Metabolic Engineering 12 (2010) 518–525

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid

Andreas M. Raab [a,b], Gabi Gebhardt [c], Natalia Bolotina [b], Dirk Weuster-Botz [c], Christine Lang [a,b,*]

[a] Berlin University of Technology, Institute for Biotechnology, Department of Microbiology and Genetics, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany
[b] ORGANOBALANCE GmbH, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany
[c] Technische Universität München, Institute of Biochemical Engineering, Boltzmannstr. 15, D-85748 Garching, Germany

ARTICLE INFO

Article history:
Received 1 April 2010
Received in revised form
5 August 2010
Accepted 17 August 2010
Available online 18 September 2010

Keywords:
Yeast
Saccharomyces cerevisiae
Succinic acid
Metabolic engineering
Production

ABSTRACT

The production of bio-based succinic acid is receiving great attention, and several predominantly prokaryotic organisms have been evaluated for this purpose. In this study we report on the suitability of the highly acid- and osmotolerant yeast *Saccharomyces cerevisiae* as a succinic acid production host. We implemented a metabolic engineering strategy for the oxidative production of succinic acid in yeast by deletion of the genes *SDH1*, *SDH2*, *IDH1* and *IDP1*. The engineered strains harbor a TCA cycle that is completely interrupted after the intermediates isocitrate and succinate. The strains show no serious growth constraints on glucose. In glucose-grown shake flask cultures, the quadruple deletion strain *Δsdh1Δsdh2Δidh1Δidp1* produces succinic acid at a titer of 3.62 g L^{-1} (factor 4.8 compared to wild-type) at a yield of 0.11 mol (mol glucose)$^{-1}$. Succinic acid is not accumulated intracellularly. This makes the yeast *S. cerevisiae* a suitable and promising candidate for the biotechnological production of succinic acid on an industrial scale.

1. Introduction

Succinic acid is currently produced petrochemically and used as a surfactant, an ion chelator, and as an additive in the pharmaceutical and food industries (McKinlay et al., 2007b). This specialty chemical market is relatively small (16.000 t in 2006) (Patel et al., 2006). The chemical synthesis is predominantly based on maleic anhydride and requires heavy metal catalysts, organic solvents, high temperatures and high pressures, which makes the conversion of maleic anhydride to succinic acid costly and ecologically questionable (Cornils and Lappe, 2002; Song and Lee, 2006). On the other hand succinate is produced naturally by many microorganisms as an intermediate of the central metabolism or as a fermentation end-product. Bio-based succinate is receiving increasing attention, as it could provide a cost-effective, ecologically sustainable alternative with a market potential of more than 270.000 t/year (Willke and Vorlop, 2004).

Numerous predominantly prokaryotic organisms have been isolated, evaluated and optimized for the production of succinate. The rumen bacteria *Actinobacillus succinogenes* and *Mannheimia succiniproducens*, recombinant *E. coli*, *Corynebacterium glutamicum* and *Anaerobiospirillum succiniproducens* are among the best studied microorganisms for succinate production (Guettler et al., 1999; Lee et al., 2008, 2002; Oh et al., 2009, 2008; Okino et al., 2005, 2008; Sanchez et al., 2005; Vemuri et al., 2002a, 2002b; Wu et al., 2007). Some of these bacteria show high productivity, since they produce succinate as a major fermentation product naturally. Succinate titers of up to 106 g L^{-1} and substrate yields close to the stoichiometric maximum (up to 1.1 g g^{-1}) were achieved with *A. succinogenes* and *E. coli* (Guettler et al., 1996, Vemuri et al., 2002a, 2002b). However, the application of these bacterial production hosts at industrial scale might be limited, as some of them are obligate anaerobic, potentially pathogenic, or show poor growth and low tolerance towards acidity, osmotic stress and high glucose levels (Cheng et al., 2003; Hippe et al., 1999; Song and Lee, 2006; Tee et al., 1998).

The yeast *Saccharomyces cerevisiae* is a robust, well-established industrial production organism (e.g. for bioethanol); it exhibits very good growth characteristics, a broad substrate spectrum and an extraordinarily high acid- and osmotolerance (Dombek and Ingram, 1987; Porro et al., 1995; van Maris et al., 2004). The high tolerance towards acidity is a major advantage over bacterial succinic acid production hosts, since it makes the use of neutralisation salts dispensable, which enormously facilitates the downstream process (Kurzrock and Weuster-Botz, 2010). Most specialty and commodity applications of succinic acid require the free acid form rather than the salt form (Song and Lee, 2006).

Additionally, *S. cerevisiae* is genome-sequenced, genetically and physiologically well characterized and tools for genetic

* Corresponding author at: Berlin University of Technology, Institute for Biotechnology, Department of Microbiology and Genetics, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany. Fax: +49 30 46307210.
E-mail address: christine.lang@campus.tu-berlin.de (C. Lang).

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doi:10.1016/j.ymben.2010.08.005

optimization are established (Goffeau, 2000). These features could make yeast particularly suitable for the biotechnological production of succinic acid. As succinate is one of the main taste components produced by yeast during sake (Japanese rice wine) fermentation (Arikawa et al., 1999a), studies focussing on succinate levels in *S. cerevisiae* predominantly derive from the scientific background of sake brewing. Arikawa et al. (1999a) reported an increased succinic acid productivity in sake yeast strains that harbor deletions of TCA cycle genes. In comparison to the wild-type, succinate levels were increased up to 2.7 fold in a strain with simultaneous disruption of a subunit of succinate dehydrogenase (*SDH1*) and fumarase (*FUM1*) under aerobic conditions. The single deletion of gene *SDH1* led to a 1.6-fold increase. These effects were not observed under strictly anaerobic or sake brewing conditions. Absence or limitation of oxygen resulted in decreased succinate productivity in *sdh1* and/or *fum1* deletion strains (Arikawa et al., 1999a, 1999b). In another study on sake yeast strains, the deletion of genes encoding for succinate dehydrogenase subunits (*SDH1, SDH2, SDH3,* and *SDH4*) also resulted in increased succinate productivity only under aerobic conditions (Kubo et al., 2000). Succinate production reduced to approximately half in comparison with the parental strain was observed for yeast strains with disruptions of isocitrate dehydrogenase subunits (*IDH1* or *IDH2*) under sake brewing conditions (Asano et al., 1999).

The disruption of succinate and isocitrate dehydrogenase activity is also part of a metabolic strategy for the oxidative production of succinic acid, which was applied in succinate producing *E. coli* strains (Lin et al., 2005b). This strategy led to succinate yields close to the stoichiometric maximum (1 mol (mol glucose)$^{-1}$) in *E. coli* (Lin et al., 2005a, 2005c) and can also be implemented in yeast.

Here we report on the construction of yeast strains for the biotechnological production of succinic acid and we evaluate the general suitability of *S. cerevisiae* for this application. The genes *SDH1*, *SDH2*, *IDH1* and *IDP1*, which encode for a mitochondrial isoenzyme of isocitrate dehydrogenase, were deleted with the aim to disrupt succinate and isocitrate dehydrogenase activity in order to redirect the carbon flux into the glyoxylate cycle and to allow succinate to accumulate as an end-product. The constructed yeast strains with up to four gene deletions (*Δsdh1 Δsdh2 Δidh1 Δidp1*) and some relevant intermediate strains were evaluated on a shake flask scale with respect to their phenotypic behavior and suitability for succinic acid production.

Table 1
S. cerevisiae strains used in this study.

Strain	Relevant genotype
AH22ura3	*MAT*a, wild-type, reference strain
AH22ura3 *Δsdh2*	*MAT*a *sdh2::loxP*
AH22ura3 *Δsdh2Δsdh1*	*MAT*a *sdh2::loxP sdh1::loxP*
AH22ura3 *Δsdh2Δidh1*	*MAT*a *sdh2::loxP idh1::loxP*
AH22ura3 *Δsdh2Δsdh1Δidh1*	*MAT*a *sdh2::loxP sdh1::loxP idh1::loxP*
AH22ura3 *Δsdh2Δsdh1Δidh1Δidp1*	*MAT*a *sdh2::loxP sdh1::loxP idh1::loxP idp1::loxP*

2. Material and methods

2.1. Strains, media and culture conditions

All yeast strains used in this study (Table 1) are derived from strain *S. cerevisiae* AH22ura3 (*MAT*a *ura3Δ leu2-3 leu2-112 his4-519 can1*), which has been described (Polakowski et al., 1998). The *E. coli* strain K12 JM109 (e14$^-$(McrA$^-$), *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17($r_K - m_{K+}$), *supE*44 *relA*1, Δ(*lac-proAB*), [F*traD*36 *proABlaqI*^{q}*ZΔM*15]) was used for maintenance and amplification of plasmid DNA (Yanisch-Perron et al., 1985). Bacteria were grown in standard LB medium at 37 °C. Yeast strains were cultivated in defined minimal WMVIII medium (Lang and Looman, 1995) with 50 g L^{-1} glucose instead of sucrose supplemented with histidine (100 mg L^{-1}), uracil (100 mg L^{-1}) and leucine (400 mg L^{-1}) on a rotary shaker (150 rpm) at 30 °C. Precultures (20 ml) were grown to the stationary phase in 100 ml shake flasks. Main cultures (50 ml) were inoculated with 1% of the preculture in baffled 250 ml shake flasks. All shake flask cultivations were carried out in at least duplicates. For colony growth assays yeast cells were grown to the early stationary phase and spotted at different dilutions ($10^{-1}-10^{-4}$) on culture plates with defined minimal WMVIII medium (Lang and Looman, 1995) with 5% glucose (instead of sucrose) supplemented with histidine (100 mg L^{-1}), uracil (100 mg L^{-1}), leucine (400 mg L^{-1}) and optionally glutamate (2 g L^{-1}). Plates were incubated for 96 h at 30 °C after spotting the dilutions.

2.2. Strain construction

The vector pUG6 (Guldener et al., 1996) was used to delete the genes *SDH1*, *SDH2*, *IDH1* and *IDP1* in *S. cerevisiae* AH22ura3. The deletion of the four genes was done successively by the same method. After plasmid preparation, a fragment of pUG6 was amplified by PCR to obtain a cassette consisting of *loxP-kanMX-loxP*. Primers were constructed (Table 2) to fuse 5′ and 3′ coding sequences of genes *SDH1*, *SDH2*, *IDH1*, *IDP1* and sequences of the *loxP* regions (underlined) of the pUG6 vector. The resulting PCR product comprises the *kanMX* gene, *loxP* sites and *SDH1*, respectively *SDH2, IDH1* and *IDP1* homologous regions for the integrative transformation in *S. cerevisiae* AH22ura3. Homologous recombination in yeast led to the deletion of the target genes. Geneticin resistance was used for selection of positive clones. The geniticin resistance was removed via transformation with pSH47 (Guldener et al., 1996). The vector carries the cre-recombinase gene to remove the *kanMX* gene flanked by *loxP* sites. To dispose pSH47, yeast strains were counter selected on 5-fluoroorotic acid (1 g L^{-1}) agar plates.

Table 2
Oligonucleotides used in this study.

Oligonucleotides	Sequence
sdh1::kanlox fw	ATGCTATCGCTAAAAAAATCAGCGCTCTCCAAGTTGACTTCCAGCTGAAGCTTCGTACGC
sdh1::kanlox rev	TTAGTAGGCTCTTACAGTTGGAGGTACGGAAGGACATTCCGCATAGGCCACTAGTGGATCTG
sdh2::kanlox fw	ATGTTGAACGTGCTATTGAGAAGGAAGGCCTTTTGTTTGGCCAGCTGAAGCTTCGTACGC
sdh2::kanlox rev	CTAGGCAAATGCCAAAGATTTCTTAATTTCAGCAATAGCCGCATAGGCCACTAGTGGATCTG
idh1::kan lox fw	ATGCTTAACAGAACAATTGCTAAGAGAACTTTAGCCACTGCCAGCTGAAGCTTCGTACGC
idh1::kan lox rev	TTACATGGTAGATAATTTGTTGATGATTTCATTCGTGAAGGCATAGGCCACTAGTGGATCTG
idp1::kan lox fw	ATGAGTATGTTATCTAGAAGATTATTTTCCACCTCTCGCCCCAGCTGAAGCTTCGTACGC
idp1::kan lox rev	TTACTCGATCGACTTGATTTCTTTTTGTAGTCTTTTTTCAGCATAGGCCACTAGTGGATCTG

2.3. Metabolite analysis

Ethanol, glycerol and acetate analysis of culture supernatants was done via GC–MS analysis, which was performed on an Agilent 6890N GC system coupled to an Agilent 5975B VL MSD quadrupol mass selective detector. An Agilent DB-FFAP column (30 m, 0.25 mm i.d., 0.25 μm film thickness) was used for the analysis. The MS was operated in scan mode (70 eV, start after 4 min, mass range 29–500 a.m.u at 3.1 scans/s). Glucose concentrations were determined with a glucose assay kit (R-Biopharm, Darmstadt, Germany). The quantification of dicarboxylic acids in culture supernatants was performed by HPLC analysis with an Ascentis Express RP-Amide column (25 cm, 0.4 cm i.d., Supelco, Munich, Germany) on an Agilent 1100 HPLC system. Dicarboxylic acids were detected at 210 nm with a fluorescence detector equipped with a diode array (UVD340S). The mobile phase consisted of 99% (v:v) 20 mM NaH_2PO_4 and 1% acetonitrile (pH 2; adjusted with phosphoric acid). Flow rate was 4 ml/min and column temperature 45 °C. For analysis of intracellular succinate, 10 ml of culture broth was immediately quenched in cold methanol (−80 °C). Cells were washed twice with water and disrupted via glass beads. After centrifugation of the cell lysate, the supernatant was lyophilized prior to derivatization of carboxylic acids with methyl-chloroformiate according to a method described by Villas-Boas et al. (2003). Methyl-chloroformiate derivates were analyzed on an Agilent 6890N GC system coupled to an Agilent 5975B VL MSD quadrupol mass selective detector with an Agilent HP-5MS (30 m, 0.25 mm i.d., 0.25 μm film thickness) column. The MS was operated in scan mode (70 eV, start after 1 min, mass range 29–500 a.m.u at 3.1 scans/s). For verification of HPLC chromatogram peaks putative dicarboxylic acids were fractioned, lyophilized and derivatized with methyl-chloroformiate according to Villas-Boas et al. (2003) and analyzed via GC–MS analysis as described above.

3. Results

3.1. Succinic acid and by-product formation

The simultaneous interruption of the citric acid cycle after the intermediates isocitrate and succinate in yeast (see Fig. 1), which, to our knowledge, has not been studied in detail before, was investigated. The constructed strains were evaluated with respect to growth characteristics and succinic acid and by-product formation. Fermentation products, such as ethanol, glycerol and acetate, but also organic di- and tricarboxylic acids, such as citrate, malate, fumarate, isocitrate and α-ketoglutarate are considered as by-products. Yeast strains were cultivated in defined minimal medium with glucose as a carbon source (5% initial glucose) in 250 ml shaking flasks with baffles. A high oxygen entry was aspired, as for the oxidative production of succinic acid the propagation of the respiratory central metabolism is likely to be beneficial and oxygen limitation could be detrimental. Strain AH22ura3 (wild-type; Fig. 2A) showed the typical 2-phase growth of a "Crabtree" positive wild-type yeast during batch fermentation on glucose. The ethanol formed during exponential growth was completely assimilated within the first three days of cultivation and was mainly converted to biomass. The wild-type accumulated succinic acid up to a concentration of ca. 0.76 g L^{-1} within 168 h (Table 3). The succinic acid concentration reached the highest level after 48 h and then remained constant until the end of fermentation.

In contrast to the wild-type, the strains AH22ura3*Δsdh2* and AH22ura3*Δsdh2Δsdh1Δidh1* (Fig. 2B and C) exhibited strong accumulation of acetate and glycerol in the culture broth. Acetate was enriched in both the exponential growth phase and throughout the further course of the fermentation and reached concentrations of 6.4 g L^{-1}. This kinetic was also observed for succinic acid, which was enriched up to a concentration of

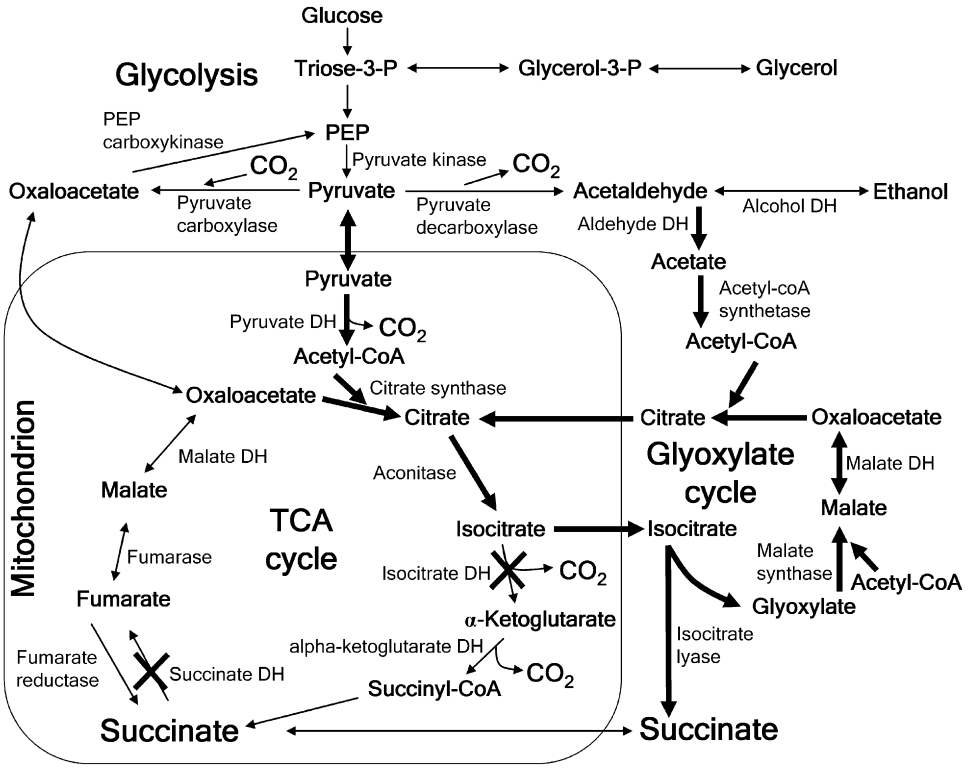

Fig. 1. Major metabolic pathways leading to the formation of succinate in *S. cerevisiae*. Bold arrows indicate a variant for succinate synthesis implemented in strains featured in this study. Crossed-out arrows indicate missing enzyme activities due to gene deletions. Arrowheads indicate reaction reversibility. Not all enzymatic steps or intermediates are shown (PEP, phosphoenolpyruvate; P, phosphate; DH, dehydrogenase).

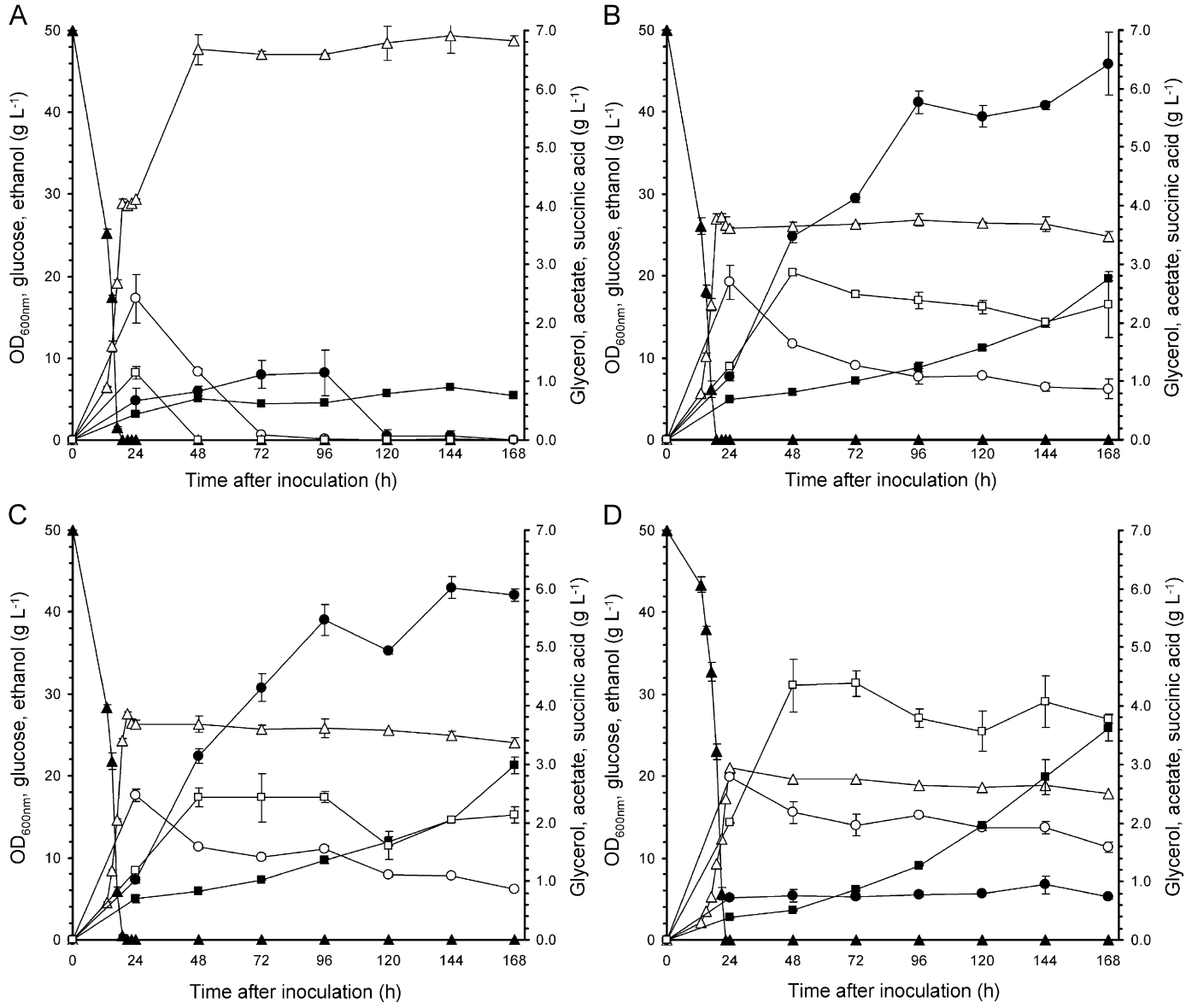

Fig. 2. Extracellular metabolites during shake flask fermentations of strains AH22ura3 (wild-type; A), AH22ura3*Δsdh2* (B), AH22ura3*Δsdh2Δsdh1Δidh1* (C) and AH22ura3*Δsdh2Δsdh1Δidh1Δidp1* (D) (defined minimal medium, 50 g L^{-1} initial glucose). Glucose (▲), ethanol (○), glycerol (□), acetate (●), succinic acid (■) and $OD_{600\ nm}$ (Δ) were measured. Mean values of biological duplicates are shown. Error bars indicate the respective standard deviations.

Table 3
Succinic acid titers and yields.

Strain	Titer (g L^{-1})[a]	Y_{xs} (mol (mol glc)$^{-1}$)[a]	% of theoretical maximum yield[b]
Wild-type	0.76	0.023	2.3
Δsdh2	2.75	0.084	8.4
Δsdh2Δsdh1	2.81	0.086	8.6
Δsdh2Δidh1	2.89	0.088	8.8
Δsdh2Δsdh1Δidh1	2.98	0.091	9.1
Δsdh2Δsdh1Δidh1Δidp1	3.62	0.111	11.1

[a] Shown are mean values of two independent shake flask fermentations (defined minimal medium, 50 g L^{-1} initial glucose and 168 h fermentation time). The standard deviation for each sample was below 5%.
[b] Under aerobic conditions (oxidative production).

2.98 g L^{-1} (strain AH22ura3*Δsdh2Δsdh1Δidh1*) after 168 h (Table 3). The pH value of the cultures at the end of fermentation (168 h) was 5.8 for the wild-type and between 4.0 and 4.5 for the deletion strains. All three deletion strains slightly consumed glycerol after the first two days of fermentation (Fig. 2B–D) and exhibited only a single growth phase on glucose and did not convert ethanol into biomass. Lytic processes are excluded for all strains, since the optical density did not significantly decrease in the course of the fermentation (Fig. 2A–D). This means that the succinic acid found in the culture broth was exported from intact cells, rather than set free due to cell lysis. Because of potential glutamate auxotrophy of strains with disruptions of isocitrate dehydrogenases, the minimal salts medium used was supplemented with 10 g L^{-1} Na-glutamate. All deletion strains exclusively consumed glutamate (approx. 20% of the initial amount of glutamate) during the exponential growth phase when biomass was formed. After the exponential growth phase the glutamate concentration in the medium remained constant at about 8 g L^{-1} (data not shown). Thus, succinate and acetate produced after the first day of fermentation (Fig. 2B–D) were not formed on glutamate as a carbon source. In contrast to the wild-type, strains AH22ura3*Δsdh2* and AH22ura3*Δsdh2Δsdh1Δidh1* did not

Table 4
Extracellular organic acid pattern.

Strain	Organic acids (mg L^{-1}) in culture supernatant[a]						
	Citrate	α-Ketoglutarate	Fumarate	Malate	Pyruvate	Glyoxylate	Isocitrate
Wild-type	175	368	190	175	n.d.	n.d.	n.d.
Δsdh2	n.d.	195	n.d.	n.d.	75	n.d.	n.d.
Δsdh2Δsdh1	n.d.	240	n.d.	n.d.	95	n.d.	n.d.
Δsdh2Δidh1	n.d.	190	n.d.	n.d.	65	n.d.	n.d.
Δsdh2Δsdh1Δidh1	n.d.	220	n.d.	n.d.	75	n.d.	n.d.
Δsdh2Δsdh1Δidh1Δidp1	10	120	n.d.	n.d.	95	n.d	n.d

[a] Mean values of two independent shake flask fermentations (defined minimal medium, 50 g L^{-1} initial glucose and 168 h fermentation time) are shown. The standard deviation for each sample was below 6%; not detected (n.d.).

completely assimilate ethanol in the culture broth. Only about 11 g L^{-1} was consumed between the first and the last day of fermentation and was converted to about 5 g L^{-1} acetate and 2 g L^{-1} succinate (Fig. 2B and C).

The additional deletion of gene *IDP1* in strain AH22ura3 *Δsdh2Δsdh1Δidh1* led to an altered behavior. The resulting strain did not form more than 0.8 g L^{-1} acetate and this was exclusively formed on glucose (Fig. 2D). No further acetate was synthesized on ethanol, and succinic acid was continuously formed up to a titer of 3.62 g L^{-1} (factor 4.8 compared to wild-type) at a yield of 0.11 mol (mol glucose)$^{-1}$. The additional deletion of *SDH1* in the *SDH2* disruptant did not further increase succinate levels (Table 3). This indicates that succinate dehydrogenase activity is completely abolished in the *SDH2* disruptant. The additional deletion of genes *IDH1* and *IDP1* resulted in higher succinate levels (Table 3), indicating a flux through the glyoxylate cycle, which seems to be beneficial for succinate production.

Glyoxylate and isocitrate were not detected in the culture broth of either the wild-type or the deletion strains after 168 h of cultivation (Table 4). At least for the wild-type glyoxylate was not expected to be accumulated, as it is highly reactive and toxic in biological systems (Behnam et al., 2006). While citrate, fumarate and malate were produced and exported by the wild-type, these organic acids were not detected in culture supernatants of the deletion strains. This might indicate a downregulation of oxidative metabolism and a reduced flux through the TCA and the glyoxylate cycle in SDH disruptants. It has previously been reported that *SDH3* deletion strains show mainly fermentative metabolism when cultivated on glucose (Cimini et al., 2009). α-Ketoglutarate was observed as the main extracellular organic dicarboxylic acid besides succinate and was formed by the wild-type and to a minor degree also by all deletion strains (Table 4). The high levels of extracellular α-ketoglutarate could be due to glutamate being supplemented to the culture medium, since α-ketoglutarate is the direct deamination product of glutamate. In contrast to the wild-type, pyruvate was only detected in culture supernatants of the deletion strains.

3.2. Growth characteristics

It was of particular interest to see whether deletion of the genes *SDH1, SDH2, IDH1* and *IDP1* leads to a complete loss of growth or potential growth restrictions, which would not be tolerable for a production strain. Growth rates and biomass formation of the deletion strains were determined during batch fermentation in shake flasks in defined minimal medium (5% initial glucose). The growth rates of strains AH22ura3*Δsdh2* and AH22ura3*Δsdh2Δsdh1* were identical and, with a value of 0.26 h^{-1}, not below the wild-type growth rate (0.25 h^{-1}; Table 5). The additional deletion of *SDH1* in strain AH22ura3*Δsdh2* had no effect on the growth behavior regarding the growth rate and the biomass concentration after a cultivation period of 168 h. The additional deletion of the gene *IDH1* in strains AH22ura3*Δsdh2* and AH22ura3*Δsdh2Δsdh1* resulted in a reduced growth rate of 0.22 h^{-1} (approximately 15% reduced compared to the *IHD1* parent), while the biomass concentration measured after 168 h was not affected (Table 5). The deletion of an additional isozyme of isocitrate dehydrogenase (Idp1p) in strain AH22ura3*Δsdh2Δsdh1Δidh1* led to a further reduction of the growth rate to 0.20 h^{-1}. The wild-type showed only a slight decrease in dry biomass between the third day and the end of the fermentation (0.4 g L^{-1}), while the dry biomass concentration for all deletion strains decreased significantly during this period (for strain AH22ura3*Δsdh2* by up to 3 g L^{-1}). This was not due to lytic processes, as the reduction of the optical density during this period was negligible (Fig. 2). Rather it could indicate a consumption of intracellular reserve carbohydrates, such as glycogen and trehalose, in this fermentation period. The dry biomass concentration of the deletion strains at the end of the fermentation reached about half of the wild-type biomass concentration. This was expected since strains with SDH disruptions are respiratory deficient and exhibit only a single growth phase on glucose, and the ethanol produced cannot be converted into biomass (Arikawa et al., 1999a, 1999b; Lemire and Oyedotun, 2002; Tzagoloff and Dieckmann, 1990).

Table 5
Growth rates and biomass concentrations.

Strain	μ_{max} (h^{-1})[a]	Dry yeast biomass (g L^{-1})[a]	
		After 72 h	After 168 h
Wild-type	0.25	13.1	12.7
Δsdh2	0.26	9.1	6.1
Δsdh2Δsdh1	0.26	7.3	6.0
Δsdh2Δidh1	0.22	7.0	5.9
Δsdh2Δsdh1Δidh1	0.22	8.5	6.1
Δsdh2Δsdh1Δidh1Δidp1	0.20	7.0	4.9

[a] Shown are mean values of two independent shake flask fermentations (defined minimal medium and 50 g L^{-1} initial glucose). The standard deviation for each sample was below 5%.

The growth behavior in medium without glutamate is of particular interest for strains lacking isoenzymes of isocitrate dehydrogenase, since glutamate is a central and essential metabolite synthesized from α-ketoglutarate, the product of isocitrate dehydrogenase (Haselbeck and McAlister-Henn, 1993). When grown on plates with minimal medium lacking glutamate strains with deletion of gene *IDH1* are showing growth restrictions, while the quadruple disruptant did not grow at all (Fig. 3; left side). Apparently the mitochondrial isoenzyme Idp1p compensates for the loss of activity of the Idhp complex with respect to glutamate auxotrophy. On plates with minimal

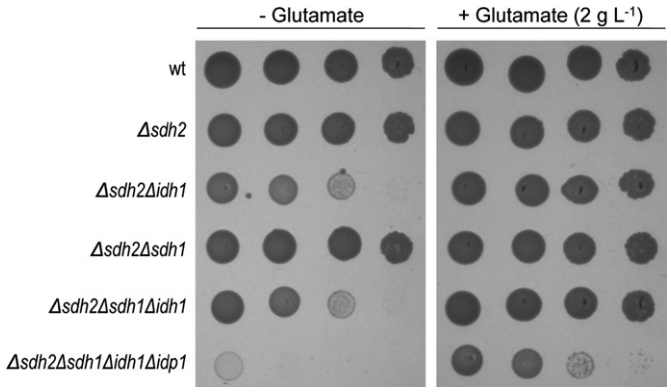

Fig. 3. Colony growth assay of wild-type (wt) and indicated deletion strains on culture plates (defined minimal medium WMVIII, 50 g L^{-1} glucose) in the absence of glutamate (left side; indicated at the top) and in the presence of glutamate (2 g L^{-1}; right side; indicated at the top). Each row represents a dilution series of cells (10^{-1}–10^{-4}). Plates were incubated for 96 h after spotting the dilutions.

medium supplemented with glutamate-only strain AH22ura3 *Δsdh2Δsdh1Δidh1Δidp1* showed restricted growth (Fig. 3; right side). It seems that the glutamate present in the medium cannot completely compensate the inability to produce endogenous glutamate in this strain. Disruption of Idp1p and Idhp leads to glutamate auxotrophy during growth on glucose, which cannot be compensated by the isoenzymes Idp2p and Idp3p, which are localized in the cytosol and the peroxisome. This observation is consistent with the literature (McCammon and McAlister-Henn, 2003).

3.3. Intracellular vs. extracellular succinic acid

A key question when evaluating the suitability of *S. cerevisiae* as a host for the production of succinic acid is whether *S. cerevisiae* is able to efficiently export succinic acid into the culture supernatant. Therefore the ratio of exported to intracellular succinate was determined for the strain AH22ura3*Δsdh2*. The intracellular amount of succinic acid accumulated by a defined number of cells was compared to the amount of succinic acid, which was exported by those cells during a 168 h shake flask cultivation. This resulted in a ratio of $1{:}239 \pm 8$ (data represent the average of biological duplicates), which shows that yeast exports succinic acid quantitatively into the culture supernatant.

4. Discussion

Interestingly, the simultaneous disruption of succinate and isocitrate dehydrogenase activity did not result in serious growth constraints on glucose (Fig. 2; Table 5). This is especially true for growth rates as only the deletion of *IDH1* and *IDP1*, but not *SDH1* and *SDH2*, led to detrimental effects on growth rates. Even the quadruple deletion strain AH22ura3*Δsdh2Δsdh1Δidh1Δidp1* exhibited a growth rate reduced by only 20% compared to the wild-type. The observation that disruption of isoenzymes of the isocitrate dehydrogenase has a stronger impact on growth than the deletion of subunits of the succinate dehydrogenase correlates with the reduced ability of the IDH deletion mutants to endogenously synthesize glutamate. Yeast possesses four isoenzymes of isocitrate dehydrogenase (Keys and McAlister-Henn, 1990; McCammon and McAlister-Henn, 2003; Panisko and McAlister-Henn, 2001). It was reported that eliminating Idhp and Idp1p activity leads to glutamate auxotrophy on glucose and that growth rates are decreased by 6% in *Δidh2* and by about 30% in *Δidh2Δidp1* deletion strains when grown in complex media with glucose (Haselbeck and McAlister-Henn, 1993). This is consistent with the results of the present study. Apparently, the simultaneous disruption of isocitrate and succinate dehydrogenase activity does not lead to synergistic detrimental effects concerning growth rates on glucose. The glutamate auxotrophy of strain AH22ura3*Δsdh2Δsdh1Δidh1Δidp1* indicates a complete loss of isocitrate dehydrogenase activity and a complete interruption of the TCA cycle after the intermediate isocitrate, which is essential for redirecting the carbon flux into the glyoxylate cycle. The use of the glyoxylate cycle instead of the TCA cycle for the synthesis of succinate results in higher yields (no oxidative decarboxylation, see Fig. 1) and also prevents potential problems with mitochondrial transport of succinate, since the glyoxylate cycle is located outside the mitochondria (Duntze et al., 1969; Kunze et al., 2002; Regev-Rudzki et al., 2005). The additional deletion of genes *IDH1* and *IDP1* in SDH disruptants even resulted in higher succinic acid levels. Strain AH22ura3 *Δsdh2Δsdh1Δidh1Δidp1* formed succinic acid continuously up to a titer of 3.62 g L^{-1} (factor 4.8 compared to wild-type and factor 1.3 compared to AH22ura3*Δsdh2*) at a yield of 0.11 mol (mol glucose)$^{-1}$. This indicates that redirection of the carbon flux into the glyoxylate cycle occurs and that this is beneficial for succinic acid production. This is inconsistent with the previous results of Asano et al. (1999), who observed that succinate production was reduced to approximately half in strains with disruption of the genes *IDH1* or *IDH2* in comparison with the parental strain. However, in the study by Asano et al. yeast strains were cultivated under anaerobic sake brewing conditions, which might lead to a non-functional glyoxylate cycle (Wu et al., 2006) and a decreased succinate productivity of IDH disruptants. The deletion of the SDH subunits Sdh1p and Sdh2p resulted in significantly increased succinic acid levels compared to the wild-type (about factor 3–4). This is consistent with the data of different studies in the field of sake brewing. By disruption of SDH activity an increase of succinate levels was observed in these studies, which was positively influenced by a higher oxygen entry (Arikawa et al. 1999a, 1999b, Kubo et al., 2000).

We propose that SDH disruptants are still capable of oxidative phosphorylation, as the formation of acetate and succinic acid from ethanol, glycerol and possibly intracellular reserve carbohydrates observed during fermentation of SDH disruptants requires the re-oxidation of NADH, which is formed by the reactions catalyzed by alcohol and acetaldehyde dehydrogenase. Results of other studies have also indicated that complex II (succinate dehydrogenase) of the respiratory chain in yeast is an electron donor in addition to the NADH dehydrogenases (Luttik et al., 1998; Marres et al., 1991) and not essential for oxidative phosphorylation (Robinson et al., 1991). In contrast to the wild-type, a strong reduction of the dry biomass was observed for all SDH deletion strains after glucose was depleted in the medium. It has previously been reported that mitochondrial respiratory mutants accumulated significantly higher amounts of glycogen than wild-type cells during the fermentative growth on glucose. After glucose was exhausted in the medium, these mutants mobilized their glycogen content completely, contrary to wild-type cells, which only transiently degraded this polymer (Enjalbert et al., 2000). Thus, it is likely that succinic acid and acetate produced by the SDH disruptant strains after glucose depletion were not exclusively formed on ethanol, but on intracellular reserve carbohydrates and glycerol, which were consumed within this fermentation period. The amount of succinic acid produced after glucose depletion was 3–4 times higher than during fermentation on glucose. The productivity is expected to be higher in absence of glucose, because under such conditions the respiratory central metabolism is not repressed, or at least to a lesser extent (Gancedo, 1998; Haurie et al., 2001; Santangelo, 2006). However, the SDH disruption strains did not

show complete derepression of the respiratory central metabolism, as the assimilated ethanol was not completely converted to succinate via the glyoxylate shunt or the oxidative part of the TCA cycle. Rather, a negative "feedback" regulation seems to occur after the metabolite acetate, which significantly accumulates in strains AH22ura3*Δsdh2Δsdh1* and AH22ura3*Δsdh2Δsdh1Δidh1*. Romano and Kolter (2005) also observed a formation of acetate in *SDH1* and *SDH4* deletion strains on ethanol. This might be due to repression of oxidative metabolism. A strong repression of the oxidative metabolism and respiratory functionality, including the TCA- and glyoxylate cycle and the respiratory chain, under aerobic, anaerobic and glucose limited conditions, has been described for *SDH3* disruptants (Cimini et al., 2009). Thus, the derepression of the respiratory metabolism – either by genetic means or by process control – could hold great potential for improving succinate productivity of the yeast strains constructed in this study. (13)C-metabolic flux analysis as performed for succinic acid producing *A. succinogenes* strains (McKinlay et al., 2007a; McKinlay and Vieille, 2008) could help to better understand the carbon flux distribution to succinate and alternative products also in yeast. Nevertheless, a succinic acid titer of 3.62 g L^{-1} is, to the best of our knowledge, the highest titer ever reported for yeast.

The analysis of intracellular and extracellular distribution of succinate shows that yeast quantitatively exports succinic acid into the culture broth. Thus yeast is suitable for an industrial production of succinic acid also in this respect. An intracellular accumulation of succinate would rapidly limit the production capacity of the cell and requires cell disruption, which is not desired at industrial scale. The transport mechanism responsible for the import and export of succinate in *S. cerevisiae* is not known. It has been suggested that either dicarboxylic acids are transported via diffusion (Camarasa et al., 2001; Volschenk et al., 2003) or that a dicarboxylate transport protein is localized in the plasma membrane exhibiting a transport mechanism that is not proton- but sodium-dependent (Aliverdieva et al., 2006).

5. Conclusion

The present study shows that the yeast *S. cerevisiae* is capable of synthesizing significant amounts of succinic acid, which is exported quantitatively into the culture broth, rather than being accumulated intracellularly. The constructed yeast strains with disruptions in the TCA cycle after the intermediates isocitrate and succinate produce succinic acid at a titer of up to 3.62 g L^{-1} at a yield of 0.11 mol (mol glucose)$^{-1}$ in glucose-grown shake flask cultures and do not exhibit serious growth constraints on glucose. Yeast is highly acid- and osmotolerant compared to prokaryotic organisms competing as a host for the industrial production of succinic acid. Various oxidative and reductive metabolic production strategies can be implemented in *S. cerevisiae*. This makes *S. cerevisiae* a suitable and promising candidate for the biotechnological production of succinic acid on an industrial scale.

Acknowledgments

The authors gratefully acknowledge financial support from the Deutsche Bundesstiftung Umwelt (AZ 13180). Andreas Raab thanks Jeffrey Schultchen for the support in questions concerning analytics. Gabi Gebhardt acknowledges the mentoring of the TUM Graduate School.

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