1. Introduction

Succinic acid is a valuable platform chemical for the production of bio-based polymers such as nylons and polyesters but is also valuable itself as surfactant, chelator, and as an additive in the agricultural, food, and pharmaceutical industry (Ahn et al., 2016; Bozell and Petersen, 2010; Mazière et al., 2017). Succinic acid is conventionally synthesized petrochemically with an overall market size of about 60 kt in 2015 (Jansen and van Gulik, 2014; marketsandmarkets.com, 2016) and highly optimistic projections of more than 600 kt exist for 2020 (Choi et al., 2015; Pinazo et al., 2015). Conventional, large-scale, centralized chemical production plants inhibit modernization of process design and create hurdles for start-ups and new incomers (Clomburg et al., 2017). Instead, start-ups turn to biotechnological fermentation-based strategies with lower requirements for capital investment thus allowing for more dynamic market adaptations and better adjustment to niche requirements (Clomburg et al., 2017). As a consequence, bio-based production of succinic acid is advancing in recent years, and to date, a variety of microorganisms has been engineered for the synthesis of succinic acid from sugars, glycerol or acetate (Ahn et al., 2016; Becker et al., 2015; Pinazo et al., 2015; Valderrama-Gomez et al., 2016). Current production hosts include 

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value-added products. Acetogens in the bacterial Clostridia class are frequently used for carbon fixation using CO₂/H₂ or syngas mixtures under anaerobic conditions (Daniell et al., 2012; Phillips et al., 2017). Due to energetic challenges, these gas fermentations are limited to the production of short-chain chemicals (Fast and Papoutsakis, 2012). To overcome this limitation, strategies are developed to convert acetate from the anaerobic first fermentation to the desired long chain products in a second, aerobic fermentation. For example, the acetate generated in a fermentation of C. ljungdahlii with syngas was converted to malate with Aspergillus oryzae in a second cultivation within the same fermenter (Oswald et al., 2016). Similarly, Sporomusa ovata was used for anaerobic acetate generation, followed by conversion to palmitate by Acinetobacter baylyi (Lehtinen et al., 2017). Finally, a continuous process has been developed in which Moorella thermoacetica fixes CO₂ to acetate, which is continuously pumped into a second aerobic fermentor for conversion to triacylglycerides by Yarrowia lipolytica (Hu et al., 2016).

### 1.2. Photosynthesis

Instead of feeding hydrogen, the energy required for CO₂ reduction can also be harvested from sunlight by photosynthesis. Cyanobacteria belong to the most important photosynthetic organisms and have consequently been developed for biotechnological production of chemicals. The product spectrum at lab-scale consists of alkanes, fatty acids and fatty alcohols, terpenes, squalene, sugars, and more complex compounds (Angermayr et al., 2015; Oliver et al., 2016). The currently achieved titers have already been pushed above 1 g/l, e.g., for ethanol or 2,3 butanediol with 5.5 g/l and 3 g/l, respectively. Yet, titers are often far lower (Angermayr et al., 2015; Oliver et al., 2016). Furthermore, a separation of growth and production phase has been recommended to increase genetic stability and cultivation performance (Savakis and Hellingwerf, 2015). However, due to the low titers and productivities, competitive production with cyanobacteria might currently only be achieved for fine chemicals (Savakis and Hellingwerf, 2015). Succinate production faces particular challenges, because the cyanobacterial TCA cycle has a low activity and the conversion of 2-oxoglutarate to succinate is low (Hendry et al., 2017; Young et al., 2011; Zhang and Bryant, 2011).

### 1.3. Methylotrophy

Methanol is a promising candidate as energy storage compound and chemical feedstock and may form the basis of a future methanol economy (Olah, 2005). Indeed, carbon capture and utilization techniques of converting CO₂ to methanol are already in industrial use (Bansode and Urakawa, 2014; Pérez-Fortes et al., 2016; Pontzen et al., 2011; Van-Dal and Bouallou, 2013), and methanol is used as a substrate for various biotechnological fermentation processes (Clomburg et al., 2017; Looser et al., 2015; Pfeifenschneider et al., 2017; Schrader et al., 2009). For poly-hydroxybutyrate high titers of 130 g/l and productivities of 1.86 g/l/h have been reached (Kim et al., 1996). The methylotrophic yeast Pichia pastoris has been widely used for heterologous protein production and the metabolic capabilities are well characterized (Jorda et al., 2014; Krainer et al., 2012; Zahril et al., 2017). Given that methylotrophic yeasts offer various advantages, like broad pH and thermostolerance, a yeast-based conversion of methanol to value added chemicals is imminent.

### 1.4. Synthetic carbon fixation

Implementation of (synthetic) carbon fixation pathways in naturally heterotrophic organisms offers an alternative to improving existing autotrophic strains. Examples of pathway transplantation include the reductive pentose-phosphate pathway (Antonovsky et al., 2017; Guadalupe-Medina et al., 2013; Parikh et al., 2006), methylotrophy (Müller et al., 2015; Whitaker et al., 2017), and to a limited extend the 3-hydroxypropionate bicycle (HP-bicycle) (Cheng et al., 2016; Keller et al., 2013; Mattozzi et al., 2013). Apart from transplanting existing CO₂ fixation pathways between organisms, completely novel, synthetic pathways have been computationally screened (Bar-Even et al., 2010). Among those candidates was the crotonyl-coenzyme A (CoA)/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) pathway, which was subsequently selected by Schwander et al. (2016) to perform an experimental proof of principle of an artificial carbon fixation mechanism.

In this article, we evaluated the economic feasibility of microbial CO₂ conversion to succinic acid via four different metabolic pathways: (i) the reductive pentose-phosphate (Calvin-Basham-Benson) cycle, (ii) the reductive acetyl-CoA (Wood-Ljungdahl) pathway, (iii) methylotrophy in yeast, and (iv) a synthetic pathway expressed in Saccharomyces cerevisiae (Fig. 1). Based on published, physiological data or assumed metabolic or technical capabilities, we calculated maximal yields and productivities of the alternative routes using stoichiometric modeling. We further projected the capacities of CO₂ fixation pathways to industrial scale and evaluated the efficiency and profitability of the different approaches.

### 2. Materials and methods

#### 2.1. Metabolic pathway selection and simulation conditions

Published genome-scale models of Synechocystis sp. PCC6803 (iJN678), Clostridium ljungdahlii ATCC 55383 (iHN637), Escherichia coli K-12 MG1655 (iJO1366), Pichia pastoris (iMM102v63), and Saccharomyces cerevisiae (Yeast 7.5) were used to simulate the C₁ conversion routes shown in Fig. 1. The simulations were performed in Matlab®2017a (The Mathworks®, Inc., Natick, MA, USA) using the Flux Balance Analysis function of the COBRA toolbox 2.0 (Schellenberger et al., 2011) and Gurobi® 5 solver by maximizing a chosen objective
function while minimizing the taxicab norm. The non-growth associated maintenance (NGAM) demand was modeled as the hydrolysis of ATP:

$$ATP + H_2O \rightarrow ADP + H^+ + P_i$$

(1)

The NGAM value is typically derived by extrapolation from growth and substrate uptake rates (Varma and Palsson, 1995) and default values of the models were used for simulations of growing cells. However, it has been reported that the NGAM of non-growing cells is significantly lower (Drews and Kraume, 2007; Rebenegger et al., 2016). To account for this difference in simulations with zero growth, we calculated an NGAM from experimental data of non-growing cell acetate uptake and succinate production rate were inferred from the simulations of exponentially growing C. ljungdahlii were always optimized for biomass production.

To enable the generation of reduction equivalents from hydrogen gas in this autotrophic S. cerevisiae strategy, a hydrogenase reaction was added to the Yeast 7.5, which reduces NAD+ by oxidation of molecular hydrogen:

$$H_2 + NAD^+ \rightarrow NADH + H^+$$

(3)

The hydrogen uptake rate was set to 30 mmol/gCDW/h and the CO2 reduction rate to 10 mmol/gCDW/h (Mourato et al., 2017). Since the CETCH pathway utilizes NADPH, a transhydrogenase reaction was added to exchange electrons between NADP+ and NAD+: 

$$NADPH + NAD^+ \rightarrow NADP^+ + NADH$$

(4)

Furthermore, we adapted the S. cerevisiae model to resemble a pyruvate decarboxylase negative strain, effective for pyruvate and reduced byproducts synthesis (van Maris et al., 2004). This was done by disabling fluxes through the pyruvate decarboxylase reaction (Yeast 7.5 reaction ID: r_0959). Growth of this strain was simulated with glucose as carbon source and aerobic conditions. A glucose uptake rate of 10 mmol/gCDW/h and unconstrained O2 uptake rate (1000 mmol/gCDW/h) were used and the objective function defined to maximize biomass formation. Additional constraints used in the FBA simulations are provided in Table 2.

### 2.2. Calculation of fermentation time and substrate requirements

The genome-scale models were employed to interrogate the production properties during a two-stage fermentation process (Fig. 2), in which the first stage serves to allow exponential growth from the starting biomass concentration $N_0$ of 0.01 gCDW/l to the organism-specific final working biomass concentration $N_f$ (Table 2). The growth rate $\mu$ of each organism was either estimated with FBA (Eq. (7)).

$$N(t) = N_0 e^{\mu t}$$

(5)

To calculate volumetric resource requirements and by-product formation during the growth phase, Eq. (5) was integrated over the fermentation time (Eq. (6)) and the resulting biomass-time integral $O_b$ multiplied with the specific consumption/production rates derived from flux distributions calculated with FBA (Eq. (7)).

$$O_b = \frac{N_0}{\mu} (e^{\mu t_f} - 1)$$

(6)

$$c_i = v_i \cdot O_b$$

(7)

The variable $c_i$ in Eq. (7) represents the concentration of metabolite $i$ (CO2, O2, H2, photons) computed as the product of the cell specific production rate ($v_i$) and the biomass-time integral $O_b$. Note, that succinate is generated only by non-growing cells. The final succinate concentration of 50 g/l ($c_{fi}$ was 423.7 mM) was chosen, and the produc-

### Table 1

Feedstock prices and cost for process requirements.

| Feedstock  | Price  | Source |
|------------|--------|--------|
| H2         | 1500 EUR/t | http://heshydrogen.com/hydrogen-fuel-cost-vs-gasoline/ |
| Methanol   | 450 EUR/t  | http://www.indexmundi.com/commodities/?commodity=sugar&currency=eur (reference date 02/17) |
| Electricity| 0.119 EUR/kWh | electricity prize in EU-28 region, http://ec.europa.eu/energy/energy_price_statistics |
| Agitation + Aeration, E/c | 8.925 x 10^-4 EUR/l/h | Energy cost for stirred-tank reactors combined with current electricity prices (Zhuang and Herrgard, 2015) |
| Agitation + Aeration, E/c | 2.975 x 10^-4 EUR/l/h | Energy cost for tubular photo-bioreactors combined with current electricity prices (Jorquera et al, 2010) |
| Sterilization, S | 1.05 x 10^-2 EUR/l | Zhuang and Herrgard (2015), combined with current electricity prices |

*Note: Prices and cost may vary depending on location and time.*
was calculated based on a constant biomass concentration \( N_0 \) of 2 g CDW/l.

The succinate specific production rate \( v_{\text{Suc}} \) was computed by FBA with the genome-scale models described in Section 2.1, constrained by the carbon uptake rates given in Table 2 and optimized for succinate production.

Succinate production via the reductive acetyl-CoA pathway was simulated with a 2-organism/2-fermenter concept, in which acetate production was controlled by the biomass density and was matched to the acetate production rate. The production time included accumulation of acetate by \( C. \) ljungdahlii and succinate by \( E. \) coli. Experimental evidence showed an acetate to succinate conversion efficiency in \( E. \) coli of 80% (Li et al., 2016). Therefore, the overall amount of acetate required was set to 62.5 g/l (1059.3 mmol/l). The procedure to determine the overall fermentation time was as follows:

1. The biomass-time integral \( O_N^{\text{prod}} \) (gCDW/h) required to generate the target acetate concentration was calculated with Eq. (7) using a time-invariant acetate production rate of 14.65 mmol/gCDW/h and 30 mmol/gCDW/h for a realistic and an optimistic scenario, respectively.

2. The duration of the exponential growth phase \( t_{\text{growth}} \) was determined with Eq. (5) assuming a constant working biomass concentration of \( N = 2 \text{ gCDW/l} \).

3. The biomass-time integral of the exponential growth phase was calculated with Eq. (6).

\[
O_N^{\text{prod}} = O_N^{\text{growth}} + O_N^{\text{prod}}
\]

4. The overall biomass-time integral \( O_N^{\text{tot}} \) was determined with Eq. (9).

5. The total process duration was calculated with Eq. (10), in which

\[
t_{\text{tot}} = t_{\text{growth}} + t_{\text{prod}}
\]
the second term defines the duration of the production phase and \(N_t\) the working biomass concentration.

\[
l_{tot} = l_{growth} + \frac{Q_{tot}^{Bio}}{N_t}
\tag{10}
\]

The steps (1)–(5) were combined to give Eq. (11):

\[
l_{tot} = \frac{\ln \left( \frac{N_t}{N_0} \right)}{\mu} + \frac{c_{Sub} - \frac{N_0}{\mu} (e^{\mu l_{growth}} - 1)}{N_t}
\tag{11}
\]

2.3. Cost calculations and sensitivity analysis

The calculated costs were normalized to the final succinate concentration. The two-fermenter strategy employing \(C. ljungdahlii\) and \(E. coli\), was designed as a retentostat with a constant outflow of the succinate-containing, cell-free fermentation broth. In this scenario, we used the amount of succinate produced relative to the biomass concentrations and fermenter volumes of \(C. ljungdahlii\) and the \(E. coli\) instead of the absolute succinate concentration. Process duration, biomass concentrations, and feedstock requirements to synthesize the target volumetric succinate amount of 50 g/l were calculated as described in Section 2.2. Table 1 shows process and commodity prices used for the calculations.

The total volumetric succinate production costs \(Cost_{Suc}\) comprised the demand of substrates \(Cost_{Sub}\), energy per time and per volume \((E_{vi})\), and sterilization per volume \((\epsilon_{vi})\) (Eq. (12)) and was divided by the succinate concentration to yield the overall costs per metric ton succinate \(\rho_{Suc}\) (Eq. (13)).

\[
Cost_{Suc} = Cost_{Sub} + E_{vi} \cdot \epsilon_{vi} + \frac{S}{c_{Suc}}
\tag{12}
\]

\[
\rho_{Suc} = \frac{Cost_{Suc}}{c_{Suc}}
\tag{13}
\]

The expenditures for the substrates, \(H_2\), methanol or glucose, were calculated using Eq. (14).

\[
Cost_{Sub} = c_{Sub} \cdot M_{Sub} \cdot \frac{price_{Sub}}{10^9}
\tag{14}
\]

c_{Sub} was calculated using Eq. (7). \(M_{Sub}\) is the molar mass, and \(price_{Sub}\) the substrate price given in Table 1.

The volumetric agitation and aeration costs were obtained by multiplying the energy demands from literature (Table 1) with the total fermentation time (Table 3).

The cyanobacterial strategy was designed to be operated with sunlight assuming 10 h per day of sufficient irradiation for \(CO_2\) fixation. Succinate production was assumed to take place in the period of 2.4. Additional calculations for the two-organism/two-fermenter strategy

The proposed \(C. ljungdahlii/E. coli\) strategy used two connected fermentations, each equipped with cell retention, in which the medium from the first fermenter is continuously fed into the aerated second fermenter. Variation of the reactor volume \(V\) (l) and the dilution rate of the \(C. ljungdahlii\) fermentation \(D = \frac{q}{V}\) (l/h), with \(q\) being the volume flow rate (l/h), affects the acetate concentration, which in turn requires adaptation of the \(E. coli\) biomass concentration in the second fermenter to guarantee complete and immediate acetate conversion. The concentration of acetate in the \(C. ljungdahlii\) fermenter was calculated via Eq. (15).

\[
\frac{dc_{Ac}}{dt} = \frac{c_{Ac} N_{Ac}^{Clj}}{V_{Ac}} - D_{Fl} c_{Ac}^{Clj} = 0
\tag{15}
\]

\[
N_{Ac}^{Clj} = \frac{c_{Ac}^{Clj} A_{Prn}}{\frac{\rho_{Ac}}{\rho_{Clj}}}
\tag{16}
\]

The subscript index \(Ac\) and the superscript index \(Clj\) denote acetate and \(C. ljungdahlii\), respectively. The volume flow rate is equal in both coupled fermenters. The variable \(APRn\) represents the volumetric molar acetate production rate (mmol/l/h).

The biomass of \(E. coli\) \(N_{Ecol}\) required to fully convert the incoming acetate was determined with Eq. (16). The superscript \(Ecol\) stands for \(E. coli\). \(N_{Ecol}\) is a function of the volume ratio of the \(C. ljungdahlii – E. coli\) fermenters, which was varied between 1 and 20 in the sensitivity analysis while the \(APRn\) was kept constant.

\[
\frac{dc_{Ecol}}{dt} = \frac{c_{Ecol} N_{Ecol}^{Ecol}}{V_{Ecol}} - D_{Fl} c_{Ecol}^{Ecol} = 0
\tag{17}
\]

\[
N_{Ecol}^{Ecol} = \frac{c_{Ecol}^{Ecol} A_{Prn}}{\frac{\rho_{Ecol}}{\rho_{Ecol}}}
\tag{18}
\]

The concentration of succinate \(c_{Suc}\) in the fermentation broth released from the \(E. coli\) fermenter during the steady state production phase was determined with Eq. (17).

\[
\frac{dc_{Suc}}{dt} = \frac{c_{Suc} N_{Suc}^{Ecol}}{V_{Suc}} - D_{Fl} c_{Suc} = 0
\tag{19}
\]

\[
\rho_{Suc} = \frac{c_{Suc}^{Ecol} A_{Prn}}{\frac{\rho_{Suc}}{\rho_{Suc}}}
\tag{20}
\]

In the realistic and optimistic scenarios, we assumed a dilution rate \(\frac{q}{V}\) of 10/h, which corresponds to the condition that the volume flow rate is 10-fold the reactor volume. This dilution rate is high, but still in

| Table 3 Properties of the simulated strategies with a target succinate concentration of 50 g/l and for the optimistic scenario. Substrate requirements were calculated as the sum of growth and production phase according to Eqs. (10) and (11). Because \(E. coli\) growth on glucose is rapid, we assumed it to take place within the \(C. ljungdahlii\) growth phase. Cell densities and productivities were according to the optimistic scenarios in Table 2 and black stars in Fig. 3. Parameters of cost calculation are shown in Table 1. Glc, Glucose. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Strategy        | Synechocystis   | \(C. ljungdahlii – E. coli\) | \(P. pastoris\) | \(S. cerevisiae\) | \(S. cerevisiae\) (Glc) | |
| growth phase + production time, h | 73 + 42 = 115 | 38 + 5 = 43 | 56 + 17 = 73 | 8 + 11 = 19 | 9 + 6 = 15 | |
| overall product yield, C-mol/C-mol | 81% | 72% | 68% | 64% | 35% | |
| product yield during production, C-mol/C-mol | 100% | 72% | 100% | 100% | 100% | |
| Substrate cost EUR/l | - | 1.4·10^{-2} (H2) | 3·10^{-2} (MeOH) | 1.3·10^{-2} (H2) | 6.6·10^{-2} (Glc) | |
| Energy cost EUR/l | 0.1 | 4·10^{-2} | 6.5·10^{-2} | 1.7·10^{-2} | 1.3·10^{-2} | |
| rel. substrate cost | 0% | 28% | 32% | 49% | 74% | |
| Production cost EUR/t (50 g/l) | 1847 | 1136 | 2107 | 1078 | 1785 | |
| Production cost EUR/t (100 g/l) | 1226 | 828 | 1405 | 776 | 1172 | |
the order of achievable rates reported, for example, for immobilized cells (Qureshi et al., 2005).

The energy requirement of the C. ljungdahlii – E. coli fermentation is a function of the two fermenter volumes. For E. coli, the volume of the E. coli fermenter is inversely proportional to the biomass concentration according to Eq. (16). The total energy requirement is calculated by combining Eq. (16) with the total fermentation time and volumes as derived from Eq. (18):

$$E_{tot}^{cis} = t_{tot} \cdot E_{ri} \cdot \frac{V_{Clju} + V_{Eco}}{V_{Clju}}$$

$$E_{tot}^{cis} = t_{tot} \cdot E_{ri} \left(1 + \frac{\Delta \text{P�R}}{\frac{A}{S_c} \frac{S}{N}}\right)$$

(18)

3. Results

3.1. Definition of realistic and optimistic simulation scenarios

We tested the succinate productivity of five different production strategies: (i) light driven CO$_2$ fixation in cyanobacteria, (ii) anaerobic CO$_2$ reduction to acetate by C. ljungdahlii and subsequent aerobic conversion of acetate to succinate by E. coli, (iii) methanol conversion to succinate by P. pastoris, (iv) synthetic CO$_2$ reduction pathway in S. cerevisiae, and (v) a heterotrophic succinate production from glucose in S. cerevisiae. The simulations were performed in two contexts: ‘realistic’ parameter sets, based on experimental data or assumed to be achievable without substantial metabolic or process engineering efforts; and ‘optimistic’ parameter sets that require extensive genetic modifications. We based our analysis on the production of a succinate titer of 50 g/l, which is within the tolerance range of most organisms and lower than maximum concentrations currently reached (Cui et al., 2017; Zhu et al., 2014), but we also tested price levels with final titers of 100 g/l.

The realistic and optimistic scenarios are specific for each strategy and represent key properties of the production. In the cyanobacterial strategy, the difference between the scenarios was the CO$_2$ uptake rate. In the C. ljungdahlii/E. coli strategy, we defined the optimistic scenario as having a doubled specific acetate production rate, a tripled growth rate and higher maximum biomass concentration for C. ljungdahlii compared to the realistic scenario, which was based on published experimental data (Straub et al., 2014). For E. coli, the specific acetate uptake was increased six-fold from the realistic case (Li et al., 2016) to the optimistic case. The realistic scenarios of P. pastoris and S. cerevisiae strategies were based on a 25% yield (C-mol/C-mol) on carbon source while carbon was fully converted to succinate in simulations of the optimistic scenario.

3.2. Succinate productivity and cost assessment

An important benchmark for the economic viability of bio-based succinate production is the volumetric productivity, which should surpass 2.5 g/l/h, according to estimates of the U.S. Department of Energy (Werpy et al., 2004). We determined the volumetric productivity for the succinate producing fermentation phase, i.e. the production phase of the two-stage fermentations and the total time of the one-stage fermentation. Whereas this limit was not met by any strategy for the realistic constraints, most strategies exceeded the productivity limit in the optimistic scenario, except of the cyanobacterial strategy (Table 2). An additional noteworthy point are the high carbon uptake rates in the C. ljungdahlii strategy (> 60 mmol/gCDW/h, optimistic scenario). The accordingly high molecular hydrogen uptake rate of the strain (> 130 mmol/gCDW/h, optimistic scenario) will require specialized bioreactors allowing very high k$_{H2}$ values, for example hollow-fiber membrane reactors for which mass transfer coefficients above 100/h have been reported (Orgill et al., 2013), and pressurized systems to increase the H$_2$ solubility (Phillips et al., 2017).

The production times required to achieve a succinate titer of 50 g/l varied greatly, from 42 h for the cyanobacteriological strategy to 11 h for the yeast-based process relying on the synthetic CETCH pathway (Table 3). Note, that with the C. ljungdahlii/E. coli strategy succinate is produced already during the growth phase of C. ljungdahlii. For slow growing organisms like the cyanobacteria and C. ljungdahlii, but also P. pastoris growing on methanol, the exponential growth phase to reach the final biomass concentration was a large contributor to the total fermentation time. The growth phase was shortest for S. cerevisiae expressing the CETCH pathway, but still comprised approximately 60% of the overall fermentation time. The overall product yield on assimilated and dissimilated carbon was greatest for the cyanobacterial strategy (80% (C-mol/C-mol)), hence carbon substrate requirements were lowest. The synthetic carbon fixation in S. cerevisiae had the lowest yield, which, however, was still higher than that of the heterotrophic strategy using glucose (Table 3).

The most cost effective autotrophic production was achieved with the engineered S. cerevisiae strategy, which is explained by the low H$_2$ cost, and the low energy requirement due to the short process time resulting in production costs of 1078 EUR/t. Note, however, that the accumulation of biomass during exponential phase is fueled by glucose (Table 3, see Material and Method section for details on the calculations). The two-stage fermentation of C. ljungdahlii – E. coli was similarly cost-effective. The highest cost was estimated for the P. pastoris strategy because of the high methanol price, which contributed half of the production cost. Also, the long fermentation time resulted in a high energy demand. Compared with a conventional fermentation of S. cerevisiae with glucose, the strategies employing methanol-driven P. pastoris and light-driven cyanobacteria were more expensive.

We also tested performance with an increased final titer of 100 g/l instead of 50 g/l. The associated, reduced volumetric costs were most prominent for the P. pastoris strategy, for which the expenses dropped by 33% to 1405 EUR/t. This saving is generated due to the reduced fraction of carbon used for the synthesis of biomass, the concentration of which was kept constant. Note, however, that downstream processing and investments costs are excluded from the analysis. The downstream cost will be similar for cyanobacteria, P. pastoris, and S. cerevisiae strategies because of identical final succinate titers. The C. ljungdahlii/E. coli two-step strategy, by contrast, is an open system with a constant outflow of a more dilute succinate concentration, which will ultimately raise the product purification cost.

3.3. Sensitivity of production cost to the biomass concentration of the microbial host

Higher biomass concentrations do not by default increase profitability. A trade-off exists between high volumetric productivities requiring high biomass concentrations (denominator of Eq. (8)) and substrate costs, which increase with the biomass concentration (Eqs. (5)–(7)). In addition, higher biomass concentrations require a longer growth phase. Consequently, the biomass concentration is a parameter that needs to be optimized to minimize both overall production time and costs. We calculated the overall production cost for a broad range of possible biomass concentrations to evaluate the sensitivity of the production cost to changes of this parameter (Fig. 3). A hyperbolic, decreasing trend of production costs for increasing biomass concentrations of cyanobacteria and S. cerevisiae was observed and biomass concentrations above 10 g/l resulted only in marginal savings of the production cost. Overall production costs of the P. pastoris based process dropped with increasing biomass because of a stark decrease of the energetic costs but increased again because of the increasing substrate expenses (Fig. 3C). For a biomass concentration of 10 g/l, the production rate exceeded the 2.5 g/l/h threshold and was therefore chosen for the cost analysis.
The optimistic scenario, the inlet synthetic CETCH pathway in U.W. Liebal et al. Metabolic Engineering Communications 7 (2018) e00075 conversion to succinate by (C) conversion of methanol to succinate by methylotrophic yeast, the optimistic scenario is achieved by increasing the succinate yield and productivity (\(v_{\text{suc}}\)), D) \(\text{CO}_2\) growth rate, biomass concentration, and specificity (Fig. 4). The highest impact on the acetate synthesis rate was observed by 1.4 mmol/gCDW/h to nearly 30 mmol/gCDW/h, resulting in a reduction of and simultaneously increasing the specificity further reduction of the production time was achieved by substantially increasing the growth rate of \(C. \text{ljungdahlii}\), the optimistic scenario is achieved by increasing the specific biomass concentration a limited impact on lowering the total production time was concurrently increased (Fig. 4C).

We examined in more detail the sensitivity of the acetate production rate to changes of the parameters for which we had chosen the current market price (Table 1). \(\text{CO}_2\) conversion to methanol is already conducted in conditions with cheap electricity and proximity to \(\text{CO}_2\) intense industries (Carbon Recycling Inc, BASF), and the future methanol price is expected to decrease to 100 EUR/t (Pfeifenschneider et al., 2017), i.e., to nearly one quarter of the current price. That being the case, the production cost of the methylotrophic strategy would decrease to 1100–1600 EUR/t succinic acid.

Dependence of volumetric costs for energy, substrate, and sterilization, and the total cost to produce 1 t succinate on the biomass concentration. Calculations were based on a final succinate titer of 50 g/l. Lines represent results for the realistic scenario, and dashed lines represent the optimistic scenario. The black stars denote the optimal process conditions listed in Tables 2 and 3. (A) Succinate production from sunlight, \(\text{CO}_2\) and water with cyanobacteria for the realistic scenario and improvements following increased \(\text{CO}_2\) uptake \((v_{\text{CO}_2})\) in the optimistic scenario, the inlet figure shows the total costs above 45,000 EUR/t, (B) \(C. \text{ljungdahlii} - E. \text{coli}\) strategy with \(\text{CO}_2\) fixation to acetate via the reductive acetyl-CoA pathway and conversion to succinate by \(E. \text{coli}\). Here, biomass concentration refers to \(E. \text{coli}\), which can be varied by simultaneously adjusting the volume ratios of the two coconcentrating fermenters, (C) conversion of methanol to succinate by methylotrophic yeast, the optimistic scenario is achieved by increasing the succinate yield and productivity \((v_{\text{suc}})\), D) \(\text{CO}_2\) fixation via the synthetic CETCH pathway in \(S. \text{cerevisiae}\), again the optimistic scenario is achieved by increasing the succinate yield and productivity \((v_{\text{suc}})\).

**Fig. 3.** Dependence of volumetric costs for energy, substrate, and sterilization, and the total cost to produce 1 t succinate on the biomass concentration. Calculations were based on a final succinate titer of 50 g/l. Lines represent results for the realistic scenario, and dashed lines represent the optimistic scenario. The black stars denote the optimal process conditions listed in Tables 2 and 3. (A) Succinate production from sunlight, \(\text{CO}_2\) and water with cyanobacteria for the realistic scenario and improvements following increased \(\text{CO}_2\) uptake \((v_{\text{CO}_2})\) in the optimistic scenario, the inlet figure shows the total costs above 45,000 EUR/t, (B) \(C. \text{ljungdahlii} - E. \text{coli}\) strategy with \(\text{CO}_2\) fixation to acetate via the reductive acetyl-CoA pathway and conversion to succinate by \(E. \text{coli}\). Here, biomass concentration refers to \(E. \text{coli}\), which can be varied by simultaneously adjusting the volume ratios of the two coconcentrating fermenters, (C) conversion of methanol to succinate by methylotrophic yeast, the optimistic scenario is achieved by increasing the succinate yield and productivity \((v_{\text{suc}})\), D) \(\text{CO}_2\) fixation via the synthetic CETCH pathway in \(S. \text{cerevisiae}\), again the optimistic scenario is achieved by increasing the succinate yield and productivity \((v_{\text{suc}})\).

4. Discussion

Here, we analyzed the performance of different \(\text{CO}_2\) to succinate conversion strategies and evaluated the conditions required for an economically viable process. With production costs in the range of 1100–2100 EUR/t (Table 3), the costs of succinic acid from \(\text{CO}_2\) and methanol are comparable to glucose-based bioprocesses. The most cost-effective processes are achievable with the strategies involving acetogens (\(C. \text{ljungdahlii}/E. \text{coli}\) and the engineered carbon-fixing \(S. \text{cerevisiae}\), whereas expenses are higher for the cyanobacterial and methylotrophic strategy. Our assessment of production costs also support the results of Comer et al. (2017) that the biggest contributor to the expense for the methylotrophic strategy is the methanol price, for which we had chosen the current market price (Table 1). \(\text{CO}_2\) conversion to methanol is already conducted in conditions with cheap electricity and proximity to \(\text{CO}_2\) intense industries (Carbon Recycling Inc, BASF), and the future methanol price is expected to decrease to 100 EUR/t (Pfeifenschneider et al., 2017), i.e., to nearly one quarter of the current price. That being the case, the production cost of the methylotrophic strategy would decrease to 1100–1600 EUR/t succinic acid.

All evaluated strategies require substantial optimization of the strain performance to increase production rates to the limit of commercial competitiveness (Fig. 5). The blue circles in Fig. 5 indicate that production rates achievable with standard metabolic optimization (i.e., optimization of co-factor supply, suppression of competitive reaction pathways) that mainly affect the product yield are insufficient because productivities would remain below the minimum of 2.5 g/l/h defined by the US Department of Energy (Fig. 5, green circles) (Werpy et al., 2004). Our simulations have shown that high-cell density fermentations can increase volumetric productivity but do not result
in a reduction of overall production costs because of reduced overall product yield and consequently increased substrate costs. Rather, significant research must be devoted to optimize the substrate uptake and succinate production rates of the simulated optimistic scenarios to achieve economic succinate production from C1 carbon sources.

One major drawback of the cyanobacteria-based process is the low specific carbon uptake and fixation rate at increased biomass concentrations, which needs to be addressed to elevate the productivity. The carbon uptake rate of cyanobacterial fermentations is generally high during the initial growth rate, but decreases at steady state when biomass concentrations of approx. 3 gCDW/l are reached (Zhang et al., 2015). The decrease in carbon uptake is probably caused by shadow-casting of cells at higher biomass concentrations resulting in photon-limited growth. This condition is accounted for in the realistic scenario, in which the carbon uptake has been limited to experimental rates of 0.5 mmol/gCDW/h (Gong et al., 2015) and photon fluxes to 6 mmol/gCDW/h (Table 2). To increase the carbon uptake at high biomass concentrations, new bioreactor designs are required. We have simulated the cyanobacterial strategy assuming steady metabolism during the assumed 10 h of sunlight, during which all carbon is converted into succinic acid, and no metabolic activity in the dark. A more realistic representation of a cyanobacterial strategy requires the more accurate inclusion of diurnal cycles with different rates of production. Consequently, the predictions for cyanobacteria might be particularly optimistic and the effective potential of the cyanobacterial process be lower than reported here (Knap and Steuer, 2015; Savakis and Hellingwerf, 2015).

Similarly, the *C. ljungdahlii* fermentation is limited by the transfer rate of the energy carrier H2 in the realistic scenario and significantly elevated gas transfer rates are required to increase the productivity beyond the productivity limit. Such an increase can be achieved by using pressurized systems along with an optimization of the bioreactor configuration for improved gas mass transfer (kLa) (Kantzow et al., 2015; Orgill et al., 2013). Further potential bottlenecks caused by enzymatic capacities need to be identified and targeted by metabolic or protein engineering. Optimization of acetate production in *C. ljungdahlii* is demanding because acetate production is coupled to growth with the consequent use of acetyl-CoA for anabolism (Abubackar et al., 2015; Cotter et al., 2009). Hence, achieving higher yields of acetate production requires complex deregulation and decoupling from cell growth.

The methylo trophic yeast *P. pastoris* is a model organism, for which a range of genetic engineering tools exist facilitating metabolic engineering efforts. The most important genetic modifications are likely to be associated to the engineering of a switch that changes the metabolic objective from growth to succinic acid production, while still maintaining high metabolic fluxes. Difficulties in enabling this transition has led Klamt et al. (2017) to conclude that two-stage fermentations are not outperforming one-stage fermentation strategies. However, in some organisms nitrogen limitation causes growth arrest while C-sources are still metabolized at high rate (Zambanini et al., 2016). Also, *E. coli* has been manipulated to show high glucose throughput in resting conditions (Michalowski et al., 2017), thus enabling more favorable two-stage fermentation strategies. The *P. pastoris* strategy had in our simulations a very long growth phase, because it was assumed to take place with methanol. Adaptive laboratory evolution to select faster growing mutants or the use of alternative methylo trophic organisms with higher growth rates, e.g., *Pseudomonas methylotropha* (*Methylobacillus glycoxogenes*) (Goldberg et al., 1976) might overcome this barrier.

The biotechnological use of synthetic autotrophic *S. cerevisiae* is very promising but still years away. The stable integration of more than twenty heterologous genes and expression of the encoding heterologous enzymes in active form is a great molecular biology challenge. Furthermore, the expression levels and reaction fluxes must be fine-tuned and harmonically integrated into the host metabolism. The feasibility of the synthetic autotrophs would have to be re-evaluated once the basic challenges of synthetic transplantation of trophic modes is solved.

The basic economic analysis of autotrophic succinic acid production conducted here was based on minimal input of experimental data and excluded downstream processing and investment costs. Further, more detailed economic evaluations are necessary to evaluate sustainability and competitiveness similar to Zhuang and Herrgard (2015) who included land use, and agricultural features of the feedstock, impact of the energy and market sector, metabolic details of the applied strain and process designs. If similarly adapted for autotrophic succinic acid production, it is possible to more precisely identify optimal environmental and regulatory application niches.

5. Conclusion

The evaluation of the economic performance of microbial conversion of CO2 to succinate is an important step in identifying the most
applicable solutions and the knowledge and engineering gaps that have to be addressed (Köhler et al., 2015). The applied FBA-based method quantitatively explores the metabolic capabilities based on technical and physiological constraints and thus allows a first screening and evaluation of process alternatives at very early development stages, at which no detailed data and insight is available.

Whereas conventional petrochemical and fermentative succinic acid production based on glucose will provide the bulk of succinic acid because of ubiquitous substrate availability, simple process design and high productivities, our analysis suggests that also CO₂ can be a sustainable feedstock for succinic acid production if readily available or efficiently being converted to methanol, and if technical barriers such as limiting gas transfer rates or shadow-casting in photobioreactors can be overcome.

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Appendix A Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micron.2018.e00075.

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