Metabolic Engineering 42 (2017) 74–84

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

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

Jillian Marc[a], Estelle Grousseau[a], Eric Lombard[a], Anthony J. Sinskey[b], Nathalie Gorret[a], Stéphane E. Guillouet[a,*]

[a] LISBP, Université de Toulouse, CNRS, INRA, INSA, 135 avenue de Rangueil, 31077 Toulouse CEDEX 04, France
[b] Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts avenue, Cambridge, MA 02139, USA

ARTICLE INFO

Keywords:
Ralstonia eutropha
Cupriavidus necator
Isopropanol
Carbon dioxide
Hydrogen
Metabolic engineering
GroEL/S
Chaperones
Gas fermentation
Autotrophy

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.

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., 1988) 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

* Corresponding author.
E-mail address: stephane.guillouet@insa-toulouse.fr (S.E. Guillouet).

http://dx.doi.org/10.1016/j.ymben.2017.05.007
Received 23 December 2016; Received in revised form 30 March 2017; Accepted 31 May 2017
Available online 04 June 2017

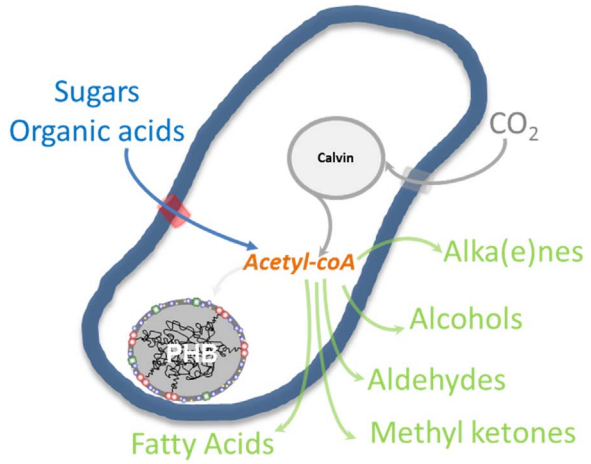

Fig. 1. Reported metabolic routes for the production of interest molecules through the engineering of the natural metabolism of a *Cupriavidus necator* strain deleted for the PHB synthesis pathway.

containing 67.7% P(3HB) in 40 h 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 (Fig. 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 80 s (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 (Fig. 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 mg l^{-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).

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-*groESL* inserted after the MCS (Kan^r), P_{Bad}	This work
pEG23	pBBad with *phaA*-RBS-*ctfAB*-RBS-*adc**-RBS-*adh** sequence inserted into MCS and P_{LAC}-RBS-*groESL* 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 2351–3406) 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).

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.

2. Material and methods

2.1. Strains and medium

2.1.1. 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 Fig. 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.