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ABSTRACT

Yarrowia lipolytica strains PGC01003 and PGC202 engineered for succinic acid production were studied and compared to the wild type strain W29. For the first time, these two strains were characterized in a chemically defined medium. Strain growth and organic acid production were investigated in fed-batch mode with glycerol as carbon and energy source. This study evaluated the impact of nitrogen deficiency strategy to redirect carbon flux toward succinic acid synthesis. Strain PGC01003 produced 19 g L^{-1} succinic acid with an overall yield of 0.23 g g^{-1} and an overall productivity of 0.23 g L^{-1} h^{-1}, while strain PGC202 produced 33 g L^{-1} succinic acid with an overall yield of 0.12 g g^{-1} and a productivity of 0.57 g L^{-1} h^{-1}. Nitrogen limitation effectively stopped biomass growth and increased succinic acid yield of PGC01003 and PGC202 by 18% and 62%, respectively. However, the specific succinic acid production rate was reduced by 77% and 66%, respectively.

Keywords

Succinic acid; Yarrowia lipolytica; Nitrogen deficiency; Fed-batch cultivation; Minimal medium

Abbreviations

SA, succinic acid; CA, citric acid; AA, acetic acid; FA, fumaric acid; PA, pyruvic acid; MA, malic acid; OA, organic acid; gly, glycerol; totlip, total lipids; storlip; storage lipids; Xcat, catalytic biomass; CDW, cell dry weight; OD, optical density; DO, dissolved oxygen; q, specific consumption or production rate; Y, yield; YPG, yeast peptone glycerol; CM, chemical medium; Ylsdh5, *Yarrowia lipolytica* gene encoding the fifth subunit of succinate dehydrogenase ; Ylach1, *Y. lipolytica* gene encoding acetyl-CoA hydrolase ; Ylscs2, *Y. lipolytica* gene encoding succinyl-CoA synthetase; Scpck, *Saccharomyces cerevisiae* gene encoding pyruvate carboxykinase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; ICD, isocitrate dehydrogenase; TCA, tricarboxylic acid.

1. INTRODUCTION

Succinic acid (butanedioic acid) is a commodity chemical used in various applications such as biomaterials (e.g., polybutylene succinate, polyurethanes), food and dietary supplements (e.g., acidulants, flavoring and antimicrobial agents), detergents and surfactants, ion chelators, and pharmaceuticals. This building block can also be transformed into other derivatives such as 1,4-butanediol, succinic and maleic anhydrides, γ-butyrolactone, 2-pyrrolidone, tetrahydrofuran, and adipic, malic, fumaric, and itaconic acids, which further expands its scope of applications (Beauprez et al., 2010; Nurmi et al., 2018). In the 2000s, succinic acid was identified as one of the most promising biobased building blocks in two major publications by the US Department of Energy and the BREW Project (Patel et al., 2006; Werpy and Petersen, 2004).

Succinic acid is mainly produced by the catalytic hydrogenation of maleic acid and maleic anhydride, which are both produced by oxidation of C4 hydrocarbons issued from oil. In the 2010s, the high production costs of petrochemical synthesis and environmental concerns promoted the emergence of several biobased succinic acid producing companies, such as BioAmber, Myriant, Succinity, and Reverdia. In 2013, the biotechnological production of succinic acid, with 50 kt produced worldwide (a market price of 2,800 – 3,000 $ per ton), had even overtaken the petrobased route that represented 30 kt (2,400 – 2,600 $ per ton) (Nurmi et al., 2018; Weastra, 2012). However, the lack of cost-competitiveness of most of the biobased processes developed, attributed to the high recovery costs of succinic acid from the culture broth (Jansen and van Gulik, 2014; Jansen and Verwaal, 2011), has led to the successive shutdown of BioAmber, Myriant, and Succinity. Although the succinic acid bioproduction process at low pH developed by Reverdia (a joint-venture of DSM and

Roquette) is exploited in Cassano, Italy, R&D is still needed to improve the competitiveness of bioprocesses for succinic acid production (McCoy, 2019).

These industrial developments were accompanied by scientific research to design efficient producing hosts through metabolic and culture engineering, and to improve the production and recovery processes. Literature on succinic acid bioproduction has been compiled and scrutinized in several review articles (Ahn et al., 2016; Beauprez et al., 2010; Cheng et al., 2013; Pateraki et al., 2016). Natural succinic acid producers such as *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Basfia succiniciproducens*, *Anaerobiospirillum succiniciproducens*, *Bacteroides fragilis*, and *Corynebacterium glutamicum* were considered for their ability to produce high amounts of succinic acid through the reductive branch of the tricarboxylic acid (TCA) cycle under anaerobic conditions (Isar et al., 2007; Lee et al., 2003; Meynial-Salles et al., 2008; Okino et al., 2005; Scholten and Dägele, 2008; Shen et al., 2018). Natural producers were improved by metabolic engineering (Becker et al., 2013; Lee et al., 2016; Litsanov et al., 2012), and so were the model hosts *Escherichia coli*, *Saccharomyces cerevisiae*, and the strictly aerobic yeast *Yarrowia lipolytica* (Jansen and Verwaal, 2011; Lin et al., 2005; Raab et al., 2010; Yuzbashev et al., 2010).

Although unable to accumulate considerable amounts of succinic acid in nature, *Y. lipolytica* has been studied as a potential succinic acid producer because of the high activity of the oxidative branch of its TCA cycle (Ermakova et al., 1986; Kamzolova and Morgunov, 2017; Markham and Alper, 2018) and for the possibility to perform cultures at low pH, which facilitates the recovery of succinic acid during the downstream process (Jansen and van Gulik, 2014; Jansen and Verwaal, 2011; Yuzbashev et al., 2010). A first study demonstrated

90 the feasibility of succinic acid production with *Y. lipolytica* at pH of 3 (Yuzbashev et al., 2010),

91 and other works followed this path with remarkable results (Table 1).

Table 1: Performance indicators of *Y. lipolytica* as a host for succinic acid bioproduction using glycerol or crude glycerol as carbon and energy source reported in the literature and in the present study.

Strain	Description[a] Parent; modifications	Culture medium and strategy[b]					$Y_{SA/Gly}$[c] (g_{SA} g_{gly}^{-1})	[SA][c] (g L^{-1})	r_{SA}[c] (g L^{-1} h^{-1})	Ref.
Y-3314	Po1f; Δ*sdh2*, +*ura3*	B	Gly	YE, Pep, $CaCO_3$	-	pH 5.5	0.36	45.5	0.27	(Yuzbashev et al., 2010)
				YE, Pep,	-	pH 3.2	0.28	17.4	0.10	
Y-3460	Y-3314; chemical mutagenesis	F	Gly	Def	-	pH 2.7	0.32	40.5	1.12	(Yuzbashev et al., 2016)
H222-AZ2	H222-SW2; *pot1-sdh2*, +*ura3*	B	Gly	Def	-	pH 5	0.04	4.0	0.02	(Jost et al., 2015)
					$LimO_2$		0.26	25.1	0.15	
PGC01003	Po1f; Δ*sdh5*, +*ura3*	F	cGly	YE, Try	$LimO_2$	pH 6	0.40	160	0.40	(Gao et al., 2016)
		F	Gly		$LimO_2$		0.42	198.2	0.83	(Li et al., 2017)
		B	cGly		$LimO_2$		0.45	53.6	1.45	(Li et al., 2018)
		F					nd	209.7	0.65	
		F	Gly	Def	LimN	pH 5.5	0.23	19	0.23	This study
PGC202	Po1f; Δ*sdh5*, Δ*ach1*, + *Scpck*, +*Ylscs2*, +*leu2*, +*ura3*	F	Gly	YE, Try	$LimO_2$	pH 3.4	0.53	110.7	0.80	(Cui et al., 2017)
		F	Gly	Def	LimN	pH 5.5	0.12	33	0.57	This study

[a] Δ : deletion; + : addition or overexpression. *sdhZ*: succinate dehydrogenase subunit Z (gene); *ach1*: acetyl-CoA hydrolase 1 (gene); *Scpck*: pyruvate carboxykinase from *S. cerevisiae* (gene); *Ylscs2*: succinyl-CoA synthetase from *Y. lipolytica* (gene); *ura3*: orotidine 5'-phosphate decarboxylase (marker); *leu2*: 3-isopropylmalate dehydrogenase (marker); *pot1*: inducible promoter of the 3-ketoacyl-CoA thiolase.

[b] B: batch; F: fed-batch. Gly: glycerol; cGly: crude glycerol. YE: yeast extract; Pep: peptone; Try: tryptone; Def: defined medium (i.e., no complex source of nitrogen and carbon was added). limO2: oxygen limitation was used, limN: nitrogen limitation was used. pH: final culture pH.

[c] $Y_{SA/gly}$: production yield of succinic acid relative to the substrate consumed (glycerol); [SA]: succinic acid titer reached; r_{SA}: overall productivity of succinic acid. The values selected for the yields, concentrations, and productivities are not the maxima reached for each indicator, but correspond to a single moment of the cultivation when these indicators are optimal with a priority given to the indicators following this order: yield > titer > productivity. nd: not determined.

111 *Y. lipolytica* has also been studied as a host for producing heterologous proteins, carotenoids
112 (e.g., lycopene, β-carotene, astaxanthin, zeaxanthin), erythritol, erythrulose, campesterol,
113 linalool, and organic acids such as citrate, isocitrate, α-ketoglutarate, pyruvate, and
114 itaconate (Abdel-Mawgoud et al., 2018; Barth and Gaillardin, 1997; Markham and Alper,
115 2018). As an oleaginous yeast, its ability to produce and store high amounts of lipids and
116 polyunsaturated fatty acids (e.g., linolenic acid, docosahexaenoic acid, and arachidonic acid)
117 has been exploited for single-cell oil production (Beopoulos et al., 2009; Papanikolaou and
118 Aggelis, 2009). Accumulation of lipid and citric acid can be triggered by mineral nutrient
119 deficiency (e.g., nitrogen, phosphorus, sulfur, or magnesium) associated with a carbon
120 overflow. Such mineral deficiencies prevent biomass growth and entail a redirection of the
121 carbon fluxes towards other compounds, which can result in higher yields of the
122 accumulation products. Among the mineral limitation strategies, nitrogen limitation is the
123 most commonly applied strategy for lipid and citrate production because of its efficiency and
124 convenience (Beopoulos et al., 2009; Morgunov et al., 2013). Despite its usefulness for lipid
125 and citric acid bioproduction, the impact of nitrogen deficiency condition on succinic acid
126 production by *Y. lipolytica* has never been evaluated yet.

127 Therefore, two strains of *Y. lipolytica* engineered for succinic acid production were selected:
128 the PGC01003 strain (Gao et al., 2016), which has been shown to produce very high succinic
129 acid titer (up to 210 g L^{-1}, see Table 1), and the derived improved strain PGC202 (Table 2)
130 (Cui et al., 2017; Gao et al., 2016) also able to produce high succinic acid titer (up to
131 110 g L^{-1}, see Table 1) and which was optimized to avoid acetate synthesis as a co-product.
132 The wild type, model strain, W29 was chosen as the reference strain (parental strain of the
133 two engineered strains, see Table 2). These three *Y. lipolytica* strains were characterized
134 using fed-batch cultivation on chemically defined medium containing glycerol as the sole

carbon and energy source, at pH 5.5. The cultivation strategy relied on a growth step performed under non-limiting conditions, and an organic acid production step performed under nitrogen deficiency conditions. This study explored the impact of nitrogen deficiency on the carbon distribution towards biomass, organic acids, lipids, and carbon dioxide.

2. MATERIAL AND METHODS

2.1. Strains, media, and chemicals

The wild-type strain W29 (ATCC 20460, CBS 7504, CLIB 89) was purchased from CIRM-Levures (France). Strain Po1f derived from W29 (Table 2) (Madzak et al., 2000). Strains PGC01003 and PGC202 were constructed in previous studies (Cui et al., 2017; Gao et al., 2016). Briefly, strain PGC01003 was engineered from Po1f by deletion of gene *Ylsdh5* coding for the fifth sub-unit of succinate dehydrogenase (Table 2). Strain PGC202 was engineered from PGC01003 by deletion of gene *Ylach1* coding for acetyl-coA hydrolase, followed by the incorporation of the *S. cerevisiae* pyruvate carboxykinase *Scpck* gene and overexpression of the succinyl-CoA synthetase *Ylscs2* gene (Table 2).

Table 2: Comparison of *Y. lipolytica* strains used in this study and intermediate strains obtained along their molecular construction.

Strain	Parental strain	Genotype	Phenotype	Source
W29 (Wild type)	X	MatA	Suc^-	(Wickerham et al., 1970)
Po1f[a]	(derived from W29)	MatA, xpr2-322, axp-2, leu2-270, ura3-302, *suc2*	Suc^+, Leu^-, Ura^-, ΔAEP, ΔAXP,	(Madzak et al., 2000)
PGC01003	Po1f	MatA, xpr2-322, axp-2, leu2-270, ura3-302, *suc2,* Δ*sdh5::URA3*	Suc^+, **Leu^-**, ΔAEP, ΔAXP, **ΔSDH5**	(Gao et al., 2016)
PGC11505[a]	PGC01003	MatA, xpr2-322, axp-2, leu2-270, ura3-302, *suc2, Δsdh5::URA3*, *Δach1::LEU2*	Suc^+, ΔAEP, ΔAXP, ΔSDH5, ΔACH1	(Cui et al., 2017)
PGC52[a]	PGC11505	MatA, xpr2-322, axp-2, leu2-270, ura3-302, *suc2, Δsdh5::loxP*, *Δach1::loxP*	Suc^+, Leu^-, Ura^-, ΔAEP, ΔAXP, ΔSDH5, ΔACH1	(Cui et al., 2017)
PGC62[a]	PGC52	MatA, xpr2-322, axp-2, leu2-270, ura3-302, *suc2, Δsdh5::loxP*, *Δach1::loxP, Scpck*	Suc^+, Leu^-, ΔAEP, ΔAXP, ΔSDH5, ΔACH1, ScPCK	(Cui et al., 2017)
PGC202	PGC62	MatA, xpr2-322, axp-2, leu2-270, ura3-302, *suc2, Δsdh5::loxP*, *Δach1::loxP, Scpck, Ylscs2*	Suc^+, ΔAEP, ΔAXP, **ΔSDH5, ΔACH1, ScPCK, YlSCS2**	(Cui et al., 2017)

[a]*: Strains not used in the present study, but which are intermediate construction strains between W29, PGC01003, and PGC202.*

MatA: haploid cells type a; Xpr2-322: defective *xpr2* gene encoding an inducible neutral-alkaline extracellular Protease (resulting in ΔAEP phenotype); Axp2: defective axp2 gene encoding an acidic extracellular Protease (resulting in ΔAXP phenotype); Leu2-270: defective leucine gene; Ura3-302: defective ura sequence; *sdh5*: gene encoding succinate dehydrogenase subunit 5; *ach1*: gene coding for the acetyl-CoA hydrolase 1; *Scpck*: gene encoding pyruvate carboxykinase from *S. cerevisiae*; *Ylscs2*: gene coding for Succinyl-CoA synthetase from *Y. lipolytica*.

160 The strains were stored at -80 °C in glycerol stocks composed of 50% (v/v) glycerol and 50%
161 (v/v) Yeast extract – Peptone – Glycerol (YPG) medium.

162 YPG medium contained (in g L^{-1}): glycerol, 10; peptone, 20; yeast extract, 10. It was
163 heat-sterilized (115 °C, 15 min).

164 The chemically defined medium (CM) was used for the second seed culture and for
165 fed-batch cultures. CM medium contained (in g L^{-1}): Glycerol, 10 (seed culture) or 40 (initial
166 concentration for fed-batch cultures); KH_2PO_4, 2.3; $(NH_4)_2SO_4$, 3.0; $Na_2HPO_4.2H_2O$, 3.9;
167 $MgSO_4.7H_2O$, 1; $ZnSO_4.7H_2O$, 4.0×10^{-2}; $CuSO_4.5H_2O$, 9.0×10^{-4}; $MnSO_4.H_2O$, 3.8×10^{-3};
168 $CoCl_2.6H_2O$, 5.0×10^{-4}; $CaCl_2.2H_2O$, 2.3×10^{-2}; $FeSO_4.7H_2O$, 1.6×10^{-2}; $Na_2MoO_4.2H_2O$, 6.0×10^{-5};
169 H_3BO_3, 3.0×10^{-3}; 4-aminobenzoic acid, 2.0×10^{-4}; myo-inositol, 2.5×10^{-2}; nicotinic acid,
170 1.0×10^{-3}; panthotenic acid, 1.0×10^{-3}; pyridoxine, 1.0×10^{-3}; thiamine HCl, 1.0×10^{-3}; biotin,
171 5.0×10^{-5}. Leucine (6.6×10^{-1} g L^{-1}) was added for cultivation performed with strain PGC01003
172 for complementation of auxotrophy. Solutions of glycerol, KH_2PO_4, $(NH_4)_2SO_4$, and Na_2HPO_4
173 were heat-sterilized by autoclave (115 °C, 15 min), while all other solutions (trace elements
174 and vitamins) were filtrated through sterile cellulose acetate filters with 0.22 µm pore
175 diameter.

176 Glycerol and KH_2PO_4 were purchased from VWR. NH_4OH, $(NH_4)_2SO_4$, $Na_2HPO_4.2H_2O$,
177 $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $MnSO_4.H_2O$, $CoCl_2.6H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$,
178 $Na_2MoO_4.2H_2O$, H_3BO_3, H_3PO_4, nicotinic acid, pyridoxine, aminobenzoic acid, panthotenic
179 acid, L-leucine, and biotin were ordered from Sigma-Aldrich. Myo-inositol and thiamine HCl
180 were obtained from Fluka AG. KOH was purchased from Merck. Antifoam Biospumex 153K
181 was ordered from PMC Ouvrie, France.

2.2. Seed cultures

For each seed culture, one glycerol stock was used to inoculate a culture, grown for 12 h in a 100 mL erlenmeyer flask with 10 mL of liquid YPG medium at 28 °C at 180 rpm. This culture was streaked on an YPG medium Petri dish (YPG with an addition of 15 g L^{-1} agar) and the plate was incubated for 24 h to 48 h at 28 °C. One colony was then used to inoculate the first seed culture, grown for 24 h in a 100 mL shake flask with 10 mL of liquid YPG medium at 28 °C at 180 rpm. The first seed culture was used to inoculate a second one that was grown for 24 h in 100 mL of CM medium in a 1 L shake flask at 28°C and 180 rpm. This latter culture was used to inoculate the bioreactor.

2.3. Fed-batch cultivation conditions and bioreactor system

Fed-batch cultivations were performed at 28 °C in a 2 L (working volume) instrumented bioreactor (SGI Setric, Toulouse, France) on CM medium containing initially 40 g L^{-1} glycerol. The pH value (pH probe InPro 3030/200, Mettler Toledo, Greifensee, Switzerland) was maintained at 5.5 with automatic addition of 6.7 M, 7.4 M or 14 M NH_4OH during the growth step of W29, PGC01003, and PGC202 cultivations, respectively, and with 14 M KOH during the organic acid production step of all cultivations (to ensure nitrogen deficiency conditions). Aeration rate with air and/or pure oxygen (Mass-Flowmeter type 8713, Bürkert, Ingelfingen, Germany) and stirring were adjusted manually to keep dissolved oxygen (DO) above 20% of the O_2 solubility in the cultivation medium at 28°C from air aeration (Optical O_2 sensor, VisiFerm DO, Hamilton, Bonaduz, Switzerland). Foam formation was avoided by automatic addition of a 10% (v/v) aqueous solution of Biospumex 153K antifoam. O_2 and CO_2 content of the gas output from the reactor were measured by a gas analyzer (Abiss (Anéolia, France) for cultivation of strain PGC01003, and TanDem (Analytic biosystems) for cultivation of W29 and PGC202).

Pure glycerol was added manually when DO shifted suddenly from stationary to high value of O_2 saturation for PGC01003 cultivation, and when glycerol concentration dropped below a concentration of 5 g L^{-1} for W29 and PGC202 cultivations. Pure glycerol, KOH solution, and antifoam feeding systems were heat-sterilized (115 °C, 15 min). NH_4OH solution was transferred in the corresponding feeding system under sterile conditions.

The bioreactor, the pure glycerol and the base feeding systems were placed on scales. Real-time total nitrogen concentration in the culture medium was calculated from the initial $(NH_4)_2SO_4$ concentration in the culture medium and the weight loss of NH_4OH feeding solution. The evolution of the culture broth volume was calculated by taking into account sampling and the addition of pure glycerol and alkali solution.

2.4. Biomass characterization

2.4.1. Total Biomass

The quantification of total biomass was done offline and online. Both cell dry weight (CDW) measurements and OD at 600 nm (OD_{600}) measurements were determined offline as follows:

- For CDW concentrations, a weighed volume of culture broth was filtered through 0.45 µm cellulose acetate filters (previously dried and weighed), rinsed twice with the same volume of physiological saline solution (9 g L^{-1} NaCl), and dried at 105°C until a constant weight was reached.
- OD_{600} was measured with a spectrophotometer (PRIM, SECOMAM, France) in a cuvette with an optical path of 1 cm after appropriate dilutions of the broth with physiological saline solution. OD_{600} was expressed as CDW equivalents and used to confirm the $OD_{>850}$ measurements with the correlations shown in Figure A.1.

OD from 850 nm ($OD_{>850}$) was monitored online with a broadband (from 850 nm) NIR optical probe with a 5 mm optical path (Model BT65, WedgeWood Analytical, USA) and using the model 653 absorbance monitor (Wedgewood, USA). $OD_{>850}$ was then expressed as CDW equivalents with the correlations shown in Figure A.2.

2.4.2. Catalytic biomass

Catalytic biomass (X_{cat}) was calculated by subtracting storage lipids from the total amount of biomass (i.e., CDW). The elementary composition (w/w) of *Y. lipolytica* catalytic biomass was assumed to be $C_1H_{1.744}O_{0.451}N_{0.132}$ with an ash content of 7.5% (w/w), which corresponds to a C molecular weight of 24.7 g_X $Cmol_X^{-1}$, according to Ochoa-Estopier and Guillouet (2014).

2.4.3. Total lipid content

Samples of the culture broth were centrifuged for 10 min at 4,500 $\times g$. The pellets were washed with physiological saline solution and centrifuged (10 min, 4,500 $\times g$) twice. The washed pellets were frozen at -20°C, then freeze-dried (Christ LMC-2, Osterode am Harz, Germany).

Total lipids were quantified from the freeze-dried pellets according to Folch et al. (1957), as follows. Pellets were crushed with a spatula as a fine powder and homogenized. A sample was suspended in a chloroform-methanol (2:1, v/v) solution to a concentration of 20 g L^{-1}. The suspension was dispersed by ultrasonication in a bath for 2 min, then stirred for 1.5 h at room temperature and filtered through a 0.22 µm polyvinylidene fluoride (PVDF) membrane (Merck). The retentate was resuspended in the same volume of chloroform-methanol solution for another extraction, then immediately filtered through a 0.22 µm PVDF membrane. The two filtrates were pooled, and an aqueous solution of NaCl (1 g L^{-1}) corresponding to 25% (v/v) of the filtrates gathered was added. The solutions obtained were

vigorously shaken for 2 min, then centrifuged for 10 min at 3,000 $\times g$ to accelerate the phase separation. The upper aqueous layer was discarded and the lower organic layer was dried under vacuum. The products remaining after solvent evaporation were weighed to assess the total lipid content of cell biomass. A second identical extraction procedure was performed on all biomass residue samples resulting from the first extraction round.

A third extraction round performed on five biomass residue samples showed that the lipids remaining after two rounds of extraction accounted for less than 1% w/w of biomass residues, and less than 5% of the lipid content obtained with the first two extractions. Therefore, the results of the third extraction were neglected, and the total lipid content of cell biomass was determined as the sum of the lipid content obtained with the first two extraction rounds divided by the amount of freeze-dried cells used.

2.4.4. Structural lipids content and storage lipids content

The total lipids quantified from freeze-dried biomass samples were considered as the sum of structural lipids and storage lipids. Structural lipids, assessed from the total lipid content of biomass during the exponential growth phase, were found to represent 6% of the CDW for the W29 and PGC202 cultivations, and 7% for the PGC01003 cultivation (Figure A.3). This is in accordance with structural lipid values reported for *Y. lipolytica* (Robles-Rodriguez et al., 2017). Storage lipids were calculated as the difference between total lipids and structural lipids.

The average fatty acid composition (w/w) of *Y. lipolytica* was assumed to be 51% linoleate, 28% oleate, 11% palmitate, 6% palmitoleate, 1% stearate, and 1% linolenate, according to Beopoulos et al. (2009). Lipids were assumed to be composed of triacylglycerols, because *Y. lipolytica* stores more than 90% of its lipids under this form (Beopoulos et al., 2009;

Robles-Rodriguez et al., 2017). Therefore, the average lipid molecule was considered to possess a C molecular weight of 15.5 g_{lip} $Cmol_{lip}^{-1}$. The lipid composition of *Y. lipolytica* was assumed to remain equal all along the cultivation experiment.

2.5. Glycerol and organic acid quantifications

The filtrate from cell dry weight measurement was used for glycerol and organic acid quantification by high-performance liquid chromatography (HPLC).

The concentrations of glycerol, succinic acid (SA), citric/isocitric acid (CA), acetic acid (AA), fumaric acid (FA), pyruvic acid (PA), and malic acid (MA) were determined by liquid chromatography using a Shimadzu LC-10 ADVP HPLC system equipped with an Aminex HPX-87H column (300 × 7.8 mm, Biorad, Hercules, CA, USA) maintained at 50 °C. The eluent was an H_2SO_4 (2.5 mM) aqueous solution at a flow rate of 0.5 mL min^{-1}. The system was coupled with a Shimadzu RID-10A refractometer (Shimadzu, Kyoto, Japan) and a SPD-20A UV-ViS Detector (Shimadzu, Kyoto, Japan) measuring the absorbance at 210 nm.

Conductivity of the culture broth was monitored online by a BIOMASS system (Fogale nanotech, Nîmes, France) and was correlated to HPLC measurements of acids as follows.

The concentration of total carboxylate groups ($[carboxylate]_{tot}$ in mol m^{-3}) was calculated from HPLC measurements using the following equation:

$$[carboxylate]_{tot} = \sum_{OA} \frac{[OA] \times 1000 \times r_{OA}}{M_{OA}} \qquad \text{(Equation 1)}$$

Where [OA] is the concentration (in g L^{-1}) of organic acid measured by HPLC, r_{OA} is the theoretical molar ratio of carboxylate groups per mole of organic acid in water at pH 5.5 (r_{CA} = 2 $mol_{carboxylate}$ mol_{CA}^{-1}, r_{PA} = 1 $mol_{carboxylate}$ mol_{PA}^{-1}, r_{MA} = 1.71 $mol_{carboxylate}$ mol_{MA}^{-1}, r_{SA} = 1.5 $mol_{carboxylate}$ mol_{SA}^{-1}, r_{AA} = 0.85 $mol_{carboxylate}$ mol_{AA}^{-1}), and M_{OA} is the molecular mass of the organic acid.

Based on Kohlrausch's law, experimental measurements of the conductivity of the culture broth (σ in mS m^{-1}) were related to total acid concentration (determined by HPLC and expressed in total carboxylate equivalents) according to the following linear model (Equation 2 and Figure A.4) :

$$\sigma = 4.45 \times [carboxylate]_{tot} - 155 \qquad \text{(Equation 2)}$$

Online conductivity measurements could thus be used as an estimator of the concentration of predominant organic acids throughout W29 and PGC01003 cultivations, in between offline HPLC analyses. In the case of W29 cultivation, citrate prevailed over other organic acids. Therefore, citrate concentration ([CA] in g L^{-1}) was calculated according to Eq. 3, where σ is the conductivity of the culture broth (in mS m^{-1}), M_{CA} is the molecular mass of citric acid (M_{CA} = 192 g.mol^{-1}), r_{CA} is the theoretical molar ratio of carboxylate groups per mole of citrate in water at pH 5.5 (r_{CA} = 2 $mol_{carboxylate}$ mol_{CA}^{-1}), and $\overline{x_{carb,CA}}$ is the average molar fraction of carboxylate groups originating from citrate throughout W29 cultivation ($\overline{x_{carb,CA}} \approx 1$).

$$[CA] = \frac{\sigma+155}{4.45} \times \frac{M_{CA}}{r_{CA} \times 1000} \times \overline{x_{carb,CA}} \qquad \text{(Equation 3)}$$

In the case of PGC01003 cultivation, succinate was the main product, but other organic acids were not negligible. Succinate concentration ([SA] in g L^{-1}) was calculated according to Eq. 4, where σ is the conductivity of the culture broth (in mS m^{-1}), M_{SA} is the molecular mass of succinic acid (M_{SA} = 118 g mol^{-1}), n_{SA} is the theoretical mean number of carboxylate groups in a molecule of succinate in water at pH 5.5 (n_{SA} = 1.5), and $\overline{x_{carb,SA}}$ is the average molar fraction of carboxylate groups originating from succinate throughout cultivation phases IIa and IIb.

$$[SA] = \frac{\sigma+155}{4.45} \times \frac{M_{SA}}{n_{SA} \times 1000} \times \overline{x_{carb,SA}} \quad \text{(Equation 4)}$$

The average molar fraction of carboxylate groups originating from succinate ($\overline{x_{carb,SA}}$) was calculated according to Eq. 5, where x_{SA} is the molar fraction (in % mol) of succinate relatively to organic acids (OA; including succinate, citrate, acetate, pyruvate, and malate), x_{OA} is the molar fraction (in % mol) of organic acids, and n_{OA} is the theoretical mean number of carboxylate groups in a molecule of OA in water at pH 5.5. The mean was calculated with every data point included in cultivation phases IIa and IIb (i.e., between 25 h and 80 h of cultivation) of PGC01003 cultivation.

$$\overline{x_{carb,SA}} = Mean\left[\frac{x_{SA} \times n_{SA}}{\sum(x_{OA} \times n_{OA})}\right]_{IIa,IIb} \quad \text{(Equation 5)}$$

2.6. Yields and specific rates calculations

Overall yields were calculated as the total amount of organic acid produced (in g) divided by the total amount of glycerol consumed (in g) after a given cultivation time.

The production yields of catalytic biomass ($Y_{Xcat/gly}$), organic acids ($Y_{OA/gly}$), total lipids ($Y_{totlip/gly}$), storage lipids ($Y_{storlip/gly}$), and carbon dioxide ($Y_{CO2/gly}$) were calculated by linear regression on the relationship between the corresponding amounts produced per cultivation phase against the glycerol amount consumed per cultivation phase (Figures A.5 to A.7). The production yields per cultivation phases are expressed as carbon molar ratios (Cmol $Cmol^{-1}$).

The specific growth rates (μ) were obtained by linear regression of the logarithm of the amount of catalytic biomass as a function of time (Figure A.8). The specific glycerol uptake rates (q_{gly}), the specific organic acid production rates (q_{OA}), and the specific lipid production rates (q_{lip}) were obtained by dividing the production rates (i.e., the derivative function of production as a function of time) by the instantaneous amount of catalytic biomass.

All amounts were calculated by taking into account both the volumetric medium feed rate and the withdrawal volume from the sampling.

3. RESULTS AND DISCUSSION

3.1. Overview of strain performances in fed-batch cultivations

3.1.1. Cultivation strategy and definition of cultivation phases

Figure 1 shows the evolution of the concentrations of glycerol, organic acids, storage lipids, and catalytic biomass (i.e., biomass including a basal amount of 6-7% (w/w) structural lipids; see section 2.4. Biomass characterization for measurements and calculations) throughout the fed-batch cultivations of strains W29, PGC01003, and PGC202 separately. Fed-batch cultivations were performed following a dual-step strategy. In the first step (i.e., Phase I in Figure 1), cells were grown exponentially under non-limiting conditions until the targeted biomass concentration was reached (i.e, 15 g_{Xcat} L^{-1} for PGC01003 cultivation and 50 g_{Xcat} L^{-1} for W29 and PGC202 cultivations). In the second step (Phase II in Figure 1), cell growth was progressively stopped and organic acids accumulated under nitrogen-deficient conditions. The transition from the first to the second step was triggered by replacing the ammonia solution used for pH regulation by potassium hydroxide solution. The nitrogen nutrient limitation strategy was implemented to limit biomass growth under a defined concentration, and to redirect the carbon fluxes towards the production of organic acids to increase their production yield (Beopoulos et al., 2009; Morgunov et al., 2013). During Phase IIa, biomass growth slowed down and reached a plateau; during Phase IIb, the amount of biomass was constant or slowly decreasing and organic acids were produced; Phase IIc was separated from Phase IIb when a change in organic acid production rate was observed.

The organic acid concentrations calculated from the conductivity measurements of the culture broth fits well with the concentrations measured by HPLC (Figure 1, black dots

representing citrate and succinate concentrations for W29 and PGC01003 cultivations, respectively). Conductivity is thus an efficient and non-destructive indicator for the online monitoring of organic acid production.

3.1.2. Comparison of fermentation performance for strains W29, PGC01003 and PGC202

W29 was selected as a reference strain because it is the wild-type from which the engineered strains PGC01003 and PGC202 derive (Table 2) (Cui et al., 2017; Gao et al., 2016; Madzak et al., 2000). During the growth phase (I), strain W29 showed a specific growth rate of 0.28 h^{-1}, which is in accordance with the values comprised between 0.20 h^{-1} and 0.32 h^{-1} obtained with various *Y. lipolytica* wild-type strains in the presence of glycerol (Kamzolova et al., 2015; Papanikolaou et al., 2002; Sabra et al., 2017; Workman et al., 2013). At the end of the growth phase, a catalytic biomass concentration of 49 g_{Xcat} L^{-1} was reached, and organic acids did not accumulate. Under nitrogen deficiency condition, strain W29 accumulated mainly citric acid and lipids. The resultant citrate concentration of 67 g L^{-1} was reached in 72 h of culture (i.e., the overall citrate productivity was 0.92 g L^{-1} h^{-1}), with an overall yield of 0.33 g_{CA} g_{gly}^{-1}. At the same time, total lipid concentration was 7 g L^{-1}, which represented 20% (w/w) of total biomass. No succinic acid accumulation was observed throughout the fermentation. The overall carbon balance for this cultivation was about 0.95 Cmol $Cmol^{-1}$ (Figure A.9).

These results are consistent with the literature on lipid and citric acid production by wild-type strains of *Y. lipolytica* using a nitrogen limitation strategy (Kamzolova et al., 2015; Levinson et al., 2007).

Strain PGC01003 was engineered from strain Po1f by deletion of the gene encoding the fifth subunit of succinate dehydrogenase (*Ylsdh5*) (Gao et al., 2016). Such a modification is usual

388 to make succinic acid an end-product of the oxidative branch of the TCA cycle (Cheng et al.,
389 2013; Gao et al., 2016; Lin et al., 2005; Yuzbashev et al., 2010). Under the same cultivation
390 conditions for strain W29 (excepted leucine supplementation, necessary to alleviate leucine
391 auxotrophy), strain PGC01003 showed a specific growth rate of 0.19 h^{-1}, representing a 32%
392 decrease compared to the specific growth rate of W29. The decrease in growth rate is
393 associated with a carbon redirection from biomass to organic acids. Contrary to strain W29,
394 PGC01003 growth (i.e., Phase I of the culture) was associated with succinic acid biosynthesis
395 (up to 3 g L^{-1}) and of citrate and acetate (1 g L^{-1} and 2 g L^{-1}, respectively). This cultivation
396 confirmed that a strictly aerobic *Y. lipolytica* strain was able to grow on glycerol with a SDH
397 subunit knocked-out (Jost et al., 2015; Yuzbashev et al., 2016, 2010).

398 A succinic acid concentration of 19 g L^{-1} was reached in 82 h, with an overall productivity of
399 0.23 g L^{-1} h^{-1} and an overall yield of 0.23 g_{SA} g_{gly}^{-1}. A coproduction of citrate (7 g L^{-1}), acetate
400 (5 g L^{-1}), malate (5 g L^{-1}), pyruvate (3 g L^{-1}), and lipids (1.3 g L^{-1} of total lipids) was also
401 observed. The overall carbon balance for this cultivation was about 0.95 Cmol $Cmol^{-1}$
402 (Figure A.9), showing that no major other by-product was generated.

403 Since a lower catalytic biomass concentration was produced with PGC01003 than with W29
404 and PGC202 cultivations (i.e., 12, 49 and 49 g_{Xcat} L^{-1}, respectively), succinic acid production
405 and overall productivity obtained with strain PGC01003 cannot be compared directly with
406 the other cultivations. Thus, the performances of the three strains are evaluated more
407 comprehensively based on yields and specific production rates in the next sections
408 3.2. Kinetic characterization and 3.3. Stoichiometric characterization.

409 **Strain PGC202** was engineered from strain PGC01003 by deletion of the gene coding for
410 acetyl-CoA hydrolase (*Ylach1*) to reduce acetic acid synthesis, followed by the incorporation

411 of the *S. cerevisiae* gene encoding phosphoenolpyruvate carboxykinase (*Scpck)*, and the
412 overexpression of the gene encoding succinyl-CoA synthetase (*Ylscs2*) to improve succinic
413 acid production (Cui et al., 2017). It is noteworthy that strain PGC202 was selected for its
414 efficiency to produce succinic acid in low pH culture medium (i.e., pH from 3 to 4.3) (Cui et
415 al., 2017), while in the present study a pH of 5.5 was used. Under the culture conditions
416 applied here, strain PGC202 showed a specific growth rate of 0.17 h^{-1}, similar to the one
417 obtained with PGC01003 (0.19 h^{-1}). Like with this latter strain, succinic acid production was
418 associated with the growth of PGC202. Succinic concentration reached 3 g L^{-1} at the end of
419 Phase I and 33 g L^{-1} after 59 h of cultivation. The overall succinic acid productivity was thus
420 0.57 g L^{-1} h^{-1}, with an overall yield of 0.12 g_{SA} g_{gly}^{-1}. A total lipid production of 8 g L^{-1} was also
421 achieved, which represented 17% (w/w) of total biomass. Contrary to strain PGC01003 that
422 coproduced high amounts of organic acids, strain PGC202 produced succinic acid as the sole
423 organic acid detected by our analytical method. However, carbon was not efficiently
424 redirected towards succinic acid, the yield representing only nearly half of that from
425 PGC01003 cultivation. The incomplete carbon balance in PGC202 cultivation (0.60 to
426 0.85 Cmol $Cmol^{-1}$, Figure A.9) indicates an additional leak in carbon utilization that could
427 originate from mannitol or erythritol production as measured by Cui et al. (2017).

428 **3.1.3. Comparison of fermentation performance of strains W29, PGC01003 and PGC202**
429 **with other *Y. lipolytica* strains reported in literature**

430 The fermentation performance of strains PGC01003 and PGC202 in this study was close to
431 the one reported by Jost et al. (2015) in terms of yields and concentrations, but lower than
432 most of the other reported results obtained with *Y. lipolytica* strains engineered for succinic
433 acid production (Table 1), including previous investigations performed with strains
434 PGC01003 and PGC202. However, it is worth noting that most of these studies involved

culture media containing complex nutrients such as yeast extract or corn steep liquor. These nutrients contain additional sources of carbon and nitrogen, which are generally not taken into account in the yield calculations (Beauprez et al., 2010).

As shown in Table 1, two other studies have been performed using minimal media (Jost et al., 2015; Yuzbashev et al., 2016). Yuzbashev et al. (2016) engineered strain Po1f by deleting gene *sdh2* and adapting it to low pH conditions by chemical mutagenesis. The strain Y-3460 obtained was characterized in fed-batch cultivations performed in glycerol minimal medium without pH regulation (final pH was 2.4). Jost et al. (2015) constructed strain H222-AZ2 by replacing the *sdh2* promoter of strain H222-SW2 for the *pot1* promoter, which is inducible by dissolved oxygen. Strain H222-AZ2 was characterized in fed-batch cultivations performed in glycerol minimal medium with a pH regulated at 5 and under oxygen and potassium limitation conditions.

The succinic acid yield of 0.23 g_{SA} g_{gly}^{-1} obtained in the present work with PGC01003 is close to the yield of 0.26 g_{SA} g_{gly}^{-1} obtained with the strain H222-AZ2, but it remains 30% lower than that obtained with strain Y-3460 (0.32 g_{SA} g_{gly}^{-1}). The overall succinic acid productivities displayed herein for PGC01003 and PGC202 cultivations (0.23 g_{SA} L^{-1} h^{-1} and 0.57 g_{SA} L^{-1} h^{-1}, respectively) were higher than those of H222-AZ2 cultivations (0.15 g_{SA} L^{-1} h^{-1} under the optimal conditions), but at least half of that obtained with strain Y-3460 (1.12 g_{SA} L^{-1} h^{-1}).

3.2. Kinetic characterization of fermentation performances in fed-batch cultivation

The results of the fed-batch cultivations given in the previous part of the study were further analyzed. The yields (Y), the specific consumption or production rates (q), and the maximum concentrations of catalytic biomass, organic acids and total and storage lipids were

458 calculated for each phase of the fed-batch cultivations performed with strains W29,

459 PGC01003, and PGC202 (Table 3).

Table 3: Stoichiometric and kinetic performance indicators for fed-batch cultivations performed with strains W29, PGC01003, and PGC202.

	W29				PGC01003			PGC202			
Performance indicators	**I**	**IIa**	**IIb**	**IIc**	**I**	**IIa**	**IIb**	**I**	**IIa**	**IIb**	**IIc**
$[X_{cat}]_{max}$ (g L^{-1})	17	45	49	34	7	12	12	17	49	48	44
$[SA]_{max}$ (g L^{-1})	-	-	-	-	3	10	19	3	26	33	37
$[CA]_{max}$ (g L^{-1})	0	9	43	67	1	4	7	-	-	-	-
$[AA]_{max}$ (g L^{-1})	-	-	-	-	2	3	5	-	-	-	-
$[MA]_{max}$ (g L^{-1})	0	0	1	5	0	1	5	-	-	-	-
$[PA]_{max}$ (g L^{-1})	-	-	-	-	0	1	3	-	-	-	-
$[totlip]_{max}$ (g L^{-1})	1	4	7	8	0	1	1	2	6	7	8
%totlip in X (g $100g^{-1}$)	6	6 to 8	8 to 18	18 to 20	7	7 to 9	9 to 12	6	6 to 12	12 to 15	15 to 17
$Y_{Xcat/gly}$ (Cmol $Cmol^{-1}$)	0.52	0.58	0	0	0.43	0.31	0.00	0.49	0.31	0	0
$Y_{SA/gly}$ (Cmol $Cmol^{-1}$)	-	-	-	-	0.17	0.24	0.20	0.08	0.16	0.13	0.05
$Y_{CA/gly}$ (Cmol $Cmol^{-1}$)	0	0.06	0.56	0.46	0.06	0.10	0.05	-	-	-	-
$Y_{AA/gly}$ (Cmol $Cmol^{-1}$)	-	-	-	-	0.13	0.05	0.03	-	-	-	-
$Y_{MA/gly}$ (Cmol $Cmol^{-1}$)	0	0	0.01	0.08	0.01	0.02	0.07	-	-	-	-
$Y_{PA/gly}$ (Cmol $Cmol^{-1}$)	-	-	-	-	0.02	0.04	0.06	-	-	-	-
$Y_{storlip/gly}$ (Cmol $Cmol^{-1}$)	0	0.04	0.09	0	0	0.01	0.01	0	0.04	0.03	0.04
$Y_{totlip/gly}$ (Cmol $Cmol^{-1}$)	0.05	0.09	0.09	0	0.05	0.05	0.01	0.04	0.07	0.03	0.04
$Y_{CO2/gly}$ (Cmol $Cmol^{-1}$)	0.19	0.43	0.25	0.29	0.26	0.26	0.27	0.18	0.23	0.25	0.28
μ (h^{-1})	0.28	-	-	-	0.19	-	-	0.17	-	-	-
q_{gly} (mCmol $CmolX_{cat}^{-1}$ h^{-1})	-700 to -200	-200 to -100	-100 to -80	-50	-800 to -400	-400 to -100	-80 to -100	-400 to -200	-200 to -100	-80	-50
q_{SA} (mCmol $CmolX_{cat}^{-1}$ h^{-1})	-	-	-	-	60 to 70	70 to 30	30 to 10	30	30 to 20	10	2
q_{CA} (mCmol $CmolX_{cat}^{-1}$ h^{-1})	0	0 to 40	40	40 to 20	30 to 20	20 to 5	5 to 6	-	-	-	-
q_{AA} (mCmol $CmolX_{cat}^{-1}$ h^{-1})	-	-	-	-	90 to 50	10 to 5	5	-	-	-	-
q_{MA} (mCmol $CmolX_{cat}^{-1}$ h^{-1})	0	0	0 to 4	4	0 to 2	2 to 6	6 to 7	-	-	-	-
q_{PA} (mCmol $CmolX_{cat}^{-1}$ h^{-1})	-	-	-	-	0 to 8	9 to 5	5 to 6	-	-	-	-
$q_{storlip}$ (mCmol $CmolX_{cat}^{-1}$ h^{-1})	0	4	6	0	0	0 to 3	1	0	1	2	2
q_{totlip} (mCmol $CmolX_{cat}^{-1}$ h^{-1})	60	10	6	0	20	5 to 8	1	20	2	2	2

$[]_{max}$: maximum concentration reached during the cultivation phase; %totlip in X: total lipid content in biomass; $Y_{A/B}$: production yield of A relative to B consumed; μ: specific growth rate; q: specific consumption/production rate. X: biomass (CDW); X_{cat}: catalytic biomass; gly:

463 glycerol; SA: succinic acid; CA: citric acid; AA: acetic acid; MA: malic acid; PA: pyruvic acid; totlip: total lipids; storlip: storage lipids; CO2: carbon
464 dioxide.

For strain **W29**, the specific citrate production rate reached 40 mCmol $Cmol_{Xcat}^{-1}$ h^{-1} during Phase IIa and stayed at this value during Phase IIb, which is equivalent to a specific succinic acid production rate of 27 mCmol $Cmol_{Xcat}^{-1}$ h^{-1}, considering that one mole citrate can lead to one mole succinic acid and two moles of CO_2 according to the oxidative branch of the TCA cycle. Contrary to strain W29, where citrate was produced only during the nitrogen-limited phase, succinic acid was produced by the two engineered strain since the beginning of the cultivation because of the SDH knock-out. A specific succinic acid production rate of 60 to 70 mCmol $Cmol_{Xcat}^{-1}$ h^{-1} (Table 3) was obtained with strain **PGC01003** over Phase I of the fed-batch cultivation (Figure 1). Acetate was produced at a rate ranging from 90 to 50 mCmol $Cmol_{Xcat}^{-1}$ h^{-1} during the same phase of the same cultivation. During Phase IIa, the specific succinic acid production rate decreased from 70 to 30 mCmol $Cmol_{Xcat}^{-1}$ h^{-1}, which is 1.1 to 2.9 times higher than the citrate production rate obtained with W29 during the same phase. The specific succinic acid production rate decreased from 30 to 10 mCmol $Cmol_{Xcat}^{-1}$ h^{-1} (Phase IIb), corresponding to 1.1 to 0.4 times the potential registered with W29.

For the **PGC202** strain, a specific succinic acid production rate of 30 mCmol $Cmol_{Xcat}^{-1}$ h^{-1} (Table 3) was obtained during Phase I of the fed-batch cultivation. During Phase IIa, the specific succinic acid production rate decreased from 30 to 20 mCmol $Cmol_{Xcat}^{-1}$ h^{-1}, which is close to the expected one when comparing to strain W29.

Overall, engineered strains showed good kinetics performances compared to the potential of the wild type strain.

3.3. Stoichiometric characterization of strain performance in fed-batch cultivations and impact of nitrogen deficiency on carbon distribution

3.3.1. Citric acid, succinic acid and other organic acid production yields

The **wild-type strain W29** produced up to 43 g L^{-1} citrate with a yield of 0.56 Cmol $Cmol^{-1}$ during Phase IIb of the cultivation (Table 3), which is the highest organic acid production yield reported in this study. This yield represents 56% of the theoretical citrate production yield from glycerol (1 Cmol $Cmol^{-1}$). This strain was unable to accumulate succinic acid, acetate or pyruvate. Only malate (0.08 Cmol $Cmol^{-1}$) was also accumulated, especially during the last phase of W29 cultivation. If citrate flux was totally redirected toward succinic acid (oxidative branch of the TCA cycle), a yield of 0.37 Cmol $Cmol^{-1}$ could thus be reached by the engineered strains. Carbon flux redirections are discussed in section 3.3.3. Impact of nitrogen deficiency on carbon distribution.

Strain PGC01003 showed an average succinic acid yield of 0.24 Cmol $Cmol^{-1}$ over Phase IIa and 0.20 Cmol $Cmol^{-1}$ over Phase IIb of the corresponding fed-batch cultivation (Table 3). This represents 30-36% of the theoretical succinic acid production yield (0.67 Cmol $Cmol^{-1}$) from glycerol considering that succinic acid is produced through the oxidative branch of the TCA or the glyoxylate cycle, and corresponds to 54-65% of the potential of the reference strain W29. Succinic acid production by strain PGC01003 was accompanied by a high coproduction of acetate, but also citrate, malate and pyruvate, showing that the deletion of sdh probably led to redox imbalance that must be equilibrated by the synthesis of other co-products. A better balance of redox reactions could have led to higher yields.

With **strain PGC202** engineered from PGC01003 to limit the formation of by-products, only 35-43% (0.13 to 0.16 Cmol $Cmol^{-1}$) of the potential of the reference strain was obtained (Phases IIa and IIb in Table 3). Although strain PGC202 did not accumulate any organic acid

analyzed, a lower succinic acid yield was reached compared to PGC01003. The low carbon balance (60-80 %, Cmol $Cmol^{-1}$, Figure A.9) obtained with the corresponding cultivation indicates that other by-products were generated, as explained previously (section 3.1. Overview of strain performances), and that metabolism is still imbalanced for succinic acid synthesis.

3.3.2. Lipid production

The **wild-type strain W29** accumulated 20% (w/w) total lipids with an overall yield of 0.08 Cmol $Cmol^{-1}$. Here the feeding was not optimized for lipid production, but for citrate production. This yield was higher than with strains **PGC202** (0.05 Cmol $Cmol^{-1}$; 17% total lipids) and **PGC01003** (0.03 Cmol $Cmol^{-1}$; 12% total lipids). Comparatively, the theoretical lipid production yield on glycerol is about 0.30 g_{lip} g_{gly}^{-1} (0.55 Cmol $Cmol^{-1}$). About 0.16 Cmol $Cmol^{-1}$ was reached with the wild type strain LGAM S(7)1 (Papanikolaou and Aggelis, 2002). The maximum production yields of storage lipids were 0.09 Cmol $Cmol^{-1}$ during Phase IIb of W29 cultivation, 0.04 Cmol $Cmol^{-1}$ during Phases IIa and IIc of PGC202 cultivation, and 0.01 Cmol $Cmol^{-1}$ during Phases IIa and IIb of PGC01003 cultivation. The highest production yields of storage lipids, citrate (strain W29) and succinic acid (strains PGC01003 and PGC202) were observed at the same cultivation stage (Table 3).

3.3.3. Impact of nitrogen deficiency on carbon distribution

The impact of nitrogen deficiency on carbon distribution was studied by comparing the yields under non-limiting cultivation Phase I and nitrogen-deficient Phase IIb (Figure 2).

The carbon allocated to biomass during the exponential growth phase of strain W29 was completely reassigned during Phase IIb to citrate and, to a lesser extent, to storage lipids. These results confirm the efficiency of nitrogen limitation as a strategy to promote citrate

and lipid accumulation by oleaginous yeasts such as *Y. lipolytica* (Beopoulos et al., 2009; Morgunov et al., 2013; Sabra et al., 2017). The mechanisms behind this metabolic shift have been described in literature. Nitrogen deficiency induces the activation of adenosine monophosphate (AMP) deaminase, which catalyzes the conversion of AMP into ammonium and inosine monophosphate. As AMP is an allosteric activator of isocitrate dehydrogenase (ICD), the TCA cycle is thus interrupted at this step, resulting in an accumulation of isocitrate and citrate in the mitochondria (Boulton and Ratledge, 1983; Ratledge and Wynn, 2002). The NAD(P)H/NAD(P) ratio has also been reported to increase under nitrogen deficiency conditions, leading to the same consequences as for AMP since NAD(P) is a cofactor of the NAD(P)-dependent ICD (Morgunov et al., 2004).

Lipids can also accumulate because citrate acts as an acetyl-CoA shuttle in oleaginous eukaryotes (Abdel-Mawgoud et al., 2018). The citrate accumulated in mitochondria is exported to the cytosol via malate-citrate antiporters. Cytosolic citrate is converted into oxaloacetate and acetyl-CoA by ATP citrate lyase. The oxidation of oxaloacetate by malate dehydrogenase regenerates malate, which can join the citrate exportation cycle again. On the other side, cytosolic acetyl-CoA is at the basis of fatty acid and lipid biosynthesis. Therefore, the accumulation of citrate caused by nitrogen deficiency is prone to lead to the accumulation of lipids in oleaginous microorganisms. From previous studies and our own results (Figure 1, W29), it appears that when the nitrogen depletion goes further, lipid production ceases due to NADPH limitation and only citrate accumulates (Beopoulos et al., 2009).

Such a redirection of carbon from biomass to citrate and lipids was not observed with the engineered strains used in this study. With strain PGC01003, the carbon dedicated to

555 catalytic biomass and acetate during Phase I was partially redirected towards succinic acid
556 and other organic acids (malate and pyruvate) instead of citrate and lipids under nitrogen
557 deficiency conditions (Table 3 and Figure 2). The part of carbon allocated to succinic acid
558 increased from 17% during growth phase to 20% during Phase IIb (after reaching a maximum
559 of 24% during Phase IIa), while the part directed towards citrate remained similar (6% during
560 Phase I and 5% during Phase IIb). It is important to note that some by-products could be
561 missing since during Phase IIb the carbon balance was about 70% (against 95% at the end of
562 the culture as stated in section 3.1. Overview of strain performances).

563 With strain PGC202, a part of the carbon flow was redirected towards carbon dioxide,
564 succinic acid, lipids and unidentified by-products instead of biomass. The carbon allocated to
565 succinic acid increased from 8% during growth phase to 13% during Phase IIb, after reaching
566 a maximum of 16% during Phase IIa.

567 These results show that nitrogen deficiency conditions promoted carbon redirection towards
568 succinic acid. However, succinic acid yield and production rate were the highest during
569 Phase IIa of the cultivations. The favorable conditions of Phase IIa could be maintained by
570 keeping constant the C/N ratio of the medium throughout the succinic acid production step
571 (i.e., by implementing a strategy based on nitrogen limiting flux instead of nitrogen
572 deficiency).

573 Considering the metabolic modifications carried by strains PGC01003 and PGC202, in
574 particular the deletion of *sdh5*, succinic acid accumulation was expected under the aerobic
575 and non-limiting conditions of cultivation Phase I. Nitrogen deficiency conditions applied in
576 Phase II should have led to ICD inhibition and citrate accumulation, because ICD is located
577 upstream SDH in the oxidative branch of the TCA cycle. Citrate indeed accumulated with

strain PGC01003 under non-limiting ($Y_{CA/gly}$ yield of 0.06 Cmol $Cmol^{-1}$) and nitrogen-deficient conditions ($Y_{CA/gly}$ yield of 0.05 to 0.10 Cmol $Cmol^{-1}$), but to a much lesser extent than with strain W29. The accumulation of succinic acid with these strains under nitrogen deficiency conditions could be explained by the functioning of another pathway converting citrate to succinic acid or by the only partial inhibition of ICD.

Under the first hypothesis, the alternative pathway generating succinic acid from citrate could be the glyoxylate cycle. According to Cui et al. (2017) who designed strain PGC202, the overexpression of enzymes involved in the glyoxylate cycle (i.e., isocitrate lyase, malate synthase, and ATP citrate lyase) in strain PGC52 (Table 2) did not enhance succinic acid production. Moreover, the modifications that enabled strain PGC202 to be selected as the best succinic acid-producing host, in particular the overexpression of succinyl-CoA synthetase, indicate that succinic acid was mainly produced via the oxidative branch of the TCA cycle. Nevertheless, these cultivations were performed under non-limiting conditions and cannot fully explain our results obtained under nitrogen-deficient conditions.

Under the second hypothesis, the reduced activities of most of the TCA cycle enzymes (and especially ICD) under nitrogen-deficient conditions compared to non-limiting conditions should lead to decreased specific succinic acid production rates (Ermakova et al., 1986), while the succinic acid yields could theoretically be enhanced by redirection of carbon from biomass. This possibility matches well with the results obtained for the engineered strains.

CONCLUSIONS

This study characterized the impact of nitrogen deficiency on the redirection of the metabolic carbon flux toward succinic acid synthesis in *Y. lipolytica* engineered strains (PGC01003 and PGC202) during fed-batch cultivations in a chemically defined medium. This strategy was successful by increasing yield of succinic acid of by 18% and 62%, respectively.

Nevertheless, a cultivation strategy relying on nitrogen limiting flux instead of nitrogen deficiency could improve further the succinic acid yield and specific production rate. The strain PGC01003 was shown to be the best succinic acid producer under the conditions applied.

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AUTHOR CONTRIBUTIONS

Guillaume Billerach: Conceptualization, Formal analysis, Investigation, Writing – Original draft – Review & editing, Vizualization; **Laurence Preziosi-Belloy**: Formal analysis, Writing – Review & editing, Resources; **Carol Sze Ki Lin**: Resources, Funding acquisition, Writing – Review & editing; **Hélène Fulcrand**: Supervision, Project Administration, Funding acquisition, Resources; **Eric Dubreucq**: Conceptualization, Supervision, Writing – Review & editing, Project Administration, Funding acquisition, Resources; **Estelle Grousseau**: Conceptualization, Supervision, Formal analysis, Investigation, Writing –– Review & editing, Project Administration, Resources

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REFERENCES

Abdel-Mawgoud, A.M., Markham, K.A., Palmer, C.M., Liu, N., Stephanopoulos, G., Alper, H.S., 2018. Metabolic engineering in the host *Yarrowia lipolytica*. Metab. Eng., Metabolic engineering host organism special issue 50, 192–208. https://doi.org/10.1016/j.ymben.2018.07.016

Ahn, J.H., Jang, Y.-S., Lee, S.Y., 2016. Production of succinic acid by metabolically engineered microorganisms. Curr. Opin. Biotechnol. 42, 54–66. https://doi.org/10.1016/j.copbio.2016.02.034

Barth, G., Gaillardin, C., 1997. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. FEMS Microbiol. Rev. 19, 219–237. https://doi.org/10.1111/j.1574-6976.1997.tb00299.x

Beauprez, J.J., De Mey, M., Soetaert, W.K., 2010. Microbial succinic acid production: Natural versus metabolic engineered producers. Process Biochem. 45, 1103–1114. https://doi.org/10.1016/j.procbio.2010.03.035

Becker, J., Reinefeld, J., Stellmacher, R., Schäfer, R., Lange, A., Meyer, H., Lalk, M., Zelder, O., Abendroth, G. von, Schröder, H., Haefner, S., Wittmann, C., 2013. Systems-wide analysis and engineering of metabolic pathway fluxes in bio-succinate producing *Basfia succiniciproducens*. Biotechnol. Bioeng. 110, 3013–3023. https://doi.org/10.1002/bit.24963

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.-L., Molina-Jouve, C., Nicaud, J.-M., 2009. *Yarrowia lipolytica* as a model for bio-oil production. Prog. Lipid Res. 48, 375–387. https://doi.org/10.1016/j.plipres.2009.08.005

Boulton, C.A., Ratledge, C., 1983. Use of transition studies in continuous cultures of *Lipomyces starkeyi*, an oleaginous yeast, to investigate the physiology of lipid accumulation. Microbiology, 129, 2871–2876. https://doi.org/10.1099/00221287-129-9-2871

Cheng, K.-K., Wang, G.-Y., Zeng, J., Zhang, J.-A., 2013. Improved succinate production by metabolic engineering. BioMed Res. Int. https://doi.org/10.1155/2013/538790

Cui, Z., Gao, C., Li, J., Hou, J., Lin, C.S.K., Qi, Q., 2017. Engineering of unconventional yeast *Yarrowia lipolytica* for efficient succinic acid production from glycerol at low pH. Metab. Eng. 42, 126–133. https://doi.org/10.1016/j.ymben.2017.06.007

Ermakova, I.T., Shishkanova, N.V., Melnikova, O.F., Finogenova, T.V., 1986. Properties of *Candida lipolytica* mutants with the modified glyoxylate cycle and their ability to produce citric and isocitric acid. Appl. Microbiol. Biotechnol. 23, 372–377. https://doi.org/10.1007/BF00257036

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226, 497–509.

Gao, C., Yang, X., Wang, H., Rivero, C.P., Li, C., Cui, Z., Qi, Q., Lin, C.S.K., 2016. Robust succinic acid production from crude glycerol using engineered *Yarrowia lipolytica*. Biotechnol. Biofuels 9, 179. https://doi.org/10.1186/s13068-016-0597-8

Isar, J., Agarwal, L., Saran, S., Kaushik, R., Saxena, R.K., 2007. A statistical approach to study the interactive effects of process parameters on succinic acid production from *Bacteroides fragilis*. Anaerobe 13, 50–56. https://doi.org/10.1016/j.anaerobe.2006.12.002

Jansen, M.L., van Gulik, W.M., 2014. Towards large scale fermentative production of succinic acid. Curr. Opin. Biotechnol. 30, 190–197. https://doi.org/10.1016/j.copbio.2014.07.003

Jansen, M.L.A., Verwaal, R., 2011. Dicarboxylic acid production by fermentation at low pH. US 2011/0229945 A1.

Jost, B., Holz, M., Aurich, A., Barth, G., Bley, T., Müller, R.A., 2015. The influence of oxygen limitation for the production of succinic acid with recombinant strains of *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 99, 1675–1686. https://doi.org/10.1007/s00253-014-6252-z

Kamzolova, S.V., Morgunov, I.G., 2017. Metabolic peculiarities of the citric acid overproduction from glucose in yeasts *Yarrowia lipolytica*. Bioresour. Technol. 243, 433–440. https://doi.org/10.1016/j.biortech.2017.06.146

Kamzolova, S.V., Vinokurova, N.G., Lunina, J.N., Zelenkova, N.F., Morgunov, I.G., 2015. Production of technical-grade sodium citrate from glycerol-containing biodiesel waste by *Yarrowia lipolytica*. Bioresour. Technol. 193, 250–255. https://doi.org/10.1016/j.biortech.2015.06.092

Lee, J.W., Yi, J., Kim, T.Y., Choi, S., Ahn, J.H., Song, H., Lee, M.-H., Lee, S.Y., 2016. Homo-succinic acid production by metabolically engineered *Mannheimia succiniciproducens*. Metab. Eng. 38, 409–417. https://doi.org/10.1016/j.ymben.2016.10.004

Lee, P.C., Lee, S.Y., Hong, S.H., Chang, H.N., 2003. Batch and continuous cultures of *Mannheimia succiniciproducens* MBEL55E for the production of succinic acid from whey and corn steep liquor. Bioprocess Biosyst. Eng. 26, 63–67. https://doi.org/10.1007/s00449-003-0341-1

Levinson, W.E., Kurtzman, C.P., Kuo, T.M., 2007. Characterization of *Yarrowia lipolytica* and related species for citric acid production from glycerol. Enzyme Microb. Technol. 41, 292–295. https://doi.org/10.1016/j.enzmictec.2007.02.005

Li, C., Gao, S., Yang, X., Lin, C.S.K., 2018. Green and sustainable succinic acid production from crude glycerol by engineered *Yarrowia lipolytica* via agricultural residue based *in situ* fibrous bed bioreactor. Bioresour. Technol. 249, 612–619. https://doi.org/10.1016/j.biortech.2017.10.011

Li, C., Yang, X., Gao, S., Wang, H., Lin, C.S.K., 2017. High efficiency succinic acid production from glycerol via *in situ* fibrous bed bioreactor with an engineered *Yarrowia lipolytica*. Bioresour. Technol. 225, 9–16. https://doi.org/10.1016/j.biortech.2016.11.016

Lin, H., Bennett, G.N., San, K.-Y., 2005. Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate. Biotechnol. Bioeng. 89, 148–156. https://doi.org/10.1002/bit.20298

Litsanov, B., Brocker, M., Bott, M., 2012. Toward homosuccinate fermentation: Metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate. Appl. Environ. Microbiol. 78, 3325–3337. https://doi.org/10.1128/AEM.07790-11

Madzak, C., Tréton, B., Blanchin-Roland, S., 2000. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. J. Mol. Microbiol. Biotechnol. 2, 207–216.

Markham, K.A., Alper, H.S., 2018. Synthetic biology expands the industrial potential of *Yarrowia lipolytica*. Trends Biotechnol. 36, 1085–1095. https://doi.org/10.1016/j.tibtech.2018.05.004

McCoy, M., 2019. Succinic acid, once a biobased chemical star, is barely being made. Chem. Eng. News. https://cen.acs.org/business/biobased-chemicals/Succinic-acid-once-biobased-chemical/97/i12 (accessed January 15, 2021).

Meynial-Salles, I., Dorotyn, S., Soucaille, P., 2008. A new process for the continuous production of succinic acid from glucose at high yield, titer, and productivity. Biotechnol. Bioeng. 99, 129–135. https://doi.org/10.1002/bit.21521

Morgunov, I.G., Kamzolova, S.V., Lunina, J.N., 2013. The citric acid production from raw glycerol by *Yarrowia lipolytica* yeast and its regulation. Appl. Microbiol. Biotechnol. 97, 7387–7397. https://doi.org/10.1007/s00253-013-5054-z

Morgunov, I.G., Solodovnikova, N.Yu., Sharyshev, A.A., Kamzolova, S.V., Finogenova, T.V., 2004. Regulation of NAD+-dependent isocitrate dehydrogenase in the citrate producing yeast *Yarrowia lipolytica*. Biochem. Mosc. 69, 1391–1398. https://doi.org/10.1007/s10541-005-0086-3

Nurmi, L., Jaatinen, S., Saleem, F., Asikainen, M., Kalliola, A., Mori, E.-L.H., Karinen, R., Lehtonen, J., Linnekoski, J., Murzin, D., 2018. From biomass to value-added furan-based platform chemicals: FURCHEM and CatBio roadmap. VTT Technical Research Centre of Finland.

Ochoa-Estopier, A., Guillouet, S.E., 2014. D-stat culture for studying the metabolic shifts from oxidative metabolism to lipid accumulation and citric acid production in *Yarrowia lipolytica*. J. Biotechnol. 170, 35–41. https://doi.org/10.1016/j.jbiotec.2013.11.008

Okino, S., Inui, M., Yukawa, H., 2005. Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. Appl. Microbiol. Biotechnol. 68, 475–480. https://doi.org/10.1007/s00253-005-1900-y

Papanikolaou, S., Aggelis, G., 2009. Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid by *Yarrowia lipolytica*. Lipid Technol. 21, 83–87. https://doi.org/10.1002/lite.200900017

Papanikolaou, S., Aggelis, G., 2002. Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresour. Technol. 82, 43–49. https://doi.org/10.1016/S0960-8524(01)00149-3

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., Marc, I., 2002. *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. J. Appl. Microbiol. 92, 737–744. https://doi.org/10.1046/j.1365-2672.2002.01577.x

Patel, M., Crank, M., Dornburg, V., Hermann, B., Roes, L., Hüsing, B., Overbeek, L., Terragni, F., Recchia, E., 2006. Medium and long-term opportunities and risk of the biotechnological production of bulk chemicals from renewable resources: the potential of white biotechnology : the BREW project. Utrecht University, Technology and Society (STS)/Copernicus Institute, Utrecht, the Netherlands.

Pateraki, C., Patsalou, M., Vlysidis, A., Kopsahelis, N., Webb, C., Koutinas, A.A., Koutinas, M., 2016. *Actinobacillus succinogenes*: Advances on succinic acid production and prospects for development of integrated biorefineries. Biochem. Eng. J. 112, 285–303. https://doi.org/10.1016/j.bej.2016.04.005

Raab, A.M., Gebhardt, G., Bolotina, N., Weuster-Botz, D., Lang, C., 2010. Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid. Metab. Eng. 12, 518–525. https://doi.org/10.1016/j.ymben.2010.08.005

Ratledge, C., Wynn, J.P., 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms, in: Advances in applied microbiology. Academic press, pp. 1–52.

745 Robles-Rodriguez, C.E., Bideaux, C., Guillouet, S.E., Gorret, N., Cescut, J., Uribelarrea, J.-L., Molina-
746 Jouve, C., Roux, G., Aceves-Lara, C.A., 2017. Dynamic metabolic modeling of lipid accumulation and
747 citric acid production by *Yarrowia lipolytica*. Comput. Chem. Eng. 100, 139–152.
748 https://doi.org/10.1016/j.compchemeng.2017.02.013

749 Sabra, W., Bommareddy, R.R., Maheshwari, G., Papanikolaou, S., Zeng, A.-P., 2017. Substrates and
750 oxygen dependent citric acid production by *Yarrowia lipolytica*: insights through transcriptome and
751 fluxome analyses. Microb. Cell Factories 16. https://doi.org/10.1186/s12934-017-0690-0

752 Scholten, E., Dägele, D., 2008. Succinic acid production by a newly isolated bacterium. Biotechnol.
753 Lett. 30, 2143–2146. https://doi.org/10.1007/s10529-008-9806-2

754 Shen, N., Zhang, H., Qin, Y., Wang, Q., Zhu, J., Li, Y., Jiang, M.-G., Huang, R., 2018. Efficient production
755 of succinic acid from duckweed (*Landoltia punctata*) hydrolysate by *Actinobacillus succinogenes*
756 GXAS137. Bioresour. Technol. 250, 35–42. https://doi.org/10.1016/j.biortech.2017.09.208

757 Weastra, 2012. BioConSepT EU funded R&D project, deliverable of WP8.1 "Determination of market
758 potential for selected platform chemicals, itaconic acid, succinic acid, 2,5-furandicarboxylic acid."

759 Werpy, T., Petersen, G., 2004. Top value added chemicals from biomass: Volume I -- Results of
760 screening for potential candidates from sugars and synthesis gas (No. DOE/GO-102004-1992).
761 National Renewable Energy Lab., Golden, CO (US). https://doi.org/10.2172/15008859

762 Wickerham, L.J., Kurtzman, C.P., Herman, A.I., 1970. Sexual reproduction in *Candida lipolytica*.
763 Science 167, 1141–1141. https://doi.org/10.1126/science.167.3921.1141

764 Workman, M., Holt, P., Thykaer, J., 2013. Comparing cellular performance of *Yarrowia lipolytica*
765 during growth on glucose and glycerol in submerged cultivations. AMB Express 3, 58.
766 https://doi.org/10.1186/2191-0855-3-58

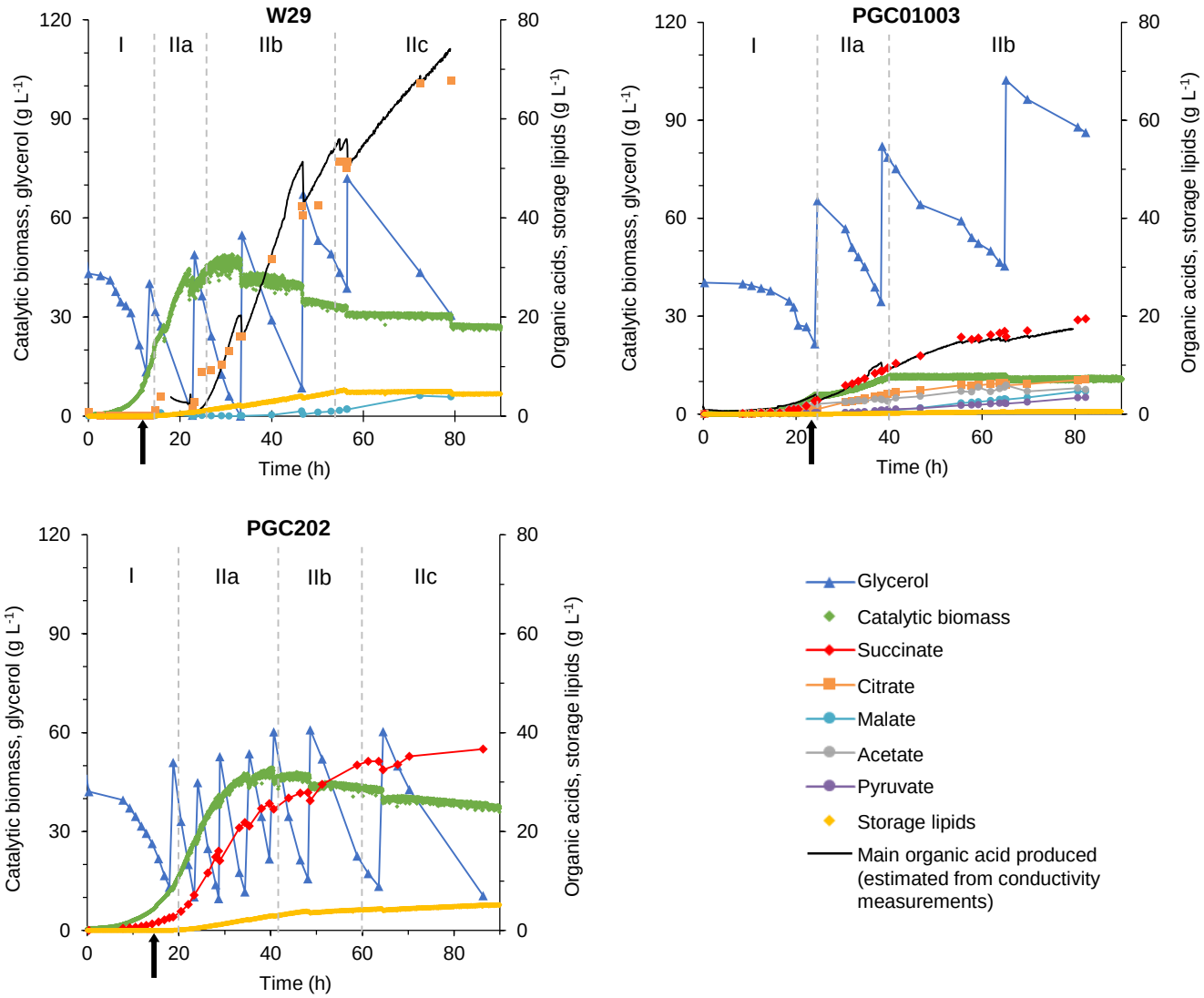
767 Yuzbashev, T.V., Bondarenko, P.Y., Sobolevskaya, T.I., Yuzbasheva, E.Y., Laptev, I.A., Kachala, V.V.,
768 Fedorov, A.S., Vybornaya, T.V., Larina, A.S., Sineoky, S.P., 2016. Metabolic evolution and 13C flux
769 analysis of a succinate dehydrogenase deficient strain of *Yarrowia lipolytica*. Biotechnol. Bioeng. 113,
770 2425–2432. https://doi.org/10.1002/bit.26007

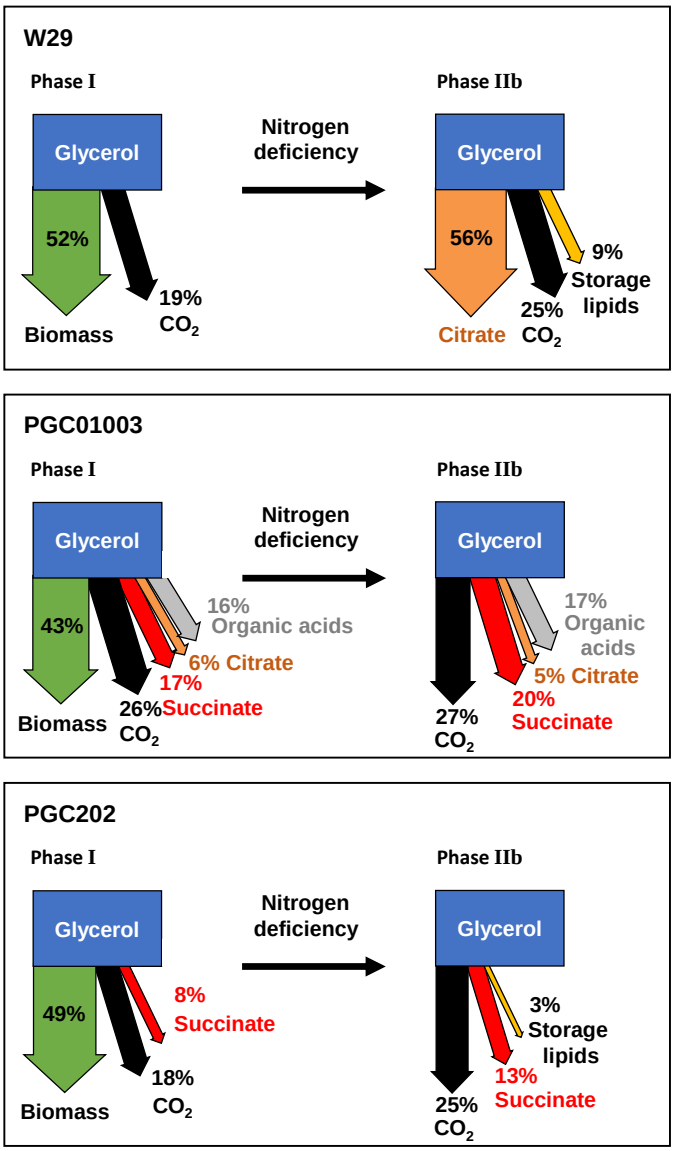
771 Yuzbashev, T.V., Yuzbasheva, E.Y., Sobolevskaya, T.I., Laptev, I.A., Vybornaya, T.V., Larina, A.S.,
772 Matsui, K., Fukui, K., Sineoky, S.P., 2010. Production of succinic acid at low pH by a recombinant
773 strain of the aerobic yeast *Yarrowia lipolytica*. Biotechnol. Bioeng. 107, 673–682.
774 https://doi.org/10.1002/bit.22859

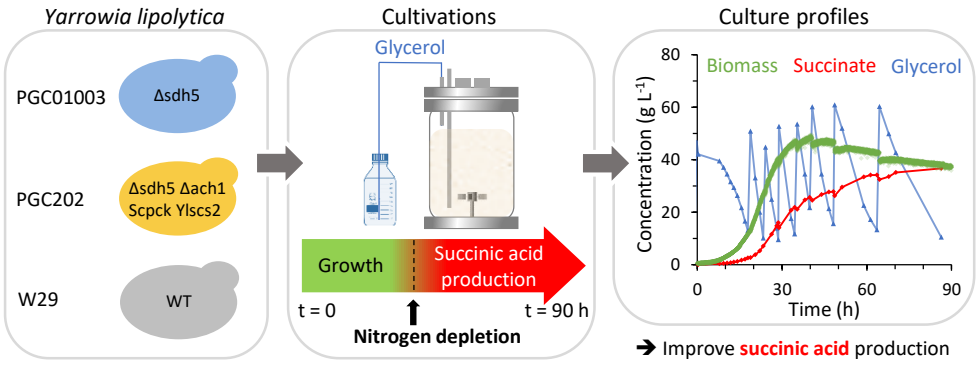
FIGURE CAPTIONS

Figure 1: Fermentation profile obtained with strains W29, PGC01003, and PGC202 for the fed-batch cultivations performed on glycerol CM medium, at 28 °C, pH 5.5, with DO maintained above 20% of saturation, including a growth step (i.e., Step I) under non-limiting conditions and an organic acid production step (i.e., Step II) under nitrogen deficiency. Culture phases (i.e., Phases I, IIa, IIb, IIc) are separated by grey dotted lines. The switch of alkali solution (NH_4OH replaced by KOH) for pH regulation is indicated by a black arrow.

Figure 2: Carbon distribution for the strains W29, PGC01003, and PGC202 under the nitrogen-deficient cultivation conditions.


W29
I
IIa
IIb
IIc
Catalytic biomass, glycerol (g L-1)
Organic acids, storage lipids (g L-1)
Time (h)
PGC01003
PGC202
Glycerol
Catalytic biomass
Succinate
Citrate
Malate
Acetate
Pyruvate
Storage lipids
Main organic acid produced (estimated from conductivity measurements)


W29
Phase I
Glycerol
52%
Biomass
19%
CO2
Nitrogen
deficiency
Phase IIb
Glycerol
56%
Citrate
25%
CO2
9%
Storage
lipids
PGC01003
Phase I
Glycerol
43%
Biomass
26%
CO2
16%
Organic acids
6% Citrate
17%
Succinate
Nitrogen
deficiency
Phase IIb
Glycerol
27%
CO2
17%
Organic
acids
5% Citrate
20%
Succinate
PGC202
Phase I
Glycerol
49%
Biomass
18%
CO2
8%
Succinate
Nitrogen
deficiency
Phase IIb
Glycerol
25%
CO2
3%
Storage
lipids
13%
Succinate


Yarrowia lipolytica
PGC01003
Δsdh5
PGC202
Δsdh5 Δach1
Scpck Ylscs2
W29
WT
Cultivations
Glycerol
Growth
Succinic acid production
t = 0
t = 90 h
Nitrogen depletion
Culture profiles
Biomass
Succinate
Glycerol
Concentration (g L-1)
Time (h)
0
20
40
60
80
0
30
60
90
➔ Improve succinic acid production