Review article

Succinic acid biosynthesis by *Corynebacterium glutamicum*

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Abstract: *Succinic acid is one of the most desirable raw materials. Currently, it is obtained mainly by hydrogenation of maleic acid derived from the C4 petroleum fraction. Corynebacterium glutamicum is considered as a possible microbiological producer of this acid. Under oxygen deprivation conditions this bacteria secretes L-lactate, succinate and acetate. Succinic acid is an intermediate of the Krebs cycle, in order to achieve high efficiency of its biosynthesis, metabolic engineering of C. glutamicum is required. The best producers described so far are C. glutamicum R ΔldhA pCRA717 (146 g/L) and C. glutamicum BOL-3/pAN6-gap (133 g/L). Succinic acid biosynthesis can also be achieved under aerobic conditions. C. glutamicum ZX1 (pEacsAgltA) produces aerobically 21.7 g/L of succinate. Genetic engineering is also necessary to enable the use of low-cost, waste carbon source such as glycerol or starch. The paper also presents and discusses examples of modifications of bacterial cells, allowing them to use these two carbon sources.*

Keywords: *Corynebacterium glutamicum, succinic acid, metabolic engineering.*

Introduction

Succinic acid (SA, 1.4- butanediolic acid) is one of 12 chemicals that could be produced from sugars, through microbial fermentation [1]. It was estimated that it is a component or substrate for more than 25 mln tons of industrial products per year. It is used for chemical synthesis of 1.4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, succinate salts, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate polyamides. It is also widely applied in the production of coatings, plasticizers, surfactants, detergents or foaming agents, as a component of ion chelators, various green solvents and biodegradable plastics. It could be exploited in food industry as acidulant, flavouring or anti-microbial agent as well as in health-related products (Figure 1) [2-7].

The global market for bio-succinate is currently worth about $ 200 million. It is predicted that its value will reach 496 million dollars in 2016. Rapid increase in demand for this raw material, from current 40 thousand tons per year to more than 2 million tons in 2020 is anticipated [5, 7].

At present, succinic acid is produced mainly by hydrogenation of maleic acid or its anhydride (1), which is synthesized chemically or obtained as a product of oxidation of n-butane (2), derived from the C4 petroleum fraction.

$$C_2H_2(CO)_2O + H_2 + H_2O \rightarrow C_4H_6O_4 \qquad (1)$$

$$3CH_3CH_2CH_2CH_3 + 14.5O_2 \rightarrow C_2H_2(CO)_2O + 4CO_2 + 4CO + 14H_2O \qquad (2)$$

Cost of succinic acid production is highly dependent on its purity, country of origin, cost of substrates and ranges from 6 to 9 $/kg [8]. The rising prices of the fossil resources have limited the use of petrochemical succinate for wide range applications. Therefore, there is a need to develop a cost-effective fermentation process. Environmental aspects of microbiological production of succinate are also very important. Since its introduction, CO_2 emission will be significantly reduced [9].

Only a few companies produce succinic acid in biotechnological way. BioAmber, which has recently launched production plants in France and Canada is the current market leader. Likewise, other bio-based bulk chemicals such as butanediol and tetrahydrofuran are produced by this company. Its main competitors are Myriant Technologies LLC and Royal DSM NV cooperating with Roquette Frères SA. Probably the biggest plant in Europe will be opened in Italy by Royal DSM NV. It will produce approximately 10.000 tonnes of succinic acid. BASF with the CSM are going to start production of succinate with *Basfia succiniciproducens* in the near future [10-11].

Metabolic basis of succinic acid production by *Corynebacterium glutamicum*

There are a number of microorganisms which could be used in industrial production of bio-based succinic acid. The most frequently mentioned are: *Actinobacillus succinogenes* [5, 12-16] *Anaerobiospirillum succiniciproducens* [17-21], *Mannheimia succiniciproducens* [22-24], *Corynebacterium glutamicum* [25-38], *Escherichia coli* [39-42] and *Yarrowia lipolytica* [43-44]. However, efficient and cost-effective method, which is competitive with the current applied chemical process is still sought. It was estimated that the biosynthesis will be profitable if the concentration of the final product reaches ~ 150 g/L or the productivity of 5 g/L/h [5].

One of the most promising producers of succinic acid is *Corynebacterium* glutamicum. This microorganism was isolated in 1957 by Kinoshita and co-workers. Kyowa Hakko Kogyo Co., Ltd., Tokyo, was searching for microorganisms able to produce and secrete large amounts of glutamic acid. Isolated then the wild strain of *C. glutamicum* has been described as a mesophilic, nonmotile, non-spore forming, Gram-positive, catalase-positive, morphologically variable soil microorganisms [45].

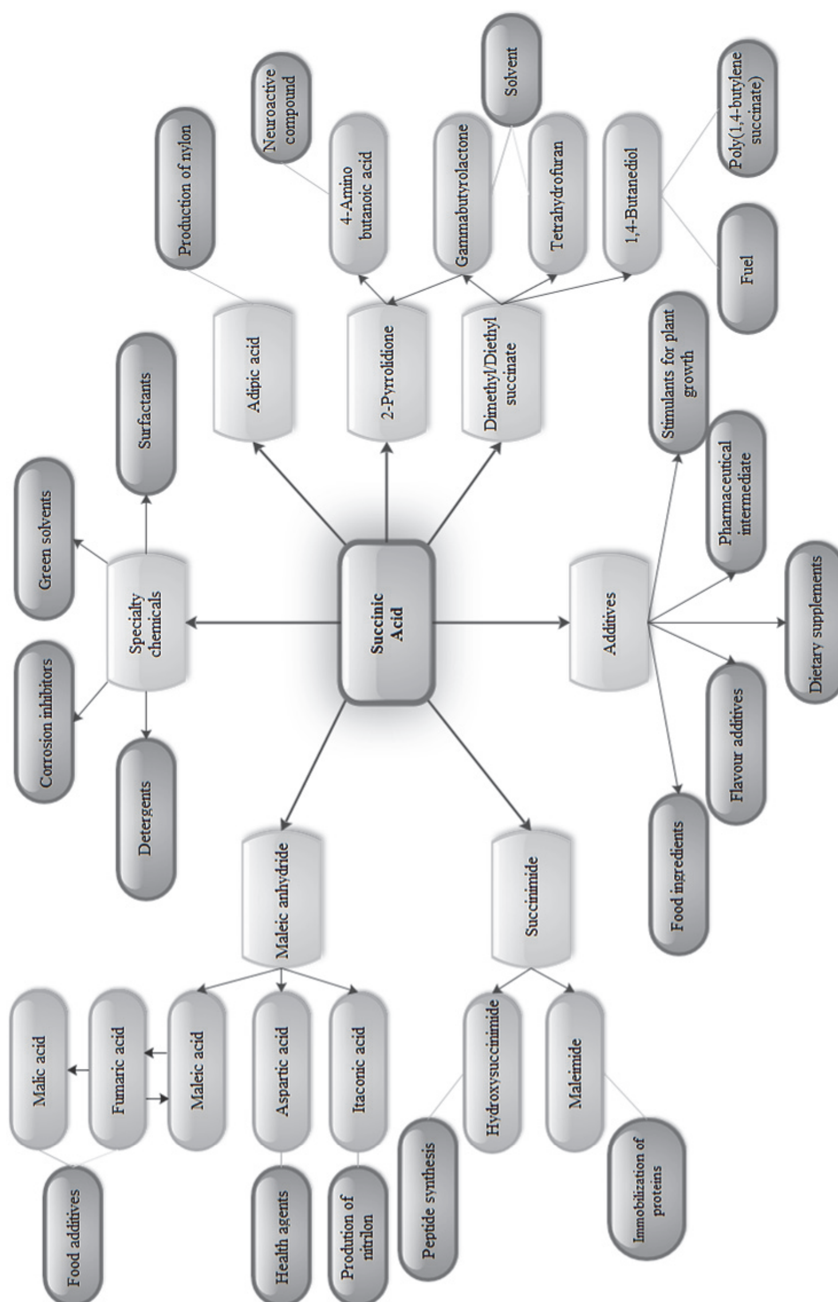

Figure 1. Applications of succinic acid

Due to the simple mechanisms of regulation of the primary metabolites biosynthesis, they are used in the biotechnology industry for the production of amino acids and nucleosides [46]. Knowledge about the genomes of several *C. glutamicum* strains has allowed for a better understanding of their physiology and to extend the range of fermentation products possible to obtain [35, 47, 48]. Modifications of the metabolism allow to produce a wide range of organic acids such as L- and D-lactic acid [49, 50], succinic acid [25-38], pyruvic acid [51], 2-ketoisovaleriate and 2-oxoglutarate [52, 53].

As a result of the oxygen and nitrogen sources (final electron acceptors) limitation, growth of *C. glutamicum* is inhibited [25, 26]. Despite the adverse conditions cells retain the ability to ferment carbohydrates. For the first time this property was described by Dominguez et al. (1993), who placed a thick suspension of *C. glutamicum* cells in mineral medium. After a few hours under oxygen deprivation conditions they observed organic acids formation. L-lactic acid was the main product (15.7 g/L), in addition small amounts of succinic and acetic acid were detected [25]. Preliminary studies in which *C. glutamicum* able to grow at temperatures above 40°C were used, shows that these strains, after 24-hour incubation in mineral medium are able to produce about 20 g/L of lactic acid. Among the fermentation products also acetic and succinic acids were found (unpublished results). This thesis shows that production of succinic acid by *C. glutamicum* requires not only the development of appropriate culture conditions, but first of all the genetic modifications of these microorganisms.

C. glutamicum is able to utilize monosaccharides such as glucose, fructose and ribose, disaccharides: sucrose, mannose, and maltose, alcohols: inositol or ethanol, organic acids: pyruvic acid, propionic acid, lactic acid, acetic acid and gluconic acid, and amino acids: L-glutamic acid and L-glutamine [54]. Different carbon sources derived from the environment are introduced at different levels to the central metabolic pathways such as glycolysis (Embden-Meyerhof-Parnas pathway, EMP), pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA) and glyoxylate cycle [55]. The most important central metabolite is pyruvate. It is produced mainly by glycolysis. It can be converted by lactate dehydrogenase (LDH), pyruvate carboxylase (PC), or pyruvate:quinone oxidoreductase (POQ) to L-lactate, oxaloacetate and acetate, respectively (Figure 2) [56]. Another important central metabolite is phosphoenolopyruvate (PEP). In oxygen deprivation conditions and in presence of CO_2 also phosphoenolpyruvate is carboxylated by PEPC to oxaloacetate. During succinic acid production the metabolism of bacteria requires to be directed towards transformation of pyruvate and phosphoenolpyruvate to oxaloacetate by appropriate carboxylases.

Inui et al. (2004) identified enzymes involved in the biosynthesis of organic acids and established their importance for cell metabolism. In their studies, they have considered: L-lactate dehydrogenase (LDH, gene *ldhA*),

Figure 2. Succinic acid production pathway in *Corynebacterium glutamicum*
AK (*ack*) – acetate kinase, CtfA (*cat*) – CoA transferase A, FDH (*fdh* from *Mycobacterium vaccae*) – formate dehydrogenase, Fum (*fum*) – fumarase, GAP– glyceraldehyde-3P, GAPDH (*gapA*) – glyceraldehyde-3P dehydrogenase, ICD (*icd*) – isocitrate dehydrogenase, ICL (*aceA*) – isocitrate dehydrogenase, LDH (*ldhA*) – L-lactate dehydrogenase, MctC (*mctC*) – monocarboxylic acid transporter, Mdh (*mdh*) – malate dehydrogenase, MQO (*mqo*) – malate:quinone oxidoreductase, MS (*aceB*) – malate synthase, ODHC (*odhA*, *aceF*, *lpd*) – oxoglutarate dehydrogenase complex, PC (*pyc*) – pyruvate carboxylase, PEP – phosphoenolpyruvate, PEPC (*ppc*) – PEP carboxylase, PTA – phosphotransacetylase, PQO (*pqo*) – pyruvate: quinone oxidoreductase, PTS – phosphotransferase system, Pyc (*pyk)* – pyruvate kinase, SDH (*sdhCAB*) – succinate dehydrogenase, SucE (*sucE*) – succinate exporter

phosphoenolpyruvate carboxylase (PEPC, gene *ppc*), pyruvate carboxylase (PC, gene *pyc*), phosphoenolpyruvate carboxykinase (PEPCK, gene *pckA*) fumarase (FUM, gene *fum*) and isocitrate lyase (ICL, gene *aceA*) [26]. Bacterial strains with induced deletion of certain genes were constructed. Comparison of enzyme activity, maintaining the balance of NAD^+ and NADH, and the glucose consumption rate between wild-type and recombinant strains, with induced deletion of certain genes can lead to some interesting conclusions. The *ΔldhA* recombinant showed no lactate dehydrogenase activity, therefore it can be deduced that there are no LDH isoenzymes in the genome of *C. glutamicum*. Elimination of lactate dehydrogenase effectively blocked the biosynthesis of lactic acid, but also it disturbed balance between NAD^+ and NADH. In consequence glycolysis and glucose consumption is inhibited and expected increase in the concentration of succinic acid is not observed. Constructing of *C. glutamicum* strains: *Δppc*, *Δpyc*, *ΔppcΔpyc*, *ΔpckA* allowed to determine the importance of enzymes involved in anaplerotic reactions which direct the carbon flux into the reducing arm of the TCA cycle. In absence of PEPC activity, radical reduction in the amount of succinic and lactic acid is noted. Also the ratio between NAD^+/NADH has been disturbed. The lack of this enzyme inhibits the subsequent reactions in TCA cycle reductive arm in which NADH is oxidized. This causes inhibition of GAPDH and following on from this the whole glycolysis. Deletions of other genes did not significantly affect the metabolism. The carbon source consumption rate and concentrations of co-substrates were similar to wild-type strain. Studying *ΔldhAΔppc* double mutants it was observed that the lack of activity of PEPC and LDH seriously impairs cell functioning. In limited oxygen conditions PEPC activity is critical for the regulation of the very significant metabolic node. This enzyme is responsible for providing the substrate – oxaloacetate for the reductive TCA cycle arm, whose function includes regeneration of NAD^+. The proof this statement strains with fumarase deletion were constructed. Lack of this enzyme also disrupts glycolysis [26].

An important factor, influencing the course of the metabolism, is the oxygen concentration in the fermentation medium. Its limitation affects the proportions between the available cofactors. Elevation of the amount of oxidized dinucleotide induces increase in the activity of certain enzymes involved in glycolysis, for example: glyceraldehyde-3P dehydrogenase (GAPDH), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK) and already mentioned anaplerotic enzymes responsible for the biosynthesis of lactate and succinate, such as phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) [26, 49, 50].

In *C. glutamicum* cells, glycolysis is regulated by a kind of a cascade involving enzymes responsible for the oxidation and reduction of cofactors. Carbon dioxide is formed by the oxidation of pyruvate to acetyl-CoA and acetate or isocitrate to 2-oxoglutarate. It is used for carboxylation of pyruvate or phosphoenolpyruvate whereby oxaloacetate, the substrate for the reductive arm

of TCA cycle is formed (Figure 2). Nicotinamide adenine dinucleotide (NADH – reduced form, NAD^+ – oxidized form) is a cofactor and component regulating multiple metabolic pathways. In the tricarboxylic acid cycle oxaloacetate is converted to malate by malate dehydrogenase, the reaction requires presence of the reduced form of NADH or NADPH, whereas conversion of fumarate to succinate by succinate dehydrogenase involves the MQH_2 availability. Regeneration of the oxidized menaquinone requires the presence of NADH. The basic way of sugars assimilation is glycolysis. The activity of enzymes involved in glycolysis depends on maintaining the balance between the cofactors. The oxidized form of NADH is used by GAPDH, the excess of the cofactor reduced form inhibits the metabolism of sugars. Fermentation process can be directed by deletion of certain genes, introduction of more active isoforms of enzymes or providing additional, specific substrates for the anaplerotic reactions responsible for the regeneration of the co-substrates, but all this changes may affect the activity of the glycolytic pathway [26, 57].

Genetic modification of *Corynebacterium glutamicum* towards overproduction of succinic acid

The first papers concerning the production of organic acids by *C. glutamicum* were focused on the main product of fermentation – L- lactic acid. Wild strains of these microorganisms possess only L-lactate dehydrogenase, so that it is possible to obtain high purity L-lactate (> 99.9%) [26]. As it was already mentioned, in oxygen deprivation condition succinic acid is produced in small amounts, therefore metabolic paths have to be directed toward reducing arm of the Krebs cycle, where succinate is synthesized. Inui et al. (2004) used *C. glutamicum* R, which was proliferated to a high density, then biomass were separated and placed in the fermentation medium, supplemented with sodium bicarbonate as an additional source of CO_2 for PEPC and PEP. Under these conditions, activation of the reductive arm of the TCA cycle, an increase in the amount of NAD^+ and intensification of glycolysis were observed [26]. In another experiment the same group used thick suspension of bacterial cells (30 $g_{d.w.}$/L) in fed batch fermentation. As a result 52 g/L L-lactate, small amount of succinate (1.18 g/L) and acetate were received [27]. The addition of CO_2 source (sodium bicarbonate) to the fermentation medium leads to an increase in the total concentration of organic acids, and changed the proportions between them. The more bicarbonate is added the more glucose is transformed into succinic acid. In fed batch fermentation in the presence of 400 mmol bicarbonate 90 g/L L-lactate, 22.7 g/L succinate and 3 g/L acetate were obtained [27].

In oxygen deprivation conditions L-lactic acid is generated as a main product. Pyruvate is formed as the final product of glycolysis and is a key central metabolite used for synthesis of succinate, lactate and acetate as well as L-alanine or L-valine (Figure 2) [48, 57].

Table 1. Biotechnological production of succinic acid by different bacterial species

Stain	Medium	Succinic acid titre g/L (mmol/L)	Succinic acid yield mol succinate/mol substrate (g/g)	Productivity mmol/L/h (g/L/h)	By-products	References
***C. glutamicum* R *ΔldhA* pCRA717**	Mineral, with glucose and bicarbonate	146,3 (1240)	1,40 (0,92)	27 (3,1)	acetate	[27]
C. glutamicum* BOL-3/pAN6-*gap	Mineral, with glucose bicarbonate and formate	133,8 (1134)	1,67 (1,09)	21 (2,48)	2-oxoglutarate, acetate, fumarate, malate	[30]
C. glutamicum* BL-1 pVWEx1-*glpFKD	Mineral, with glycerol	9,3 (79)	0,21 (0,27)	3,6 (0,42)	acetate	[31]
***C. glutamicum* BL-1/pAN6pyc^{P458S}ppc**	Mineral, with glucose	10,6 (90)	0,45 (0,30)	0,8 (0,09)	2-oxoglutarate, acetate	[29]
***C. glutamicum* ZX1** (pE*acsAgltA*)	Mineral, with glucose	27,1 (241)	0,63 (0,42)	3,55 (0,32)	pyruvate	[64]
C. glutamicum* ATCC13032/pCC-*pgsA-amyA	Mineral, with starch	14 (119)	1,33 (0,88)	-	lactate, acetate	[65]
***A. succinogenes* FZ53**	Complex, with glucose	105,8 (897)	1,26 (0,83)	0,18 (1,3)	formate, acetate	[10]
***M. succiniciproducens* LPK7**	Complex, medium with glucose	52,4 (444)	0,35 (0,76)	15 (1,75)	acetate, formate, lactate	[28]
***E.coli* AFP111(pTrc99A-*pyc*)**	Complex with glucose and bicarbonate	99,2 (837)	1,68 (1,10)	11 (1.30)	acetate, pyruvate	[39]

For this reason Litsanov et al. (2012) assumed that the key issues relating to the production of succinic acid by *C. glutamicum* are: elimination of lactic and acetic acid biosynthesis, maintaining the appropriate balance between the $NADH/NAD^+$ and provide access to large amounts of CO_2 [30, 35].

C. glutamicum R *ΔldhA* pCRA717 was devoid of LDH activity and exhibiting an overexpression of native pyruvate carboxylase (PC). It was used in two-stage batch fermentation previously proposed by Okino et al. (2005). The biomass of bacterial cells were placed in mineral salts medium the concentration of about 50 g/L. Using the periodic addition of glucose and sodium bicarbonate, after 46 hours of fermentation 146 g/L succinic acid was obtained in a yield of $Y_{P/S}$ 1.4 mol succinate per mol of glucose (Table 1). Unfortunately, also the by-products were formed, the main of them was acetic acid (1.8 g/L) [27].

Litsanov et al. (2012) used *the C. glutamicum* ATCC 13032 to construct a new strain with repressed expression of the main enzymes responsible for the biosynthesis of lactic and acetic acid. Production of L-lactate was stopped due to deactivation of *ldhA* gene. Blocking the expression of several genes encoding acetylo-CoA:CoA transferase (*cat*), pyruvate:menaquinone oxidoreductase (*pqo*), fosfotransacetylase and acetate kinase (*pta-ackA*) caused a reduction of acetic acid biosynthesis. The new strain *C. glutamicum ΔcatΔpqoΔpta-ackAΔldh* was called BOL-1 [30].

As previously mentioned, the bottleneck of succinic acid biosynthesis is carboxylation of pyruvate and phosphoenolpyruvate. Plasmid pAN6-pyc^{P458S} contains homologous pyruvate carboxylase which activity is increased by exchange of the proline at the position of 458 to serine [59]. Gene pyc^{P458S} under the control of a strong, constitutive promoter *Ptfu* (encoding translation elongation factor Tu) [60] was integrated into the BOL-1 bacterial chromosome, in previously deleted coding region of the gene *pta-ack*. As a result strain *C. glutamicum ΔcatΔpqoΔpta-ack::*P_{tfu} pyc^{P458S}*ΔldhA* (BOL-2) was created [30].

Strains *C.glutamicum ΔldhA*/pAN6-pyc^{P458S}, BOL-1/pAN6-pyc^{P458S} and BOL-2 were tested for their ability to the production of succinic acid. To provide adequate amount of CO_2 for carboxylation, 200 mM bicarbonate was added [30]. After 21 hours fermentation with *C. glutamicum ΔldhA*/pAN6-pyc^{P458S} succinic acid concentration reached 15.3 g/L and $Y_{P/S}$ of up to 1.16 mol of succinic acid per mol of glucose. Aside from succinate small amounts of acetate (3 g/L), pyruvate, malate, fumarate and 2-ketoglutarate as by-product were formed. BOL-1/pAN6-*pyc*P^{458S} produced 90% less acetate than the reference strain. Glucose consumption and production of succinic acid (13.6 g/L) was also about 38% lower. *C. glutamicum* BOL-2 showed similar characteristics to BOL-1/pAN6-pyc^{P458S}, however the rate of glucose consumption and the production of succinic acid were more than 80% higher than during fermentation with strain BOL-2 [30].

Efficient succinic acid biosynthesis requires the presence of NADH, but the excess inhibits one of the enzymes involved in glycolysis. Formate dehydrogenase,

encoded by a gene *fdh* derived from *Mycobacterium vaccae* was introduced under the control of a strong promoter *Ptfu* into *C. glutamicum* cells. This enzyme catalyses oxidation of formate to carbon dioxide, donating the electrons to NAD^+ (Figure 2) [60]. A single copy of the *fdh* gene was incorporated into BOL-2 bacterial chromosome, in a place of the locus *Δpqo*. As a result *C. glutamicum* $\Delta pqo::P_{tfu}fdh\Delta pta\text{-}ack::P_{tfu}pyc^{P458S}\Delta ldhA$ (BOL-3) was obtained. Constitutively expressed *fdh* gene allows to provide additional portions of CO_2 and NADH. Carbon dioxide is a substrate for appropriate carboxylase during biosynthesis of oxaloacetate. Additional portions of NADH are used in the reductive arm of the TCA cycle, which is responsible for NAD^+ generation. In result glycolysis should be stimulated, but to high FDH activity is the reason for the inhibition of glycolysis. In further modifications of the strain BOL-3 homologous gene *gapA* encoding glycerol-3-P dehydrogenase (GAPDH) was used. Overexpression of the gene *gap* was achieved by introducing it in pAN6-*gap* plasmid. Thus, increased specific activity of GAPDH compensated the inhibition of this enzyme caused by an excess of NADH. Strains were tested for their ability to production of succinic acid and formation of undesirable metabolites, such as acetic and lactic acid. Fermentations were carried out in a medium containing bicarbonate and sodium formate. If the tested cells had no FDH activity, formate caused decrease in the amount of succinate and glucose consumption rate. After 3 hours of fermentation strain BOL-3 reached stationary phase, which lasted for several hours and then again started to consume glucose and generate succinic acid. This phenomenon could be explained as the effect of formic acid. In undissociated form it penetrates the cytoplasmic membrane and distorts ion gradient which is pH depended [30, 61]. Finally, 16.6 g/L succinate was obtained with yield $Y_{P/S}$ 1.26 mol succinate per mol of glucose. The concentration of TCA cycle intermediates such as malate and fumarate increased while the amounts of undesired metabolic products such as acetic acid were reduced. Fermentation using strain BOL-3/pAN6-*gap* had a slightly different course. During the fermentation stationary phase was not observed, after 20 hours 17.8 g/L succinic acid and the yield $Y_{P/S}$ 1.41 mol succinate per mol of glucose were obtained. Again, in the fermentation broth malate and fumarate were identified. BOL-3/pAN6-*gap* strain was also tested during the fed-batch fermentation. Production medium contained sodium formate and bicarbonate, which were added periodically. The cells remain metabolically active for more than 2 days. After addition of the last formate portion, fermentation was stopped. Its high concentration of about 35 g/L probably has toxic effects on cell metabolism. After 53 hours of fermentation 133 g/L succinic acid were formed, at this time the cells used 679 mmol/L glucose and 772 mmol/L formate (Table 1). The yield of product relative to substrate reached: 1.67 mol succinate per mol of glucose, this is the highest score achieved so far for the anaerobic fermentation of succinic acid with *C. glutamicum* [30]. Unfortunately, the use of

formate increases the cost of production and purification of succinic acid, and therefore the method may be prohibitive in large scale.

Zhu et al. 2014 presented a dual route for anaerobic succinate production by *C. glutamicum*. In the first step they eliminated genes encoding L-lactate dehydrogenase (*ldhA*), enzymes responsible for acetate synthesis (*pqo, cat, ackA*) and pyruvate carboxylase (*pyc*). Phosphoenolpyruvate carboxylase (*ppc*) responsible for oxaloacetate synthesis and genes coding for enzymes involved in the glyoxylate cycle, such as isocitrate lyase (*aceA*), malate synthase (*aceB*) and citrate synthase (*gltA*) were overexpressed. In order to ensure effective export of succinate to fermentation medium, putative exporter encoded by *sucE* gene was up-regulated. Described strain *C. glutamicum* SA5 was used in dual phase, fed-batch fermentation including an aerobic growth and anaerobic fermentation. Anaerobic phase was carried out with 27.5 g dry cell/L. After 98 h of fed-batch fermentation succinic acid concentration reached 109 g/L, with $Y_{P/S}$ 1.32 mole succinate per mole of glucose. Acetate and small amounts of the TCA cycle intermidients such as α-ketoglutarate, pyruvate, malate and fumarate were formed [37].

Aerobic conditions have many advantages over anaerobic processes, among them is worth to mention about higher biomass generation, faster carbon throughput and product formation. Litsanov et al. (2012) proposed aerobic production of succinic acid. Under these conditions an intensive growth of *C. glutamicum* biomass is observed. To cause inhibition of growth and conversion of carbon sources mainly to organic acids, nitrogen sources have to be limited. The production of succinic acid in aerobic culture was achieved by construction of *C. glutamicum ΔsdhCAB* strain, which was devoid of succinate dehydrogenase (SDH), the Krebs cycle was inhibited at the stage of conversion of succinic acid to fumaric acid. Further modifications were aimed at reducing acetic acid biosynthesis. The mutant *ΔpqoΔpta-ackAΔsdhCABΔcat* was called *C. glutamicum* BL-1. Deletions of the enzymes responsible for the production of acetic acid such as pyruvate: quinone oxidoreductase (PQO, *pqo*), CoA transferase (CoAT, *cat*), phosphotransacetylase (PTA) and acetate kinase (AK), which are under the control of the *pta-ack*, do not eliminate the formation of acetate completely [29]. It is not clear which other proteins are involved in the formation of acetate. Anaplerotic reactions providing oxaloacetate to the TCA cycle have an influence on the succinic acid biosynthesis. To increase its availability strain *C. glutamicum* BL-1/pAN6-pyc^{P458S}*ppc* was constructed. Genes pyc^{P458S} and *ppc* introduced under the control of a strong promoter triggered a considerable increase in the production of succinic acid. Finally 10.6 g/L of succinic acid were produced with a yield $Y_{P/S}$ 0.45 mole succinate per mole of glucose [29].

Zhu et al. (2013) were engineered *C. glutamicum* ATCC 13032 to increase succinate production and eliminate acetate accumulation. Genes encoding L-lactate dehydrogenase (*ldhA*), succinate dehydrogenase complex (*sdhCAB*) and

enzymes responsible for acetate synthesis (*pqo, pta, cat*) were disturbed. Native promoters of the genes coding pyruvate and phosphoenolpyruvate carboxylase (*pyc*, *ppc*) were replaced by the strong promoter of the superoxide dismutase (*sod*). The resulting strain was named *C. glutamicum* ZX1. To avoid the accumulation of acetate expression of several enzymes responsible for the conversion of acetyl-coA to acetate should be blocked [62-64]. Alternatively, introduction of more efficient acetate assimilation pathway could solve this problem. The assimilation pathway from *Bacillus subtilis*, catalyzed by AMP-forming acetyl-CoA synthetase (*acsA*) was expressed in strain ZX1. New strain ZX1 (pE*acsA*) did not accumulate acetate at the end of fermentation, this time pyruvate was the main by-product. Reduction of its accumulation was obtained by citrate synthase overexpressing. Therefore, the gene encoding the native citrate synthase (*gltA*) was introduced into strain ZX1 using plasmid pE*acsAgltA*. The resulting strain ZX1 (pE*acsAgltA*). To assess the ability of the resulting strain for succinic acid production under aerobic conditions, fed-batch cultures were carried out. Strain *C. glutamicum* ZX1 (pE*acsAgltA*) produced 21.7 g/L succinate with $Y_{P/S}$ 0.63 mole succinate per mole of glucose (Table 1). The major by-product was pyruvate [64].

As it was shown in Table 1 the highest succinic acid, production described so far, was obtained by strains *C. glutamicum* R *ΔldhA* pCRA717 and *C. glutamicum* BOL-3/pAN6-*gap* which produce in oxygen deprivation conditions, respectively 146 and 133 g/L of succinic acid [27, 30], whereas *A. succinogenes* FZ53 produces 105 g/L [12] and *E. coli* AFP111(pTrc99A-*pyc*) 100 g/L [35]. Both of the *C. glutamicum* strains exhibit excellent properties. Alas, they are still not suitable for use in industrial scale. However, the concentration of succinic acid achieved during fermentation with any other microorganism is significantly lower. Likewise, aerobic succinate production involving *C. glutamicum* is not possible. Described above *C. glutamicum* ZX1 (pE*acsAgltA*) synthesized only 21.7 g/L of succinate (Table 1) [64].

Succinate production from alternative carbon sources by *Corynebacterium glutamicum*

The primary carbon source in described process was glucose, additionaly formate or carbonate were added in some cases. To reduce the cost of organic acids production, waste substances from different industries could be used. However, the metabolic abilities of *C. glutamicum* wild strains do not allow using many cheap and available carbon sources. Numerous strains able to utilize alternative substrates, such as starch [65-69], lactose [70-71], and galactose [71], arabinose [72-75], xylose [76-80], cellobiose [80-81]; dicarboxylic acids [82-83] or glycerol [31, 84, 85] have been constructed. We would like to present only the two most interesting examples.

Previously described *C. glutamicum* BL-1 was used to create a new strain able to utilize glycerol, waste substance generated during the biodiesel production.

The gene operon *glpFKD* from *E. coli* encoding proteins responsible for transport and metabolism of glycerol has been cloned into the *C. glutamicum* BL-1. As a result, strain *C. glutamicum* BL-1 pVWEx1-*glpFKD* was constructed. It was able to aerobic production of about 79 mol/L (9.3 g/L) succinic acid from glycerol with a yield $Y_{P/S}$ 0.21 mole succinate per mole of glycerol (Table 1) [31].

Biosynthesis of organic acids from starch is possible by using cell surface-engineered *C. glutamicum* ATCC 13032. Gene *amyA*, encoding α-amylase from *Streptococcus bovis* 148, was fused with C-terminal region of an anchor *pgsA* from *Bacillus subtilis*. The construct was inserted into pCC plasmid and cloned into *C. glutamicum* cells. The recombinant strain exhibited the ability to express the PgsA-anchored α-amylase on the cell surface, degraded starch and produced organic acids. Fermentations, with high-density cell suspensions were carried out in three different temperatures: 30, 35 and 40°C. Maximum for organic acid concentration was observed in 35°C. Unfortunately the higher temperature the more significant decrease in activity of the α-amylase was observed. After five 10 h fermentation cycles with biomass recycle, *C. glutamicum* ATCC13032/pCC-*pgsA-amyA* produced 107.8 g/L of total organic acids, including 88,9 g/L (0.99 M) lactate and 14.0 g/L (119 mmol/L) succinate (Table 1) [65].

Conclusion and future prospects

Bio-succinate is a very desirable product due to the large number of applications. For several years, potential producers of this acid have been sought. Among the most frequent mentioned bacterial species, which may be used for the biosynthesis of the acid are *Escherichia coli*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens* and *Corynebacterium glutamicum* (Table 1).

Technologies with *C. glutamicum* have many advantages. Among them it is worth to remark the possibility of using cheap, mineral media which not only reduce the manufacturing costs at the stage of fermentation, but also during isolation and purification of the final product. Lack of cells growth under anaerobic conditions enables the carbon sources to be completely transformed into succinate. There is also the possibility of biomass recycling and using it again in the next cycle of fermentation, which further reduces costs and increases efficiency of the process. However, to achieve effective production of succinate it is necessary to introduce a number of improvements. Succinic acid production with *C. glutamicum* requires high concentration of biomass in the fermentation medium. It may entail technological problems. Therefore, it is necessary to increase succinate production rates. Economical relevant and sustainable biosynthesis of organic acids in an industrial scale are dependent on the use of low-cost carbon sources, in particular from renewable resources. However, *C. glutamicum* cannot utilize many of them. Especially glycerol, starch, whey, straw or hemi- and lignocellulose are widely available and potentially attractive sources of renewable feedstock. Therefore *C. glutamicum* needs to be genetically modified to expand the spectrum of sugars that can be utilized. Other genetic

modifications are necessary to limit the production of metabolic waste products, especially acetate. To make this possible, we need to deepen the knowledge about the metabolism and cell function.

Bio-succinate large scale production can significantly reduce the use of non-renewable carbon sources and greenhouse gas emission, so we believe that worthwhile and competitive technology can be established in the near future.

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