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 C₄-dicarboxylate offering interesting prospects for the chemical industry as feedstock for the production of a large variety of important bulk chemicals, such as tetrahydrofuran (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...
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 ΔsdhCAB (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 ΔsdhCAB 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 C3 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).

**Results and discussion**

**Utilization of glycerol for aerobic succinate production by C. glutamicum ΔsdhCAB/pVWE1-glpFKD**

We previously showed that the strains C. glutamicum ΔsdhCAB 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 pVWE1-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...
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 \textit{pO}_2 > 30\% saturation. During cultivation growth (\textit{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 \textit{C. glutamicum \Delta sdhCAB} and \textit{C. glutamicum BL-1} (Fig. 2, Table 1). Except for the carbon source, the cultivation conditions were the same as previously published (Litsanov \textit{et al.}, 2012b).

\textit{Corynebacterium glutamicum \Delta sdhCAB/pVWE\textit{x}1-glplFKD} 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 \textit{C. glutamicum \Delta sdhCAB} [32 mmol carbon g (cdw)\(^{-1}\) h\(^{-1}\)]. The different uptake mechanisms may play a role in this context. Whereas

**Fig. 2.** Biomass formation (A), substrate consumption (B), succinate production (C) and acetate production (D) of \textit{C. glutamicum \Delta sdhCAB/pVWE\textit{x}1-glplFKD} (filled squares, \(n = 3\)) and \textit{C. glutamicum BL-1/pVWE\textit{x}1-glplFKD} (filled circles, \(n = 3\)) on glycerol and of \textit{C. glutamicum \Delta sdhCAB} (open squares, \(n = 4\)) and \textit{C. glutamicum BL-1} (open circles, \(n = 2\)) on glucose. The values for strains \textit{\Delta 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 \textit{pO}_2 > 30\% using a Multifors bioreactor system as described previously (Litsanov \textit{et al.}, 2012b). The results displayed are average data including standard deviation from at least three experiments except for \textit{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.

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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 (D-glucose concentration multiplied by factor of 6, glycerol concentration multiplied by factor of 3).

| Substrate | Biomass consumed (mmol carbon (cdw) h⁻¹) | Substrate uptake rate (mmol g (cdw) h⁻¹) | Final succinate titre (mM) | Yield succinate/substrate (mol mol⁻¹) | Succinate production rate [mmol g⁻¹ (cdw) h⁻¹] | Substrate consumption rate [mmol g⁻¹ (cdw) h⁻¹] | PEP concentrations in the cell, a situation which was 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 substrate for anaplerotic C₃ carboxylation via phosphoenolpyruvate carboxylase (PEPCase) 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 availa-
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 (ΔG^° = −162.84 kJ mol⁻¹ C) compared with glucose (ΔG^° = −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 ΔsdhCAB 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 pqq, ackA, pta and cat coding for pyruvate : menaquinone oxidoreductase (PQQ), acetate kinase (AK), phosphotransacetylase (PTA), and acetyl-CoA : CoA transferase (CoAT), respectively, reduced acetate formation during growth on glycerol by 61% compared with strain ΔsdhCAB/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 ΔsdhCAB/pVWEx1-glpFKD. The production results of strain BL-1/pVWEx1-glpFKD on glycerol were comparable to that of strain BL-1/pAN6-ycp^ppc which represents the currently best strain of C. glutamicum for aerobic succinate production from glucose (Litsanov et al., 2012b). Plasmid pAN6-ycp^ppc^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 anaerobic C₃ 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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