Minireview

Bio-based production of organic acids with Corynebacterium glutamicum

Stefan Wieschalka,1† Bastian Blombach,2† Michael Bott3 and Bernhard J. Eikmanns1*
1Institute of Microbiology and Biotechnology, University of Ulm, D-89069 Ulm, Germany.
2Institute of Biochemical Engineering, University of Stuttgart, D-70569 Stuttgart, Germany.
3Institute for Bio- und Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, D-52425 Jülich, Germany.

Summary
The shortage of oil resources, the steadily rising oil prices and the impact of its use on the environment evokes an increasing political, industrial and technical interest for development of safe and efficient processes for the production of chemicals from renewable biomass. Thus, microbial fermentation of renewable feedstocks found its way in white biotechnology, complementing more and more traditional crude oil-based chemical processes. Rational strain design of appropriate microorganisms has become possible due to steadily increasing knowledge on metabolism and pathway regulation of industrially relevant organisms and, aside from process engineering and optimization, has an outstanding impact on improving the performance of such hosts. Corynebacterium glutamicum is well known as workhorse for the industrial production of numerous amino acids. However, recent studies also explored the usefulness of this organism for the production of several organic acids and great efforts have been made for improvement of the performance. This review summarizes the current knowledge and recent achievements on metabolic engineering approaches to tailor C. glutamicum for the bio-based production of organic acids. We focus here on the fermentative production of pyruvate, L- and D-lactate, 2-ketoisovalerate, 2-ketoglutarate, and succinate. These organic acids represent a class of compounds with manifold application ranges, e.g. in pharmaceutical and cosmetics industry, as food additives, and economically very interesting, as precursors for a variety of bulk chemicals and commercially important polymers.

Introduction
The depletion of earth’s fossil energy resources, accompanied by the strong impact of their use on the environment, particularly in form of higher CO2 emissions, raises the demand and the consumer pull for sustainable, safe and efficient substitution of hitherto crude oil derived chemicals and chemical building blocks from renewable resources. Besides chemical manufacturing of renewable feedstocks to valuable compounds, biotechnological processes afford more and more opportunities to produce fuels, building blocks, and solvents in a cost-effective way from biomass (Bozell and Petersen, 2010). Chemical building blocks, such as some organic acids, serve as precursors for a variety of bulk chemicals and commercially important polymers (Werpy and Petersen, 2004). The cost-effective bio-based production of these chemicals is a most relevant goal for the future and has to meet economic and environmental requirements. Therefore, the microbial production systems have to perform excellent with regard to yield, productivity, product purity and flexibility to substrate consumption.

Corynebacterium glutamicum is a Gram-positive facultative anaerobic organism that grows on a variety of sugars, organic acids, and alcohols as single or combined carbon and energy sources (Eggeling and Bott, 2005; Liebl, 2006; Nishimura et al., 2007; Takeno et al., 2007). The organism is generally regarded as safe (GRAS status) and is traditionally employed for large scale production of amino acids, such as L-glutamate (> 2 million t/a) and L-lysine (> 1.4 million t/a) (Eggeling and Bott,
Production of pyruvate

Pyruvate is broadly used as ingredient or additive in food, cosmetics and pharmaceuticals, but also for the synthesis of various chemicals and polymers (Li et al., 2001; Zhu et al., 2008). Chemical production of pyruvate is realized by dehydration and decarboxylation of tartaric acid, but in a cost-ineffective way (Howard and Fraser, 1932; Li et al., 2001). Different approaches were made for pyruvate production with eukaryotic microorganisms like multi-auxotrophic yeasts (reviewed in Li et al., 2001); however, prokaryotic microorganisms, such as E. coli and C. glutamicum, also were successfully engineered to produce pyruvate.

Pyruvate is a central intermediate in the carbon and energy metabolism (see Fig. 1) in all organisms and thus, for construction of an efficient pyruvate-producing C. glutamicum strain, the major pyruvate-drawing reactions had to be downregulated or even eliminated. In the course of the molecular analysis of the pyruvate dehydrogenase complex (PDHC), Schreiner and colleagues (2005) inactivated this complex in C. glutamicum by deletion of the aceE gene, encoding the E1p subunit of the PDHC. The resulting strain C. glutamicum ΔaceE required acetate or ethanol as an additional carbon source for growth on glucose (Schreiner et al., 2005; Blombach et al., 2009). In an approach to engineer C. glutamicum for L-valine production, Blombach and colleagues (2007) observed that C. glutamicum ΔaceE showed a relatively high intracellular concentration of pyruvate and, when acetate was exhausted from the medium and growth stopped, secreted significant amounts of L-alanine (30 mM), L-valine (30 mM), and pyruvate (30 mM) from glucose. In subsequent studies, the PDHC-deficient strain turned out to be an excellent starting point to engineer C. glutamicum for the efficient production of L-valine (Blombach et al., 2007; 2008; 2009; Krause et al., 2009), isobutanol (Blombach et al., 2011), and also of 2-ketoisovalerate and 2-ketogluutarate (Takahashi et al., 2012). The additional inactivation of the pyruvate:quinone oxidoreductase (PQO) and NADH-dependent L-lactate dehydrogenase (L-LDH) significantly improved pyruvate formation (Wieschalka et al., 2012). In shake-flask experiments, C. glutamicum ΔaceE Δpqo ΔldhA accumulated in a growth-decoupled manner about 50 mM pyruvate with a substrate-specific product yield (YP/s) of 0.48 mol per mol of glucose, aside from L-alanine (29 mM) and L-valine (21 mM) as by-products (Wieschalka et al., 2012). To abolish overflow metabolism towards L-valine, the native acetylcoenzyme A synthase (AHAS) was substituted by a leaky variant (ΔC-T llnV) leading to an almost threefold increased YP/s of 1.36 mol pyruvate per mol of glucose, and a strong increase of pyruvate production (up to 193 mM), while L-valine and L-alanine formation were reduced to 1 mM and 9 mM respectively (Wieschalka et al., 2012). Additional deletion of the genes encoding alanine aminotransferase (AlaT) and valine-pyruvate aminotransferase
**Organic acid production with Corynebacterium glutamicum**

**Fig. 1.** Schematic presentation of the central carbon metabolism of *C. glutamicum* including pathways for the degradation of carbon sources (glucose, glycerol, D-cellobiose, L-arabinose, D-xylose, mannose, formate, acetate) used for the production of pyruvate, D,L-lactate, 2-ketoisovalerate, 2-ketoglutarate and succinate. Ellipses represent enzymes and transport systems present in *C. glutamicum*. Rectangles represent heterologous enzymes.

**Abbreviations:** Coding genes are given in brackets. 6PG, 6P-gluconate; 6PGDH (*gnd*), 6PG dehydrogenase; AHAIR (*ilvC*), acetohydroxyacid isomeroreductase; AHAS (*ilvBN*), acetohydroxyacid synthase; AK (*ack*), acetate kinase; AlaT (*alaT*), alanine aminotransferase; AraA (*araA* from *E. coli*), arabinose isomerase; AraB (*araB* from *E. coli*), ribulokinase; AraD (*araD* from *E. coli*), L-ribulose-5-phosphate 4-epimerase; AraE (*araE* from *E. coli*), L-arabinose transporter; AvtA (*avtA*), valine-pyruvate aminotransferase; BglA (*bglA1*, *bglA2*), phospho-β-glucosidases; BglF (*bglF*V317A), mutated PTS permease enabling D-cellobiose import; CtfA (*cat*), CoA transferase A; DHAD (*ilvD*), dihydroxyacid dehydratase; DHAP, dihydroxyacetone-P; F1,6P, fructose-1,6P; F6P, fructose-6P; FDH (*fdh* from *Mycobacterium vaccae*), formate dehydrogenase; Fum (*fum*), fumarase; GAP, glyceraldehyde-3P; GAPDH (*gapA*), GAP dehydrogenase; GlpD (*glpD* from *E. coli*), glycerol-3P dehydrogenase; GlpF (*glpF* from *E. coli*), glycerol facilitator; GlpK (*glpK* from *E. coli*), glycerol kinase; G6P, glucose-6P; G6PDH (*zwf*, *opcA*), G6P dehydrogenase; ICD (*icd*), isocitrate dehydrogenase; ICL (aceA), isocitrate lyase; LDH (native *ldhA* or *ldhA* from *L. delbrueckii*), L- and D-lactate dehydrogenase respectively; MalE (*malE*), malic enzyme; MctC (*mctC*), monocarboxylic acid transporter; Mdh (*mdh*), malate dehydrogenase; MDO (*mqo*), malate:quinone oxidoreductase; MS (*aceB*), malate synthase; ODHC (*odhA*, *aceF*, *lpd*), 2-oxoglutarate dehydrogenase complex; ODx (*odx*), oxaloacetate decarboxylase; PCx (*ppc*), pyruvate carboxylase; P, phosphate; PDHC (aceE, aceF, *lpd*), pyruvate dehydrogenase complex; PEPCk (*pck*), PEP carboxykinase; PEPCx (*ppc*), PEP carboxylase; Pyk (*pyk*), pyruvate kinase; PMI (*manA*), phosphomannose isomerase; PQO (*pqo*), pyruvate: quinone oxidoreductase; PTA (*pta*), phosphotransacetylase; PTS (*ptsG*, *hpr*, *ptsI*), phosphotransferase system; Rpe (*rpe*), ribulose-5-phosphate epimerase; SDH (sdhABC), succinate dehydrogenase; TA (*ilvE*), transaminase B; XylA (*xylA* from *E. coli*), xylose isomerase; XylB (*xylB* from *E. coli*), xylulokinase.

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(AvtA) resulted in cumulative reduction of L-alanine as undesired by-product by 50% (Wieschalka et al., 2012). With the final strain *C. glutamicum* `ΔaceE Δpqo ΔldhA ΔC-T ilvN ΔalaT ΔavtA` (designated as *C. glutamicum* ELB-P; see Fig. 2) up to 200 mM pyruvate were formed in shake-flask experiments, with a Y_{P/S} of 1.49 mol per mol of glucose. The yields of the by-products L-alanine and L-valine were evanescent low with 0.03 and 0.01 mol per mol of glucose respectively (Wieschalka et al., 2012). To study the relevance for industrial applications, fed-batch fermentations were performed with *C. glutamicum* ELB-P. When *C. glutamicum* ELB-P was cultivated with a constant pO₂ of about 30% a twofold lower glucose consumption rate (0.28 mmol g cell dry weight⁻¹ h⁻¹) and a significantly lower Y_{P/S} (0.8 mol pyruvate per mol of glucose) were observed when compared with shake-flask experiments (0.58 mmol g(CDW⁻¹ h⁻¹) and 1.49 mol pyruvate per mol of glucose respectively). Implementation of low oxygen tension from the middle until the end of growth phase restored the production performance and led to the formation of more than 500 mM (45 g l⁻¹) pyruvate with a Y_{P/S} of 0.97 mol pyruvate per mol of glucose in the production phase (Wieschalka et al., 2012). In comparison, the best pyruvate-producing *E. coli* strains (*E. coli* YYC202 and ALS1059) produced under optimized process conditions about 720 mM (63 g l⁻¹) and 1 M (90 g l⁻¹) pyruvate, with Y_{P/S} of 1.74 and 1.39 mol pyruvate per mol of glucose respectively (Zelic et al., 2003; Zhu et al., 2008). Since the yield of *C. glutamicum* ELB-P in shake-flask experiments is in the same range as in these *E. coli* strains, further process optimization might disclose the whole potential of *C. glutamicum* ELB-P for a further improved pyruvate production process.

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Production of lactate

Lactate is widely used as both D- and L-isomers for pharmaceutical, cosmetic, leather and textile, chemical, biomedical and food industries, as well as for green solvent and biodegradable fibre and polymer production (Hofvendahl and Hahn-Hägerdal, 2000; Bozell and Petersen, 2010; Okano et al., 2010). Especially the latter, in form of the stereocomplex of D- and L-polyactic acid is a fully biodegradable substitute for polyethylene terephthalates and therefore, of great economical interest (Lorenz and Zinke, 2005; Dodds and Gross, 2007; Fukushima and colleagues, 2003b) (see Table 1). However, these bacteria have a demand for complex media, which makes the cultivation of the organisms and the purification of the product relatively cost-intensive. Therefore, other less fastidious organisms, such as metabolically engineered E. coli, Saccharomyces cerevisiae and C. glutamicum have also been developed for efficient L- and D-lactic acid production (Okano et al., 2010).

Corynebacterium glutamicum is facultatively anaerobic and grows aerobically and anaerobically in the presence of oxygen and nitrate respectively (Nishimura et al., 2007; Takeno et al., 2007). The lack of oxygen or nitrate as external electron acceptors results in growth arrested cells, which still have the capability to ferment C6 sugars to L-lactate and succinate as major products. Dominguez and colleagues (1993) firstly reported that C. glutamicum forms lactate, succinate and acetate at small amounts when oxygen is limited during aerobic growth. Inui and colleagues (2004a) further studied this phenomenon in an attempt to utilize corynebacterial properties for the industrial production of lactate and succinate. These authors reported of organic acid production with C. glutamicum strain R and described that the bacteria showed no growth under oxygen-deprivation conditions, but produced significant amounts of L-lactate (~220 mM) and succinate (~20 mM) from about 130 mM glucose. Addition of bicarbonate to the medium led to an increase of the NAD+/NADH ratio and, probably as consequence of a derepression of the glyceraldehyde-3-phosphate dehydrogenase gene gapA, to an increased glucose consumption (Inui et al., 2004a). Furthermore, the addition of bicarbonate led to an altered product spectrum, i.e. the formation of succinate and lactate increased by a factor of two to four and significant concentrations (about 10 mM) of acetate were formed (Inui et al., 2004a; Okino et al., 2005). In a high cell density [30 g dry cell weight (DCW) l−1] fed-batch system, C. glutamicum R already produced 574 mM L-lactate (i.e. 53 g l−1), with only small amounts (<10 mM) of succinate and acetate as side-products (Okino et al., 2005). Addition of 400 mM bicarbonate raised the L-lactate concentration to more than 1 M (97.5 g l−1), but also the concentrations of the by-products succinate (192 mM) and acetate (50 mM) (Okino et al., 2005). Even without genetic modification of C. glutamicum, the resulting L-lactate titre from glucose and the YPS of 1.79 mol L-lactate per mol of glucose (i.e. 0.90 g g−1) are highly competitive as e.g. the best known metabolically engineered L-lactate-producing E. coli strain SZ85 (pflB, frdBC, adhE, ackA, ldhA::idhL, overexpressed ldhL gene from Pediococcus acidilactici) accumulated 505 mM L-lactate (i.e. 46 g l−1) with an optical purity of >99% and a YPS of 1.9 mol per mol of glucose (i.e. 0.95 g g−1; Zhou et al., 2003b) (see Table 1).

For D-lactate production with C. glutamicum, a L-LDH-deficient mutant was constructed, expressing the D-lactate dehydrogenase (D-LDH) from Lactobacillus delbrueckii (Okino et al., 2008b). Under oxygen-deprivation conditions, this mutant (C. glutamicum R ΔldhA/pCRB204) produced in a high cell density system (60 g DCW l−1) about 1.34 M D-lactate (i.e. 120 g l−1) within 30 h with an optical purity of >99.9% and a YPS of 1.73 mol per mol of glucose. But also significant amounts of succinate (146 mM) and acetate (52 mM) were formed, underlining product purity as major problem (Okino et al., 2008b). However, C. glutamicum R ΔldhA/pCRB204 produced more D-lactate than E. coli JP203 (pta, ppc) (Chang et al., 1999) and SZ63 (W3110; pflB, frdBC, adhE, ackA) (Zhou et al., 2003a) (Table 1), the best known genetically defined D-lactate producing E. coli strains, harbouring the native D-LDH of E. coli. With about 690 mM (62 g l−1) and 530 mM (48 g l−1), these strains formed approximately half of the titre obtained with C. glutamicum R ΔldhA/pCRB204, however, with comparable YPS of between 1.76 and 1.92 mol D-lactate per mol of glucose (0.90–0.99 g D-lactate per g of glucose; Chang et al., 1999; Zhou et al., 2003a).

It has to be noted that all described C. glutamicum and E. coli processes have to compete with those using recombinant yeast strains (Saccharomyces and Kluyveromyces) that produce L-lactic acid with titres of up to 1.3 M, optical purity of >99.9%, and YPS of up to 1.6 mol L-lactate per mol of glucose (Saitoh et al., 2005; Okano et al., 2010).

Production of 2-ketoisovalerate and 2-ketoglutарате

In nature, 2-ketoisovalerate (3-methyl-2-oxobutanoic acid) is a precursor for L-valine, L-leucine, and pantothenate synthesis in bacteria and plants. In these organisms, it is formed from two molecules of pyruvate via the reactions catalysed by AHAS, acetohydroxyacid isomerodeuctase (AHAIR), and dihydroxyacid dehydratase.
Table 1. Maximal titres, substrate-specific yields \((Y_{\text{P/S}})\), productivities, by-products and the respective references of the so far most efficient processes for organic acid production with \(C.\ glutamicum\) and \(E.\ coli\) strains.

| Strain          | Medium                                      | Maximal titre \((\text{mM})\) \((\text{g l}^{-1})\) | \(Y_{\text{P/S}}\) \((\text{mol product per mol substrate})\) \((\text{g g}^{-1})\) | Productivity\(^a\) \((\text{mM h}^{-1})\) \((\text{g l}^{-1} \text{ h}^{-1})\) | By-products\(^b\) | Reference                      |
|-----------------|---------------------------------------------|-----------------------------------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-------------------|--------------------------------|
| Pyruvate        |                                             |                                                     |                                                                                |                                                                                |                   |                                 |
| \(C.\ glutamicum\) ELB-P | minimal medium, glucose                      | 512 (44.5)                                          | 1.49 (0.72)                                                                    | 5.6 (0.49)                                                            | --                | Wieschalka et al. (2012)        |
| \(E.\ coli\) ALS1059 | minimal medium, glucose, L-isoleucine, betaine | 1022 (88.9)                                        | 1.39 (0.67)                                                                    | 23.9 (2.08)                                                            | --                | Zhu et al. (2008)              |
| \(E.\ coli\) YYC202 | minimal medium, glucose                      | 720 (62.6)                                          | 1.74 (0.84)                                                                    | 37.0 (3.22)                                                            | --                | Zelic et al. (2003)            |
| \(C.\ glutamicum\) R | minimal medium, glucose                      | 574 (51.1)                                          | 1.42 (0.70)                                                                    | 71.8 (6.39)                                                            | acetate, succinate | Okino et al. (2005)            |
| \(E.\ coli\) YYC202 | minimal medium, glucose, bicarbonate         | 1061 (94.4)                                         | 1.79 (0.89)                                                                    | 176.8 (15.74)                                                          | acetate, succinate | Okino et al. (2005)            |
| \(E.\ coli\) S285 | minimal medium, glucose                      | 505 (44.9)                                          | 1.90 (0.94)                                                                    | 7.2 (0.64)                                                             | --                | Zhou et al. (2003b)            |
| \(C.\ glutamicum\) Beta-lactate | minimal medium, glucose                    | 1340 (119.3)                                         | 1.73 (0.86)                                                                    | 44.5 (3.96)                                                            | acetate, succinate | Okino et al. (2008b)           |
| \(E.\ coli\) JP203 | complex medium, glucose                      | 691 (61.5)                                          | 1.80 (0.89)                                                                    | 11.6 (1.03)                                                            | --                | Chang et al. (1999)            |
| \(E.\ coli\) S263 | minimal medium, glucose                      | 528 (47.0)                                          | 1.92 (0.95)                                                                    | 9.8 (0.87)                                                             | --                | Zhou et al. (2003a)            |
| \(2\)-Ketoglutarate |                                             |                                                     |                                                                                |                                                                                |                   |                                 |
| \(C.\ glutamicum\) \(\Delta\) \(\text{ldhA}\) pCRB204 | minimal medium, glucose                    | 188 (21.8)                                           | 0.56 (0.36)                                                                    | 4.6 (0.53)                                                            | L-valine          | Krause et al. (2010)           |
| \(E.\ coli\) JP203 | complex medium, glucose                      | 82 (9.1)                                            | n.s.\(^\text{d}\)                                                               | 2.7 (0.39)                                                             | --                |                                 |
| \(C.\ glutamicum\) \(\Delta\) \(\text{aceE}\) \(\Delta\) \(\text{pqo}\) \(\Delta\) \(\text{ilvE}\) \(\text{pJC4ilvBNCD}\) | minimal medium, glucose, yeast extract | 325 (47.5)                                          |                                                                                  |                                                                                |                   |                                 |
| Succinic acid \((\text{anaerobic})\) |                                             |                                                     |                                                                                |                                                                                |                   |                                 |
| \(C.\ glutamicum\) \(\Delta\) \(\text{gdh}\) \(\Delta\) \(\text{gltB}\) \(\Delta\) \(\text{aceA}\) \(\text{pCRA717}\) | minimal medium, glucose, bicarbonate | 1240 (146.3)                                         | 1.40 (0.92)                                                                    | 27 (3.19)                                                             | acetate           | Okino et al. (2008a)           |
| \(C.\ glutamicum\) \(\text{ELB-P}\) | minimal medium, glucose                      | 330 (38.9)                                          | 1.02 (0.67)                                                                    | 5.6 (0.66)                                                             | pyruvate          | S. Wieschalka and B.J. Eikmanns, own unpubl. data |
| \(C.\ glutamicum\) \(\text{BOL-3/pAN6-gap}\) | saline, glucose, formate, bicarbonate   | 1134 (133.8)                                         | 1.67 (1.09)                                                                    | 21 (2.48)                                                             | 2-oxoglutarate, acetate, fumarate, malate acetate, formate  | Litsanov et al. (2012b) |
| \(E.\ coli\) SBS550MG/pHL413 | complex medium, glucose                    | 330 (38.9)                                          | 1.61 (1.06)                                                                    | 10 (1.18)                                                             | acetate, pyruvate, malate acetate, pyruvate, malate      | Sánchez et al. (2005) |
| \(E.\ coli\) KJ134 | minimal medium, glucose                      | 606 (71.5)                                          | 1.53 (1.00)                                                                    | 6.4 (0.76)                                                             | acetate           | Jantama et al. (2008)  |
| Succinic acid \((\text{aerobic})\) |                                             |                                                     |                                                                                |                                                                                |                   |                                 |
| \(C.\ glutamicum\) \(\text{BL-1/pAN6-pyc}\) \(\Delta\) \(\ppc\) | minimal medium, glucose          | 90 (10.6)                                            | 0.45 (0.30)                                                                    | 0.8 (0.09)                                                             | 2-oxoglutarate, acetate, pyruvate acetate, pyruvate pyruvate | Litsanov et al. (2012a) |
| \(C.\ glutamicum\) BL-1 \(\text{pVWEx1-glKFKD}\) | minimal medium, glycerol                | 79 (9.3)                                             | 0.21 (0.27)                                                                    | 3.6 (0.42)                                                             | acetate           | Litsanov et al. (2012c)        |
| \(E.\ coli\) HLS1276k(pKK313) | complex medium, glucose, bicarbonate | 70 (8.3)                                             | 1.09 (0.71)                                                                    | 1.2 (0.14)                                                             | acetate, pyruvate, malate acetate, pyruvate, malate      | Lin et al. (2005) |
| \(E.\ coli\) HLS2765k(pKK313) | complex medium, glucose, bicarbonate | 60 (7.1)                                             | 0.95 (0.62)                                                                    | 2.3 (0.27)                                                             | acetate           | Lin et al. (2005)              |

\(^a\) During production phase.
\(^b\) Significant concentrations above 10 mM.
\(^c\) -- = byproducts below 10 mM.
\(^d\) n.s. = not specified.
(DHAD) (see Fig. 1). 2-Ketoisovalerate is used as substitute for L-valine or L-leucine in chronic kidney disease patients (Teschan et al., 1998; Feiten et al., 2005; Aparicio et al., 2009; 2012) and also has been used in therapy for uremic hyperphosphatemia (Schafer et al., 1994). To our knowledge, 2-ketoisovalerate for these purposes is mainly synthesized chemically by different methods (Cooper et al., 1983) and only very recently, directed fermentative production of 2-ketoisovalerate with microorganisms has been reported for the first time (Krause et al., 2010; see below).

Since 2-ketoisovalerate stems from two molecules of pyruvate (see Fig. 1) and a PDHC-deficient C. glutamicum secreted significant amounts of pyruvate and L-valine (see above), C. glutamicum ΔaceE was an excellent basis to engineer C. glutamicum for the production of this 2-ketoacid. To avoid transamination of 2-ketoisovalerate to L-valine, the ilvE gene encoding transaminase B was deleted, leading to an auxotrophy for branched chain amino acids. Aerobically, C. glutamicum ΔaceE ΔilvE formed about 76 mM pyruvate, 25 mM L-alanine, and 40 mM 2-ketoisovalerate in a growth-decoupled manner from glucose (Krause et al., 2010). Overexpression of the AHAS, AHAIR and DHAD genes shifted the product spectrum towards 2-ketoisovalerate and the resulting strain C. glutamicum ΔaceE ΔilvE Δpqo (pJC4ilvBNCD) produced in fed-batch fermentations about 85 mM 2-ketoisovalerate with a volumetric productivity of 1.9 mM h−1 and a YP/S of about 0.38 mol per mol of glucose. Although the PQO has been found to be dispensable for growth and a deletion was only slightly beneficial on L-valine production (Schreiner et al., 2006; Blombach et al., 2008), PQO inactivation turned out to be highly beneficial for 2-ketoisovalerate production. Compared with the parental strain, C. glutamicum ΔaceE ΔilvE Δpqo (pJC4ilvBNCD) showed in fed-batch fermentations more than two times higher final titres (up to 220 mM = 25.5 g l−1) and volumetric productivities of 4.6 mM h−1 (Krause et al., 2010; Table 1).

It is noteworthy to mention that the 2-ketoisovalerate-producer C. glutamicum ΔaceE ΔilvE Δpqo (pJC4ilvBNCD) was used as a basis for the generation of a series of C. glutamicum strains producing isobutanol via the so-called ‘Ehrlich pathway’ (Blombach and Eikmanns, 2011; Blombach et al., 2011). The most promising strain of this series, C. glutamicum Iso7, carries additional deletions of the δ-LDH and malate dehydrogenase genes (ΔmdhA and ΔmdhC respectively) and overexpresses additionally the E. coli transhydrogenase genes pntAB, the Lactococcus lactis ketoacid decarboxylase gene kivD, and the homologous alcohol dehydrogenase gene adhA (Blombach et al., 2011).

2-Ketogluutarate is an intermediate of the tricarboxylic acid (TCA) cycle and the precursor for the synthesis of glutamate and the glutamate family of amino acids. 2-Ketogluutarate is used in dairy industry (Banks et al., 2001; Gutiérrez-Mendéz et al., 2008) and also is suitable to treat chronic renal insufficiency in hemodialysis patients (Riedel et al., 1996). An enzymatic process to synthesize 2-ketoglutarate from glutamate via the coupled reactions of glutamate dehydrogenase and NADH oxidase has been established (Ödmann et al., 2004), however, this bioconversion seems not very efficient. Therefore, Jo and colleagues (2012) very recently used a glutamate-overproducing mutant of C. glutamicum for the construction of a 2-ketogluutarate-producer. Inactivation of the genes encoding glutamate dehydrogenase, glutamate synthase and isocitrate lyase (gdh, gitB, and aceA respectively) led to a drastic reduction of glutamate formation (< 10 mM) and concomitantly to 2-ketogluutarate accumulation to concentrations of up to 325 mM (47.5 g l−1) after 120 h of cultivation in medium containing glucose, molasses, glutamate, and soybean hydrolysate (Jo et al., 2012). To our knowledge, there were no other approaches to produce 2-ketogluutarate by fermentation with any other bacterium. However, Zhou and colleagues (2012) recently reported efficient 2-ketogluutarate production (up to about 380 mM) with a recombinant (‘non-conventional’) yeast strain of Yarrowia lipolytica with enhanced acetyl-CoA availability.

**Production of succinate**

The C4 dicarboxylic succinate has been denoted as ‘a LEGO® of chemical industry’ (Sauer et al., 2008) and as such, can be used as precursor for known petrochemical bulk products, such as 1,4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, maleic anhydride, various n-pyrrolidinones, and linear aliphatic esters (Zeikus et al., 1999; Sauer et al., 2008; Bozell and Petersen, 2010). Moreover, succinate (or succinic acid) is directly used as surfactant, ion chelator, and as an additive in pharmaceutical, and food industry (McKinlay et al., 2007). The market potential for succinic acid and its direct derivatives has been estimated to be 245 000 tons per year, that for succinic acid-derived polymers about 25 000 000 tons per year, and with the transition to cost-efficient bio-based production of succinate or succinic acid, the market is predicted to steadily increase (Werpy and Petersen, 2004; Bozell and Petersen, 2010).

Aside from L-lactate and acetate, succinate is a natural fermentative end-product of the wild type of C. glutamicum, when incubated with glucose under oxygen deprivation (Dominguez et al., 1993; Inui et al., 2004a). Under these conditions, succinate is formed via glycolysis, carboxylation of phosphoenolpyruvate (PEP) or pyruvate to oxaloacetate (OAA) by PEP carboxylase (PEPCx) and/or pyruvate carboxylase (PCx), and subsequent conversion
of OAA by malate dehydrogenase (Mdh), fumarase (Fum), and succinate dehydrogenase (SDH) (Inui et al., 2004a; see Fig. 1).

A two-stage succinate production process with *C. glutamicum* strain R was developed by Okino and colleagues (2008a), using a derivative devoid of LDH activity and overexpressing the native PCx gene (*pyc*), *C. glutamicum* R ΔldhA PCRA717. In a first step, cells of this strain were grown under fully aerobic conditions. Then, the cells were harvested, washed and transferred to closed bottles, to give a high cell density of about 50 g(DCW) l⁻¹. With repeated intermittent addition of glucose and sodium bicarbonate, a final titre of 1.24 M succinate (146 g l⁻¹) was obtained within 46 h, with a Y_{PS} of 1.4 mol per mol of glucose (Okino et al., 2008a). The cells did not form any lactate; however, they produced significant amounts of acetate (0.3 M = 16 g l⁻¹) as by-product.

Recently, also Litsanov and colleagues (2012b) engineered *C. glutamicum* ATCC 13032 for high yield succinate production by further extending the experimental approach by Okino et al. (see above). Deletion of the LDH gene, chromosomal integration of an allele for a deregulated PCx (pyc²₅₄₈₈⁵) and deletion of the genes encoding enzymes responsible for acetyl synthesis (∆act, ∆pgo, ∆pta-ack) resulted in *C. glutamicum* BOL-2, that produces up to 116 mM succinate with a Y_{PS} of 1.03 mol per mol of glucose, and pyruvate (23 mM) as well as 2-ketoglutarate (12 mM) as major by-products (Litsanov et al., 2012b). To increase NADH and CO₂ availability and to increase the glycolytic flux, the authors then integrated the formate dehydrogenase gene *fdh* from *Mycobacterium vaccae* into the genome of *C. glutamicum* BOL-2 and additionally overexpressed the homologous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (*gapA*) from plasmid. In a fed-batch fermentation with glucose, fumarate and bicarbonate as substrates, the ultimate strain *C. glutamicum* BOL-3/pAN6-gap (see Fig. 3) produced 1.13 M succinate (134 g l⁻¹) with a Y_{PS} of 1.67 mol per mol of glucose (Litsanov et al., 2012b). Aside from succinate, 2-ketoglutarate (35 mM), malate (33 mM), acetate (20 mM), fumarate (13 mM), and pyruvate (6 mM) were formed as by-products.

In a further approach, the pyruvate-producing strain *C. glutamicum* ELB-P (see above and Fig. 2) was employed for succinate production (S. Wieschalka and B. J. Eikmanns, unpublished). Due to the inactivation of the PDHC, PQQ, and LDH, this strain does not form significant amounts of acetate or lactate as by-products under any aerobic and anaerobic condition tested (Wieschalka et al., 2012). In contrast to the two-stage processes described above (i.e. aerobic growth in complex or minimal media and, after harvest of the cells and resuspension in new medium, transfer to sealed bottles or fermenters respectively; Okino et al., 2008a; Litsanov et al., 2012b), a one-stage fed-batch fermentation process with *C. glutamicum* ELB-P was established, combining biomass formation and succinate production in a single bioreactor. This process includes three phases: (i) an aerobic growth phase on glucose plus acetate, (ii) a self-induced microaerobic phase at the end of the exponential growth by minimal aeration, and (iii) an anaerobic production phase, realized by gassing the fermenter with CO₂ (Fig. 4). This optimized process led to growth-decoupled succinate production of more than 330 mM (i.e. 39 g l⁻¹) with a Y_{PS} of 1.02 mol succinate per mol of glucose. The final Y_{PS} obtained, together with the formation of pyruvate (about 30 mM) as by-product, however, still indicates a limitation, which might be overcome by increasing the carbon flux from PEP/pyruvate to OAA or by integration of the *M. vaccae* fdh gene and the use of formate as an additional substrate for reduction equivalents, as described above by Litsanov and colleagues (2012b).

The experimental setup of a one-stage process (consecutive aerobic growth and anaerobic production in a single bioreactor) as done with *C. glutamicum* ELB-P, see above) represents an industrially feasible process. However, a recent study on isobutanol production with *C. glutamicum* disclosed the differences in the production performance between two-stage fermentations (aerobic growth in complex or minimal media and anaerobic production in different containments, see above) and one-stage fermentations in a single bioreactor: The isobutanol Y_{PS} in the one-stage fermentation was significantly lower (0.48 mol vs 0.77 mol of isobutanol per mol of glucose), indicating that the transition from the aerobic environment (growth phase) to the anaerobic environment (production phase) has a strong impact on the overall production behaviour (Blombach and Eikmanns, 2011; Blombach et al., 2011). Similarly, Martinez and colleagues (2010) recently observed that introducing a microaerobic phase at the end of the aerobic growth phase of an *E. coli* succinate-producer led to an adjustment of the enzymatic machinery and to improved succinate production under anaerobic conditions. To our knowledge, the physiological changes of *C. glutamicum* during a (slow or fast) shift from aerobic to anaerobic conditions have so far not been investigated. However, it can be foreseen that the insight into the metabolic adaptation of the cells to such alternating culture conditions will help to further optimize organic acid production by novel metabolic engineering approaches and also by applying optimally adapted process conditions.

The Y_{PS} of the most efficient *E. coli* strains producing succinate under anaerobic conditions, *E. coli* SBS550MG/pHL413 and *E. coli* KJ134, were 1.60 mol and 1.53 mol succinate per mol of glucose respectively (Sánchez et al., 2005; Jantama et al., 2008; Table 1).
Thus, both recombinant E. coli strains and in particular, C. glutamicum BOL-3/pAN6-gap (Table 1) showed higher Y_{PS} than all known natural succinate-producing bacteria, such as Anaerospirillium succiniproducens (1.37 mol\,mol\,-^{1}\,of\,glucose; Glassner and Datta, 1992) or Mannheimia succiniproducens (1.16 mol\,mol\,-^{1}\,glucose; Lee et al., 2006). A further advantage of employing the recombinant C. glutamicum or E. coli strains is the potential use of mineral media, keeping production and purification costs lower than with Mannheimia or Anaerospirillum, which both require complex media. C. glutamicum BOL-3/pAN6-gap and C. glutamicum R \,\Delta\,ldhA pCRA717 produced about threefold higher succinate titres than E. coli SBS550MG/pHL413 (Table 1) and thus, Corynebacterium seems to be the superior organism for succinate production.

Very recently, Litsanov and colleagues (2012a) reported also on aerobic succinate production with C. glutamicum for the first time. Deletion of the SDH genes initiated aerobic succinate production in C. glutamicum via glycolysis, PEP and/or pyruvate carboxylation, the oxidative branch of the TCA cycle, and the glyoxylate shunt. Acetate formation was mostly prohibited by shutdown of the known pathways for acetate synthesis, resulting in C. glutamicum BL-1 (genotype: \Delta\,sdhCAB, \Delta\,cat, \Delta\,pqo, \Delta\,pta-ack; Litsanov et al., 2012a). To reduce carbon-loss into cell mass, nitrogen-limited growth conditions were established, forcing the cells into a resting state after a certain period. With additional, plasmid-bound overproduction of both PEPCx and the PCx^{45885}-variant, final succinate titres and Y_{PS} of up to 90\,mM and 0.45 mol succinate per mol of glucose, respectively, were observed.
Concerning the specific productivity of 1.6 mmol g (CDW)⁻¹ h⁻¹, *C. glutamicum* BL-1/pAN6-pycP458Sppc showed the highest value described so far for aerobic succinate production with bacteria. In comparison to other bacterial succinate producers, *C. glutamicum* BL-1/pAN6-pycP458Sppc is exceedingly competitive in aerobic succinate production (Table 1). Lin and colleagues (2005) described various *E. coli* strains approaching the maximal theoretical Y_{P/S} of about 1 mol succinate per mol of glucose under aerobic conditions. *Corynebacterium glutamicum* BL-1/pAN6-pycP458Sppc did not reach this high Y_{P/S}, but the recombinant *C. glutamicum* strains produced significantly higher final succinate titres in minimal instead of complex media (Table 1).

Production of organic acids with *C. glutamicum* from alternative substrates

Economical relevant and sustainable production of organic acids with microorganisms in an industrial scale is dependent on the use of low-cost carbon sources, in particular from renewable resources. So far, we focused in this review on the fermentative organic acid production from pretreated and purified carbon sources, such as glucose and glucose plus formate, since the most promising attempts to produce organic acids with *C. glutamicum* were made with these substrates. To simplify feedstock purchase and to improve the economic efficiency, utilization of alternative, crude materials is of great interest. However, *C. glutamicum* naturally cannot utilize certain industrially relevant substrates, such as glycerol, starch (from corn, wheat, rice, or potato), whey, straw, or hemicellulose and lignin, is a widely abundant and potentially attractive source of renewable feedstock. Hemicellulose, consisting largely of cellulose, hemicellulose, and lignin, is a widely abundant and potentially attractive source of renewable feedstock. Hemicellulose, consisting mainly of glucose but also to a significant portion of C5 sugars (xylose and arabinose) (Wiselogel *et al.*, 1996; Aristidou and Penttilä, 2000), can be depolymerized by chemical or enzymatic processes, and the resulting sugar mixtures are also of interest as alternative feedstock for *C. glutamicum*. Whereas some organisms (e.g. *E. coli*) are naturally able to consume the majority of sugars in the mixtures resulting from saccharification from hemicellulose, *C. glutamicum* needs metabolic engineering to expand the spectrum of sugars that can be utilized. Thus, the extension of the substrate spectrum of *C. glutamicum* to cheap, easily accessible and renewable monomeric and polymeric carbon sources is desired and therefore, an ongoing field of intensive research (Wendisch *et al.*, 2006b; Blombach and Seibold, 2010; Rumbold *et al.*, 2010; Okano *et al.*, 2010; Becker and Wittmann, 2011).

Several attempts have been made to broaden the natural substrate spectrum of *C. glutamicum* towards...
starch (Seibold et al., 2006; Tateno et al., 2007), whey (Barret et al., 2004), rice straw and wheat bran hydrolysates (Gopinath et al., 2011), grass and corn silages (Neuner et al., 2012), glucosides and D-cellobiose (Kotbra et al., 2003), glycerol (Rittmann et al., 2008), amino sugars (Gruteser et al., 2012; Ude et al., 2012) or pentose sugars for growth and for the production of amino acids or other value-added products (Blombach and Seibold, 2010; Jojima et al., 2010; Buschke et al., 2011; Schneider et al., 2011; Gopinath et al., 2012). The first approaches to extend the substrate spectrum especially for organic acid production were performed by Kawaguchi and colleagues (2006; 2008) and Sasaki and colleagues (2008; 2009). Plasmid-bound introduction of the xylose isomerase and xylulokinase genes (xylA and xylB respectively) from E. coli into C. glutamicum R enabled both aerobic growth on xylose as sole carbon source and production of L-lactate and succinate with resting cells under oxygen deprivation conditions (Kawaguchi et al., 2006). Although the sugar consumption rate and the specific productivity of the recombinant C. glutamicum CRX2 was lower with xylose than with glucose, the YPS for succinate was even higher on xylose (0.42 mol mol\(^{-1}\)) than on glucose (0.23 mol mol\(^{-1}\)). In contrast, the YPS for L-lactate was lower with xylose as substrate (1.06 and 1.36 mol mol\(^{-1}\) respectively; Kawaguchi et al., 2006). A similar behaviour was shown for succinate and L-lactate production from arabinose with C. glutamicum CRA1, which expresses the E. coli genes araA, araB and araD (encoding L-arabinose isomerase, L-ribulokinase, and L-ribulose-5-phosphate 4-epimerase respectively) and therefore is able to metabolize this C5 sugar (Kawaguchi et al., 2008). In this case, with 200 mM arabinose as substrate, the YPS for succinate and L-lactate were 0.67 mol mol\(^{-1}\) and 0.75 mol mol\(^{-1}\) respectively (Kawaguchi et al., 2008).

Co-utilization of different C5 sugars with C6 sugars was investigated to study catabolite repression effects in C. glutamicum and to expand sugar utilization on conditioned hemi- and lignocellulosic biomass hydrolysates (Sasaki et al., 2008). These efforts resulted in a C. glutamicum strain harbouring xylA and xylB as well as bglF\(^{317A}\) and bgIA (encoding PTS \(\beta\)-glucoside-specific enzyme IIbCA component and phospho-\(\beta\)-glucosidase respectively). This strain produced from a mixture of D-cellobiose (10 g l\(^{-1}\)), glucose (40 g l\(^{-1}\)), and D-xylose (20 g l\(^{-1}\)) about 460 mM L-lactate, 110 mM succinate, and 30 mM acetate under anaerobic conditions, with a combined yield of 0.85 g acids per g of sugar (Sasaki et al., 2008). A combined strain, containing all named modifications for D-xylose, L-arabinose and D-cellobiose consumption, and additionally overexpressing the arabinose transporter gene araE from C. glutamicum ATCC31831, was even able to consume glucose (35 g l\(^{-1}\)), D-xylose (17.5 g l\(^{-1}\)), L-arabinose (7 g l\(^{-1}\)), and cellobiose (7 g l\(^{-1}\)) simultaneously and completely under oxygen-deprived conditions within 14 h (Sasaki et al., 2009).

Recently, Sasaki and colleagues (2011) developed a C. glutamicum strain overexpressing the mannose 6-phosphate isomerase and fructose permease genes manA and ptsF respectively. This strain consumed mannose and glucose simultaneously and produced about 400 mM L-lactate, 100 mM succinate and 30 mM acetate from a sugar mixture of 200 mM glucose and 100 mM mannose under oxygen deprivation conditions (Sasaki et al., 2011).

Litsanov and colleagues (2012c) very recently showed aerobic succinate production with glycerol as sole carbon source, by plasmid-bound transfer of the glycerol utilizing genes glpFKD from E. coli into C. glutamicum BL-1. Glycerol is a main by-product of biodiesel and bioethanol production (Yazdani and Gonzalez, 2007) and using this carbon source for the production of value-added chemicals (such as succinate), the economic efficiency of these biofuel production processes can be increased (Wendisch et al., 2011). Plasmid pVWEx1-glpgFKD has previously been shown to enable growth and amino acid production of C. glutamicum on glycerol as sole carbon source (Rittmann et al., 2008). Consequently, using the conditions established for C. glutamicum BL-1/pAN6-pyc\(^{458S}\)ppc (see above), C. glutamicum BL-1 (pVWEx1-glpgFKD) aerobically produced up to 79 mM succinate (9.3 g l\(^{-1}\)) with a YPS of 0.21 mol per mol of glycerol (Litsanov et al., 2012c). The specific succinate productivity of C. glutamicum BL-1 pVWEx1-glpgFKD on glycerol was as high as for C. glutamicum BL-1/pAN6-pyc\(^{458S}\)ppc on glucose with 1.6 mmol g\(\text{(CDW)}^{-1}\) h\(^{-1}\). However, the volumetric productivity of 3.59 mM h\(^{-1}\) is the highest productivity so far described for aerobic succinate production (Litsanov et al., 2012c; Table 1).

In summary, the above mentioned studies showed the feasibility to expand the substrate spectrum of C. glutamicum to the main C5 and C6 sugars found in agricultural residues, in hydrolysed hemicellulose and lignocellulosic biomass, and to glycerol. For directed production of organic acids from hemicellulose feedstock, the modifications made for broadening the substrate spectrum and those made for optimal carbon flux to a desired organic acid must be combined. The successful aerobic production of succinate from glycerol instead of glucose by introduction of the glycerol utilizing genes from E. coli to C. glutamicum (Litsanov et al., 2012c; see above), is one such example and promises the feasibility of such approaches.

**Summary and outlook**

Driven by old and new knowledge and genome-based metabolic and genetic engineering strategies, C. glutami-
*Corynebacterium glutamicum* has become a major candidate as platform organism for bio-based, industrial production of a variety of organic acids from renewable biomass. As outlined above, and highlighted in Table 1, titres, YP/S, and productivities of recently developed *C. glutamicum* producer strains are highly competitive, in several cases already superior in comparison to other bacterial, well-established production systems. From current studies on transcriptome, proteome, metabolome, and intracellular fluxes (Vertes et al., 2012), from recent advances in evolutionary engineering tools (Becker and Wittmann, 2011), and from recent development of plasmid addiction systems (Schneider et al., 2012) and of single cell approaches (Binder et al., 2012; Mustafi et al., 2012), it can be expected that a variety of further metabolic (or genetic) targets for strain development/improvement will be identified in *C. glutamicum*. Future approaches to optimize organic acid production certainly will not only aim at substrate flexibility (low cost and eco-efficient feedstocks), product extension (e.g. fumarate, malate, or itaconate), and/or the paths from a substrate or substrate mixtures to the desired products (i.e. substrate uptake, central metabolism, precursor supply, synthetic pathways, and export of the respective organic acid). They will also focus on maintenance of a well-balanced redox state within the cells, on optimal adaptation of the cells to alternating culture conditions (e.g. shift from aerobic to anaerobic conditions), on strain robustness, and on an increased acid-resistance of the producer strains. Tolerance to organic acid stress represents a highly relevant factor for process design and downstream processing of large-scale production processes, since organic acid recovery from low pH fermentation broth in general is more cost-efficient than from neutral broth. However, the achievements obtained in the last 6 years and the wealth of new knowledge about the physiology, the metabolism and its regulation, and the proven production capabilities of *C. glutamicum* bode well for the implementation of this organism as a platform for new and even more (cost-)efficient processes for the production of a variety of organic acids and also of other specialty, fine and bulk chemicals.

**Conflict of interest**

None declared.

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