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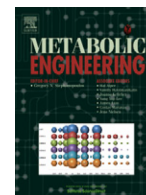

New options to engineer biofuel microbes: Development and application of a high-throughput screening system

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ABSTRACT

The number of recent efforts on rational metabolic engineering approaches to increase butanol production in *Clostridium acetobutylicum* are quite limited, demonstrating the physiological complexity of solventogenic clostridia. Since multiple largely unknown parameters determine a particular phenotype, an inverse strategy to select a phenotype of interest can be useful. However, the major constraint for explorative or combinatorial metabolic engineering approaches is the availability of a feasible screening method to select the desired phenotype from a large population in a high-throughput manner. Therefore, a semi-quantitative assay was developed to monitor alcohol production in microtiter cultures of *C. acetobutylicum*. The applicability of the screening system was evaluated by two examples. First, *C. acetobutylicum* ATCC 824 was chemically mutagenized and subjected to high butanol concentrations as a pre-selection step. Screening of the butanol-tolerant population resulted in the identification of mutants with $>20\%$ increased butanol production as compared to the wildtype. The second application example was based on a pre-engineered *C. acetobutylicum* strain with low acetone biosynthetic activity, but concomitantly reduced butanol titer. After chemical mutagenesis, a total of 4390 clones was analyzed and mutants with significantly increased butanol concentrations and similarly low acetone levels as the parental strain were selected. Thus, the suitability of the semi-quantitative screening system was validated, opening up new perspectives for combinatorial strategies to improve solventogenic clostridia and other biofuel microbes.

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1. Introduction

Employing solventogenic clostridia for biotechnological *n*-butanol production has a long tradition and plays an important role during the first half of the last century prior to petrochemical production processes (Jones and Woods, 1986; Green, 2011). According to the renewed interests in biofuel sources, *Clostridium acetobutylicum* and related strains obtained much attention recently (Jang et al., 2012a; Tracy et al., 2012). However, suitable tools and protocols for genetic manipulation of clostridia are limited and thus, metabolic engineering approaches are quite rare as compared to other model organisms such as *Escherichia coli* (Desai and Papoutsakis, 1999; Heap et al., 2012; Kuehne and Minton, 2012). In addition to the industrial perspective, solventogenic clostridia represent an interesting group of bacteria because of their unique physiology, no other organism is known to naturally synthesize acetone and butanol. The life cycle of *C. acetobutylicum* comprises a typical biphasic fermentation pattern. During exponential growth, acetic and butyric acids are formed, which is accompanied by the evolution of molecular hydrogen. When the cells enter the stationary growth phase, the metabolism switches to acid re-assimilation and the neutral solvents acetone, ethanol and butanol are produced while the environmental pH increases again. Subsequently, the cells form endospores which are finally released to survive unfavorable environmental conditions (Paredes et al., 2005).

In general, the branched metabolism of *C. acetobutylicum* is quite complex and previous studies aimed to increase the metabolic flux towards butanol as a desired product by inactivation of competing pathways. For example, targeting the acetone biosynthetic pathway reduced or eliminated the unwanted byproduct, but however, the butanol titer was also lower than in the wildtype strain (Jiang et al., 2009; Lehmann et al., 2012a; Jang et al., 2012b). Interestingly, butyrate-negative mutants shifted the metabolic profile towards ethanol production (Lehmann and Lütke-Eversloh, 2011; Lehmann et al., 2012b; Cooksley et al., 2012). These recent systematic strategies clearly indicated that manipulation of the central metabolism might not necessarily result in an optimized production strain with improved titer, yield and productivity. Therefore, a better understanding of the physiological events in solventogenic clostridia and the related regulatory mechanisms will be required for successful rational metabolic

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engineering approaches (Papoutsakis, 2008; Lütke-Eversloh and Bahl, 2011).

On the other hand, inverse strategies to engineer biofuel producing microbes constitute an alternative option, because multiple parameters often determine a particular phenotype, which are usually unknown (Bailey et al., 2002; Santos and Stephanopoulos, 2008). Nevertheless, the implementation of such combinatorial or explorative approaches strictly depends on the availability of a respective screening method to analyze a large population and to identify an improved phenotype of interest (Dietrich et al., 2010). Since butanol and other biofuels are colorless compounds excreted into the culture medium, detection in a high-throughput manner can be difficult. The most frequently employed screening technique for solventogenic clostridia was the exposition to high butanol concentrations in order to select butanol-tolerant strains, which is often but not necessarily related to increased production capacities (e.g. Hermann et al., 1985; Matta-el-Ammouri et al., 1986; Borden and Papoutsakis, 2007; Mann et al., 2012). Screening populations in the presence of suicide substrates such as allyl alcohol or bromobutyrate was used to select solvent-negative mutants (Dürre et al., 1986; Rogers and Palosaari, 1987; Clark et al., 1989; Medkor et al., 2010). More recently, flow cytometry was employed to analyze morphological cell types of *C. acetobutylicum* and *Clostridium pasteurianum* (Tracy et al., 2008; Tracy et al., 2010; Linhová et al., 2010; Linhová et al., 2012).

The objective of this study was the development of a suitable screening platform to monitor biofuel production in microbial cultures, strictly uncoupled from any product tolerance-related phenotype. The colorimetric nitroblue tetrazolium assay was transferred to the microtiter scale to visualize butanol and ethanol production of clostridial cultures in 96-well microtiter plates. The applicability of this assay for strain-specific butanol production was demonstrated in two independent screenings of mutant libraries.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

All strains used in this study are listed in Table 1. *C. acetobutylicum* ATCC 824 and derived mutants thereof were cultivated anaerobically at 37 °C without shaking. Strains were maintained on reinforced clostridial agar (RCA, Oxoid Deutschland GmbH, Wesel, Germany) or were stored as spore suspensions at −70 °C. Procedures requiring strictly anaerobic conditions were done in an anaerobic chamber with 90% N_2 and 10% H_2 (MG1000, Meintrup DWS, Lähden-Holte, Germany).

Liquid cultures were performed in Hungate tubes (Ochs GmbH, Bovenden, Germany), serum bottles (Müller & Krempel AG, Bülach, Switzerland) or 96-well microtiter plates (Sarstedt AG & Co., Nürnbrecht, Germany) and pretreated according to Breznak and Costilow (1994). Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) was added at a concentration of 1 mg/l as a redox indicator for anaerobiosis and when necessary, residual oxygen was removed from the medium prior to inoculation with titanium (III) nitrilotriacetic acid (NTA) solution (1.3 M NaOH, 0.16 M NTA, 0.27 M Na_2CO_3 and 1.3% $TiCl_3$). Inoculation and sample drawing were conducted using disposable plastic syringes (B. Braun AG, Melsungen, Germany).

Cultures were inoculated with spores in clostridial growth medium (CGM) containing per liter 0.75 g KH_2PO_4, 0.75 g K_2HPO_4, 0.71 g $MgSO_4 \times 7H_2O$, 0.01 g $MnSO_4 \times 7H_2O$, 0.01 g $FeSO_4 \times 7H_2O$, 1 g NaCl, 2 g $(NH_4)_2SO_4$, 5 g yeast extract, 2 g asparagin and 1 mg resazurin; 50 g glucose were added after autoclaving (modified from Roos et al., 1985). Prior to cultivation, the CGM cultures were pasteurized for 10 min at 80 °C to inactivate vegetative cells. For *C. acetobutylicum adhE1*::int(158), *C. acetobutylicum adc*::int(180) and its chemical mutants, erythromycin was added at a concentration of 25 μg/ml.

Cultivation experiments for phenotypic characterization were performed in MS-MES medium with the following composition per liter: 0.55 g KH_2PO_4, 0.55 g K_2HPO_4, 0.22 g $MgSO_4 \times 7H_2O$, 0.011 g $FeSO_4 \times 7H_2O$ and 2.3 ml acetic acid; after the pH was adjusted to 6.6 with NH_4OH, 40 μg *p*-aminobenzoic acid, 0.32 μg biotin, 1 mg resazurin, 21.3 g 2-(*N*-morpholino) ethanesulfonic acid (MES) and 60 g/l glucose were added (modified from Monot et al., 1982). Growth in microtiter plates and on agar plates was performed in an anaerobic chamber (MG1000, Meintrup DWS, Lähden-Holte, Germany).

2.2. Chemical mutagenesis

Random mutagenesis of *C. acetobutylicum* was performed as described previously (Murray et al., 1983; Clark et al., 1989). Briefly, 50 ml of a CGM culture was harvested at $OD_{600}=1$, washed with CGM without glucose and incubated at 37 °C for 2 h in CGM without glucose to enable endogenous nutrient depletion. The cells were subjected to 500 μg/ml N′-methyl-N′-nitrosourea (MNU, Sigma-Aldrich Chemie GmbH, München, Germany) for 30 min, which resulted in a survival rate of approximately 1% (Singer and Kusmierek, 1982). The cells were harvested, washed twice and incubated in 10 ml CGM comprising 5% (w/v) glucose over night for regeneration. The mutant library was spread on agar plates and single colonies were subjected to the screening as described in Section 3.2.

2.3. Nitroblue tetrazolium (NBT) assay

Ethanol and butanol were detected in clostridial cultures by an alcohol dehydrogenase-coupled conversion of nitroblue tetrazolium chloride (NBT) to insoluble formazan (Fibla and Gonzàlez-Duarte,

Table 1
Bacterial strains used in this study.

Strain	Relevant characteristics	Reference
C. acetobutylicum ATCC 824	Wildtype	American type culture collection
C. acetobutylicum adc::int(180)	ClosTron mutant with disrupted *adc* gene (CAP0165), Erm^R	(Lehmann et al., 2012a)
C. acetobutylicum adhE1::int(158)	ClosTron mutant with disrupted *adhE1* gene (CAP0162), Erm^R	(Lehmann, 2012)
C. acetobutylicum 2D8	MNU mutant of ATCC 824	this study
C. acetobutylicum 2E8	MNU mutant of ATCC 824	this study
C. acetobutylicum 3E8	MNU mutant of ATCC 824	this study
C. acetobutylicum 8F9	MNU mutant of ATCC 824	this study
C. acetobutylicum a13B5	MNU mutant of *adc*::int(180)	this study
C. acetobutylicum a14E6	MNU mutant of *adc*::int(180)	this study
C. acetobutylicum a20A7	MNU mutant of *adc*::int(180)	this study

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