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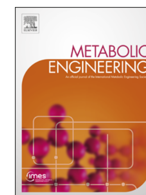

Metabolic engineering of *Corynebacterium glutamicum* for the production of itaconate

Q1 Andreas Otten, Melanie Brocker*, Michael Bott**

IBG-1: Biotechnology, Institute of Bio- and Geosciences, Forschungszentrum Jülich, 52425 Jülich, Germany

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The capability of *Corynebacterium glutamicum* for glucose-based synthesis of itaconate was explored, which can serve as building block for production of polymers, chemicals, and fuels. *C. glutamicum* was highly tolerant to itaconate and did not metabolize it. Expression of the *Aspergillus terreus CAD1* gene encoding *cis*-aconitate decarboxylase (CAD) in strain ATCC13032 led to the production of 1.4 mM itaconate in the stationary growth phase. Fusion of CAD with the *Escherichia coli* maltose-binding protein increased its activity and the itaconate titer more than two-fold. Nitrogen-limited growth conditions boosted CAD activity and itaconate titer about 10-fold to values of 1440 mU mg^{-1} and 30 mM. Reduction of isocitrate dehydrogenase activity via exchange of the ATG start codon to GTG or TTG resulted in maximal itaconate titers of 60 mM (7.8 g l^{-1}), a molar yield of 0.4 mol mol^{-1}, and a volumetric productivity of 2.1 mmol l^{-1} h^{-1}.

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1. Introduction

Itaconic acid (2-methylidenebutanedioic acid) is an unsaturated dicarboxylic acid which has gained considerable interest in recent years as it was reported to be one of the top 12 building block chemicals that can be produced from biomass and replace fossil-based chemicals (Werpy and Peterson, 2004). It is used in industry e.g. for the synthesis of resins, lattices, fibres, detergents, cleaners and bioactive compounds (Okabe et al., 2009; Willke and Vorlop, 2001). The demand for itaconic acid is estimated at 30,000 t per year compared to a worldwide production capacity of about 50,000 t per year (Steiger et al., 2013). Although a number of fungi are capable of itaconic acid synthesis, including *Ustilago*, *Candida*, and *Rhodotorula* species, *Aspergillus terreus* is still the dominant production host reaching titers of >80 g l^{-1} (Okabe et al., 2009; Willke and Vorlop, 2001). The production of itaconic acid in *A. terreus* occurs via the decarboxylation of *cis*-aconitate, an intermediate of the aconitase reaction in the tricarboxylic acid cycle, by the enzyme *cis*-aconitate decarboxylase (EC 4.1.1.6) (Bentley and Thiessen, 1957c; Dwiarti et al., 2002), which is encoded by the *CAD1* gene (Kanamasa et al., 2008).

Despite the fact that the production costs were reduced in the past decade from about 4 US $ kg^{-1} in 2001 (Willke and Vorlop, 2001) to about 2 US $ kg^{-1} (Steiger et al., 2013), they are still too high to allow the application of itaconic acid as substitute for acrylic or methacrylic acid used for the production of plastics. This situation has motivated a variety of research efforts to improve itaconic acid production (for recent reviews see Klement and Büchs, 2013; Steiger et al., 2013). These included e.g. the optimization of production with *A. terreus* (Hevekerl et al., 2014; Kuenz et al., 2012; Tevz et al., 2010), the development of processes with other natural itaconate producers such as *Ustilago maydis* (Carstensen et al., 2013; Klement et al., 2012; Maassen et al., 2014; Panakova et al., 2009; Voll et al., 2012), or the use of *Aspergillus niger* as production host (Blumhoff et al., 2013; Li et al., 2011, 2012, 2013; van der Straat et al., 2013, 2014). *A. niger* is a highly efficient citric acid producer, reaching titers above 200 g l^{-1}, but does not naturally produce itaconic acid and thus requires heterologous genes, e.g. from *A. terreus*. Besides *A. niger*, also other heterologous hosts were tested for itaconic acid production, such as *Escherichia coli* (Li et al., 2011; Liao and Chang, 2010) or *Saccharomyces cerevisiae* (Blazeck et al., 2014). In all cases, the itaconic acid titers reached were far lower than those obtained with *A. terreus*.

In this study, we explored the potential of *Corynebacterium glutamicum* for itaconate production. *C. glutamicum* is the favorite host for industrial acid production of L-glutamate and L-lysine and has become a model organism in industrial biotechnology (Burkovski, 2008; Eggeling and Bott, 2005; Yukawa and Inui, 2013). In the past decades, *C. glutamicum* strains were developed for a broad spectrum of other metabolites (Becker and Wittmann, 2012). Besides various amino acids, such as L-serine (Stolz et al., 2007), L-valine (Blombach et al., 2007; Hasegawa et al., 2013; Radmacher et al., 2002), L-isoleucine (Vogt et al., 2014c), or L-leucine (Vogt et al., 2014a), this spectrum also included organic acids (Wieschalka et al., 2013), such as D-lactate (Okino et al., 2008b), succinate (Litsanov et al., 2012a, 2012b, 2013,

Q2 * Corresponding author.Tel.: +49 2461 61 5168.
** Corresponding author. Tel.: +49 2461 613294; Fax: +49 2461 61 2710.
E-mail addresses: m.brocker@fz-juelich.de (M. Brocker), m.bott@fz-juelich.de (M. Bott).

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2014; Okino et al., 2008a), pyruvate (Wieschalka et al., 2012), 2-ketoisovalerate (Krause et al., 2010), or 2-ketoisocaproate (Bückle-Vallant et al., 2014; Vogt et al., 2014b). The production parameters of several of these strains were highly competitive with alternative production hosts or represented best-in-class examples, which motivated us to study itaconate production with *C. glutamicum*. As outlined above, itaconate is derived from *cis*-aconitate, an intermediate of the TCA cycle, which has been intensively studied in *C. glutamicum* (Bott, 2007; Bott and Eikmanns, 2013; Eikmanns, 2005), also with respect to aconitase, the enzyme responsible for *cis*-aconitate formation (Baumgart and Bott, 2011; Baumgart et al., 2011; Emer et al., 2009; Garcia-Nafria et al., 2013; Krug et al., 2005). Heterologous expression of the *cis*-aconitate decarboxylase gene from *A. terreus* in *C. glutamicum* wild type resulted in the production of low mM concentrations of itaconate from glucose. By optimization of CAD activity, cultivation conditions, and reduction of isocitrate dehydrogenase activity, itaconate concentrations up to 7.8 g l^{-1} were obtained with a yield of 0.29 g g^{-1} glucose and a maximal volumetric production rate of 0.27 g l^{-1} h^{-1}.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture and cultivation conditions

All bacterial strains and plasmids used or constructed in this study are listed in Table 1. *C. glutamicum* was routinely pre-cultured in Brain Heart Infusion medium (Difco Laboratories, Detroit, USA) (37 g l^{-1}) supplemented with 91 g l^{-1} sorbitol (BHIS) and afterwards pre- and main-cultured in mCGXII medium (5 g l^{-1} $(NH_4)_2SO_4$, 5 g l^{-1} urea, 1 g l^{-1} KH_2PO_4, 1 g l^{-1} K_2HPO_4, 42 g l^{-1} 3-morpholinopropanesulfonic acid (MOPS), 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 10 mg l^{-1} $CaCl_2$, 10 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 0.1 mg l^{-1} $MnSO_4 \cdot H_2O$, 1 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.2 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 20 μg l^{-1} $NiCl_2 \cdot 6H_2O$, 0.2 mg l^{-1} biotin) with 4% (w/v) glucose as carbon and energy source. Before autoclaving, the pH of the medium was adjusted to pH 7 with solid KOH. When the medium was supplemented with itaconate, an itaconic acid solution (1 M) was adjusted to pH 7 by addition of NaOH and sterile-filtered before addition to the autoclaved medium. For establishing nitrogen-limited conditions, the urea concentration was reduced to 1 g l^{-1} and $(NH_4)_2SO_4$ was entirely omitted. *E. coli* DH5α strains were routinely cultured in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl). When appropriate, kanamycin (25 μg l^{-1} for *C. glutamicum* or 50 μg ml^{-1} for *E. coli*) was added to the cultures. For strain construction, BHIS (*C. glutamicum*) and LB (*E. coli*) agar plates were used containing 15 g l^{-1} agar. For all itaconate production experiments, a 5 ml BHIS pre-culture was routinely inoculated with a single colony of the desired *C. glutamicum* strain from a fresh streaked agar plate and incubated on a rotary shaker at 170 rpm for 8 h at 30 °C. A second pre-culture was incubated overnight in a 500 ml baffled shake flask filled with 60 ml mCGXII medium containing 4% (w/v) glucose. After approximately 14 h incubation at 30 °C and 120 rpm the cells were harvested, washed with fresh mCGXII medium and used to inoculate the 60 ml mCGXII main culture to an optical density at 600 nm (OD_{600}) of approximately 1. Under the cultivation conditions chosen, *C. glutamicum* wild type transiently accumulates lactate as major and acetate, succinate and malate as minor by-products. The excretion of these organic acids starts when the cells become oxygen-limited and they are later consumed again (Koch-Koerfges et al., 2012). To induce overexpression of plasmid-encoded target genes, 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG) was added to the culture. Growth of the bacterial strains was monitored by measuring the OD_{600} with an Ultrospec 500pro spectrophotometer (Amersham Biosciences, Freiburg, Germany). The biomass was calculated with an experimentally determined correlation factor of 0.25 g (cdw) l^{-1} for an OD_{600} of 1 (Kabus et al., 2007).

2.2. Recombinant DNA work

All oligonucleotides used in this work were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table S1. PCR was performed according to standard protocols (Sambrook and Russell, 2001) with KOD Hot Start polymerase (Novagen, Darmstadt, Germany). Restriction enzymes used for molecular cloning were

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
C. glutamicum ATCC 13032	Wild type, biotin-auxotrophic	Abe et al. (1967)
C. glutamicum AO1	ATCC 13032 derivative carrying an exchange of the native ATG start codon of the isocitrate dehydrogenase gene *icd* (cg0766) to GTG	Zahoor et al. (2014)
C. glutamicum AO2	ATCC 13032 derivative carrying an exchange of the native ATG start codon of *icd* to TTG	This work
E. coli DH5α	F$^-$ φ80*dlac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*) *U169 endA1 recA1 hsdR17* (r_K^-, m_K^+) *deoR thi-1 phoA supE44* λ$^-$ *gyrA96 relA1*	Invitrogen
pEKEx2	Kan^R; P_{tac}, *lacI*^{q}, pBL1 *ori*$_{C. glutamicum}$, pUC18 *ori*$_{E. coli}$; *C. glutamicum*/*E. coli* shuttle-vector for regulated gene expression	Eikmanns et al. (1991)
pEKEx2-*cad*$_{opt}$	Kan^R; pEKEx2 derivative containing the synthetic codon-optimized *CAD1* gene of *A. terreus* derived from pMK-RQ-*cad* under control of the *tac* promotor	This work
pEKEx2-*cad*$_{nat}$	Kan^R; pEKEx2 derivative containing the native *CAD1* gene of *A. terreus* derived from pMK-RQ-*cad*$_{nat}$ under control of the *tac* promotor	This work
pEKEx2-*malE*	Kan^R; pEKEx2 derivative containing the *malE* gene from *E. coli* without signal peptide and stop codon under control of the *tac* promotor for fusion proteins	This work
pEKEx2-*malEcad*$_{opt}$	Kan^R; pEKEx2-*malE* derivative containing the synthetic *cad*$_{opt}$ gene fused to the 3′-end of the *malE* gene from *E. coli*	This work
pK19*mobsacB*	Kan^R; pK18 *ori*$_{E. coli}$, *sacB lacZ*α; vector for allelic exchange in *C. glutamicum*	Schäfer et al. (1994)
pK19*mobsacB-icd*	Kan^R; pK19*mobsacB* derivative containing a 1.2 kb fragment covering the *icd* gene of *C. glutamicum*	This work
pK19*mobsacB-icd* (A1T)	Kan^R; derivative of pK19*mobsacB-icd* with an exchange of the ATG start codon of *icd* to TTG	This work
pK19*mobsacB-icd* (A1G)	Kan^R; derivative of pK19*mobsacB-icd* with an exchange of the ATG start codon of *icd* to GTG	This work
pMal-c	Amp^R; pBR322 origin; *lacI*; expression vector for constructing fusion proteins with the *E. coli* maltose-binding protein lacking its signal peptide and expression under control of the *tac* promoter	New England Biolabs
pMK-RQ-*cad*$_{opt}$	Kan^R; ColE1 origin, containing the *CAD1* gene from *A. terreus* codon-optimized for *C. glutamicum*	Life Technologies
pMK-RQ-*cad*$_{nat}$	Kan^R; ColE1 origin, containing the *CAD1* gene from *A. terreus* with its native sequence	Life Technologies

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