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Brief report

Glycerol as a substrate for aerobic succinate production in minimal medium with *Corynebacterium glutamicum*

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Summary

***Corynebacterium glutamicum*, an established microbial cell factory for the biotechnological production of amino acids, was recently genetically engineered for aerobic succinate production from glucose in minimal medium. In this work, the corresponding strains were transformed with plasmid pVWEx1-*glpFKD* coding for glycerol utilization genes from *Escherichia coli*. This plasmid had previously been shown to allow growth of *C. glutamicum* with glycerol as sole carbon source. The resulting strains were tested in minimal medium for aerobic succinate production from glycerol, which is a by-product in biodiesel synthesis. The best strain BL-1/pVWEx1-*glpFKD* formed 79 mM (9.3 g l^{-1}) succinate from 375 mM glycerol, representing 42% of the maximal theoretical yield under aerobic conditions. A specific succinate production rate of 1.55 mmol g^{-1} (cdw) h^{-1} and a volumetric productivity of 3.59 mM h^{-1} were obtained, the latter value representing the highest one currently described in literature. The results demonstrate that metabolically engineered strains of *C. glutamicum* are well suited for aerobic succinate production from glycerol.**

Introduction

Glycerol is a main by-product of biodiesel and bioethanol production (Yazdani and Gonzalez, 2007). By utilizing glycerol for the production of value-added chemicals, such as 1,3-propanediol, ethanol, amino acids or succinate, the economic efficiency of these biofuel production processes can be significantly increased (Wendisch *et al*., 2011). Succinate is a C_4-dicarboxylate offering interesting prospects for the chemical industry as feedstock for the production of a large variety of important bulk chemicals, such as tetrahydrofurane (THF), 1,4-butanediol, γ-butyrolactone or maleic anhydride, with a total annual production of more than 500 000 tons (Zeikus *et al*., 1999; McKinlay *et al*., 2007).

Bio-based succinate production from glycerol has been described for a number of natural succinate producers, such as *Basfia succiniciproducens* (Scholten and Dagele, 2008) and *Anaerobiospirillum succiniciproducens* (Lee *et al*., 2001), as well as for metabolically engineered *Escherichia coli* strains (Blankschien *et al*., 2010; Zhang *et al*., 2010). The described processes are anaerobic ones (with exception of one microaerobic process) and allow a maximal succinate yield of 1 mol succinate mol^{-1} glycerol, whereas aerobic processes can maximally yield 0.5 mol mol^{-1}. The slow anaerobic growth with glycerol, attributed to a redox imbalance resulting from the use of intermediates for biosynthesis (Zhang *et al*., 2010), and the low energy yield during anaerobic utilization of glycerol (Gonzalez *et al*., 2008) result in a low volumetric productivity (0.7–1.7 mM h^{-1}) (Blankschien *et al*., 2010; Zhang *et al*., 2010). This disadvantage of all described processes hinders their biotechnological implementation (Zhang *et al*., 2010). Alternatively, aerobic succinate production from glycerol with the yeast *Yarrowia lipolytica* was recently examined (Yuzbashev *et al*., 2010). It accumulated succinate with relatively high volumetric productivity (2.29 mM h^{-1}) under optimal conditions (pH 7). However, complex media additives were necessary for growth and product formation which can complicate downstream processing and increase the production costs. Thus, none of the so far described succinate production processes from glycerol is perfectly suitable for industrial application.

Corynebacterium glutamicum is a Gram-positive soil bacterium with GRAS status (generally regarded as safe). It is used industrially for the large-scale production of

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more than 2 million tons of L-glutamate and about 1.4 million tons of L-lysine annually (Ajinomoto Co., 2010; 2011). In addition, *C. glutamicum* strains were designed for the production of many other industrially relevant products, such as L-serine (Stolz *et al*., 2007) and D-serine (Stäbler *et al*., 2011), L-valine (Blombach *et al*., 2008), 2-ketoisovalerate (Krause *et al*., 2010), putrescine (Schneider and Wendisch, 2010), isobutanol (Smith *et al*., 2010; Blombach *et al*., 2011), diaminopentane (Mimitsuka *et al*., 2007), D-lactate (Okino *et al*., 2008a) or ethanol (Inui *et al*., 2004). The genome of *C. glutamicum* is known (Ikeda and Nakagawa, 2003; Kalinowski *et al*., 2003; Yukawa *et al*., 2007) and reliable tools for genetic engineering exist (Kirchner and Tauch, 2003). Moreover, extensive knowledge on the metabolism of *C. glutamicum* is available due to 60 years of research on amino acid production (Eggeling and Bott, 2005; Burkovski, 2008). Recently metabolically engineered *C. glutamicum* strains have been described which belong to the most efficient microorganisms for succinate production from glucose either under anaerobic (Okino *et al*., 2008b; Litsanov *et al*., 2012a) or aerobic conditions (Litsanov *et al*., 2012b). Under aerobic conditions *C. glutamicum* was able to synthesize succinate in minimal medium with a high specific productivity [1.6 mmol g (cdw)$^{-1}$ h^{-1}]. The strain *C. glutamicum* Δ*sdhCAB* (Litsanov *et al*., 2012b) devoid of the operon coding for succinate dehydrogenase showed relatively high succinate production but also formed large amounts of acetate as a by-product. When the genes for all known metabolic routes for acetate synthesis were deleted in the Δ*sdhCAB* background, the resulting strain *C. glutamicum* BL-1 showed an 82% reduction in acetate accumulation and a 65% increase in succinate production. Further improvements were obtained by increasing C_3 carboxylation and by using growth-decoupled conditions (Litsanov *et al*., 2012b).

Naturally, *C. glutamicum* ATCC 13032 is not able to utilize glycerol as carbon source, but expression of the *E. coli glpFKD* operon allows the organism to grow on glycerol as sole carbon and energy source (Rittmann *et al*., 2008). Based on our previous study on aerobic succinate production from glucose we now explored the possibility of aerobic succinate production from glycerol with *C. glutamicum* (Fig. 1).

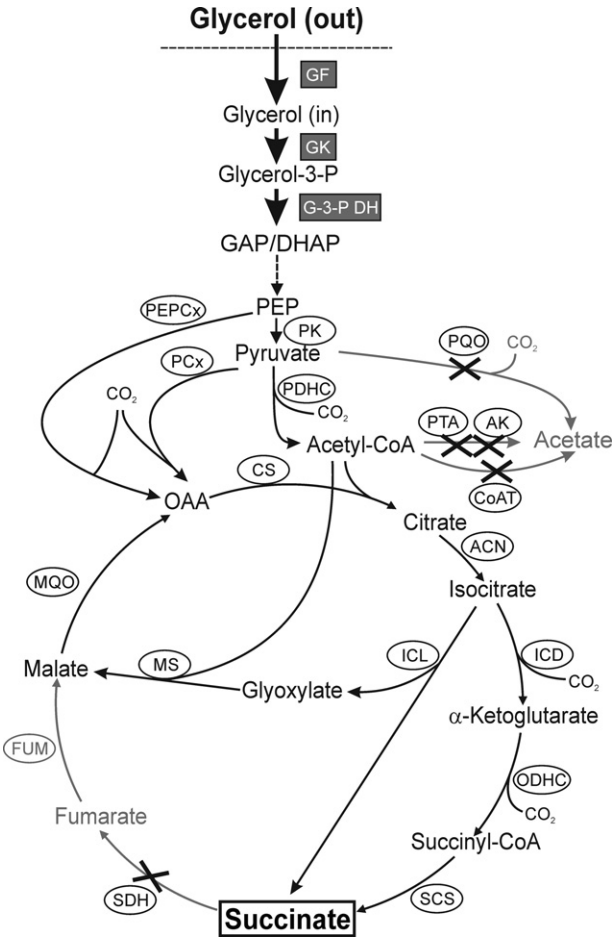

Fig. 1. Scheme of the central metabolism of *C. glutamicum* tailored for aerobic succinate production from glycerol. Enzymes whose genes were deleted are indicated by 'X'. The reactions affected by these deletions and their products are displayed in grey. Enzymes whose genes were overexpressed are highlighted in grey boxes and the arrows for the corresponding reactions are thickened. Abbreviations: ACN, aconitase; AK, acetate kinase; CoAT, acetyl-CoA : CoA transferase; CS, citrate synthase; FUM, fumarase; GF, glycerol facilitator (from *E. coli*); GK, glycerol kinase (from *E. coli*); G-3-P DH, glycerol-3-phosphate dehydrogenase (from *E. coli*); ICD, isocitrate dehydrogenase; ICL, isocitrate lyase; MQO, malate : menaquinone oxidoreductase; MS, malate synthase; OAA, oxaloacetate; ODHC, 2-oxoglutarate dehydrogenase complex; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PEPCx, PEP carboxylase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PTA, phosphotransacetylase; PQO, pyruvate : menaquinone oxidoreductase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase.

Results and discussion

Utilization of glycerol for aerobic succinate production by C. glutamicum ΔsdhCAB/*pVWEx1*-glpFKD

We previously showed that the strains *C. glutamicum* Δ*sdhCAB* and *C. glutamicum* BL-1 are capable of aerobic succinate production from glucose (Litsanov *et al*., 2012b). To give these strains the ability to use glycerol as carbon source, they were transformed with plasmid pVWEx1-*glpFKD*, coding for a glycerol facilitator, glycerol kinase and glycerol-3-phosphate dehydrogenase from *E. coli* (Rittmann *et al*., 2008). The resulting strains were tested for aerobic succinate production from glycerol (Fig. 1). Three independent batch cultivations with each strain in glycerol minimal medium were performed in a parallel bioreactor system. Since elevated glycerol concentrations had been shown to inhibit growth (Rittmann *et al*., 2008), the experiments were performed with

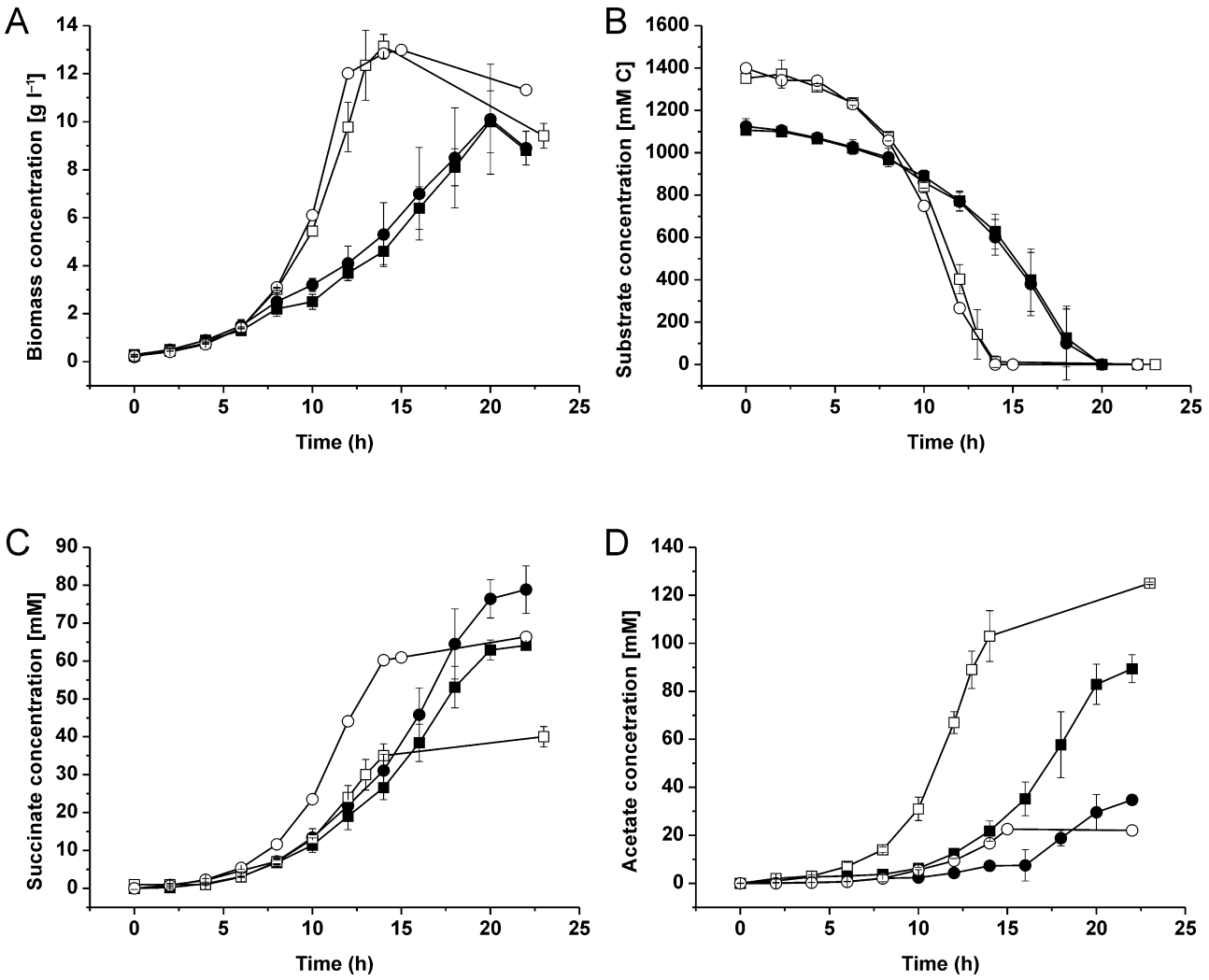

Fig. 2. Biomass formation (A), substrate consumption (B), succinate production (C) and acetate production (D) of *C. glutamicum* Δ*sdhCAB*/pVWEx1-*glpFKD* (filled squares, $n = 3$) and *C. glutamicum* BL-1/pVWEx1-*glpFKD* (filled circles, $n = 3$) on glycerol and of *C. glutamicum* Δ*sdhCAB* (open squares, $n = 4$) and *C. glutamicum* BL-1 (open circles, $n = 2$) on glucose. The values for strains Δ*sdhCAB* and BL-1 on glucose were taken from Litsanov and colleagues (2012b) The experiments were performed as aerobic batch cultivations in modified CGXII medium containing 400 mM (1200 mM carbon) glycerol or 222 mM (1332 mM carbon) glucose under constantly controlled conditions of pH 7.0 and $pO_2 > 30\%$ using a Multifors bioreactor system as described previously (Litsanov *et al.*, 2012b). The results displayed are average data including standard deviation from at least three experiments except for *C. glutamicum* BL-1, where only mean values from two independent experiments are presented. Glucose and organic acids were quantified by HPLC as described previously. Glycerol was also analysed by HPLC and quantified by an Agilent 1100 Refractive Index Detector.

400 mM glycerol as sole carbon source. With respect to carbon (1200 mM), this concentration was slightly lower than the one (1332 mM carbon) used previously for succinate production from glucose (222 mM), but in the same range to allow a comparison. To prevent acidification of the medium by the organic acid production, the pH was kept constant at 7.0 by addition of 3 M KOH. Oxygen limitation was avoided by keeping pO_2 at > 30% saturation. During cultivation growth (OD_{600}), glycerol consumption and organic acid production were measured and compared with data previously published for aerobic succinate production from glucose by the strains *C. glutamicum* Δ*sdhCAB* and *C. glutamicum* BL-1 (Fig. 2, Table 1). Except for the carbon source, the cultivation conditions were the same as previously published (Litsanov *et al.*, 2012b).

Corynebacterium glutamicum Δ*sdhCAB*/pVWEx1-*glpFKD* grew on glycerol with a growth rate of 0.19 h^{-1} and completely consumed glycerol (369 mM) within 22 h. The specific substrate uptake rate for glycerol [23 mmol carbon g $(cdw)^{-1}$ h^{-1}] was 28% lower than the specific glucose uptake rate of strain *C. glutamicum* Δ*sdhCAB* [32 mmol carbon g $(cdw)^{-1}$ h^{-1}]. The different uptake mechanisms may play a role in this context. Whereas

Table 1. Growth and organic acid production parameters of different *C. glutamicum* succinate producer strains during aerobic cultivation on 222 mM glucose or 400 mM glycerol as carbon source in modified CGXII medium using a Multifors bioreactor system at pH 7.0 and $pO_2 > 30\%$ saturation.

Substrate	*C. glutamicum* strain	Growth rate (h^{-1})	Biomass [g (cdw) l^{-1}]	Substrate consumed (mM carbon)[a]	Substrate uptake rate [mmol carbon g (cdw)$^{-1}$ h^{-1}]	Final acetate titre (mM)	Final succinate titre (mM)	Succinate production rate [mmol g^{-1} (cdw) h^{-1}]	Yield succinate/substrate (mol mol^{-1})	Succinate yield (%)[b]
Glucose	Δ*sdhCAB*	0.31 ± 0.01	13.1 ± 0.5	1351 ± 23	32 ± 1	125 ± 11	40 ± 3	0.75 ± 0.03	0.18 ± 0.01	18 ± 1
	BL-1	0.33	13.0	1399	31	22	66	1.39	0.28	29
Glycerol	Δ*sdhCAB*/pVWEx1-*glpFKD*	0.19 ± 0.00	10.0 ± 1.3	1107 ± 10	23 ± 2	89 ± 6	64 ± 1	1.30 ± 0.05	0.17 ± 0.00	35 ± 1
	BL-1/pVWEx1-*glpFKD*	0.22 ± 0.02	10.1 ± 2.3	1125 ± 35	25 ± 2	35 ± 2	79 ± 6	1.55 ± 0.23	0.21 ± 0.02	42 ± 4

a. Glucose concentration multiplied by factor of 6, glycerol concentration multiplied by factor of 3.
b. Based on the maximal theoretical succinate yields from glucose (1 mol succinate mol^{-1} glucose) or glycerol (0.5 mol succinate mol^{-1} glycerol) calculated for non-growing cells metabolizing the substrate exclusively via glycolysis and the oxidative TCA cycle.
All parameters describing rates were calculated for the exponential growth phase of the cultures. The data are average values including standard deviation from at least three experiments except for *C. glutamicum* BL-1 where only mean values from two independent experiments are presented. The values for strains Δ*sdhCAB* and BL-1 on glucose were taken from Litsanov and colleagues (2012b).

glucose is transported actively into the cell by a high-affinity phosphotransferase system (PTS) (Mori and Shiio, 1987), glycerol enters cells of *C. glutamicum* carrying pVWEx1-*glpFKD* via facilitated diffusion mediated by the glycerol facilitator GlpF (Rittmann *et al*., 2008). Remarkably, succinate production by strain *C. glutamicum* Δ*sdhCAB*/pVWEx1-*glpFKD* on glycerol was significantly increased compared with that of strain *C. glutamicum* Δ*sdhCAB* on glucose. A succinate titre of 64 mM (+60%) was reached with a succinate production rate of 1.3 mmol g (cdw)$^{-1}$ h^{-1} (+73%). The final succinate yield (0.17 mol mol^{-1} glycerol) represented 35% of the maximal theoretical yield of 0.5 mol succinate mol^{-1} glycerol. The calculation of the maximal yield was based on resting cells which catabolized glycerol exclusively via glycolysis and the oxidative branch of the TCA cycle (Fig. 1). *Corynebacterium glutamicum* Δ*sdhCAB* reached only 18% of the maximal theoretical succinate yield (1 mol mol^{-1} glucose) during growth on glucose (Litsanov *et al*., 2012b). This difference in the relative yields could at least partially be due to the fact that 29% less acetate was formed on glycerol (Table 1). These results suggest that the utilization of glycerol favours succinate production and lowers acetate production.

Glycerol dissimilation is initiated by its ATP-dependent phosphorylation via glycerol kinase (Durnin *et al*., 2008; Rittmann *et al*., 2008). In contrast, glucose is phosphorylated by the glucose-specific PTS to glucose 6-phosphate with phosphoenolpyruvate (PEP) as phosphoryl donor. High glucose uptake rates favour high pyruvate and low PEP concentrations in the cell, a situation which was shown to be unfavourable for aerobic and anaerobic succinate production by *E. coli*, because PEP is the subtrate for anaplerotic C_3 carboxylation via phosphoenolpyruvate carboxylase (PEPCx) or phosphoenolpyruvate carboxykinase (PEPCk) (Lin *et al*., 2005; Zhang *et al*., 2009). In contrast, pyruvate is the major precursor for by-product synthesis in the corresponding strains. Inactivation of the PTS system in the aerobic and anaerobic succinate producers of *E. coli* improved succinate production and decreased the accumulation of by-products (Lin *et al*., 2005; Zhang *et al*., 2009). *Corynebacterium glutamicum* possesses two carboxylation enzymes, phosphoenolpyruvate carboxylase (Mori and Shiio, 1985; Eikmanns *et al*., 1989) and pyruvate carboxylase (Peters-Wendisch *et al*., 1998). Although pyruvate carboxylase is the predominant carboxylation enzyme during aerobic growth of the wild type, PEP carboxylase also contributes to the anaplerotic flux (Petersen *et al*., 2000). In our previous study we showed that oxaloacetate formation by PEP carboxylase becomes more important during aerobic succinate production, since overexpression of the *ppc* gene led to an increased succinate yield (Litsanov *et al*., 2012b). Based on these observations an improved avail-

ability of PEP for PEP carboxylation and a reduced level of pyruvate might be responsible for the better succinate yield and reduced acetate formation from glycerol compared with glucose. Furthermore, the higher energy content of glycerol ($\Delta G_f^0 = -162.84$ kJ mol^{-1} C) compared with glucose ($\Delta G_f^0 = -152.87$ kJ mol^{-1} C) (Thauer *et al.*, 1977) due to its higher reduction status and the resulting higher ATP yield might positively influence succinate formation. The conversion of glycerol to dihydroxyacetone phosphate (DHAP) yields reducing equivalents in the form of menaquinol which can drive ATP synthesis via oxidative phosphorylation (Bott and Niebisch, 2003). In contrast, conversion of glucose to DHAP via the Embden–Meyerhof pathway is not accompanied by formation of NADH or menaquinol.

Utilization of glycerol for aerobic succinate production by C. glutamicum *BL-1/pVWEx1*-glpFKD

Corynebacterium glutamicum BL-1/pVWEx1-*glpFKD*, a derivative of strain Δ*sdhCAB* which lacks all known genes for acetate formation (Litsanov *et al.*, 2012b), grew on glycerol with a growth rate of 0.22 h^{-1} and completely consumed 375 mM glycerol within 22 h. Absence of the genes *pqo*, *ackA*, *pta* and *cat* coding for pyruvate : menaquinone oxidoreductase (PQO), acetate kinase (AK), phosphotransacetylase (PTA) and acetyl-CoA : CoA transferase (CoAT), respectively, reduced acetate formation during growth on glycerol by 61% compared with strain Δ*sdhCAB*/pVWEx1-*glpFKD* (Table 1). Remarkably, the final acetate concentration in strain BL-1/pVWEx1-*glpFKD* on glycerol was higher than that of strain BL-1 on glucose, a result which cannot be explained yet. Strain BL-1/pVWEx1-*glpFKD* achieved a 23% higher succinate titre (79 ± 6 mM), a 19% higher specific succinate productivity [1.55 mmol g (cdw)$^{-1}$ h^{-1}] and a 24% higher yield (0.21 mol mol^{-1}) than strain Δ*sdhCAB*/pVWEx1-*glpFKD*. The production results of strain BL-1/pVWEx1-*glpFKD* on glycerol were comparable to that of strain BL-1/pAN6-*pyc*^{P458S}*ppc*, which represents the currently best strain of *C. glutamicum* for aerobic succinate production from glucose (Litsanov *et al.*, 2012b). Plasmid pAN6-*pyc*^{P458S}*ppc* improves PEP/pyruvate carboxylation by combined overexpression of the genes for pyruvate carboxylase (with a P458S exchange) and PEP carboxylase.

Strain BL-1/pVWEx1-*glpFKD* showed a volumetric succinate productivity of 3.59 mM h^{-1}, which to our knowledge represents the highest value described in literature for aerobic succinate producers from glycerol. Additional improvements of this strain are possible by increasing anaplerotic C_3 carboxylation and applying growth-limiting conditions, as shown before for aerobic succinate production from glucose (Litsanov *et al.*, 2012b). Furthermore, the genomic integration of the *E. coli glpFKD* genes under control of a strong constitutive promoter such as the *tuf* promoter (Litsanov *et al.*, 2012a) could have a positive effect by eliminating the requirements for plasmid maintenance and improving strain stability. For industrial application, the *C. glutamicum* strains have to be tested and if necessary improved with respect to their tolerance to raw glycerol, which often contains growth-inhibitory impurities (Moon *et al.*, 2010).

In summary, the present study describes for the first time the efficient use of glycerol as substrate for aerobic succinate production with metabolically engineered *C. glutamicum*. The described process shows two unique features: (i) the highest known volumetric productivity of all currently described microbial strains for aerobic succinate production from glycerol and (ii) the use of minimal medium. Taken together, these two advantages and the opportunities for strain and process optimization makes the current process an interesting alternative to anaerobic fermentations.

Conflict of interest

None declared.

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