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Improved fermentative production of the compatible solute ectoine by *Corynebacterium glutamicum* from glucose and alternative carbon sources

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ABSTRACT

The cyclic amino acid ectoine is a compatible solute serving as a protective substance against osmotic stress. Ectoine finds various applications due to its moisturizing effect. To avoid the disadvantages of the prevailing so-called "bacterial milking ectoine production process" caused by the high salt concentration, low salt fermentation strategies are sought after. As L-lysine and ectoine biosynthesis share L-aspartate-semialdehyde as common precursor, L-lysine producing strains can be converted to ectoine producing strains. *Corynebacterium glutamicum*, which is used for L-lysine production in the million-ton-scale, was engineered for ectoine production by heterologous expression of the ectoine biosynthesis operon *ectABC* from *Chromohalobacter salexigens*. Derepression of glucose metabolism by deletion of the regulatory gene *sugR* and avoiding L-lactate formation by deletion of the lactate dehydrogenase gene *ldhA* increased ectoine productivity. In bioreactor fed-batch cultivations an ectoine titer of 22 g L^{-1} and a volumetric productivity of 0.32 g L^{-1} h^{-1} were obtained. The ectoine yield of 0.16 g g^{-1}, to the best of our knowledge, exceeded previously reported yields. Moreover, ectoine production from the alternative carbon sources glycerol, glucosamine, xylose, arabinose, and soluble starch was achieved.

1. Introduction

Ectoine is a compatible solute that was first discovered in *Halorhodospira halochloris,* but is widespread among γ-proteobacteria like *Halomonas* and *Chromohalobacter*, actinobacteria like *Brevibacterium* and *Streptomyces* or firmicutes like *Bacillus* and *Marinococcus* (Galinski et al., 1985; Kuhlmann and Bremer, 2002; Louis and Galinski, 1997; Onraedt et al., 2005). In three enzymatic steps ectoine is synthesized from L-aspartate-semialdehyde, an intermediate of L-lysine biosynthesis. Glutamate is the amino group donor for the transamination of L-aspartate-semialdehyde by diaminobutyrate transaminase (EctB) to diaminobutyrate, which is subsequently acetylated by diaminobutyrate acetyl transferase (EctA). In the last step, ring closure of acetyl diaminobutyrate by ectoine synthase (EctC) yields ectoine. Hydroxylation of ectoine by ectoine hydroxylase (EctD) leads to hydroxyectoine, a related compatible solute which, for example, plays an important role in heat stress protection (García-Estepa et al., 2006). Ectoine has several protective functions on macromolecules, cells, or tissues since it was shown to improve protein folding and activity or to decrease the melting temperature of DNA. It is used in sun blockers to quench singlet oxygen derived from ultraviolet light, and was shown to moisturize human skin (Botta et al., 2008; Buenger and Driller, 2004; Heinrich et al., 2007; Pastor et al., 2010). Ectoine has been commercialized and is produced with *Halomonas elongata* by a process called "bacterial milking". The natural producer *H. elongata* is cultivated under high salt conditions to induce ectoine synthesis to increase water activity within the cell. After reaching an appropriate cell density, a hypoosmotic shock is applied by addition of distilled water after reduction of the culture volume. This hypoosmotic shock induces the release of ectoine to the culture medium in order to reduce the water activity within the cell. The product is separated from the cells which are recycled for repeated growth in fresh medium under high salt conditions (Sauer and Galinski, 1998). Subsequently, a two-reactor system was developed for *Chromohalobacter salexigens*, which maintains cells under optimal growth conditions in one bioreactor and constantly drains off culture to a second bioreactor, where the hypoosmotic shock is applied (Fallet et al., 2010). Both "bacterial milking" techniques have several drawbacks arising from the high salt media, thus, there was a need to develop a low-salt fermentation approach.

A recombinant approach was possible since the biosynthesis of ectoine was studied in detail in *H. halochloris*, *H. elongata* and *C.*

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salexigens (Cánovas et al., 1998; Ono et al., 1999; Peters et al., 1990). The ectoine biosynthesis genes *ectA*, *ectB* and *ectC* are typically clustered on the genome co-transcribed as operons. Moreover, the basic *ectABC* gene cluster is frequently associated with an aspartokinase gene (Pastor et al., 2010; Widderich et al., 2014). If in addition to the basic *ectABC* gene cluster a gene encoding ectoine hydroxylase (*ectD*) is present in the organism, it may be part of the *ectABC* operon or may be present as a separate transcription unit somewhere else in the genome as in the case of *C. salexigens*. Since ectoine biosynthesis starts from L-aspartate-semialdehyde, an intermediate of L-lysine biosynthesis, L-lysine overproducing strains of the Gram-positive *Corynebacterium glutamicum* were considered as ectoine production hosts. *C. glutamicum* is genetically amenable and has been already engineered for the production of a very wide range of metabolites such as vitamins (Hüser et al., 2005), alcohols (Inui et al., 2004; Jojima et al., 2015; Siebert and Wendisch, 2015; Wendisch et al., 2006), diamines like cadaverine (Mimitsuka et al., 2007) and putrescine (Schneider and Wendisch, 2010), organic acids (Litsanov et al., 2012; Okino et al., 2005; Tsuge et al., 2015; Wieschalka et al., 2013), and non-proteinogenic amino acids (Jorge et al., 2016a, 2016b; Kim et al., 2013; Pérez-García et al., 2016, 2017). Since decades, *C. glutamicum* is used for the biotechnological amino acid production and especially for production of L-glutamate and L-lysine (Eggeling and Bott, 2015; Hermann, 2003; Lee et al., 2016; Wendisch et al., 2016). For example, the annual production of L-lysine exceeds 2 million tons (Hirasawa and Shimizu, 2016; Wendisch et al., 2016). Not surprisingly, *C. glutamicum* was used as a basis to engineer strains for the production of L-lysine-derived products such as cadaverine (Mimitsuka et al., 2007), L-pipecolic acid (Pérez-García et al., 2016) or δ-aminovalerate (Rohles et al., 2017; Jorge et al., 2017). Recently, also an ectoine producing *C. glutamicum* strain has been described (Becker et al., 2013). In this work, *C. glutamicum* wild type was used as a basis and *ectABCD* from *Pseudomonas stutzeri* was integrated into the genome disrupting *ddh*, encoding the diaminopimelate dehydrogenase. Introduction of the amino acid exchange T311I in the *lysC* gene led to feedback-resistant aspartokinase and deletion of *lysE* avoided L-lysine export. In a fed-batch cultivation, this strain produced 4.5 $g\ L^{-1}$ ectoine with a volumetric productivity of 0.28 $g\ L^{-1}\ h^{-1}$ (Becker et al., 2013).

Here, we describe ectoine producing *C. glutamicum* strains based on the previously described L-lysine producing strains DM1800 and DM1729 (Georgi et al., 2005). Both, DM1800 and DM1729 possess L-lysine feedback-resistant aspartokinase (encoded by $lysC^{T311I}$) (Kalinowski et al., 1991; Schrumpf et al., 1992) and a variant of pyruvate carboxylase (encoded by pyc^{P458S}) for increased provision of oxaloacetate as precursor for L-lysine biosynthesis (Peters-Wendisch et al., 2001). In addition, strain DM1729 possesses a mutated version of the homoserine dehydrogenase (encoded by hom^{V59A}). The resulting reduced homoserine dehydrogenase enzyme levels and reduced threonine concentrations improve L-lysine production and avoid conversion of L-aspartate-semialdehyde towards threonine and methionine biosynthesis (Eikmanns et al., 1991; Follettie et al., 1988). To increase the glycolytic flux deletion of the genes coding for transcriptional repressor SugR (Engels et al., 2008; Engels and Wendisch, 2007; Gaigalat et al., 2007) and fermentative L-lactate dehydrogenase (Engels et al., 2008; Toyoda et al., 2009) were chosen as a suitable strategy (Pérez-García et al., 2016) (Fig. 1). Upon expression of the ectoine biosynthetic genes from *C. salexigens* in this strain, efficient production of ectoine from glucose and the alternative carbon sources glycerol, glucosamine, xylose, arabinose, and soluble starch was established.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains and plasmids employed in this study are listed in Table 1. *Escherichia coli* DH5α (Hanahan, 1983) was used for the genetic engineering work of vector construction. *C. glutamicum* and *E. coli* were routinely grown in Brain-heart infusion broth (BHI) (ROTH®) in 500-mL baffled flasks at 30 °C or 37 °C inoculated from a fresh LB agar plate. For growth experiments of *C. glutamicum* strains 50 mL of CGXII medium (Eggeling and Bott, 2005) with glucose 40 $g\ L^{-1}$ was inoculated to an optical density (OD_{600}) of 1 and routinely incubated at 30 °C and 120 rpm (shaking diameter: 16.5 cm) in 500 mL baffled flasks. Growth was followed by measuring the optical density using V-1200 Spectrophotometer at 600 nm (VWR, Radnor, PA, USA). *C. glutamicum* biomass calculations were done according to the correlation CDW = 0.353 OD (Bolten et al., 2007). *E. coli* cultures were routinely incubated at 37 °C and 200 rpm (shaking diameter: 16.5 cm) in 500 mL baffled flasks. When necessary, the growth medium was supplemented with kanamycin (25 $\mu g\ mL^{-1}$), spectinomycin (100 $\mu g\ mL^{-1}$), and/or tetracycline (5 $\mu g\ mL^{-1}$). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added when necessary to the minimal medium to induce gene expression from the vector pVWEx1 (Peters-Wendisch et al., 2001).

2.2. In-frame deletion of sugR, ldhA, and lysE

All *in-frame* deletions of this work were performed following the two-steps homologous recombination procedure (Rittmann et al., 2003). For that purpose the vectors pK19mobsacB-Δ*sugR* (Engels and Wendisch, 2007), pK19mobsacB-Δ*ldhA* (Blombach et al., 2011), and pK19mobsacB-Δ*lysEG* (Vrljic et al., 1996) were used as described elsewhere (Engels and Wendisch, 2007). The deletions were verified by PCR using the primers Δ*sugR*-Ver-fw (GTTCGTCGCGGCAATGATTGACG), Δ*sugR*-Ver-rv (CTCACCACATCCACAAACCACGC), Δ*ldhA*-Ver-fw (TGATGGCACCAGTTGCGATGT), Δ*ldhA*-Ver-rv (CCATGATGCAGGATGGAGTA), Δ*lysE*-Ver-fw (CGCGAGCAAGGAGAGTACG), and Δ*lysE*-Ver-rv (AAATCAAGCAGCACTACTACA).

2.3. Molecular genetic techniques and strains construction

Standard molecular genetic techniques were carried out as described elsewhere (Sambrook et al., 1989). *E. coli* were transformed by heat shock (Sambrook et al., 1989) and *C. glutamicum* by electroporation (Eggeling and Bott, 2005).

The operon *ectABC* was amplified from *C. salexigens* DSM 3043T genomic DNA using the primers *ectA*-fw (CAGGGATCC*GAAAGGAG*GCCCTTCAGATGACGCCTACAACCGAG) and *ectC*-rv (CAGGGATCCTCAATCGACCGGTGCG) which carry the restriction site sequence for BamHI (underlined); *ectA*-fw also carries the RBS sequence (italics). The PCR product and the vector pVWEx1 were treated with BamHI and ligated as described elsewhere (Sambrook et al., 1989). The vector pVWEx1-*ectABC* was further used to transform *C. glutamicum* strains.

2.4. Analytical procedures

For the quantification of extracellular amino acids, carbohydrates, and ectoine a high-pressure liquid chromatography system was used (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). The supernatants of the cell cultures were collected by centrifugation (13,000 × *g*, 10 min), and further used for analysis.

For detection of L-lysine and other amino acids, samples were derivatised with *ortho*-phthaldialdehyde (OPA) (Schneider and Wendisch, 2010). The amino acid separation was performed by a precolumn (LiChrospher 100 RP18 EC-5 μ (40 mm × 4 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a column (LiChrospher 100 RP18 EC-5 μ (125 mm × 4 mm), CS Chromatographie Service GmbH). The detection was carried out with a fluorescence detector (FLD G1321A, 1200 series, Agilent Technologies) with the excitation and emission wavelengths of 230 nm and 450 nm, respectively.

For quantification of carbohydrates, a column for organic acids (300 mm × 8 mm, 10 μm particle size, 25 Å pore diameter, CS

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