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Isopropanol production with engineered *Cupriavidus necator* as bioproduction platform

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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 CO_2. 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 towards 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 $Cmole.Cmole^{-1}$) corresponded to more than 60% of the respective theoretical performance. Moreover, the overall isopropanol production yield (0.24 $Cmole.Cmole^{-1}$) 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.

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 towards 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 titre 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 titre 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 titre of 27 mg. L^{-1} in batch was achieved, although not directly from CO_2 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 CO_2 (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 H_2- and CO_2-based chemolithoautrophic 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 et al.), 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 80s (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 towards 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; 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 titre, yield and specific rate.

Material and Methods

Strain

Cupriavidus 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^r).

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, 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 $mg.L^{-1}$ 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 48h 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_{600nm} of 0.4 was centrifuged in a 15 mL falcon tube (1900xg, 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 Liter flask). 20 $g.L^{-1}$ of fructose and 0.38 g. L^{-1} of NH_4Cl were used as carbon and nitrogen sources respectively. The nitrogen amount corresponded to the amount necessary to produce about 0.7 $g.L^{-1}$ of biomass cell dry weight (CDW) considering the following biomass formula: $C_1H_{1.77}O_{0.44}N_{0.25}$, 4% ashes, MW=25.35 $g.Cmole^{-1}$ (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, Valencia, CA, USA). Restriction enzymes used were from New England Biolabs (Ipswich, MA, USA).

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 Inc., 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 (TTGACAATTAATCATCGGCTCGTATAATG) 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 except for pEG7c. For pEG7c, pBBad (Fukui *et al.* 2009) was digested with *Kpn*I and *Xba*I, and the fragments containing the isopropanol production pathway genes were purified from pEG7a via *Kpn*I and *Xba*I 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 *Xho*I and *Cla*I 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 (Invitrogen^{TM}, 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, 300x7.8 mm, Bio-Rad, Hercules, CA, USA) protected with a guard column (Cation H+ cartridge, 30x4.6 mm, Bio-Rad, Hercules, CA, USA) and coupled to a RI detector and an UV detector (λ=210 nm). The column was eluted with 2.5 mM H_2SO_4 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_{600nm}) using a visible spectrophotometer (Spectronic GENESYS 20 Visible Spectrophotometer) with a 1 cm path length absorption PS semi-micro cuvette (VWR, Radnor, PA, USA).

Enzymatic assay

Cell pellets after centrifugation of 5 mL culture broth (taken at 24 h of cultures) in a 15 mL Falcon tube (1900xg, 10 min, Centrifuge 5804R Eppendorf AG, Hamburg, Germany) 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 (16000xg, 10 min, Microcentrifuge 1816, VWR, Radnor, PA, USA). Cell lysates were filtrated using 0.45 µm syringe filters (PES, VWR, Radnor, PA, USA) 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, Hercules, CA, USA) 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, Santa Clara, CA, USA). 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 TrisHCl (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 CO_2. 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, $Y_{S,Isoprop}^{theo} = 0.5\,Cmole.Cmole^{-1}$. The experimental isopropanol yield ($Y_{S,Isoprop}$) was the ratio of isopropanol produced and substrate consumed during a time interval (t_2-t_1):

$$Y_{S,Isoprop} = \left| \frac{Isoprop_{t2} - Isoprop_{t1}}{S_{t2} - S_{t1}} \right|.$$

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 towards pyruvic acid (up to 2.61 ± 0.19 $g.L^{-1}$, Fig. 3.a 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), respectively coding for a β-ketothiolase A (THL) and the two subunits of a CoA-Transferase (CTF). Additionnaly, the plasmid also contained the heterologous genes from *Clostridium* species *adc* (CA_P0165) and *adh* (AF157307 nt 2351 to 3406), respectively coding for an acetoacetate decarboxylase (ADC) and an alcohol dehydrogenase (ADH) (Fig. 1, Fig. 2). The resulting strain Re2133/pEG2 produced 0.22 ± 0.07 $g.L^{-1}$ of isopropanol (Fig. 3.a). The titre reached was very low and up to 2.44 ± 0.14 $g.L^{-1}$ 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 towards 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, respectively at 0.75 ± 0.84 $U.mg^{-1}$ and 0.05 ± 0.01 $U.mg^{-1}$, while the specific activities of the native *C. necator* enzymes THL and CTF were 5.47 ± 0.62 $U.mg^{-1}$ and 9.89 ± 1.28 $U.mg^{-1}$ 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*, *C. acetobutylicum* and *C. 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, for example, 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 $U.mg^{-1}$ and 0.72 ± 0.04 $U.mg^{-1}$ (Table 1) were respectively determined for ADC and ADH. As a result, the strain bearing pEG7a produced 1.95 ± 0.18 $g.L^{-1}$ of isopropanol in 88 h (Fig. 3.a), 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 $Cmole.L^{-1}$, Fig. 4) to products (biomass, pyruvic acid, isopropanol and acetone) in Re2133/pEG7a compared to Re2133/pEG2 (0.10 $Cmole.L^{-1}$, 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 88h of the culture time,the peak isopropanol production time, a pyruvic acid concentration of 1.52 ± 0.29 $g.L^{-1}$ and an acetone concentration of 0.09 ± 0.09 $g.L^{-1}$ were detected for the strain Re2133/pEG7b suggesting that further redirection from pyruvic acid towards the production of isopropanol can be achieved.

Suitability of alcohol dehydrogenase (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 towards 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, Steinbuchel et al. 1988) and by comparison to the whole genome sequence (Pohlmann, Fricke et al. 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. 3.a) compared to strain Re2133/pEG7a *(*1.95 ± 0.18 $g.L^{-1}$ isopropanol). Large amounts of acetone (~1.31 $g.L^{-1}$) and pyruvic acid (~2.00 $g.L^{-1}$) 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 towards acetone as a substrate, 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) (Fig. 2 and Fig. 3.a).

The activity of β-ketothiolase , encoded by *phaA*, in cell extracts increased with the increase in the plasmid gene copy number (Fig. 5.a). β-ketothiolase activity of 3.2 ± 2.2 $U.mg^{-1}$ 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. 3.a). 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. 3.a). 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. 3.a). 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. 5.b). 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 ADH was demonstrated (Fig. 5.b). 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}, P_{phaP}), P_{TAC} promoter was the strongest and demonstrated 1.5-2.0 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 (TTTACACTTTATGCTTCCGGCTCGTATGTTG) 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 ± 0.07 $g.L^{-1}$ isopropanol (Fig. 3.b), 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 titre, 0.19 $Cmole.L^{-1}$ were produced by the strain Re2133/pEG7b compared to the 0.17 $Cmole.L^{-1}$ 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 g per g of biomass in the strain Re2133/pEG7a and 1.14 $\pm$ 0.01 $g.g^{-1}$ 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 OD_{600nm} of 1.5, corresponding to 75% of the maximum OD_{600nm} reached with the amount of nitrogen supplied. The maximal growth rate was 0.17 ± 0.01 h^{-1}, which was the same as the strain with the empty plasmid pBBR1MCS-2. Isopropanol production by Re2133/pEG7c reached 3.44 ± 0.14 $g.L^{-1}$ (Fig. 3b and Fig. 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 (Fig. 3b and Fig. 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 $\pm$ 0.18 $g.L^{-1}$) 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. 3.a), an exploration of other potentially suitable ADHs from the *C. necator* genome could be fruitful. No native ADC encoding gene or ADC activity were 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. 3.b). 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 h^{-1} 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 ± 0.14 $g.L^{-1}$ of isopropanol (Fig. 3.b and Fig. 6) and no more pyruvic acid after L-arabinose induction(Fig. 4, Fig. 3.b and Fig. 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 titre (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 ± 0.14 $g.L^{-1}$ of isopropanol. In batch mode, natural producers from the *Clostridium* family such as *C. isopropylicum* were able to produce titres up to 4.6 $g.L^{-1}$ with immobilized cells (Matsumura *et al.* 1992). Higher titre were reached with engineered strains: 4.9 $g.L^{-1}$ by *E. coli* (Hanai *et al.* 2007), 8.8 $g.L^{-1}$ by *Clostridium 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 titre of 3.44 ± 0.14 $g.L^{-1}$ 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 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 ± 0.006 $g.g^{-1}.h^{-1}$ (0.016 ± 0.001 $g.L^{-1}$. $OD_{600nm}{}^{-1}.h^{-1}$, Table 2). The instantaneous maximal specific productivity of Re2133/pEG7c after induction by arabinose was 0.093 $g.g^{-1}.h^{-1}$ which corresponded to 62% of the maximum theoretical isopropanol production specific rate (Table 2). The maximum theoretical specific rate was calculated 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-producting *E. coli* strain (Hanai *et al.* 2007) and the engineered isopropanol-producting *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 $Cmole.Cmole^{-1}$).

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 ± 0.14 $g.L^{-1}$) considering the low concentration of *C. necator* biomass used as catalyst (0.82 ± 0.02 $g.L^{-1}$).

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.

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Tables

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

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

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		*E. coli*	*C. acetobutylicum*			*Candida utilis*	*C. necator*	*C. necator*
Isopropanol	$g.L^{-1}$	4.9	8.8	5	3.5	9.5	3.44 ± 0.14	nc
Biomass	OD_{600nm}	20	nr	nr	20	nr	2.25 ± 0.05	nc
Biomass	$g.L^{-1}$	nr	nr	16	nr	nr	0.82[a] ± 0.01	nc
Time	h	30.5	45	20	60	52	96.3	nc
Overall specific productivity	$g.L^{-1}.OD_{600nm}^{-1}.h^{-1}$	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

[a] the CDW was calculated using the relationship: 1 OD_{600nm} =0.363 $g.L^{-1}$

[b] Calculated with kinetic modeling from Grousseau *et al.* 2013 considering a null growth rate

[c] See Material and methods, 2.6. Yield calculation

nr: not reported, nc: not calculated

Figure Captions

Fig. 1 Engineered isopropanol production pathway in *C. necator.* Acetate, Fructose, Glucose and CO_2 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. CO_2 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) respectively 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)

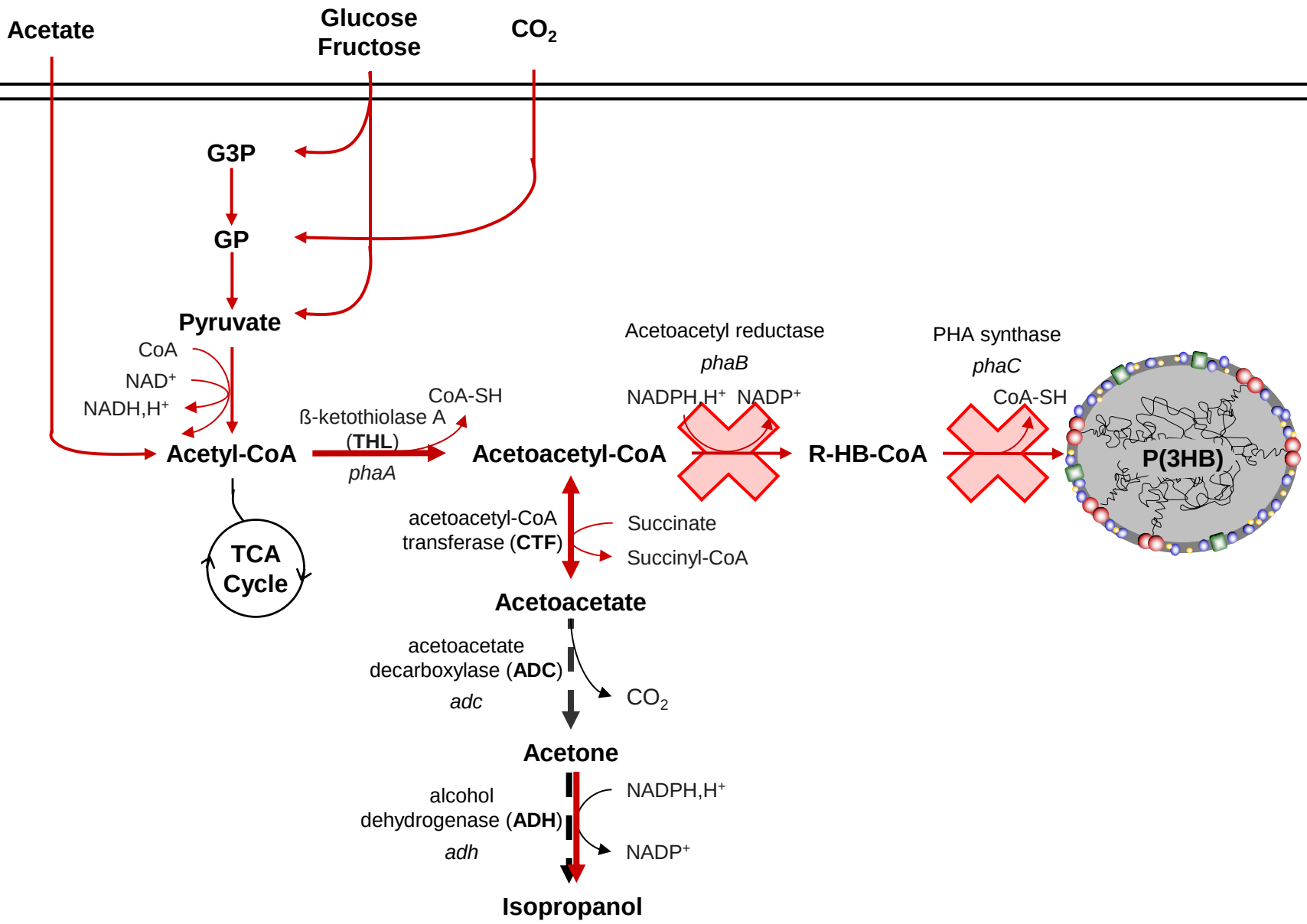
Fig. 2 Schematic of isopropanol production pathways constructed and plasmid utilized. Each plasmid was incorporated into strain Re2133 (H16 Δ*phaB1B2B3C1* (Gen^r), Budde *et al.* 2011)

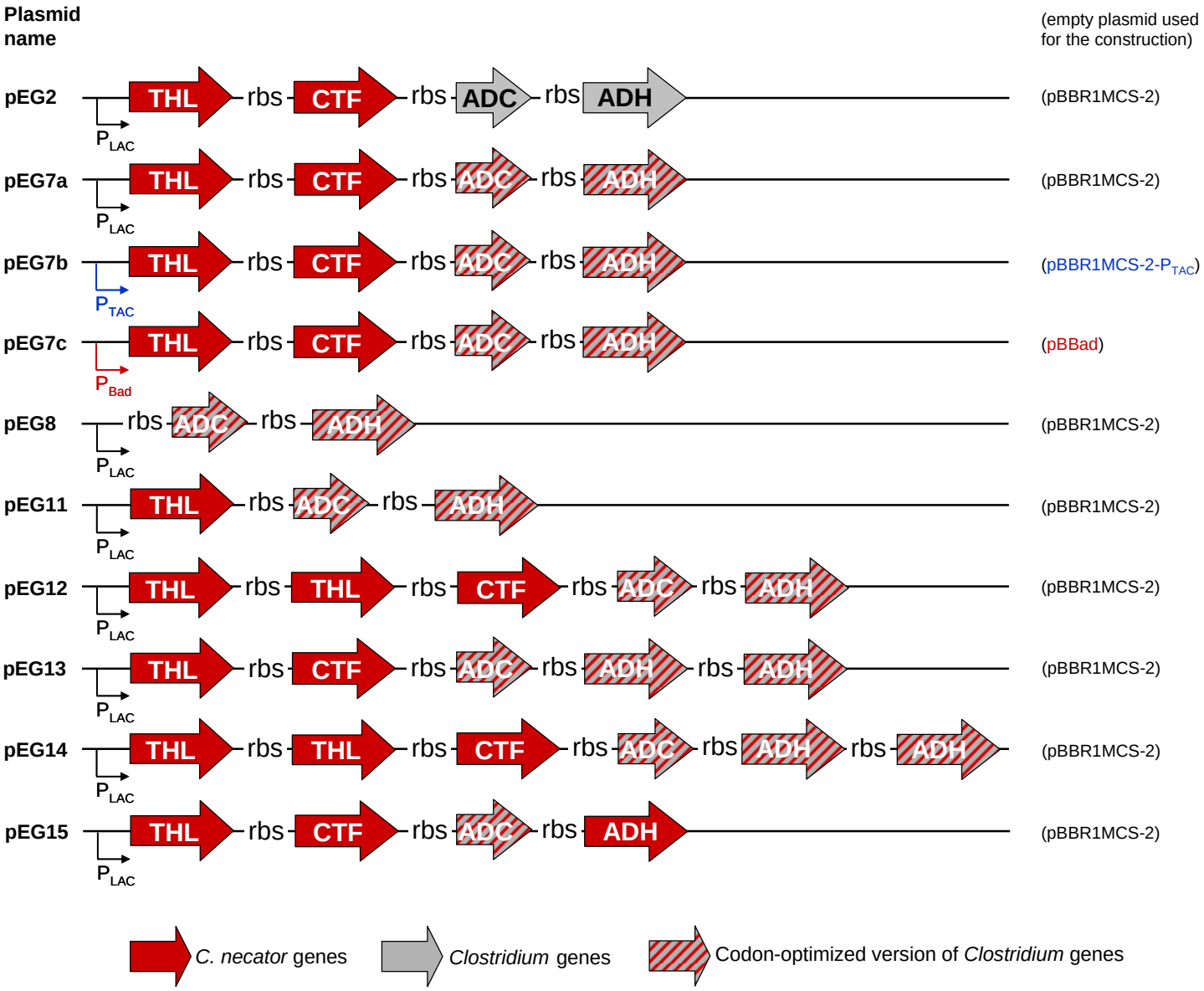
Fig. 3 Time point in which maximum isopropanol concentration was produced; in addition pyruvic acid and acetone concentrations produced at the same time point are depicted. (a) Coding sequence evaluation with the plasmid pBBR1MCS-2 incorporated in the strain Re2133. Composition of the plasmid is indicated 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

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

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 get for Re2133/pEG2 (n=3), Re2133/pEG7a (n=3), Re2133/pEG11 (n=3), Re2133/pEG13 (n=3). For 2 copies, average of values get for 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

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


Acetate
Glucose
Fructose
CO_2
G3P
GP
Pyruvate
CoA
NAD^+
$NADH,H^+$
Acetyl-CoA
TCA Cycle
CoA-SH
ß-ketothiolase A (THL)
phaA
Acetoacetyl-CoA
Acetoacetyl reductase
phaB
$NADPH,H^+$ $NADP^+$
R-HB-CoA
PHA synthase
phaC
CoA-SH
P(3HB)
acetoacetyl-CoA transferase (CTF)
Succinate
Succinyl-CoA
Acetoacetate
acetoacetate decarboxylase (ADC)
adc
CO_2
Acetone
alcohol dehydrogenase (ADH)
adh
$NADPH,H^+$
$NADP^+$
Isopropanol



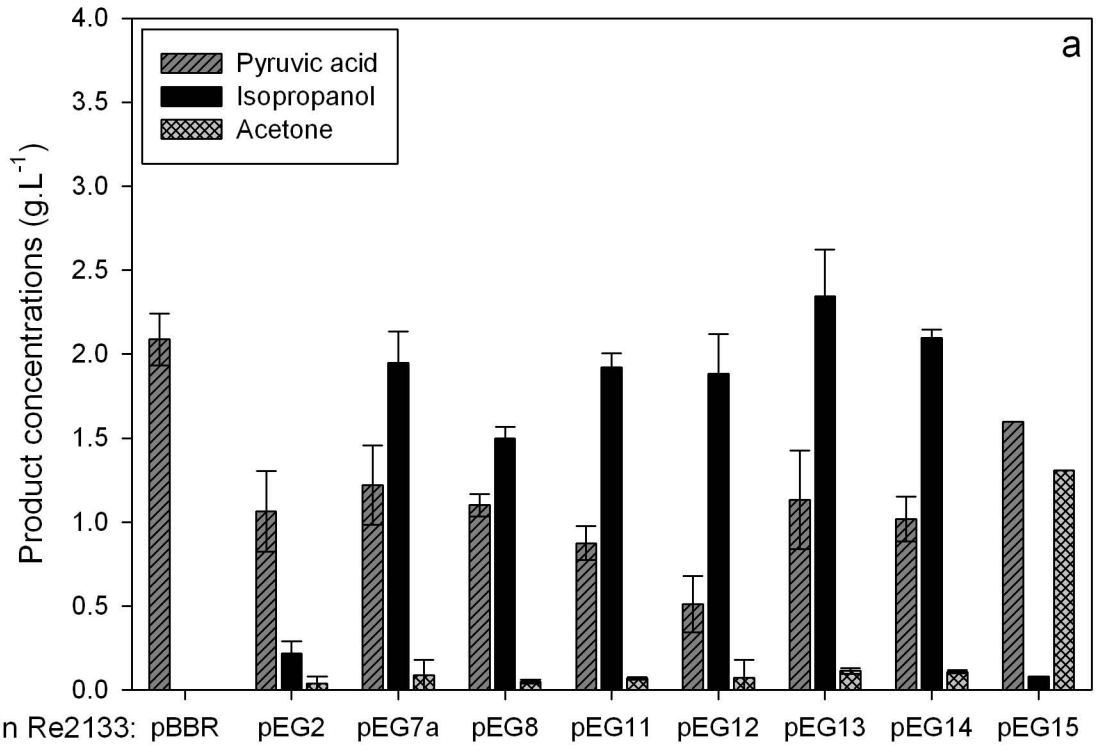
Abbr.	Name	Locus tag	Microorganism	Enzyme
THL:	*phaA*	(H16_A1438)	from *C. necator,*	coding for a β-ketothiolase
CTF:	*ctfAB*	(H16_A1331 and H16_A1332)	from *C. necator,*	coding for a succinyl-CoA transferase
ADC:	*adc*	(CA_P0165)	from *C. acetobutylicum*, coding for an acetoacetate decarboxylase	
ADH:	*adh*	(AF157307 nt 2351 to 3406)	from *C. beijerinckii*,	coding for an alcohol dehydrogenase
ADH:	*adh*	(H16_A0757)	from *C. necator*,	coding for an alcohol dehydrogenase

rbs: Ribosome Binding Site and nucleotide linker sequence: AAAGGAGGACAACC (Lu *et al.* 2012a)

pBBR1MCS-2: Broad-Host-Range cloning vector (Kan^r), P_{Lac} (Kovach *et al.* 1995)

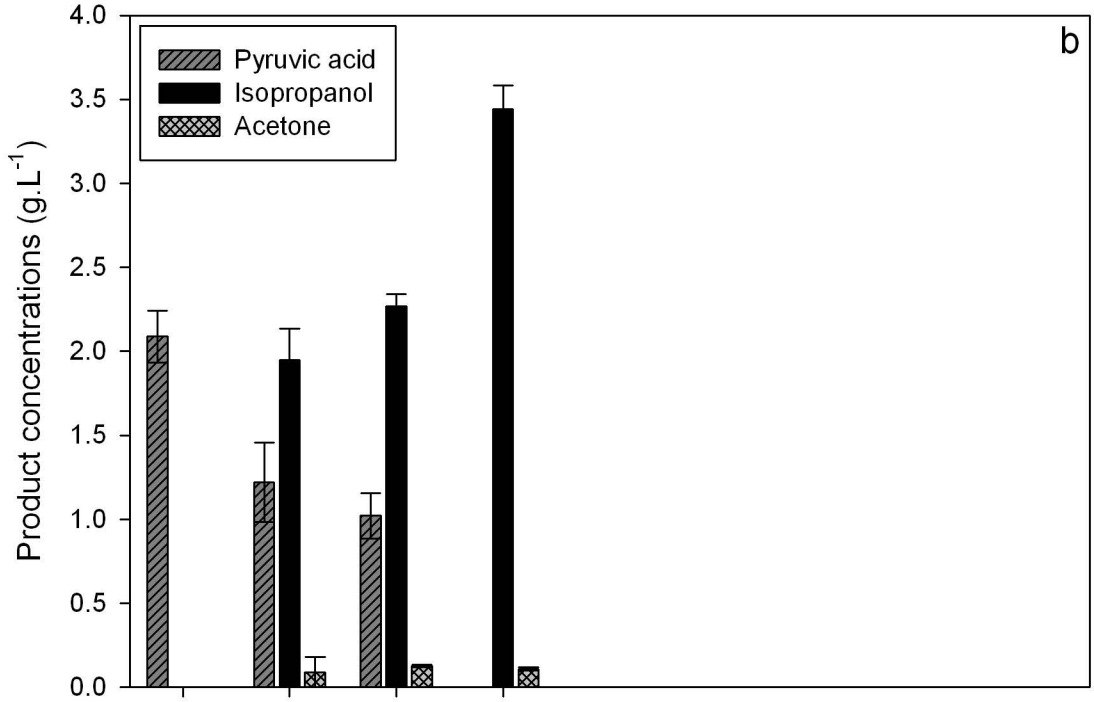
pBBR1MCS-2-P_{Tac}: pBBR1MCS-2 with P_{Tac} promoter instead of P_{Lac} (Kan^r)

pBBad: pBBR1MCS-2 derivative with L-Arabinose inducible system P_{Bad} (Fukui *et al.* 2010)



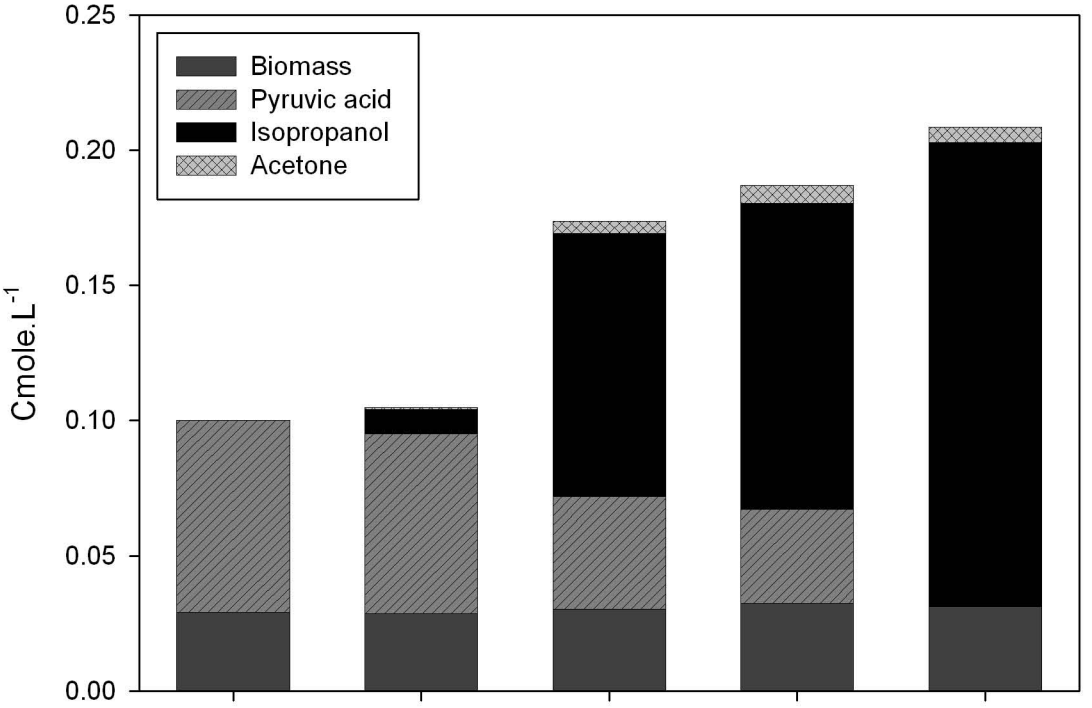
plasmid incorporated in Re2133:			pBBR	pEG2	pEG7a	pEG8	pEG11	pEG12	pEG13	pEG14	pEG15
gene copy numbers on the plasmid	THL	*phaA* (H16_A1438)	0	**1**	**1**	0	**1**	**2**	**1**	**2**	**1**
	CTF	*ctfAB* (H16_A1331 H16_A1332)	0	**1**	**1**	0	0	**1**	**1**	**1**	**1**
	ADC	*adc* (CA_P0165)	0	**1**	**1***	**1***	**1***	**1***	**1***	**1***	**1***
	ADH	*adh* (AF157307 nt 2351 - 3406)	0	**1**	**1***	**1***	**1***	**1***	**2***	**2***	0
	ADH	*adh* (H16_A0757)	0	0	0	0	0	0	0	0	**1**

* are codon-optimized version of *Clostridium* genes

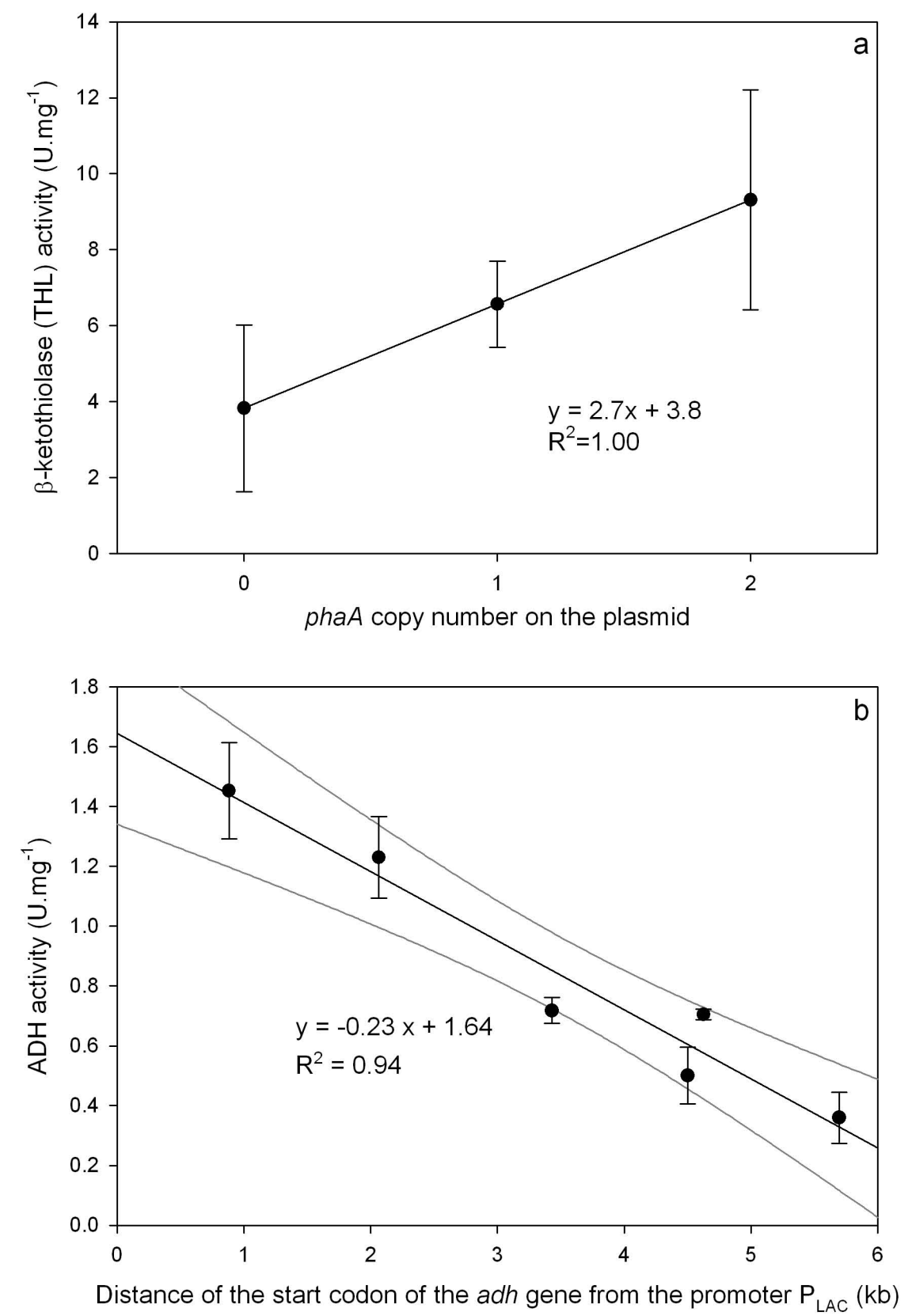


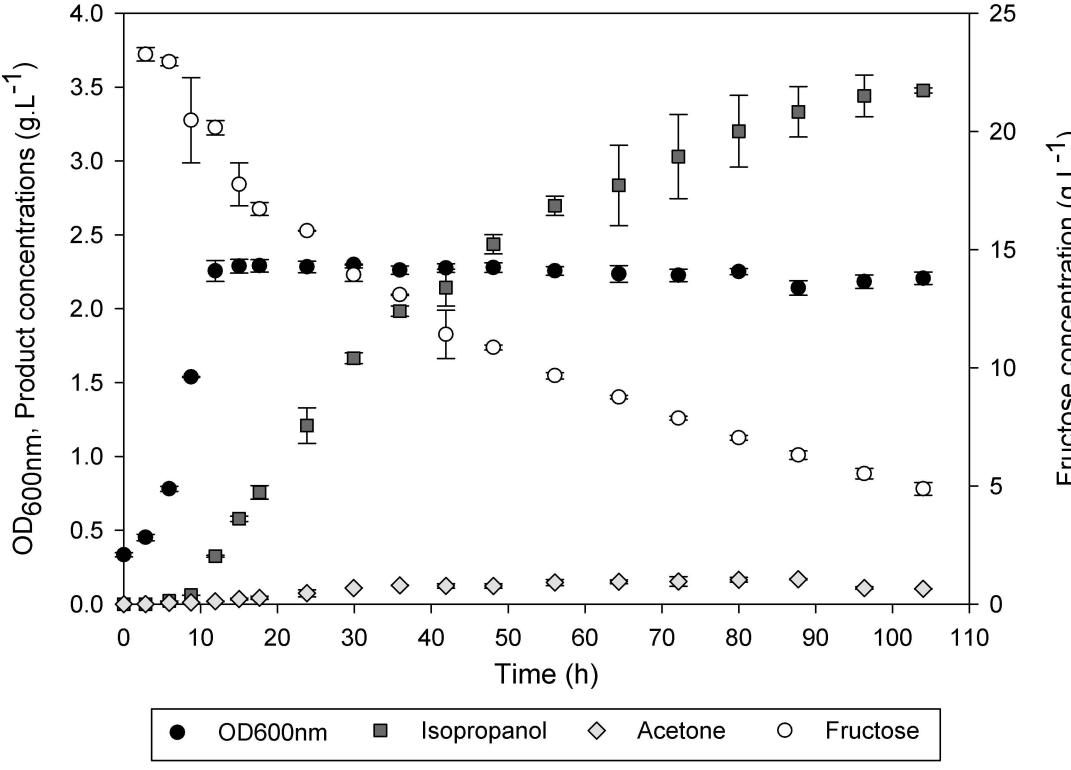
plasmid incorporated in Re2133:	pBBR	pEG7a	pEG7b	pEG7c	
Promoter	P_{LAC}	P_{LAC}	P_{TAC}	P_{Bad}	
Inducible	×	×	×	✓	
Isopropanol pathway	×	✓	✓	✓	(*phaA*, *ctfAB*, *adc**, *adh**)

* are codon-optimized version of *Clostridium* genes



Plasmid inserted in Re2133:	pBBR1MCS-2	pEG2	pEG7a	pEG7b	pEG7c
Promoter	P_{LAC}	P_{LAC}	P_{LAC}	P_{TAC}	P_{Bad}
Inducible	×	×	×	×	✓
Isopropanol operon	×	✓	✓	✓	✓
Codon optimization	na	×	✓	✓	✓
Time	24 h	30 h	88 h	88 h	96 h


a
β-ketothiolase (THL) activity (U.mg-1)
14
12
10
8
6
4
2
0
0
1
2
phaA copy number on the plasmid
y = 2.7x + 3.8
R2=1.00
b
ADH activity (U.mg-1)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Distance of the start codon of the adh gene from the promoter PLAC (kb)
y = -0.23 x + 1.64
R2 = 0.94


OD600nm, Product concentrations (g.L-1)
Fructose concentration (g.L-1)
Time (h)
OD600nm
Isopropanol
Acetone
Fructose