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Key Process Conditions for Production of C_4 Dicarboxylic Acids in Bioreactor Batch Cultures of an Engineered *Saccharomyces cerevisiae* Strain[▽]

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A recent effort to improve malic acid production by *Saccharomyces cerevisiae* by means of metabolic engineering resulted in a strain that produced up to 59 g liter^{-1} of malate at a yield of 0.42 mol (mol glucose)$^{-1}$ in calcium carbonate-buffered shake flask cultures. With shake flasks, process parameters that are important for scaling up this process cannot be controlled independently. In this study, growth and product formation by the engineered strain were studied in bioreactors in order to separately analyze the effects of pH, calcium, and carbon dioxide and oxygen availability. A near-neutral pH, which in shake flasks was achieved by adding $CaCO_3$, was required for efficient C_4 dicarboxylic acid production. Increased calcium concentrations, a side effect of $CaCO_3$ dissolution, had a small positive effect on malate formation. Carbon dioxide enrichment of the sparging gas (up to 15% [vol/vol]) improved production of both malate and succinate. At higher concentrations, succinate titers further increased, reaching 0.29 mol (mol glucose)$^{-1}$, whereas malate formation strongly decreased. Although fully aerobic conditions could be achieved, it was found that moderate oxygen limitation benefitted malate production. In conclusion, malic acid production with the engineered *S. cerevisiae* strain could be successfully transferred from shake flasks to 1-liter batch bioreactors by simultaneous optimization of four process parameters (pH and concentrations of CO_2, calcium, and O_2). Under optimized conditions, a malate yield of 0.48 ± 0.01 mol (mol glucose)$^{-1}$ was obtained in bioreactors, a 19% increase over yields in shake flask experiments.

In recent years, biologically produced 1,4-dicarboxylic acids (succinate, malate, and fumarate) have attracted great interest as more sustainable replacements for oil-derived commodity chemicals, such as maleic anhydride (50). Malate is currently mainly produced via petrochemical routes for use in food and beverages (18). Development of a biotechnological production process started in the early 1960s with the investigation of the natural malate producer *Aspergillus flavus* (2). Although process improvements eventually resulted in high product yields and productivities (6), the potential production of aflatoxins (20) prevented the use of this filamentous fungus in industry. Other investigated natural malate-producing fungi (listed in reference 51) produced insufficient malate for industrial use. With the rational design options of metabolic engineering, microorganisms that do not naturally produce large amounts of malic acid may also be considered as production platforms. Wild-type *Saccharomyces cerevisiae* strains produce little if any malate but would be an interesting starting point for the construction of an efficient malate producer. This yeast has a relatively high tolerance to organic acids and low pH, and due to its role as a model organism in research, a well-developed metabolic engineering toolbox is available. In addition, wild-type *S. cerevisiae* strains have GRAS (Generally Regarded As Safe) status, so that modified strains are more likely to be allowed in the production of food-grade malic acid.

One of the main challenges in the development of an organic acid-producing strain of *S. cerevisiae* has been the elimination of ethanol formation, which in wild-type strains occurs even under aerobic conditions when glucose concentrations are high (45). Deletion of the pyruvate decarboxylase-encoding genes was found to prevent ethanolic fermentation (17). After evolutionary engineering to remove the growth defects usually associated with pyruvate decarboxylase-negative *S. cerevisiae* strains, a strain was obtained that produced large amounts of pyruvate, a direct precursor to malate, when grown on glucose (42). Subsequent overexpression of the anaplerotic enzyme pyruvate carboxylase, a cytosolically relocalized malate dehydrogenase and a heterologous malate transporter from *Schizosaccharomyces pombe* led to a strain that produced significant amounts of malate (51). Cultivation in calcium carbonate ($CaCO_3$)-buffered shake flasks resulted in malate titers of up to 59 g liter^{-1} at a yield of 0.42 mol (mol glucose)$^{-1}$.

There are many differences between cultivation in shake flasks and cultivation in (laboratory or industrial) bioreactors. As shake flask cultures lack online pH monitoring and control, there is often significant pH variation over time. The pH is of particular importance. If the yeast can be persuaded to produce organic acids at lower pH values, this reduces the need for active neutralization and thereby reduces by-product formation such as gypsum. However, thermodynamic constraints on acid export, as well as increased stress levels from (undissociated) acid and the low pH, often limit the ability of the microorganisms to produce acids at low pH (32, 43). For this reason, the poorly soluble compound $CaCO_3$ has traditionally been used to maintain a near-neutral pH in malic acid-producing microbial cultures (6, 29, 51). Adding $CaCO_3$ also gives increased concentrations of bicarbonate (and thereby CO_2), a substrate for pyruvate carboxylase in the carboxylation of pyru-

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vate (a C_3 carbon molecule) to oxaloacetate (C_4 carbon), as well as calcium. Calcium is known to be involved in cellular signaling pathways (22, 26, 33, 46) and to influence pyruvate carboxylase activity (21, 24). Finally, oxygen transfer rates in shake flasks are often poor compared to those in stirred (laboratory) bioreactors. The formation of significant concentrations (25 g liter^{-1}) of glycerol, a well-known redox sink in *S. cerevisiae* (41), in shake flask cultures of the engineered malate-producing strain (51) was a strong indication of oxygen limitation.

Initial experiments in aerobic, pH-controlled bioreactor cultures of the malate- and succinate-producing *Saccharomyces cerevisiae* strain RWB525 yielded only low concentrations of these C_4 dicarboxylic acids. The goal of the present study was to identify process parameters that explain the different production levels in shake flask and bioreactor cultures. To this end, we analyzed, both separately and in combination, the impact of culture pH and concentrations of calcium, carbon dioxide, and oxygen on the production of malate and succinate.

MATERIALS AND METHODS

Strain and maintenance. The malate-producing strain RWB525 (51) is derived from the *S. cerevisiae* CEN.PK strain family (40). Stock cultures were prepared from shake flask cultures grown on 100 ml synthetic medium consisting of demineralized water, 20 g liter^{-1} glucose, 5 g liter^{-1} $(NH_4)_2SO_4$, 3 g liter^{-1} KH_2PO_4, 0.5 g liter^{-1} $MgSO_4 \cdot 7H_2O$, vitamins, and trace elements (44), pH 6 (set with KOH). Round-bottom shake flasks (500 ml) were incubated at 30°C and 200 rpm in a rotary shaker. After addition of glycerol (20% vol/vol), 2-ml aliquots were stored at −80°C.

Shake flask cultivations. Inocula for batch fermentations were obtained by inoculating preculture shake flasks with 2 ml frozen stock culture. The preculture medium was identical to the stock culture medium, except that urea (2.3 g liter^{-1}) was used instead of $(NH_4)_2SO_4$ and 6.6 g liter^{-1} K_2SO_4 was added. After 48 h of incubation, the biomass was centrifuged and resuspended in 10 ml demineralized water. The cell suspension was then evenly distributed over two shake flasks with fresh medium, in order to obtain more biomass. After an additional 24 h, biomass was again collected by centrifuging, resuspended in demineralized water, and used to inoculate the bioreactors at an initial dry weight of ca. 0.25 g liter^{-1}. Calcium carbonate-buffered shake flask cultures for malic acid production were performed as described earlier (51).

Bioreactor batch fermentations. Aerobic batch cultivation was done at 30°C in 2-liter bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1 liter. The pH was controlled by the automatic titration of base (10 M KOH was used to minimize dilution of the fermentation broth). For fermentations run at a pH below that of the medium (pH 4.8, low buffering capacity), no correction was made by acid addition (here the desired pH was attained within the first few hours after inoculation). The bioreactors were sparged with 0.5 liter gas per minute and stirred at 800 rpm, which ensured dissolved oxygen concentrations above 30% of air saturation, as measured by an oxygen electrode for non-oxygen-limited fermentations. For CO_2-enriched fermentations, pure CO_2 was mixed with air, except when gaseous CO_2 concentrations above 15% were needed. In those cases, a blend of 21% O_2 and 79% CO_2 was mixed with air to maintain a sufficiently high concentration of dissolved oxygen. The medium was identical to the stock culture medium, except for the nitrogen source (1 g liter^{-1} urea), the glucose concentration (100 g liter^{-1}), the addition of K_2SO_4 (6.6 g liter^{-1}), and, where indicated, $CaCl_2$. Silicon antifoam (BDH, Poole, England) was added to control foaming. Glucose was autoclaved separately (110°C for 20 min), while urea and vitamins were filter sterilized.

Dry weight determination. Culture samples (5 ml) were filtrated over oven-dried and weighed nitrocellulose filters (0.45-μm pore size; Gelman Sciences) and washed with demineralized water. After being dried in a microwave oven for 20 min at 360 W, the filters were weighed again. The weight increase was used to calculate biomass dry weight concentration. Samples from cultures with added $CaCl_2$ were diluted 1:1 with 1 M HCl shortly before filtration, to dissolve precipitates.

Metabolite analysis. Extracellular concentrations of acetate, ethanol, fumarate, glucose, glycerol, lactate, and succinate were determined by high-pressure liquid chromatography (HPLC), using a Bio-Rad Aminex HPX-87H column eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} and at 60°C. Acetate, fumarate, and lactate were detected by a Waters 2487 dual-wavelength absorbance detector at 214 nm. Ethanol, glucose, glycerol, and succinate were detected with a Waters 2410 refractive index detector. Malate concentrations were determined by enzymatic analysis (Enzyplus L-malic acid kit no. EZA786; BioControl Systems, Inc.). Pyruvate was assayed enzymatically with a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 0.17 mM NADH and diluted culture supernatant. Pyruvate was determined by measuring NADH consumption after addition of lactate dehydrogenase (6 U ml^{-1}). To support HPLC analysis, glucose was determined enzymatically (EnzyPlus D-glucose kit no. EZS781). All metabolite and biomass concentrations were corrected for dilution by titration of the cultures with KOH. Precipitates were found only for fermentations supplemented with $CaCl_2$, and dissolving the precipitates by acidification did not result in increased carboxylic acid titers. Data from independent duplicate cultures are presented as averages and standard deviations from the means.

RESULTS

Aerobic bioreactor batch cultures of the malate-producing, engineered *S. cerevisiae* strain RWB525 (51) were grown on a synthetic medium with 98 ± 1 g liter^{-1} (542 ± 4 mM) glucose as the sole carbon and energy source (Fig. 1A). Temperature and pH were maintained at 30°C and 5.0, respectively, which are routinely used conditions for batch cultivation of *S. cerevisiae*. In these cultures, growth ceased about 25 h after inoculation. The final biomass concentration of 10 ± 0 g liter^{-1} reached in the bioreactors was higher than the biomass concentration of 6.0 g liter^{-1} obtained with calcium carbonate-buffered shake flasks cultures with 100 ± 1 g liter^{-1} (555 ± 6 mM) glucose, even though bioreactors were inoculated with only 0.25 g liter^{-1} biomass dry weight, versus 1 g liter^{-1} for shake flasks. Fermentation times were similar, with glucose depletion occurring after 83 ± 2 h in bioreactors and after ca. 72 h in shake flasks.

When the glucose became depleted, the malate and succinate concentrations in the bioreactor cultures had reached 77 ± 4 and 27 ± 1 mM, respectively (Fig. 1A). The malate yield on glucose in the bioreactor cultures was a third of that previously found in calcium carbonate-buffered shake flask cultures (0.14 ± 0.01 and 0.41 ± 0.04 mol mol^{-1}, respectively). The succinate yield in the bioreactor cultures was half that in the shake flasks (0.05 ± 0.00 and 0.11 ± 0.03 mol mol^{-1}, respectively). Concentrations of fumarate, already low in shake flask cultures (5 ± 1 mM), were only 1 ± 0 mM in duplicate bioreactor batch cultures.

In the bioreactor cultures, half of the substrate carbon was diverted to pyruvate production (565 ± 2 mM) (Fig. 1A) rather than to dicarboxylate production. The resulting high pyruvate yield of 1.0 ± 0.0 mol mol^{-1} was about twice as high as that in $CaCO_3$-buffered shake flasks (0.56 ± 0.04 mol mol^{-1}). Conversely, the glycerol yield on glucose was much lower in the bioreactor cultures than in the shake flask cultures (0.08 ± 0.00 and 0.18 ± 0.02 mol mol^{-1}, respectively). This suggested that the efficient aeration in the bioreactors increased the reoxidation of NADH formed in glycolysis via mitochondrial respiration while decreasing the reoxidation via reduction of dihydroxyacetone phosphate to glycerol.

After glucose had been depleted, the succinate and fumarate concentrations continued to increase slowly, while glycerol, pyruvate, and malate concentrations slowly decreased (Fig. 1A).

Strong pH dependency of malate productivity. The average pH of approximately 6 in calcium carbonate-buffered shake

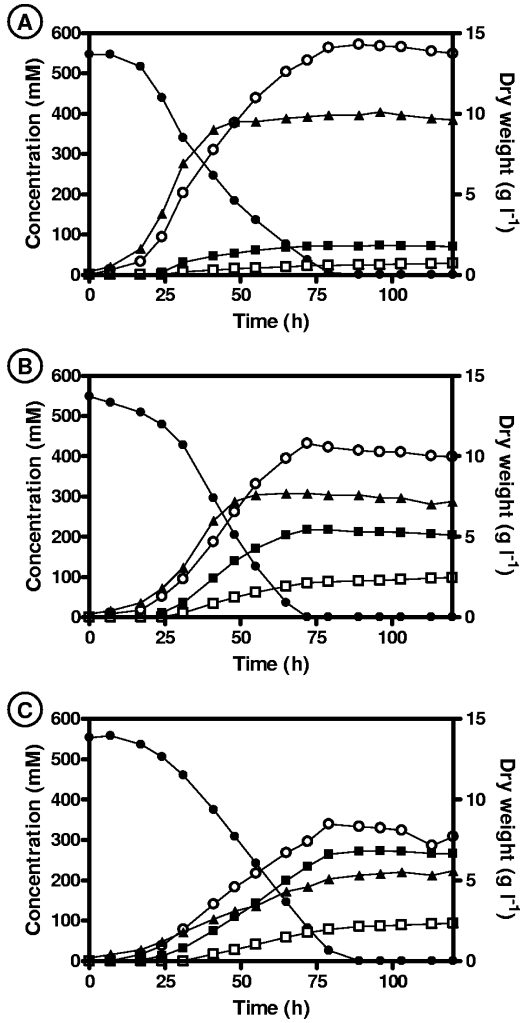

FIG. 1. Extracellular concentrations of glucose and metabolites during aerated bioreactor batch cultivation of the engineered malate production strain *S. cerevisiae* RWB525 on 100 g liter^{-1} glucose at 30°C. (A) Culture grown at pH 5, sparged with air; (B) culture grown at pH 6.8, sparging with air/CO_2 mixture containing 15% CO_2; (C) culture grown at pH 6.8 supplemented with 10 mM $CaCl_2$, sparged with an air/CO_2/N_2 mixture containing 15% CO_2 and a limiting O_2 concentration of 3%. Symbols: ●, glucose; ○, pyruvate; ■, malate; □, succinate; ▲, biomass (right axis).

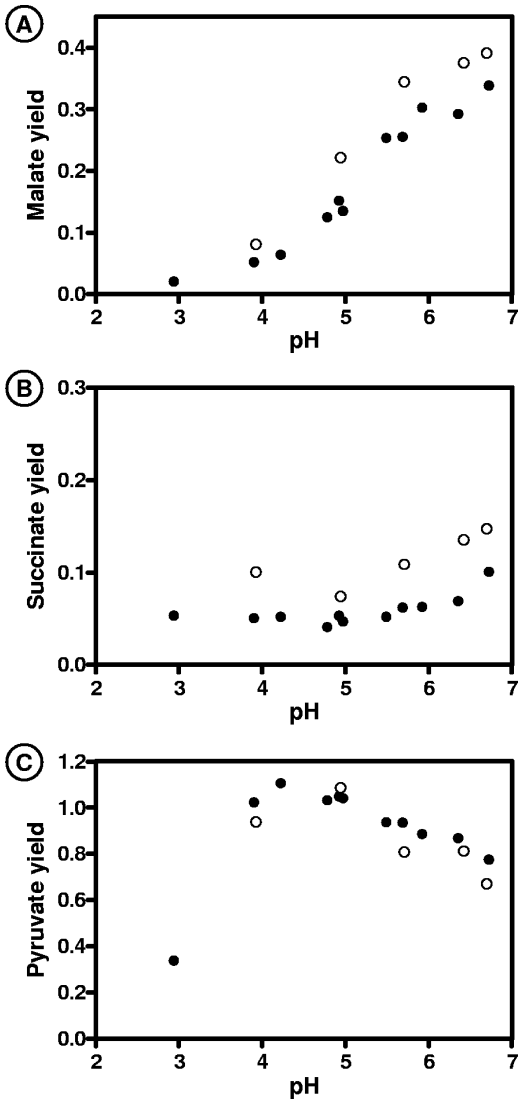

FIG. 2. Molar yields on glucose of malate (A), succinate (B), and pyruvate (C) for aerobic bioreactor batch cultivations of *S. cerevisiae* RWB525 on 100 g liter^{-1} glucose as a function of pH (each data point represents an independent fermentation). Yields were determined for the time point at which the highest malate concentration was observed. Closed and open symbols indicate sparging with air and with air enriched to 10.8 ± 0.4% CO_2, respectively.

flasks (51) differed significantly from the KOH-titrated pH of 5.0 used in the reference bioreactor cultures. As culture pH has a major impact on weak organic acid transport (43), tolerance to organic acids (30), and the process economy of downstream processing, the impact of pH on malate production by *S. cerevisiae* RWB525 in aerobic bioreactor cultures was studied over a pH range from 2.9 to 6.7.

At higher culture pH values, concentrations of dicarboxylic acids increased (Fig. 2A and B), while pyruvate production fell (Fig. 2C). The pH dependency of malate production was much more pronounced than that of succinate production. Final malate and succinate titers in cultures grown at pH 6.7 were 191 mM and 57 mM, respectively (corresponding to malate and succinate yields of 0.34 and 0.10 mol [mol glucose]$^{-1}$, respectively). In cultures grown at a pH of 3.9, only 28 mM malate and 27 mM succinate were produced. Glycerol and fumarate titers showed positive correlations with pH (21 and 1 mM at pH 3.9 and 52 and 13 mM at pH 6.7, respectively). In contrast, the final pyruvate concentration at pH 6.7 (438 mM) was lower than that at pH 3.9 (556 mM). Complete consumption of glucose took considerably longer at low pH, with fermentation times increasing to 101 h at pH 4.2 and 160 h at pH 3.9. At pH 2.9, the lowest pH tested, fermentation became stuck after 96 h, with half the glucose left unconsumed. Carbon balances of the bioreactor batch experiments showed carbon recoveries of 95% ± 4%.

Impact of carbon dioxide on C_4 dicarboxylic acid production. The previously discussed fermentations were sparged with air, with CO_2 concentrations in the off gas averaging 0.4 ±

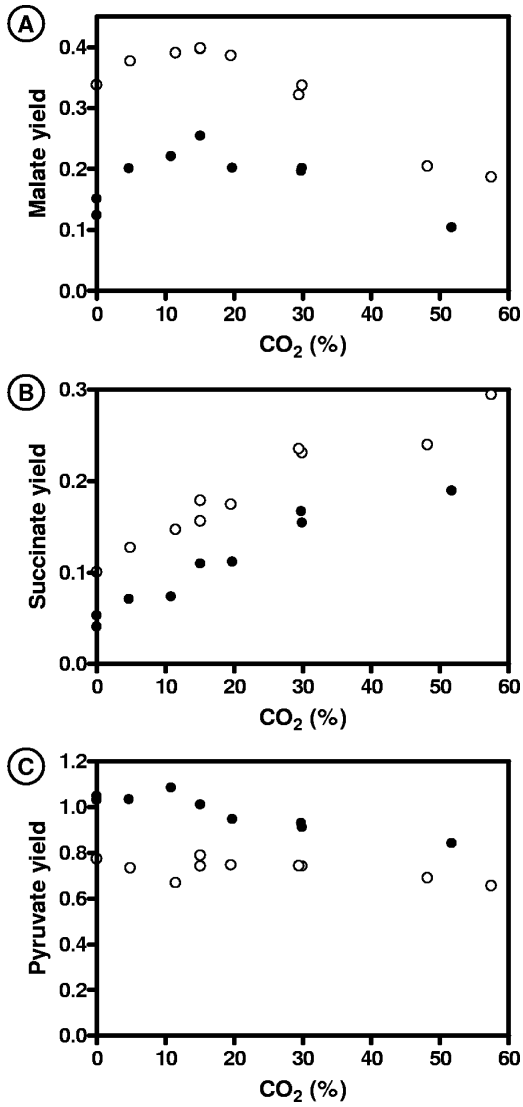

FIG. 3. Molar yields on glucose of malate (A), succinate (B), and pyruvate (C) for aerobic bioreactor batch cultivations of *S. cerevisiae* RWB525 on 100 g liter^{-1} glucose as a function of the CO_2 concentration in the inlet gas (each data point represents an independent fermentation). Yields were determined for the time point at which the highest malate concentration was observed. Closed and open symbols indicate cultivation at pH 5 and at pH 6.8, respectively.

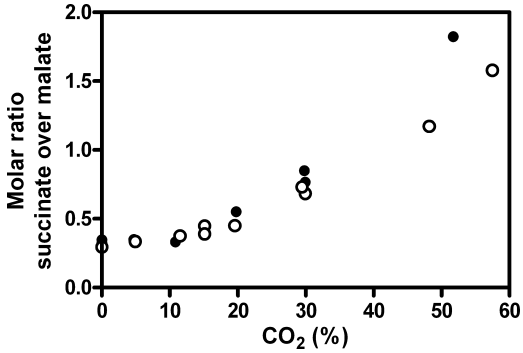

FIG. 4. Molar ratio of succinate to malate as a function of CO_2 concentration in the inlet gas for aerobic bioreactor batch cultivations of *S. cerevisiae* RWB525 on 100 g liter^{-1} glucose (each data point represents an independent fermentation). Ratios were determined for the time point at which the highest malate concentration was observed. Closed and open symbols indicate cultivation at pH 5 and pH 6.8, respectively.

0.1% over the fermentation experiments and peaking at 1.1 ± 0.3% at the end of the exponential growth phase. We subsequently investigated whether the concentration of CO_2 affects dicarboxylic acid yields, for example by influencing the carboxylation of pyruvate. To this end, fermentations were run at different pH values, while bioreactors were sparged with air enriched with 10.8 ± 0.4% CO_2. The CO_2 enrichment increased malate and succinate yields on glucose over the entire range of pH values tested (Fig. 2A and B), while pyruvate (Fig. 2C) and glycerol titers slightly decreased. At pH 3.9, sparging with CO_2-enriched air extended the fermentation time to 191 h, an effect not observed at higher pH values.

After the positive effect of CO_2 on dicarboxylic acid production had been established using a fixed CO_2 concentration in the inlet gas, additional cultures were run at pH values of 5 and 6.8 to identify the optimal CO_2 concentration for malate and succinate production. At both pH values, a gaseous CO_2 concentration of about 15% gave the highest malate yields (Fig. 3A). Higher levels of CO_2 resulted in lower malate yields, extended fermentation duration (104 to 115 h at CO_2 concentrations of 50% and above), and reduced biomass yields. The latter effect was more pronounced at pH 6.8, where, when 57% CO_2 was used, the final biomass concentration was only 6 g liter^{-1}.

In contrast to the negative impact on malate production of CO_2 levels above 15%, succinate titers continued to increase with the CO_2 concentration in the sparging gas (Fig. 3B). At CO_2 concentrations above 50%, succinate even became the dominant dicarboxylic acid at both culture pH values investigated (Fig. 4). In the culture grown at 57% CO_2 and pH 6.8, succinate and malate yields of 0.29 and 0.19 mol (mol glucose)$^{-1}$ were obtained. Glycerol and fumarate yields did not substantially change over the tested range of CO_2 concentrations (data not shown), while pyruvate showed a slight negative trend with increasing CO_2 levels (Fig. 3C).

On the basis of the observed effects of pH and CO_2 enrichment, duplicate aerobic bioreactor batch cultures on 100 ± 1 g liter^{-1} (553 ± 5 mM) glucose, grown at pH 6.8 and with a CO_2 concentration in the inlet gas of 15%, were analyzed in detail (Fig. 1B). Compared to the reference cultures (pH 5, sparging with air), a substantially higher malate titer was observed (219 ± 2 mM, corresponding to a malate yield of 0.40 ± 0.00 mol [mol glucose^{-1}]), while the pyruvate titer decreased to 422 ± 9 mM (0.8 ± 0.0 mol mol^{-1}). Glycerol levels remained around 45 ± 2 mM (0.08 ± 0.00 mol mol^{-1}), while succinate and fumarate increased significantly to 92 ± 7 (0.17 ± 0.01 mol mol^{-1}) and 13 ± 3 mM (0.02 ± 0.01 mol mol^{-1}), respectively. Fermentation time (75 ± 3 h) was slightly shorter than in the reference cultures, and the final biomass concentration (8 ± 0 g liter^{-1}) was about 20% lower.

Effects of calcium on malate production. Although malate production was greatly improved by optimizing the pH and CO_2-concentration in the sparging gas, the malate yield of 0.40 mol mol^{-1} did not surpass the yields obtained in $CaCO_3$-

buffered shake flasks. As well as increasing pH and CO_2 availability, the use of $CaCO_3$ gives a higher Ca^{2+} concentration. In *S. cerevisiae*, Ca^{2+} is involved in signaling pathways, stress responses, and maintenance of cellular integrity (22, 26, 46). Ca^{2+} might also affect production of organic acids by chelation (37). Finally, Ca^{2+} has been found to influence pyruvate carboxylase activity in rat liver mitochondria (21, 48) and in the yeast *Torulopsis glabrata* (24).

To investigate the possible impact of Ca^{2+} on dicarboxylic acid production by *S. cerevisiae* RWB525, concentrations of up to 100 mM $CaCl_2$ were tested in bioreactor batch cultures grown at pH 6.8 and sparged with 15% CO_2. Over the range of tested calcium concentrations, a modest increase (ca. 5%) in malate yield was observed only when 5 or 10 mM $CaCl_2$ was added (data not shown). Subsequent fermentation experiments were therefore carried out with 10 mM $CaCl_2$.

Positive effect of oxygen limitation on malate production. As mentioned above, oxygen transfer capacities of (unbaffled) shake flasks are much lower than those of laboratory bioreactors. The batch cultivation experiments discussed above all had dissolved oxygen concentrations above 30% of air saturation. To investigate the possible effect of oxygen limitation on malate production, the oxygen concentration in the sparging gas was reduced by using mixtures of air, CO_2, and N_2 while keeping the culture pH at 6.8 and the CO_2 concentration in the inlet gas at 15%. Although complete depletion of oxygen in the off gas did not occur, the reduced oxygen supply did result in oxygen limitation, as was evident from near-zero dissolved oxygen concentrations. Oxygen limitation did not occur at the start of the fermentation, when biomass concentration was still low, or after glucose had been depleted. With this setup, it was observed that with ingoing oxygen concentrations of 2 to 4%, the total fermentation time was about 100 h, with oxygen limitation occurring during a period of ca. 50 h in the middle of the fermentation. Although the severity of oxygen limitation varied, oxygen limitation in three separate cultures clearly resulted in lower pyruvate yields (0.57 ± 0.08 mol mol glucose^{-1}) and higher malate yields (0.48 ± 0.01 mol mol glucose^{-1}). More severe oxygen limitation, such as that achieved by a very low oxygen concentration of 0.4% in the ingoing gas, led to an earlier onset of oxygen limitation but severely lengthened the fermentation: after 144 h, during which biomass dry weight had increased linearly to only 2 g liter^{-1}, just one-fifth of the glucose initially present had been consumed. For further analysis, an oxygen percentage of 3% in the ingoing gas was used.

To analyze the combined impact of all 4 parameters (pH, CO_2, Ca^{2+}, and O_2 limitation), duplicate batch experiments were carried out with 100 ± 1 g liter^{-1} (556 ± 3 mM) glucose, pH 6.8, CO_2-enriched (15%) air, 10 mM $CaCl_2$, and a reduced oxygen concentration (3%) in the inlet gas (Fig. 1C). Compared to cultures without $CaCl_2$ addition and oxygen limitation, the greatest changes were observed for malate titers, which increased from 219 ± 2 mM to 268 ± 5 mM (corresponding to a yield on glucose of 0.48 ± 0.01 mol mol^{-1}), and for pyruvate titers, which decreased from 422 ± 9 mM to 347 ± 18 mM (0.6 ± 0.0 mol mol^{-1}). Final titers of the other metabolites remained at similar levels: 92 ± 2 mM glycerol (0.17 ± 0.00 mol mol^{-1}), 86 ± 1 mM succinate (0.15 ± 0.00 mol mol^{-1}), and 18 ± 0 mM fumarate (0.03 ± 0.00 mol mol^{-1}). The total fermentation time was slightly longer (82 ± 1 h), and biomass dry weight fell to 6 ± 0 g liter^{-1}.

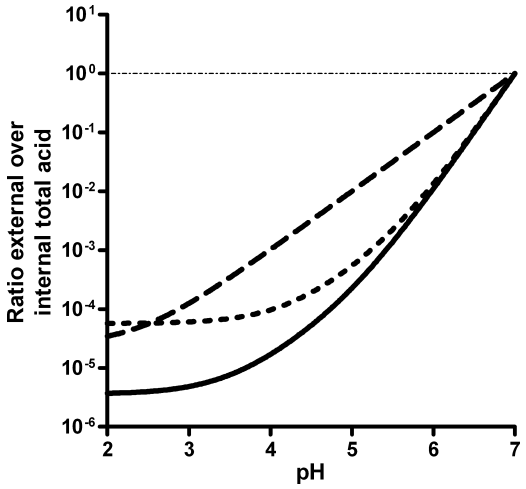

FIG. 5. Theoretical ratios of extracellular to intracellular concentrations of malate (solid line), succinate (dotted line), and pyruvate (dashed line). Equilibrium ratios were calculated on the assumption that all three compounds are exported via monoanion proton symport, on a proton motive force across the yeast plasma membrane of −150 mV and an intracellular pH of 7 and on the following acid dissociation (pK_a) constants: malate, 3.46 and 5.10; pyruvate, 2.39; succinate, 4.16 and 5.61. Calculations were performed as described earlier (1, 43).

DISCUSSION

Impact of culture pH on organic acid production. A strong positive correlation between increasing culture pH and malate yields was found in bioreactor batch cultures of the engineered *S. cerevisiae* strain RWB525 (Fig. 2A). This observation might be explained by the equilibrium thermodynamics of product export (8, 43). Export of malate and succinate in *S. cerevisiae* RWB525 has been shown to be strongly dependent on expression of the heterologous malate transporter SpMae1 (51), which seems to catalyze electroneutral proton-coupled symport of the monoanion species of these dicarboxylates (9, 36). Export via this transport mechanism would become progressively more difficult as the extracellular pH decreases (Fig. 5). Due to the different acid dissociation constants of the two dicarboxylates, this pH dependency is predicted to be more pronounced for malate production than for succinate (Fig. 5). This is consistent with the experimental observation that succinate production was much less affected by culture pH than malate production (Fig. 2).

Production of pyruvate, the major by-product of all bioreactor fermentations in this study and the key precursor of malate production via the engineered pyruvate-carboxylase-dependent pathway in *S. cerevisiae* RWB525, occurred even at low pH. In cultures grown at pH 3.9, the final extracellular pyruvate concentration exceeded 0.5 M. Jen1p, the only pyruvate transporter that has hitherto been characterized in *S. cerevisiae*, is essential for pyruvate uptake (4, 25) and catalyzes electroneutral anion-proton symport (12, 13). However, this mode of transport appears to be incompatible with efficient pyruvate export at low pH (Fig. 5). Even if the cytosolic pH is

strongly reduced, pyruvate concentrations would have to be several orders of magnitude higher intracellularly than extracellularly. It is therefore likely that, at least at low pH values, pyruvate is exported via different mechanisms, presumably by as-yet-unidentified ABC transporters.

Not only does the culture pH impact the thermodynamics of product export, but also it has been reported that a low extracellular pH, combined with the presence of organic acids, can decrease the cytosolic pH (11, 28). With a pK_a of 6.35, the equilibrium between carbon dioxide and bicarbonate would be strongly influenced by changes in the intracellular pH. This in turn might influence pyruvate carboxylase (14), a key carboxylating enzyme in *S. cerevisiae* RWB525 for the production of malate (51).

Effects of carbon dioxide on dicarboxylate production. It has been shown that C_4 dicarboxylic acid production by bacteria can benefit from supplementation with either CO_2 (16, 31, 34) or bicarbonate salts (6, 27, 38, 39). This effect can be explained by more favorable kinetics or thermodynamics of the carboxylation reactions in C_4 acid production or by improved pH buffering. Furthermore, it is probably not a coincidence that natural succinate producers are often isolated from high-CO_2 environments, such as the rumen (35).

The most efficient C_4 dicarboxylic acid-producing pathways require a net input of CO_2. However, all cultures of the engineered *S. cerevisiae* strain RWB525 showed a net production of CO_2 due to respiratory glucose dissimilation. The positive effect of CO_2 enrichment on C_4 acid production can therefore be entirely attributed to kinetic effects. The conversion between CO_2 and bicarbonate occurs spontaneously but is also catalyzed by carbonic anhydrase (3). To assess the impact of the extracellular CO_2 concentration on C_4 acid production, we assumed that CO_2 diffuses freely over the plasma membrane (19) and that intracellular bicarbonate and CO_2 are in equilibrium. The increase of the extracellular CO_2 concentration from approximately 0.4% to 11% (Fig. 2) would then decrease the free energy change of bicarbonate-dependent pyruvate carboxylation by 8 kJ mol^{-1}, thus stimulating formation of oxaloacetate.

S. cerevisiae RWB525 was engineered with the aim of increasing yields and titers of malate. Interestingly, cultivation at CO_2 concentrations above 15% gave lower malate production but strongly stimulated succinate production, with yields and titers of succinate that are the highest known for *S. cerevisiae*. This differential effect of CO_2 on malate and succinate production must be due to different transport mechanisms or metabolic pathways involved in the production of these dicarboxylic acids.

Wild-type *S. cerevisiae* is unable to efficiently transport malate across the plasma membrane (47). In previous experiments with $CaCO_3$-buffered shake flask cultures, production of both malate and succinate transport by *S. cerevisiae* RWB525 was shown to strongly depend on functional expression of the heterologous SpMae1 transporter. Unless a succinate-specific native exporter is induced at high CO_2 concentrations, it seems unlikely that the differential effect of CO_2 on the production of the two dicarboxylates originates at the level of transport.

In the engineered *S. cerevisiae* strain RWB525, ^{13}C-labeling experiments in shake flasks suggested that malate production predominantly occurred via the overexpressed fermentative pathway in which oxaloacetate, formed by carboxylation of pyruvate, is reduced to malate (51). Intriguingly, succinate dehydrogenase, which catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid (TCA) cycle, has been shown to be inhibited by bicarbonate in several organisms (7, 15, 49). This inhibition could limit the (re)oxidation of succinate to malate, thereby explaining the observed effect of CO_2 on the production of succinate and malate. However, the exact route of succinate production remains to be investigated. *In vitro* assays to verify inhibition of succinate dehydrogenase (SDH) in *S. cerevisiae*, and ^{13}C-labeling studies to ascertain the origin of the produced succinate would likely prove valuable. In light of this, it is interesting that deletion of *SDH* genes has previously been shown to increase succinate titers in aerobic *S. cerevisiae* cultures (5, 10, 23), presumably via interruption of the TCA cycle.

From shake flask to bioreactor. The metabolically engineered, malate-producing *S. cerevisiae* strain RWB525 was initially characterized in calcium carbonate-buffered shake flask cultures for which malate yields corresponding to 21% of the theoretical maximum were obtained (51). Reproducing these results in bioreactor batch cultures proved to be a nontrivial exercise. On the basis of over 50 controlled 1-liter bioreactor experiments, culture pH and CO_2 and O_2 levels were identified as key process parameters for C_4 dicarboxylic acid production by the engineered yeast strain, while an increased calcium ion concentration had an additional, minor impact on malate production. However, optimizing these parameters gave only a modest improvement (19%) of the malate yield on glucose, compared to the shake flask cultures; further strain engineering is clearly required to reach malate yields that are compatible with industrial application. The high-level production of pyruvate by the engineered strain indicates that sufficient precursor is available for further improvement of C_4 dicarboxylate production.

The results in this paper will contribute to further strain optimization in two ways. First, rational strain improvement will benefit from the availability of a bioreactor-based fermentation system, especially where C_4 acid production at low pH, quantitative analysis, and interpretation of genome- and metabolome-wide analyses are concerned. Second, the results from the bioreactor experiments indicate that $CaCO_3$-buffered batch cultures provide favorable conditions for malate production at the current yields and titers and therefore provide a useful platform for high-throughput screening in classical strain improvement and/or metabolic engineering.

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