. Minimal medium used for the cultures was previously described by Lu et al. 2012b with addition of gentamycin (10 mg l⁻¹) and kanamycin (100 mg l⁻¹).

The amount of kanamycin was reduced compared to the precultures to decrease the toxic effect of kanamycin on growth. In the literature, the amount of kanamycin used for *C. necator* is between 50 and 450 mg l⁻¹ (Kusian et al. 2002; Pötter et al. 2002; Aneja et al. 2009; Park et al. 2010; Wahl et al. 2012).

One glycerol stock was streaked on a rich medium Petri dish (rich medium with addition of 20 g l⁻¹ agar A). The plate was incubated for 48 h at 30 °C. One colony was used to inoculate the seed culture, which was grown for 24 h in culture tube at 30 °C on a roller-drum with 10 ml of rich medium. Then the volume of broth needed to inoculate a flask with an initial OD_{600 nm} of 0.4 was centrifuged in a 15 ml falcon tube (1,900×g, 10 min, Centrifuge 5804R Eppendorf AG, Hamburg, Germany). The cell pellet was then resuspended in the mineral media used for the flask culture (100 ml in 1 l flask). Fructose (20 g l⁻¹) and NH₄Cl (0.38 g l⁻¹) were used as carbon and nitrogen sources, respectively. The nitrogen amount corresponded to the amount necessary to produce about

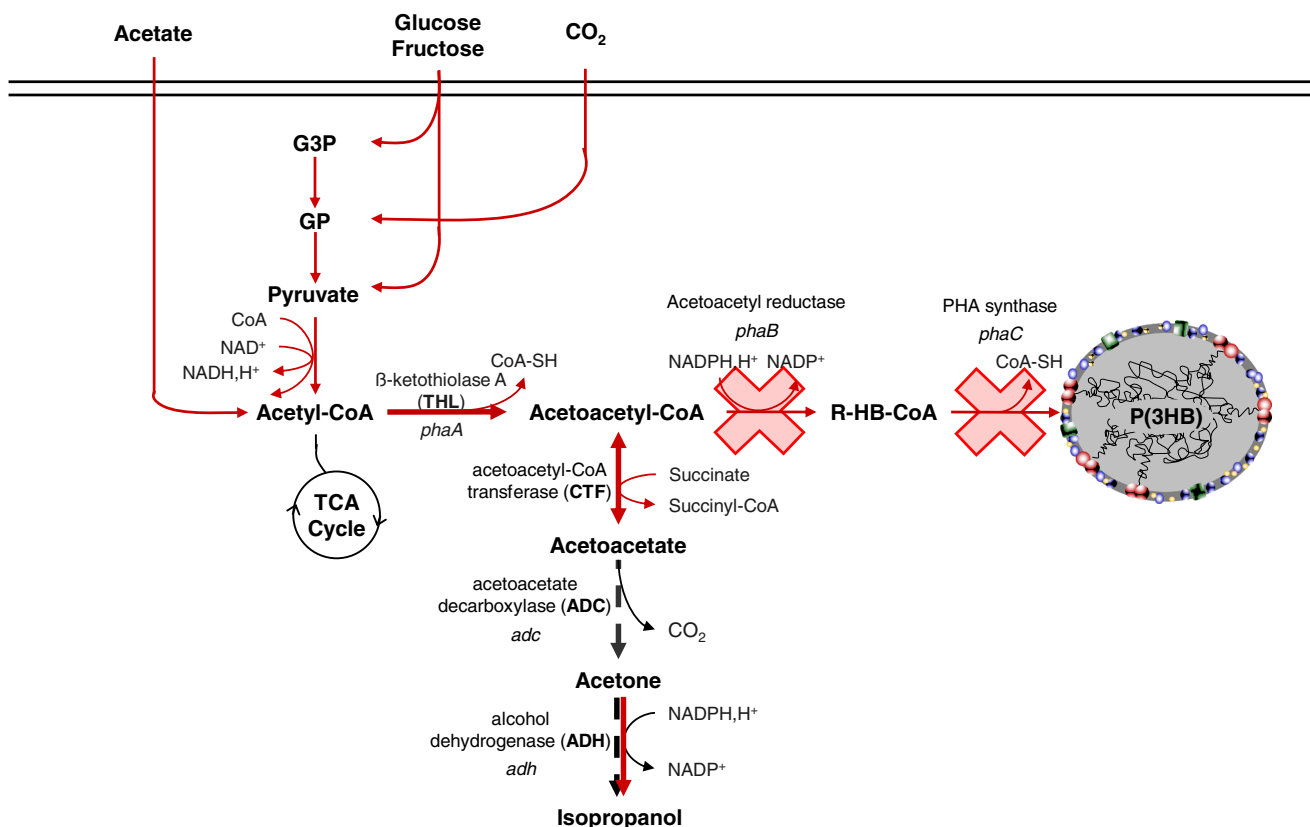


Fig. 1 Engineered isopropanol production pathway in *C. necator*. Acetate, fructose, glucose and CO₂ are potential carbon sources, which can be used for isopropanol production. Dashed arrows depict steps which are missing in *C. necator* for the production of isopropanol. Carbohydrates are catabolized through Entner-Doudoroff pathway and lead to glyceraldehyde-3-phosphate (G3P) and pyruvate. CO₂ is assimilated through the Calvin cycle and lead to glycerate-3-phosphate (GP). G3P, pyruvate, GP, and acetic acid can all lead to the production of acetyl-CoA. Acetyl-CoA can be directed toward the tricarboxylic acid (TCA) cycle or toward isopropanol or polyhydroxybutyrate (P(3HB)) synthesis. Isopropanol is synthesized by condensation of two acetyl-CoA molecules

into acetoacetyl-CoA via the β -ketothiolase enzyme. Next, acetoacetyl-CoA transferase (CTF) transfers the CoA moiety from acetoacetyl-CoA to succinate (provided by the TCA cycle) for the formation of acetoacetate. Acetoacetate is then decarboxylated with the aid of acetoacetate decarboxylase (ADC) to form acetone, before an alcohol dehydrogenase (ADH) finally reduces acetone to isopropanol. The last two steps of P(3HB) synthesis must be removed by the deletion of *phaB* and *phaC* genes (Re2133), encoding for the NADPH-dependent acetoacetyl-reductase [performing the reduction of acetoacetyl-CoA into R-hydroxybutyryl-CoA (R-HB-CoA)] and the PHA synthase (performing the polymerization), respectively

0.7 g l⁻¹ of biomass cell dry weight (CDW) considering the following biomass formula: C₁H_{1.77}O_{0.44}N_{0.25}, 4 % ashes, MW=25.35 g Cmol⁻¹ (Aragao 1996). The baffled flasks were continuously shaken in a 30 °C incubator at 200 rpm to ensure proper oxygen transfer. Culture samples were taken regularly for analysis as described below.

Plasmid and strain constructions

DNA sequence amplification was achieved using Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Ipswich, MA, USA). QIAQuick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) was used for gel purification of all DNA products. Plasmid extractions were carried out using the QIAprep Spin Miniprep Kit (QIAGEN). Restriction enzymes used were from New England Biolabs.

Synthetic ribosome-binding site (RBS) and a nucleotide linker sequence were incorporated between each gene as shown in Fig. 2. The RBS and linker sequences used were tested and described by Lu et al. (2012a). The synthesized codon-optimized genes (GenScript USA, Piscataway, NJ, USA) were received in pUC57-Kan (sequence of codon-optimized genes in Online Resource 1, NCBI accession number: KF975390). The plasmid assemblies were achieved by one-step isothermal DNA assembly protocol (Gibson et al. 2009), except when stated otherwise. pBBR1MCS-2-*P*_{TAC} was constructed by replacing the *P*_{LAC} promoter region (TTTACACTTTATGCTTCCGGCTCGTATGTTG) of the broad-host vector pBBR1MCS-2 (Kovach et al. 1995) with the *P*_{TAC} promoter (TTGACAATTAATCATCGGCTCGTAATATG) using primers listed in Online Resource 2. Empty vectors digested by *Cla*I and *Xho*I were used as backbone DNA for the assembly of all isopropanol production plasmids

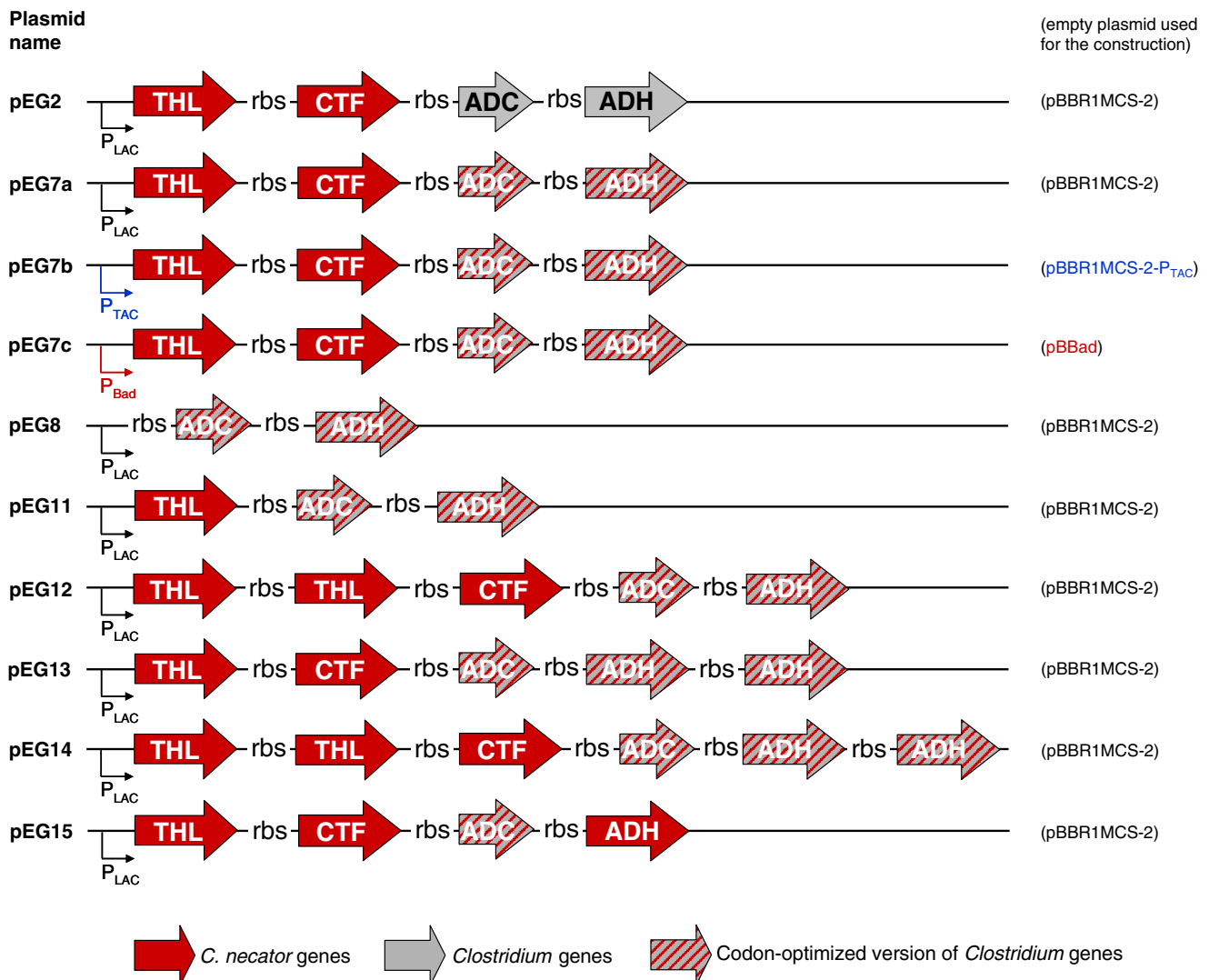


Fig. 2 Schematic of isopropanol production pathways constructed and plasmid utilized. Each plasmid was incorporated into strain Re2133 (H16 Δ *phaB1B2B3C1* (Gen⁺), Budde et al. 2011)

except for pEG7c. For pEG7c, pBBad (Fukui et al. 2009) was digested with *KpnI* and *XbaI*, and the fragments containing the isopropanol production pathway genes were purified from pEG7a via *KpnI* and *XbaI* digestion. The pathway genes were then inserted into pBBad to create pEG7c. For pEG7b, the fragment with pathway genes from the digestion of pEG7a with *XhoI* and *ClaI* was isolated and purified. The fragment was then ligated with the corresponding digested vector. All ligation and one-step isothermal assembly products were transformed into high efficiency *E. coli* Top10 chemical competent cells (InvitrogenTM, Life technologies). Colonies were screened by diagnostic digestion after plasmid extraction. Correct gene insertions on plasmids were confirmed by sequencing. All constructed plasmids are described in Fig. 2. Each confirmed plasmid was transformed into *E. coli* S17-1 (ATCC 47055) by electroporation (Simon et al. 1983). *E. coli* S17-1 harboring the plasmid was then used to introduce the plasmid into *C. necator* Re2133 by conjugative transfer (Slater et al. 1998).

Analytical procedures

Culture supernatants were obtained by filtration (0.2 µm PTFE or PES syringe filters, VWR, Radnor, PA, USA) of the flask broth samples and used for substrate and products determination. The residual substrate and product concentrations were quantified by high-performance liquid chromatography (HPLC). The HPLC instrument (Series 1100, Agilent, Santa Clara, CA, USA) was equipped with an ion-exchange column (Aminex HPX-87H, 300×7.8 mm, Bio-Rad, Hercules, CA, USA) protected with a guard column (Cation H+ cartridge, 30×4.6 mm, Bio-Rad) and coupled to a RI detector and an UV detector (λ=210 nm). The column was eluted with 2.5 mM H₂SO₄ as a mobile phase at 50 °C at a flow rate of 0.5 ml min⁻¹.

Biomass growth was monitored by measuring the optical density at 600 nm (OD_{600 nm}) using a visible spectrophotometer (Spectronic GENESYS 20 Visible Spectrophotometer) with a 1 cm path length absorption PS semi-micro cuvette (VWR).

Enzymatic assay

Cell pellets after centrifugation of 5 ml culture broth (taken at 24 h of cultures) in a 15 ml Falcon tube (1,900×g, 10 min, Centrifuge 5804R Eppendorf AG) were frozen at -80 °C until time of enzymatic assays.

Cell pellets were thawed on ice and resuspended in 0.8 ml of the buffer associated with each assay. Then cells were lysed with beads (0.6 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) in 2 ml screw top plastic vial) at 4 °C using FastPrep-24 (MP Biomedicals, Solon, OH, USA) at 6 m s⁻¹ for 40 s. Three cycles of Fast-Prep with 5 min rest in

between were carried out. Cell debris was removed by microcentrifugation (16,000×g, 10 min, Microcentrifuge 1816, VWR). Cell lysates were filtrated using 0.45 µm syringe filters (PES, VWR) prior to enzyme activity assay.

The protein content of the cell lysates was determined by the standard procedure of the Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin (BSA) as a standard. All measurements including standards were repeated three times.

All enzymatic assays were performed at 25 °C with the Agilent 8453 spectrophotometer (Agilent 8453 UV-Visible Kinetic Mode, Agilent). β-ketothiolase (THL) assay was performed according to Budde et al. (2010). CoA-Transferase (CTF) assay was conducted according to Cary et al. (1990) with succinic acid disodium salt 0.15 mole l⁻¹ as the substrate. Acetoacetate decarboxylase (ADC) assay was performed by a method adapted from Yu et al. (2011). In brief, the buffer used for cell resuspension was 5 mM potassium phosphate buffer (pH 7.3). The assay buffer was 20 mM acetate buffer (pH 4.8) containing 70 µM bromocresol green and 10 mM lithium acetoacetate. The reaction was initiated with addition of 50 µl of appropriately diluted cell extract and the increase in absorbance at 620 nm was monitored. Alcohol dehydrogenase (ADH) assay was adapted from Ismaiel et al. (1993), Hanai et al. (2007), and Shen et al. (2011). Briefly, crude extracts were prepared in 130 mM of Tris-HCl (pH 7.5). ADH activities were measured by following the reduction of acetone (200 mM) with NADPH (OD_{340nm}=6.2 mM⁻¹ cm⁻¹). The assay mixture (1 ml) contained 100 mM Tris-Cl buffer (pH 7.5), 5 mM dithiothreitol (DTT), and 0.2 mM NADPH.

One enzyme unit is defined as 1 µmole of product formed per minute.

Yield calculations

All yields were expressed as carbon ratio. The theoretical isopropanol yield ($Y_{S,Isoprop}^{theo}$) was calculated on a carbon basis considering the pathway shown in Fig. 1. One mole of fructose is converted to 2 moles of acetyl-CoA and 2 moles of CO₂. Then the two molecules of acetyl-CoA ligate into one acetoacetate, which is subsequently decarboxylated and reduced to form one mole of isopropanol. A 6-carbon molecule leads to a 3-carbon molecule, $Y_{S,Isoprop}^{theo}=0.5$ Cmole Cmol⁻¹. The experimental isopropanol yield ($Y_{S,Isoprop}$) was the ratio of isopropanol produced and substrate consumed during a time interval (t_2-t_1):

$$Y_{S,Isoprop} = |(\text{Isoprop}_{t_2} - \text{Isoprop}_{t_1}) / (S_{t_2} - S_{t_1})|.$$

All data are presented as means ± SD from three independent experiments except when stated otherwise.

Results

Coding sequence optimization

First demonstration of isopropanol production in C. necator strain Re2133/pEG2

In order to redirect the carbon flow from P(3HB) into isopropanol pathway, the strain Re2133 (Budde et al. 2011) was used as the parent strain since genes coding for acetoacetyl-CoA reductases (*phaB1B2B3*) and for the PHA synthase (*phaC1*) were deleted (Fig. 1). The strain Re2133 transformed with the empty plasmid pBBR1MCS-2 was used as a reference strain during this study. This strain was cultivated on fructose as the sole carbon source in a mineral medium designed to reach a biomass concentration of about 0.7 g l^{-1} once nitrogen was depleted, as stated in Section 2.2. After nitrogen depletion, the carbon excess from fructose was directed toward pyruvic acid (up to $2.61 \pm 0.19 \text{ g l}^{-1}$, Fig. 3a and Online Resource 3).

The entire isopropanol production pathway from acetyl-CoA was inserted in the plasmid pBBR1MCS-2 to construct the plasmid pEG2. pEG2 plasmid harbored the native *C. necator* genes *phaA* (H16_A1438) and *ctfAB* (H16_A1331 and H16_A1332) coding for a β -ketothiolase A (THL) and the two subunits of a CoA-Transferase (CTF), respectively. Additionally, the plasmid also contained the heterologous genes from *Clostridium* species *adc* (CA_P0165) and *adh* (AF157307 nt 2351 to 3406) coding for an acetoacetate decarboxylase (ADC) and an alcohol dehydrogenase (ADH), respectively (Figs. 1, 2). The resulting strain Re2133/pEG2 produced $0.22 \pm 0.07 \text{ g l}^{-1}$ of isopropanol (Fig. 3a). The titer reached was very low, and up to $2.44 \pm 0.14 \text{ g l}^{-1}$ pyruvic acid by-product was still produced (Online Resource 3), indicating that the expression of the isopropanol pathway genes was not significant enough to shunt all the P(3HB) precursors toward isopropanol.

Cell extracts of Re2133/pEG2 were analyzed in terms of enzyme specific activities for the heterologous enzymes ADC and ADH. The specific activities of both heterologous enzymes were very low, at 0.75 ± 0.84 and $0.05 \pm 0.01 \text{ U mg}^{-1}$, respectively, while the specific activities of the native *C. necator* enzymes THL and CTF were 5.47 ± 0.62 and $9.89 \pm 1.28 \text{ U mg}^{-1}$, respectively (Table 1).

The poor expression of the heterologous genes from *Clostridium* species (*adc* and *adh*) necessary for isopropanol production in *C. necator* (Fig. 1) could be explained by differences in genome GC content between the *Clostridium* species (about 30 % GC) and *C. necator* (about 66 % GC) as depicted in Online Resource 4. The differences in GC content between the two species could impact the transcriptional and translational efficiencies since GC content is known to drive codon usage. Codon bias of the host organism has been

reported as a major limiting factor in the production yield of a desired protein (Behura and Severson 2012). A comparative table of the codon usage in *C. necator*, *Clostridium acetobutylicum*, and *Clostridium beijerinckii* was constructed (Online Resource 5) from the database <http://www.kazusa.or.jp/codon/> (Nakamura et al. 2000). The codon usage between these three organisms is very different. For example, the codon UUA for Leucine is not used at all by *C. necator*, whereas it is used 51–53 % of the time by *C. acetobutylicum* and *C. beijerinckii*. Codon optimization of the heterologous genes appears to be necessary to improve protein expression in *C. necator*.

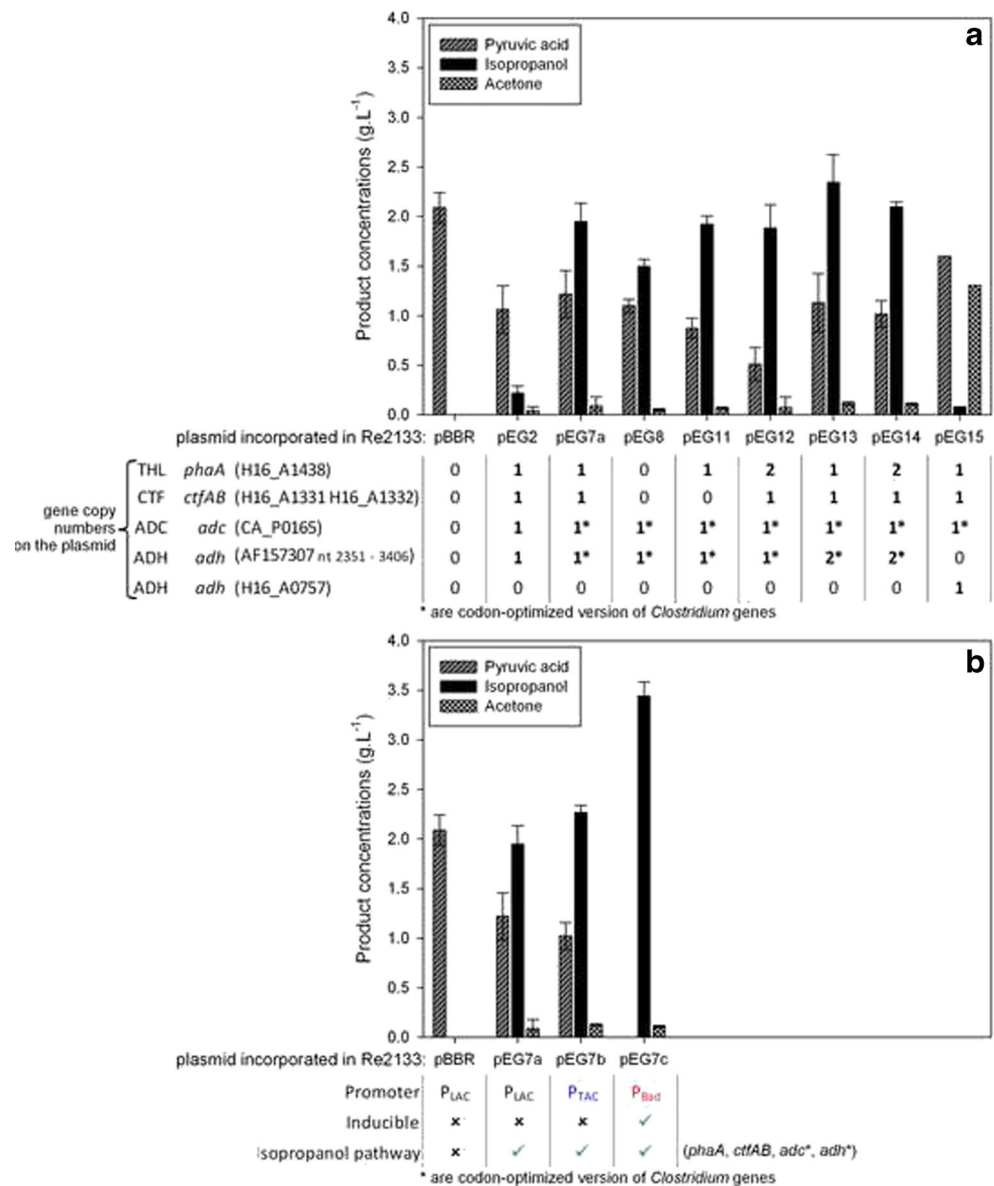
Codon optimization to overcome the poor expression of heterologous genes from Clostridium species

The plasmid pEG7a was constructed with the codon-optimized version of the *adc* and *adh* heterologous genes (NCBI accession number KF975390) of *Clostridium* species (Fig. 2) and incorporated into Re2133. Utilization of codon optimized genes successfully led to an increase in ADC and ADH activities in Re2133/pEG7a cell extracts. An activity of 8.89 ± 1.30 and $0.72 \pm 0.04 \text{ U mg}^{-1}$ (Table 1) were determined for ADC and ADH, respectively. As a result, the strain bearing pEG7a produced $1.95 \pm 0.18 \text{ g l}^{-1}$ of isopropanol in 88 h (Fig. 3a), which corresponded to an 8.9 ± 3.0 -fold increase compared to Re2133/pEG2. There is a global increase in the carbon conversion ($0.17 \text{ C mole l}^{-1}$; Fig. 4) to products (biomass, pyruvic acid, isopropanol, and acetone) in Re2133/pEG7a compared to Re2133/pEG2 ($0.10 \text{ C mole l}^{-1}$; Fig. 4). This indicated that in the strain Re2133/pEG7a more carbon was driven through the entire pathway instead of only the conversion of pyruvic acid (produced by the strain Re2133/pEG2) to isopropanol. At 88 h of the culture time, the peak isopropanol production time, a pyruvic acid concentration of $1.52 \pm 0.29 \text{ g l}^{-1}$, and an acetone concentration of $0.09 \pm 0.09 \text{ g l}^{-1}$ were detected for the strain Re2133/pEG7b, suggesting that further redirection from pyruvic acid toward the production of isopropanol can be achieved.

Suitability of ADH from C. necator for isopropanol production

An alternative to codon optimization of heterologous ADH genes was to identify and use a native ADH gene from *C. necator* that is active toward acetone. One native ADH has been previously identified in *C. necator* and tested (Steinbüchel and Schlegel 1984). This ADH was reported to be a very unspecific enzyme regarding its substrates. Isopropanol was reported as one of its wide spectrum of substrates (Steinbüchel and Schlegel 1984). The sequence of *adh* has been published (Jendrossek et al. 1988) and by comparison to the whole genome sequence (Pohlmann et al.

Fig. 3 Time point in which maximum isopropanol concentration was produced; in addition, pyruvic acid and acetone concentrations produced at the same time point are depicted. **a** Coding sequence evaluation with the plasmid pBBR1MCS-2 incorporated in the strain Re2133. Composition of the plasmid is indicated below each plasmid name. $n=3$, except for strains Re2133/pEG12 and Re2133/pEG14, where $n=2$ was performed, and Re2133/pEG15, where $n=1$ was performed. **b** Promoter evaluation with the same set of genes [*phaA* (H16_A1438), *ctfAB* (H16_A1331 H16_1332), codon-optimized *adc* and *adh* (NCBI accession number: KF975390)]. The promoter on the plasmid is indicated below each plasmid name. $n=3$, except for Re2133/pEG7c, where $n=2$ was performed



2006), locus H16_A0757 was identified. In the wild-type strain, this native ADH is only expressed under restricted supply of oxygen (Steinbüchel and Schlegel 1984). Nevertheless, mutant strains of *C. necator* with constitutive expression of *adh* have been isolated (Steinbüchel et al. 1987), indicating that *adh* expression under a constitutive promoter should lead

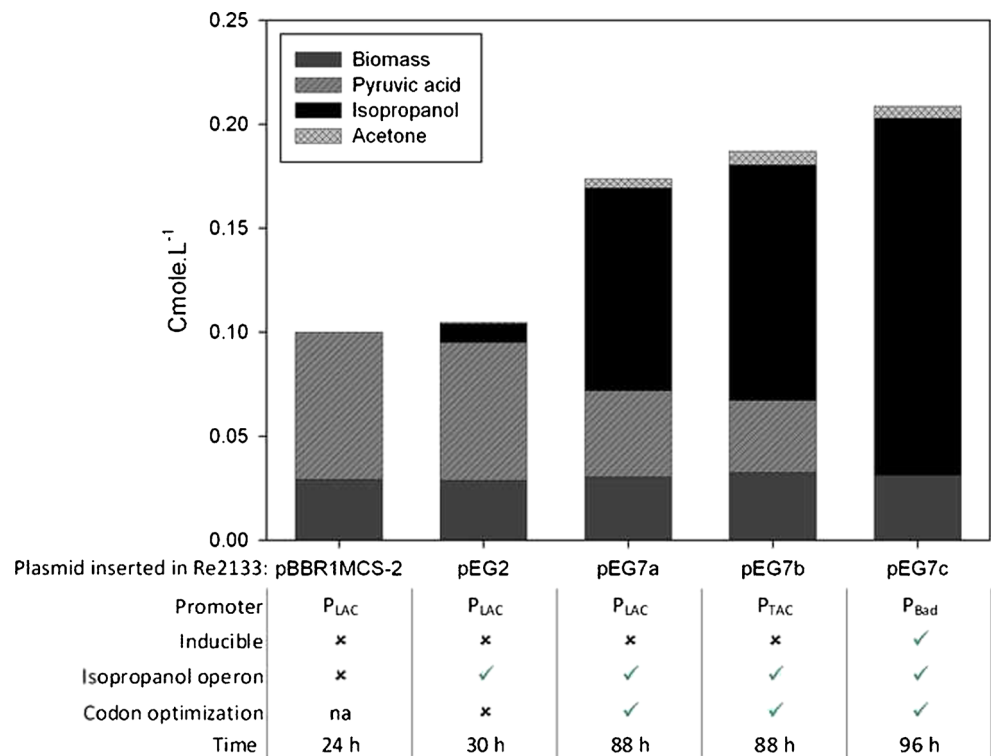
to ADH enzyme activity even under aerobic conditions. Thus, another plasmid, pEG15 was constructed with the *C. necator adh* (H16_A0757) instead of the *C. beijerinckii adh* (Fig. 2). The resulting strain Re2133/pEG15 produced a small amount of isopropanol (about 0.8 g l⁻¹, Fig. 3a) compared to strain Re2133/pEG7a (1.95±0.18 g l⁻¹ isopropanol). Large amounts

Table 1 Enzymatic activities of β -ketothiolase (THL), acetoacetyl-CoA transferase (CTF), acetoacetate decarboxylase (ADC), and alcohol dehydrogenase (ADH) in cell extracts of the strains Re2133/pBBR1MCS-2, Re2133/pEG2, and Re2133/pEG7a

Strain	THL activity (U mg ⁻¹)	CTF activity (U mg ⁻¹)	ADC activity (U mg ⁻¹)	ADH activity (U mg ⁻¹)
Re2133/pBBR1MCS-2	2.34±0.73	8.68±0.79	-0.36±0.40	0.03±0.03
Re2133/pEG2	5.47±0.62	9.89±1.28	0.75±0.84	0.05±0.01
Re2133/pEG7a	6.54±0.34	23.18±5.05	8.89±1.30	0.72±0.04

Samples were taken at 24 h of culture. Each value represents the mean ± standard deviation on $n=3$

Fig. 4 Carbon distribution in Cmole l^{-1} of the products (biomass, pyruvic acid, isopropanol, and acetone) for the strains Re2133/pBBR1MCS-2, Re2133/pEG2, Re2133/pEG7a, Re2133/pEG7b, and Re2133/pEG7c. Cumulative data for the culture time point corresponding to the maximum concentration of total products



of acetone ($\sim 1.31 \text{ g l}^{-1}$) and pyruvic acid ($\sim 2.00 \text{ g l}^{-1}$) were detected in the supernatant of Re2133/pEG15. Although there was a slight ADH activity toward acetone, the expression of the ADH from *C. beijerinckii* led to 25 times higher isopropanol production. This indicates that ADH from *C. necator* is not specific toward acetone as a substrate and, as a result, is not suitable for the production of isopropanol.

Influence of the pathway gene copy number on isopropanol production

In order to further direct the carbon flow from pyruvic acid to isopropanol, copy numbers of three pathway genes (*phaA*, *ctfAB*, and *adh*) were investigated, since an increase in gene copy number could result in an increase in protein expression level and thus a higher enzymatic activity (Schendel et al. 1989). For this purpose, plasmids pEG8, pEG11, pEG12, pEG13, and pEG14 were constructed, which included various copy numbers of *phaA* (0–2), *ctfAB* (0–1), and *adh* (1–2) (Figs. 2 and 3a).

The activity of β -ketothiolase, encoded by *phaA*, in cell extracts increased with the increase in the plasmid gene copy number (Fig. 5a). β -ketothiolase activity of $3.2 \pm 2.2 \text{ U mg}^{-1}$ in cell extract was found in strains with 0 copy of *phaA* on the plasmid. An addition of about 2.7 U mg^{-1} was detected with each additional copy of *phaA* on the plasmid. The increase in activity resulting from additional gene copies led to an increase in isopropanol production, although only for the addition of one gene copy (Re2133/pEG11 and Re2133/pEG8

shown on Fig. 3a). These results indicated that the reaction performed by the β -ketothiolase was no longer the limiting step when one copy of *phaA* was overexpressed on the plasmid.

The overexpression of native CoA-transferase (*ctfAB*) on the plasmid was not necessary since it did not improve the isopropanol production (Re2133/pEG11 vs. Re2133/pEG7a on Fig. 3a). Hence, the CoA-transferase was not a rate-controlling step when compared to the other enzymes in the pathway of interest.

The addition of a second copy of the codon-optimized *adh* gene on the plasmid slightly increased the production of isopropanol by 1.11 ± 0.14 -fold (Re2133/pEG14 vs. Re2133/pEG12) and by 1.20 ± 0.18 -fold (Re2133/pEG13 vs. Re2133/pEG7a), depending on the plasmid construction. An additional copy of *adh* gene seemed to lead to a smaller increase in isopropanol production with the plasmid pEG14 than with the plasmid pEG13 (Fig. 3a). The difference between these two constructions was the addition of a second *phaA* gene copy on the plasmid pEG14, thus increased the distance between the promoter and the second copy of *adh* from 4.4 to 5.6 kb. A previous study demonstrated that expression was always greater for the gene that is closest to the promoter (Lim et al. 2011). To determine if such conclusion applied in this case, ADH-specific activities obtained in the constructed strains were compared to the distance of the furthest *adh* gene from the promoter (Fig. 5b). In accordance with the findings of Lim et al. (2011), an inverse linear relationship between the distance from the promoter and the activity of the associated

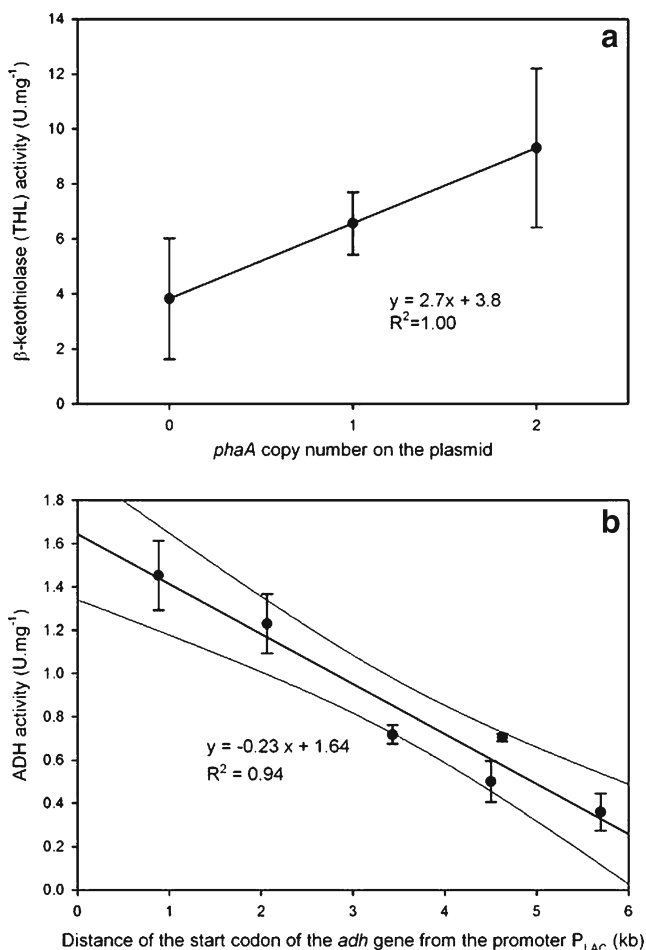


Fig. 5 **a** β -ketothiolase (THL) activity vs. the copy number of *phaA* on the plasmid. For 0 copy of *phaA*, average of values from Re2133/pBBR1MCS-2 ($n=3$) and Re2133/pEG8 ($n=3$). For 1 copy, average of values from Re2133/pEG2 ($n=3$), Re2133/pEG7a ($n=3$), Re2133/pEG11 ($n=3$), Re2133/pEG13 ($n=3$). For two copies, average of values from Re2133/pEG12 ($n=2$) and Re2133/pEG14 ($n=2$). **b** Alcohol dehydrogenase (ADH) activity associated to the expression of one *adh* gene copy vs. the distance between the promoter and the *adh* gene start codon. The activity associated to the second *adh* gene (for the strains Re2133/pEG13 and Re2133/pEG14) was calculated by subtracting the activity of the cell extract measured for Re2133/pEG7a and Re2133/pEG13, respectively

ADH was demonstrated (Fig. 5b). The addition of *adh* gene copies on the same plasmid did not seem to be an optimal strategy to increase ADH expression and activity to improve isopropanol production.

Promoter comparison

Promoter strength is another important parameter for pathway gene expression. We evaluated several promoter systems for isopropanol production, in which two are constitutively expressed promoters (P_{LAC} and P_{TAC}) and one of which is an inducible promoter (P_{BAD}). All three promoters were evaluated for the production of isopropanol in *C. necator*.

Comparison of the two constitutive promoters P_{LAC} and P_{TAC}

Fukui et al. (2010) evaluated several promoters to regulate gene expression in *C. necator*. Among the constitutive promoters identified (P_{LAC} , P_{TAC} , P_{phaC} , and P_{phaP}), P_{TAC} promoter was the strongest and demonstrated 1.5-fold to 2.0-fold higher read-through expression when compared with P_{LAC} . To test the effect of different promoter strength on isopropanol production, the P_{LAC} promoter region (TTTACACTTTATGC TTCCGGCTCGTATGTTG) of the broad-host vector pBBR1MCS-2 was exchanged with the P_{TAC} promoter region (TTGACAATTAATCATCGGCTCGTATAATG) leading to the plasmid pBBR1MCS-2- P_{TAC} . Then the synthetic operon with four genes encoding for isopropanol production pathway enzymes were digested from pEG7a and inserted into pBBR1MCS-2- P_{TAC} , resulting in plasmid pEG7b.

Strain Re2133 harboring pEG7b plasmid produced $2.27 \pm 0.07 \text{ g l}^{-1}$ isopropanol (Fig. 3b), corresponding to a 1.16 ± 0.12 -fold increase compared to the strain Re2133/pEG7a with the P_{LAC} promoter. In terms of total carbon titer, $0.19 \text{ C mole l}^{-1}$ were produced by the strain Re2133/pEG7b compared to the $0.17 \text{ C mole l}^{-1}$ produced by the strain Re2133/pEG7a (Fig. 4). Constitutive expression of isopropanol pathway led to a growth-associated isopropanol production of $0.98 \pm 0.06 \text{ g g}^{-1}$ of biomass in the strain Re2133/pEG7a and $1.14 \pm 0.01 \text{ g g}^{-1}$ in the strain Re2133/pEG7b. Consequently, a decrease in the maximal growth rate by 2.70 ± 0.07 times (Re2133/pEG7a) and by 3.19 ± 0.16 times (Re2133/pEG7b) compared to Re2133/pBBR1MCS-2 (0.17 h^{-1}) was observed. Low maximal growth rate of the strains harboring pEG7a and pEG7b (between 0.05 and 0.06 h^{-1}) is not attractive for an efficient culture strategy. To overcome the poor growth due to the growth-associated isopropanol production, an inducible promoter was evaluated.

Study of an inducible promoter: P_{BAD}

Fukui et al. (2010) demonstrated that broad-host vector pBBad harboring P_{BAD} with *araC* regulator gene responding to the addition of L-arabinose was functional in *C. necator*. This system was utilized by inserting the synthetic isopropanol production pathway from pEG7a into the pBBad plasmid, which led to the plasmid pEG7c. Re2133/pEG7c flask cultures were induced with 0.1 % L-arabinose at an $\text{OD}_{600 \text{ nm}}$ of 1.5, corresponding to 75 % of the maximum $\text{OD}_{600 \text{ nm}}$ reached with the amount of nitrogen supplied. The maximal growth rate was $0.17 \pm 0.01 \text{ h}^{-1}$, which was the same as the strain with the empty plasmid pBBR1MCS-2. Isopropanol production by Re2133/pEG7c reached $3.44 \pm 0.14 \text{ g l}^{-1}$ (Figs. 3b and 6), which was an increase by 1.76 ± 0.18 -fold compared to the strain Re2133/pEG7a. The P_{BAD} promoter system with 0.1 % L-arabinose induction allowed for a higher production of isopropanol than the two constitutive

promoter systems tested (P_{LAC} and P_{TAC} with plasmids pEG7a and pEG7b, respectively). Moreover, no extracellular pyruvic acid was detected (Figs. 3b and 6), indicating that the increase in promoter strength was sufficient to pull carbon through the isopropanol production pathway from fructose consumption. In Re2133/pEG7c, up to 0.21 mole l^{-1} of total carbon (biomass with addition of isopropanol and acetone) were produced, which corresponded to a significant increase of carbon flow into the products formation comparatively to the other engineered strains (Fig. 4). Slight promoter leakiness was detected which resulted in only 0.15 g of isopropanol produced per g of biomass in the absence of arabinose induction.

Discussion

This study presents the first demonstration of isopropanol production by *C. necator*. A rational design of isopropanol production plasmids and production evaluation in batch culture were performed.

Codon usage differences between the host *C. necator* and *Clostridium* species was proven to be a critical design factor. Strain Re2133/pEG7a with codon-optimized version of the *Clostridium* genes *adc* and *adh* produced isopropanol that was 8.9±3.0-folds higher ($1.95\pm0.18\text{ g l}^{-1}$) than Re2133/pEG2. Another strategy to avoid poor expression of heterologous genes was to directly use host genes coding for ADH. Although the expression of a native *adh* gene from *C. necator* was not appropriate for isopropanol production (Fig. 3a), an exploration of other potentially suitable ADHs from the *C. necator* genome could be fruitful. No native ADC encoding gene or ADC activity was reported in *C. necator* to date. However, acetone excretion was reported by some mutant strains of *C. necator* partially or completely lacking the ability to synthesize P(3HB) (Vollbrecht et al. 1978), indicating that a native decarboxylase gene may exist. Such gene remains to be identified, evaluated, and tested for isopropanol production.

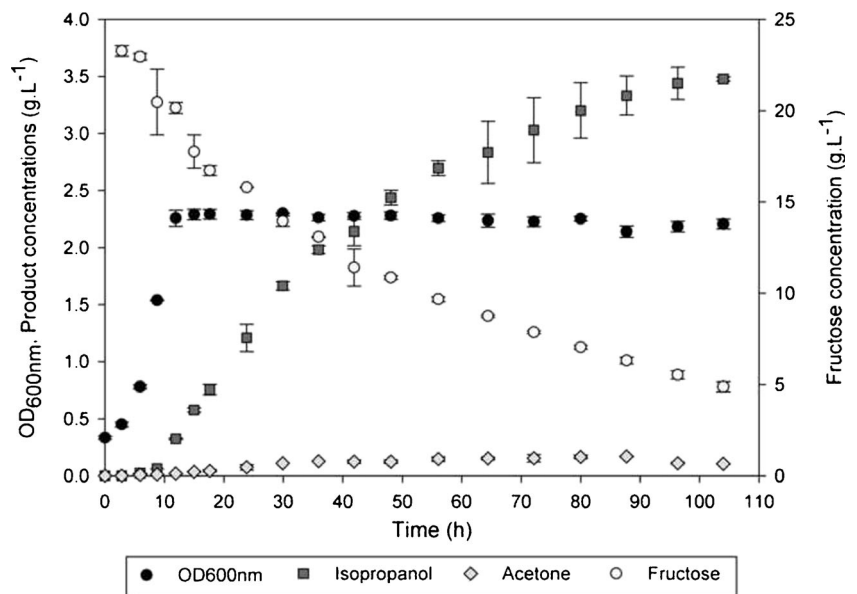
In order to further direct the carbon flow from pyruvic acid to isopropanol in strain Re2133/pEG7a, gene copy number, and distance of the gene from the promoter were investigated. The addition of a copy of *adh* gene improved the isopropanol production. Considering that acetone was still detected in the culture broth, the alcohol dehydrogenase (ADH) may be the limiting step and may require a fine-tuning of expression to further increase isopropanol production. However, in order to enhance enzyme activity via an increase in gene dosage, the distance between the added genes and the promoter should be reduced to a minimum (Fig. 5b).

Increasing the promoter strength further improved the isopropanol production. In accordance with Fukui et al. (2010), the use of P_{TAC} promoter instead of P_{LAC} promoter

increased the isopropanol concentration produced by 1.16±0.12-fold (strain Re2133/pEG7b vs. Re2133/pEG7a, Fig. 3b). These two promoters led to a constitutive production (Fukui et al. 2010) of isopropanol. As a consequence, the growth rate is strongly reduced (0.05 to 0.06 h^{-1} instead of 0.17 h^{-1}), and such low growth rates were not realistic for scaled-up industrial production. The use of an inducible promoter P_{BAD} successfully overcame this problem. In addition, the constructed strain Re2133/pEG7c produced up to $3.44\pm0.14\text{ g l}^{-1}$ of isopropanol (Figs. 3b and 6) and no more pyruvic acid after L-arabinose induction (Figs. 4, 3b and 6), indicating that the increase in promoter strength was significant enough to increase the isopropanol pathway production to the level of the fructose consumption. One disadvantage of the inducible system P_{BAD} is the need to add an inducer molecule. It would be ideal to utilize a system that is auto-upregulated when a nutrient is depleted as it is for PHA production. Fukui et al. 2010 evaluated a vector system with P_{phaP} promoter along with the *phaR* gene coding for PhaR regulator, which was a useful expression vector enabling autoregulation of gene expression linked with P(3HB) biosynthesis. However, this system relies on the presence of P(3HB) in the cells (Pötter et al. 2002; York et al. 2002) and cannot be used for the production of other targeted molecules from P(3HB) precursors with PHB⁻ mutants strains such as Re2133. Other inducible systems dependent on nutrient depletion could be used such as two-component signal transduction systems (Ninfa et al. 2007).

The key features for industrial production of isopropanol are high titer (to reduce recovery process costs), high yield (close to the theoretical production yield), high volumetric productivity, and cheap carbon source. To fulfil these requirements, the production strains must be selected for high specific productivity and high production yields. Titre and volumetric productivity will then depend on the cultivation monitoring. To assess the suitability of the best isopropanol production strain (Re2133/pEG7c) constructed in this study, the performances of this strain were compared to those of other engineered strains cultivated in similar conditions, i.e. batch cultures. The Re2133/pEG7c *C. necator* strain was able to produce up to $3.44\pm0.14\text{ g l}^{-1}$ of isopropanol. In batch mode, natural producers from the *Clostridium* family, such as *C. isopropyllicum*, were able to produce titers up to 4.6 $g l^{-1}$ with immobilized cells (Matsumura et al. 1992). Higher titers were reached with engineered strains: 4.9 $g l^{-1}$ by *E. coli* (Hanai et al. 2007), 8.8 $g l^{-1}$ by *C. acetobutylicum* (Collas et al. 2012), and 9.5 $g l^{-1}$ by *Candida utilis* (Tamakawa et al. 2013; Table 2). Nevertheless, in this work, the maximum titer of $3.44\pm0.14\text{ g l}^{-1}$ of isopropanol was reached with only 0.82±0.02 $g l^{-1}$ of biomass under conditions of nitrogen depletion. Comparatively in *E. coli* batch culture (Hanai et al. 2007), nitrogen was not limiting which enabled a higher protein synthesis and biomass concentration (Table 2) considering

Fig. 6 Evaluation of substrate (fructose) and products (biomass, pyruvic acid, acetone, and isopropanol) over cultivation time of Re2133/pEG7c



the OD_{600 nm} reported (dry cell weights were not estimated in the paper by Hanai et al.). The specific productivity and yields were calculated to better compare the performances of the strains. The overall specific productivity of the strain Re2133/pEG7c was $0.044 \pm 0.006 \text{ g g}^{-1} \text{ h}^{-1}$ ($0.016 \pm 0.001 \text{ g l}^{-1}$

OD_{600 nm}⁻¹h⁻¹; Table 2). The instantaneous maximal specific productivity of Re2133/pEG7c after induction by arabinose was $0.093 \text{ g g}^{-1} \text{ h}^{-1}$ which corresponded to 62 % of the maximum theoretical isopropanol production specific rate (Table 2). The maximum theoretical specific rate was calculated

Table 2 Comparison of this work with other engineered isopropanol production organisms in batch culture

References	Hanai et al. 2007	Collas et al. 2012	Dusséaux et al. 2013	Jang et al. 2013	Tamakawa et al. 2013	This work	Theoretical maximum
Microorganism	<i>Escherichia coli</i>	<i>Clostridium acetobutylicum</i>	<i>Clostridium acetobutylicum</i>	<i>Clostridium acetobutylicum</i>	<i>Candida utilis</i>	<i>Cupriavidus necator</i>	<i>C. necator</i>
Isopropanol (g l ⁻¹)	4.9	8.8	5	3.5	9.5	3.44 ± 0.14	nc
Biomass (OD _{600 nm})	20	nr	nr	20	nr	2.25 ± 0.05	nc
Biomass (g l ⁻¹)	nr	nr	16	nr	nr	$0.82^a \pm 0.01$	nc
Time (h)	30.5	45	20	60	52	96.3	nc
Overall specific productivity (g l ⁻¹ OD _{600 nm} ⁻¹ h ⁻¹)	0.008	nr	nr	0.003	nr	0.016 ± 0.001	nc
Overall specific productivity (g g ⁻¹ h ⁻¹)	nr	nr	0.016	nr	nr	0.044 ± 0.006	nc
Maximum specific productivity (g g ⁻¹ h ⁻¹)	nr	nr	nr	nr	nr	0.093 ± 0.004	0.15 ^b
Overall yield (Cmole Cmole ⁻¹)	0.18	nr	0.13	0.07	nr	0.24 ± 0.01	nc
Maximum yield (Cmole Cmole ⁻¹)	0.22	nr	nr	nr	nr	0.32 ± 0.01	0.50 ^c
By-products	Ethanol, acetone	Ethanol, butanol, acetate, butyrate, acetoïn, acetone, 2,3 butanediol	Ethanol, butanol, acetate, butyrate, acetoïn	Ethanol, butanol, acetate, butyrate	Ethanol, acetate	Acetone	None

nr not reported, nc not calculated

^a The CDW was calculated using the relationship: $1 \text{ OD}_{600 \text{ nm}} = 0.363 \text{ g l}^{-1}$

^b Calculated with kinetic modeling from Grousseau et al. (2013) considering a null growth rate

^c See “Materials and methods” and “Yield calculations” sections

considering the model developed by Grousseau et al. (2013), where the limiting rate of product formation was defined by the NADPH synthesis rate.

The overall yields of Re2133/pEG7c were respectively 1.3 and 1.8 times higher (Table 2) compared to the engineered isopropanol-producing *E. coli* strain (Hanai et al. 2007) and the engineered isopropanol-producing *C. acetobutylicum* strain (Dusséaux et al. 2013). The maximum yield reached by the strain Re2133/pEG7c corresponded to 64 % of the theoretical yield ($0.5 \text{ Cmole Cmole}^{-1}$).

As reported here, *C. necator* is an excellent host for isopropanol production:

- (1) after deletion of genes encoding for the P(3HB) synthesis (*phaB1B2B3* and *phaC*), the expression of two heterologous genes (*adc* and *adh*) and two native genes (*phaA* and *ctfAB*) was sufficient to divert carbon from P(3HB) precursors to isopropanol
- (2) the intrinsic performances of the strain (specific production rate and yield) corresponded to more than 60 % of the theoretical performances
- (3) isopropanol concentrations produced were significant ($3.44 \pm 0.14 \text{ g l}^{-1}$) considering the low concentration of *C. necator* biomass used as catalyst ($0.82 \pm 0.02 \text{ g l}^{-1}$)

The strain Re2133/pEG7c will be further evaluated for the scale-up production of isopropanol from various carbon sources. High cell density culture associated with a product recovery system with a controlled supply of nitrogen or any other limiting elements would be beneficial.

Acknowledgments This work was supported by grants from the MIT-France Seed Fund and U.S. Department of Energy, Advanced Research Projects Agency-Energy (ARPA-E). Dr. Estelle Grousseau was funded by a Post-Doctoral grant from the French National Center for Scientific Research (CNRS) and the French Ministry of Higher Education and Research following the France-MIT Energy Forum in June 29, 2011. We thank Dr. Jens K. Plassmeier and Dr. Christopher J. Brigham for the helpful discussions. We thank Mr. Stephan Grunwald for his assistance, and Mr. John W. Quimby for the review of this manuscript. We also thank Dr. Toshiaki Fukui from the Tokyo Institute of Technology for the generous gift of the pBBad plasmid.

References

- Aneja KK, Ashby RD, Solaiman DK (2009) Altered composition of *Ralstonia eutropha* poly(hydroxyalkanoate) through expression of PHA synthase from *Allochromatium vinosum* ATCC 35206. *Biotechnol Lett* 31(10):1601–1612
- Aragao GMF (1996) Production de poly-beta-hydroxyalkanoates par *Alcaligenes eutrophus*: caractérisation cinétique et contribution à l'optimisation de la mise en oeuvre des cultures. Institut National des Sciences Appliquées de Toulouse, Thèse no d'ordre:403
- Araki S, Fukuhara H, Isaka T, Matsunaga F, Yasuhara M (1993) Preparation of propylene by dehydration of isopropanol in the

- presence of a pseudo-boehmite derived gamma alumina catalyst. US5227563A
- Behura SK, Severson DW (2012) Codon usage bias: causative factors, quantification methods and genome-wide patterns: with emphasis on insect genomes. *Biol Rev Camb Philos* 88(1):49–61
- Bruno TJ, Wolk A, Naydich A (2009) Composition-explicit distillation curves for mixtures of gasoline with four-carbon alcohols (butanols). *Energy Fuel* 23:2295–2306
- Budde CF, Mahan AE, Lu JN, Rha C, Sinskey AJ (2010) Roles of multiple acetoacetyl coenzyme A reductases in polyhydroxybutyrate biosynthesis in *Ralstonia eutropha* H16. *J Bacteriol* 192(20):5319–5328
- Budde CF, Riedel SL, Willis LB, Rha C, Sinskey AJ (2011) Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. *Appl Environ Microbiol* 77(9):2847–2854
- Cary JW, Petersen DJ, Papoutsakis ET, Bennett GN (1990) Cloning and expression of *Clostridium acetobutylicum* ATCC-824 acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A transferase in *Escherichia coli*. *Appl Environ Microbiol* 56(6):1576–1583
- Chen JS, Hiu SF (1986) Acetone-butanol-isopropanol production by *Clostridium beijerinckii* (Synonym, *Clostridium butylicum*). *Biotechnol Lett* 8(5):371–376
- Collas F, Kuit W, Clement B, Marchal R, Lopez-Contreras A, Monot F (2012) Simultaneous production of isopropanol, butanol, ethanol and 2,3-butanediol by *Clostridium acetobutylicum* ATCC 824 engineered strains. *AMB Expr* 2(1):45
- Connor MR, Liao JC (2009) Microbial production of advanced transportation fuels in non-natural hosts. *Curr Opin Biotechnol* 20(3):307–315
- Doi Y, Kawaguchi Y, Nakamura Y, Kunioka M (1989) Nuclear magnetic-resonance studies of poly(3-hydroxybutyrate) and polyphosphate metabolism in *Alcaligenes eutrophus*. *Appl Environ Microbiol* 55(11):2932–2938
- Dürre P (1998) New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. *Appl Microbiol Biotechnol* 49(6):639–648
- Dusséaux S, Croux C, Soucaille P, Meynial-Salles I (2013) Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for the high-yield production of a biofuel composed of an isopropanol/butanol/ethanol mixture. *Metab Eng* 18:1–8
- Friedrich CG, Bowien B, Friedrich B (1979) Formate and oxalate metabolism in *Alcaligenes eutrophus*. *J Gen Microbiol* 115(Nov):185–192
- Fukui T, Suzuki M, Tsuge T, Nakamura S (2009) Microbial synthesis of poly((R)-3-hydroxybutyrate-co-3-hydroxypropionate) from unrelated carbon sources by engineered *Cupriavidus necator*. *Biomacromolecules* 10(4):700–706
- Fukui T, Ohsawa K, Mifune J, Orita I, Nakamura S (2010) Evaluation of promoters for gene expression in polyhydroxyalkanoate-producing *Cupriavidus necator* H16. *Appl Microbiol Biotechnol* 89(5):1527–1536
- Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6(5):343–345
- Grousseau E, Blanchet E, Deleris S, Albuquerque MGE, Paul E, Uribealarea J-L (2013) Impact of sustaining a controlled residual growth on polyhydroxybutyrate yield and production kinetics in *Cupriavidus necator*. *Bioresour Technol* 148:30–38
- Hanai T, Atsumi S, Liao JC (2007) Engineered synthetic pathway for isopropanol production in *Escherichia coli*. *Appl Environ Microbiol* 73(24):7814–7818
- Inokuma K, Liao JC, Okamoto M, Hanai T (2010) Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping. *J Biosci Bioeng* 110(6):696–701

- Ismail AA, Zhu CX, Colby GD, Chen JS (1993) Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. J Bacteriol 175(16):5097–5105
- Jang YS, Malaviya A, Lee J, Im JA, Lee SY, Lee J, Eom MH, Cho JH, Seung DY (2013) Metabolic engineering of *Clostridium acetobutylicum* for the enhanced production of isopropanol-butanol-ethanol fuel mixture. Biotechnol Prog 29(4):1083–1088
- Jendrosseck D, Steinbüchel A, Schlegel HG (1988) Alcohol dehydrogenase gene from *Alcaligenes eutrophus*: subcloning, heterologous expression in *Escherichia coli*, sequencing, and location of Tn5 insertions. J Bacteriol 170(11):5248–5256
- Johnson BF, Stanier RY (1971) Dissimilation of aromatic compounds by *Alcaligenes eutrophus*. J Bacteriol 107(2):468
- Jojima T, Inui M, Yukawa H (2008) Production of isopropanol by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol 77(6):1219–1224
- Kibby CL, Hall WK (1973) Studies of acid catalyzed reactions: XII. Alcohol decomposition over hydroxyapatite catalysts. J Catal 29(1):144–159
- Koller M, Atlé A, Dias M, Reiterer A, Brauneegg G (2010) Microbial PHA production from waste raw materials. In: Chen GQ (ed) Plastics from bacteria: natural functions and applications. Microbiol Monogr 14:85–119
- Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd, Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166(1):175–176
- Krouwel PG, Vanderlaan WFM, Kossen NWF (1980) Continuous production of normal-butanol and isopropanol by immobilized, growing *Clostridium butylicum* cells. Biotechnol Lett 2(5):253–258
- Kusakabe T, Tatsuke T, Tsuruno K, Hirokawa Y, Atsumi S, Liao JC, Hanai T (2013) Engineering a synthetic pathway in cyanobacteria for isopropanol production directly from carbon dioxide and light. Metab Eng 20:101–108
- Kusian B, Sultemeyer D, Bowien B (2002) Carbonic anhydrase is essential for growth of *Ralstonia eutropha* at ambient CO₂ concentrations. J Bacteriol 184(18):5018–5026
- Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS (2008a) Fermentative butanol production by clostridia. Biotechnol Bioeng 101(2):209–228
- Lee WH, Loo CY, Nomura CT, Sudesh K (2008b) Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-hydroxyvalerate precursors. Bioresour Technol 99(15):6844–6851
- Lim HN, Lee Y, Hussein R (2011) Fundamental relationship between operon organization and gene expression. Proc Natl Acad Sci U S A 108(26):10626–10631
- Lu J, Brigham C, Gai C, Sinskey A (2012a) Studies on the production of branched-chain alcohols in engineered *Ralstonia eutropha*. Appl Microbiol Biotechnol 96(1):283–297
- Lu J, Brigham CJ, Rha C, Sinskey AJ (2012b) Characterization of an extracellular lipase and its chaperone from *Ralstonia eutropha* H16. Appl Microbiol Biotechnol 97(6):2443–2454
- Matsumura M, Takehara S, Kataoka H (1992) Continuous butanol isopropanol fermentation in down-flow column reactor coupled with pervaporation using supported liquid membrane. Biotechnol Bioeng 39(2):148–156
- Nakamura Y, Gojobori T, Ikemura T (2000) Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res 28(1):292
- Ninfa AJ, Selinsky S, Perry N, Atkins S, Song QX, Mayo A, Arps D, Woolf P, Atkinson MR (2007) Using two-component systems and other bacterial regulatory factors for the fabrication of synthetic genetic devices. In: Simon MI, Crane BR, Crane A (eds) component signaling systems, Pt A. Elsevier Academic, San Diego, 422:488–512
- Park JS, Park HC, Huh TL, Lee YH (1995) Production of poly-beta-hydroxybutyrate by *Alcaligenes eutrophus* transformants harboring cloned *phbCAB* genes. Biotechnol Lett 17(7):735–740
- Park JM, Jang YS, Kim TY, Lee SY (2010) Development of a gene knockout system for *Ralstonia eutropha* H16 based on the broad-host-range vector expressing a mobile group II intron. FEMS Microbiol Lett 309(2):193–200
- Peoples OP, Sinskey AJ (1989) Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16 — identification and characterization of the PHB polymerase gene (*PhbC*). J Biol Chem 264(26):15298–15303
- Peralta-Yahya PP, Keasling JD (2010) Advanced biofuel production in microbes. Biotechnol J 5(2):147
- Pharkya P, Burgard AP, Robin E, Burk MJ, Sun J (2011) Microorganisms and Methods for the co-production of isopropanol with primary alcohols, diols, and acids. US 2011/0201068 A1
- Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering C, Pötter M, Schwartz E, Strittmatter A, Voss I, Gottschalk G, Steinbüchel A, Friedrich B, Bowien B (2006) Genome sequence of the bioplastic-producing Knallgas bacterium *Ralstonia eutropha* H16. Nat Biotechnol 24(10):1257–1262
- Pötter M, Madkour MH, Mayer F, Steinbüchel A (2002) Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. Microbiology 148(Pt 8):2413–2426
- Reinecke F, Steinbüchel A (2009) *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. J Mol Microbiol Biotechnol 16(1–2):91–108
- Repaske R, Mayer F (1976) Dense autotrophic cultures of *Alcaligenes eutrophus*. Appl Environ Microbiol 32(4):592–597
- Schendel FJ, Baude EJ, Flickinger MC (1989) Determination of protein expression and plasmid copy number from cloned genes in *Escherichia coli* by flow injection analysis using an enzyme indicator vector. Biotechnol Bioeng 34(8):1023–1036
- Schlegel HG (1990) *Alcaligenes eutrophus* and its scientific and industrial career. NATO Adv Sci I E-App 186:133–141
- Schwartz E, Henne A, Cramm R, Eitinger T, Friedrich BR, Gottschalk G (2003) Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H₂-based lithoautotrophy and anaerobiosis. J Mol Biol 332(2):369
- Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC (2011) Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl Environ Microbiol 77(9):2905–2915
- Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nat Biotechnol 1(9):784
- Slater S, Houmiel KL, Tran M, Mitsky TA, Taylor NB, Padgett SR, Gruys KJ (1998) Multiple beta-ketothiolases mediate poly(beta-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J Bacteriol 180(8):1979–1987
- Steinbüchel A, Schlegel HG (1984) A multifunctional fermentative alcohol-dehydrogenase from the strict aerobe *Alcaligenes eutrophus* — purification and properties. Eur J Biochem 141(3):555–564
- Steinbüchel A, Fründ C, Jendrosseck D, Schlegel HG (1987) Isolation of mutants of *Alcaligenes eutrophus* unable to derepress the fermentative alcohol-dehydrogenase. Arch Microbiol 148(3):178–186
- Survase SA, Jurgens G, van Heiningen A, Granstrom T (2011) Continuous production of isopropanol and butanol using *Clostridium beijerinckii* DSM 6423. Appl Microbiol Biotechnol 91(5):1305–1313
- Tamakawa H, Mita T, Yokoyama A, Ikushima S, Yoshida S (2013) Metabolic engineering of *Candida utilis* for isopropanol production. Appl Microbiol Biotechnol 97(14):6231–6239

- Tanaka K, Ishizaki A, Kanamaru T, Kawano T (1995) Production of poly(D-3-hydroxybutyrate) from CO₂, H₂, and O₂ by high cell-density autotrophic cultivation of *Alcaligenes eutrophus*. Biotechnol Bioeng 45(3):268–275
- Vollbrecht D, Elnawawy MA, Schlegel HG (1978) Excretion of metabolites by hydrogen bacteria: 1. Autotrophic and heterotrophic fermentations. Eur J Appl Microbiol Biotechnol 6(2):145–155
- Wahl A, Schuth N, Pfeiffer D, Nussberger S, Jendrossek D (2012) PHB granules are attached to the nucleoid via PhaM in *Ralstonia eutropha*. BMC Microbiol 12(1):262
- Wilde E (1962) Untersuchungen über wachstum und speicherstoffsynthese von *Hydrogenomonas*. Arch Microbiol 43(2):109
- York GM, Stubbe J, Sinskey AJ (2002) The *Ralstonia eutropha* PhaR protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. J Bacteriol 184(1):59–66
- Yu K, Hu S, Huang J, Mei L-H (2011) A high-throughput colorimetric assay to measure the activity of glutamate decarboxylase. Enzym Microb Technol 49(3):272–276