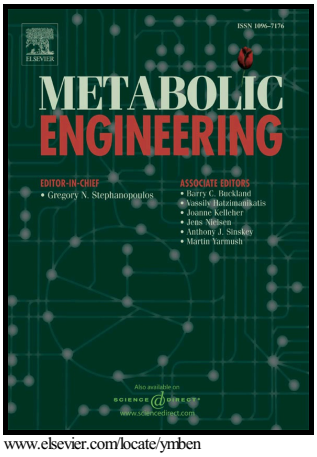

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PII: S1096-7176(16)30277-4
DOI: http://dx.doi.org/10.1016/j.ymben.2017.05.007
Reference: YMBEN1253

To appear in: *Metabolic Engineering*

Received date: 23 December 2016
Revised date: 30 March 2017
Accepted date: 31 May 2017

Cite this article as: Jillian Marc, Estelle Grousseau, Eric Lombard, Anthony J Sinskey, Nathalie Gorret and Stéphane E. Guillouet, Over expression of GroESI in *Cupriavidus necator* for heterotrophic and autotrophic isopropanol productior *Metabolic Engineering,* http://dx.doi.org/10.1016/j.ymben.2017.05.007

Over expression of GroESL in *Cupriavidus necator* for heterotrophic and autotrophic isopropanol production

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ABSTRACT

We previously reported a metabolic engineering strategy to develop an isopropanol producing strain of *Cupriavidus necator* leading to production of 3.4 $g.L^{-1}$ isopropanol. In order to reach higher titers, isopropanol toxicity to the cells has to be considered. A toxic effect of isopropanol on the growth of *C. necator* has been indeed observed above a critical value of 15 $g.L^{-1}$. GroESL chaperones were first searched and identified in the genome of *C. necator*. Native *groEL* and *groES* genes from *C. necator* were over-expressed in a strain deleted for PHA synthesis. We demonstrated that over-expressing *groESL* genes led to a better tolerance of the strain towards exogenous isopropanol. *GroESL* genes were then over-expressed within the best engineered isopropanol producing strain. A final isopropanol concentration of 9.8

$g.L^{-1}$ was achieved in fed-batch culture on fructose as the sole carbon source (equivalent to 16 $g.L^{-1}$ after taking into account evaporation). Cell viability was slightly improved by the chaperone over-expression, particularly at the end of the fermentation when the isopropanol concentration was the highest. Moreover, the strain over-expressing the chaperones showed higher enzyme activity levels of the 2 heterologous enzymes (acetoacetate carboxylase and alcohol dehydrogenase) of the isopropanol synthetic operon, translating to a higher specific production rate of isopropanol at the expense of the specific production rate of acetone. Over-expressing the native chaperones led to a 9-18% increase in the isopropanol yield on fructose.

Finally, isopropanol production was demonstrated up to 250 $mg.L^{-1}$ in only 12 hours under autotrophic conditions from CO_2 as the sole carbon source, following a growth phase on fructose.

KEYWORDS: *Ralstonia eutropha*; *Cupriavidus necator*; Isopropanol; carbon dioxide; hydrogen; metabolic engineering; GroEL/S; chaperones; gas fermentation; autotrophy

1 INTRODUCTION

Increasing awareness of global warming caused by carbon dioxide (CO_2) emission has stimulated interest in microbial production of biofuel and chemical synthons from renewable carbon sources. Progress in metabolic engineering and synthetic biology enables chemical production in various microorganisms. Key features for engineering microorganism for industrial metabolite production gathered together (i) robustness towards industrial process conditions (ii) ability to grow with minimal nutrient supplement for cost effectiveness issues (iii) ability to grow on cheap substrates (iv) capability to express heterologous genes and (v) robustness towards the targeted product. To develop the future bioprocesses for the bio-based chemical synthesis using the cheap CO_2 sustainable carbon source, the bacterium *Cupriavidus necator* appears to be as a good candidate meeting most of those requirements. This facultative chemolithoautotrophic bacterium, also known as *Ralstonia eutropha*, is a metabolically versatile organism, since it is metabolically capable of utilizing many simple and complex carbon sources under simple nutrient requirement (no vitamin requirement for instance). These renewable carbon sources can be derived from agro-industrial waste streams, for example: oils (Budde et al., 2011; Lee et al., 2008), fatty acids (Friedrich et al., 1979; Johnson and Stanier, 1971; Wilde, 1962), organic acids (Doi et al. 1989) and CO_2 (Repaske and Mayer, 1976; Tanaka et al., 1995; Wilde, 1962). Particularly, *C. necator* has the ability to grow at high growth rates (in the range of 0.3 h^{-1}) to high cell densities on gas mixture $CO_2/H_2/O_2$ (Tanaka et al., 1995). This is very appealing in the current context where non-photosynthetic CO_2 fixation routes are gaining more and more interest and could be more effective than photosynthetic routes (Hawkins et al., 2013). In addition to H_2- and CO_2-based

chemolithoautrophic metabolism, *C. necator* is a model for the study of polyhydroxyalkanoates (PHA) biopolymers (Schlegel 1990; Reinecke and Steinbüchel, 2009 ; Budde et al., 2010). *C. necator* is able to divert a significant amount of its carbon flow into poly-3-hydroxybutyrate (P(3HB)) under nutrient limitation conditions (oxygen, nitrogen, phosphorus) in excess of carbon (Koller et al., 2010). A production of 91 $g.l^{-1}$ of *C. necator* cells containing 67.7% P(3HB) in 40 hours was reported under autotrophic and O_2-limited conditions in high gas–liquid-mass-transfer bioreactor (Tanaka et al., 1995). This natural ability to store excess carbon in the form of P(3HB) is of huge interest as numerous interesting chemicals share the same production pathway precursor, acetyl-coA (Figure 1), which implies that fewer genetic modifications would be required to divert P(3HB) precursors into the final product. Moreover, basic genetic tools are available to manipulate *C. necator* since the end of the 80s (Peoples and Sinskey, 1989) (Jendrossek *et al.* 1988; Park *et al.* 1995) and its genome has been fully sequenced (Pohlmann et al., 2006; Schwartz et al., 2003) allowing metabolic engineering applications to be developed in this very versatile organism.

Recently with the advances in the development of genetic engineering tools in *C. necator*, the range of metabolites that it can produce has enlarged. This bacterium was successfully engineered for the production of relatively low amounts of isopropanol (3.44 $g.l^{-1}$; Grousseau *et al.*, 2014), hydrocarbons (670 mgl^{-1}, Crepin *et al.*, 2016) isobutanol (250 $mg.l^{-1}$ Lu *et al.*, 2012), methyl ketones (65 $mg.l^{-1}$; Müller *et al.*, 2013) , free fatty acids (62 $mg.l^{-1}$; Chen et al., 2015) and alka(e)nes (5.7 $mg.l^{-1}$; Bi *et al.*, 2013) under heterotrophic conditions from fructose as carbon source (Figure 1). Some of those engineered strains showed also their ability to produce chemicals under autotrophic conditions from $CO_2/H_2/O_2$ mixture. Up to 180 $mg.l^{-1}$ of methyl ketones (Müller et al., 2013) and 4.4 mgl^{-1} alka(e)nes (Crepin et al., 2016) were obtained under autotrophic conditions. One of the strains developed by Grousseau and

colleagues (2014) produced up to 216 mg.l^{-1} isopropanol from CO_2 in a hybrid microbial–water-splitting catalyst system (Torella et al., 2015). An electromicrobial conversion of CO_2 up to 100 mg.l^{-1} isobutanol was demonstrated by Li et al. (2012).

One of the main issues that has to be considered when producing non-natural compounds by engineered strains is the potential product toxicity. There are several specific mechanisms involved in the tolerance improvement such as expression of efflux pumps, heat shock proteins, membrane modifying proteins, and activation of general stress response genes. Some of them were already reported in the literature (Dunlop 2011 ; Mukhopadhyay 2015). Among those, expressing the GroESL chaperones (heat shock protein family) proved to be useful to decrease solvent toxicity. Overexpression of such proteins in *Clostridium acetobutylicum* reduced the growth inhibition by butanol and acetone (Mann et al., 2012; Tomas et al., 2003) and increased these solvent titer by 30 and 38%, respectively (Tomas et al., 2003). Overexpression of GroESL in *Lactobacillus paracasei* and *Lactococcus lactis* led to an improvement of butanol tolerance during the growth of the two non-producing strains (Desmond et al., 2004). GroESL overexpression in *Escherichia coli* enhanced the cell growth in the presence of various alcohols (ethanol, n-, i- butanol and 1,2,4 butanetriol) (Zingaro and Papoutsakis, 2013).

We previously engineered a *C. necator* strain for the production of isopropanol that was able to produce up to 3.44 g l^{-1} isopropanol from fructose as sole carbon source with a high yield (60% of the maximal theoretical yield). In this paper, we further engineered this strain to improve the isopropanol production by tackling alcohol toxicity through the overexpression of native GroESL chaperones and evaluated the strains under heterotrophic and autotrophic conditions.

Note for editor: Figure 1 is a single-column fitting image

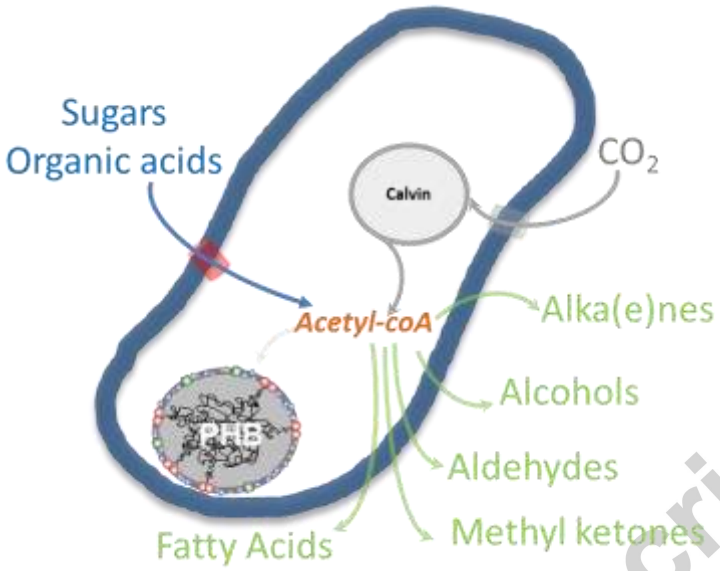


Figure 1: Reported metabolic routes for the production of interest molecules through the engineering of the natural metabolism of a *Cupriavidus necator* strain deleted for the PHB synthesis pathway.

2 MATERIAL AND METHODS

2.1 Strains and medium

Strains and Plasmids

Relevant characteristics of plasmids and strains are listed in Table 1. *C. necator* Re2133 (Budde *et al.* 2011; Table 1) was used as the expression strain. The construction of strains Re2133/pBBR1MCS-2 and Re2133/pEG7c was detailed in (Grousseau et al., 2014). The construction of the strain Re2133/pEG23 is described below.

The plasmid assemblies were achieved by one-step isothermal DNA assembly protocol (Gibson et al., 2009). The primers used to amplify each DNA fragment are listed in supplementary material (Supplementary Table 1). DNA sequence amplification was achieved using Phusion High-Fidelity PCR Master Mix with GC Buffer from NEB (New England Biolabs, Ipswich, Massachusetts, USA). QIAQuickGel Extraction Kit (QIAGEN, Valencia, California, USA) was used for gel purification of all DNA PCR products.

For the construction of pEG23, the isopropanol operon was amplified from the plasmid pEG7c and the plasmid backbone from pEG19. Then the one-step isothermal DNA assembly was performed with the two DNA fragments. Plasmid maps are available in Supplementary Figure 1.

For the construction of the plasmid pEG19, the constitutive P_{Lac} promoter was amplified from pBBR1MCS-2 (Kovach et al., 1995) and groESL (H16_A0705 and H16_A0706) was amplified from *C. necator* H16. A fusion PCR was done to assembly the P_{Lac} promoter and groESL. The plasmid backbone was amplified from pBBad (Fukui et al., 2011). Then the one-step isothermal DNA assembly was performed with the two DNA fragments.

The one-step isothermal assembly products were transformed into high efficiency NEB 10-beta competent *E. coli* cells. Colony PCR with OneTaq Hot Start 2X Master Mix with GC Buffer from NEB was used to screen colonies for correct gene insertion. The correct gene insertions on plasmids were confirmed by sequencing, after plasmid extraction using the QIAprep Spin Miniprep Kit (QIAGEN).

The plasmid pEG23 was inserted into Re2133 by electroporation following the protocol below adapted from (Taghavi et al., 1994).

To obtain electrocompetent cells of *C. necator*, overnight cultures of strain Re2133 were grown in TSB medium (3 mL in 10 mL tubes) at 30°C with vigorous shaking. Then 1 mL of the culture were transferred in flask of 250 mL with 25 mL of TSB medium and grown at 30°C with shaking at 200 RPM, until OD_{600nm} reached 0.5-0.6. The cells were harvested and washed twice with 25 mL of ice-cold washing buffer (10% glycerol, 90% water [$^{vol}/_{vol}$]). After the washing steps, the cells were concentrated in washing buffer solution to a final OD_{600nm} of 50 and aliquoted by 150 µL of electrocompetent cells. 2–5 µL containing 100-150 $ng.\mu L^{-1}$ of plasmid DNA were added to the cell mixture. To mix cells and DNA, the tubes were carefully flicked 4–5 times. The mixture was placed on ice for 5 minutes and then transferred to chilled electroporation cuvette. Electroporation was performed with the following settings: voltage, 2.5 kV; capacity, 25 µF, external resistance 200 Ω. Immediately after electroporation, the cells were mixed with 1 mL of modified SOC medium (SOC + fructose, store at 4°C for Re2133/pEG23) and left for 2 h at 30°C before being plated on warm selection plates (TSB medium with addition of 10 $mg.L^{-1}$ gentamycin and 200 $mg.L^{-1}$ kanamycin).

Note for editor: Table 1 is a 2-column fitting table

Table 1: Strains and plasmids used in this work.

Strains	**Relevant characteristics**	**References**
C. necator H16	Wild type, gentamicin resistant (Gen^r)	ATCC17699
C. necator Re2133	H16 Δ*phaB1B2B3C1* (Gen^r)	(Budde et al., 2010)
Plasmids	**Relevant characteristics**	**References**
pBBR1MCS-2	Broad-Host-Range cloning vector (Kan^r), P_{Lac}	(Kovach *et al.*, 1995)
pBBad	pBBR1MCS-2 derivative with L-Arabinose inducible system P_{Bad}	(Fukui et al., 2009)
pEG7c	pBBad with *phaA*-RBS-*ctfAB*-RBS-*adc**-RBS-*adh** sequence inserted into the multiple cloning site (MCS) (Kan^r), P_{Bad}	(Grousseau et al., 2014)
pEG19	pBBad with P_{LAC}-RBS-g*roESL* inserted after the MCS (Kan^r), P_{Bad}	This work
pEG23	pBBad with with *phaA*-RBS-*ctfAB*-RBS-*adc**-RBS-*adh** sequence inserted into MCS and P_{LAC}-RBS-g*roESL* inserted after the MCS (Kan^r), P_{Bad}	This work

phaA (H16_A1438) from *C. necator,* coding for a β-ketothiolase A (THL)

ctfAB (H16_A1331 and H16_A1332) from *C. necator,* coding for the two subunits of a CoA-Transferase (CTF)

*adc** - RBS – *adh** (NCBI accession number KF975390.1) are the codon-optimized version of the *adc* (CA_P0165) and *adh* (AF157307 nt 2351 to 3406) heterologous genes of Clostridium species, respectively coding for an acetoacetate decarboxylase and an alcohol dehydrogenase

groESL (H16_A0705 and H16_A0706) from *C. necator,* coding for the co-chaperonin GroES and the chaperonin GroEL

RBS : RBS and nucleotide linker sequence: AAAGGAGGACAACC (Lu et al., 2012)

Media

For strains constructions, cells were cultivated in TSB medium, consisted of 27.5 g.L^{-1} dextrose-free tryptic soy broth (TSB, Becton Dickinson, Sparks, Maryland, USA). The cells were stored at -80°C in 1.5 mL aliquots, from a one-step cell amplification on TSB medium with addition of 10 mg.L^{-1} gentamycin and 200 mg.L^{-1} kanamycin with 20% glycerol. Minimal medium (A) was previously described by Lu et al., (2013). It was used for the seed cultures with addition of 10 mg.L^{-1} gentamycin and 200 mg.L^{-1} kanamycin, and for the

microplate cultures with addition of 100 mg.L^{-1} kanamycin. Minimal medium (B) used for the bioreactors cultivations on fructose contained per litre: $(NH_4)_2SO_4$, 2,39 g; $MgSO_4,7H_2O$, 0.75 g; phosphorus ($Na_2HPO_4,12H_2O$, 1.5 g; KH_2PO_4, 0.25 g); nitrilotriacetic acid, 0.285 g; ammonium iron(III) citrate (28%), 0.9 g; $CaCl_2$, 0.015 g; trace elements (H_3BO_3, 0.45 mg; $CoCl_2,6H_2O$, 0.3 mg; $ZnSO_4,7H_2O$, 0.15 mg; $MnCl_2,4H_2O$, 0.045 mg; $Na_2MoO_4,2H_2O$, 0.045 mg; $NiCl_2,6H_2O$, 0.03 mg; $CuSO_4$, 0.015 mg); kanamycin, 0.1 g. Minimal medium (C) used for the bioreactors cultivations on gaseous substrates was equal to medium B, without $(NH_4)_2SO_4$.

2.2 Seed cultivations

After thawing at room temperature, one glycerol stock was streaked on a medium (A)-Petri dish (with the addition of agar at 15 g.L^{-1}). The plate was incubated for 36 h at 30°C. One isolated colony was used to inoculate the first seed culture grown for 24 h with 10 mL of TSB supplemented with 10 mg.L^{-1} gentamycin and 200 mg.L^{-1} kanamycin, in a 100 mL baffled Erlenmeyer flask. Two more steps of propagation were carried out for 12 h each one (30 mL and 300 mL of medium A, respectively in 250 mL and 2 L baffled Erlenmeyer flasks). Each seed culture was grown at 30°C, 100 RPM and was used to inoculate the next-step and experiments at 10% ($^{vol}/_{vol}$).

2.3 Microplate cultivations on fructose

Microplate cultivations were carried out in batch mode in 3527 Costar 24 wells plates (Corning, New-York, USA), filled with 1 mL of medium (A) and inoculated from the TSB first step of seed cultures. Isopropanol was initially added to the medium in a range from 0 to 24 g.L^{-1}. Cultures were grown at 30°C under continuous agitation in a Synergy HT microplate

reader (Biotek, Winooski, Vermont, USA), under a glass cover to avoid isopropanol or acetone evaporation and OD_{600nm} was measured every 10 minutes. Each condition was achieved in triplicate.

2.4 Fed-batch cultivations on fructose

Fed-batch cultures on fructose were carried out in two phases. The first phase consisted of a non-limited growth until depletion of initial nitrogen, to reach 4 $g.L^{-1}$ of biomass. The second phase consisted of a N-limited growth coupled with isopropanol production. In this second phase, nitrogen was fed exponentially from a 56 $g.L^{-1}$ NH_3 solution. This second phase was initiated by a pulse of L-arabinose (1 $g.L^{-1}$) to induce the expression of the isopropanol operon.

Theses cultures were performed in 5 L bioreactors BDCU B.Braun with a working volume of 3 L, managed with the MFCS/win 2.0 software. The temperature was regulated at 30°C and pH at 7.0 by the addition of a 2.5 M KOH solution. Air flow and stirring rate were adjusted to maintain fully aerobic condition, *i.e.* a dissolved oxygen concentration above 20% of saturation, with priority to stirring to restrain isopropanol and acetone evaporation. Initial fructose concentration was 50 $g.L^{-1}$ for all experiments. When fructose concentration reached 10 $g.L^{-1}$ in the bioreactor, pulses of a 600 $g.L^{-1}$ fructose solution were achieved to reach 50 $g.L^{-1}$. In order to avoid any nutrient limitation, pulses of phosphorus and trace elements were achieved every 10 $g.L^{-1}$ biomass, by adding the equivalent of the initial concentration of the medium (B) from, respectively, 150 and 2000 fold concentrated stock solutions. The same strategy of pulses was used with kanamycin to ensure the selection pressure, by adding the equivalent of 50 $mg.L^{-1}$ every 5 $g.L^{-1}$ biomass from a 50 $g.L^{-1}$ stock solution.

2.5 Fed-batch cultivations on gaseous substrates

Fed-batch culture on gaseous substrates was carried out in three phases. The first phase consisted on a controlled-growth phase on fructose to reach 0.9 $g.L^{-1}$ of biomass at a constant $0.16h^{-1}$ specific growth rate by controlling an exponential feeding of ammonia solution. After fructose depletion, the second phase was performed to enable the adaptation of the cell metabolism to gaseous substrates. It was started by switching the air flow to a $H_2/O_2/CO_2$ (60:2:10) mix flow. The third phase consisted on a N-limited growth on gaseous substrates coupled with isopropanol production. This phase was initiated by a 1 $g.L^{-1}$ pulse of L-arabinose to induce the expression of the isopropanol operon. Nitrogen was fed exponentially from a 56 $g.L^{-1}$ NH_3 solution, to control a residual specific growth rate of $0.02h^{-1}$.

This culture was performed in 1.4 L bioreactors BDCU B.Braun with a working volume of 1 L, managed with the MFCS/win 2.0 software. The temperature was regulated at 30°C and pH at 7.0 by the addition of a 2.5 M KOH solution. In the first phase, air flow and stirring rate were adjusted to maintain fully aerobic condition, *i.e.* a dissolved oxygen concentration above 20% of saturation. In the second and third phases, the bioreactor was ventilated with a 0.22 $L.min^{-1}$ flow of a commercial $H_2/O_2/CO_2/N_2$ mix (molar %: 60:2:10:28; Air Liquide, Paris, France). Dissolved oxygen level in the reactor was controlled below 4 % of air saturation by varying the stirring rate in order to restrict the inhibition of *C. necator*'s hydrogenases by O_2 concentration.

2.6 Characterization of biomass

In order to conduct experiments, spectrophotometric measurements at 600 nm were performed with a DR3900 spectrophotometer (Hach, Loveland, Colorado, USA) after a calibration against cell dry weight measurements to evaluate cell growth. For cell dry weight

determination, culture medium was harvested and filtrated on 0.2 µm pore-size polyamide membranes (Sartorius AG, Göttingen, Germany), which were then dried to a constant weight at 60°C under partial vacuum (200 mm Hg, *i.e.* approximately 26.7 kPa).

Viability of cells was monitored with a BD Accuri C6® flow cytometer (BD Biosciences). The instrument was equipped with blue (488 nm) and red (640 nm) excitation lasers. Light scatter was collected at two angles: forward (FSC; 0°, ±13) and side (SSC; 90°, ±13) scatter. Fluorescence intensity was collected at FL1 (533/30 nm), FL2 (585/40 nm), FL3 (>670 nm), and FL4 (675/25 nm) detectors. Propidium iodide (PI) dye (Molecular probes, Invitrogen, USA) was used to monitor membrane integrity as indicators of viability. Single staining was performed during the time course of fermentations. PI was supplied by the manufacturer as 20 mM in dimethyl sulfoxide solvent (DMSO), and was used as the working solutions at a concentration of 1 $\mu L.mL^{-1}$ of cell suspension. Before staining, culture samples were diluted to reach approximately 10^6 $cells.mL^{-1}$ and were then incubated in the dark for 20 min at 37 °C. 70% isopropanol-treated cells (70% isopropanol for 1h at room temperature) were used as the 100 % dead-cell control. A sample volume of 20 µL was analyzed at the slow flow-rate setting (14 $\mu L\ min^{-1}$) using MilliQ water as sheath fluid. The FSC signal (threshold value of 10,000) and SSC signal (threshold value of 2,000) were used as a trigger channel. The red fluorescence of the PI was collected in the FL3. Data acquisition was processed using BD Accuri CFlow® software. Sample data were further analyzed with FlowJo software (Tree Star)

2.7 Metabolites analysis

Metabolites (isopropanol, acetone, pyruvic and acetic acids) and substrate (fructose) were quantified by chromatography as described by Grousseau (2014).

2.8 Off-gas analysis

For fed-batch cultures on fructose, inlet and outlet gas composition were analysed by an infra-red photoacoustic gas monitor. Analyses were performed every 2 min for O_2, CO_2 and CH_x using a fermentation gas monitor system (Lumasense Technologies inc., Santa Clara, California, USA). The instrument combines a multipoint sampler 1309 (INNOVA 1309) with a gas analyzer (INNOVA 1313). Based on the boiling point of the metabolites detected in the culture medium, isopropanol (82.5°C) and acetone (56.05°C) were assumed to be the only ones to be retrieved in the gaseous phase. Thus, their evaporation was determined by identically processing the CHx signal of the gas analyser previously calibrated with a standard ethanol gas bottle. A coefficient of distribution between isopropanol and acetone in the gas phase was determined, based on the principles of thermodynamics for ideal gas law. The treatment of the CHx signal was validated in regard with carbon and the degree of reduction balances. The equivalent isopropanol concentration was calculated assuming that the amount of evaporated isopropanol was dissolved in the liquid phase.

For fed-batch culture on gaseous substrates, the outlet O_2 and CO_2 composition was analysed by an ATEX infra-red and electrochemical sensor. Analyses were performed every 20 sec for O_2 and CO_2 with a BlueInOne (BlueSens, Herten, Germany).

Aeration started few hours after inoculation to avoid CO_2 stripping from the medium and then prevent lag phase. CO_2 production rate and O_2 consumption rate were calculated as described

by Poilpre (2002) considering the liquid and gas volume evolution, the inlet airflow, the temperature and the pressure

2.9 Enzymatic assays

Enzymatic assays of β-ketothiolase (THL), coA-transferase (CTF), acetoacetate decarboxylase (ADC) and alcohol dehydrogenase (ADH) were performed as described by Grousseau (2014), except for protein determination. The protein content of the cell lysates was determined by the Bradford technique (Bradford, 1976), according to the standard procedure of a commercial Bradford reagent (Sigma-Aldrich, Saint-Louis, Missouri, USA) with bovine serum albumin (BSA) as a standard. All enzymatic assays were performed with a DR5000 spectrophotometer (Hach, Loveland, Colorado, USA)

3 RESULTS & DISCUSSION

3.1 GroESL impact on cell tolerance to isopropanol

In previous work (Grousseau et al., 2014), it was observed that the constitutive expression of the isopropanol operon led to growth inhibition suggesting isopropanol inhibition of growth. Literature reported an improved tolerance to various alcohols by overexpression of native *groESL* genes in several bacteria (Desmond et al., 2004; Tomas et al., 2003; Zingaro and Papoutsakis, 2013). Thus, the potentiality of improving cell tolerance to isopropanol by the over-expression of *groESL* genes in *C. necator* was investigated.

One co-chaperonin GroES has been identified in *C. necator* genome (H16_A0705) and two chaperonins GroEL (H16_A0706 and H16_A1997). H16_A0705 and H16_A0706 were found adjacent. A protein sequence alignment was done to compare *C. necator*'s GroESL system to *E. coli*'s (groES locus tag b4142 (NCBI Gene ID: 948655), groEL locus tag b4143 (NCBI Gene ID: 948665)) and *C. acetobutylicum*'s (CA_C2704, CA_C2703) systems. *C. necator*'s GroES was about 50% identical and 70% similar to the other systems. *C. necator*'s GroEL from the locus H16_A0706 was closer to *E. coli* and *C. acetobutylicum* (from 76 to 86% of similarity, Table 1) than the one from the locus H16_A1997. Moreover the GroES H16_A0705 and GroEL H16_A0706 were located in the same operon. Therefore, it was chosen to over-express *C. necator's groESL* (H16_A0705 and H16_A0706) under the control of a constitutive promoter P_{Lac} downstream the MCS of pBBad and upstream the terminator (Supplementary Figure 1) leading to the pEG19 plasmid.

Firstly, the native *groESL* genes were overexpressed in the non-isopropanol producing strain Re2133 after transformation of the pEG19 plasmid. The obtained Re2133/pEG19 and

Re2133/pBBR1MCS-2 strains (as the reference) were cultivated in small scale batches on fructose in triplicate in presence of different concentrations of exogenous isopropanol.

Without the addition of isopropanol, the GroESL over-expressing strain showed a 95% similar maximal growth rate compared to the reference strain, respectively 0.17 h^{-1} ± 0.01 compared to 0.18 h^{-1} ± 0.01 , suggesting no metabolic burden due to the expression of *groESL* genes.

Moreover, cell growth was clearly less impacted by increasing concentration of isopropanol under GroESL overexpression (Figure 2). The decrease in the maximal growth rate in the presence of isopropanol was 24% lower in Re2133/pEG19 strain (0.050 h^{-1} per $g_{Isopropanol}.L^{-1}$) compared to the reference strain (0.065 h^{-1} per $g_{Isopropanol}.L^{-1}$). This difference resulted in a higher critical concentration of isopropanol (i.e. the concentration of isopropanol leading to no growth (μ=0)), at 20 $g.L^{-1}$ for the *groESL* overexpressing cells compared to 15 $g.L^{-1}$ in Re2133/pBBR1MCS-2.

Note for editor: Figure 2 is a single-column fitting image

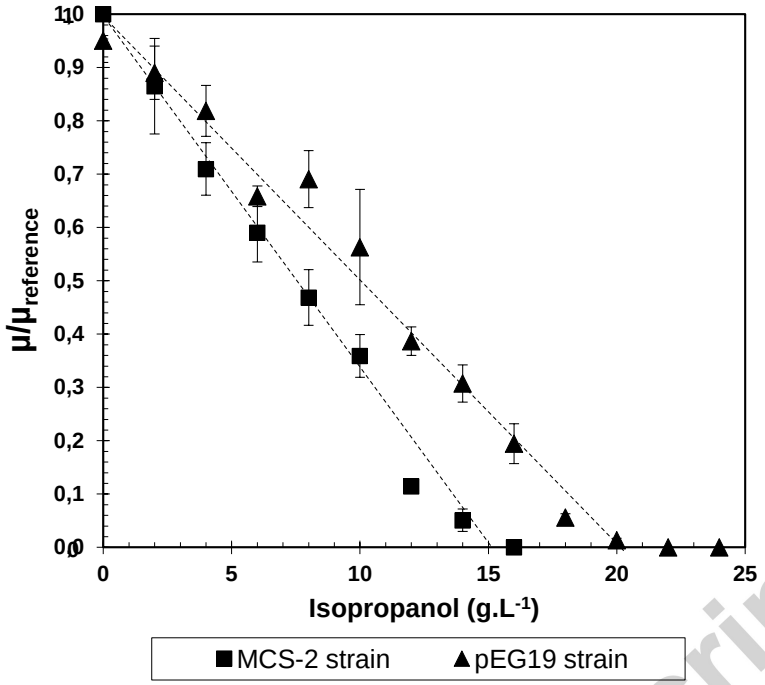


Figure 2: Maximal growth rate as a function of isopropanol concentration of Re2133/pBBR1MCS-2 (■) and Re2133/pEG19 (▲) strains ($\mu_{reference}$ = maximal growth rate of the Re2133/pBBR1MCS-2 strain without isopropanol; error bars represent two SD; dotted lines are linear regression of the no null values).

These observations are consistent with expectation from literature, which reported a positive impact of GroESL chaperones on cell tolerance to other alcohols, such as butanol and ethanol (Tomas et al., 2003; Desmond et al., 2004; Zingaro et al., 2013). GroESL has been shown to be upregulated under alcoholic stress as well (Tomas et al., 2004).

However the impact of over-expression of GroESL was observed here on the growth of a non-producing strain towards exogenously added isopropanol. Tolerance towards produced isopropanol in a producing strain may imply different mechanisms. *GroESL* were therefore overexpressed in the best producing strain Re2133/pEG7c (Grousseau et al., 2014).

3.2 Fed-batch isopropanol production on fructose

The impact of *groESL* over-expression on the cell metabolism was further characterized under isopropanol production conditions. Thus, Re2133/pBBR1MCS-2 and Re2133/pEG7c were used as no-producing and isopropanol producing reference strains, respectively. Then Re2133/pEG23, bearing the plasmid with the isopropanol operon (as in pEG7c) and the *groESL* genes, was used to study the chaperones impact under isopropanol production.

The strain characterization was performed in a controlled environment in fed-batch experiments on fructose in bioreactor experiments. The cultures were planned in two phases. In the first phase, only biomass was produced with no nutrient limitation, involving a growth at the maximal rate until reaching nitrogen depletion. Then, the second phase was started by applying a pulse of arabinose (1 $g.L^{-1}$) to induce the heterologous gene expression. The aim of this second phase was to redirect Carbon flow from growth towards isopropanol production. This was performed by limiting the growth via Nitrogen limitation. The nitrogen limiting flux was calculated to ensure a growth rate of about 0.04 h^{-1}, which was shown to be the optimum in terms of specific production rate and in term of yields for PHA production (Aragao, 1996; Grousseau et al., 2013).Nitrogen limiting condition was verified by measurement of residual ammonium concentration.

In the 1st phase of no-limited growth, Re2133/pBBR1MCS-2 strain showed a 0.25 h^{-1} growth rate (Table 2). Compared to previous work in flask cultivations, this result represented a 45% increase of specific growth rate by better controlling cell environment (Grousseau et al., 2014). Re2133/pEG7c strain showed a 0.22 h^{-1} growth rate (Table 2), which is 30% higher than the specific growth rate obtained in flask (Grousseau et al., 2014). Re2133/pEG23 strain showed the same maximal growth rate than Re2133/pEG7c (Table 2). Thus, over-expressing

groESL did not enhance the growth capacity without isopropanol, which was consistent with previous observations in microplate cultures.

Note for editor: Figure 3 is a 2-column fitting image

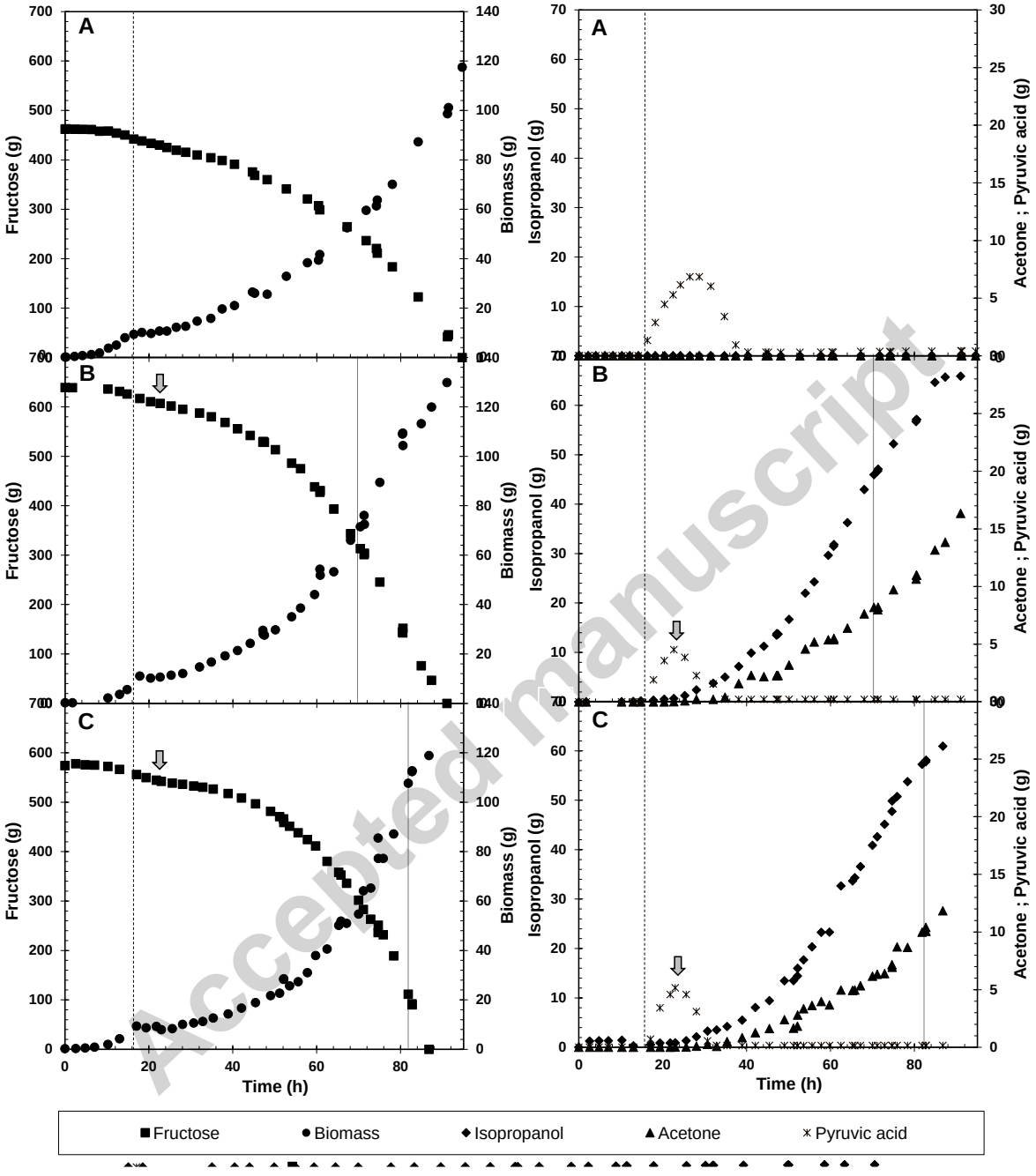


Figure 3: Evolution of cumulated masses of biomass (●), fructose (■), isopropanol (◆), acetone (▲) and pyruvate (✶) during fed-batch cultivations on fructose of the strains bearing the plasmids Re2133/pBBR1MCS-2 (A), Re2133/pEG7c (B) and Re2133/pEG23 (C) (dotted

line = full consumption of nitrogen, i.e. end of the first phase, start of the second phase; full line = maximal isopropanol concentration in the medium; arrow = induction of isopropanol production by arabinose).

After induction by arabinose, the strains bearing pEG7c and pEG23 started isopropanol and acetone production under N limitation, at a controlled growth rate of 0.042 h^{-1}.

A maximal isopropanol titer of 8.5 $g.L^{-1}$ was reached 47 h after induction with the pEG7c bearing strain (Figure 3). Moreover, this metabolite was produced at a global and constant yield of 0.159 $g.g^{-1}$ throughout the production phase (Table 2). This is higher than the yields reported in the literature with other bacterial species (Dusséaux et al., 2013; Hanai et al., 2007; Jang et al., 2013). Compared to previous work under N-depletion with the same strain, its value was equal to the overall yield obtained by Grousseau *et al.* (2014) (0.24 $Cmol.Cmol^{-1}$ / 0.16 $g.g^{-1}$), but lower than its maximal yield, reaching 0.21 $g.g^{-1}$ (0.32 $Cmol.Cmol^{-1}$) when considering only the production phase under N deficiency. The N feeding strategy applied in this work could explain this difference. The biomass and isopropanol produced in the second phase represented about the same amount of carbon from fructose, respectively 2.33 and 2.30 C-moles. Finally, the yield of conversion obtained with Re2133/pEG7c under N limitation reached 48% of the maximal theoretical yield (0.33 $g\ g^{-1}$), versus 64% under N deficiency, but it was obtained in only 70 h when flask cultivations needed 96 h.

GroESL over-expression (pEG23 plasmid) maintained an increasing isopropanol titer until 59 h after induction, reaching 9.1 $g.L^{-1}$, corresponding to a 7% enhancement compared to Re2133/pEG7c strain (Figure 3).This concentration was close to the highest one that can be found with similar strategies of engineering (Collas et al., 2012; Dusséaux et al., 2013; Hanai et al., 2007; Jang et al., 2013). Only one engineered strain of *Candida utilis*, known to show a

natural tolerance to ethanol, reached a higher value, at 9.5g.L^{-1} (Tamakawa et al., 2013). However, taking into account the strong isopropanol evaporation encountered under the experimental conditions, an equivalent of 16 gl^{-1} was produced. Nevertheless, the maximal titer obtained in the liquid phase during fed-batch remained under the critical inhibitory concentration (20 g.L^{-1}) observed with pEG19 bearing strain, in the presence of exogenous isopropanol stress (Figure 2).

The chaperone expression in the Re2133/pEG23 strain also permitted to improve the global conversion yield by 9%, at 0.174 g.g^{-1}, and the maximal conversion yield by 12 % compared to those obtained with the Re2133/pEG7c strain (Table 2).

Table 2: Parameters of fed-batch cultivations of strains bearing plasmids pBBR1MCS-2 (control), Re2133/pEG7c and Re2133/pEG23 on fructose and gaseous substrates (na: not applicable ; nd: not detected)

					Y isopropanol		**Y acetone**	
Plasmids	**Carbon source**	μ_{max} (h^{-1})	μ_{prod} (h^{-1})	$[Isop]_{max}$ ($g.L^{-1}$)	**overall** ($g.g^{-1}$)	**maximal** ($g.g^{-1}$)	**overall** ($g.g^{-1}$)	**maximal** ($g.g^{-1}$)
pBBR1MCS-2	Fructose	0.25	0.03		-	-	-	-
pEG7c (IPO)	Fructose	0.22	0.042	8.5 ± 0.1 [70 h]	0.159 ± 0.005 [23 – 70 h]	0.159 ± 0.005 [23 – 70 h]	0.029 ± 0.002 [23 – 70 h]	0.038 ± 0.002 [23 – 55 h]
pEG23 (GroESL)	Fructose	0.22	0.042	9.1 ± 0.1 [82 h]	0.174 ± 0.005 [23 – 82 h]	0.187 ± 0.005 [23 – 70 h]	0.023 ± 0.002 [23 – 82 h]	0.029 ± 0.002 [23 – 65 h]
pEG23 (GroESL)	Fructose/CO_2	n.a.	0.025	0.25 ± 0.02 [28 h]	0.093 ± 0.013 [19 – 28 h]	0.093 ± 0.013 [19 – 28 h]	n.d.	n.d.

Note for editor: Table 2 is a 2-column fitting table

Acetone, an intermediate molecule in the isopropanol biosynthesis pathway (Figure 1), was also produced concomitantly with isopropanol in the producing strains. Re2133/pEG7c excreted 0.029 gram of acetone per gram of fructose consumed when considering the whole production phase, against 0.023 $g.g^{-1}$ for the Re2133/pEG23 (Table 2). This represented a 21% decrease in the conversion yield.

The maximal amount of pyruvate was also reduced in the isopropanol producing strains, but its entire re-consumption by all of the strains limited its importance in term of carbon conversion. This transient phenomenon is probably due to a carbon overflow because of the switch between N-unlimited to N-limited growth phases as long as the isopropanol operon was not induced by arabinose addition.

Cellular viability was also tracked during fed-batch cultures by flow cytometry upon labelling bacteria with propidium iodide, as a membrane integrity marker (Figure 4). Re2133/pBBR1MCS-2 strain maintained a population with membrane damaged around 5% all along the culture, whereas this population increased constantly with the Re2133/pEG7c strain to reach the highest value of 20% at the end of the culture when the isopropanol concentration was highest. The membrane damaged population was kept below 10% with the strain overexpressing the chaperones, suggesting their positive impact on alcohol tolerance as observed with the exogenous presence of isopropanol.

Note for editor: Figure 4 is a 2-column fitting image

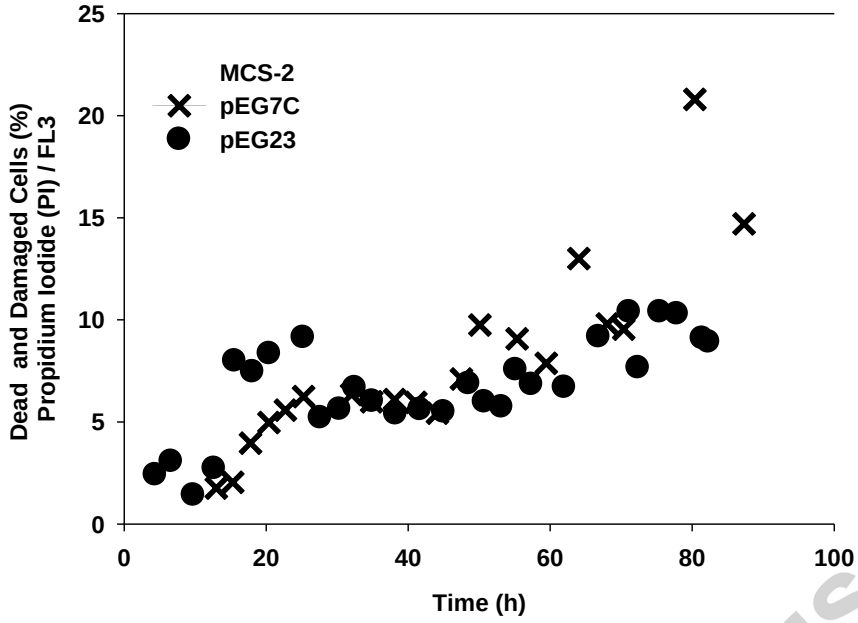


Figure 4: Evolution of the percentage of "Dead and damaged cells" subpopulation during the fed-batch cultures for the strains bearing the plasmid MCS-2 (∇), pEG7c (×) or pEG23 (●).

3.3 Enzymatic activities and carbon redirection

The impact of GroESL was further investigated by measuring the *in vitro* specific activities of the four enzymes of the synthetic isopropanol operon throughout the fed-batch cultures on fructose.

In Re2133/pBBR1MCS-2 strain, which do not bear the isopropanol operon, the specific activities of the two native enzymes, β-ketothiolase A (THL) and acetoacetyl-coA transferase (CTF)slightly increased in the last stage of the culture (Figure 5).

The expression of the isopropanol operon in the pEG7c bearing strain led to very different profiles after induction by L-arabinose. The specific activities of ADC and CTF were widely

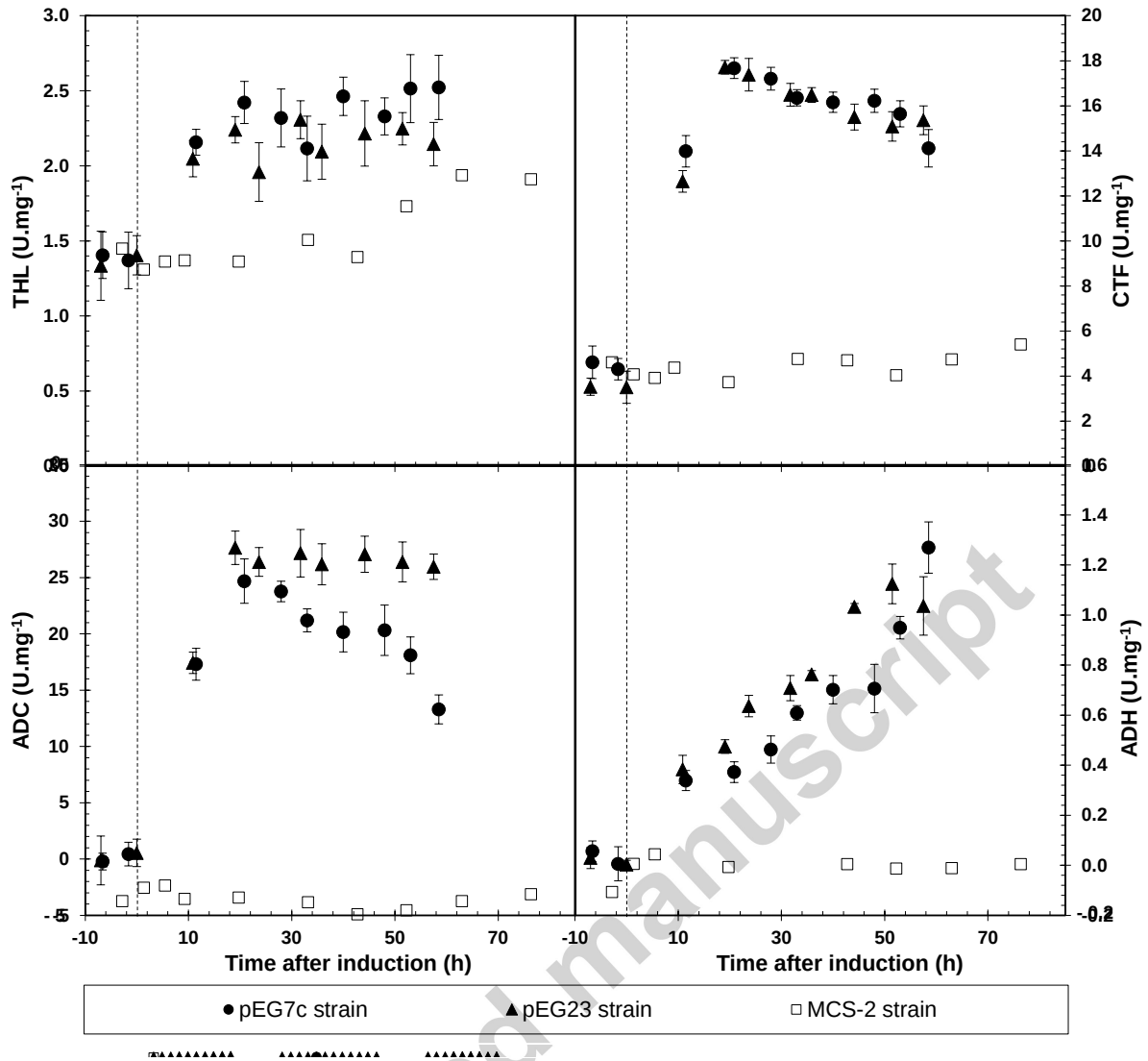
increased during the first twenty hours after induction, from 0 to 25 $U.mg^{-1}$ and from 4 to 18 $U.mg^{-1}$ respectively (Figure 5). Then, these activities decreased slightly, but continuously until the end of the cultures, mostly for the heterologous ADC. THL showed a similar profile, with an increased activity during the first twenty hours after induction, from 1.4 to 2.3 $U.mg^{-1}$; but it was maintained for the rest of the culture. The profile of ADH, the fourth key enzyme, was singular compared to the other ones: its specific activity increased linearly from 0 to 1.1 $U.mg^{-1}$ (Figure 5). Thus, ADH seemed to follow a different pattern of regulation than ADC, THL and CTF. This would not be explained by a differential expression of the coding gene inserted into the plasmid, since it is part of the isopropanol operon like the genes coding for the other key enzymes. A differential pattern of protein maturation or stability could rather explain it. Nonetheless, the additional expression of the native ADH gene caused by the alcohol stress could not be excluded, even if the activity of this enzyme was demonstrated to be very low with isopropanol (Jendrossek et al., 1988) and with acetone (Grousseau *et al.*, 2014) as substrates.

The strain bearing the pEG23 plasmid showed similar profiles of specific activities compared to Re2133/pEG7c strain for the native enzymes (Figure 5). The overproduced endogenous enzymes THL and CTF particularly followed the same dynamic during the whole culture, at very close values. However the specific activities of the two heterologous enzymes ADC and, to a lesser extent, ADH showed different evolution profiles between Re2133/pEG7c and Re2133/pEG23 strains. The amount of ADC maintained constant at a high value in Re2133/pEG23 strain after 20 hours, when it dramatically dropped in Re2133/pEG7c strain. The differences in the evolution of the amount of ADH were much lower between the two strains, even though the specific activity of ADH was slightly higher in Re2133/pEG23 strain compared to Re2133/pEG7c strain after induction time. These chaperones seemed to be

dispensable for the expression of the endogenous enzymes, since no difference in enzymatic activity was observed.

Thus, such gain of specific enzymatic activity could be explained by the mechanism of GroESL, whose activity enhance stabilization and activity of up to 50% of soluble enzymes by increasing the stabilization of their quaternary structure (Wang and Chen, 2003). GroEL and GroES act together for a correct folding of protein indeed, even under stress conditions (Georgopoulos and Welch, 1993). First, the GroEL tetradecamer, forming two back-to-back rings, binds the unfolded or misfolded proteins. After the binding of an ATP, which causes the opening of the structure, GroES closes it, leading to the protein folding. Then the protein is released and the GroESL system is free to act upon another protein. (Martin et al., 1991; Wang and Chen, 2003; Zingaro and Papoutsakis, 2012). Thus, over-expressing *GroESL* in a strain engineered for isopropanol production could lead to enhanced performances of metabolite production and fructose uptake, as already observed in butanol production and glucose consumption by *Clostridium acetobutylicum* (Tomas et al., 2003). Fructose metabolism and uptake related genes present in *Cupriavidus* species, as ABC transporters, phosphotransferase systems or 6-phosphofructokinase, were indeed shown to be upregulated by such chaperonin in bacteria (Dills et al., 1980; Tomas et al., 2004; von Rozycki et al., 2005). Moreover, it has been reported that GroES,GroEL and GroESL like chaperones were implied in preventing thermal denaturation and enhancing thermostability of ADH in *Saccharomyces cerevisiae* (Yan et al., 1997), and other organisms (Mendoza et al., 1996) .Finally, it has been reported examples where co-overexpression of GroESL improved the heterologous protein expression in *E. coli* (Kelson et al., 1996 ; Luo and Hua, 1998 ; Lee et al., 2002) and in yeasts (Jariyachawalid et al., 2012 ; Guadalupe-Medina et al., 2013). However, here the effect of native GroESL co-overexpression resulted in the stabilization of

the amount of the heterologous ADC (and in a much lesser extent ADH) among the four proteins expressed in the isopropanol operon. It was reported that the majority of *E. coli* GroELS substrates were proteins smaller than 55kDA in size (Ewalt et al. 1997 ; Houry et al. 1999). It has been also mentioned that GroEL substrates consisted preferentially of several αβ domains with buried hydrophobic β –sheets (Houry et al. 1999). Size of the two heterologous unfolded ADC and ADH are 33and 40 kDa, respectively, and their structure complies with αβ domains rich proteins (Ho et al. 2009 ; Khorkhin et al., 1998), suggesting that the two heterologous proteins could be good substrates for GroEL. Therefore explanation of the different effect upon GroELS over-expression here seems more complicated and would need further investigation.



Note for editor: Figure 5 is a 2-column fitting image

Figure 5: Activities of enzymes involved in the isopropanol metabolic pathway from pyruvate, during fed-batch cultivations on fructose of Re2133/pBBR1MCS-2 (□), Re2133/pEG7c (●) and Re2133/pEG23 (▲) strains (dotted line = induction of isopropanol production by arabinose; error bars represent SD between triplicates).

The positive effect of chaperones over-expression on the isopropanol tolerance and on the heterologous enzyme activities was reflected in the redirection of the carbon flux. Indeed, the

maximal specific rate of isopropanol production was improved by about 30% in the pEG23 bearing strain compared to the pEG7c one, from 0.036 to 0.047 $g.g^{-1}.h^{-1}$ (Figure 6). With a global view of the culture, the integral of the specific rate of isopropanol production over time was also higher in Re2133/pEG23 cells. In parallel, the specific rate of acetone production had been always slightly lower in the Re2133/pEG23 strain (Figure 6). A partially lifted bottleneck in the hydrogenation of acetone by a higher ADH activity could explain the lesser production of acetone, for the benefit of isopropanol.

Thus, carbon from fructose was redirected from the undesired co-metabolite, acetone, to the targeted product, isopropanol, by over-expressing the GroESL chaperones. In conclusion, increased levels of GroES and GroEL may stabilize the ADC and ADH specific activities, leading to a better coordination within the isopropanol operon by pulling further the carbon flow towards isopropanol.

Note for editor: Figure 6 is a single-column fitting image

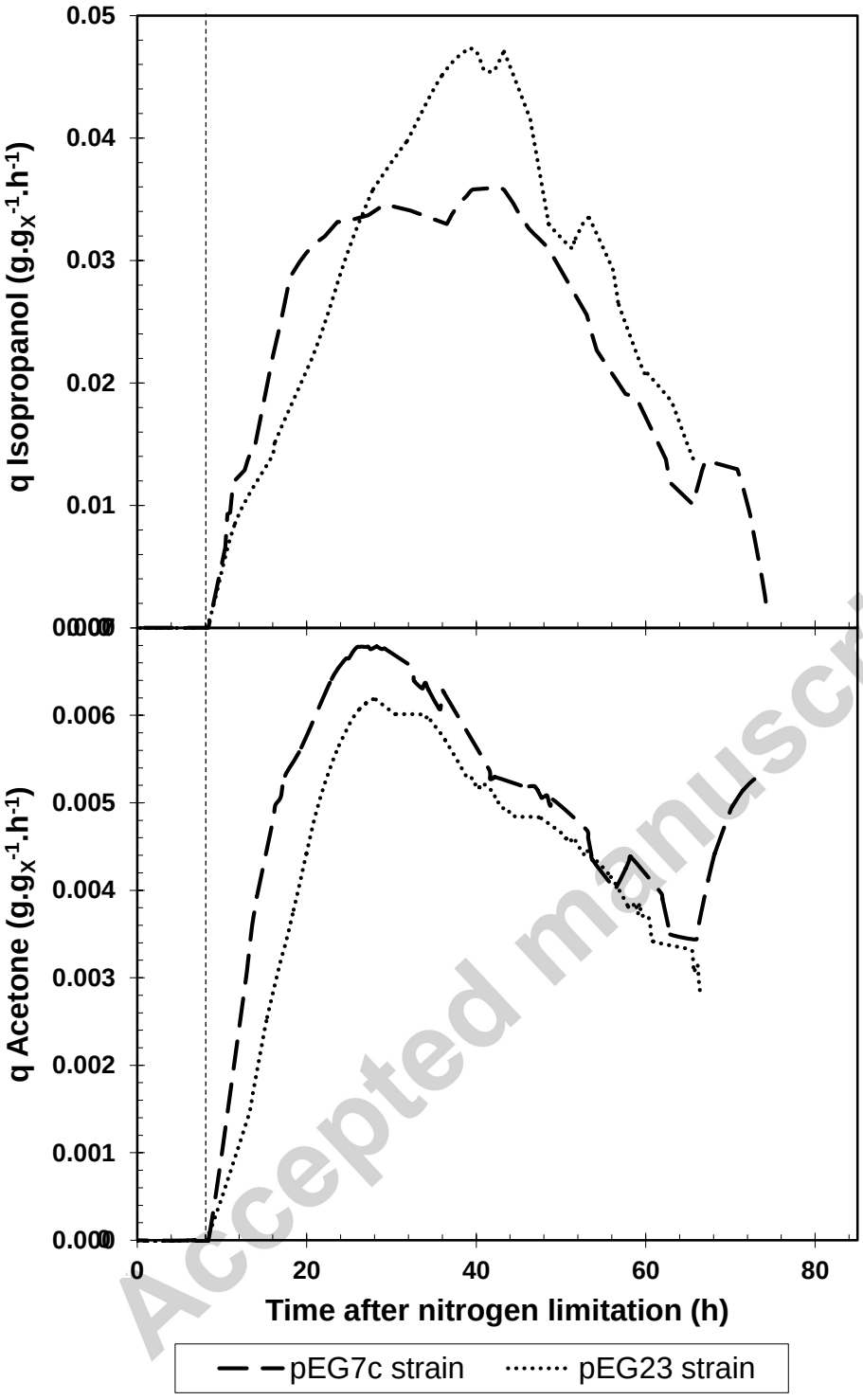


Figure 6: Specific production rates (q) of isopropanol and acetone during fed-batch cultivations on fructose of the strains bearing the plasmids pEG7c (dashed curve) and pEG23 (dotted curve) (dotted line = induction of isopropanol production by arabinose).

3.4 Isopropanol production under autotrophic conditions

One of the isopropanol engineered strain Re2133/pEG12 (Grousseau et al.,2014) was recently evaluated under autotrophic culture conditions using H_2 from water splitting to reduce carbon CO_2 and led to a production of 216 $mg.L^{-1}$ in 120 hours (Torella et al., 2015). Here, the strain over-expressing the isopropanol operon and the *groESL* genes (Re2133/pEG23) was cultivated in a bioreactor, uncoupling biomass production on fructose, then isopropanol production on CO_2 as the sole carbon source. During the growth phase, fructose was fed to control the specific growth rate at 0.16 h^{-1} and nitrogen was initially provided to reach 0.8 $g.L^{-1}$ of cell dry weight. Immediately after nitrogen depletion, fructose feeding was stopped, and nitrogen was fed to support a residual specific growth rate at 0.02 h^{-1}. CO_2 feeding was performed by bubbling of a gas mixture containing H_2, O_2 and CO_2 (60:2:10). Cultivation was performed under O_2-limited condition in order to both avoid the risk of explosion and restrict the inhibition of *C. necator* hydrogenases by O_2 concentration. Though *C. necator* [Ni-Fe] hydrogenases are among the most O_2 tolerant ones, various studies reported different levels of inhibiting oxygen concentrations between 4 and 20% O_2 suggesting strain dependency. Phenomenological models reported inhibition constant (K_i^{O2}) in the range of 0.11-0.14 mM corresponding to around 10% of the O_2 saturation (Siegel and Ollis, 1984). It was reported inhibition of the membrane bound hydrogenase by O_2 concentrations as low as 0.05 mM O_2 (4% of dissolved oxygen in the reactor) with a K_i^{O2} = 0.11 mM (9.5% of dissolved oxygen in the reactor) at pH 5.5 and 30°C (Cracknell et al., 2009). After six hours under gaseous substrates to ensure the diauxic shift, isopropanol production was induced by the addition of 0.1 % of arabinose. Arabinose was measured through the whole fermentation and was not consumed by the bacteria. After 12 h of nitrogen limitation and production phase on CO_2, the final level of biomass was 1.1 $g.L^{-1}$. The production phase on CO_2 of Re2133/pEG23 resulted

in an isopropanol production of 250 mg.L^{-1} in only 12 hours (Figure 7). During the production phase, the strain reached a maximal specific production rate of isopropanol of 0.035 Cmole.Cmole^{-1}.h^{-1}. It represented nearly 60% of the one obtained from fructose (0.062 Cmole.Cmole^{-1}.h^{-1}). The isopropanol yield on CO_2 was constant over the 12 hours of the production phase at 0.093 g.g^{-1}, representing only 20% of the theoretical yield. These results are very encouraging as autotrophic conditions were not optimal: (i) the gas mixture $H_2/O_2/CO_2$ (60:2:10) was far from the optimal stoichiometry for growth (63/23/9) (Morinaga et al., 1978), (ii) reactor design was not optimal in terms of gas transfer capacity and (iii) and in addition the production was performed at a low cell density (around 1 g.L^{-1}). These parameters could be easily improved within an optimal bioreactor dedicated for autotrophic conditions with better gas transfer capacity.

Note for editor: Figure 7 is a 2-column fitting image

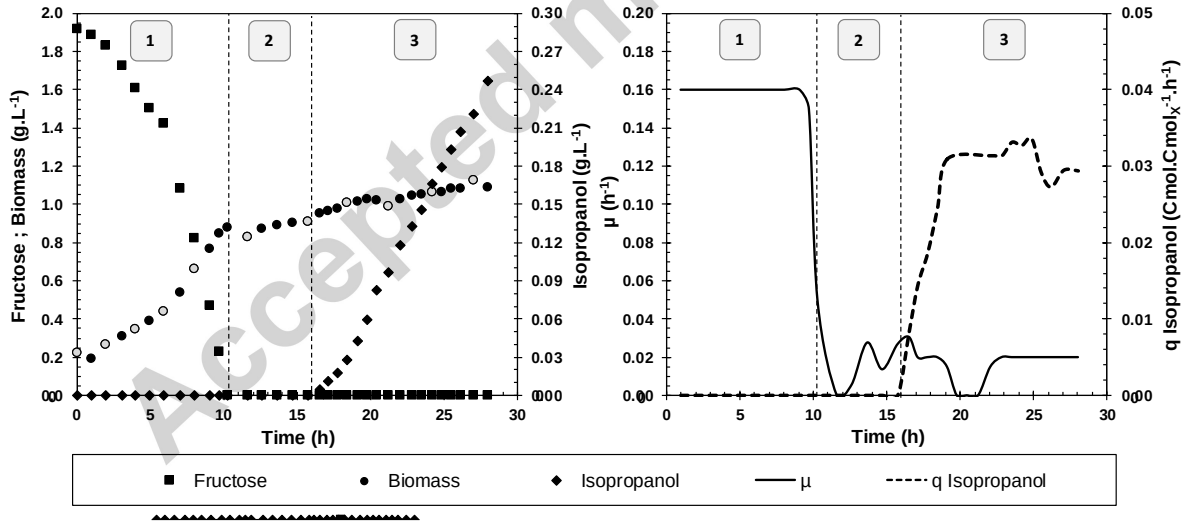


Figure 7: Evolution of cumulated masses of biomass (dry cell weight: ○; Optical density: ●), fructose (■) and isopropanol (◆) and evolution of specific growth (full line) and isopropanol production (dashed lines) rates, during fed-batch cultivations on gaseous substrates of Re2133/pEG23 strain (phase 1: N-limited growth on fructose; phase 2: adaptation of cell metabolism switch; 3: isopropanol production on gaseous substrates).

4 CONCLUSIONS

In this study, *groESL* genes in the *C. necator* genome were identified and over-expressed in *C. necator* Re2133 strain deleted for the PHB synthesis pathway. Over-expression of *groESL* in Re2133 strain led to an increase of cell's tolerance towards isopropanol characterized by the ability to grow in the presence of exogenous isopropanol. Over-expression of *groESL* in an engineered isopropanol producing strain (Re2133/pEG23) led to slightly improve the capacity to produce isopropanol on fructose in terms of final titer (+ 7%) and conversion yield (+ 9-18%) concomitantly with a reduction in acetone production. In *groESL* over-expressing strain, highest levels of the two heterologous enzymes (ADC and, in a lesser extent, ADH) were observed that translated in higher carbon fluxes towards isopropanol and lower one towards acetone. In conclusion, increased levels of GroES and GroEL may have held the ADC and ADH in a more stabilized state leading to a better coordination within the isopropanol operon by pulling further the carbon flow towards isopropanol.

Finally, Re2133/pEG23 strain was able to produce 250 $mg.L^{-1}$ in 12 hours under autotrophic conditions demonstrating that *C. necator* is a very promising strain to produce isopropanol or other chemicals from CO_2.

ACKNOWLEDGMENT

This work was supported by grants from the MIT-France Seed Fund. Drs Estelle Grousseau and Jillian Marc were funded by a postdoctoral 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. Toshiaki Fukui from the Tokyo Institute of Technology for the generous gift of the pBBad plasmid. Last, we thank BlueSens company for lending us the BlueInOne sensor.

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Highlights

- Over-expression of the native *groEL* and *groES* genes led led to a better tolerance of the *C. necator* strain towards exogenous isopropanol
- Over-expression of *groEL* and *groES* genes in an engineered isopropanol producing *C. necator* strain led to improve the production isopropanol on fructose
- Isopropanol production from CO_2 as sole C-source was demonstrated using our engineered C. necator strain in bioreactor.