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Metabolic Evolution and ^{13}C Flux Analysis of a Succinate Dehydrogenase Deficient Strain of *Yarrowia lipolytica*

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ABSTRACT: Bio-based succinic acid production can redirect industrial chemistry processes from using limited hydrocarbons to renewable carbohydrates. A fermentation process that does not require pH-titrating agents will be advantageous to the industry. Previously, a *Yarrowia lipolytica* strain that was defective for succinate dehydrogenase was constructed and was found to accumulate up to 17.5 g L^{-1} of succinic acid when grown on glycerol without buffering. Here, a derivative mutant was isolated that produced 40.5 g L^{-1} of succinic acid in 36 h with a yield of 0.32 g g^{-1} glycerol. A combination approach of induced mutagenesis and metabolic evolution allowed isolation of another derivative that could utilize glucose efficiently and accumulated 50.2 g L^{-1} succinic acid in 54 h with a yield of 0.43 g g^{-1}. The parent strain of these isolated mutants was used for [1,6-$^{13}C_2$]glucose assimilation analysis. At least 35% glucose was estimated to be utilized through the pentose phosphate pathway, while ≥84% succinic acid was formed through the oxidative branch of the tricarboxylic acid cycle.

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KEYWORDS: *Yarrowia lipolytica*; *SDH2*; succinic acid; metabolic evolution; low pH

Introduction

Since 1857, several attempts were made by Louis Pasteur to analyze the products of alcoholic fermentation in detail (Pasteur, 1860). He soon found two other components that "make the wine from the water"; these were glycerol and succinic acid besides the alcohol. Following Gay-Lussac, who had deduced the equation of alcoholic fermentation, Pasteur tried to calculate the material balance of the conversion of glucose into these products. It is now apparent that at least three independent pathways, namely the reductive cytosolic pathway, the oxidative branch of the TCA cycle, and the glyoxylate cycle, might be responsible for succinic acid formation during the fermentation process (Camarasa et al., 2003; Rezaei et al., 2015). However, even after a century and a half, the pathways or combination thereof which is the most efficient for succinic acid biosynthesis by yeast cells, remains to be determined.

In last decade, the microbial synthesis of succinic acid as a prospective building block chemical has attracted special attention (Cukalovic and Stevens, 2008; McKinlay et al., 2007; Sauer et al., 2008). The majority of engineered strains that are capable of accumulating substantial amounts of succinate with both high yield and a high rate are of bacterial origin (Patent application US 5573931; Jantama et al., 2008b; Okino et al., 2008; Vemuri et al., 2002). However, most bacteria do not perform well in strong acidic environments, and it is therefore necessary to conduct the fermentation process at a moderate pH. This results in wastage of large amounts of neutralizing agents and affects the cost of the bio-based product. Furthermore, the succinic acid salt that is produced needs to be re-converted into the free acid form in a downstream process. In this regard, there is need for an advanced technology that is based on a strain that remains viable and productive despite high concentrations of succinic acid in the medium. Yeasts are the most appropriate candidates for such a purpose, since they show a number of strains that are naturally predisposed to survival under adverse conditions. This property was noticed by largest manufacturers who have directed their research toward the elaboration of yeast strains that produce succinic acid per se instead of its salt (Jansen and van Gulik, 2014).

Natural yeast strains with a few exceptions only produce succinic acid in small amounts, and as a by-product (Goldberg et al., 2006;

Abbreviations: PP pathway, pentose phosphate pathway; SDH, succinate dehydrogenase; TCA cycle, tricarboxylic acid cycle.

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Lupianez et al., 1974; Taing and Taing, 2007). A particular case is that of *Candida brumptii* IFO 0731, which accumulates up to 24 g L^{-1} of succinate in the medium (Sato et al., 1972). This process requires neutral pH and an inappropriate carbon source such as n-paraffins, which is disadvantageous for the industry. It is believed that a more advantageous process should be a cultivation with a final pH 3.0 or less (Jansen and van Gulik, 2014). Nevertheless, very few studies describe the strains designed for succinic acid production under such conditions and these include *Saccharomyces cerevisiae* SUC-297 disclosed by DSM (Patent application WO 2011064151) and *Issatchenkia orientalis* 13723 elaborated by Cargill (Patent application WO 2013112939). Both inventions were based on the reconstruction of the cytosolic reductive pathway and inactivation of the competing fermentation routes. A significant result was demonstrated with *I. orientalis* 13723 that accumulated 47.4 g L^{-1} of succinic acid with yield 0.55 g g^{-1} of glucose after 90 h of cultivation with a final pH 3.0 that was maintained by addition of alkali.

The current approaches in metabolic engineering are generally based on the introduction of directed modifications. However, the state of the art has not allowed taking into account a number of minor metabolic reactions and the complexity of the regulatory network. A rough redirection of the natural biochemical pathways by gene manipulation, as well as accumulation of induced mutations, typically leads to reduced metabolic activity and growth retardation. An approach that allows compensation for such rough metabolic rearrangements through the adaptation of an organism by spontaneous mutagenesis is known as “metabolic evolution” (Jantama et al., 2008a). The advantage of this approach is the possibility of stepwise accumulation of multiple mutations, each of which has little effect individually, but in conjunction, they compensate the rising imbalance. While selective pressure improves the growth rate, the latter may not be accompanied by increasing of productivity. Therefore, for application of this approach, an accumulation of a product should be inextricably linked with an elevation of the growth rate. This is essentially the case of *SDH2* gene deletion in the aerobic yeast *Yarrowia lipolytica* (Yuzbashev et al., 2010, 2011).

Y. lipolytica obligatorily used the citric acid cycle to generate the reducing equivalents required for the respiratory chain. An interruption of the TCA cycle at the succinate dehydrogenase (SDH) reaction resulted in accumulation of succinic acid as the main product of substrate oxidation along with carbon dioxide. There is no another pathway to convert succinate to fumarate except the SDH reaction, as well as the deletion of *SDH2* gene cannot revert. Therefore, the viability of the strain strongly depends on adaptation to the operation of TCA cycle in such interrupted manner. This fact allowed using a simple approach of direct selection for improving viability and therefore enhancing succinic acid production under conditions desired. Induced mutagenesis allowed isolation of some derivatives that accumulated high levels of succinic acid on the media with glycerol under conditions without buffering. However, glucose, which is a favorable raw material in the industry, was found to be a less preferred substrate for the isolated strains. Attempts to improve growth rate and production on glucose media by means of induced mutagenesis did not give proper results. Therefore, the metabolic evolution approach based on long-term cultivation in steady-state (chemostat) culture was applied. The resultant strain efficiently utilized glucose and accumulated succinic acid at high titer without any pH control. Finally, an assimilation study of selectively labeled glucose allowed the quantitative evaluation of the participation of different metabolic pathways in the formation of succinic acid by the isolated cells.

Materials and Methods

Strains and Media

The *Y. lipolytica* strains used in this study are summarized in Table I. The minimal medium for routine manipulations was YNB (0.67% yeast nitrogen base without amino acids, Difco, Detroit, MI) supplemented with glucose, glycerol, succinic acid, or sodium succinate at various concentrations as stated in the text. All isolated strains were derived from Po1f (*MatA leu2-270 ura3-302 xpr2-322 axp2-deltaNU49 XPR2::SUC2*) and carried a lesion in the *LEU2* gene, therefore 0.1 g L^{-1} L-leucine was added to YNB for complementation of auxotrophy. The rich media included YPD (10 g L^{-1} yeast extract, 10 g L^{-1} bacto-peptone with various concentrations of glucose) or YPG (10 g L^{-1} yeast extract, 10 g L^{-1} bacto-peptone with various concentrations of glycerol). For solid media, 15 g L^{-1} agar was added. When maintenance of neutral pH was required, sterile $CaCO_3$ was added up to 10 g L^{-1} to the solid media or 25 g L^{-1} in the liquid media. All cultivations were performed at 30°C. Flasks and test tubes were incubated on a rotary shaker at 250 rpm.

DM1 media was used for seed cultures as well as for fed-batch cultivations. The composition of DM1 media was (per liter): 2.38 g Na_2HPO_4, 0.71 g KH_2PO_4, 11.77 g NH_4Cl, 0.65 g $(NH_4)_2SO_4$, 0.32 g $MgSO_4$, 1.8 g citric acid, 1.0 g L-leucine, 0.11 g $CaCl_2$, 0.2 mg D(+)-biotin, 1.0 mg thiamine-HCl, and 4.6 mL of trace element solution (pH 7.0 adjusted by NH_4OH). The trace element solution comprised (per liter) 3.84 g CuSO4, 1.88 g $MnSO_4$, 0.2 g H_3BO_3, 0.52 g $CoCl_2$, 23.58 g $ZnSO_4$, 35.52 g $FeSO_4$, 88.0 mg KI, and 5 mL of concentrated H_2SO_4. Glycerol was used as the carbon source for strains Y-3460, while glucose was used for strains Y-3753 and Y-4215.

For long-term steady-state cultivation, DM2 media was used which comprised (per liter) 6.6 g $(NH_4)_2HPO_4$, 11.5 g $NH_4H_2PO_4$, 1.8 g citric acid, 1.0 g KCl, 0.5 g NaCl, 0.32 g $MgSO_4$, 1.0 g L-leucine, 0.11 g $CaCl_2$, 0.2 mg D(+)-biotin, 1.0 mg thiamine-HCl, and 4.6 mL of the trace element solution (pH 5.5).

Test-Tube Cultivation

The *Y. lipolytica* strains were cultivated in 50-mL test tubes with vigorous shaking in 10 mL of YPD or YPG media with various concentrations of glucose or glycerol, respectively. Sterile $CaCO_3$ was added after 24 h of incubation, when maintenance of neutral pH was required. The medium was inoculated with approximately 5×10^5 cells mL^{-1} as was determined using a hemocytometric chamber under a light microscope. After 96–168 h of cultivation, biomass was precipitated by centrifugation and the concentration of succinic acid in the culture broth was analyzed. If $CaCO_3$ was used, the crystals were dissolved before centrifugation by mixing the culture with an equal volume of 1.0 N HCl. Test-tube fermentation

Table I. Conditions used for screening and isolation of *Y. lipolytica* mutants.

Step#	Strain[a]	Parental strain	Mutagenesis (survival rate %)	Medium for selection	Screening conditions in test-tube (final titer, pH, and time)	Alternative conditions in test-tube (final titer[b] and time)	Reference
1	Y-3314	Y-3312	Chemical (2%)	YNB, glycerol 25.2 g L^{-1}	YPG glycerol 63.1 g L^{-1} (17.4 g L^{-1}, pH 3.2, 168 h)	YPD glucose 50 g L^{-1}, $CaCO_3$ (13.4 ± 0.1 g L^{-1}, 120 h)	Yuzbashev et al. (2010)
2	Y-3351	Y-3314	Chemical (1.8%)	YNB, glycerol 63.1 g L^{-1}, SA 30 g L^{-1} (pH 2.3)	YPG glycerol 63.1 g L^{-1} (28.2 g L^{-1}, pH 2.9, 168 h)	YPD glucose 50 g L^{-1}, $CaCO_3$ (24.9 ± 0.6 g L^{-1}, 120 h)	This study
3	Y-3460	Y-3351	Chemical (3.8%)	YNB, glycerol 63.1 g L^{-1}, SA 70 g L^{-1} (pH 3.5)	YPG glycerol 189.3 g L^{-1} (47.6 g L^{-1}, pH 2.6, 120 h)	YPD glucose 50 g L^{-1}, $CaCO_3$ (13.1 ± 0.8 g L^{-1}, 120 h)	This study
4	Y-3854	Y-3351	Chemical (0.2%)	YNB, yeast extract 2.0 g L^{-1}, glucose 20 g L^{-1}, SA 1.0 g L^{-1} (pH 3.0)	YPD glucose 150 g L^{-1} (30.3 g L^{-1}, pH 2.9, 120 h)	ND	This study
5	Y-3751	Y-3854	Chemical (0.4%)	YNB, yeast extract 2.0 g L^{-1}, glucose 20 g L^{-1}, SA 10 g L^{-1} (pH 2.5)	YPD glucose 150 g L^{-1} (42.4 g L^{-1}, pH 2.7, 120 h)	ND	This study
6	Y-3753	Y-3751	Chemical (0.5%)	YNB, glucose 250 g L^{-1}, SA 10 g L^{-1} (pH 5.0)	YPD glucose 110 g L^{-1} (52.3 g L^{-1}, pH 2.6, 96 h)	ND	This study
7	Y-4215	Y-3753	Spontaneous	DM2 (chemostat)	ND[c]	ND	This study

[a]All strains obtained in this study are deposited in BRC VKPM and are indicated with collection numbers.
[b]The values are means ± standard deviations from three independent experiments.
[c]ND, not determined.

experiments were performed in triplicate except for the screening procedure after chemical mutagenesis.

Fed-Batch Cultivation

The *Y. lipolytica* strains were inoculated into 10 mL of DM1 medium with 25 g L^{-1} of the carbon source and were grown for 48 h in 50-mL test tubes with shaking. The culture was transferred to a 750-mL flask with 90 mL of the same medium and was grown for 24 h with shaking. This volume was then used for inoculation of a 3.0-L bioreactor KF 103 (ProInTech, Pushchino, Russia), which contained 1.0 L of DM1 medium with 100 g L^{-1} of the carbon source. pH 5.5 was automatically maintained by adding 25% (w/w) aqueous ammonia solution. The culture was agitated at 700 rpm and aerated at 1.0 volume per volume min^{-1}. The antifoaming agent Propinol B-400 (Barva, Ivano-Frankovsk, Ukraine) was added as required. The seed culture was grown to OD_{600} of about 40, which observed near the end of the exponential phase.

Approximately, 100 mL of this culture was transferred to a sterile bioreactor with 0.9 L of DM1 medium for attaining an OD_{600} of 4.0, while cell dry weight (CDW) was estimated about 2.5 g L^{-1}. Fed-batch cultivations were performed under conditions similar to the seed culture, but without any pH control. Initial concentration of the carbon source was 50 g L^{-1}. During the entire process, the carbon source was maintained within the range of 30–80 g L^{-1} by feeding with a 700 g L^{-1} solution.

A total of 3-mL aliquots of the culture broth were sampled periodically under sterile conditions. The biomass was precipitated by centrifugation, washed twice with distilled water, lyophilized, and CDW was determined. The supernatant was used for measuring the concentrations of the carbon source and succinic acid. All fed-batch cultivation experiments were repeated at least thrice and the results of representative experiments were shown.

Long-Term Steady-State Cultivation

The overnight culture of Y-3753 grown in a 750-mL flask with 100 mL of YPD (25 g L^{-1} of glucose) was used for inoculation of the bioreactor containing 0.9 L of DM2 medium with 120 g L^{-1} of glucose. The same medium was used for the continuous feeding process. pH was controlled automatically by addition of 25% (w/w) aqueous ammonia solution. In general, the experimental conditions were similar to those described for fed-batch cultivation. Selection pressure was regulated by setting two parameters, namely maintenance of pH and dilution rate.

Chemical Mutagenesis

The *Y. lipolytica* strains were mutagenized as previously described, with minor modifications (Yuzbashev et al., 2010). The actual concentration of N-methyl-N′-nitro-N-nitrosoguanidine varied between 40 and 200 μg mL^{-1}, whereas the incubation time was in the range of 60–120 min. The survival rates in individual experiments along with selective media and screening conditions are indicated in Table I.

Metabolite Assays

The concentrations of succinic acid and glycerol in the supernatant were measured by high-performance liquid chromatography (Yuzbashev et al., 2010). Residual glucose was determined by a glucose oxidase enzymatic kit (Diakom, Moscow, Russia) as recommended by the manufacturer.

Small-Scale Cultivation With Labeled Glucose

Y-3351 was cultivated in 30-mL test tubes in 1.0 mL YNB medium with 50 g L^{-1} of [1,6-$^{13}C_2$]glucose (cat# 453196, Sigma–Aldrich,

Miamisburg, OH) as a single carbon source. The initial titer was 2×10^5 cell mL^{-1}. The cultivation was carried out in a rotary shaker specifically equipped to prevent evaporation. After the first 24 h of incubation, sterile $CaCO_3$ was added and cultivation was continued for another 144 h. After this, an equal volume of 1.0 N HCl was added, and biomass was separated. Succinic acid was exhaustively extracted with ethyl acetate. The organic phase was dried under vacuum to the constant weight and used for NMR analysis.

NMR Spectroscopy

^1H and ^{13}C NMR spectra were recorded in D_2O at 25°C on a Bruker Avance II 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm inverse Z-gradient broadband probe. Spectra were referenced to the chemical shift of carbon atoms (−1.29 ppm) and protons (0.00 ppm) in a solution of 0.1 g L^{-1} sodium trimethylsilyl propionate-d4 (internal standard) in water. Quantitative ^{13}C-spectra were recorded using a 90° excitation pulse, inverse gated broadband ^1H decoupling, and a 55 s relaxation delay. The spectra were accumulated in 1,024 scans with 256 K data points and an acquisition time of 4.9 s. ^1H spectra were recorded using a 30° pulse, 16 scans, 3 s delay, and 2.7 s acquisition time. A total of 64 K data points were acquired for each spectrum. ^{13}C and ^1H spectra were processed using Topspin 2.1 software with exponential multiplication (LB 1.0 and 0.3 for ^1H and ^{13}C, respectively) and baseline correction before integration.

Calculations

For evaluation of fluxes participating in succinic acid formation, a model was proposed that included glycolysis, oxidative and non-oxidative branches of the PP pathway, pyruvate carboxylase and pyruvate dehydrogenase reactions, glyoxylate cycle, reductive cytosolic pathway from oxaloacetate to succinate, and the TCA cycle interrupted at the SDH activity. The model also considered a highly active exchange of intermediates between subcellular compartments (see Supplementary material for details).

The fraction of glucose assimilated through the oxidative branch of the PP pathway was quantified as follows:

$$F_{pp}(\%) \geq \left(6 - \frac{11.88}{2.99 - 2 \times P_{2,3}}\right) \times 100 \qquad (1)$$

wherein $P_{2,3}$ being part of the ^{13}C-labeled atoms among total carbon at the C-2/C-3 positions as determined from ^1H NMR spectroscopy.

The fraction of succinic acid synthesized in the mitochondria from the oxidative branch of the TCA cycle was quantified as follows:

$$F_{mit}(\%) \geq \left(\frac{R_{1,4/2,3} \times P_{2,3} - 0.01}{P_{2,3} - 0.01}\right) \times 100 \qquad (2)$$

wherein $R_{1,4/2,3}$ being the ratio of ^{13}C-isotope at C-1/C-4 positions to ^{13}C-isotope at C-2/C-3 positions experimentally observed by ^{13}C NMR spectroscopy.

Results and Discussion

Improving Productivity of the Strain by Induced Mutagenesis

In the previous work, a *Y. lipolytica* strain Y-3314 was isolated by a single mutagenesis step from strain Y-3312, with the deletion of *SDH2* gene (Yuzbashev et al., 2010). Y-3314, that could accumulate 17.5 g L^{-1} of succinic acid without buffering, was used in this study as the starting material for an adaptive selection at low pH conditions (Table I). For each selection step, a threshold concentration of succinic acid that completely blocked the growth of the parent strain was chosen. After mutagenesis, between 30 and 60 single clones were isolated from selective plates and screened for production in test tubes under the conditions indicated (Table I). A strain with the best production level was mutagenized in the subsequent selection step. Two consecutive steps (steps 2 and 3) allowed improvement of productivity at low pH on the rich media with glycerol. The isolated strains Y-3351 and Y-3460 accumulated 28.2 and 47.6 g L^{-1} of succinic acid in test tubes, respectively.

The strain Y-3460 was cultivated in minimal DM1 medium in a bioreactor under fed-batch conditions (Fig. 1A). As observed, Y-3460 accumulated 40.5 g L^{-1} of succinic acid in 36 h. The calculated yield was 0.32 g g^{-1} of glycerol. No pH-titrating agents were added and the final pH dropped to a value of pH 2.4.

Previously, it was observed that the parent strain Y-3314 did not grow on glucose as a carbon source (Yuzbashev et al., 2010). This property was also inherited by the newly isolated strains, Y-3351 and Y-3460. However, it has been found that this obstacle can be partially overcome by supplying the medium with pH-buffering agents. This could be explained by the fact that a buffer prevented acidification, which had blocked an already weak growth on glucose media. Three glycerol-utilizing strains were tested for succinic acid production from glucose in the presence of $CaCO_3$ (Table I). The strain Y-3351 demonstrated a better result and was chosen for further selection steps on media containing glucose. Steps 4 and 5 involved elevation of succinic acid concentration and lowering of pH, while step 6 was used for improving tolerance to excess glucose. As a result, three isolated strains Y-3854, Y-3751, and Y-3753 demonstrated a gradual increase in succinic acid production in test tubes on the rich medium with glucose, *ca*. 30.3, 42.4, and 52.3 g L^{-1}, respectively (Table I).

Strain Y-3753 was cultivated in the bioreactor under fed-batch conditions without pH control, and with glucose as a carbon source (Fig. 1B). The maximal succinic acid titer 42.1 g L^{-1} was obtained in 54 h. A final pH 2.9 was observed, and a yield of 0.39 g g^{-1} of glucose was estimated.

Improving Productivity of the Strain by the Metabolic Evolution

Subsequent attempts to increase productivity by means of induced mutagenesis did not lead to improvement in the properties of the strain Y-3753 (data not shown). Therefore, a long-term cultivation of Y-3753 in a steady-state culture was performed (Fig. 2). Initially, pH was maintained at value 4.0, and dilution rate was set up at 0.02, 0.05, 0.06, and 0.07 h^{-1}. At 0.07 h^{-1}, the cells were

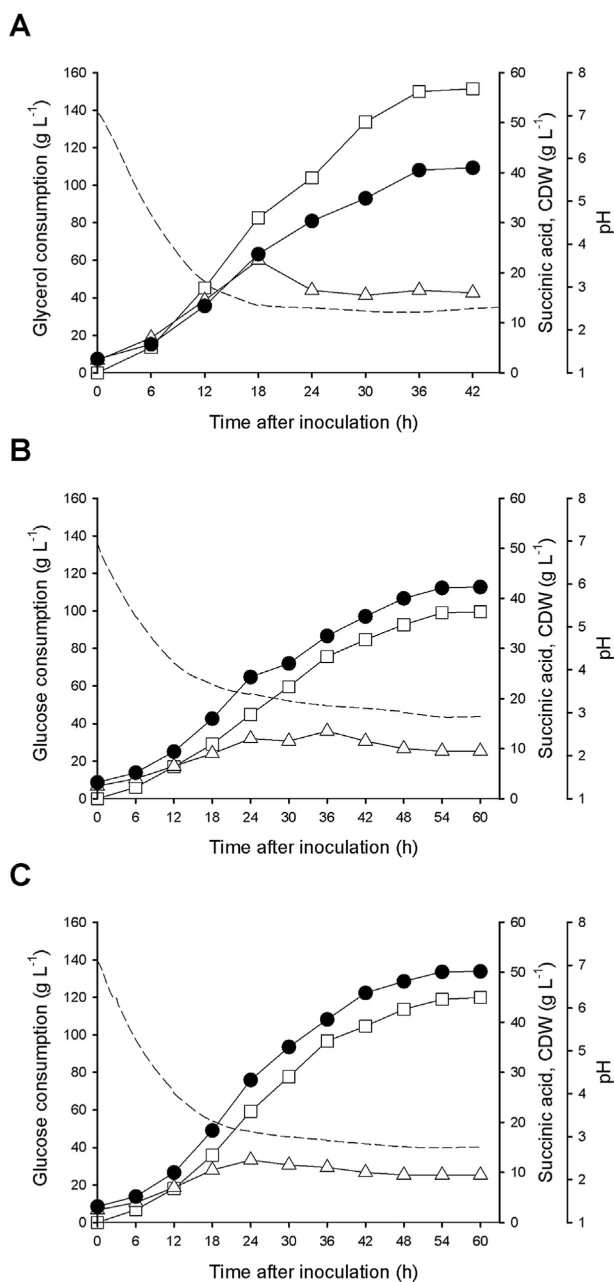

Figure 1. Fed-batch cultivation of SDH deficient *Y. lipolytica* strains. The strain Y-3460 (**A**) was cultivated on media with glycerol, while glucose was used for strains Y-3753 (**B**) and Y-4215 (**C**). The growth of the strains was controlled by measuring CDW (open triangles). Accumulated succinic acid (filled circles) and consumed carbon source (open squares) were analyzed every 6 h. The pH of the culture medium is depicted by the dashed line.

washed out indicating that under the conditions used, the maximum growth rate for this strain was between 0.06 and 0.07 h^{-1}. Further, the dilution rate was reduced to 0.035 h^{-1}, and steady-state culture was obtained with 20 $g L^{-1}$ of constant CDW. Under these conditions, succinic acid titer was maintained at 50.5 $g L^{-1}$, and glucose was the growth-limiting factor. Thereafter, pH was lowered to a value of 3.8 resulting in a CDW reduction of up to 17.9 $g L^{-1}$. This pH level was maintained for about 100 h until both CDW, and succinic acid titer were restored to the values of 20 and 50 $g L^{-1}$, respectively. Subsequently, the procedure with decreasing pH followed by restoration of the growth rate was repeated three more times. Each time, the pH was reduced by a value of 0.15. At the end of the process, pH was maintained at 3.3, while CDW and succinic acid titer were estimated as 19.5 and 50.4 $g L^{-1}$, respectively. The total cultivation time was counted as 840 h. The final culture was plated to obtain separate colonies, and a single isolated clone was designated as Y-4215.

Succinic acid production by strain Y-4215 was analyzed in the bioreactor (Fig. 1C) under the same conditions used for Y-3753. At 54 h of cultivation, succinic acid titer reached 50.2 $g L^{-1}$ with a yield of 0.43 $g g^{-1}$, and the final pH dropped to a value of 2.7. In order to compare three isolated strains, the maximal growth rates in fed-batch experiments were estimated on the basis of CDW. The comparison of strain Y-4215 with the parent strain Y-3753 revealed some improvement of the maximal growth rate (0.09 and 0.07 h^{-1}, respectively). This result is still far from the growth rate of 0.13 h^{-1} calculated for strain Y-3460 in medium containing glycerol (Fig. 1A). At the same time, at 36 h of cultivation both strains Y-3460 and Y-4215 accumulated equal amounts of succinic acid (40.5 $g L^{-1}$), while the latter continued to produce it up to 54 h.

Therefore, the application of the metabolic evolution in this particular case allowed improvement of the growth rate of the strain, as well as an increase in the final titer of succinic acid accumulated at low pH conditions. The data also suggest that an obligate aerobic culture could effectively adapt to the operation of the interrupted TCA cycle, and was able to secrete succinic acid as the final product of glucose oxidation. The isolated mutant Y-4215 should be a suitable basic strain for construction of an industrial producer. It should be noted that the most critical parameter for industrialization is the product yield based on the substrate consumed. Hence, it is of greatest interest to investigate the possibility of combining the present synthetic pathway with other metabolic routes for improving succinic acid yield.

Contribution of Metabolic Pathways in Succinic Acid Formation

To evaluate the participation of metabolic pathways in succinic acid formation, the experiment was set up with [1,6-$^{13}C_2$]glucose as the substrate. The contribution of pathways was evaluated based on tracing of positional ^{13}C-labeling. Disruption of the *SDH2* gene prevented randomization of labeling in the TCA cycle and thus allowed distinction between succinic acid molecules synthesized by different routes (Fig. 3). The strain Y-3351 was selected since it was the common precursor for both lineages of the strains adapted for media with both glycerol and glucose. After 168 h of small-scale cultivation of Y-3351 on the minimal medium with [1,6-$^{13}C_2$] glucose and $CaCO_3$, succinic acid was extracted and analyzed by ^{1}H and ^{13}C NMR spectroscopy.

^{1}H NMR spectra allowed determination of the portion of labeled carbon in the methylene (C-2/C-3) groups (Table II). Assuming that [3-^{13}C]pyruvate is the sole precursor for succinic acid synthesis, this portion should be not less than 0.5 (Fig. 3). The difference between the estimated value of 0.5 and the experimentally observed value of 0.4432 allowed evaluation of the amount of unlabeled

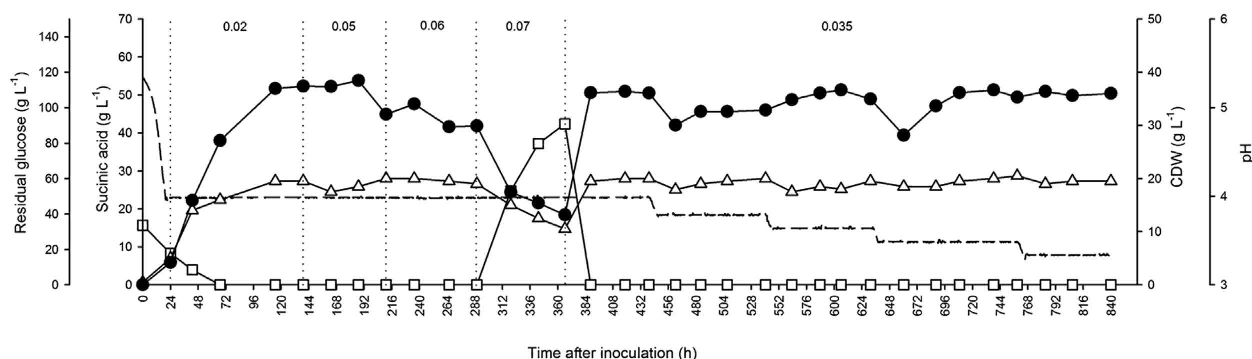

Figure 2. A long-term cultivation of the strain Y-3753. CDW (open triangles), succinic acid titer (filled circles), and residual glucose concentration (open squares) were analyzed once a day. The pH of the culture medium is depicted by the dashed line. The vertical dotted lines indicate time points when dilution rate has been switched. The exact values of dilution rate (h^{-1}) are indicated above corresponding time periods.

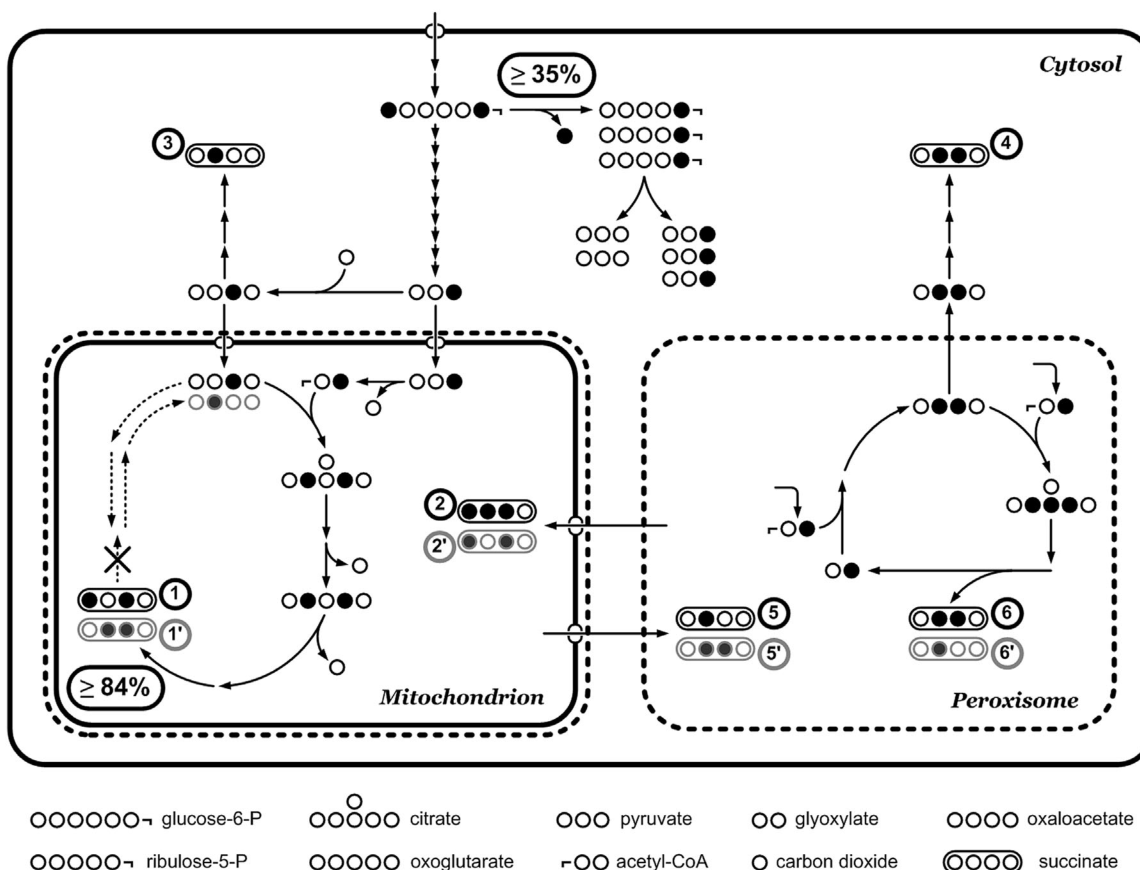

Figure 3. A metabolic model for ^{13}C-tracing in synthesis of succinic acid isotopomers from [1,6-$^{13}C_2$]glucose by the SDH deficient *Y. lipolytica* strain. Main pathways of the central metabolism including glycolysis, PP pathway, TCA cycle, glyoxylate cycle, and reductive cytosolic pathway of succinic acid biosynthesis are schematically depicted. Filled circles and open circles indicate ^{13}C- and ^{12}C-atoms of carbon, respectively. All chemical compounds are represented such that the left atom corresponds to the first carbon by IUPAC nomenclature. Isotopomers of succinic acid were outlined. For each synthetic pathway, a specific product number was assigned. Products 2, 2′, 5, and 5′ can be formed if intensive exchange of TCA cycle intermediates exists between the compartments. The appearance of minor products 1′, 2′, 5′, and 6′ required the interconversion of [3-^{13}C] oxaloacetate and [2-^{13}C]oxaloacetate due to activities of malate dehydrogenase and fumarase. The scheme was not described for the participation of unlabeled pyruvate in biosynthesis. The values represent specific fluxes calculated from experimental data (see Results and discussion section).

Table II. Data of NMR analysis of succinic acid produced from $[1,6\text{-}^{13}C_2]$glucose by the strain Y-3351.

Source of data	Evaluated parameter	Value[a]
^{1}H NMR	$P_{2,3}$[b]	0.4432 ± 0.0008
^{13}C NMR	$R_{1,4/2,3}$[c]	0.844 ± 0.003

[a]Data are means ± standard errors obtained from two independent experiments.

[b]$P_{2,3}$ is the experimentally observed proportion of ^{13}C-isotope at C-2/C-3 among the total carbon at these positions.

[c]$R_{1,4/2,3}$ is the experimentally observed ratio of ^{13}C-isotope at C-1/C-4 positions to the ^{13}C-isotope at C-2/C-3 positions of succinic acid.

pyruvate formed. Based on Equation (1), the minimal fraction of glucose assimilated through the PP pathway was quantified as 35%. These data are in agreement with the range 30–52% as was previously reported for the parental *Y. lipolytica* wild-type strain W29 (Blank et al., 2005; Wasylenko et al., 2015). The high activity of this pathway can be easily explained as due to apparently being the major source that supplies reduced NADPH for anabolic reactions in *Y. lipolytica* (Ratledge, 2014).

^{13}C NMR spectroscopy allowed estimating the distribution of ^{13}C-isotopes between the carboxyl (C-1/C-4) and methylene (C-2/C-3) groups in the succinic acid produced. It should be mentioned that this ratio did not change by the appearance of unlabeled pyruvate from the PP pathway. In fact, the ^{13}C-isotope was distributed almost equally between the methylene and carboxyl groups (Table II). Such a distribution was only possible if the main component of the mixture was $[1,3\text{-}^{13}C_2]$succinate, because for other expected products the labeling was significantly shifted toward the central positions (C-2/C-3). Based on Equation (2), the minimal fraction of $[1,3\text{-}^{13}C_2]$succinate in the mixture was estimated to be 84%. Among all, only two products (products 1 and -2′) had the appropriate structure. Both were formed in the mitochondria and originated from the oxidative branch of TCA cycle.

Despite the early data suggesting that, the glyoxylate cycle is active on media with glucose (Flores and Gancedo, 2005; Matsuoka et al., 1980), the provided analysis clarifies that the contribution of this pathway to succinic acid synthesis if any, is not significant. Both pathways, the oxidative branch of TCA cycle and the glyoxylate cycle, provide equal yield (one mole of succinic acid per mole of glucose). However, oxidative pathway supplies more energy that should be crucial for a strain with the TCA cycle interruption. Hence, such a distribution of the fluxes may be a consequence of specific properties of the strain.

The results obtained also raise the question of what transporter is responsible for the efflux of succinate from mitochondria. It seems unlikely that one of the two previously characterized in *S. cerevisiae* succinate mitochondrial carriers is suitable for this role (Palmieri et al., 2006). The transport by succinate-fumarate carrier Sfc1p proceeds via a strict counter-exchange mechanism. Thus, for the efflux of succinate Sfc1p requires an influx of equal amounts of fumarate from cytoplasm that is obviously impossible for a succinic acid producing strain. The dicarboxylate carrier Dic1p performs a strict counter-exchange of succinate for inorganic phosphate. In this case, an efflux of succinate requires an excess of inorganic phosphate in cytoplasm. The latter, however, is maintained at high level in the mitochondrial matrix.

Recently, several *Y. lipolytica* permeases participating in uptake of carboxylic acids were identified (Dulermo et al., 2015). Disruption of *YLJEN1* gene solely prevented growth of the strain on a medium with succinate as single carbon source. Surprisingly, all six identified permeases when overexpressed were capable fulfilling the succinate uptake function. It would be of a particular interest to determine a role of these transporters in succinic acid production in the isolated *sdh2* derivative strains. Another question remains to be clarified is what transporter is responsible for efflux of succinic acid from the cell. Further study should shed light on a mechanism that enables an efficient succinic acid transport in spite of high concentration of this component and low pH of media.

Conclusions

The results clearly demonstrated that the combination approach of induced mutagenesis and metabolic evolution enhanced the industrially valuable properties of the *Y. lipolytica* mutant deficient for SDH activity. The isolated strain Y-4215 efficiently utilized glucose, grew well without pH-titrating agents, and accumulated up to $50.2\,g\,L^{-1}$ of succinic acid in 54 h with a yield $0.43\,g\,g^{-1}$. The ^{13}C-label tracing experiment with parent strain Y-3351 demonstrated that at least 35% of glucose was utilized through the PP pathway, while 84% or more of succinic acid formed from the oxidative branch of the TCA cycle.

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