Engineering the central carbon metabolism of *Saccharomyces cerevisiae* for succinic acid production from glycerol

Joeline Xiberras

A thesis submitted in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy
in
Biochemical Engineering

Approved Dissertation Committee

Prof. Dr. Elke Nevoigt
Jacobs University Bremen, Germany

Prof. Dr. Sebastian Springer
Jacobs University Bremen, Germany

Prof. Dr. Pau Ferrer
Autonomous University of Barcelona, Spain

Date of Defense: 17th January 2020

Department of Life Sciences & Chemistry

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Family Name, Given/First Name	Xiberras Joeline
Matriculation number	20331383
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SUMMARY

Glycerol is an attractive carbon source in industrial biotechnology because its conversion into the glycolytic intermediate pyruvate via the glycolytic pathway produces twice the amount of reducing equivalents (per carbon equivalent) compared to glucose. This enables higher maximum theoretical yields of metabolic products whose production pathways require the more reducing equivalents in the form of NADH than ethanol. One example for such a product of high commercial interest is succinic acid (SA). SA, a 1,4-dicarboxylic acid, is regarded as one of the most important bio-based platform chemicals due to its numerous potential applications, such as its conversion to industrially valuable chemicals including adipic acid and tetrahydrofuran.

Saccharomyces cerevisiae is a well-established platform production organism in industrial biotechnology. It is the preferred host for dicarboxylic acid production such as SA due to its tolerance towards low pH values accompanied by the possibility to produce the protonated form of the acid which significantly facilitates downstream processing. Three metabolic routes exist by which SA can naturally be formed in *S. cerevisiae*: i) the glyoxylate cycle, ii) the oxidative and iii) the reductive branch of the TCA cycle. The latter is a fermentative pathway and it generates the highest theoretical SA yield if established in the cytosol.

The amount of SA naturally formed in wild-type *S. cerevisiae* is relatively low and extensive metabolic engineering is necessary if an economically viable SA production process is envisaged. With regard to the use of glycerol as a carbon source in synthetic medium, the knowledge about the central carbon metabolism was relatively fragmentary at the beginning of this thesis. Therefore, an extensive review of the current knowledge about the central carbon catabolism during growth of *S. cerevisiae* on

glycerol was written before engineering *S. cerevisiae* for the production of SA from glycerol. A number of uncertainties particularly regarding the role of the glyoxylate cycle, the subcellular localization of the respective enzymes, the contributions of mitochondrial transporters and the active anaplerotic reactions during growth on glycerol were identified. In addition, the current state of the art with regard to the production of fermentation products from glycerol was summarized in the respective review.

Anaplerotic reactions are very important for any accumulation of dicarboxylic acids such as SA. In addition, net ATP generation and co-factor balance of the cell strongly depend on the anaplerotic route that is mainly used for the production of SA. Notably, it was unclear at the beginning of this thesis which is the main anaplerotic route active during growth in synthetic glycerol medium. On glucose (C6) or ethanol (C2), the main anaplerotic route during growth of *S. cerevisiae* has been previously identified to be pyruvate carboxylase and the glyoxylate cycle, respectively. One of the goals of the current work was therefore to investigate the importance of the two isoenzymes of pyruvate carboxylase (encoded by *PYC1* and *PYC2*) and one of the key enzymes of the glyoxylate cycle (encoded by *ICL1*) for growth on glycerol (C3) as a sole carbon source. A reverse engineered CEN.PK derivative which shows a maximum specific growth rate of 0.14 h^{-1} on glycerol was used as the baseline strain. Results suggest that both the pyruvate carboxylase and the glyoxylate cycle mainly contribute to the anaplerotic reactions on glycerol and can partly replace each other in case one route is abolished.

A central part of the current thesis was the establishment of a redox-balanced, fermentative pathway from glycerol to SA, i.e. glycerol catabolism via the NAD^+-dependent pathway and SA production through the cytosolic reductive branch of the

TCA cycle (referred to as 'SA module') present in the cytosol. Strain CEN.PK113-1A *UBR2*$_{CBS}$ *gut1*::*mod2* (referred here to as *UBR2*$_{CBS}$-DHA) was chosen as the baseline strain for this part. This strain catabolizes glycerol via the NAD^+-dependent glycerol catabolic pathway and shows a maximum growth rate (μ_{max}) of 0.26 h^{-1}. The three genetic modifications constituting a cytosolic path from oxaloacetate to SA ('SA module', referred to as SA in strain name) comprise the overexpression of a cytosolic form of the endogenous peroxisomal malate dehydrogenase, the heterologous expression of fumarase and of a cytosolic form of a heterologous fumarate reductase as well as expression of the heterologous dicarboxylic acid transporter *An*DCT-02, resulting in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02. In shake flask cultures, this strain produced a maximum titre of 10.7 g/L SA corresponding to a yield of 0.22 ± 0.01 g/g glycerol. Thus, the current study is the first demonstrating significant SA accumulation in *S. cerevisiae* from glycerol as the sole carbon source. The data obtained strongly indicate that the enzymes of the cytosolic `SA module´, the endogenous pyruvate dehydrogenase bypass and the glyoxylate cycle act together in a highly synergistic manner supporting SA overproduction in the engineered strain. Deletion of *SDH1* further increased the maximum SA yield to 0.43 ± 0.02 g/g glycerol. As all experiments have been conducted in shake-flask cultivations without pH control or carbon dioxide sparging so far, it is assumed that there is room for further improving SA production by bioprocess engineering in the future.

The 'SA module' is the only pathway which allows redox-neutral SA production accompanied by carbon dioxide fixation. However, the SA formed by the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 was obviously not only produced via this pathway. Another part of the thesis was the attempt to force the metabolic flux via the envisaged route by means

of increasing the flux from either pyruvate or phosphoenolpyruvate to oxaloacetate by overexpressing either *PYC1, PYC2* or *PCK1*. Surprisingly, each of the individual genetic modifications led to a reduction in SA production and an onset of ethanol formation as well as improved biomass formation and glycerol consumption. The strain with *PYC2* overexpression produced up to 13 g/L of ethanol during the first 72 h of cultivation while the overexpression of *PYC1* and *PCK1* resulted in the generation of ~ 6 g/L ethanol after 120 h. Although a number of hypothesis have been discussed, the exact route by which ethanol is being formed in the engineered strains remains to be clarified in the future.

To conclude, the results presented in this thesis contribute to the understanding of the central carbon catabolism during glycerol utilization by *S. cerevisiae.* They also show the potential for the production of valuable fermentation products from glycerol such as SA which require more reducing power than the formation of ethanol. The results also demonstrate that the metabolism of *S. cerevisiae* during growth on glycerol is still far from completely understood and further studies are required to fill the knowledge gaps.

ACKNOWLEDGEMENTS

First and foremost, I am indebted to Professor Dr. Elke Nevoigt for her exceptional dedication and constant support throughout this whole journey. No words can describe my gratitude towards her.

I am also grateful to Dr. Mathias Klein. His enthusiasm and humour have brought much joy to my work. It was a pleasure working with him.

A special thanks goes to Max and Ping-Wei for their help during the start of my PhD thesis. I am also particularly thankful to Zahabiya for proof reading some parts of this thesis. In addition, I am indebt to my current and previous colleagues, Dorthe, Andreea, Federica, Solvejg, Mehmet, Frederico, Uschi, Celina, and Raquel for their constant support.

I would like to express my deepest gratitude to Prof. Dr. Jack Pronk for allowing me to perform bioreactor experiments in his lab under the supervision of Erik de Hulster and Dr. Roberts Mans. I sincerely thank them for all their support and nice discussions during my stay at TU Delft, in the Netherlands. A special thanks goes to Prof. Dr. Sebastian Springer and Prof. Dr. Pau Ferrer for accepting to be part of my PhD committee.

My appreciation also goes to my family and friends in Malta. In particular, I would like to thank Michael and Vicky for their constant understanding and encouragement in all my endeavours. Lastly, I would like to thank Karl-Wolfgang Lindenblatt and Baloo (my dog) for their endless patience and support throughout this thesis.

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Chapter 1. INTRODUCTION

1.1 The yeast *Saccharomyces cerevisiae* - a popular production organism in industrial biotechnology

The yeast *S. cerevisiae* has become a favourite microbial cell factory in industrial biotechnology for several reasons. One attribute for its popularity is undoubtedly due to its classification as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration (FDA). In addition, the long-standing usage of this species in traditional biotechnology and microbial fermentation such as for production of wine (at least as early as 3150 BC (Cavalieri et al., 2003)), beer and bread led to an accumulation of in-depth knowledge regarding to its biochemistry, genetics and physiology (Barnett et al, 2008). In contrast to microbial fermentations using bacteria, yeast cultures are not vulnerable to bacteriophage infection. Furthermore, *S. cerevisiae* is also highly tolerant towards high sugar and ethanol concentrations as well as low pH and many industrial processes profit from these characteristics (Nevoigt, 2008).

Another important attribute that particularly boosted the popularity of *S. cerevisiae* in industrial biotechnology is the development of genome editing tools which have facilitated the genetic manipulation of this species (Kavscek et al., 2015). Examples of traditional genetic tools for *S. cerevisiae* include expression vectors, efficient transformation methods, genetic selectable markers as well as Cre recombinase system (Akada et al., 2002; Gietz & Woods, 2002; Gueldener et al., 2002; Jinek et al., 2012). In the last years, new tools like zinc finger nucleases (Carroll, 2011), transcription activator like (TAL) effector nucleases (Bogdanove & Voytas, 2011) and the clustered regularly interspaced short palindromic repeats (CRISPR-Cas) system (Jinek et al., 2012) have been developed. In comparison to the traditional genetic tools, the latter tools have increased the accuracy, efficiency and speed with which *S. cerevisiae* can be

manipulated. Currently, the CRISPR-Cas system is the most promising tool for genome engineering, whose applicability in *S. cerevisiae* has been reported for the first time by DiCarlo et al. (2013). Furthermore, metabolic engineering of *S. cerevisiae* has benefited from the advancement in modelling and synthetic biology tools, which led to faster construction of efficient and robust cell factories (Borodina & Nielsen, 2014; Nielsen & Jewett, 2008). In fact, these tools led to a system-level insight into production strains which enabled predictable metabolic engineering (Hansen et al., 2017). Example of compounds which have been successfully produced in *S. cerevisiae* using model-aided approaches include succinic acid (SA) (Agren et al., 2013; Otero et al., 2013), vanillin (Brochado et al., 2010)and ethanol (Bro et al., 2006).

S. cerevisiae favours homologous recombination (HR) for DNA repair while other non-conventional yeast species show preference for non-homologous end joining (NHEJ) (Da Silva & Srikrishnan, 2012). The extraordinary high efficiency of HR in *S. cerevisiae* is an attribute used by the CRISPR-Cas system to considerably facilitate the stable genomic incorporation of multiple expression cassettes with short homologous flanking regions as often required for extensive metabolic engineering endeavours (Mans et al., 2015; Salsman & Dellaire, 2017).

1.2 Glycerol is a promising feedstock for microbial processes

1.2.1 The potential of glycerol and its market

A crucial challenge for the utilization of sugar-based substrates for the production of chemicals is that their degree of reduction per carbon atom is significantly lower compared to starting materials that are based on fossil resources, which inevitably results in low carbon yield (Burk & Van Dien, 2016). In this context, it is interesting to note that although glycerol is still less reduced than hydrocarbons derived fossil

resources, its degree of reduction per carbon atom (4.7) is higher compared to common sugars such as glucose (4.0) and xylose (4.0) (Yazdani & Gonzalez, 2007). In fact, the conversion of glycerol to the intermediate pyruvate (via the lower glycolysis route) generates double the amount of reducing equivalents (per carbon atom) when compared to sugars. This is particularly advantageous for production of chemicals whose biosynthesis has a high demand for reducing equivalents such as SA and 1,2-propandiol (Yazdani & Gonzalez, 2007). As a result, higher yields can be achieved with glycerol as sole carbon source.

In the last decade, glycerol was abundant due to the fast development of biodiesel industry. Biodiesel is synthesized by transesterification of vegetable oils and fats with alcohol (mainly methanol), in the presence of a catalyst, and is accompanied by the production of crude glycerol as the main by-product (Fig. 1) (Clomburg & Gonzalez, 2013). Over the last years, the price of crude glycerol has faced strong falling trend and is presently sold at a $200-220 per ton (Wong, 2019). When discussing glycerol as a carbon source for industrial processes, one cannot ignore the fact that the glycerol market is unpredictable because the current and future biodiesel industry has to face a number of uncertainties due to changing crude oil prices, politics and governmental priorities, as reviewed by Naylor & Higgins (2017). Therefore, it would be advisable to explore novel routes for the efficient direct or indirect generation of glycerol from carbon dioxide in order to provide a sustainable supply of this valuable carbon source.

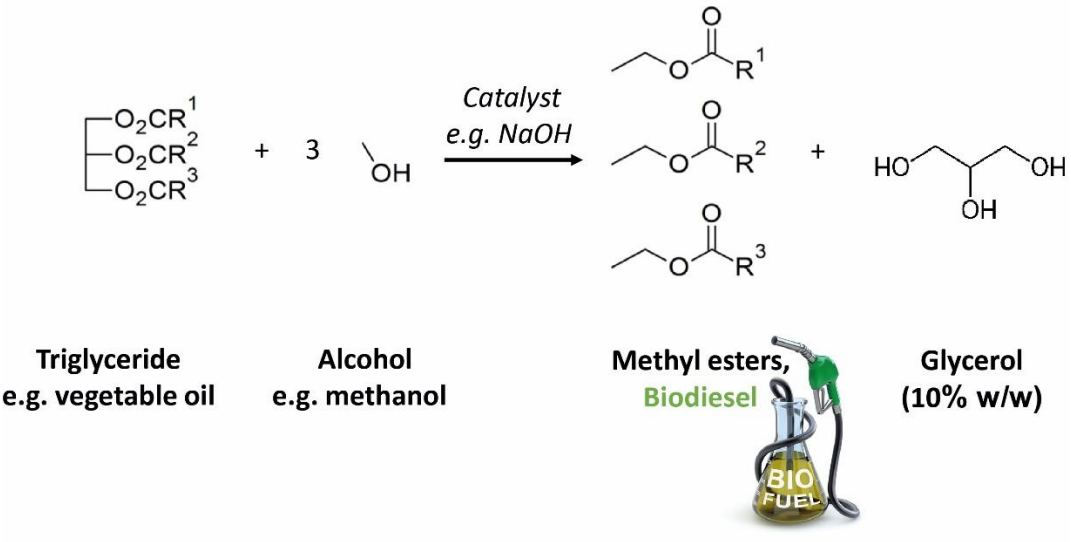


Fig. 1: Glycerol is generated as the main by-product during biodiesel production.

1.2.2 Valorisation routes of crude glycerol

The glycerol produced from the transesterification reaction (Fig. 1) is in crude form and it is a mixture consisting mainly of glycerol and impurities such as inorganic salts, water, matter organic non-glycerol (MONG) and triglycerides (Cornejo et al., 2017, Fig. 2). Over the last decade, the increase in biodiesel industry has led to a surplus production of crude glycerol, making it a waste with a disposal cost for biodiesel plants (Chol et al., 2018). Therefore, several routes for the valorisation of crude glycerol have emerged to increase the economic viability of biodiesel industry (Luo et al., 2016; Tan et al., 2013).

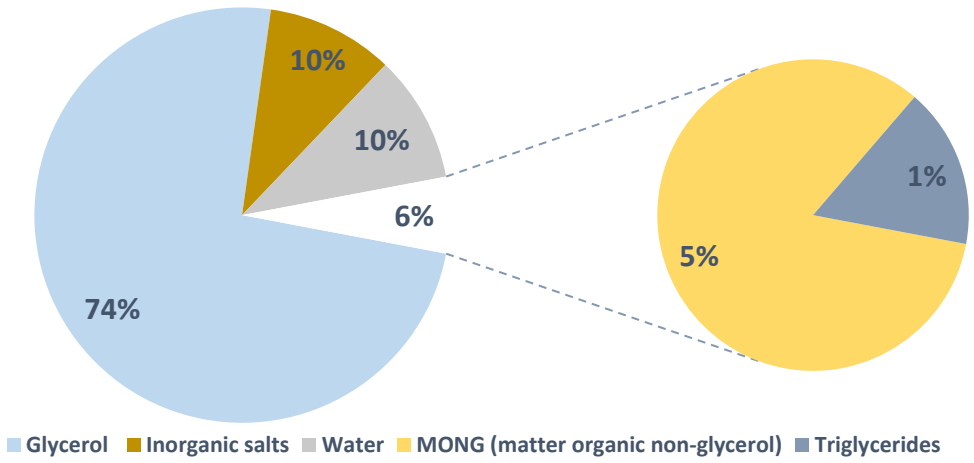


Fig. 2. Composition of the crude glycerol derived from the transesterification reaction (Cornejo et al., 2017).

One route for the valorisation of crude glycerol is its conversion into pure glycerol via refining processes, such as the 'Universal Recovery strategy' (Chol et al., 2018; Luo et al., 2016). This process consists of three steps; (1) neutralization to remove salts and soap and adjust pH; (2) vacuum evaporation to remove water and methanol; and (3) deep refining to further enhance the purity of glycerol to > 95% (Galanakis, 2012; Luo et al., 2016; Wu et al., 2016). Purified glycerol can be used in a wide range of industrial applications including personal care, food and pharmaceuticals as well as cosmetics (Clomburg & Gonzalez, 2013; Quispe et al., 2013; Tan et al., 2013). However, a major weakness of this route is the high cost of the refining process and is therefore not economically viable for small to medium sized companies (Luo et al., 2016).

Another alternative is the conversion of crude glycerol into high value-added chemicals such as oxygenated chemicals (e.g. acrolein) (Cornejo et al., 2017), hydrogen and syngas (Dou et al., 2009; Dou et al., 2010) by chemical approaches. In addition to chemical transformation, biological approaches are also an avenue for production of high value-added chemicals from crude glycerol, since a number of microorganism can metabolize this carbon source (Almeida et al., 2012; Quispe et al., 2013; Tan et al., 2013). As a result, extensive research has been carried out in recent years on trying to metabolically engineer microorganisms for the bio-based production of chemicals from crude glycerol as reviewed in detail by Chen and Liu (2016). For example, *Lactobacillus reuteri*, *Escherichia coli* and *Corynebacterium glutamicum* have been genetically engineered to produce 1,3-propandiol (Vaidyanathan et al., 2011), lactate (Wang et al., 2015) and SA (Litsanov et al., 2013) from glycerol, respectively.

1.3 *S. cerevisiae* and glycerol utilization

1.3.1 Current knowledge about glycerol utilization

S. cerevisiae shows intraspecies diversity with regard to growth on glycerol. Without the addition of complex medium supplements, most strains either grow poorly or not at all on glycerol as a carbon source (Barnett et al., 2000). Although specific glycerol growth rates have been reported for certain *S. cerevisiae* strains (Ferreira et al., 2005; Lages & Lucas, 1997; Ronnow & Kielland-Brandt, 1993; Taccari et al., 2012), they are not useful for strain comparisons because different medium compositions were used in the different studies. In addition, these studies added supplements in the medium, which largely influences the glycerol growth (Swinnen et al., 2013).

The problem that commonly used *S. cerevisiae* strains do not grow at all in synthetic glycerol medium has been overcome by several strategies. In a previous study conducted in our lab, 52 prototrophic natural and industrial *S. cerevisiae* strains were screened for growth on glycerol as the sole carbon source in defined medium without complex supplements (Swinnen et al., 2013). Out of these 52 strains, many commonly used laboratory and well-known industrial *S. cerevisiae* strains indeed did not grow at all, including W303-1A, S288C, and CEN.PK, and Ethanol Red. On the contrary, the prototrophic yeast strain CBS 6412 was able to grow and showed the shortest lag phase and one of the highest growth rates among the growing strains (Swinnen et al., 2013). This strain has formerly been described as a particular sake yeast strain, but it has been later reported that this description may be incorrect (Hubmann et al., 2013) and thus, its description still has to be elucidated (CBS – KNAW Fungal Biodiversity Centre, the Netherlands). A selected haploid isolate of the aforementioned strain (CBS 6412-13A) showed a maximum specific growth rate (μ_{max}) of ~0.13 h^{-1} and a lag phase of ~18 h

(Swinnen et al., 2013). A major genetic determinant for the enhanced glycerol growth of CBS 6412-13A (compared to the non-growing popular laboratory strain CEN.PK113-1A) was shown to be its *UBR2* allele (referred to as $UBR2_{CBS}$) (Swinnen et al., 2016). The identification of the crucial mutation has served as a basis for a reverse engineering approach, which consisted of transferring the entire $UBR2_{CBS}$ allele from CBS 6412-13A to the strain CEN.PK113-1A. The resulting strain CEN.PK113-1A $UBR2_{CBS}$ grew with a μ_{max} of ~0.04 h^{-1} after a lag phase of 25 h (Swinnen et al., 2016). The *UBR2* gene encodes a cytoplasmic ubiquitin-protein ligase, which has a function in the ubiquitin proteasome system of *S. cerevisiae* (Finley et al., 2012). There is not much information available for the specific biological role of *UBR2* gene so far. The only function which has been experimentally proven is the proteasomal degradation of Rpn4, which is a transcription factor that regulates the expression of almost all genes encoding proteasome subunits (Mannhaupt et al., 1999; Salin et al., 2008; Teixeira et al., 2008; Wang et al., 2004). This regulation is important for a wide range of cellular processes, including cell cycle division, genomic stability, DNA damage, response to heat shock and ageing (Akiyoshi et al., 2013; Gonzalez-Ramos et al., 2013; Hahn et al., 2006). Nevertheless, the actual mode of action of the identified $UBR2_{CBS}$ allele and why it established glycerol growth in CEN.PK113-1A still has to be investigated.

In order to improve the growth on glycerol of the laboratory *S. cerevisiae* strains CBS 8066 and CEN.PK 113-7D, as well as of the industrial strain Ethanol Red (i.e. strains that do not grow at all on glycerol as sole carbon source), adaptive laboratory evolution (ALE) has been employed as an alternative approach (Ho et al., 2017; Ochoa-Estopier et al., 2011; Strucko et al., 2018). ALE is a strain improvement strategy which relies on the plasticity of microbial genomes by applying cultivation conditions that favour an

advantage to mutants with an industrial relevant trait (Dragosits & Mattanovich, 2013; Mans et al., 2015; Sauer, 2001). The mutants harbouring a selective advantage in relation to other cells in the population are selected based on a lower death rate, increased retention in the culture, and/or higher μ_{max}. In particular, Ochoa-Estopier et al. (2011) reported an improvement in the μ_{max} from ~0.01 h^{-1} up to ~0.22 h^{-1} of the CEN.PK113-7D-derived *S. cerevisiae* strain JL1. The genetic determinants underlying this phenotype are two-point mutations in the coding sequences of the genes *UBR2* and *GUT1* (Ho et al., 2017), which have been confirmed by the aforementioned reverse engineering strategy. The study by Ho et al. (2017) reinforced the importance of exchanging the *UBR2* allele of CEN.PK113-1A for growth on glycerol.

1.3.2 Glycerol transport and catabolic pathways

In *S. cerevisiae*, glycerol is transported into the cell by a glycerol/H^+-symporter encoded by *STL1* (Fig. 3). It has been previously shown that deletion of *STL1* in the *S. cerevisiae* strain CBS 6412-13A leads to complete abolishment of its growth on glycerol (Ferreira et al., 2005; Swinnen et al., 2013). Although *S. cerevisiae* also contains a native aquaglyceroporin encoded by *FPS1*, its contribution to glycerol uptake is limited (Oliveira et al., 2003). Instead, it controls the release of intracellularly accumulated glycerol during osmoregulation (Tamas et al., 1999).

Once inside the cell, *S. cerevisiae* naturally utilizes the L-glycerol 3-phosphate (L-G3P) pathway for glycerol catabolism (Fig. 3). In this pathway, glycerol is phosphorylated by a glycerol kinase encoded by *GUT1* which results in the formation of L-glycerol 3-phosphate (Pavlik et al., 1993; Sprague & Cronan, 1977). This intermediate is then oxidized by an FAD-dependent glycerol-3-phosphate dehydrogenase encoded by *GUT2* to DHAP (Ronnow & Kielland-Brandt, 1993; Sprague & Cronan, 1977). Gut2p is located

at the outer surface of the inner mitochondrial membrane, and the electrons are directly transferred via $FADH_2$ to ubiquinone in the mitochondrial electron transport chain.

In addition to the L-G3P pathway, an alternative NAD^+-dependent glycerol catabolic pathway (referred to as dihydroxyacetone (DHA) pathway) has been suggested to exist in a number of microbial species including certain yeasts and filamentous fungi (Matsuzawa et al., 2010). Although both pathways generate different intermediates, eventually, both produce dihydroxyacetone phosphate (DHAP), which is then converted to glyceraldehyde-3-phosphate (GA3P) by the glycolytic enzyme triose phosphate isomerase (Compagno et al., 2001). In the DHA pathway, glycerol is first oxidized by a glycerol dehydrogenase (GDH) to DHA and then phosphorylated to DHAP by a native dihydroxyacetone kinase (DAK) encoded by *DAK1/2* (Fig. 3). Unlike the L-G3P pathway, there is no definite proof for the presence of a functional native DHA pathway in *S. cerevisiae*. In fact, both a *gut1*Δ and a *gut2*Δ deletion strain showed completely abolished growth on glycerol, which indeed puts the importance of the DHA pathway into question (Swinnen et al., 2013). While *DAK* activity, mediated by *DAK1* and *DAK2* has been confirmed to be detectable in *S. cerevisiae*, it is still uncertain whether the several genes proposed to encode GDHs such as the native *GCY1*, are active (Jung et al., 2012). As such, *GCY1* is not likely to be responsible for the conversion of glycerol to DHA as reviewed by Klein et al. (2017). Therefore, GDH was not included in Fig. 3.

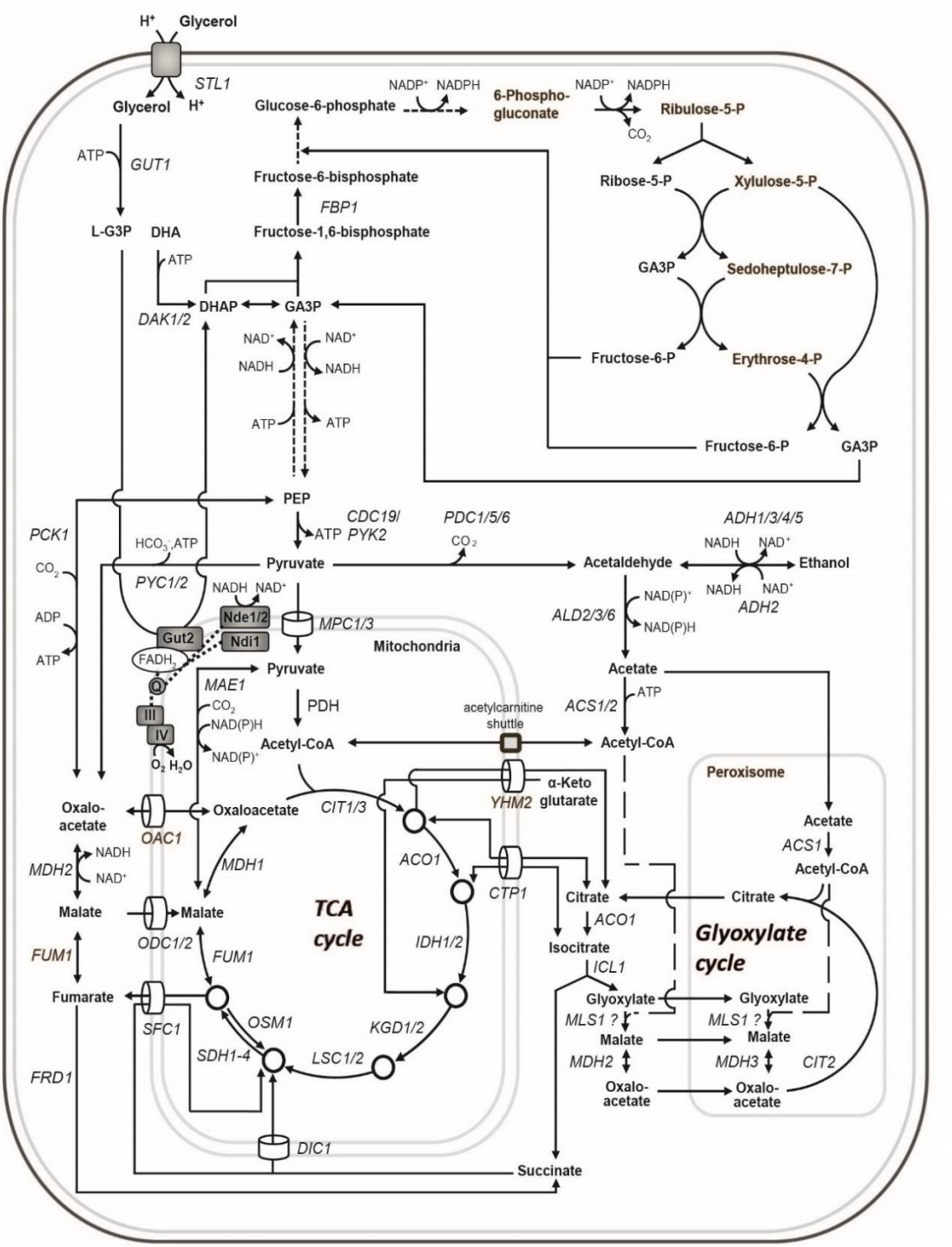


Fig. 3. Central metabolic pathways in *S. cerevisiae* during growth on glycerol. The two possible localizations of Mls1 are shown with long dash lines and question mark. Abbreviations: L-G3P: L-glycerol-3-phosphate, DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde-3 phosphate, P: phosphate, PEP: phosphoenolpyruvate, *STL1*: glycerol/H^+ symporter, *GUT1*: glycerol kinase, Gut2: FAD-dependent glycerol-3-phosphate dehydrogenase, *DAK1/2*: dihydroxyacetone kinase, *FBP1*: fructose 1,6-bisphosphatase; *CDC19*/*PYK2*: pyruvate kinase, *PCK1*: phosphoenolpyruvate carboxykinase, *PYC1/2*: pyruvate carboxylase, *MDH1*/*MDH2*/*MDH3*: malate dehydrogenase, *FUM1*: fumarase, PDH: pyruvate dehydrogenase complex, *MAE1*: malic enzyme, *CIT1/2/3*: citrate synthase, *ACO1*: aconitase, *IDH1/2*: isocitrate dehydrogenase, *LSC1/2*: succinyl-CoA ligase, *KGD1/2:* α-ketoglutarate dehydrogenase, *OSM1/FRD1,* fumarate reductase, *SDH1/2/3/4*: succinate dehydrogenase, *ICL1*: isocitrate lyase, *MLS1*: malate synthase, *ACS1/2*: acetyl-coA synthetase, *PDC1/5/6*: pyruvate decarboxylase, *ADH1/3/4/5*: alcohol dehydrogenase, *ADH2*: alcohol dehydrogenase, *ALD2/3/6*: aldehyde dehydrogenase, *MPC1/3*: pyruvate carrier, *YHM2*: citrate and ketoglutarate transporter, *CTP1*:

citrate transporter, *DIC1*: dicarboxylate transporter, *SFC1*: succinate-fumarate transporter, *ODC1/2*: oxodicarboxylate carriers, *OAC1*: oxaloacetate and sulfate transporter, Nde1/2: mitochondrial external NADH dehydrogenase, Ndi1: NADH:ubiquinone oxidoreductase, Q: ubiquinone, III: complex III, IV: complex IV.

1.3.3 Knowledge regarding the central carbon catabolism during growth on glycerol

Although certain wild-type bacteria are able to anaerobically ferment glycerol and naturally produce reduced fermentation products such as 1,3-propanediol (Celińska, 2010), glycerol has always been considered a 'non-fermentable' (or respiratory) carbon source such as ethanol and acetate for *S. cerevisiae* (Schuller, 2003; Turcotte et al., 2010). In line with this, glycerol-based medium has been used to select strains for a respiration-deficient phenotype (Hampsey, 1997). Schüller (2003) and Turcotte et al. (2010) have reviewed in detail the central carbon catabolism during the utilization of non-fermentable carbon sources (including glycerol) in *S. cerevisiae*. According to these reviews, there has been a consensus that the TCA cycle, oxidative phosphorylation, the glyoxylate cycle and gluconeogenesis represent those central metabolic pathways which seem to be activated during growth on non-fermentable carbon sources but downregulated during growth on excess glucose (Fig. 3). However, previous information regarding the central carbon catabolism during growth on glycerol does not originate from strains growing on synthetic glycerol medium because strains with this capability have not been available until recently. Past data (i) originate either from studies performed with complex media containing glycerol and/or (ii) were deduced from studies conducted with other non-fermentable carbon sources, such as ethanol or acetate. Nevertheless, supplements can strongly affect growth on glycerol as discussed in **chapter 1.3.1**. In addition, glycerol is a C3 carbon source while ethanol and acetate are C2 carbon sources, and therefore their metabolism require different metabolic fluxes. A detailed study focusing on reviewing the current knowledge about

the central carbon metabolism during growth on glycerol to clarify what is known and the respective media from which this knowledge was deduced is still lacking. Such a study could lead to the identification of knowledge gaps on synthetic glycerol medium.

1.4 Previous metabolic engineering strategies for fermentative utilization of the 'non-fermentable' carbon source glycerol in *S. cerevisiae*

1.4.1 Engineering *S. cerevisiae* for utilization of glycerol through a synthetic, NAD^+-dependent route

As discussed in **chapter 1.3.2**, *S. cerevisiae* naturally uses an FAD-dependent pathway for glycerol catabolism, and therefore the electrons from glycerol are eventually transferred to the respiratory chain. However, the electrons derived from glycerol oxidation should be saved in the form of cytosolic NADH in order to be able to exploit glycerol's reducing power for the production of chemicals whose biosynthesis has a high demand for reducing equivalents such as SA. As described in the following paragraph, much effort has been made to force glycerol catabolism through a synthetic, NAD^+-dependent route.

While the presence of a natural functional DHA pathway in *S. cerevisiae* has not yet been proven (**chapter 1.3.2**), we have shown for the first time that *S. cerevisiae* can grow on glycerol exclusively using an engineered, partly heterologous NAD^+-dependent DHA pathway for glycerol catabolism (Fig. 4). As a first step, we assumed that the glycerol import might be a bottleneck for growth on glycerol because a previous study has shown that glycerol uptake in *S. cerevisiae* was 10^5 lower as compared to the non-conventional yeast species *Cyberlindnera jadinii* (Gancedo et al., 1968). Thus, glycerol transport in the natural glycerol grower strain CBS 6412-13A was improved by expression of different aquaglyceroporins from non-conventional yeast species whose

μ_{max} in synthetic glycerol medium was higher than *S. cerevisiae* (Klein et al., 2016b). For example, *PtFPS2* from *Pachysolen tannophilus* or *FPS1* from *C. jadinii* (referred to as *CjFPS1*) were tested. *PtFPS2* and *CjFPS1* improved the μ_{max} of strain CBS 6412-13A in synthetic glycerol medium from ~0.13 h^{-1} to ~0.17 h^{-1} and ~0.18 h^{-1}, respectively, while both aquaglyceroporins shortened lag phase from 18 h^{-1} to ~9 h. Afterwards, a functional NAD^+-dependent DHA pathway was established in strain CBS 6412-13A *PtFPS2* (Klein et al., 2016a). This was achieved by the expression of a heterologous glycerol dehydrogenase from *Ogatea parapolymorpha* (*gdh* gene) combined with an increase of the endogenous DAK activity (*DAK1* gene overexpression) (Fig. 4). Altogether, these modifications were able to restore growth on glycerol in a *gut1*Δ background and a μ_{max} of ~0.11 h^{-1} and a lag phase of ~10 h were obtained. The respective strain with the replaced glycerol catabolic pathway has been referred to as CBS 6412-13A *PtFPS2 DAK1$_{OE}$ gut1*Δ *Opgdh* as described by Klein et al. (2016a).

Later, the L-G3P pathway was replaced by the DHA pathway in other *S. cerevisiae* strains including CEN.PK113-1A, CEN.PK113-1A *UBR2$_{CBS}$* (see **chapter 1.3.1**), and Ethanol Red (Klein et al., 2016a). CEN.PK derivatives have been used in a number of metabolic and evolutionary engineering studies, including those aiming for the production of C4-dicarboxylic acids (Zelle et al., 2010a), pyruvate (Van Maris et al., 2004), and isoprenoids (Kwak et al., 2017). The robust industrial diploid *S. cerevisiae* strain Ethanol Red is commonly used for first- and second-generation bioethanol production (Demeke et al., 2013). To facilitate the pathway replacement, all the necessary expression cassettes were designed in a way to enable chromosomal integration at the *GUT1* locus within a single transformation step via the CRISPR-Cas9 system. The first module (module I) included expression cassettes for *Opgdh* and *DAK1* as described above, while the coding

sequence of *PtFPS2* was replaced by the slightly better *CjFPS1* (Klein et al., 2016b). In addition to those three expression cassettes, module II included a second *DAK1* expression cassette.

Interestingly, integration of the DHA pathway module I even allowed growth on glycerol of the industrial *S. cerevisiae* strain Ethanol Red and the laboratory strain CEN.PK113-1A, both of which are unable to grow in synthetic glycerol medium in their wild-type genetic constitution. In addition, module I further improved the growth on glycerol of strain CEN.PK113-1A $UBR2_{CBS}$ (from ~0.04 h^{-1} to ~0.12 h^{-1}) (Klein et al., 2016a). The implementation of the DHA pathway module II in the *S. cerevisiae* strains Ethanol Red and CEN.PK113-1A further improved their growth on glycerol compared to module I. In CEN.PK113-1A $UBR2_{CBS}$ (resulting in strain CEN.PK113-1A $UBR2_{CBS}$ *gut1::mod2*), the second *DAK1* expression cassette in module II considerably improved growth on glycerol (growth improved from a μ_{max} of ~0.12 to ~0.26 h^{-1}). The achieved growth rate of 0.26 h^{-1} is the highest growth rate ever reported for *S. cerevisiae* growing in synthetic glycerol medium (Klein et al., 2016a). The high growth rate on glycerol in the CEN.PK113-1A $UBR2_{CBS}$ carrying module II in comparison to the other strains with module II could be explained by the assumption that the $UBR2_{CBS}$ allele acts on a pathway step downstream of DHAP, and that its impact on total flux becomes only effective when the carbon flow through the DHA pathway (up to DHAP) is not rate-controlling anymore (Klein et al., 2016a).

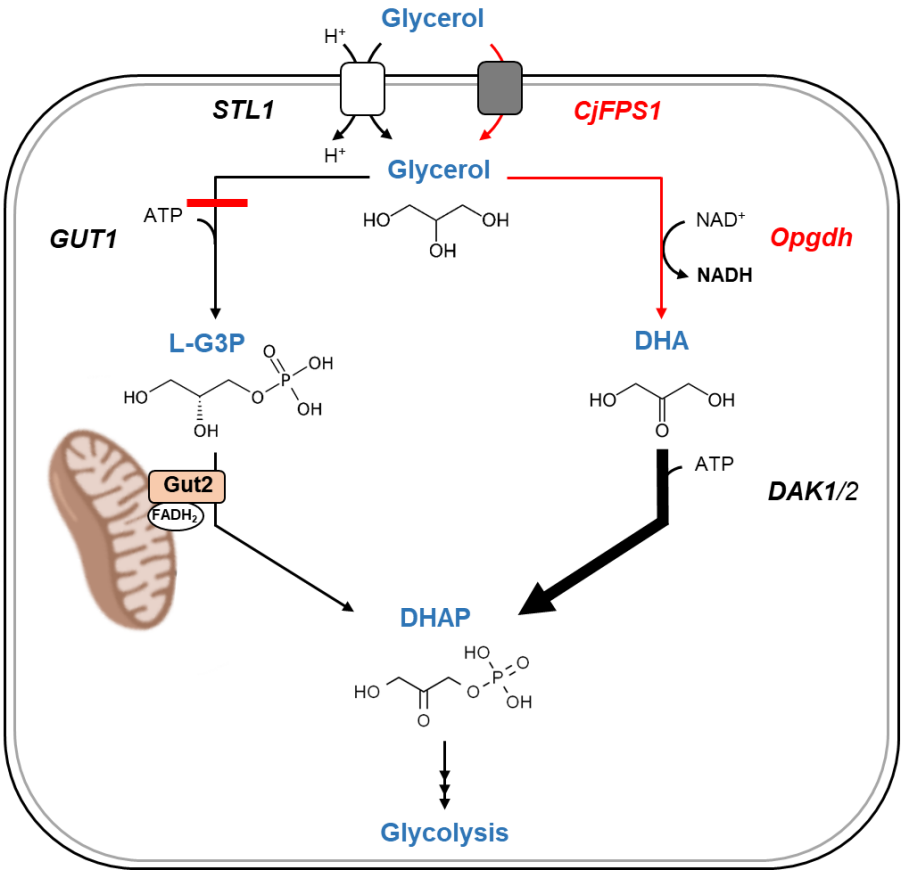


Fig. 4. The rational modifications performed to replace the L-G3P pathway by a NAD$^+$-dependent DHA pathway for glycerol utilization. Black arrows indicate native enzyme activities. Red arrows indicate enzymatic steps that were established by the expression of heterologous genes. The thick black arrow indicates increased *DAK* activity achieved by the overexpression of *DAK1*. The red bar indicates the abolishment of Gut1 enzyme activity by the deletion of *GUT1*. Enzymes/transport proteins and genes: *STL1*, homologous glycerol symporter; *CjFPS1*, heterologous aquaglycerolporin from *C. jadinii*; *GUT1*, homologous glycerol kinase; *GUT2*, homologous mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase; *Opgdh*, heterologous (NAD$^+$-dependent) glycerol dehydrogenase from *O. parapolymorpha*; *DAK1/2*, homologous dihydroxyacetone kinases. Abbreviations: L-G3P - L-glycerol 3-phosphate; DHA - dihydroxyacetone; DHAP - dihydroxyacetone phosphate.

1.4.2 Metabolic engineering to allow the formation of fermentation products (1,2-PDO and ethanol) from glycerol

1,2-propanediol (1,2-PDO) was the first fermentation product generated from synthetic glycerol medium by an engineered *S. cerevisiae* strain with an improved glycerol import (by heterologous expression of *CjFPS1*) and glycerol catabolism via the DHA pathway (Islam et al., 2017). In this study, ca. 45 g/L glycerol was converted to 3.2 g/L 1,2-PDO. Alongside 1,2-PDO, the engineered strain also produced the natural fermentation product ethanol (8 g/L). A recent study has shown that *CjFPS1* was a prerequisite in

combination with the DHA pathway for the observed ethanol formation, and thus resulting in fermentative metabolism of glycerol in *S. cerevisiae* (Asskamp et al., 2019b). Ethanol formation is thought to be related to the fact that *CjFPS1* has significantly increased the rate of glycerol uptake and cytosolic NADH generation in the DHA pathway strain leading to an overflow metabolism. This effect is very similar to the 'Crabtree effect' (Crabtree, 1929) which refers to the occurrence of alcoholic fermentation during growth in glucose-containing medium. This effect occurs when cells are grown in bath cultivation in excess glucose (long-term Crabtree effect) or when cells from a glucose-limited chemostat cultivation are subjected to a short glucose pulse (short-term Crabtree effect) (Pronk et al., 1996).

1.5 Succinic acid (SA) is a product of industrial biotechnology which is in demand and can be produced from glycerol through a fermentative redox-neutral pathway

1.5.1 SA and its applications

SA is a four-carbon dicarboxylic acid with wide industrial applications (Fig. 5). The major part of the current SA production is produced from petroleum-derived maleic anhydride. This occurs via a two-step process: (i) maleic anhydride is first hydrogenated into succinic anhydride, a reaction which is catalysed by metal supported catalysts mainly in organic solvent and then (ii) succinic anyhdride is hydrated to produce SA (Pinazo et al., 2015).

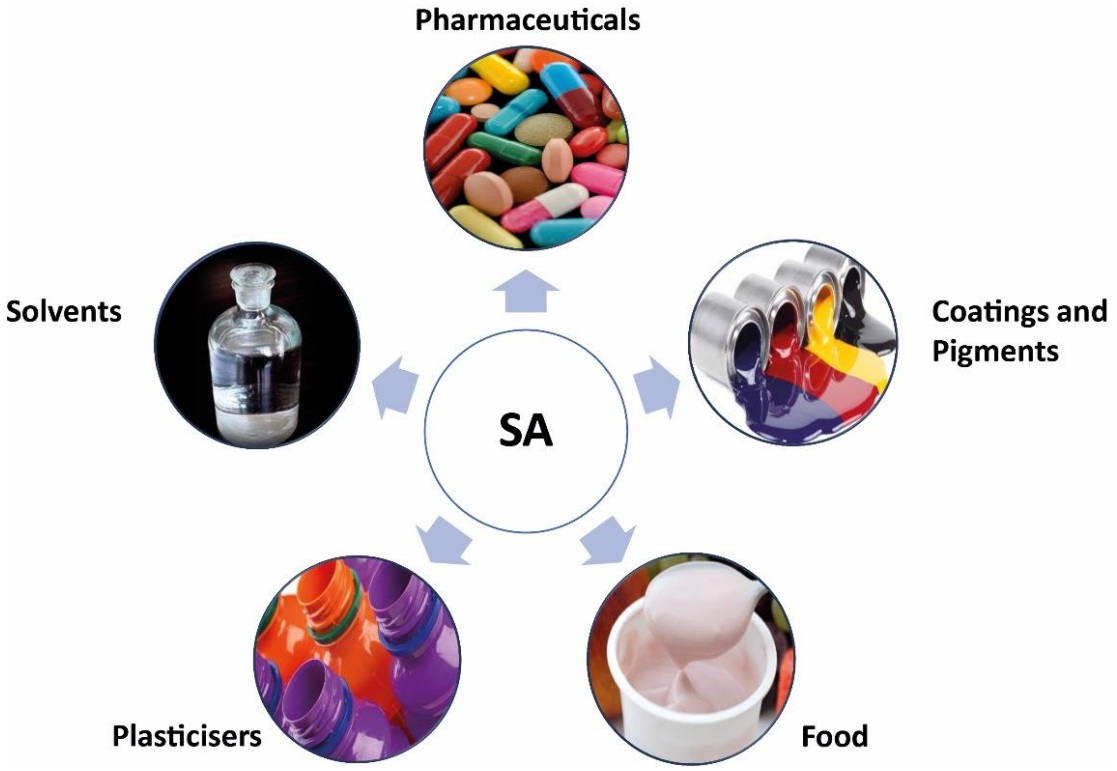


Fig. 5. Application of SA (Ahn et al., 2016).

1.5.2 Microbial SA production

Due to major concerns on climate change and environmental problems, there has been an increased effort to develop sustainable bio-based production processes for chemicals and materials starting from renewable resources. This includes the production of SA, which is regarded as one of the five most important bio-based platform chemicals due to its wide applications as discussed in **chapter 1.5.1**. As an intermediate of the TCA cycle, SA is a ubiquitous metabolite in virtually all organisms. Some organisms even produce significant amounts of SA by nature (Ahn et al., 2016). Extensive research has been carried out both in an academic and industrial setting, which led to the development of several cost-effective processes in both bacteria and yeast for SA production from renewable sources (Ahn et al., 2016). In fact, several plants for generation of SA using either bacteria or yeast as a host were established by companies such as Succinity, Michigan Biotechnology Institute, Myriant, Bioamber and Reverdia. However, currently biobased production of SA is not competitive anymore with the SA

generated from petroleum derived maleic anhydride due to the drastic decrease in price of petroleum in the last few years (McCoy, 2019). As a result, the plants run by Succinity, Myriant and Bioamber were closed in the last two years. Even though these companies don't invest anymore in production of SA from renewable carbon sources, a lot of research is still on going on an academic level. This valuable research might become interesting for industry once the price for petroleum increases again.

1.5.2.1 SA production in bacteria

Great efforts have been made to produce SA using different bacteria including *Mannheimia succiniciproducens*, *Basfia succiniciproducens*, *Actinobacillus succinogenes, E. coli* and *Corynebacterium glutamicum* (Ahn et al., 2016). In contrast to *E. coli* and *C. glutamicum,* rumen bacteria such as *M. succiniciproducens*, *B. succiniciproducens*, and *A. succinogenes* produce SA as a major fermentation product of their natural metabolism. A disadvantage of using these wild-type facultative anaerobic, gram-negative bacteria for SA formation is the production of metabolic by-products, such as formic, acetic, and lactic acids, which reduces the SA yield (Ahn et al., 2016). Therefore, they have been extensively engineered to improve their performance by reducing the formation of metabolic by-products. For example, an engineered *M. succiniciproducens* has been reported to produce 90.68 g/L of SA, with yield and overall productivity of 1.15 mol/mol glucose and 3.49 g/L/h, respectively (Choi et al., 2016). *B. succiniciproducens* has also been rationally engineered for SA production by deletion of pyruvate-formate lyase and lactate dehydrogenase. The double deletion mutant strain revealed a SA yield of 1.08 mol/mol glucose, corresponding to a 45 % improvement over the wild-type strain (Becker et al., 2013). Another study showed that a metabolically engineered *A. succinogenes* strain can produce 105.8 g/L of SA with a

productivity and yield of 105.8 g/L/h and 1.22 mol/mol glucose, respectively (Guettler et al., 1996). In addition, it has been reported that an engineered *C. glutamicum* resulted in the production of 109 g/L of SA with a yield and overall productivity of 1.32 mol/mol glucose and 1.32 g/L/h (Zhu et al., 2014). In engineered *E. coli*, yields of up to 1.6 mol/mol have already been demonstrated (Jantama et al, 2008a; Jantama et al., 2008b).

Although the above-mentioned bacterial species are/have been used for commercial production of bio-based SA, their use as production hosts is not optimal since they show low tolerance towards acidity and hence are unable to grow effectively at low pH (Ahn et al., 2016). Therefore, large quantities of neutralizing compounds (e.g. calcium carbonate) need to be added during the fermentation process to maintain a neutral environment. Subsequently, the respective salt (e.g. calcium succinate) rather than the desired SA (in its protonated form) is secreted by the cells. Afterwards, the succinate is converted to the free SA by a cost-intensive acidification process (i.e. addition of sulphuric acid) (Jansen & van Gulik, 2014). Gypsum, i.e. calcium sulphite dehydrate, is formed as a by-product with little commercial value. The gypsum has to be separated from the desired product SA using a cost intensive precipitation process. Therefore, other hosts which show higher tolerance towards low pH were also considered for SA production to reduce the costs of the downstream process.

1.5.2.2 SA production in yeast

The yeast *P. kudriavzevii* (*Issatchenkia orientalis*) and *S. cerevisiae* do not naturally produce SA as a major fermentation product. However, unlike bacterial SA producers, yeast is highly tolerant to low pH values and more robust towards phage infection, making it superior for SA production (Ahn et al., 2016). Production of SA at low pH values is preferred because it markedly reduces the demand for alkaline neutralizers and allows

to generate SA in the acid form. As a result, the above-mentioned formation of high amounts of gypsum can be eliminated in the downstream process. These advantages were the main drive behind establishing the commercial bio-based SA production in the yeast *P. kudriavzevii* (*Issatchenkia orientalis*) and *S. cerevisiae* by Bioamber and Reverdia, respectively.

1.5.3 Previous metabolic engineering strategies leading to SA production from glucose in *S. cerevisiae*

As *S. cerevisiae* does not naturally produce SA at high levels, extensive metabolic engineering including cofactor engineering and rerouting of metabolism is necessary to increase SA production. The pathways leading to the generation of SA in *S. cerevisiae* are the glyoxylate cycle, the oxidative and the reductive branch of the TCA cycle (Fig. 6), all of which have been already engineered for improvement of SA production from glucose (Table 1). Compared to the other two routes, the reductive branch of the TCA cycle allows the highest possible maximum theoretical yield (1.71 mol/mol glucose), since it is accompanied by the net fixation of carbon dioxide (Beauprez et al., 2010).

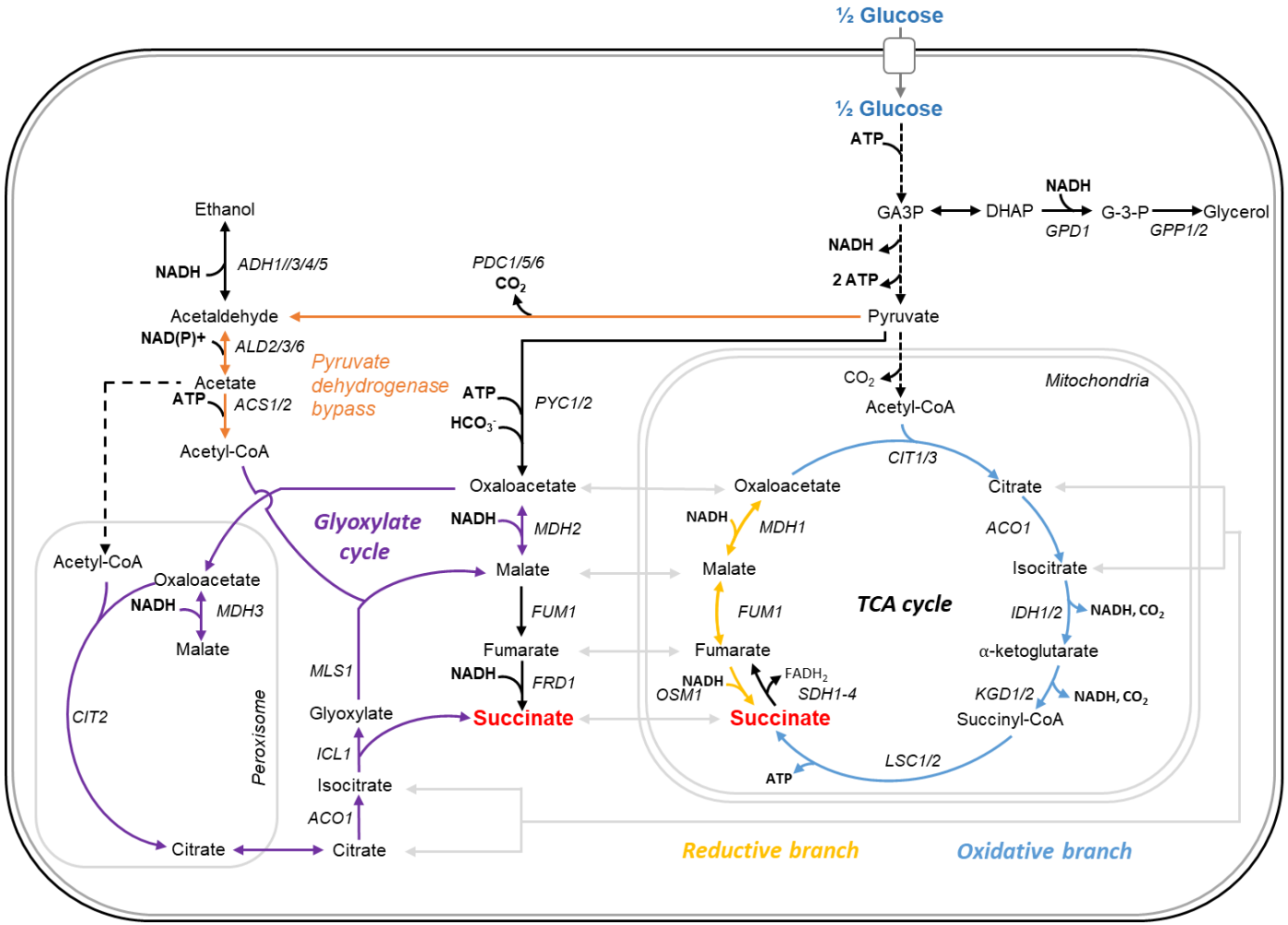


Fig. 6. Pathways leading to generation of SA in *S. cerevisiae*. Abbreviations. DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde-3-phosphate, *GPD1*: glycerol 3-phosphate dehydrogenase, *GPP1/2*: glycerol-3-phosphate phosphatase, *CIT1/2/3*: citrate synthase; *ACO1*: aconitase, *IDH1/2*: isocitrate dehydrogenase, *KGD1/2:* α-ketoglutarate dehydrogenase, *LSC1/2*: succinyl-CoA ligase, *SDH1/2/3/4*: succinate dehydrogenase, *OSM1/FRD1*, fumarate reductase, *FUM1*: fumarase, *MDH1*/*MDH2*/*MDH3*: malate dehydrogenase, *PYC1/2*: pyruvate carboxylase, *PDC1/5/6*: pyruvate decarboxylase, *ADH1/3/4/5*: alcohol dehydrogenase, *ALD2/3/6*: aldehyde dehydrogenase, *ACS1/2*: acetyl-coA synthetase, *ICL1*: isocitrate lyase, *MLS1*: malate synthase.

1.5.3.1 Glyoxylate cycle

The glyoxylate cycle, also called glyoxylate shunt, is essential for growth on two-carbon compounds such as ethanol and acetate (Kunze et al., 2006). The cycle is comprised of many of the same reactions as the TCA cycle, but it does not include the two decarboxylation reactions, i.e. *IDH1/2* and *KGD1/2* (Fig. 6). Subsequently, the two-carbon substrates, which enter the cycle as acetyl-CoA, are converted to four-carbon compounds, which in turn can be further metabolized to create sugars and other essential organic compounds. It can therefore be considered as the anaplerotic route in the absence of C3 compounds. Although many of the reactions of the glyoxlyate and TCA cycle are identical, but many of them are catalysed by different isoenzymes in different cellular compartments. In fact, the glyoxylate cycle occurs in the peroxisome and cytoplasm, while the TCA cycle occurs in the mitochondria (Kunze et al., 2006).

Raab et al. (2010) engineered the glyoxylate cycle in *S. cerevisiae* for SA production. First, the α-ketoglutarate formation has been hindered by deleting the two mitochondrial isocitrate dehydrogenase isozymes, encoded by *IDH1* and *IDH*2 (Fig. 6). To this end, glutamate that is essential for cell growth could not be synthetized. Therefore, additional supplementation of glutamate was required to overcome the glutamate auxotrophy. Additionally, two of the four subunits of succinate dehydrogenase, encoded by *SDH1* and *SDH2* (Fig. 6) were also disrupted resulting in a strain with up to four deletions *(sdh1*Δ *sdh2*Δ *idh1*Δ *idh2*Δ) to redirect the carbon flux from the oxidative branch of the TCA cycle into the glyoxylate cycle and allow to accumulate SA as an end-product. The cultivation conditions and achieved yield and titre as well as biomass specific SA production rate are shown in Table 1.

1.5.3.2 Oxidative branch of the TCA cycle

The generation of SA through the oxidative TCA pathway in the mitochondria involves the condensation of oxaloacetate and acetyl-coenzyme A (acetyl-CoA) to citric acid by citrate synthase (encoded by *CIT1/3*), followed by its oxidation to SA via aconitase (encoded by *ACO1*), isocitrate dehydrogenase (encoded by *IDH1/2*), α-ketoglutarate dehydrogenase (encoded by *KDG1/2*), and succinyl-CoA synthetase (encoded by *LSC1/2*) (Fig. 6). This pathway has also been previously engineered for SA production (Table 1). However, the main disadvantage of using this pathway is the presence of succinate dehydrogenase (Fig. 6) because it oxidizes SA to fumaric acid, and thereby reduces the SA yield. This enzyme is composed of four subunits encoded by *SDH1*, *SDH2*, *SDH3,* and *SDH4*. As a result, Ito et al., (2014) disrupted *SDH1* and *SDH2* genes to enhance SA production via the oxidative branch of the TCA cycle. The resulting *sdh1*Δ *sdh2*Δ *S. cerevisiae* strain produced high levels of ethanol, and therefore, the effect of eliminating the ethanol biosynthesis pathway on SA production was investigated by deleting five alcohol dehydrogenase genes (*ADH1-ADH5*). These genetic modifications increased SA production and glycerol production while the cell growth was reduced. The resulting strain was further analysed with metabolic profiling which revealed that SA accumulated inside the cell. Henceforth, the *Schizosaccharomyces pombe* dicarboxylic acid transporter (exports malic acid and SA) was introduced, which further increased the production of SA. However, the final titer and yield achieved were lower compared to that obtained by Raab et al. (2000) as shown Table 1.

1.5.3.3 Reductive branch of the TCA cycle

The reductive branch of the TCA cycle consists of pyruvate carboxylation, oxaloacetate reduction, conversion of malic acid to fumaric acid, and fumaric acid reduction (Fig. 6).

The maximum theoretical SA yield via this pathway on glucose is 1.71 mol/mol glucose. Achieving high SA yields via this pathway from glucose is challenging because the generation of 2 mol of SA consumes 4 mol of NADH while only 2 mol of NADH are generated (Yan et al., 2014). Using glycerol as a source of carbon could solve this problem. As described in **chapter 1.2.1**, glycerol could release double the amount of NADH per carbon atom (compared to glucose) provided that the DHA pathway is used for catabolism (see **chapter 1.4.1).** These NADH molecules can be used to produce SA via the reductive branch of the TCA cycle, thus resulting in a fermentative redox-neutral pathway with a maximum theoretical yield of 2 mol/mol glycerol. Interestingly, the production of SA in *S. cerevisiae* using glycerol (and carbon dioxide) as sole carbon source has not been investigated so far.

In order to achieve high-yield SA production through the reductive branch of the TCA cycle from glucose, previous metabolic engineering strategies have focused on eliminating pathways that compete with the SA pathway for the available cytosolic NADH. One of these strategies involved the use of a pyruvate decarboxylase (pdc)-deficient *S. cerevisiae* strain (TAM strain) (Van Maris et al., 2004) for the establishment of the enzymatic steps of the reductive branch of the TCA cycle in the cytosol. As a result, the NADH produced from glycolysis was used to produce SA instead of ethanol as the fermentation product (Yan et al., 2014). The genetic modifications for SA accumulation included the overexpression of *PYC2*, which encodes for one of the isoenzymes of *S. cerevisiae* pyruvate carboxylase and a cytosolically retargeted form of malate dehydrogenase, encoded by *MDH3*. The native fumarase, encoded by *FUM1*, which mainly converts fumaric acid to malic acid in the TCA pathway was deleted to reduce the flux from fumaric acid to malic acid. Instead, the *E. coli* fumarate hydratase, encoded

by *FumC,* together with *S. cerevisiae* fumarate reductase, encoded by *FRD1*, were overexpressed in the cytosol (Fig. 6). Moreover, glycerol-3-phosphate dehydrogenase, encoded by *GPD1*, which controls glycerol synthesis (consuming cytosolic NADH) under aerobic condition was deleted to avoid spilling reduction potential and carbon reserves (Fig. 6). This engineered strain was cultured in glucose-grown shake flask cultures supplemented with $CaCO_3$, biotin, and urea, and produced 10 g/L SA with a yield of 0.32 mol/mol glucose (Yan et al., 2014). Afterwards, the strain was further characterized in a bioreactor. The values for the achieved titre and yield as well as the biomass-specific SA production rate are shown in Table 1.

The commercial bio-based production of SA using glucose as the sole carbon source was established by Reverdia (Van De Graaf et al., 2015, Patent No. US20150057425A1, Priority date 24.11.2009, Table 1). As shown in Table 1, this company has envisaged the cytosolic reductive branch of the TCA cycle for SA production. Except for $PYC2_{OE}$ and *MDH3-R*, all the other employed genetic modifications were different from that of Yan et al. (2014). Jansen et al. (2017, Patent No. US9624514B2, Priority date 1.07.2011) have demonstrated that the expression of dicarboxylic acid transporters from *Aspergillus niger* (encoded by *An*DCT-02) or *SpMAE1* were crucial for obtaining high SA titres and yields (Table 1). These transporters obviously allow an efficient export of the overproduced SA into the medium.

Table 1. Previously published studies aiming at the production of SA in *S. cerevisiae* from glucose as the carbon source. Abbreviations: *ADH1-5*, alcohol dehydrogenase; *SDH1/2*, succinate dehydrogenase; *PDC1/5/6*, pyruvate decarboxylase; *GPD1*; glycerol-3-phosphate dehydrogenase; *FUM1*; fumarase; *PYC2*, pyruvate carboxylase; *MDH3*R, peroxisomal malate dehydrogenase retargeted to the cytosol; *EcfumC*, fumarase from *Escherichia coli*; *FRD1*, fumarate reductase; *MspckA*, PEP carboxykinase from *M. succiniproducens*; *GSH1*, gamma glutamylcysteine synthetase*; CYS3*, cystathionine gamma-lyase*; GLR1,* glutathione oxidoreductase*; RofumR*, fumarase from *Rhizopus oryzae*; *TbFRD*g-*R*, peroxisomal fumarate reductase from *Trypanosoma brucei* retargeted to the cytosol; *SpMAE1*, dicarboxylic acid transporter from *S. pombe*; *An*DCT-02, dicarboxylic acid transporter from *A. niger*; *IDH1/2*, isocitrate dehydrogenase. OE, overexpression; Δ*,* deletion; N/A, not applicable.

	Raab et al., 2010	**Ito et al., 2014**	**Yan et al., 2014**	**Van De Graaf et al., 2015; Jansen et al., 2017**
***S. cerevisiae* strain**	AH22ura3	S149	CEN.PK113-7D	CEN.PK113-7D
Genetic modifications	*sdh1*Δ, *sdh2*Δ, *idh1*Δ, *idp2*Δ	*adh1-5*Δ, *sdh1*Δ, *sdh2*Δ	*pdc1*Δ, *pdc5*Δ, *pdc6*Δ, *gpd1*Δ, *fum1*Δ, $PYC2_{OE}$, *MDH3*-R_{OE}, *EcfumC*, $FRD1_{OE}$	*adh1/2*Δ, *gpd1*Δ, *MspckA*, $GSH1_{OE}$, $CYS3_{OE}$, *GLR1*, $PYC2_{OE}$, *MDH3*-R_{OE}, *RofumR*, *TbFRDg*-R_{OE}
Envisaged metabolic route	glyoxylate cycle	oxidative branch of the TCA cycle	cytosolic reductive branch of the TCA cycle	cytosolic reductive branch of the TCA cycle
Expression of a heterologous transporter	No	*SpMAE1*	No	*SpMAE1*/ *An*DCT-02
Cultivated in	Shake flasks	Shake flasks	Aerobic fed-batch	Aerobic fed-batch, dual phase
Temperature	30 °C	30 °C	30 °C	30 °C
Dissolved oxygen	N/A	N/A	>30 %	First phase 20 %
pH	N/A	N/A	3.8	3.0
Media	Defined	Defined	Defined	Defined
P_{co2} (Bar)	N/A	N/A	0.10	0.50
Initial glucose (mM)	279	111	555.55	N/A
Cell density (OD_{600}) at measured titer	20	20	48	N/A
Titer (g/L)	3.62	0.43	13	43
Yield (mol succinate/mol glucose)	0.11	0.03	0.21	N/A
Cultivation time (h)	168	120	120	N/A
Biomass specific SA production rate (mmol/DCW/h)	0.07	0.01	0.05	N/A

1.5.4 Considerations with regard to the transport and energetics of dicarboxylic acid transport

Dicarboxylic acids are charged molecules and therefore, cannot diffuse easily through the membranes. The equilibrium between the three forms of dicarboxylic acids, i.e. the undissociated acid (H_2A) and dissociated anions (HA^- and A^{2-}) is pH dependent (Abbott et al, 2009; Jamalzadeh, 2013; Shah et al., 2016). At low pH, the undissociated form predominates and this is favourable for the recovery of organic acids during downstream processing in the course of a production process. Still, the undissociated form has a high membrane permeability and therefore, can re-enter the cell via passive diffusion. Afterwards, the near neutral pH of the *S. cerevisiae* cytosol leads to dissociation of the previously undissociated form, resulting in dissociated form which cannot readily diffuse across the cell membrane. The dissociated form can only be exported via a specific transporter.

Membrane transporters more or less specific for SA have been described in several fungal species such as *A. niger* and *S. pombe* (Jansen et al., 2017) and bacterial species such as in *C. glutamicum* (Fukui et al., 2019; Zhu et al., 2014) and *E. coli* (Chen et al., 2014). In *S. cerevisiae*, no specific transport mechanism responsible for the export of SA in has been identified so far. It has been suggested that in *S. cerevisiae* SA might also be transported via a dicarboxylate transport protein localized in the protein membrane via a sodium-dependent mechanism (Aliverdieva et al, 2006). Such a transport system might explain why Raab et al. (2010) and Yan et al. (2014) achieved relatively high SA production even without expressing a heterologous dicarboxylic acid transporter (Table 1). Although a native dicarboxylic acid transporter might be present in *S. cerevisiae*, it is less efficient than the tested heterologous dicarboxylic acid

transporters. In fact, the heterologous expression of *SpMAE1* and/or *An*DCT-02 (Ito et al., 2014; Jansen et al., 2017, Table 1) improved the SA titre in the respective strains engineered for SA production.

Export of SA from cells by membrane transporters costs ATP because the transport of divalent species requires the export of protons to maintain pH and charge gradient. This can, especially at low pH, have a huge negative impact on the net ATP generation in cells engineered for strong production of SA from the substrate and consequently on the maximum product titres and yields of SA (or other dicarboxylic acids) produced in biotechnological processes.

Chapter 2. SCOPE OF THE THESIS

The goal of this study was to construct a *S. cerevisiae* strain that is able to convert glycerol to SA via a fermentative pathway. In comparison to glucose, the use of glycerol as the sole carbon source allows a higher theoretical yield since the envisaged pathway from the substrate to the product is redox-neutral. Apart from testing a novel approach for SA production, the knowledge gained from this study will improve our understanding of how glycerol is metabolized by *S. cerevisiae* strains equipped with a NADH-generating pathway for glycerol utilization (DHA pathway). This will also facilitate the exploitation of glycerol's reducing power for the production of small molecules other than SA.

As wild-type *S. cerevisiae* strains do not naturally produce SA at sufficiently high levels, extensive metabolic engineering is necessary. Such metabolic engineering endeavor that includes re-routing of central carbon metabolism require comprehensive understanding about the carbon fluxes during growth on glycerol. As SA can be formed via different native metabolic routes in different cellular compartments such as the cytosol, the mitochondria and the peroxisome, it is also important to acquire knowledge about how the relevant pathway intermediates are transported between these compartments. The first part of this thesis is a review about the central carbon catabolism of *S. cerevisiae* during growth on glycerol (**chapter 3.3**). The respective literature survey revealed that the knowledge regarding the *in vivo* fluxes during growth of *S. cerevisiae* on glycerol is far from complete. Although a few previous studies exist that have been performed in glycerol medium, one has to be careful when interpreting the data because the experiments were conducted in complex medium and the addition of supplements can significantly affect growth on glycerol as demonstrated by our lab. Therefore, a number of uncertainties were identified which are highly relevant when it comes to the goal of the current thesis focusing on SA production from glycerol. These

uncertainties include the role of the glyoxylate cycle and the localization of the respective (iso)enzymes as well as mitochondrial transporters and the anaplerotic reactions active on glycerol. The insights summarized in **chapter 3.3** highlight the need to collect novel experimental data from cells growing in synthetic medium glycerol in order to fill the numerous knowledge gaps.

Anaplerotic routes are extremely important for the production of TCA cycle intermediates such as SA. It is also important to consider which of the different possible routes is used because this choice can strongly influence net ATP generation and co-factor balance. After compiling **chapter 3.3**., it remained an open question which anaplerotic route is mainly active during growth in synthetic glycerol medium in wild-type and engineered *S. cerevisiae*. The second part of this thesis (**chapter 3.4**) aimed at delivering an answer to this question. Pyruvate carboxylase and the glyoxylate cycle have been experimentally identified to be the main anaplerotic routes during growth of *S. cerevisiae* on glucose (C6) and ethanol (C2), respectively. In **chapter 3.4**., the importance of the two aforementioned anaplerotic reactions was investigated during growth on glycerol by analyzing the impact of suitable gene deletions on the maximum growth rate. A reverse engineered CEN.PK derivative showing a maximum specific growth rate of 0.14 h^{-1} was used as the baseline strain. The results showed that the pyruvate carboxylase and the glyoxylate cycle can interchangeably act as the main anaplerotic reaction on glycerol. This is in clear contrast to the situation on glucose and ethanol, where only one major route is used.

Chapter 3.5 deals with establishing a redox-balanced pathway from glycerol to SA in the cytosol of *S. cerevisiae*. Compared to the oxidative branch of the TCA pathway and the glyoxylate cycle (in which SA is also an intermediate), the reductive branch of

the TCA pathway is a fermentative pathway because the electrons are transferred to organic metabolic intermediates resulting in SA formation. Fermentative (redox-balanced) metabolism of glycerol to SA is needed to achieve a high product yield. Therefore, we used a strain engineered for catabolizing glycerol via the NAD^+-dependent DHA pathway as the starting point for this study. More precisely, the previously constructed strain CEN.PK113-1A *UBR2*$_{CBS}$ *gut1*::*mod2* (referred here to as *UBR2*$_{CBS}$-DHA) with a maximum growth rate (μ_{max}) of 0.26 h^{-1} was selected as the baseline strain. The reductive branch of the TCA pathway (referred to as 'SA module', SA in strain name) was established in the cytosol of the selected baseline strain by targeting the peroxisomal malate dehydrogenase to the cytosol (1); and heterologous cytosolic expression of fumarase (2) and fumarate reductase (3). The export of SA into the culture supernatant was facilitated by the heterologous expression of a gene from *A. niger* encoding a dicarboxylic acid transporter (*An*DCT-02). The resulting strain, *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 produced a maximum SA titre of 10.7 g/L corresponding to a yield of 0.22 ± 0.01 g/g glycerol. Results strongly suggest that the enzymes of the cytosolic `SA module´, the pyruvate dehydrogenase bypass and the endogenous glyoxylate cycle act together in a highly synergistic manner supporting SA overproduction in the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02. Additional deletion of *SDH1* encoding a mitochondrial succinate dehydrogenase subunit increased the maximum SA yield to 0.43 ± 0.02 g/g glycerol in shake flasks cultivations, but the strain showed a severe growth defect after initially showing a biomass accumulation that was even faster than the reference strain.

The study described in **chapter 3.5** provided a baseline strain (*UBR2*$_{CBS}$-DHA-SA-*An*DCT-02) to further force metabolic flux via the 'SA module'. This was the goal of the work described in **chapter 3.6** of this thesis. As the first step, the flux from either PEP or

pyruvate to oxaloacetate was increased by different approaches. For example, overexpression of both *PYC1* and *PYC2* was tested. These genes encode for the isoenzymes of pyruvate carboxylase in *S. cerevisiae*. In addition, overexpression of *PCK1* was tested. This gene encodes for phosphoenolpyruvate (PEP) carboxykinase which is known to reversibly convert PEP to oxaloacetate. Interestingly, each of the three genetic modifications led to a reduction in SA production and an onset of ethanol formation as well as improved biomass formation and glycerol consumption. In particular, *PYC2* overexpression led to the formation of up to 13 g/L of ethanol during the first 72 h of cultivation while the overexpression of *PYC1* and *PCK1* resulted in the formation of up to ~ 6 g/L after 120 h. Several hypotheses are discussed which could explain the ethanol formation in the engineered strains. The results obtained in this chapter demonstrate the complexity of the central carbon metabolism during growth of *S. cerevisiae* on glycerol and reflect our incomplete understanding of the metabolic fluxes during growth on glycerol as discussed in **chapter 3.3.**

Chapter 4 provides an overall discussion of all findings collected throughout the course of this thesis and suggests avenues for future research.

Chapter 3. MANUSCRIPTS

3.1 List of manuscripts

I. Xiberras, J., Klein, M., Nevoigt, E. (2019). Glycerol as a substrate for *Saccharomyces cerevisiae* based bioprocesses – knowledge gaps regarding the central carbon catabolism of this 'non-fermentable' carbon source. *Biotechnology Advances*. *37*(6).

II. Xiberras, J., Klein, M., Prosch, C., Malubhoy, Z., Nevoigt, E. (2019). Anaplerotic reactions active during growth of *Saccharomyces cerevisiae* on glycerol. *FEMS Yeast Research.*

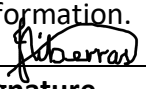
III. Xiberras, J., Klein, M., Nevoigt, E. (2019). An engineered reductive pathway and endogenous central metabolic pathways synergistically enhance succinic acid production using glycerol as carbon source in *Saccharomyces cerevisiae*. *Metabolic Engineering. Submitted.*

IV. Xiberras, J., Klein, M., Nevoigt, E. (2019). Understanding ethanol formation in a *Saccharomyces cerevisiae* strain engineered for succinic acid production from glycerol. *To be submitted.*

3.2 Statement of the author's contribution to the manuscripts

Manuscript	Contribution	Please provide the following information about the article (if applicable)				
		Title	**Co-authors**	**Journal**	**Status (submitted, accepted, published)**	**Date of submission/ acceptance**
I (chapter 3.3)	I reviewed the current literature about *S. cerevisiae* growing on glycerol and identified the knowledge gaps mentioned in the critical review. I wrote the draft of the manuscript.	Glycerol as a substrate for *Saccharomyces cerevisiae* based bioprocesses – knowledge gaps regarding the central carbon catabolism of this 'non-fermentable' carbon source	Mathias Klein Elke Nevoigt	*Biotechnology Advances*	Accepted	26.03.2019
II (chapter 3.4)	I participated in the design of the study and helped in strain construction and quantitative and qualitative growth analysis of the strains. I analyzed the data and wrote the draft of the manuscript.	Anaplerotic reactions active during growth of *Saccharomyces cerevisiae* on glycerol	Mathias Klein Celina Prosch Zahabiya Malubhoy Elke Nevoigt	*FEMS Yeast Research*	Accepted	03.12.2019
III (chapter 3.5)	I participated in the design of the study and carried out the strain construction, physiological characterization of the strains and HPLC metabolite analysis. I analyzed the data and wrote the draft of the manuscript.	An engineered reductive pathway and endogenous central metabolic pathways synergistically enhance succinic acid production using glycerol as carbon source in *Saccharomyces cerevisiae*	Mathias Klein Elke Nevoigt	*Metabolic Engineering*	Submitted	12.07.2019
IV (chapter 3.6)	I participated in the design of the study and did all wet lab work including strain construction, physiological characterization of the strains and HPLC metabolite analysis. I analyzed the data and wrote the draft of the manuscript.	Understanding ethanol formation in a *Saccharomyces cerevisiae* strain engineered for succinic acid production from glycerol	Mathias Klein Elke Nevoigt	--	To be submitted	--

In lieu of oath, I herewith declare that the information above is correct and that all co-authors and my PhD Advisor agree with the given information.



Bremen, 11.02.2020

Signature **Place, Date**

3.3 Manuscript I

Glycerol as a substrate for *Saccharomyces cerevisiae* based bioprocesses – knowledge gaps regarding the central carbon catabolism of this 'non-fermentable' carbon source

Joeline Xiberras[1], Mathias Klein[1] and Elke Nevoigt[1*]

[1] Department of Life Sciences and Chemistry, Jacobs University Bremen gGmbH, Campus Ring 1, 28759, Bremen, Germany

* Corresponding author:
E-mail: e.nevoigt@jacobs-university.de

Short title: Glycerol catabolism in *Saccharomyces cerevisiae*

Abstract

Glycerol is an interesting alternative carbon source in industrial bioprocesses due to its higher degree of reduction per carbon atom compared to sugars. During the last few years, significant progress has been made in improving the well-known industrial platform organism *Saccharomyces cerevisiae* with regard to its glycerol utilization capability, particularly in synthetic medium. This provided a basis for future metabolic engineering focusing on the production of valuable chemicals from glycerol. However, profound knowledge about the central carbon catabolism in synthetic glycerol medium is a prerequisite for such incentives. As a matter of fact, the current assumptions about the actual *in vivo* fluxes active on glycerol as the sole carbon source have mainly been based on *omics* data collected in complex media or were even deduced from studies with other non-fermentable carbon sources, such as ethanol or acetate. A number of uncertainties have been identified which particularly regard the role of the glyoxylate cycle, the subcellular localisation of the respective enzymes, the contributions of mitochondrial transporters and the active anaplerotic reactions under these conditions. The review scrutinizes the current knowledge, highlights the necessity to collect novel experimental data using cells growing in synthetic glycerol medium and summarizes the current state of the art with regard to the production of valuable fermentation products from a carbon source that has been considered so far as 'non-fermentable' for the yeast *S. cerevisiae*.

Keywords: Yeast, *Saccharomyces cerevisiae*, Glycerol, Carbon source, Catabolism, Glyoxylate cycle, Anaplerotic reactions, Mitochondrial transporters

1. Introduction

The yeast *Saccharomyces cerevisiae* has become a popular production organism in industrial biotechnology for several obvious reasons. First of all, it is a simple, fast growing and single-cell eukaryotic model organism, already having a relatively long history in large-scale industrial use. Moreover, *S. cerevisiae* is robust with regard to low pH values and high alcohol concentrations and many industrial applications profit from these characteristics. In contrast to microbial fermentations using bacteria, yeast cultures are not vulnerable to bacteriophage infection. However, the property that particularly boosted the popularity of *S. cerevisiae* in industrial biotechnology in the last decades is its highly efficient homologous recombination, which considerably facilitates the stable genomic incorporation of multiple expression cassettes as often required for extensive metabolic engineering endeavors.

As virtually every natural microorganism, wild-type *S. cerevisiae* also has its weaknesses when considered as a platform organism for industrial biotechnology. Its relatively narrow substrate spectrum is limiting in the view of using renewable feedstocks, which contain a broad spectrum of potential carbon sources. Apart from hexose sugars, wild-type *S. cerevisiae* cannot use many other constituents of polymers present in 'energy crops' or agricultural waste streams, such as pentose sugars or sugar acids. For this reason, huge efforts have been undertaken to extend the substrate spectrum of baker's yeast by metabolic engineering. Particularly *S. cerevisiae* strains carrying pathways for pentose sugar utilization have been developed with great success (Kim et al., 2013; Kwak and Jin, 2017; Lane et al., 2018). Another carbon source that can be obtained from renewable resources, especially from raw materials rich in oils and fats, is glycerol. This valuable substrate has been neglected as a carbon source for *S. cerevisiae*-based

bioprocesses so far, which can be attributed to its rather inefficient utilization by most commonly used wild-type strains of this yeast species.

A major advantage of using glycerol as a carbon source in industrial bioprocesses is its higher degree of reduction per carbon compared to sugars (4.7 versus 4.0), which is accompanied by higher maximum theoretical yields of reduced target molecules (Clomburg and Gonzalez, 2013). Furthermore, the additional reducing power of glycerol can support the (co-)fermentation of compounds more oxidized than glucose, such as acetic acid (de Bont et al., 2013). Currently, the major part of the available glycerol results from biodiesel production, where it is generated as an inevitable by-product. In fact, the remarkable growth of the biodiesel industry about 15 years ago initiated a huge interest in glycerol valorization in order to improve the economics of the biodiesel production process (Yazdani and Gonzalez, 2007). Several biotechnological processes (including commercial ones) based on glycerol as raw material and microorganisms other than *S. cerevisiae* have already been developed (Chen and Liu, 2016).

The fact that *S. cerevisiae* barely grows on glycerol as a carbon source holds for media in which growth-supporting supplements, such as amino acids and nucleic bases, are omitted (Swinnen et al., 2013). Indeed, many relevant industrial and laboratory strains do not grow at all under these conditions. However, when considering the industrial conversion of glycerol into products of relatively low market price, the addition of complex supplements is not economically viable. Therefore, our group has scrutinized the growth of 52 *S. cerevisiae* strains with regard to growth in synthetic glycerol medium without any supplements. Interestingly, a few wild-type isolates were identified that are able to grow up to a maximum specific growth rate of ~0.15 h^{-1} (Swinnen et al., 2013). A haploid meiotic segregant of one strain showing superior growth on glycerol was

characterized in more detail (Swinnen et al., 2013). This segregant referred to as CBS 6412-13A has been used as a major baseline strain in our metabolic engineering endeavors for exploiting glycerol's reducing power for the production of valuable chemicals (see section 6). Apart from using natural glycerol-utilizing wild-type isolates, it has been relatively straightforward to apply adaptive laboratory evolution (ALE) for the establishment of glycerol utilization in strains unable to grow in synthetic glycerol medium by nature. Indeed, several virtually non-growing *S. cerevisiae* strains have been evolved for this phenotype so far (Ho et al., 2017; Merico et al., 2011; Ochoa-Estopier et al., 2011; Strucko et al., 2018). The identification of the genetic determinants underlying natural and artificial diversity with respect to the glycerol growth phenotype within the species *S. cerevisiae* has also provided the basis for reverse engineering approaches. In this methodology, the crucial genetic determinants identified in one strain with a superior phenotype are transferred to another strain showing an inferior phenotype as comprehensively reviewed by Oud et al. (2012). Reverse engineering was successfully applied for establishing growth on glycerol in the popular (non-growing) laboratory strain CEN.PK via different strategies (Ho et al., 2017; Strucko et al., 2018; Swinnen et al., 2016).

As reviewed by Klein et al. (2017), *S. cerevisiae* naturally catabolizes glycerol via the so-called L-G3P pathway. This pathway is composed of three proteins: one transporter (a glycerol/H^+ symporter) encoded by *STL1*, a glycerol kinase encoded by *GUT1* and a L-glycerol-3-phosphate dehydrogenase encoded by *GUT2* (Ferreira et al., 2005; Klein et al., 2016a; Ronnow and Kielland-Brandt, 1993; Sprague and Cronan, 1977; Swinnen et al., 2013). Gut2 is located at the outer face of the inner mitochondrial membrane. By the action of this FAD-dependent enzyme, the electrons originating from glycerol

oxidation end up in the ubiquinone pool of the respiratory chain and are finally transferred to the inorganic electron acceptor oxygen. The product of this reaction is dihydroxyacetone phosphate (DHAP) that is further channeled into the central carbon metabolism (glycolysis and gluconeogenesis) as depicted in Fig. 1.

For any metabolic engineering endeavor aiming at small molecule production from glycerol, a profound knowledge about the actual metabolic fluxes active in synthetic glycerol medium and their regulation is crucial. As discussed later, glycerol belongs to the carbon sources that have been considered non-fermentable for *S. cerevisiae*. Comprehensive reviews about the central carbon catabolism during the utilization of non-fermentable carbon sources by *S. cerevisiae* have been provided by Schüller (2003) and Turcotte et al. (2010). However, a closer look reveals that the majority of our assumptions about the fluxes results from transcriptome studies, a fact that has to be considered with caution. Moreover, published experimental data are rare when it comes to the utilization of true synthetic glycerol media (i.e. media without amino acid or nucleic base supplements). Even studies that have focused on complex glycerol media are relatively low in number compared to other non-fermentable carbon sources such as ethanol, acetate or oleate. Several fundamental studies have used mixtures of ethanol and glycerol, and a few crucial experimental data have not been collected at all on glycerol as the sole carbon source. The many uncertainties we came across when searching for details regarding the central carbon catabolism of glycerol in *S. cerevisiae* motivated us to write this critical review. We would like to stimulate scientific discussion as well as basic research to clarify the open questions.

We first critically discuss the general assumptions about the central carbon catabolism on non-fermentable carbon sources (including glycerol) which are mainly based on

transcriptome and proteome studies. Afterwards, we particularly scrutinize the role of the glyoxylate cycle during growth on glycerol and what is known about the localisation of several enzymes contributing to the activity of this pathway. Moreover, we address intriguing experimental results regarding mitochondrial transporters and anaplerotic reactions active on glycerol. The last section is devoted to the potential biotechnological exploitation of glycerol's reducing power for the production of small molecules including promising results obtained during our own recent research activities.

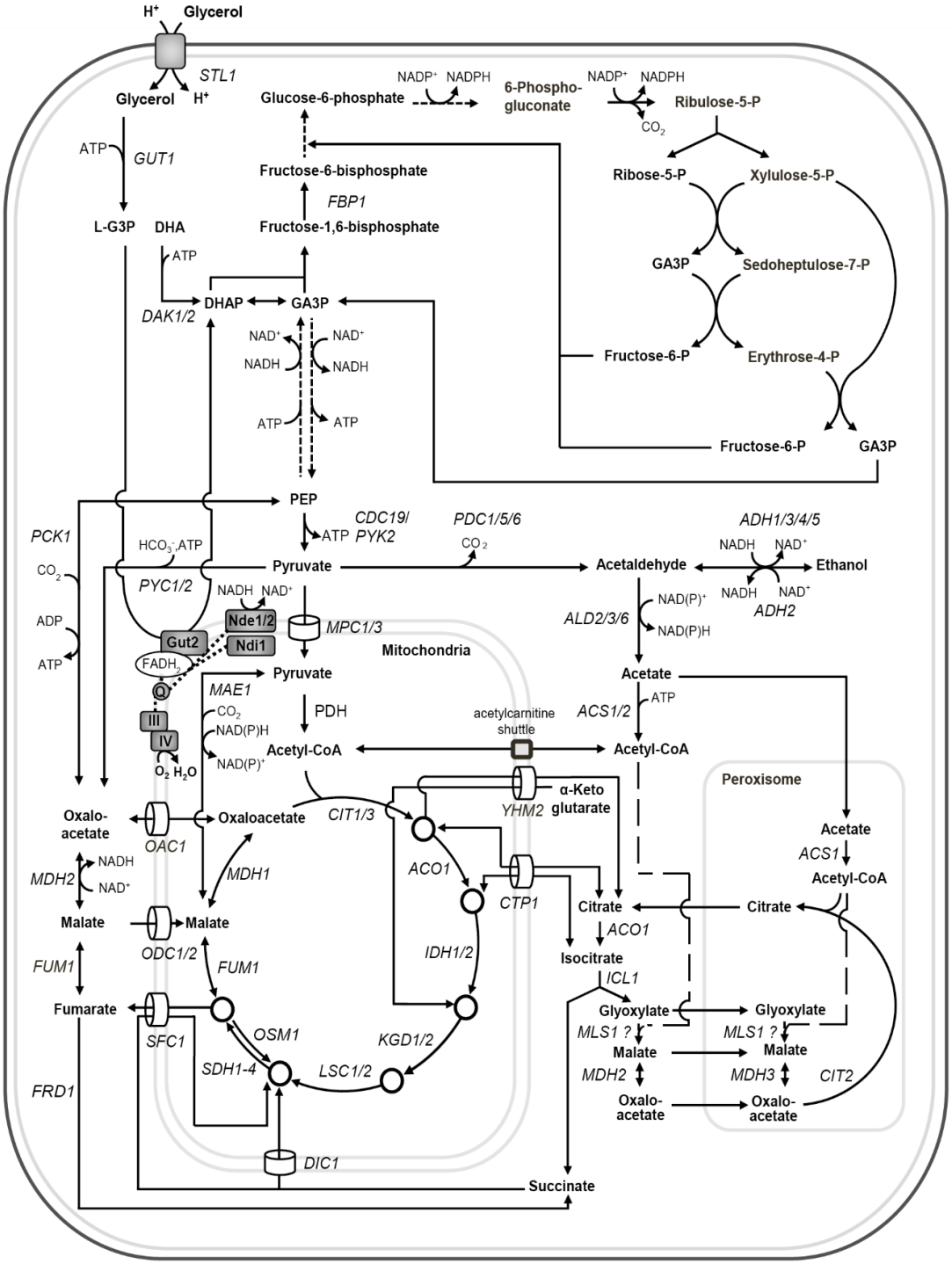


Fig. 1. Central metabolic pathways in *S. cerevisiae* during growth on glycerol. The two possible localizations of Mls1 are shown with long dash lines and question mark. Abbreviations: L-G3P: L-glycerol-3-phosphate, DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde-3-phosphate, PEP: phosphoenolpyruvate, P: phosphate, *STL1*: glycerol/H^+ symporter, *GUT1*: glycerol kinase, Gut2: FAD-dependent glycerol-3-phosphate dehydrogenase, *DAK1/2*: dihydroxyacetone kinase, *FBP1*: fructose-1,6-bisphosphatase, *CDC19*/*PYK2*: pyruvate kinase, *PCK1*: phosphoenolpyruvate carboxykinase, *PYC1/2*: pyruvate carboxylase, *MDH1*/*MDH2*/*MDH3*: malate dehydrogenase, *FUM1*: fumarase, PDH: pyruvate dehydrogenase complex, *MAE1*: malic enzyme, *CIT1/2/3*: citrate synthase, *ACO1*: aconitase, *IDH1/2*: isocitrate dehydrogenase, *KGD1/2:* α-ketoglutarate dehydrogenase, *LSC1/2*: succinyl-CoA ligase, *SDH1/2/3/4*: succinate dehydrogenase, *ICL1*: isocitrate lyase, *MLS1*: malate synthase, *ACS1/2*: acetyl-coA synthetase, *PDC1/5/6*: pyruvate decarboxylase, *ADH1/3/4/5*: alcohol dehydrogenase, *ADH2*: alcohol dehydrogenase, *ALD2/3/6*: aldehyde dehydrogenase, *MPC1/3*: pyruvate carrier, *YHM2*: citrate and ketoglutarate transporter, *CTP1*: citrate transporter, *DIC1*: dicarboxylate

transporter, *SFC1*: succinate-fumarate transporter, *ODC1/2*: oxodicarboxylate carriers, *OAC1*: oxaloacetate and sulfate transporter, Nde1/2: mitochondrial external NADH dehydrogenase, Ndi1: NADH:ubiquinone oxidoreductase, Q: ubiquinone, III: complex III, IV: complex IV.

2. Assumptions regarding the central carbon fluxes on non-fermentable carbon sources including glycerol

In lack of any experimental data with regard to a ^{13}C flux analysis and comprehensive *in vitro* enzyme activity measurements for *S. cerevisiae* cells exponentially growing on glycerol as the sole source of carbon, to date any assumption about the active metabolic fluxes on glycerol can only be considered hypothetical. According to previous reviews regarding the metabolism of *S. cerevisiae* on non-fermentable carbon sources, there has been a consensus that the TCA cycle, oxidative phosphorylation, the glyoxylate cycle and gluconeogenesis represent those central metabolic pathways which seem to be activated during growth on non-fermentable carbon sources but downregulated during growth on excess glucose (Carlson, 1999; Schüller, 2003; Turcotte et al., 2010). It has, however, to be recognized that most of these well-accepted assumptions have been mainly based on experimental data obtained from comparing transcript and protein abundances from cells growing on non-fermentable carbon sources and comparing them to those obtained from cells growing on glucose. We have summarized the most important of these studies in Table 1. First of all, it has to be emphasized that it is not correct to directly translate mRNA or protein abundances into metabolic fluxes since such an approach would ignore post-transcriptional modifications and/or metabolic regulation (e.g. allosteric regulation). Both levels of regulation can have a significant impact on the actual *in vivo* fluxes. Moreover, one has to realize that most of the respective *omics* studies have been conducted on non-fermentable carbon sources other than glycerol. Although all carbon sources tested in these studies have been considered to be catabolized in a fully respiratory manner, glycerol is a C3 carbon source

while ethanol and acetate are C2 carbon sources whose metabolism require different metabolic fluxes. In the following, we want to scrutinize the available experimental data in more detail. We also want to alert the reader about the fact that it is still somewhat unclear how much of the global picture obtained from experiments in ethanol/glycerol mixtures or ethanol can actually be transferred to the situation when glycerol is the only carbon source, particularly if synthetic medium is considered.

In most transcriptome studies focusing on non-fermentable carbon sources, the respective transcript levels have been compared to the transcript levels detected in cells growing in excess glucose. Working with glucose excess as a reference condition inherently leads to difficulties when making interpretations about the metabolic pathways/enzymes active or inactive on the non-fermentable carbon source under investigation. The reasons have been comprehensively summarized by Daran-Lapujade et al. (2004). First, glucose excess leads to glucose repression of genes involved in the utilization of alternate carbon sources, gluconeogenesis, respiration and peroxisomal functions (Carlson, 1999). Therefore, it is impossible to dissect the transcriptional changes that have been caused by i) the relief from glucose repression and ii) the presence of the non-fermentable carbon source. Another difficulty arises from the fact that toxic compounds, such as ethanol and acetic acid, are formed in batch cultivations with excess glucose, causing a stress-dependent transcriptional regulation. Moreover, excess glucose is associated with a much higher maximum specific growth rate compared to those achieved with non-fermentable carbon sources. This leads to growth-rate specific differences in global transcription interfering with the real differences solely resulting from metabolizing different carbon sources. These issues should be kept in mind when discussing the transcriptome data obtained from

experiments such as those provided by DeRisi et al. (1997) and Gasch et al. (2000). The first authors performed microarray-based studies with cells harvested from batch cultivations after the diauxic shift, i.e. when excess glucose was consumed and the cells grew on a mixture of ethanol and glycerol. Gasch et al. (2000) harvested the cells from ethanol-grown batch cultures and compared the transcript levels to those obtained from exponential growth in excess glucose. DeRisi et al. (1997) detected more than 400 transcripts that were found to change by a factor of 2, while Gasch et al. (2000) identified more than 600. These two benchmarking studies shown in Table 1 have been central with regard to our understanding of metabolism when *S. cerevisiae* grows on non-fermentable carbon sources.

Nevertheless, uncertainties result from the fact that assumptions made about active metabolic fluxes which are solely based on transcriptome data inherently disregard post-transcriptional regulation. It has been well known that post-transcriptional regulation contributes to the shift from excess glucose to non-fermentable carbon sources (Schüller, 2003; Yin et al., 2000). Tripodi et al. (2015) and Chen and Nielsen (2016) summarized numerous examples for this type of regulation with regard to selected enzymes of yeast central carbon metabolism. Various studies have shown that e.g. phosphorylation of metabolic enzymes is very widespread, with two-thirds of the respective *S. cerevisiae* metabolic proteins being targets of kinase and phosphatase signalling networks (Bodenmiller et al., 2010; Breitkreutz et al., 2010; Oliveira et al., 2012; Ptacek et al., 2005). For example, it has been shown that the E1 α subunit of the pyruvate dehydrogenase complex, the glycerol-3-phosphate dehydrogenase (Gpd1) and the Pfk2 β subunit of the phosphofructose-1-kinase complex are regulated by phosphorylation (Oliveira et al., 2012).

An important indication for a significant contribution of post-transcriptional regulation between growth on glucose and non-fermentable carbon sources at the whole-genome level has been delivered by the work of Daran-Lapujade et al. (2004), who used aerobic carbon-limited chemostat cultures in order to compare both the metabolic fluxes (based on metabolic flux balancing using a compartmented stoichiometric model) and the transcriptomes in cells grown on four different carbon sources. The authors used two fermentable carbon sources (glucose and maltose) and two non-fermentable ones (the C2 compounds ethanol and acetate). Thereby, they avoided excess glucose and adjusted the growth rates to exactly the same level in all carbon sources. Notably, hexose sugars are fully respired during this mode of cultivation and any regulation caused by excess glucose is obsolete. Of course, one has to consider that continuous cultures are barely used in industrial practice. However, the study has been very useful to better understand the regulation contributing to the use of different carbon sources independent from carbon catabolite repression. The genes which were transcriptionally upregulated during growth on C2 compounds are included in Table 1. A first, very interesting result obtained in the study of Daran-Lapujade et al. (2004) has been that only 117 genes were differentially expressed at the level of transcription in a comparison between sugars and C2 compounds. This number is significantly lower than those obtained in the studies from DeRisi et al. (1997) and Gasch et al. (2000) analyzing cells after the diauxic shift. This huge discrepancy reflects the above-mentioned differences in transcriptional regulation caused by using glucose excess and batch cultivations instead of applying chemostats. Second, the study revealed that the fluxes predicted by the stoichiometric model were only partly supported by the experimental data obtained by the transcriptome analysis. In fact, only the differences in transcript levels for the

enzymes involved in the glyoxylate cycle and gluconeogenesis showed a good correlation with the predicted *in vivo* fluxes when a C6 carbon source was compared with a C2 carbon source. The latter two pathways are indeed not required during growth on glucose. As expected, the flux through acetyl-coenzyme A synthetase was also low in glucose-grown cultures but high for C2 compounds. In contrast, the model-predicted fluxes for growth on C2 carbon sources, such as a remarkably higher flux through the TCA cycle and a reduced flux through the pentose phosphate pathway, were not accordingly reflected by the transcript levels corresponding to the respective enzymes involved in these pathways. Provided that the used metabolic model was sufficiently accurate and indeed reflected the actual fluxes, the results strongly indicated that the *in vivo* fluxes in the central carbon metabolism are controlled to a large part via posttranscriptional mechanisms.

A follow-up study of Kolkman et al. (2005) on the proteome used the same strain and tested it under exactly the same conditions used in the study by Daran-Lapujade et al. (2004). In comparison to the above-mentioned 117 transcriptional differences on ethanol versus glucose (Daran-Lapujade et al. 2004), Kolkman et al. (2005) only identified 15 protein spots whose abundance significantly changed with the carbon source in their proteome study. A closer look at the results show that the key enzymes involved in the glyoxylate cycle (i.e. Mls1 and Icl1) and gluconeogenesis (Pck1), as well as most enzymes involved in the TCA cycle are regulated at the transcriptional level (Table 1). However, most glycolytic enzymes, except for Hxk1 appear to significantly rely on post-transcriptional regulation. The TCA cycle enzymes seem to be worth a particular discussion since the published results regarding the protein levels are not consistent. While Kolkman et al. (2005) identified several enzymes to be upregulated at the protein

level, a parallel study of Ohlmeier et al. (2004), who solely focused on the mitochondrial proteome, only found the expression of succinate dehydrogenase to be significantly upregulated. The latter authors even suggested that the TCA cycle enzyme abundance between glucose and non-fermentable carbon sources might be similar due to the anabolic function of this cycle in addition to its role in catabolism. It is worth mentioning in this context that the study of Kolkman et al. (2005) was carried out in defined mineral medium with ethanol in a chemostat while Ohlmeier et al. (2004) conducted their experiments on complex medium with glycerol in batch cultivations. The amino acids present in complex media (Ohlmeier et al., 2004) can provide TCA cycle intermediates for anabolic reactions and might have reduced the need to generate them from the carbon source.

Except the study from Ohlmeier et al. (2004) exclusively analyzing mitochondrial proteins, none of the *omics* studies discussed here so far provided information about the specific situation on glycerol. In contrast to the utilization of glucose and C2 non-fermentable carbon sources, glycerol specifically requires activity of the initial catabolic steps (catalyzed by Stl1, Gut1 and Gut2) in order to be channeled into the central carbon catabolism (glycolysis/gluconeogenesis) via DHAP. Interestingly, the study of Daran-Lapujade et al. (2004) conducted on ethanol and acetate showed that the expression of *STL1*, the gene that has later been demonstrated to encode the active transporter for the uptake of glycerol (Ferreira et al., 2005), is also upregulated on C2 carbon sources.

Due to the fact that glycerol is a C3 carbon source, the central metabolism on glycerol should differ from ethanol or acetate. In theory, the catabolism of glycerol neither requires the glyoxylate cycle (also see section 3) for the replenishment of TCA cycle intermediates nor the gluconeogenic enzyme phosphoenolpyruvate carboxykinase

(encoded by *PCK1*), which converts oxaloacetate to phosphoenolpyruvate (PEP). In order to compare the theoretical considerations with experimental data, results from studies on glycerol as the sole carbon source are required. In this context, Roberts and Hudson (2006) provided a transcriptome study on glycerol even though it was conducted in complex medium, i.e. YPG vs. YPD (Table 1). The study used excess glucose as a reference, and the experiments were conducted in shake flask cultivations. At the level of transcriptome, the corresponding results matched to a large part with what had been reported in the transcriptome studies during the diauxic shift conducted by DeRisi et al. (1997) and Gasch et al. (2000). Notably, the study of Roberts and Hudson (2006) conducted a parallel experiment on ethanol. The comparison of the respective data obtained with glycerol surprisingly revealed that the majority of genes was shown to be regulated in the same way on both non-fermentable carbon sources. The gene clusters encoding enzymes involved in respiration (oxidative phosphorylation and TCA cycle), the glyoxylate cycle, and gluconeogenesis were highly upregulated. Additionally, clusters encoding for proteins related to mitochondrial function, energy generation and stress responses were upregulated. Downregulated genes in the study of Roberts and Hudson (2006) clustered in functional categories such as ribosome biogenesis, transcription of RNA polymerase I and III promoters, as well as membrane lipid and sphingolipid metabolism reflecting the slower growth rate of *S. cerevisiae* on non-fermentable carbon sources compared to glucose. A closer look at the genes which are particularly required for channeling glycerol or ethanol into the central carbon metabolism revealed that *STL1*, *GUT1* and *GUT2* were strongly upregulated on glycerol but also on ethanol even though the fold changes were slightly lower on ethanol. The upregulation of *STL1* on ethanol is consistent with the respective finding of Daran-Lapujade et al. (2004).

Roberts and Hudson (2006) also identified a few differences between ethanol and glycerol. In fact, genes found in clusters covering carbohydrate and fatty acid metabolism were less upregulated on ethanol in comparison to glycerol. A rationale behind this finding was not provided.

Although the study of Ohlmeier et al. (2004) solely addressed the mitochondrial proteins on glycerol, the authors also conducted a genome-wide transcriptome analysis in parallel to just confirm that the cells had indeed undergone the metabolic shift (Table 1). The authors identified 18 mitochondrial proteins whose abundance significantly changed in glycerol but detected 4000 differentially expressed genes at the transcriptome level. The authors concluded that the discrepancy between transcript and protein levels is caused by changes in translational efficiency and protein turnover. Nevertheless, the study confirmed at the proteome level that the proteins Adh2 and Ald4 are also strongly upregulated on glycerol. Both proteins are key enzymes involved in the utilization of C2 carbon sources but are obviously also abundant during growth on glycerol. Similar results were obtained by the proteome analysis conducted by Kito et al. (2016). The obvious fact that the regulation of the metabolism seems to be strikingly similar when cells are shifted from excess glucose to either ethanol or glycerol might be attributed to the circumstance that glycerol often co-exists with ethanol in natural environments of *S. cerevisiae*. In fact, ethanol is the major fermentation product resulting from catabolism of excess glucose by *S. cerevisiae* and some glycerol is formed as a by-product for cytosolic redox balancing (Bakker et al., 2001).

A number of regulatory networks are involved in the coordinated biosynthesis of enzymes necessary for the utilization of non-fermentable carbon sources as soon as glucose repression is abolished. As already mentioned, it is generally difficult to dissect

the regulatory mechanisms involved in glucose repression from those that are related to a particular non-fermentable carbon source. We will not go into detail about the regulation of glucose repression as several comprehensive reviews have been provided in this context (Hedbacker and Carlson, 2008; Kayikci and Nielsen, 2015). Here, we only want to scrutinize whether the currently available information about transcription factors important for the regulation of genes during growth on ethanol and glycerol support the idea of common regulatory networks on both carbon sources. Table 2 summarizes a number of transcription factors for which studies in complex glycerol medium are available. The majority of tested transcription factors shows the same deletion phenotype when grown in ethanol or glycerol. However, there are two suspicious proteins, Rsf1 and Rsf2, whose mutant phenotypes indicate that they play a more specific role only on glycerol but are probably dispensable on ethanol. Indeed, the respective two haploid mutant strains showed severe growth defects on glycerol but not on ethanol (Lu et al., 2003; Lu et al., 2005). The same authors demonstrated that the expression of both *RSF1* and *RSF2* was strongly induced on glycerol, and the induction was lower on ethanol. The growth defect on glycerol of the two haploid mutants can be explained by the strong decrease in *GUT1* and *GUT2* transcript levels, the products of both genes are essential for glycerol utilization. *GUT1* and *GUT2* are not required for growth on ethanol which could explain the fact that the deletion of neither Rsf1 nor Rsf2 has an effect on ethanol-based growth. It has also been shown that the transcript levels of genes encoding products with a function in stress response were increased in the *rsf1Δ* mutant during exponential growth on glycerol but not on ethanol-based medium (Roberts and Hudson, 2009). Furthermore, loss of Rsf1 resulted in a decrease of the

transcript levels of genes encoding proteins involved in electron transport and glycerol anabolism during growth on glycerol-based, but not on ethanol-based medium.

In summary, many aspects with respect to the regulation of the transcriptional changes that are crucial for the utilization of glycerol as the sole carbon source in synthetic medium are still not understood. Apart from basic research, a more detailed knowledge about regulatory proteins could be an advantage for metabolic engineering endeavors. Several studies have demonstrated that, for example, the deletion and/or the overexpression of selected transcriptional regulators can improve industrially important strain characteristics in *S. cerevisiae* (Chen et al., 2016; Lin et al., 2014). Moreover, the random mutagenesis of regulon-specific transcriptional factors is an interesting approach to reprogram expression of genes relevant for a particular phenotype (Swinnen et al., 2017). Such approaches could be applied for strain optimization in the context of glycerol utilization as well provided that a better understanding of the crucial transcription factors is available.

Table 1. Overview about the most relevant available transcriptome and proteome studies focusing on differentially expressed genes when *S. cerevisiae* grows in ethanol/glycerol mixtures (diauxic shift), ethanol or glycerol. Cells harvested during growth on glucose were used as a reference in all studies. Notably, the experiments were conducted in different media and cultivation conditions. Thus, conclusions have to be considered with caution. Upregulated gene clusters (represented by a bold ´**+**´) as well as up- and downregulated genes encoding key enzymes in the respective cluster (represented by ´+´ and ´-´) are listed according to what has been specified by the authors of the reference publication. In all cases where a gene cluster or gene was not mentioned at all in the publication, we added a question mark (´?´). If the mRNA/protein level was not significantly affected by the carbon source, an equality sign (´=´) was used.

	Carbon source(s) used	**Ethanol and glycerol (cells harvested after diauxic shift when grown in glucose)**		**Ethanol**		**Glycerol**	
	Reference	DeRisi et al. (1997)	Gasch et al. (2000)	Daran-Lapujade et al. (2004)	Kolkman et al. (2005)	Ohlmeier et al. (2004)	Roberts & Hudson (2006)
	Medium used	Complex	Complex	Synthetic	Synthetic	Complex	Complex
	Cultivation conditions	Batch	Batch	Chemostat	Chemostat	Batch	Batch
	Type of *omics* study	Transcriptome	Transcriptome	Transcriptome	Proteome	Transcriptome	Transcriptome
Gene cluster and/or genes encoding key enzymes	**Glycerol catabolism**						**+**
	GUT1	?	?	?	?	?	+
	GUT2	?	?	?	?	?	+
	STL1	+	?	+	?	?	+
	Ethanol catabolism						
	ADH2	?	?	=	=	+	?
	ALD2	+	?	=	?	?	?
	ALD4	?	?	=	-	+	?
	Gluconeogenesis						**+**
	PCK1	+	?	+	+	?	?
	FBP1	+	?	+	not detectable	?	?
	Glyoxylate cycle		**+**				**+**
	ICL1	+	?	+	+	?	?
	MLS1	+	?	+	+	?	?
	TCA cycle		**+**				**+**
	CIT1	+	?	=	+	+	?
	ACO1	+	?	=	+	+	?
	IDH1/2	+	?	=	*IDH1* +	-	?
	KGD1/2	+	?	=	*KGD2* +	+	?
	LSC1/2	+	?	=	*LSC2* +	+	?
	SDH1/2/3/4	+	?	*SDH1/3* +	*SDH1* +	*SDH1/2/4* +	?
	FUM1	+	?	+	+	?	?
	MDH1	+	?	=	=	+	?
	Oxidative phosphorylation	**+**	**+**	**+**	**+**	**+**	**+**

Table 2. List of selected transcription factors (TF) potentially important during growth on glycerol as the sole carbon source. The selection has been based on the following criteria: (i) the DNA-binding target(s) of the respective transcriptional factor were experimentally characterized using glycerol as the sole carbon source (Hap2/3/4/5, Ino2/Ino4, Oaf1, Opi1, Pip2, Rds2, Rsf1, Rsf2) and/or (ii) the respective deletion mutant showed a clear growth impairment on glycerol (Adr1, Cat8, Rds2, Rsf1, Rsf2, and Znf1). Ert1 has been shown to bind to genes whose products are involved in gluconeogenesis on ethanol as the sole carbon source and a function in glycerol medium cannot be ruled out due to the lack of experimental data on glycerol. Notably, Roberts and Hudson (2006) identified a number of additional transcriptional factors upregulated during growth on glycerol. Some of them have not been included in the table since no experimental data have been available about their DNA-binding or deletion mutant phenotypes.

	DNA-binding studies			**Phenotype of the deletion mutant lacking the respective transcriptional factor**
TF	**Target gene(s) for which DNA binding to the respective promoter(s) was demonstrated**	**Experimental condition for which DNA binding was shown**	**Method(s) and reference(s) for the DNA binding assay(s) and target gene determination[1]**	
Adr1	Utilization of ethanol (*ADH2*, *ALD4*, *ALD5*, *ALD6*, *ACS1*)	Diauxic shift	ChIP-chip (Young et al., 2003) ChIP-chip, qRT-PCR (Tachibana et al., 2005)	Glycerol: no growth (Young et al., 2003) Ethanol: poor growth (Young et al., 2003)
	Utilization of glycerol (*GUT1*, *GUT2*)			
	Utilization of lactate (e.g. *CYB2*, *JEN1, DLD3*)			
	Mitochondrial inner membrane proteins (*CTP1*, *OAC1*, *DIC1*)			
	β-oxidation (*OAF1*)			
	Peroxisome biogenesis (*CTA1*)			
Cat8	Gluconeogenesis (*PCK1, FBP1)*	Diauxic shift	ChIP-chip, qRT-PCR (Tachibana et al., 2005)	Glycerol: no growth (Akache et al., 2001; Hedges et al., 1995) Ethanol: no growth (Gasmi et al., 2014)
	Glyoxylate shunt (*ACS1, ICL1, MDH2, MLS1*)			
	Transporters (*PUT4*, *SFC1*, *JEN1*)			
	Transcriptional factors *SIP4* and *HAP4*			
	Utilization of ethanol (*ADH2*)			
	Isocitrate dehydrogenase (*IDP2*)			
Ert1	Gluconeogenesis (*PCK1*, *FBP1, MAE1*)	Ethanol	ChIP-chip, qRT-PCR, gene reporter assay (Gasmi et al., 2014)	Glycerol: ? Ethanol: growth (Gasmi et al., 2014)
	Transporters (e.g. *SFC1, FMP43*)			

	Respiration (*HAP4*)			
	TCA cycle (e.g. *LSC2*)			
	Mitochondrial proteins (e.g. *PET9*)			
Hap2/3/4/5	Utilization of glycerol (*GUT2*)	Glycerol	Gene reporter assay (Grauslund and Ronnow, 2000)	Glycerol: no growth (Buschlen et al., 2003) Ethanol: no growth (Buschlen et al., 2003)
Ino2/Ino4	Utilization of glycerol (*GUT1*)	Glycerol	Gene reporter assay – (Grauslund et al., 1999)	Glycerol: ? Ethanol: ?
Oaf1	Fatty acids utilization (e.g. *POX1*, *FOX1*)	Glycerol	ChIP-chip, qRT-PCR (Karpichev et al., 2008)	Glycerol: growth (Rottensteiner et al., 1997) Ethanol: growth (Rottensteiner et al., 1997)
	Peroxisome biogenesis (e.g. *PEX5*, *PEX11*)			
	Transcriptional factor *PIP2*			
Opi1	Utilization of glycerol (*GUT1, GUT2*)	Glycerol	Gene reporter assay (Grauslund et al., 1999; Grauslund and Ronnow, 2000)	Glycerol: no growth (Grauslund et al., 1999) Ethanol: no growth (Grauslund et al., 1999)
Pip2	Fatty acids utilization (e.g. *POX1*, *FOX1*)	Glycerol	ChIP-chip, qRT-PCR (Karpichev et al., 2008)	Glycerol: growth (Rottensteiner et al., 1997) Ethanol: growth (Rottensteiner et al., 1997)
	Peroxisome biogenesis (e.g. *PEX5*, *PEX11*)			
Rds2	Gluconeogenesis (*PCK1, FBP1*)	Ethanol	ChIP-chip (Soontorngun et al., 2007)	Glycerol: no growth (Akache et al., 2001; Soontorngun et al., 2012) Ethanol: no growth (Soontorngun et al., 2012)
	Glyoxylate shunt (*MLS1*, *MDH2*)			
	TCA cycle (*CIT1*, *KGD2*, *SDH4*, *LSC2*)			
	Respiration (*COX6*, *CYC1*)			
	Transporter (*SFC1*)			
	Transcriptional factors *HAP4* and *SIP4*			
	Gluconeogenesis (*PCK1, FBP1)*	Glycerol	ChIP-chip, qRT-PCR (Soontorngun et al., 2012)	
	Glyoxylate shunt (*ACS1, MLS1*)			
	TCA cycle (*LSC2*)			
	Respiration (*COX4*)			
	Transporters (*SFC1*)			
	Transcriptional factors *HAP4*, *ADR1* and *SIP4*			

Rsf1	Utilization of glycerol (*GUT1, GUT2*)	Glycerol, ethanol	Y1H, qRT-PCR (Lu et al., 2003)	Glycerol: severe growth defect (Lu et al., 2003) Ethanol: growth (Lu et al., 2003)
	Respiration (*OLI1*)			
Rsf2	Utilization of glycerol (*GUT1*, *GUT2*)	Glycerol, ethanol	qRT-PCR, DNA microarray (Lu et al., 2005)	Glycerol: growth defect (Lu et al., 2005) Ethanol: growth (Lu et al., 2005)
	Mitochondrial genes (*OLI1)*			
	Respiration (*COX4*)			
Znf1	Gluconeogenesis (*FBP1, PCK1*)	Ethanol	ChIP-chip, qRT-PCR (Tangsombatvichit et al., 2015)	Glycerol: no growth (Akache et al., 2001; Tangsombatvichit et al., 2015) Ethanol: no growth (Akache et al., 2001; Tangsombatvichit et al., 2015)
	Glyoxylate shunt (*ICL1, MDH2, MLS1*)			
	TCA cycle (*ACO1*)			
	Transporters (*ADY2*, *SFC1*)			

[1] Target genes have been characterized by chromatin immunoprecipitation (ChIP-chip), by DNA microarray, by gene reporter assay, by yeast one-hybrid analysis (Y1H) and/or by quantitative RT-PCR (qRT-PCR).

3. Physiological role of the glyoxylate cycle on glycerol and subcellular localization of the respective enzymes

In *S. cerevisiae,* the glyoxylate cycle consists of five reactions catalyzed by isocitrate lyase, malate synthase, malate dehydrogenase, citrate synthase, and aconitase. Several individual reactions of this cycle are known to take place in the cytosol while others have been localized in the peroxisomes (Fig. 1). During the utilization of C2 carbon compounds and oleate, the physiological function of the glyoxylate cycle is to convert acetyl-CoA into C4 metabolites, which can then be diverted to anabolic reactions e.g. gluconeogenesis and amino acid biosynthesis (Hiltunen et al., 2003; Kunze et al., 2006). As already mentioned in section 2, the key enzymes of the glyoxylate cycle, i.e. isocitrate lyase and malate synthase, have been shown to be highly upregulated on C2 compounds as well as on oleate (Kal et al., 1999). Interestingly, the respective upregulation was also recognized in all studies available so far on glycerol at both transcriptome and proteome level (see section 2), even though the glyoxylate cycle is, at least in theory, dispensable on C3 carbon sources. It could therefore be that the upregulation of the glyoxylate cycle enzymes is simply caused by the assumed co-regulation of *S. cerevisiae*'s metabolism for the co-utilization of glycerol and ethanol. In the following, we will scrutinize this aspect by having a closer look at the experimental data obtained by studying mutants defective in glyoxylate cycle (iso)enzymes. Out of the five enzymes contributing to the cycle, only isocitrate lyase and malate synthase have a unique function in this pathway, while the other three enzyme activities can also be found in the TCA cycle. Therefore, it is inherently difficult to draw conclusions about the role of the latter three enzymes for the glyoxylate cycle. Still, we want to provide the information regarding all isoenzymes

known in *S. cerevisiae* and, if available, the phenotypes of the respective deletion mutants particularly on glycerol.

S. cerevisiae contains two actively transcribed aconitase isoenzymes called Aco1 and Aco2 (Gangloff et al., 1990; Przybyla-Zawislak et al., 1999). Deletion of *ACO1* resulted in an inability of the respective mutant to grow on glycerol (Gangloff et al., 1990). This result indicates that Aco1p is the major citric acid cycle aconitase in *S. cerevisiae.* Aco2 seems to exclusively contribute to lysine biosynthesis (Fazius et al., 2012).

Citrate synthase has three isoenzymes in *S. cerevisiae*, but only Cit2 (localized in peroxisomes) seems to be involved in the glyoxylate cycle, whereas Cit1 and Cit3 function as mitochondrial isoforms within the TCA cycle. In contrast to Cit1 and Cit2, Cit3 has been identified as a dual specificity mitochondrial citrate and methylcitrate synthase (Graybill et al., 2007). Kispal et al. (1988) have shown that individual deletions of *CIT1*, *CIT2* and *CIT3* resulted in strains that grew well on glycerol, while a double mutant strain *cit1*Δ *cit2*Δ is not viable on glycerol, glucose, and galactose. Jia et al. (1997) also tested the individual deletions of *CIT1* and *CIT3* on the same medium as Kispal et al. (1988). In contrast to Kispal et al. (1988), Jia et al. (1997) observed a small reduction in growth for the individual deletion of *CIT1*. Furthermore, a double mutant strain *cit1*Δ *cit3*Δ led to a very strong reduction in growth on glycerol. However, results regarding the growth of the double deletion strain *cit2*Δ *cit3*Δ on glycerol are still missing in order to allow a final conclusion about the requirement of the different isoenzymes for growth on glycerol.

The third enzyme with a function in both TCA cycle and glyoxylate cycle is malate dehydrogenase. Again, three isoenzymes have been identified, all of which can reversibly convert malate to oxaloacetate. Depending on the carbon sources, different isoenzymes of malate dehydrogenase seem to be essential. For instance, cells lacking

MDH2 are unable to utilize ethanol as the sole carbon source (Minard and McAlister-Henn, 1991) while cells lacking *MDH3* cannot grow on oleate (Van Roermund et al., 1995). With regard to the role of malate dehydrogenase isoenzymes active on glycerol, the published results do not allow clear conclusions because all these studies have used either complex media or minimal media supplemented with yeast extract (McAlister-Henn and Thompson, 1987; Minard and McAlister-Henn, 1991; Przybyla-Zawislak et al., 1999; Steffan and McAlister-Henn, 1992). One might argue that the anabolic function of the TCA cycle was unnecessary under the latter conditions.

The two key enzymes in the glyoxylate cycle, malate synthase (Mls1) and isocitrate lyase (Icl1), are both encoded by a single gene in *S. cerevisiae*. If the glyoxylate cycle was indeed dispensable for glycerol utilization, the growth of the respective single deletion mutants should not be negatively affected on this carbon source. Based on spot tests, Chen et al. (2012) concluded that the *mls1*Δ and *icl1*Δ mutant strains exhibited a growth defect on glycerol as the sole carbon source. It must, however, be noted that the wild-type control only formed a faint spot. This is an inherent problem when working with strains not able to grow on synthetic glycerol medium, but show weak growth as soon as the necessary amino acid(s) or nucleic base(s) is/are added in order to supplement the strain's auxotrophy/auxotrophies. It is well known that the use of auxotrophic strains together with the respective supplements can lead to misinterpretations of phenotypes (Pronk, 2002). Therefore, we strongly recommend repeating the investigation of the *mls1*Δ and *icl1*Δ mutant strains by using a prototrophic strain able to grow in liquid glycerol medium. This holds of course for many other mutant strains as well.

The localization of the (iso)enzymes contributing to the glyoxylate cycle has experimentally been carried out using cells growing on C2 compounds and oleate. According to the results, which have been summarized in Table 3, Aco1 is mainly localized in the mitochondria and a minor fraction was detected in the cytosol. Icl1 and Mdh2 were detected in the cytosol, while Cit2p and Mdh3p are localized in the peroxisomes (Fig. 1). With regard to Mls1, the subcellular localization seems to depend on the carbon source (Kunze et al., 2002). While the enzyme seems to be located in the cytosol when cells grow on ethanol, it was detected in the peroxisomes during growth on oleate. Due to this uncertainty, we allocated Mls1 to both compartments in Fig. 1 and added a question mark. The different targeting of Mls1 on ethanol and oleate has been demonstrated to depend on the peroxisomal membrane signal receptor (encoded by *PEX9*), that is involved in peroxisomal import of a subset of matrix proteins (Effelsberg et al., 2016). Pex9 is not expressed in ethanol-grown cells, but its expression is strongly induced on oleate. Therefore, in *S. cerevisiae* cells growing on oleate, Pex9 acts as a cytosolic and membrane-bound peroxisome import receptor for Mls1 (Effelsberg et al., 2016). This example demonstrates that enzyme and pathway localization in a cell is not static but may vary according to the employed conditions. Apart from the aforementioned examples, one has to note that there is currently no available information about the localization of Mls1 on glycerol.

In general, the knowledge about the subcellular localization of a particular enzyme may be required for metabolic engineering approaches. Particularly if compartmentalization of metabolic pathways is used to increase product formation, this type of information is crucial. In fact, targeting enzyme localization has been of growing interest for yeast metabolic engineering (Avalos et al., 2013; DeLoache et al., 2016; Sheng et al., 2016).

However, metabolic engineers should be aware that the localization of a particular enzyme can even change as a side effect of certain genetic modifications. For example, in the context of profiling cytosolic and peroxisomal acetyl-CoA metabolism in *S. cerevisiae* on ethanol, Chen et al. (2012) have found that, upon deletion of *ACS1* (a gene encoding one of the isoenzymes of acetyl-CoA synthetase), Mls1 unexpectedly relocated from the cytosol to the peroxisome. Such an unintended relocation may have a dramatic impact on the outcome of the experiments and it might not be trivial to determine the underlying reason.

Table 3. Localization of enzymatic activities required for the glyoxylate cycle in *S. cerevisiae*. The respective studies have been conducted in media containing C2 compounds (ethanol or acetate) or oleate as the sole carbon source.

Enzyme	Gene	ORF	C2 compounds (ethanol/acetate)		Oleate	
			Localization	Reference	Localization	Reference
Aconitase	*ACO1*	YLR304C	Cytosol (less than 5 %) and mitochondria	Regev-Rudzki et al. (2005)	Cytosol (less than 5 %) and mitochondria	Regev-Rudzki et al. (2005)
Citrate synthase	*CIT2*	YCR005C	Peroxisome	Lee et al. (2011)	Peroxisome	Lewin et al. (1990)
Isocitrate lyase	*ICL1*	YER065C	Cytosol	Chaves et al. (1997)	Cytosol	Chaves et al. (1997) Taylor et al. (1996)
Malate dehydrogenase	*MDH2*	YOL126C	Cytosol	Minard and McAlister-Henn (1991)	Cytosol	McCammon et al. (1990)
	MDH3	YDL078C	Peroxisome	Steffan and McAlister-Henn (1992)	Peroxisome	Van Roermund et al. (1995)
Malate synthase	*MLS1*	YNL117W	Cytosol	Kunze et al. (2002)	Peroxisome	Kunze et al. (2002)

4. Mitochondrial transporters active on glycerol

In eukaryotic cells such as *S. cerevisiae,* membranes often separate different metabolic pathways, which partially or completely take place in different cellular compartments. For example, the TCA cycle occurs in the mitochondrial matrix while the enzymes of the glyoxylate cycle are either located in the cytosol or in the peroxisome (see section 3). The spatial separation of the two pathways allows their differential metabolic regulation, even though both pathways partially share the same enzymatic activities. In order to translocate specific organic solutes and inorganic ions across the intracellular membranes, specific transporters are required. Knowledge about these transport mechanisms is crucial for metabolic engineering of the central carbon metabolism, especially when it relates to the target product itself or one of the necessary pathway intermediates. Here, we want to focus on known mitochondrial *S. cerevisiae* transporters that translocate main intermediates of the central carbon metabolism i.e. pyruvate, acetyl-CoA, citrate, isocitrate, succinate, fumarate, malate and oxaloacetate (Fig. 1), and scrutinize their role when cells grow on glycerol. Particularly with regard to the glyoxylate cycle intermediates, it would be of great interest to also obtain knowledge about the respective peroxisomal transport mechanisms. However, genetic screens performed in *S. cerevisiae* were not able to identify any peroxisomal transporter responsible for the transport of the relevant intermediates (Kunze and Hartig, 2013; Kunze et al., 2006). Although we still cannot completely exclude the existence of such specific peroxisomal transporters, it has been suggested that the transfer of the glyoxylate cycle intermediates might be fulfilled by peroxisomal channels, as reviewed by Antonenkov and Hiltunen (2012).

When cells grow on glycerol, they form pyruvate from DHAP similar to cells growing on glucose. One could expect that the mitochondrial pyruvate carrier complex is required for the transport of pyruvate from the cytosol into the mitochondria (Bricker et al., 2012). Two alternative carrier complexes exist that contain either Mpc1 and Mpc3 or Mpc1 and Mpc2. The former complex is required during respiratory growth on glycerol (Fig. 1), while the latter is necessary under fermentative conditions such as growth on excess glucose (Bender et al., 2015). On glycerol, a strain in which all three homologous genes (*MPC1*, *MPC2*, and *MPC3*) are deleted is still viable, even though a clearly reduced maximum specific growth rate was observed (Timon-Gomez et al., 2013). The ability of the triple mutant to still grow on glycerol may be explained by the existence of the pyruvate dehydrogenase bypass pathway (Pronk et al., 1996) (Fig. 1). In this pathway, pyruvate is first decarboxylated to acetaldehyde in the cytosol by one of the pyruvate decarboxylases (encoded by the structural genes Pdc1/5/6) (Hohmann, 1991; Hohmann and Cederberg, 1990). Afterwards, the acetaldehyde is converted to acetate by the aldehyde dehydrogenase (Ald2/3/6) and further to acetyl-CoA by acetyl-CoA synthetase (Acs1/2) in the cytosol or acetate enters the peroxisome, where it is converted to acetyl-CoA by Acs1. The cytosolic acetyl-CoA can enter the glyoxylate cycle and replenish the TCA cycle via succinic acid that is translocated into the mitochondria (Fig. 1). In principle, cytosolic acetyl-CoA can also directly enter the mitochondria via a carnitine-dependent route (Fig. 1) (Van Roermund et al., 1995). However, the latter route can only be used by *S. cerevisiae* if carnitine is added to the growth medium since it is not capable of *de novo* synthesis of carnitine (Van Roermund et al., 1999).

Notably, Timon-Gomez et al. (2013) tested their above-mentioned triple *mpc* mutant strain in synthetic glycerol medium that contained 50 mM succinic acid as a buffering

agent. Succinic acid is an intermediate of the TCA cycle and as such may have falsified the obtained result. Anyway, an *mpc1*Δ *mpc2*Δ *mpc3*Δ mutant of a glycerol-utilizing strain should be tested in pure glycerol medium to verify whether its growth phenotype can be confirmed. In theory, there could also be another route how pyruvate indirectly enters the mitochondria. Pyruvate could be converted to oxaloacetate (or malate) by pyruvate carboxylase (PYC) and malate dehydrogenase in the cytosol. These compounds can enter the mitochondria via respective transporters (Fig. 1). Malate can be finally converted to pyruvate by means of malic enzyme (Mae1) as it can be deduced from Fig. 1. To prove that such route indeed exists, one should first investigate whether an *mpc1*Δ *mpc2*Δ *mpc3*Δ mutant is able to grow on pure glycerol medium (without addition of carnitine). The question whether malic enzyme is active on glycerol is also discussed in section 5.

The dicarboxylate carrier, encoded by *DIC1*, transports dicarboxylates, such as succinate and malate, into mitochondria in exchange for internal phosphate (Palmieri et al., 1999b). In Fig. 1, only the transport of succinate is exemplarily shown. A mutant strain lacking this dicarboxylate carrier was not able to grow on ethanol or acetate, but it was viable on other non-fermentable carbon sources including glycerol, pyruvate and lactate, which are all C3 carbon sources (Palmieri et al., 1999b). As growth of the *dic1*Δ mutant strain on acetate or ethanol was only restored by addition of compounds such as oxaloacetate able to generate TCA cycle intermediates, the suggested function of this transporter is to transport cytoplasmic dicarboxylates into the mitochondrial matrix, fulfilling an essential role for the replenishment of TCA cycle intermediates (Palmieri et al., 1999b). The fact that Dic1 is not essential on C3 carbon sources could support the

hypothesis that the glyoxylate cycle is not essential as an anaplerotic route when cells grow on glycerol (see section 'Anaplerotic reactions').

The mitochondrial membrane of *S. cerevisiae* has been shown to also carry a succinate/fumarate transporter encoded by *SFC1.* This carrier exports fumarate to the cytosol in exchange with succinate. In the cytosol, fumarate is channeled into the gluconeogenic pathway (via cytosolic Fum1, Mdh2 and Pck1), i.e. a route which is indispensable for *S. cerevisiae* growth on ethanol or acetate (Palmieri et al., 1997; Palmieri et al., 2000). Consequently, an *sfc1*Δ mutant strain was neither capable to grow on ethanol nor on acetate, but was still viable on pyruvate (Fernandez et al., 1994). Although the *sfc1*Δ mutant strain was not tested in glycerol medium, we expect that this mutant is still able to grow, comparable to the situation on pyruvate. On C3 substrates, oxaloacetate can be produced from pyruvate by PYC (encoded by *PYC1/2*) in the cytosol and transported into the mitochondria via Oac1 as described in the following paragraph. In order to verify this assumption, it might be worth investigating whether a *dic1*Δ *sfc1*Δ double deletion strain can still grow in synthetic glycerol medium.

The yeast mitochondrial transporter for oxaloacetate encoded by *OAC1*, uses the proton-motive force to take up oxaloacetate produced by cytoplasmic PYC (an anaplerotic reaction) into the mitochondria as shown in Fig. 1 (Palmieri et al., 1999a). The single deletion mutant *oac1*Δ was still able to grow on rich medium containing glycerol (Colleaux et al., 1992). In line with this, it has been reported that Oac1 is not required for growth of *S. cerevisiae* on glucose, galactose, ethanol, acetate and lactate, because in the absence of Oac1, oxaloacetate can be converted to malate in the cytoplasm by Mdh2, and malate enters the mitochondrion via Dic1. Malate can subsequently be converted to oxaloacetate in the mitochondria as shown in Fig. 1

(Palmieri et al., 1999a). Indeed, an *oac1*Δ *dic1*Δ double deletion strain did neither grow on glycerol nor ethanol (Palmieri et al., 1999a). However, it has to be emphasized that the exact medium composition used to test the phenotype for the *oac1*Δ *dic1*Δ double deletion strain has not been specified. The authors only mention rich medium and synthetic complete medium containing the aforementioned carbon sources. If it was possible to confirm this phenotype on pure synthetic glycerol medium, this would prove that Dic1 plays an anaplerotic role on glycerol in the absence of the mitochondrial carrier Oac1. There have been two isoforms of another mitochondrial transporter (encoded by *ODC1* and *ODC2*) which besides transporting oxoadipate and oxoglutarate with high efficiency by a counter-exchange mechanism, also transports to a lower extent malate (Palmieri et al., 2001). However, this transporter does not seem to be capable to transport sufficient malate into the mitochondria in the absence of Oac1 and Dic1. In Fig. 1, only the transport of malate by Odc1 and Odc2 is shown.

In *S. cerevisiae*, two mitochondrial citrate transporters have been identified. The first one is the mitochondrial transporter Ctp1, which transports citric acid or isocitrate through the mitochondrial inner membrane (Kaplan et al., 1995), while the second carrier Yhm2 transports citrate out of the mitochondria in exchange of α-ketoglutarate (Castegna et al., 2010) (Fig. 1). Additionally, Yhm2 was also demonstrated to transport oxaloacetate, succinate and fumarate to a lesser extent, but not malate or isocitrate. In higher eukaryotes, the function of citric acid transport from mitochondria to the cytosol is to deliver cytosolic acetyl-CoA (via cytosolic citrate lyase) to fuel both fatty acid and sterol biosynthesis (Endemann et al., 1982; Watson and Lowenstein, 1970). In *S. cerevisiae*, the actual physiological function(s) of the two citrate transporters has/have remained unclear. In fact, *ctp1*Δ and *yhm2*Δ mutant strains were shown to be

capable to grow on ethanol (Kaplan et al., 1995), acetate (Castegna et al., 2010) and glucose (Kaplan et al., 1996; Scarcia et al., 2017). These results do not seem to be surprising. On ethanol and acetate cytosolic acetyl-CoA is directly produced from the substrates as shown in Fig. 1 and does not have to be transported out of the mitochondria. On glucose, *S. cerevisiae* synthesizes cytosolic acetyl-CoA via pyruvate decarboxylase (Pdc1/5/6), aldehyde dehydrogenase (Ald2/3/6) and acetyl-CoA synthetase (Acs2). No data have been available on glycerol, but one might speculate that described transporters are also not required for synthesis of acetyl-CoA in the cytosol and instead, *S. cerevisiae* synthesizes cytosolic acetyl-CoA via the same route as on glucose. The question of whether the two transporters have an actual physiological function remains unsolved. Nevertheless, one interesting hypothesis has been provided for the mitochondrial citrate carrier Ymh2 which transports citrate out of the mitochondria in exchange of alpha-ketoglutarate. In studies conducted on acetate, it has been demonstrated that upon deletion of *YHM2*, citrate accumulates in the mitochondria of *S. cerevisiae*. In the cytosol, citrate can be converted to isocitrate by aconitase (encoded by *ACO1*), and consequently, the latter is used to produce α-ketoglutarate and NADPH by the action of an $NADP^+$-dependent cytosolic isocitrate dehydrogenase (encoded by *IDP2*). Thus, Yhm2 could function in a shuttle mechanism which indirectly transports NADPH from the mitochondria to the cytosol (Castegna et al., 2010). In fact, production of cytosolic free fatty acids from glucose, which require NADPH for their biosynthesis, was slightly increased by overexpression of *YHM2* (Yu et al., 2018).

5. Anaplerotic reactions active during growth on glycerol

Anaplerotic reactions replenish intermediates of the TCA cycle, which is necessary to dynamically balance the reactions that steer TCA intermediates, such as α-ketoglutarate, into biosynthetic pathways. This balancing, for which evolution has developed different metabolic routes (Nelson et al., 2013), results in an almost constant concentration of citric acid cycle intermediates. In nature, there are four known anaplerotic enzymes including PYC, PEP carboxykinase, PEP carboxylase, and malic enzyme, all of which provide the C4 compounds oxaloacetate or malate from the C3 compounds pyruvate or PEP. A fifth possibility to replenish the TCA cycle (at the level of the C4 compound succinate) is the glyoxylate cycle (Fig. 1). As mentioned in section 3, this pathway is the only anaplerotic route when an organism grows on C2 carbon sources or fatty acids. From a metabolic engineer's point of view, anaplerotic reactions are of utmost importance for re-routing the central carbon metabolism towards valuable products. Obviously, they are particularly relevant if the target product is a TCA cycle intermediate (e.g. succinic acid, fumaric acid or malic acid) or whose production pathway starts from such an intermediate (e.g. glutamate and derivatives).

The actual use of the different anaplerotic routes varies between organisms and growth conditions (carbon source). Except PEP carboxylase, *S. cerevisiae* commands all enzymes mentioned that have potential anaplerotic functions. *S. cerevisiae* also possesses the glyoxylate cycle as an anaplerotic route, which has been discussed in detail in the previous sections. Similar to many of the reactions discussed in the previous sections, it is currently not completely understood which anaplerotic route(s) are the predominant ones when *S. cerevisiae* cells grow on glycerol. Therefore, we first discuss the relevance of PYC which is the major anaplerotic reaction on glucose in *S. cerevisiae* and the

glyoxylate cycle which takes over this role during growth on ethanol. Afterwards, the two remaining potentially anaplerotic reactions are discussed.

PYC consists of two cytosolic isoenzymes, encoded by the genes *PYC1* and *PYC2* (Morris et al., 1987; Stucka et al., 1991; Walker et al., 1991) (Fig. 1). Both isoenzymes catalyse the ATP-dependent carboxylation of pyruvate to oxaloacetate (Gailiusis et al., 1964). The localization of this enzyme in *S. cerevisiae* is exclusively cytosolic which is in contrast to many higher organisms where PYC is a mitochondrial enzyme (Haarasilta and Taskinen, 1977; Rohde et al., 1991; Van Urk et al., 1989; Walker et al., 1991). As mentioned, PYC seems to represent the major anaplerotic reaction in *S. cerevisiae* growing in glucose-containing media. Single deletion mutants (deleted in either *PYC1* or *PYC2*) can sustain growth on glucose, but the double deletion mutant (*pyc1*Δ *pyc2*Δ) cannot grow unless the medium is supplemented with aspartate (Blazquez et al., 1995; Brewster et al., 1994; Stucka et al., 1991). The latter is able to deliver the necessary oxaloacetate for TCA cycle replenishment. The result obtained with the double deletion strain suggests that other anaplerotic routes are not sufficiently active to take over the role of PYC on glucose. As expected, PYC is dispensable during growth on C2 compounds; a *pyc1/2* double deletion mutant was able to grow on ethanol (de Jong-Gubbels et al., 1998). Regarding the situation on glycerol, one has to consider that it is a C3 carbon source, and thereby the anaplerotic role of PYC might be similar to that on glucose. However, a *pyc1/2* double deletion mutant was able to grow on glycerol even without the requirement of aspartate (Blazquez et al., 1995). Although this study did not provide growth rates, the result suggests that PYC is not the major anaplerotic enzyme during growth on glycerol or that its function can easily be replaced by another mechanism such as the glyoxylate cycle.

During growth on ethanol (C2), the replenishment of oxaloacetate is supposed to occur via the glyoxylate cycle at the level of succinate. This has been confirmed by Schöler and Schüller (1993) who showed that an *icl1* mutant strain cannot grow on ethanol. In order to analyze the impact of deleting *ICL1* on growth in synthetic glycerol medium, we used a CEN.PK derivative that has been previously obtained by reverse engineering (see introduction) (Ho et al., 2017). The *icl1* deletion mutant of this strain was able to grow on glycerol but not on ethanol (Xiberras et al*.,* 2019). This result suggests that the glyoxylate cycle is also not the major anaplerotic route on glycerol. Another explanation might be that the cell is very flexible in using PYC and/or the glyoxylate cycle under these conditions and one route can easily be replaced by the other. It will therefore be very interesting to check whether a *pyc1*Δ *pyc2*Δ *icl1*Δ triple deletion mutant is viable in synthetic glycerol medium.

In principle, it still cannot be completely excluded that either PEP carboxykinase (*PCK1*) and/or the malic enzyme (*MAE1*) are also active as anaplerotic enzymes on glycerol (Fig. 1). Pck1 is generally considered as a decarboxylating enzyme with a function in gluconeogenesis in *S. cerevisiae* (de Torrontegui et al., 1966). However, Pck1 is the main enzyme catalysing the C3 carboxylating reaction (PEP to oxaloacetate) in *Actinobacillus succinogenes* (McKinlay et al., 2007). We have recently deleted *PCK1* in a *S. cerevisiae pyc1/2* double deletion mutant and this triple deletion mutant was still able to grow on synthetic glycerol medium (Xiberras et al., 2019). This result supports the assumption that Pck1 does not play an important role as an anaplerotic enzyme in *S. cerevisiae*, at least under these conditions. Malic enzyme catalyses the reversible oxidative decarboxylation of malate to pyruvate and (Fig. 1). However, the inability of pyruvate carboxylase negative *S. cerevisiae* strains to grow on glucose showed that malic enzyme

cannot act as a pyruvate carboxylating anaplerotic enzyme (Zelle et al., 2011). Interestingly, this enzyme provides the pyruvate required for biosynthesis in pyruvate kinase-negative strains growing on ethanol (Boles et al., 1998). Experimental data regarding a potential function of malic enzyme on glycerol are underway.

A remarkable aspect with regard to the anaplerotic reactions and its implication in biotechnological applications is the fact that the operating route strongly influences ATP and co-factor balance of the cell. Replacing a native anaplerotic reaction by an alternative one may therefore help to improve product yields (Zelle et al., 2011; Zelle et al., 2010). This approach would also be interesting for fermentative production of compounds whose synthesis require an increased ATP yield, e.g. target products whose export from the cells require cellular energy.

6. Fermentation of the 'non-fermentable' carbon source glycerol for chemical production

It is obvious that the exploitation of glycerol's reducing power for the production of small molecules requires that fermentation is the predominant route of carbon and electrons rather than respiration. Certain wild-type bacteria are able to anaerobically ferment glycerol and naturally produce reduced fermentation products such as 1,3-propanediol (Celinska, 2010). However, glycerol has always been considered a 'non-fermentable' (or respiratory) carbon source in *S. cerevisiae* (Schüller, 2003; Turcotte et al., 2010). This is also reflected by the fact that glycerol-based medium has generally been used to select strains for a respiration-deficient phenotype (Hampsey, 1997). A fermentative metabolism of glycerol (i.e. ethanol production) has even not been observed in an evolved *S. cerevisiae* strain which showed a significantly improved

glycerol utilization in synthetic medium i.e. a maximum specific growth rate of up to 0.2 h^{-1} (Ochoa-Estopier et al., 2011).

A closer look at the native pathway for glycerol catabolism in *S. cerevisiae* (L-G3P pathway) reveals that it is oxygen-dependent since two electrons from glycerol are directly transferred to the mitochondrial respiratory chain via the FAD-dependent Gut2 enzyme (Fig. 2). This respiratory route of the electrons is counterproductive in the view of generating valuable fermentation products from glycerol. We therefore recently replaced the endogenous L-G3P pathway by an artificial NAD^+-dependent pathway (the so-called DHA pathway) allowing the entrapment of the respective electrons in the form of cytosolic NADH (Klein et al., 2016a), and thus facilitating the potential formation of fermentation products (Fig. 2). Employing a CRISPR/Cas9 based strategy, the pathway swapping was achieved in various *S. cerevisiae* strains via a single transformation step. To our surprise, the DHA pathway even allowed growth on glycerol of wild-type strains originally unable to use glycerol in synthetic medium including a laboratory strain of the popular CEN.PK family (Klein et al., 2016a).

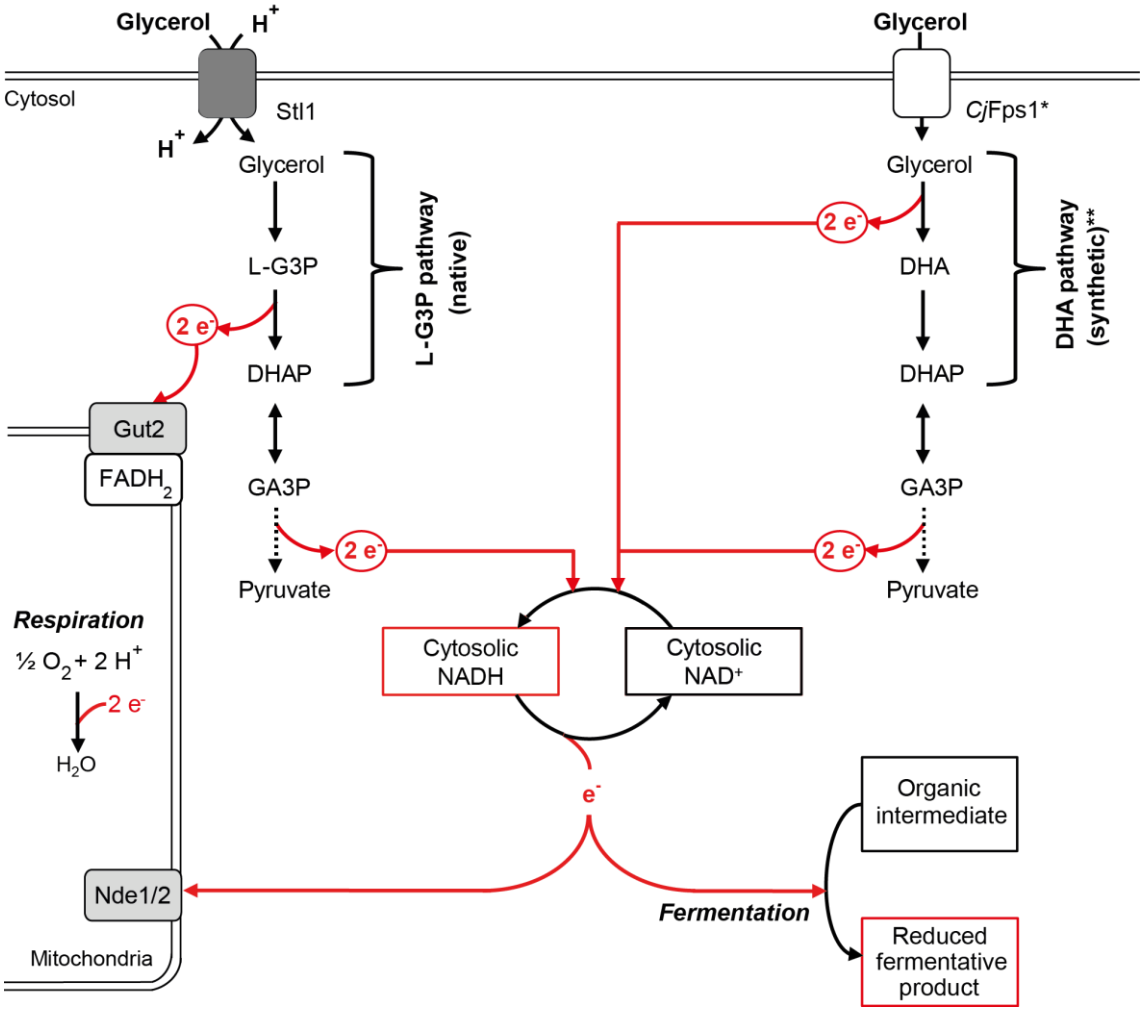


Fig. 2. Fate of electrons originating from glycerol if i) the native L-G3P pathway or ii) the engineered DHA pathway is used for glycerol catabolism. The DHA pathway allows the capturing of more electrons from glycerol in the form of cytosolic NADH. In theory, electrons from cytosolic NADH can subsequently either enter the respiratory chain (e.g. via Nde1/2) resulting in the formation of H_2O or be transferred to metabolic intermediates resulting in the formation of reduced fermentation products. *In contrast to overexpression of *S. cerevisiae*'s endogenous *FPS1* gene, the expression of a heterologous Fps1 homologue such as the one from *Cyberlindnera jadinii* (*Cj*Fps1) has been demonstrated to improve glycerol utilization in the CBS 6412-13A background (Klein et al., 2016b). **The synthetic DHA pathway consists of an NAD^+-dependent glycerol dehydrogenase from *Ogataea parapolymorpha* and the overexpression of the endogenous *DAK1* gene. The respective 'DHA pathway' strains also carry a deletion of the endogenous *GUT1* gene to abolish the native L-G3P pathway (Klein et al., 2016a). Abbreviations: L-G3P: L-glycerol 3-phosphate, DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde 3-phosphate, Gut2: FAD-dependent glycerol 3-phosphate dehydrogenase, Stl1: glycerol/H^+ symporter, Nde1/2: mitochondrial external NADH dehydrogenase.

The first fermentation product that has been generated from synthetic glycerol medium by an engineered *S. cerevisiae* strain is 1,2-propanediol (1,2-PDO). A recent study of Islam et al. (2017) reported the conversion of ca. 45 g/L glycerol to 3.2 g/L 1,2-PDO. A significant amount of the natural fermentation product ethanol was also produced (8

g/L). The same study also demonstrated that the replacement of the L-G3P pathway for glycerol catabolism by the DHA pathway was a major prerequisite for the generation of 1,2-PDO from glycerol. With regard to directing the carbon and the electrons from glycerol towards reduced small molecules (Fig. 2), there are still a number of challenges to be overcome, particularly controlling the flux distribution between respiration and fermentation. In fact, electrons captured in the form of cytosolic NADH can enter the mitochondrial respiratory chain as long as oxygen is available. *S. cerevisiae* exhibits an external NADH dehydrogenase encoded by the genes *NDE1* and *NDE2* (Fig. 2). Particularly the isoenzyme Nde1 seems to have an important function in the regeneration of cytosolic NAD^+ when *S. cerevisiae* grows in synthetic glycerol medium in shake flask cultures (Asskamp et al., 2019).

One has to note, that our previous studies included the heterologous expression of an aquaglyceroporin contributing to glycerol uptake (Islam et al., 2017; Klein et al., 2016b). The presence of this type of channel protein such as Fps1 from *C. jadinii* (Fig. 2) increased the maximum specific growth rate on glycerol of strain CBS 6412-13A from 0.13 to 0.18 h^{-1} (Klein et al., 2016b). In contrast, no improvement was visible in CBS 6412-13A after overexpression of *S. cerevisiae FPS1*. These results match the previous finding of Liu et al. (2013) that only an Fps1 homologue from another yeast species can complement a deletion of *STL1*. It is possible that the increased rate of glycerol utilization (and glycolytic flux) caused by an improved influx of glycerol via the heterologous *Cj*Fps1 was crucial for the fact that glycerol is not fully respired anymore in a strain that exclusively uses the DHA pathway (Fig. 2).

Although we see the major industrial attractiveness of glycerol in its fermentative conversion to reduced small molecules, there is also an advantage of glycerol when used

by a fully respiratory metabolism (i.e. in wild-type cells). Glycerol does not exert the so-called Crabtree effect as known from glucose (Crabtree, 1929). This favors the production of biomass and biomass-related products as discussed by Ochoa-Estopier et al. (2011). Baker's yeast strains able to efficiently utilize glycerol in a fully respiratory manner might therefore also be of great interest for industry.

When discussing glycerol as a carbon source for industrial processes, one cannot ignore the fact that the current and future biodiesel industry has to face a number of uncertainties due to changing crude oil prices, politics and governmental priorities, as recently reviewed by Naylor and Higgins (2017). It would be advisable to explore novel routes for the efficient direct or indirect generation of glycerol from carbon dioxide in order to provide a sustainable supply of this valuable carbon source.

7. Concluding remarks

As illustrated here by numerous examples, there have been a number of uncertainties with regard to the central carbon fluxes active during growth on glycerol as the sole source of carbon. They mainly result from the fact that the laboratory strains commonly used in fundamental research are unable to utilize glycerol in synthetic medium and useful strains for such experiments have not been available until recently. In fact, the previously isolated wild-type as well as evolved and reverse engineered *S. cerevisiae* strains able to grow in synthetic glycerol medium will facilitate more fundamental studies in synthetic glycerol medium in order to fill the knowledge gaps with regard to the central carbon catabolism of glycerol. Particularly, a ^{13}C metabolic flux analysis might help to identify the actual metabolic fluxes active under the relevant conditions. This information together with a profound knowledge about the active anaplerotic reactions, the localization of (iso)enzymes and the transporters active in synthetic glycerol medium

will facilitate the efficient engineering of *S. cerevisiae* for the production of useful chemicals from glycerol. Importantly, glycerol is not *per se* a 'non-fermentable' carbon source for *S. cerevisiae.* As demonstrated for 1,2-propandiol, fermentation of glycerol into useful small molecules via fermentative routes seems to be possible as soon as the native L-G3P pathway for glycerol catabolism is replaced by the DHA pathway. The results are very encouraging with regard to the exploitation of glycerol's higher reducing power in baker's yeast-based fermentative processes. Therefore, it will be important to also include DHA-pathway strains into fundamental studies regarding the central metabolic fluxes active on glycerol.

8. Acknowledgements

We thank the German Research Foundation (DFG) for funding of our research (NE 697/7-1). We are grateful to Maximilian R. Aßkamp for his support in generating Fig. 1.

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3.4 Manuscript II

Anaplerotic reactions active during growth of *Saccharomyces cerevisiae* on glycerol

Joeline Xiberras[1], Mathias Klein[1], Celina Prosch[1], Zahabiya Malubhoy[1] and Elke Nevoigt[1*]

[1] Department of Life Sciences and Chemistry, Jacobs University Bremen gGmbH, Campus Ring 1, 28759 Bremen, Germany

* Corresponding author:

E-mail: e.nevoigt@jacobs-university.de

Short title: Anaplerotic reactions during growth on glycerol

Abstract

Anaplerotic reactions replenish TCA cycle intermediates during growth. In *Saccharomyces cerevisiae,* pyruvate carboxylase and the glyoxylate cycle have been experimentally identified to be the main anaplerotic routes during growth on glucose (C6) and ethanol (C2), respectively. The current study investigates the importance of the two isoenzymes of pyruvate carboxylase (*PYC1* and *PYC2*) and one of the key enzymes of the glyoxylate cycle (*ICL1*) for growth on glycerol (C3) as a sole carbon source. As the wild-type strains of the CEN.PK family are unable to grow in pure synthetic glycerol medium, a reverse engineered derivative showing a maximum specific growth rate of 0.14 h^{-1} was used as the reference strain. While the deletion of *PYC1* reduced the maximum specific growth rate by about 38%, the deletion of *PYC2* had no significant impact, neither in the reference strain nor in the *pyc1*Δ mutant. The deletion of *ICL1* only marginally reduced growth of the reference strain but further decreased the growth rate of the *pyc1* deletion strain by 20%. Interestingly, the triple deletion (*pyc1*Δ *pyc2*Δ *icl1*Δ) did not show any growth. Therefore, both the pyruvate carboxylase and the glyoxylate cycle are involved in anaplerosis during growth on glycerol.

KEYWORDS: Glycerol, Anaplerotic reactions, Pyruvate carboxylase, Glyoxylate cycle, *Saccharomyces cerevisiae*

One sentence summary:

In comparison to glucose or ethanol as carbon sources, the yeast *S. cerevisiae* shows a higher metabolic flexibility with regard to the active anaplerotic reactions during growth on glycerol.

1. Introduction

During the last decades, the yeast *Saccharomyces cerevisiae* has become a well-established model organism for understanding fundamental cellular processes relevant to higher eukaryotes (Galao et al., 2007). In addition, it is a favourite microbial cell factory in industrial biotechnology. In fact, this organism has been extensively engineered to produce a wide range of products including bulk chemicals (e.g. succinic acid), biofuels (e.g. isobutanol) as well as nutraceuticals and pharmaceuticals (Borodina & Nielsen, 2014; Chen & Nielsen, 2016; Nevoigt, 2008).

Sugars are the preferred carbon source of *S. cerevisiae*. As a Crabtree-positive yeast, *S. cerevisiae* undergoes alcoholic fermentation when glucose in the environment exceeds a certain threshold concentration even in the presence of oxygen (Crabtree, 1929; De Deken, 1966). Only after glucose depletion and diauxic shift, the fermentation products ethanol and glycerol can also be used for the generation of energy and cellular biomass. In contrast to glucose, the latter two carbon sources are metabolized in a completely respiratory manner by wild-type *S. cerevisiae* (Schüller, 2003; Turcotte et al., 2010).

Anaplerotic reactions are pivotal in central carbon metabolism since they are responsible for the replenishment of TCA cycle intermediates during growth. In industrial biotechnology, anaplerotic reactions are of utmost importance when re-routing fluxes of the central carbon metabolism towards valuable fermentation products because the operating route strongly influences ATP and co-factor balance of the cell (Zelle et al., 2010; Zelle et al., 2011). The major anaplerotic routes in *S. cerevisiae* have been characterized when cells were grown on glucose or ethanol. On glucose, pyruvate carboxylase, which is encoded by the two isogenes *PYC1* and *PYC2* in this

species, is the main anaplerotic enzyme (Morris et al., 1987; Stucka et al., 1991; Walker et al., 1991). The enzyme catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate (Gailiusis et al., 1964) and a *pyc1Δ pyc2Δ* double deletion mutant is not able to grow in medium containing glucose as the sole carbon source unless supplemented with aspartate (Blazquez et al., 1995; Brewster et al., 1994; Stucka et al., 1991). The deamination of aspartate yields oxaloacetate, thus bypassing the need for an anaplerotic conversion of C3 to C4 compounds (De Jong-Gubbels et al., 1998). During growth on ethanol (and other C2 carbon sources), C4 carbon units for the replenishment of the TCA cycle (at the level of succinate) are synthesized from acetyl-CoA as the central intermediate via the activity of the glyoxylate cycle. This has been confirmed by Schöler and Schüller (1993) who showed that a mutant strain deleted for isocitrate lyase (encoded by *ICL1*), which is one of the key enzymes for the glyoxylate cycle, cannot grow on ethanol. Knowledge about the anaplerotic reactions occurring when *S. cerevisiae* grows on glycerol is fairly fragmentary. Although glycerol has been considered a non-fermentable carbon source in *S. cerevisiae* similar to ethanol (Schüller, 2003; Turcotte et al., 2010), the different numbers of carbon atoms (C3 vs. C2) of these two compounds might require substantially different metabolic fluxes (including anaplerotic reactions) during their metabolism as discussed by Xiberras et al. (2019). Therefore, it is questionable whether the glyoxylate cycle is indispensable during growth on glycerol.

Notably, glycerol is an attractive substrate for biotechnological processes because it is an inevitable by-product of the biodiesel industry, which generates approx. 10% (w/v) glycerol (Yazdani & Gonzalez, 2007). Apart from being abundant, glycerol also has a higher degree of reduction (4.7) compared to sugars such as glucose and xylose (4.0). This feature can generally lead to higher maximum theoretical product yields of reduced

target molecules (Mattam et al., 2013; Yazdani & Gonzalez, 2007; Zhang et al., 2013). The fact that it does not exert the above-mentioned Crabtree effect caused by glucose and other sugars can be another advantage of the 'non-fermentable' carbon source glycerol for the production of biomass and biomass-related products (Delvigne et al., 2006; Fowler and Dunlop, 1989; Ochoa et al., 2011).

The goal of the current study was to investigate the main anaplerotic routes active in *S. cerevisiae* growing in synthetic glycerol medium and particularly clarify the role of the glyoxylate cycle. A prerequisite for such a study is a strain able to grow in pure synthetic glycerol medium since growth-supporting supplements, such as amino acids and nucleic bases can interfere with the anaplerotic reactions. However, it is well known that wild-type strains of the CEN.PK family, which are popular for physiological studies in *S. cerevisiae,* are not able to grow in synthetic glycerol medium (Swinnen et al., 2013). Therefore, a reverse engineered derivative of CEN.PK113-1A constructed by Ho et al. (2017) (CEN.PK113-1A $UBR2_{CBS}$ $GUT1_{JL1}$) which grows with a maximum rate (μ_{max}) of ~0.14 h^{-1} in synthetic glycerol medium was used as a reference strain in the current study. This strain carries allele replacements for both *UBR2* (encoding a cytoplasmic ubiquitin-protein ligase (E3)) and *GUT1* (encoding the glycerol kinase involved in the glycerol catabolic pathway). The implemented *UBR2* allele originated from the natural glycerol-consuming *S. cerevisiae* isolate CBS 6412-13A (Swinnen et al., 2016) while the used *GUT1* allele was obtained from the CEN.PK113-7D derivative generated for improved glycerol utilization by adaptive laboratory evolution (Ho et al., 2017).

2. Materials and Methods

2.1. Strains, plasmids, medium composition and general cultivation conditions

The strains used in this study are listed in Table 1. Yeast cells were routinely grown on either solid YPD (*icl1*Δ, *icl1*Δ *pyc1*Δ, and *icl1*Δ *pyc2*Δ mutants), YPD with 0.5% aspartate (*icl1*Δ *pyc1*Δ *pyc2*Δ mutant) or YPE medium (*pyc1*Δ, *pyc2*Δ and *pyc1*Δ *pyc2*Δ mutants) containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose or 30 mL/L ethanol, and 15 g/L agar. Agar plates were cultivated in a static incubator at 30 °C. Media were supplemented with phleomycin (20 mg/L) or nourseothricin (100 mg/L) for selection purposes when needed.

For qualitative and quantitative growth assessment, cells were grown synthetic medium containing either 20 g/L glucose, 30 mL/L ethanol or 60 mL/L glycerol and ammonium sulfate as nitrogen source, respectively. The synthetic medium was prepared according to Verduyn et al. (1992), 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 15 mg/L EDTA, 4.5 mg/L $ZnSO_4.7H_2O$, 0.84 mg/L $MnCl_2.2H_2O$, 0.3 mg/L $CoCl_2.6H_2O$, 0.3 mg/L $CuSO_4.5H_2O$, 0.4 mg/L $NaMoO_4.2H_2O$, 4.5 mg/L $CaCl_2.2H_2O$, 3 mg/L $FeSO_4.7H_2O$, 1 mg/L H_3BO_3, and 0.1 mg/L KI. After heat sterilization of the medium, filter sterilized vitamins were added. Final vitamin concentrations were: 0.05 mg/L D-(+)-biotin, 1 mg/L D-pantothenic acid hemicalcium salt, 1 mg/L nicotinic acid, 25 mg/L myo-inositol, 1 mg/L thiamine chloride hydrochloride, 1 mg/L pyridoxine hydrochloride, and 0.2 mg/L 4-aminobenzoic acid. In case aspartate was used as a supplement, 0.5% was added to prior autoclaving. The pH of the synthetic glucose and synthetic ethanol medium was adjusted to 6.5 with 4 M KOH, while that of synthetic glycerol medium was adjusted to 4.0 with 2 M H_3PO_4.

E. coli DH5α was used for plasmid construction and isolation, and cells were routinely grown in lysogeny broth (LB) containing 10 g/L NaCl, 5 g/L yeast extract, 10 g/L peptone

and adjusted to a pH of 7.5 with 2 M NaOH (Bertani, 1951). For selection and maintenance of plasmid containing cells, 100 mg/L ampicillin was added.

Table 2. *S. cerevisiae* strains used in this study.

Strain	Genome modifications	Source or reference
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1::GUT1$_{JL1}$*	Ho et al. (2017)
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$ pyc1Δ*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1::GUT1$_{JL1}$ pyc1::loxP-ble-loxP*	This study
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$ pyc2Δ*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1::GUT1$_{JL1}$ pyc2::loxP-ble-loxP*	This study
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$ pyc1Δ pyc2Δ*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$ gut1::GUT1$_{JL1}$ pyc1::loxP pyc2::loxP-ble-loxP*	This study
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$ icl1Δ*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$ gut1::GUT1$_{JL1}$ icl1::loxP*	This study
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$ icl1Δ pyc1Δ*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$ gut1::GUT1$_{JL1}$ icl1::loxP pyc1::loxP-ble-loxP*	This study
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$ icl1Δ pyc2Δ*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$ gut1::GUT1$_{JL1}$ icl1::loxP pyc2::loxP-ble-loxP*	This study
CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$ icl1Δ pyc1Δ pyc2Δ*	*ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1::GUT1$_{JL1}$ icl1::loxP pyc1::loxP-ble-loxP pyc2::loxP-cloNAT-loxP*	This study

2.2. General molecular biology techniques

Preparative PCRs for cloning as well as for sequence determination of deletion cassettes integrated into the genome were performed using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Frankfurt am Main, Germany). PCR conditions were adapted to the guidelines of the respective manufacturer. PCR products were purified by using the GeneJET PCR Purification Kit (Thermo Fischer Scientific).

2.3. Genetic modifications of *S. cerevisiae*

Deletions of either *PYC1*, *PYC2* or *ICL1* were obtained using a disruption cassette containing either a phleomycin (*ble^r*) or nourseothricin (clonNAT) resistance marker. The deletion cassettes were amplified from either plasmid pUG66 or pUG74, respectively

(Gueldener et al., 2002) using primers listed in Table 2. The primers contained at their 5' terminal end a 60-bp sequence complementary to the region immediately upstream or downstream of the start or stop codon of the gene to be deleted. Transformation of *S. cerevisiae* with deletion cassettes was performed according to the lithium acetate method described by Gietz et al. (1995). Double and/or triple deletions were achieved either by consecutive transformations of the respective deletion cassettes or else by first removing the existing marker according to Sauer and Henderson (1988) and then transform *S. cerevisiae* with the respective deletion cassette.

Table 2. Primers used in this study.

Purpose	Primer number	Sequence (5'-3')
Deletion of *PYC1*	775	AGATAACAAAAGGAAAATCTCAGCCTCTCCCCTTCCTCTTAGACACCAGCTGAAGCTTCGTACGC
	776	AGATTCGGGTATATTATACATTATAAATGAGAACTAACCGGTCGCATAGGCCACTAGTGGATCTG
Deletion of *PYC2*	828	GGCACAGCCAGCTCTTCTGTGATTGGCAGAGAGGGGTCCTTCCAGCTGAAGCTTCGTACGC
	829	CATTAAGGAAGAAACACAGTTAGCACGTATTTCATCGAAAGCATAGGCCACTAGTGGATCTG
Deletion of *ICL1*	1189	AACAATTGAGAGAAAACTCTTAGCATAACATAACAAAAAGTCAACGAAAACCAGCTGAAGCTTCGTACGC
	1190	ATATACTTGTCAGGAAATGCCGGCAGTTCTAATGGTTAATCCTTGTCCGCATAGGCCACTAGTGGATCTG

2.4. Qualitative and quantitative analysis of glycerol growth

The qualitative and quantitative analysis of *S. cerevisiae* growth were conducted following the procedure described by Swinnen et al. (2013) with slight modifications. The main difference between the qualitative and the quantitative growth analysis was the use of the carbon source for pre- and intermediate cultures. For qualitative analysis, pre- and intermediate cultures were performed using glucose (for *icl1*Δ, *icl1*Δ *pyc1*Δ, and *icl1*Δ *pyc2*Δ mutants), glucose plus 0.5% aspartate (for the *icl1*Δ *pyc1*Δ *pyc2*Δ mutant) or ethanol (for *pyc1*Δ, *pyc2*Δ and *pyc1*Δ *pyc2*Δ mutants). For quantitative analyses the

respective cultivations were all performed using glycerol as the carbon source. In general, cells from a single colony were used to inoculate 3 mL of the synthetic medium in a 10 mL glass tube with the respective carbon source and this pre-culture was incubated at orbital shaking of 200 rpm and 30 °C for 24 h. The pre-culture was then used to inoculate 4 mL of the same medium in a 10 mL glass tube adjusting to an OD_{600} of 0.2. This culture, referred to as intermediate culture, was cultivated at the same conditions for 48 h. For qualitative analysis, the appropriate volume from the intermediate culture necessary to adjust an OD_{600} of 0.2 was centrifuged at 800 g for 5 min and the supernatant discarded. The cell pellet was then washed once by re-suspending the cells in synthetic glycerol medium, re-centrifuged and re-suspended in 4 mL of the same medium in a 10 mL glass tube. For quantitative analysis, the appropriate culture volume to adjust an OD_{600} of 0.2 was directly resuspended in 4 mL of the main culture medium in a 10 mL glass tube. The main cultures for qualitative growth analysis were incubated at orbital shaking of 200 rpm and 30 °C and growth relative to the reference strain was assessed at regular time intervals. For quantitative analysis, two aliquots (750 µL each) of the main culture were immediately transferred from the 10 mL glass tube to separate wells of a white Krystal TM 24-well clear bottom microplate (Porvair Sciences, Leatherhead, United Kingdom) and cultivated in the Growth Profiler 1152 (Enzyscreen, Haarlem, The Netherlands) at 30 °C and orbital shaking at 200 rpm. The growth profiler took a scan of the plate every 40 min. These scans were used to calculate the density of the cultures expressed as green values (G-values). Subsequently, the G-values were expressed as OD_{600} values (referred to as OD_{600} equivalents) using the following calibration curve: OD_{600} equivalent = $6.1761\ 10^{-8} * (G\text{-}value)^{3.4784}$.

3. Results and Discussion

To identify the anaplerotic reactions active on glycerol, we first focused on those routes which were known to be important on glucose and ethanol, i.e. pyruvate carboxylase and the glyoxylate cycle as mentioned in the introduction. To study the role of PYC (Fig. 1), we deleted *PYC1* and *PYC2* individually and in combination in our chosen reference strain. Growth of all constructed strains was first qualitatively assessed in liquid synthetic glycerol medium as described in Materials and Methods. As *pyc1/2* double deletion mutants are incapable of growth on glucose as the sole carbon source (Blazquez et al., 1995; Brewster et al., 1994; Stucka et al., 1991), pre-cultivations were performed in synthetic medium with ethanol. The reference strain as well as the *pyc1*Δ and *pyc2*Δ mutants were treated in the same way for a reliable comparison. The data (Table 3) show that both the *pyc1*Δ and *pyc2*Δ single and the *pyc1*Δ *pyc2*Δ double deletion strain were all able to grow in synthetic glycerol medium. Still, there was a difference with regard to the two *PYC* isogenes: mutants carrying a deletion of *PYC1* (either individually or in combination with the *PYC2* deletion) were negatively affected in their growth, while the *pyc2* single deletion strain did not show any reduction in growth compared to the reference strain. In order to quantify the strains' maximum growth rates, all mutants were pre-cultivated in synthetic glycerol medium. Main cultures were then adjusted for the same start optical density (600 nm) and the growth rates of all strains were afterwards determined as described in Materials and Methods. The results show that the deletion of *PYC2* (alone and in combination) indeed did not significantly affect growth on glycerol (Fig. 2A). In contrast, the deletion of *PYC1* remarkably reduced μ_{max} in all strains confirming the major importance of this enzyme for anaplerosis of the TCA cycle under these conditions (Fig. 2A). Our results match a previous finding showing that

PYC1 expression is highly upregulated on glycerol (Menéndez & Gancedo, 1998). The strong impact of the *PYC1* deletion on growth is very different from the situation on glucose. In fact, neither the individual deletion of *PYC1* nor of *PYC2* resulted in significant phenotypic changes on glucose (Stucka et al., 1991). In contrast to glycerol, it seems that the pyruvate carboxylase isoenzyme encoded by *PYC2* is able to effectively compensate a lack of Pyc1 activity when cells grow on glucose.

The fact that the *pyc1*Δ *pyc2*Δ double mutant strain still grows fairly well on glycerol clearly suggests that a metabolic route other than PYC significantly contributes to the replenishment of the TCA cycle intermediates on glycerol. The next hypothetical route tested was the glyoxylate cycle. This cycle, which is essential for growth on C2 compounds, could also serve as an anaplerotic route if carbon from pyruvate was channeled via the pyruvate dehydrogenase (PDH) bypass to form cytosolic acetyl Co-A (Fig. 1). We speculated that the metabolic flux via the PDH bypass might be relatively strong in glycerol. This idea is supported by the studies of Kito et al. (2016) and Dueñas-Sánchez et al. (2012) which reported a higher expression of the genes encoding for the enzymes that contribute to the PDH bypass such as Ald2, Ald3 and Acs1 either at the level of mRNA (Dueñas-Sánchez et al. 2012) or protein (Kito et al., 2016) in complex medium with glycerol in comparison to glucose. To verify this hypothesis, we abolished the activity of the glyoxylate cycle by deleting the key enzyme *ICL1* individually as well as in combination with the aforementioned *PYC1/2* deletions. The pre-cultivations to assess the qualitative growth of the mutant strains on glycerol could not be performed in synthetic ethanol medium because an active glyoxylate cycle is indispensable for growth on C2 carbon sources such as ethanol and acetate. Instead, synthetic glucose medium was used for the pre-cultivation of the *icl1*Δ, *pyc1*Δ *icl1*Δ and *pyc2*Δ *icl1*Δ

mutants and, for the *pyc1*Δ *pyc2*Δ *icl1*Δ mutant, the pre-cultivation medium was supplemented with aspartate. The data in Table 3 shows that all strains except the *pyc1*Δ *pyc2*Δ *icl1*Δ triple deletion mutant grow in synthetic glycerol medium. Further quantitative analysis of the first three strains on glycerol (with pre-cultivations on glycerol) showed that the deletion of *ICL1* only marginally reduced the growth rate of the reference strain, but it did not significantly reduce growth in the *pyc2*Δ deletion mutant (Fig. 2B). Interestingly, the same *ICL1* deletion in a *pyc1*Δ background significantly reduced the growth rate by additional 20% (Fig. 2B). The first conclusion drawn from these results is that the glyoxylate cycle is not crucial during growth on synthetic glycerol medium. This is based on the fact that the impact on growth of the sole deletion of *ICL1* was only marginal and in clear contrast to growth on ethanol. The second conclusion is that both the Pyc2 as well as the glyoxylate cycle seem to be able to replenish the TCA cycle when Pyc1 is absent (Fig. 2). This was based on the findings that i) the triple deletion mutant (*pyc1*Δ *pyc2*Δ *icl1*Δ strain) did not grow at all on glycerol, and ii) the deletion of neither *ICL1* nor *PYC2* in a *pyc1*Δ mutant strain led to growth abolishment. Another conclusion that can be drawn from our result with the triple deletion mutant (*pyc1*Δ *pyc2*Δ *icl1*Δ strain) is that there seems to be no anaplerotic route of major importance other than PYC and the glyoxylate cycle in *S. cerevisiae* growing on glycerol, and both can partly take over the function of being the main anaplerotic reaction in the absence of the other route. The latter result also implies that phosphoenolpyruvate carboxykinase (*PCK1*), which reversibly converts phosphoenolpyruvate to oxaloacetate, does not seem to be crucial as an anaplerotic route on glycerol. This finding is in line with previous reports where PEPCK is generally

considered a decarboxylating enzyme with a function in gluconeogenesis (De Torrontegui et al., 1966).

In the light of our results, it is interesting to note that there is a previous report about a *pyc1*Δ *pyc2*Δ double deletion mutant that was able to grow on glucose after mutagenesis. It turned out that the respective mutant showed a derepression of the glyoxylate cycle which allowed to overcome the necessity of supplementing the medium with C4 compounds (Blazquez et al., 1995). This metabolic situation resembles the one we assume for our strain growing on glycerol, a carbon source that does not show catabolite repression of the glyoxylate cycle. Similarly, the *pyc1*Δ *pyc2*Δ double deletion mutant was also able to grow on pyruvate (C3) but it did not grow on fructose (C6) and galactose (C6) as sole carbon source (data not shown).

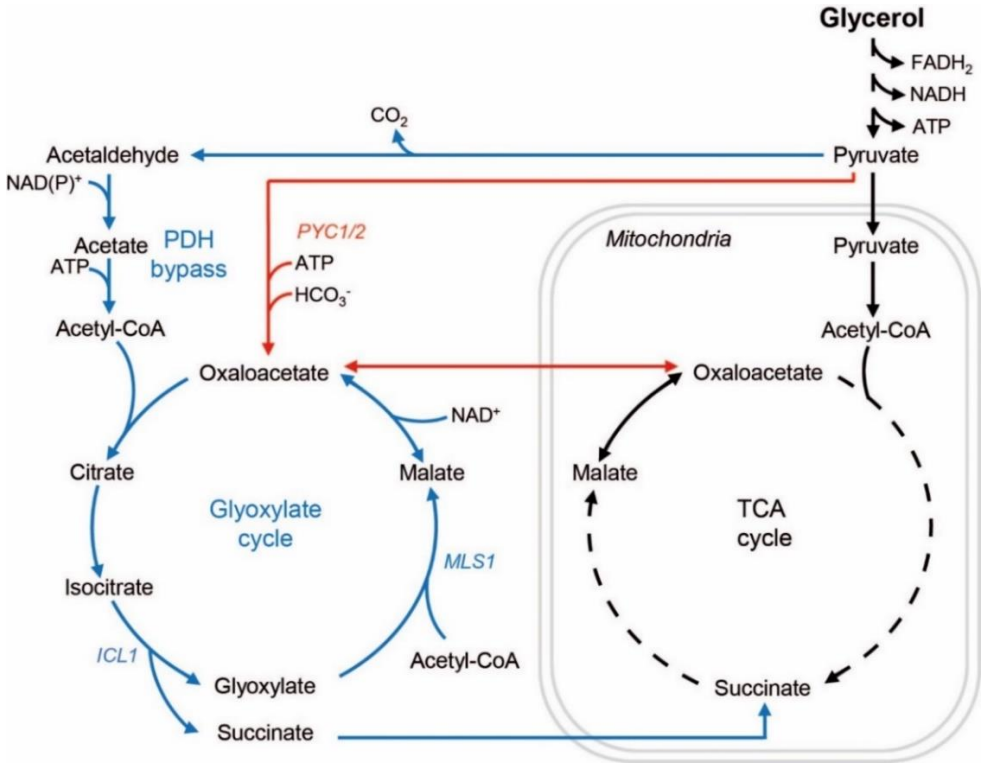

Fig. 1. The two major anaplerotic routes in *S. cerevisiae* during growth on pure synthetic glycerol medium, i) pyruvate carboxylase shown in red and (ii) the glyoxylate cycle in combination with the pyruvate dehydrogenase (PDH) bypass shown in blue. Abbreviations: *PYC1/PYC2*, pyruvate carboxylase; *ICL1*, isocitrate lyase; *MLS1*, malate synthase.

Table 3. Growth assessment of CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$* derivatives carrying individual and combined deletions in the genes *PYC1, PYC2* and *ICL1*. *PYC1* and *PYC2* code for isoforms of PYC, while *ICL1* encodes isocitrate lyase, a key enzyme of the glyoxylate cycle. The experiments were carried out in synthetic medium containing 6% (v/v) glycerol as the sole carbon source. As the deletion mutants have specific growth deficits on different carbon sources, different media were used for the pre-cultivations.

Deletion(s)	Growth in synthetic glycerol medium
-	++
*pyc1*Δ[1]	+
*pyc2*Δ[1]	++
*pyc1*Δ *pyc2*Δ[1]	+
*icl1*Δ[2]	++
*pyc1*Δ *icl1*Δ[2]	+
*pyc2*Δ *icl1*Δ[2]	++
*pyc1*Δ *pyc2*Δ *icl1*Δ[3]	-

[1] Precultivations were conducted in synthetic ethanol medium
[2] Precultivations were conducted in synthetic glucose medium
[3]Precultivations were conducted in synthetic glucose medium supplemented with aspartate
++ Growth comparable to parent strain
+ Significantly reduced growth
- No growth

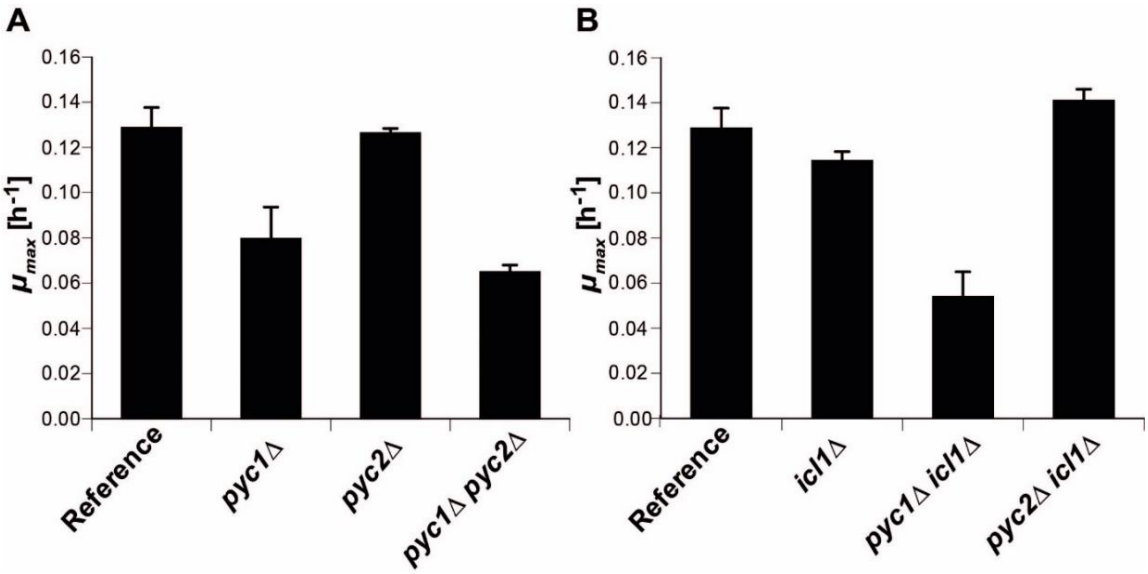

Fig. 2. μ_{max} of *S. cerevisiae* strain CEN.PK113-1A *UBR2$_{CBS}$ GUT1$_{JL1}$* and its derivatives carrying individual or combined deletions in the isogenes encoding for pyruvate carboxylase (*PYC1* and *PYC2*) (A) as well as in the gene *ICL1* (encoding isocitrate lyase a key enzyme of the glyoxylate cycle) alone or in combination with a deletion of *PYC1* or *PYC2* (B). Pre-cultures, intermediate and main cultures were performed in synthetic medium containing 6% (v/v) glycerol as the sole carbon source (SMG). Mean values and standard deviations were determined from at least three biological replicates.

As reviewed by Klein et al. (2017), *S. cerevisiae* naturally catabolizes glycerol via the so-called L-G3P pathway. We previously replaced the endogenous L-G3P pathway by an artificial NAD^+-dependent pathway referred to as 'DHA pathway' (Klein et al., 2016). This modification was meant to allow the entrapment of all electrons originating from glycerol in the form of soluble cytosolic NADH (instead of enzyme-bound $FADH_2$). We also tested some of the above-mentioned deletions in a DHA pathway strain background in order to find out whether the results are similar to those obtained with the strain CEN.PK113-1A $UBR2_{CBS}$ $GUT1_{JL1}$. At least, we have been able to show so far that individual deletions of the genes *PYC1*, *PYC2* and *ICL1* led to a similar growth reduction compared to the respective control strain (data not shown).

Glycerol is an attractive carbon source in industrial biotechnology due to its relatively high degree of reduction and its liquid nature. Moreover, it is a major by-product during biodiesel production. Our current study shows that *S. cerevisiae* shows a higher metabolic flexibility with regard to the use of anaplerotic routes on glycerol. This is different from glucose and ethanol where only one route is used and the abolishment of the respective route leads to a growth defect. Although Pyc1 seems to be the most important anaplerotic enzyme, alternative routes, i.e. Pyc2 and the glyoxylate cycle (in combination with the PDH bypass) contribute to this function.

4. Funding

We thank the German Research Foundation (DFG) for funding of our research (NE 697/7-1).

5. Acknowledgements

We thank Solvejg Sevecke for technical support.

6. Conflict of interest

The authors declare no competing interest.

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3.5 Manuscript III

An engineered reductive pathway and endogenous central metabolic pathways synergistically enhance succinic acid production using glycerol as carbon source in *Saccharomyces cerevisiae*

Joeline Xiberras[1], Mathias Klein[1], and Elke Nevoigt[1*]

[1] Department of Life Sciences and Chemistry, Jacobs University Bremen gGmbH,

Campus Ring 1, 28759 Bremen, Germany

* Corresponding author:

E-mail: e.nevoigt@jacobs-university.de

Short title: Succinic acid from glycerol in yeast

Abstract

The previously constructed *S. cerevisiae* strain *UBR2$_{CBS}$*-DHA is an engineered CEN.PK derivative that uses the synthetic dihydroxyacetone (DHA) pathway for glycerol catabolism. In theory, the additional cytosolic NADH provided by this pathway allows the establishment of a redox-neutral pathway from glycerol to succinic acid (SA). The genetic modifications for reductive SA production (`SA module´) established in *UBR2$_{CBS}$*-DHA comprise the overexpression of a cytosolic form of the endogenous peroxisomal malate dehydrogenase (*MDH3*-R), the heterologous expression of fumarase (*fumR*) from *Rhizopus oryzae* and a cytosolic form of fumarate reductase (*FRD*g-R) from *Trypanosoma brucei*. The integration of the `SA module´ together with the additional expression of the heterologous dicarboxylic acid transporter *An*DCT-02 resulted in a maximum titre of 10.7 g/L corresponding to a yield of 0.22 ± 0.01 g/g glycerol. Interestingly, the sole expression of the transporter *An*DCT-02 in strain *UBR2$_{CBS}$*-DHA increased the SA level from 0.4 to 3.2 g/L. This observation together with the fact that *ICL1* deletion decreased SA production strongly suggests that enzymes of the cytosolic `SA module´, the pyruvate dehydrogenase bypass and the endogenous glyoxylate cycle act together in a highly synergistic manner for SA production in the strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02. Deletion of *SDH1* encoding a mitochondrial succinate dehydrogenase subunit further increased the maximum SA yield to 0.43 ± 0.02 g/g. As all experiments have been conducted in shake-flask cultivations without pH control or carbon dioxide sparging so far, it can be assumed that there is much room for further improvements by bioprocess engineering.

KEYWORDS: Glycerol, Biodiesel, NADH, Succinic acid, Metabolic engineering

1. Introduction

Succinic acid (SA) has been traditionally used as surfactant, ion chelator additive in agriculture and food, as well as in pharmaceuticals (Ahn et al., 2016). Apart from these traditional applications, SA has nowadays been considered one of the most promising platform chemicals that can be produced from renewable resources (Pinazo et al., 2015). In fact, SA can be converted to a large number of chemicals and solvents including 1,4-butanediol, gamma-butyrolactone, and tetrahydrofuran (Ahn et al., 2016; Werpy and Petersen, 2004).

Certain bacteria isolated from rumen such as *Mannheimia succiniciproducens* (Lee et al., 2002; Scholten and Dägele, 2008) and *Actinobacillus succinogenes* (Guettler et al., 1999) naturally secrete significant amounts of SA into the medium. However, these organisms are auxotrophic for several vitamins and amino acids (Beauprez et al., 2010), and the use of complex media is not feasible for economic production of bulk products. Other attempts applied rational metabolic pathway engineering to well-established model bacteria such as *Escherichia coli* and *Corynebacterium glutamicum* (Jantama et al., 2008; Song and Lee, 2006). Although relatively high titres and productivities have been reported in these two organisms (Jantama et al., 2008; Okino et al., 2008), bacteria must be generally considered to be sub-optimal production hosts for SA since they are susceptible to bacteriophage infections and show low tolerance towards acidity (Los, 2012). The need to maintain the pH at a neutral level requires cumbersome downstream processing and thereby generates excessive amounts of unwanted by-products as discussed by Jansen and van Gulik (2014).

Fungal organisms are highly tolerant to low pH values and have therefore been considered ideal cell factories for the production of organic acids (Ahn et al., 2016;

Jansen and van Gulik, 2014; Raab et al., 2010). The yeast species *Saccharomyces cerevisiae* and *Pichia kudriavzevii* (*Issatchenkia orientalis*) have already been successfully engineered for commercial SA production, e.g. by the companies Reverdia and Bioamber as reviewed by Ahn et al. (2016). Notably, all attempts to produce SA in *S. cerevisiae* have been until now based on glucose as the carbon source.

Accordingly, Raab and Lang (2011) discussed three general metabolic routes that could be exploited for SA production in microorganisms including yeasts: i) the oxidative and ii) the reductive branch of the tricarboxylic acid (TCA) cycle as well as iii) the glyoxylate cycle. In order to attempt overproduction of SA in *S. cerevisiae* on glucose, all of these pathways have already been targeted by metabolic engineering as reviewed by Ahn et al. (2016). Significant SA production in yeast via the oxidative TCA branch has for example been established in strains that harbour deletions of genes encoding for succinate dehydrogenase subunits (*SDH1*, *SDH2*, *SDH3* and/or *SDH4*) (Ito et al., 2014; Kubo et al., 2000; Raab et al., 2010). In order to also redirect the carbon flux into the glyoxylate cycle, genes encoding for isocitrate dehydrogenase (*IDH1* and *IDP1*) have been deleted in addition to *SDH1* and *SDH2* (Raab et al., 2010). The production of SA via the reductive branch of the TCA cycle has been reported by Yan et al. (2014) who established the respective enzymes in the cytosol of a strain that has previously been engineered for abolishment of alcoholic fermentation (Maris et al., 2004). The authors overexpressed *PYC2* encoding for pyruvate carboxylase, retargeted the peroxisomal malate dehydrogenase (*MDH3*) to the cytosol, as well as (over)expressed the fumarase from *E. coli* (*fumC*) and the endogenous fumarate reductase (*FRD1*).

Compared to the other two routes, the reductive branch of the TCA cycle allows the highest possible maximum theoretical yield since it is accompanied by the net fixation

of carbon dioxide (Beauprez et al., 2010). The latter pathway has been established in cytosol of *S. cerevisiae* for the commercial bio-based production of SA by Reverdia using glucose as the sole carbon source (Van de Graaf et al., 2015, Patent No. US20150057425A1). The same study also demonstrated that the expression of a dicarboxylic acid transporter from *Aspergillus niger* (encoded by *An*DCT-02) is crucial for obtaining high SA titres and yields. This transporter obviously allows an efficient export of the overproduced SA into the medium (Jansen et al., 2017, Patent No. US9624514B2).

The production of SA from glucose via the cytosolic reductive branch of the TCA cycle is still not optimal since glucose catabolism does not deliver sufficient cytosolic NADH. Compared to glucose, glycerol has a higher degree of reduction (4.667 versus 4) allowing a higher maximum theoretical yield when calculated in g/g substrate consumed (i.e. 1.28 g/g glycerol versus 1.12 g/g glucose). Apart from its reducing power, glycerol is abundant because it is generated as a by-product of the biodiesel industry (app. 10 % (w/v) (Clomburg and Gonzalez, 2013).

The conversion of glycerol and carbon dioxide to SA results in a redox-neutral pathway provided that the so-called DHA pathway is used for glycerol catabolism (Fig. 1). This pathway saves two electrons from glycerol oxidation in the form of cytosolic NADH while the natural glycerol catabolic pathway, the L-glycerol 3-phosphate (L-G3P) pathway transfers the respective electrons via $FADH_2$ to the respiratory chain. Wild-type *S. cerevisiae* strains solely use the latter pathway for glycerol catabolism. Therefore, our group has successfully aimed at replacing the native L-G3P pathway by the DHA pathway in a previous work (Klein et al., 2016).

The goal of the current work was to establish the cytosolic reductive branch of the TCA cycle (referred to as `SA module´) and the *An*DCT-02 transporter in a previously generated DHA-pathway strain of *S. cerevisiae* and analyse the performance of the resulting strain for SA production from glycerol. Apart from this, a major focus was to scrutinize the role of the glyoxylate cycle for SA production from glycerol in engineered *S. cerevisiae* equipped with the `SA module´. It has been shown that the key enzymes of the glyoxylate cycle are highly upregulated at the transcriptional level on glycerol compared to glucose (Roberts and Hudson, 2006).

2. Materials & Methods

2.1. Strains and maintenance

The strains used in this study are listed in Table 1. Yeast cells were routinely grown on solid YPD medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 15 g/L agar. Agar plates were cultivated in a static incubator at 30 °C. Media were supplemented with phleomycin (20 mg/L), hygromycin B (300 mg/L) or nourseothricin (100 mg/L) for selection purposes when needed. *E. coli* DH5α was used for plasmid construction and isolation, and cells were routinely grown in lysogeny broth (LB) containing 10 g/L NaCl, 5 g/L yeast extract, 10 g/L peptone and adjusted to a pH of 7.5 with 2 M NaOH (Bertani, 1951). For selection and maintenance of plasmid containing cells, 100 mg/L ampicillin was added.

Table 3. *S. cerevisiae* strains used in this study.

Strain/modules	Genotype and genome modifications	Source or reference
CEN.PK113-1A	*MAT*a	Mating type switched as described by Ho et al. (2017)
***UBR2$_{CBS}$*-DHA**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}	Klein et al. (2016)
SA	*MATα;* YGLCτ3::P_{PGK1}-*MDH*3-R-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}	This study
***UBR2$_{CBS}$*-DHA-SA**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH*3-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}	This study
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH*3-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}	This study
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02 *sdh1*Δ**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH*3-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}; *sdh1*::*loxP-ble-loxP*	This study
***UBR2$_{CBS}$*-DHA-*An*DCT-02**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}	This study
***UBR2$_{CBS}$*-DHA-*An*DCT-02 *icl1*Δ**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}; *icl1*::*loxP-ble-loxP*	This study
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02 *icl1*Δ**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$; gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH*3-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}; *icl1*::*loxP-ble-loxP*	This study
***UBR2$_{CBS}$*-L-G3P**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; YGLCτ3::P_{TEF1}-*CjFPS1*-T_{CYC1}	Swinnen et al. (2016)
***UBR2$_{CBS}$*-L-G3P-SA-*An*DCT-02**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; YGLCτ3::P_{TEF1}-*CjFPS1*-T_{CYC1}; YPRCτ3::P_{PGK1}-*MDH*3-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}-P_{ENO2}-*AnDCT-02*-T_{DIT1}	This study

2.2. General molecular biology techniques

Preparative PCRs for cloning as well as for sequence determination of expression cassettes integrated in to the genome were performed using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Frankfurt am Main, Germany). PCR conditions

were adapted to the guidelines of the respective manufacturer. Restriction enzymes, FastAP alkaline phosphatase and T4 DNA ligase were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and used according to the manufacturer's instructions. PCR products were purified by using the GeneJET PCR Purification Kit (Thermo Fischer Scientific) and DNA fragments obtained after restriction were excised and purified using the QIAquick Gel Purification Kit (Qiagen, Hilden, Germany). Transformation of *S. cerevisiae* with plasmids as well as expression cassettes was performed according to the lithium acetate method described by Gietz et al. (1995).

2.3. Construction of expression cassettes for genomic integration

The plasmids used within this study are listed in Table S1. Codon-optimized coding sequences for *S. cerevisiae MDH3*, *Rhizopus oryzae fumR*, *Trypanosoma brucei FRDg*, and *Aspergillus niger* DCT-02*,* were kindly provided by Royal DSM N.V. (Delft, The Netherlands). Mdh3 and Frd were targeted to the cytosol by removal of the peroxisomal targeting signal (SKL) from the protein, hereafter referred to as *MDH3-R* and *Tb*FRD*g-R*, respectively (Van de Graaf et al., 2015, Patent No. US20150057425A1). The cassettes for expression of *MDH3-R* under the control of the *PGK1* promoter and the *IDP1* terminator, *RofumR* under the control of the *TEF1* promoter and the *RPL15A* terminator, *TbFRDg-R* under the control of the *TDH3* promoter and the *CYC1* terminator, and *An*DCT-02 under the control of the *ENO2* promoter and the *DIT1* terminator, were assembled in pUC18 using Gibson isothermal assembly (Gibson et al., 2009). All primers used for amplification of the respective promoters, coding sequences and terminators are listed in Table S2. All promoters and terminators were amplified from genomic DNA isolated from the *S. cerevisiae* strain S288C. One-step isothermal DNA assembly reactions contained 15 µL of the reagent-enzyme mix as described by Gibson et al. (2009),

0.05 pmol of BamHI linearized pUC18 and 3-fold excess of the inserts (promoter, coding sequence and the terminator (each 0.1 pmol) in a final volume of 20 µL. Reaction mixtures were incubated at 50 °C for 1 h and subsequently 5 µL of the reaction were directly used for transformation of *E. coli* DH5α. The resulting vectors were named pUC18-*MDH3-R*, pUC18-*RofumR*, pUC18-*TbFRDg-R*, and pUC18-*An*DCT-02 (Table S1).

2.4. Plasmids for CRISPR-Cas9 mediated genome editing in *S. cerevisiae*

For CRISPR-Cas9 mediated genome editing, the vectors p414-TEF1p-Cas9-CYC1t-nat1 (Klein et al., 2016), p426-SNR52p-gRNA.YGLCτ3-SUP4t-hphMX (Islam et al., 2017) and p426-SNR52p-gRNA.YPRCτ3-SUP4t-hphMX were used (Table S1). The vector p426-SNR52p-gRNA.YPRCτ3-SUP4t-hphMX was constructed from p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX (Klein et al., 2016) by exchanging the 20 nt sequence at the 5' end of the expressed gRNA targeting the *S. cerevisiae CAN1* gene by a 20 nt sequence targeting the long terminal repeat YPRCτ3 on chromosome XVI (Flagfeldt et al., 2009). The new target sequence was inserted by generating two overlapping PCR products of the gRNA expression cassette and their subsequent assembly in the same vector backbone according to Gibson et al. (2009). The CRISPRdirect online tool developed by Naito et al. (2015) was used to select the target sequence within the YPRCτ3 locus. Primers 463 and 596 and 460 and 597 (Table S2) were used to amplify the overlapping fragments of the gRNA expression cassette from p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX. Primers 460 and 463 generated overlaps to the ends of the PvuII linearized p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX while primers 596 and 597 generated the overlaps containing the YPRCτ3 targeting sequence. The PvuII linearized p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX was gel-purified after restriction. Gibson isothermal

assembly was done as described in section 2.3 yielding p426-SNR52p-gRNA.YPRCτ3-SUP4t-hphMX.

2.5. *S. cerevisiae* strain construction

General strategies for genomic integrations by employing the CRISPR-Cas9 system

For genomic integrations the long terminal repeats YGLCτ3 and YPRCτ3 on chromosomes VII and XVI were used (Flagfeldt et al., 2009). Expression of Cas9 in the desired strain was achieved by transformation with the plasmid p414-TEF1p-Cas9-CYC1t-nat1 (Table S1). In all strains the `SA module´ was integrated at the YGLCτ3 locus (with the help of gRNA expression from p426-SNR52p-gRNA.YGLCτ3-SUP4t-hphMX). The expression cassette for *An*DCT-02 was integrated either at the YPRCτ3 locus (using p426-SNR52p-gRNA.YPRCτ3-SUP4t-hphMX for gRNA expression) in strains *UBR2*$_{CBS}$-DHA-*An*DCT-02 and *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 or together with the `SA module´ at the YGLCτ3 locus in strain *UBR2*$_{CBS}$-L-G3P-SA-*An*DCT-02. The expression cassettes were PCR-amplified from the plasmids carrying the respective expression cassettes using the primer pairs listed in Table S2. These primers contained 5'-extensions generating 40–60 bp sequences homologous to regions directly upstream and downstream of the inserted double strand break at the integration site or to the respective adjacent expression cassette (in case several cassettes were assembled at the same locus). Co-transformation of the *S. cerevisiae* strain expressing the Cas9 endonuclease with the expression cassettes and the respective vector for gRNA expression resulted in assembly and integration of all expression cassettes at the target locus. Positive transformants were selected on YPD agar containing both nourseothricin and hygromycin B. Both vectors were subsequently removed from the resulting clone by serial transfers in YPD

medium lacking the respective antibiotics yielding the desired strain. Subsequently, all integrated expression cassettes were sequenced.

Deletions of either *SDH1* or *ICL1* were obtained using a disruption cassette consisting of the phleomycin resistance marker (*ble*^{r}) flanked by regions complementary to the upstream and downstream regions of the respective gene to be deleted. The deletion cassettes were amplified from plasmid pUG66 (Table S1) using primers 1243 and 1244 for *SDH1* deletion and primers 1189 and 1190 for *ICL1* deletion (Table S2). The primers contained at their 5' terminal end a 60-bp sequence complementary to the region immediately upstream or downstream of the start or stop codon of the gene to be deleted.

Strain SA

The expression cassettes for *MDH3-R*, *RofumR* and *TbFRDg-R* were PCR-amplified from the plasmids pUC18-*MDH3-R***,** pUC18-*RofumR* and pUC18-*TbFRDg-R* using primers pairs 690/770, 771/590, and 591/772, respectively (Tables S1 and S2). Subsequently, they were integrated at the YGLCτ3 locus of strain CEN.PK113-1A (*MATa*), by employing the CRISPR-Cas9 system, yielding strain SA. Strain CEN.PK113-1A (*MATa*) was obtained by switching the mating type of strain CEN.PK113-1A from *MAT*α to *MATa* using the same strategy as described in Ho et al. (2017).

Strain UBR2$_{CBS}$-DHA-SA

In order to bring the `SA module´ into strain *UBR2*$_{CBS}$-DHA (CEN.PK 113-1A *UBR2*$_{CBS\ 6412\text{-}13A}$ *gut1::Opgdh-DAK1-CjFPS1-DAK1*$_{OE\text{-}2}$ in Klein *et al*., 2016), the two strains were mated as shown in Fig. S1. This strategy was used to avoid homologous recombination events between the promoters and terminators, which were used for the generation of the

expression cassettes to be integrated, with those already present in strain *UBR2$_{CBS}$*-DHA upon transformation.

Mating, sporulation, and tetrad analysis were performed by procedures described by Sherman and Hicks (1991). Tetrads were dissected by using the micromanipulator from Singer Instruments co Ltd (Roadwater Watchet Somerset, UK). Mating types were determined by diagnostic PCR for the *MAT* locus (Huxley et al., 1990). Mating type PCR was performed to verify haploidy of the obtained segregants while the presence of the `DHA´ and `SA modules´ was verified by diagnostic PCR and sequencing. Afterwards, the *UBR2* in the respective haploid strain was sequenced to verify that the haploid strains contained the *UBR2* allele from CBS 6412-13A (*UBR$_{CBS}$*) (Swinnen et al., 2016).

Strains UBR2$_{CBS}$-DHA-AnDCT-02 and UBR2$_{CBS}$-DHA-SA-AnDCT-02

The expression cassette for *An*DCT-02 was PCR-amplified from the plasmid pUC18-*An*DCT-02 using primers pair 871/872 (Tables S1 and S2). The cassette was then integrated at the YPRCτ3 locus of strains *UBR2$_{CBS}$*-DHA and *UBR2$_{CBS}$*-DHA-SA, respectively, by employing the CRISPR-Cas9 system as described above, yielding strains *UBR2$_{CBS}$*-DHA-*An*DCT-02 and *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02.

Strain UBR2$_{CBS}$-L-G3P-SA-AnDCT-02

The three expression cassettes (*MDH3-R*, *RofumR* and *TbFRDg-R*) of the `SA module´ and the expression cassette for *An*DCT-02 were amplified from the plasmids pUC18-*MDH3-R***,** pUC18-*RofumR***,** pUC18-*TbFRDg-R* and pUC18-*An*DCT-02 using primers pairs 991/ 770, 771/590, 591/992, and 993/872, respectively (Table S1 and S2). All four expression cassettes for the `SA module´ and *An*DCT-02 were integrated at the YGLCτ3 locus of the previously generated strain CEN.PK113-1A *UBR2$_{CBS}$ CjFPS1* (Table 1) (Swinnen et al.,

2016) by employing the CRISPR-Cas9 system as described above, resulting in strain *UBR2*$_{CBS}$-L-G3P-SA-*An*DCT-02.

2.6. Isolation of genomic DNA from *S. cerevisiae* transformants and diagnostic PCR

Correct integration of all expression and disruption cassettes was verified by diagnostic PCR using OneTaq Quick-load DNA polymerase and buffer according to the manufacturer's guidelines (NEB). Genomic DNA was isolated according to a modified protocol from Hoffman and Winston (1987). Single colonies obtained after transformations were re-streaked on respective agar plates. Approximately 50 mg of cells from these plates were suspended in 200 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Subsequently, 300 mg of acid-washed glass beads (diameter of 0.425–0.6 mm) and 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) were added. The tubes were vortexed at maximum speed for 2 min and centrifuged at 15,700 g for 10 min. The aqueous phase (1 µL) was used as template in 20 µL PCR reactions. PCR primers were designed to bind upstream and downstream of the genomic integration sites as well as within the integrated expression/deletion cassette. For analyzing integrations of multiple expression cassettes, additional primers were designed to produce amplicons covering the junctions between the individual integrated expression cassettes. After each integration step, the presence of all previously integrated expression cassettes was verified.

2.7. Media and cultivation conditions for the production of SA from glycerol

All pre-cultures were cultured in synthetic medium-containing 20 g/L glucose and ammonium sulfate as the carbon and nitrogen source, respectively. All experiments for assessing SA production in shake-flask batch cultivation were performed in synthetic medium-containing 60 mL/L glycerol as the sole carbon source with urea as the nitrogen

source. The synthetic medium was prepared according to Verduyn et al. (1992), 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 15 mg/L EDTA, 4.5 mg/L $ZnSO_4.7H_2O$, 0.84 mg/L $MnCl_2.2H_2O$, 0.3 mg/L $CoCl_2.6H_2O$, 0.3 mg/L $CuSO_4.5H_2O$, 0.4 mg/L $NaMoO_4.2H_2O$, 4.5 mg/L $CaCl_2.2H_2O$, 3 mg/L $FeSO_4.7H_2O$, 1 mg/L H_3BO_3, and 0.1 mg/L KI. After heat sterilization of the medium, filter sterilized vitamins were added. Final vitamin concentrations were: 0.05 mg/L D-(+)-biotin, 1 mg/L D-pantothenic acid hemicalcium salt, 1 mg/L nicotinic acid, 25 mg/L myo-inositol, 1 mg/L thiamine chloride hydrochloride, 1 mg/L pyridoxine hydrochloride, and 0.2 mg/L 4-aminobenzoic acid. In case urea was used as the nitrogen source (in main culture media), an appropriate aliquot of a stock solution was added after autoclaving to obtain a final concentration of 2.8 g/L while 5 g/L ammonium sulfate was added (in pre-culture media) before heat sterilization. The pH of the synthetic glucose medium was adjusted to 6.5 with 4 M KOH, while that of synthetic glycerol medium was adjusted to 4.0 with 2 M H_3PO_4.

For pre-cultivation, cells from a single colony were used to inoculate 3 mL of the synthetic glucose medium in a 10 mL glass tube and incubated at orbital shaking of 200 rpm and 30 °C for 16 h. The pre-culture was used to inoculate 10 mL of the same medium in a 100 mL Erlenmeyer flask adjusting an OD_{600} of 0.2. This culture, hereafter referred to as intermediate culture, was cultivated at the same conditions for 48 h. The appropriate culture volume from the intermediate culture (in order to later adjust an OD_{600} of 0.2 in 100 mL of synthetic glycerol medium) was centrifuged at 800 g for 5 min and the supernatant discarded. The cell pellet was then washed once by re-suspending the cells in synthetic glycerol medium. The cell suspension was centrifuged again and re-suspended in 100 mL of the same medium in a 500 mL Erlenmeyer flask. The main

cultures were incubated at orbital shaking of 200 rpm and 30 °C and samples for OD_{600} determination and HPLC analysis were taken at regular time intervals.

2.8. Metabolite analysis by HPLC

Samples of culture supernatants (1 mL) were first filtered through 0.2 µm Minisart RC membrane filters (Sartorius, Göttingen, Germany) and if required stored at −20 °C until analysis. The concentrations of SA, glycerol and ethanol in culture media were determined using a Waters HPLC system (Eschborn, Germany) consisting of a binary pump system (Waters 1525), injector system (Waters 2707), the Waters column heater module WAT038040, a refractive index (RI) detector (Waters 2414) and a dual wavelength absorbance detector (Waters 2487). The samples were injected onto an Aminex HPX-87H cation exchange column (Biorad, München, Germany) coupled to a Micro-guard® column (Biorad) and eluted with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and a column temperature of 45 °C. Volumes of 20 µL of sample were used for injection. SA was detected using the dual wavelength absorbance detector (Waters 2487) while ethanol and glycerol were analysed with the RI detector (Waters 2414). The retention time for SA was 11.2 min, for glycerol 13.5 min and for ethanol 22.7 min. Data were processed and analyzed using the Breeze 2 software (Waters).

3. Results

3.1. Establishing `SA module´ in the cytosol of a DHA pathway strain of *S. cerevisiae*

In order to allow redox-neutral production of SA from glycerol via the `SA module´, all metabolic engineering steps were conducted in the genetic background of a CEN.PK113-1A derivative in which the native L-G3P pathway for glycerol catabolism had been replaced by the NAD-dependent DHA pathway (Klein et al., 2016). This baseline strain

named 'CEN.PK113-1A *UBR2$_{CBS}$* – DHA pathway module II' by Klein et al. (2016) and referred here to as strain *UBR2*-DHA carried the so-called DHA pathway module II comprising of cassettes for the expression of the NAD-dependent glycerol dehydrogenase from *Ogataea parapolymorpha (Opgdh)*, the overexpression of endogenous *DAK1* (two expression cassettes, each under the control of a different promoter) as well as the expression of the aquaglyceroporin Fps1 from *Cyberlindnera jadinii (CjFPS1)* for accelerated glycerol uptake (Table 1). All four expression cassettes had been integrated at the *GUT1* locus thereby abolishing the endogenous L-G3P pathway (Klein et al., 2016). The aforementioned genetic modifications will be referred to as module `DHA´ hereafter. In addition, the endogenous *UBR2* allele had been replaced in this CEN.PK derivative by the respective allele from the glycerol-utilizing wild-type isolate CBS 6412-13A (*UBR2$_{CBS}$*) as described by Swinnen et al. (2016). The latter modification proved to be crucial to achieve the reported maximum specific growth rate (μ_{max}) of ~0.26 h^{-1} in synthetic glycerol medium (Klein et al., 2016).

In order to establish the `SA module´ in the cytosol of strain *UBR2$_{CBS}$*-DHA, we (over)expressed the endogenous peroxisomal malate dehydrogenase (*MDH3*), which is responsible for oxaloacetate reduction, the heterologous cytosolic fumarase (*fumR*) from *Rhizopus oryzae* for conversion of malate to fumarate and the peroxisomal fumarate reductase (*FRD*g) from *Trypanosoma brucei* for fumarate reduction (Fig. 1). For targeting of Mdh3 and Frd to the cytosol, the peroxisomal targeting signals (SKL) were removed from the proteins (the respective genes will be referred to here as *MDH3-R* and *TbFRDg-R*) (Van de Graaf et al., 2015, Patent No. US20150057425A1). These genetic modifications concerning the `SA module´ from oxaloacetate to SA will be collectively referred to as 'SA' in the strain name throughout this study. The respective

strain was therefore named *UBR2*$_{CBS}$-DHA-SA. Subsequently, we included an expression cassette for the dicarboxylic acid transporter encoded by *An*DCT-02 resulting in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (see Materials and Methods for details). Notably, we did not abolish the ethanol formation pathway nor overexpressed *PYC2* as explained in the discussion.

An aspect to be considered when glycerol serves as the carbon source for SA production, is the fact that previously obtained transcriptome data (Roberts and Hudson, 2006) suggest a high activity of the glyoxylate cycle although evidence for the actual carbon flux is still not available (Xiberras et al., 2019). The glyoxylate cycle actually represents an alternative route to SA production in the constructed strain as further discussed in 3.2. We therefore constructed a control strain that only contained the `DHA module´ plus the expression cassette for *An*DCT-02, but no `SA module´ (*UBR2*$_{CBS}$-DHA-*An*DCT-02).

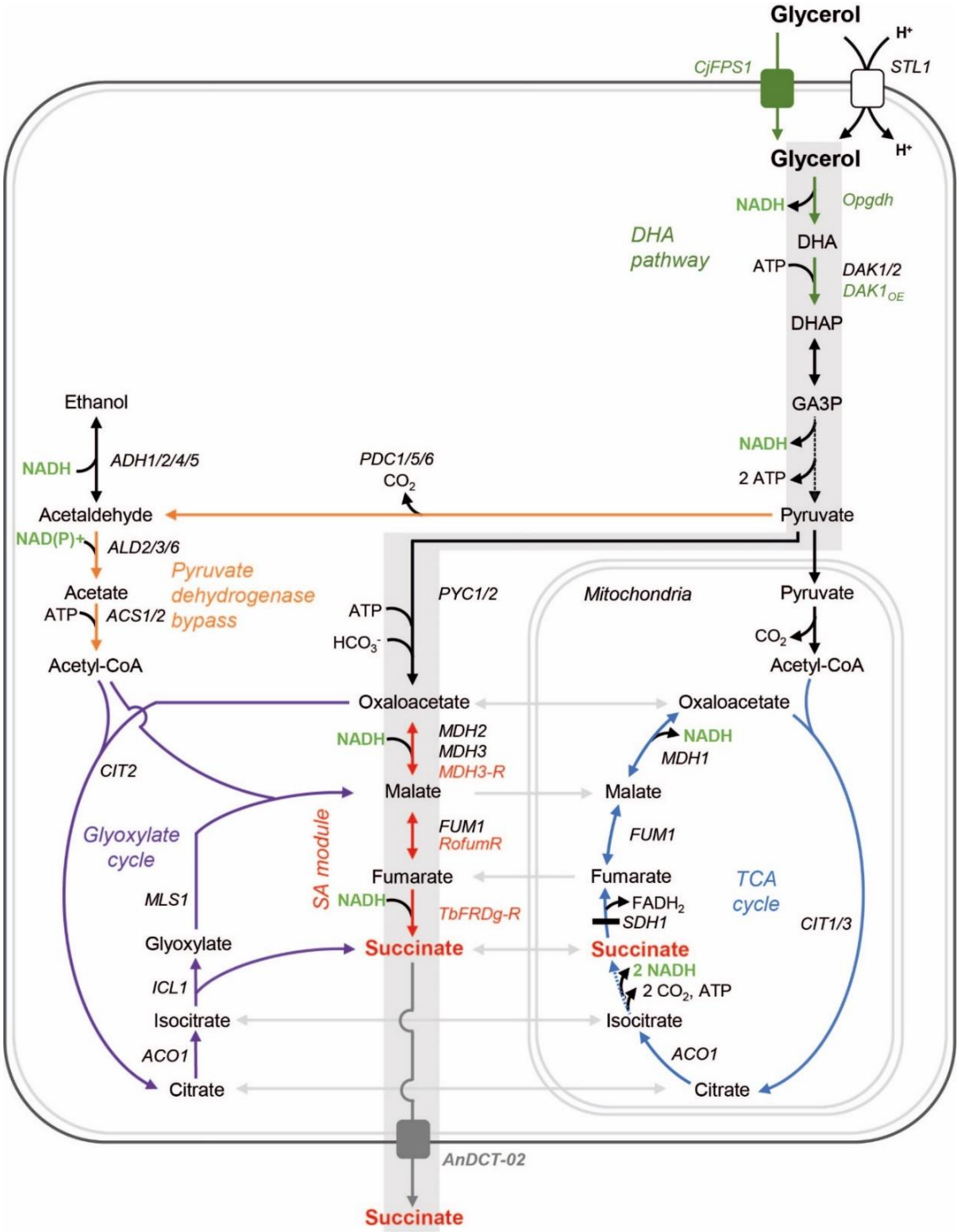

Fig. 1. Relevant metabolic pathways leading to SA formation in *S. cerevisiae* and the genetic modifications conducted for SA overproduction in the current study i.e. ´SA module´, *An*DCT-02 and *SDH1* deletion. Grey shading indicates the theoretical redox-balanced pathway from glycerol to SA. Abbreviations: DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde-3-phosphate, *STL1*: glycerol/H^+ symporter, *DAK1/2*: dihydroxyacetone kinase, *PYC1/2*: pyruvate carboxylase, *MDH1/MDH2/MDH3*: malate dehydrogenase, *FUM1*: fumarase, *CIT1/2/3*: citrate synthase, *ACO1*: aconitase, *SDH1*: subunit of succinate dehydrogenase, *ICL1*: isocitrate lyase, *MLS1*: malate synthase, *ACS1/2*: acetyl-coA synthetase, *PDC1/5/6*: pyruvate decarboxylase, *ADH1/2/4/5*: alcohol dehydrogenase, *ALD2/3/6*: aldehyde dehydrogenase, *CjFPS:* glycerol facilitator from *Cyberlindnera jadinii*; *Opgdh:* glycerol dehydrogenase from *O. polymorpha, MDH3-R*: peroxisomal malate dehydrogenase targeted to the cytosol, *RofumR: fumarase* from *Rhizopus oryzae, TbFRDg-R:* glycosomal fumarate reductase from *Trypanosoma brucei* retargeted to the cytosol*, An*DCT-02*:* dicarboxylic acid transporter from *A. niger.*

3.2 SA is produced from glycerol via the glyoxylate cycle and enzymes of the 'SA module' of the TCA cycle in a synergistic manner

The strains *UBR2*$_{CBS}$-DHA, *UBR2*$_{CBS}$-DHA-*An*DCT-02, *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (representing the strains with *ICL1* wild-type background in Fig. 2) were tested in shake flask experiments for SA production using synthetic glycerol medium. In contrast to the study of Klein et al. (2016), urea was used as a nitrogen source instead of ammonium sulfate. The metabolism of ammonia results in strong medium acidification (de Kok et al., 2012) which was assumed to be stressful for the cells, particularly since excretion of the weak acid SA was the goal.

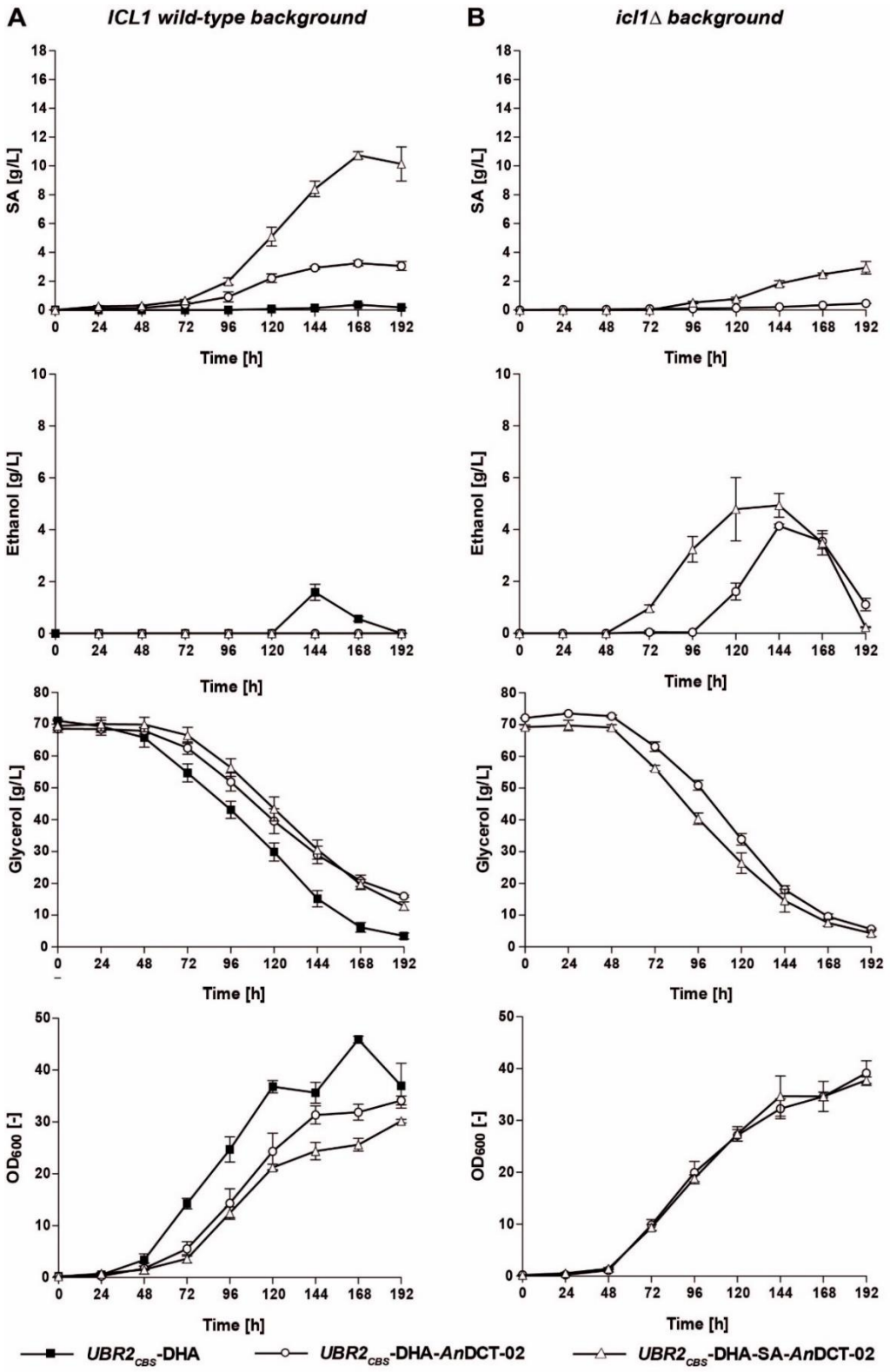

Fig. 2. SA production, biomass, ethanol and glycerol consumption in *S. cerevisiae* strains *UBR2*$_{CBS}$-DHA, *UBR2*$_{CBS}$-DHA-SA and *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (A) and in strains *UBR2*$_{CBS}$-DHA-*An*DCT-02 *icl1*Δ and *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 *icl1*Δ (B). Cultivations were performed in 500 mL shake flasks cultures containing 100 mL of synthetic medium containing 6% (v/v) glycerol as the sole carbon source and culture supernatants were analysed by HPLC for production of SA, ethanol, and the consumption of glycerol. Growth was recorded by optical density measurements at 600 nm. Mean values and standard deviations were determined from at least three biological replicates.

As expected, only marginal amounts of SA could be detected for the baseline strain *UBR2*$_{CBS}$-DHA (Fig. 2A – *ICL1* wild-type background). Instead, up to 1.6 g/L ethanol were produced. Ethanol formation from glycerol in *S. cerevisiae* seems to be caused by the DHA pathway in combination with the heterologous aquaglyceroporin as recently described by Asskamp et al. (2019). Interestingly, the sole expression of *An*DCT-02 in the baseline (DHA pathway) strain changed the metabolic profile up to 3.2 g/L SA was produced in strain *UBR2*$_{CBS}$-DHA-*An*DCT-02 while no ethanol could be detected in the cultivation medium (Fig. 2A). This result confirms that even without the `SA module´ the baseline strain was able to produce SA via an endogenous pathway when glycerol served as the carbon source. As expected, the establishment of the reductive branch of the TCA cycle in the cytosol by integration of the `SA module´ in the strain *UBR2*$_{CBS}$-DHA-*An*DCT-02 led to a remarkable increase of the SA titre up to 10.7 g/L (corresponding to a product yield of 0.216 ± 0.010 g/g). Interestingly, both strains expressing *An*DCT-02 (with and without the `SA module´) showed reduced growth and glycerol consumption when compared to the baseline strain *UBR2*$_{CBS}$-DHA (Fig. 2A).

In order to test whether the endogenous glyoxylate cycle is responsible for SA production in *UBR2*$_{CBS}$-DHA-*An*DCT-02 and also potentially contributes to SA formation in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02, we deleted *ICL1* encoding the key enzyme isocitrate lyase. In both *icl1*Δ mutants, SA formation was significantly reduced while ethanol (up to 4.9 g/L) became the predominant fermentation product (Fig. 2B). In strain *UBR2*$_{CBS}$-DHA-*An*DCT-02 *icl1*Δ, almost no SA was detectable in the medium throughout the entire cultivation (SA titres were reduced from ~3.2 g/L to ~0.3 g/L after 168 h), implying that SA is almost exclusively produced via the glyoxylate cycle route in the respective background strain *UBR2*$_{CBS}$-DHA-*An*DCT-02. Notably, the same deletion

of *ICL1* in the background of strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 resulted in a much stronger decline of SA production than expected suggesting a synergistic effect between the glyoxylate cycle and the 'SA module' (Fig. 2B). Growth and glycerol consumption were not negatively affected by the abolishment of the glyoxylate cycle (Fig. 2B). Surprisingly, the deletion of *ICL1* even seems to improve these measures in the shake flask cultures.

With regard to the proposed synergistic effect of the two pathways, a closer look at Fig. 1 reveals that 'SA module' and the glyoxylate cycle share crucial intermediates (malate and oxaloacetate). In order to check whether the malate generated from glyoxylate via the glyoxylate cycle by the activity of malate synthase (encoded by *MLS1*) was also converted to SA via the heterologous fumarase and fumarate reductase of the `SA module´, we constructed a strain lacking the *TbFRDg-R* expression cassette in the `SA module´ (strain *UBR2*$_{CBS}$-DHA-*MDH3-R-RofumR-An*DCT-02). Indeed, the absence of the last step of the `SA module´ had the same impact on SA production as the absence of the whole `SA module´ (Fig. S2). Taking the results from the *icl1*Δ mutants into account, it seems that the additional SA is produced via the lower part of the reductive branch of the TCA cycle and that carbon flux through the glyoxylate cycle is essential for this phenomenon.

SA production solely based on the `SA module´ would require more NADH compared to SA production via our proposed route (glyoxylate cycle with a contribution of *RofumR* and *TbFRDg-R* gene products) since the latter is independent of oxaloacetate conversion to malate by Mdh3-R. We therefore checked to which extent the replacement of the native FAD-dependent L-G3P pathway by the NADH delivering DHA pathway promotes SA overproduction. Implementation of the `SA module´ and *An*DCT-02 in a respective

CEN.PK113-1A derivative (catabolizing glycerol via the L-G3P pathway) resulted in strongly decreased SA formation as compared to the strain using the DHA pathway for glycerol catabolism (Fig. S3). In fact, SA titres were reduced to levels similar to those observed in *UBR2*$_{CBS}$-DHA-*An*DCT-02

(without the `SA module´) suggesting that the surplus of cytosolic NADH provided by the DHA pathway is crucial for the observed high SA levels in *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 and limits SA production in the strain catabolizing glycerol via the endogenous L-G3P pathway. The sole presence of the `SA module´ and *An*DCT-02 in a strain using the L-G3P pathway for glycerol catabolism resulted in much lower SA production compared to the same modifications carried out in a DHA pathway strain. Without the additional cytosolic NADH delivered by the DHA pathway, glycerol catabolism does not seem to cause the synergistic effect of the `SA module´ and the glyoxylate cycle on SA production.

3.3 Deletion of the endogenous succinate dehydrogenase gene *SDH1* further improved SA titer and yield

As long as *S. cerevisiae* exhibits an active mitochondrial TCA cycle, SA accumulated in the cytosol may enter the mitochondria via the transporters *SFC1* and *DIC1* as discussed by Xiberras et al. (2019) and be converted by succinate dehydrogenase to fumarate and further to oxaloacetate via the endogenous TCA cycle enzymes (Fig. 1). The abolishment of SDH activity might therefore favour SA accumulation and export by *An*DCT-02. We therefore decided to delete *SDH1* in our best SA producer *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02. Indeed, the resulting strain (*UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 *sdh1*Δ) showed faster SA production as well as faster growth and glycerol consumption during the first stage of cultivation (until 72 h) compared to strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (Fig. 3).

Surprisingly, growth and glycerol consumption ceased after this point in time. Still, the maximum SA titer and yield were significantly higher than those obtained with strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 and reached ~14.0 g/L and 0.43 ± 0.02 g/g glycerol consumed.

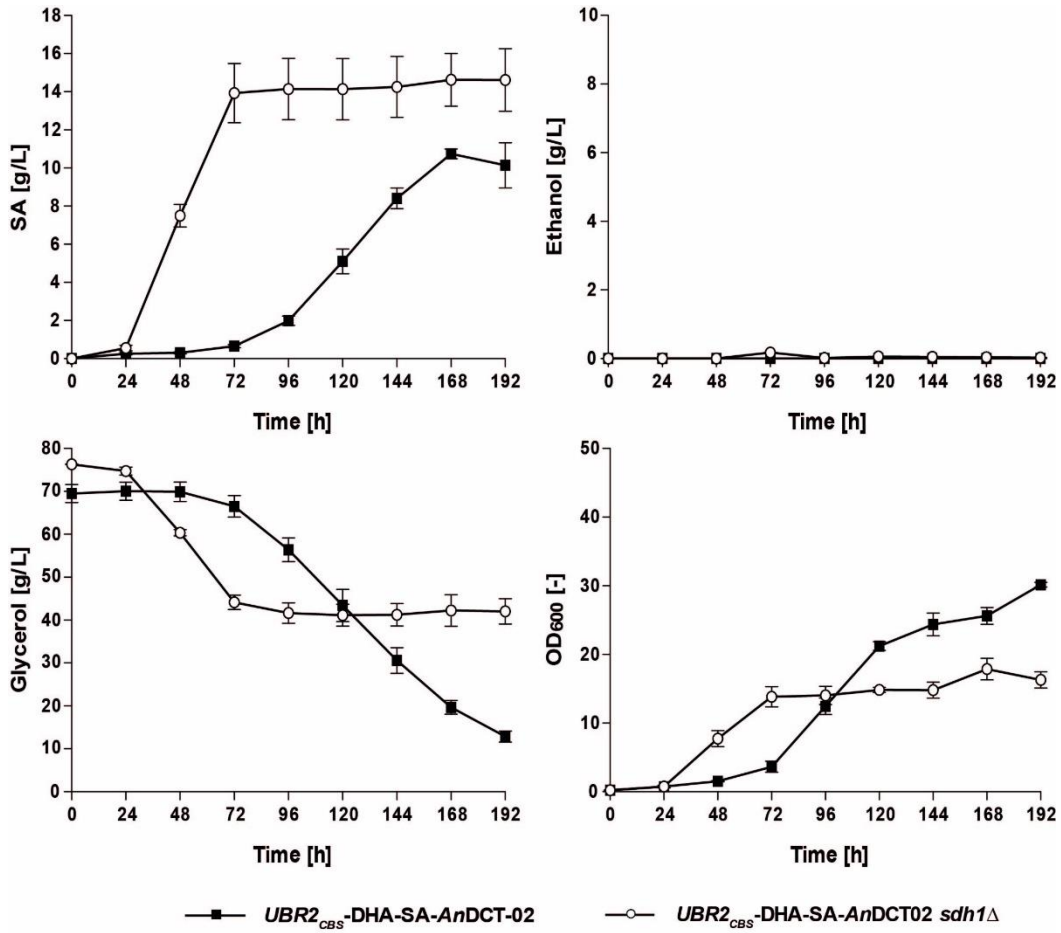

Fig. 3. Influence of *SDH1* deletion on SA production, ethanol production, glycerol consumption and growth in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02. Cultivations were performed in 500 mL shake flasks cultures containing 6% (v/v) glycerol as the sole carbon source and culture supernatants were analysed by HPLC. Growth was recorded by optical density measurements at 600 nm. Mean values and standard deviations were determined from at least three biological replicates.

4. Discussion

The initial goal of this study was to establish a pathway for redox-neutral production of SA from glycerol. For this purpose, expression cassettes encoding the enzymes for the `SA module´ were incorporated in the cytosol. The central `SA module´ was similar to

the genetic modifications used by other authors in order to establish SA production from glucose (Van de Graaf et al., 2015, Patent No. US20150057425A1). On glycerol, the respective `SA module´ also led to a significant improvement of SA production compared to the control strain (with *An*DCT-02 transporter but without the `SA module´). Still, the question arose whether the endogenous pathways such as the glyoxylate cycle and the oxidative branch of the TCA cycle also contribute to the SA produced from glycerol by our strains. As reviewed by Xiberras et al. (2019), it is generally assumed that the glyoxylate cycle and the mitochondrial TCA cycle are highly active on this carbon source in contrast to glucose. However, the respective assumptions only result from very few transcriptome studies conducted in (complex) glycerol-containing medium and it is well known that mRNA abundances do not always correlate with protein levels and, particularly in vivo enzyme activities. Interestingly, a parallel study conducted in our laboratory demonstrated that the deletion of *ICL1* in our baseline strain *UBR2*$_{CBS}$-DHA does not significantly affect growth in synthetic glycerol medium suggesting that this pathway is dispensable for growth on glycerol (Xiberras et al., manuscript in preparation). Still, this result did not exclude that the pathway might be active on glycerol. In fact, the results obtained during the current study suggest a very active flux through the glyoxylate cycle on glycerol. Abolishing the flux through the glyoxylate cycle by deleting *ICl1* in the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 reduced the maximum SA concentration by about 8.2 g/L (i.e. to ~2.5/g L). In addition, enzymatic activities of the `SA module´ contributed to SA production as SA titres increased from 3.2 g/L (in a strain without the reductive branch of the TCA cycle in the cytosol that only carries the *An*DCT-02 transporter) to 10.7 g/L SA in the isogenic strain with the `SA module´. A plausible explanation for the observed synergistic effect would be that the majority of the carbon

used for SA production in strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02 directly or indirectly originated from the pyruvate dehydrogenase bypass and the glyoxylate cycle (Fig. 1) and that the cytosolic conversion of oxaloacetate to malate was marginal. Apart from the SA that directly originates from the isocitrate lyase reaction, another significant portion of SA could be produced from the malate formed in the glyoxylate cycle (via malate synthase). This malate is presumably converted to fumarate and eventually to succinate by the (heterologous) cytosolic enzymes fumarase and fumarate reductase brought into the cell via the `SA module´ (Fig. 1).

Interestingly, both *icl1* deletion mutants constructed here (in the strain background of *UBR2$_{CBS}$*-DHA-*An*DCT-02 and *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02), showed an onset of ethanol formation and a slightly improved biomass formation compared to their respective *ICL1* wild-type counterparts. Ethanol production could be explained by the fact that cytosolic acetyl-CoA resulting from the pyruvate dehydrogenase by-pass cannot enter the glyoxylate cycle in the *icl1* deletion mutants. This might cause a redirection of carbon from acetaldehyde towards ethanol (Fig 1). The improvement of biomass formation by the *ICL1* deletion is puzzling, but could simply be caused by a significant reduction of SA production coupled to a reduced ATP dissipation during SA export and eventually a higher availability of ATP for biosynthesis processes. Additionally, cytosolic acetyl-CoA is not used in the glyoxylate cycle in the *icl1* deletion mutants. The reduced conversion of acetate to acetyl-CoA by ACS (an ATP-dependent reaction) would also result in a higher availability of ATP.

Assuming a relatively high flux through the mitochondrial TCA cycle when glycerol is used as a carbon source, the deletion of the endogenous succinate dehydrogenase gene *SDH1* was supposed to result in a further increase in SA production. The deletion of *SDH1*

indeed caused a further increase in SA titer and yield (Fig. 3). On one hand, the respective deletion might prevent the further conversion of SA accumulated in the cytosol. On the other hand, additional SA generated via the oxidative branch of the mitochondrial TCA cycle might be accumulated and exported from the mitochondria. Interestingly, the *sdh1* deletion mutant in our study grew even faster than the isogenic control strain during the first part of the cultivation. So far, we do not have a plausible explanation for this observation. It might be connected to a reduced production of NADH via the TCA cycle, which is favourable for growth

in the *sdh1* mutant. This would at least be in agreement with metabolic model predictions reported by Strucko et al. (2018). The reduced NADH generation in the TCA cycle is supposed to be accompanied by reduced ATP production, which is particularly relevant in the time phase of the fermentation when the SA concentration is high due to the described ATP dissipation caused by passive transport of the weak acid SA back into the cell. The latter is assumed to be responsible for the cessation of growth after 72 h.

As mentioned in the introduction, previously published studies aiming at the production of SA with *S. cerevisiae* via the cytosolic reductive branch of the TCA cycle (similar to the `SA module´ in this study) from glucose included an increased activity of pyruvate carboxylase which is responsible for converting cytosolic pyruvate into oxaloacetate incorporating carbon dioxide. In fact, both Yan et al. (2014) and Van de Graaf et al. (2015, Patent No. US20150057425A1) overexpressed the endogenous *PYC2* gene for this purpose. As the results of several studies indicated a significantly higher expression level of pyruvate carboxylase in wild-type *S. cerevisiae* growing on respiratory carbon sources compared to glucose (DeRisi et al., 1997; Dueñas-Sánchez et al., 2012; Huet et al., 2000;

Menéndez and Gancedo, 1998), we initially did not include PYC overexpression in our constructed strains. Nevertheless, we later also checked *PYC2* overexpression in our best producer but did not obtain an increase in SA production (data not shown). We are currently studying the significantly altered metabolic fluxes when a *PYC2* overexpression strain grows on glycerol (Xiberras et al*.,* manuscript in preparation).

Another important genetic modification for SA production from glucose has been the abolishment of ethanol formation since Yan et al. (2014) used a baseline strain with an abolished ethanol formation (TAM strain) (Maris et al., 2004). We did not engineer the ethanol formation pathway in the current study since we first wanted to check whether this is necessary at all. Most of our constructed strains indeed did not produce significant amounts of ethanol when grown in glycerol-containing synthetic medium which was in strong contrast to glucose-containing synthetic medium. In fact, strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 was also characterized in synthetic glucose medium and the strain produced huge amounts of ethanol but virtually no SA under these conditions (data not shown). Obviously, the flux from acetaldehyde into the glyoxylate cycle was so strong in glycerol-containing medium that all carbon was diverted away from ethanol production even without any genetic modification.

The results obtained in the current work confirm the important role of the dicarboxylic acid transporter *An*DCT-02 for SA production as already shown by Jansen et al. (2017, Patent No. US9624514B2) in glucose-containing medium. However, an interesting finding of this particular study conducted in glycerol-containing medium was that a remarkable amount of SA (3.2 g/L) was even secreted in a DHA pathway strain that only expressed the *An*DCT-02 transporter, i.e. a strain without the `SA module´. As an isogenic control strain without the transporter (i.e. our DHA pathway baseline strain)

did not show SA secretion, the heterologous transporter must be very efficient in redirecting carbon from the central carbon metabolism in the form of SA to the medium.

The data presented in the current manuscript demonstrate for the first time the production of SA in *S. cerevisiae* using glycerol as the sole carbon source. By establishing the `SA module´ in the cytosol of *S. cerevisiae* and expressing the dicarboxylic acid transporter *An*DCT-02, a SA titer of 10.7 g/L and a yield of 0.22 g/g were obtained from glycerol. By deleting *SDH1,* the titer and yield could be further increased to 14.0 g/L and 0.43 g/g, respectively. The yield (in g/g) is significantly higher than the 0.2 g/g achieved by Yan et al. (2014) on glucose in shake flask cultivations. It has to be noted that the strain used in the latter study carried several different additional genetic modifications apart from the cytosolic reductive branch of the TCA cycle (equivalent to the `SA module´ in this study) but no heterologous transporter for SA export. The results presented in the current work are very promising particularly since they were obtained without any carbon dioxide sparging and/or abolishment of ethanol production. The strains constructed here provide a starting point for further optimization of strain construction and process conditions for SA production from glycerol in the popular yeast *S. cerevisiae*. Future work will also focus on further metabolic engineering channelling the carbon through the initially envisaged redox-neutral pathway.

5. Funding

We thank the German Research Foundation (DFG) for funding of our research (NE 697/7-1).

6. Acknowledgements

We thank Royal DSM N.V. for kindly providing us with the plasmids pGBS414PPK-3, pGBS415FUM-3 and pGBS416DCT-2, and Celina Prosch and Solvejg Sevecke technical support. We are also grateful to Dr. Alrik Los for fruitful discussions.

7. Conflict of interest

The authors declare no competing interest.

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9. Supplementary material

Table S1. Plasmids used in this study.

Plasmid	Relevant characteristic	Source or reference
pGBS414PPK-3	Codon-optimized coding sequence for glycosomal fumarate reductase from *T. brucei* lacking the codons for the peroxisomal targeting signal (*FRDg-R*)	DSM, The Netherlands
pGBS415FUM-3	Codon-optimized coding sequence for fumarase (*fumR*) from *R. oryzae* and peroxisomal malate dehydrogenase from *S. cerevisiae* (*MDH3-R*; removal of the codons for the peroxisomal targeting signal retargets the enzyme to the cytosol)	DSM, The Netherlands
pGBS416DCT-2	Codon-optimized coding sequence for the dicarboxylic acid transporter (DCT-02) from *A. niger*	DSM, The Netherlands
p414-TEF1p-Cas9-CYCt-nat1	*natMX4,* P_{TEF1}-*cas9*-T_{CYC1}	Klein et al. (2016)
p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX	2 µm, *hphMX, SNR52p-gRNA.*CAN1.Y-*SUP4t*	Klein et al. (2016)
p426-SNR52p-gRNA.YGLCτ3- SUP4t-hphMX	2 µm, *hphMX, SNR52p-gRNA.*YGLCτ3-*SUP4t*	Islam et al. (2017)
p426-SNR52p-gRNA.YPRCτ3-SUP4t-hphMX	2 µm, *hphMX, SNR52p-gRNA.* YPRCτ3-*SUP4t*	This study
pUG66	*ble*^{r}	Gueldener et al. (2002)
pUC18	*E. coli* cloning vector	Yanisch-Perron et al. (1985)
pUC18-*DAK1*	P_{ACT1}-*DAK1*-T_{TPS1}	Klein et al. (2016)
pUC18-*DAK1*$_{OE-2}$	P_{TDH3}-*DAK1*-T_{IDP1}	Klein et al. (2016)
p41bleTEF-*Opgdh*	P_{TEF1}-*Opgdh*-T_{CYC1}	Klein et al. (2016)
pUC18-*MDH3-R*	P_{PGK1}-*MDH3*-R-T_{IDP1}	This study
pUC18-*RofumR*	P_{TEF1}-*RofumR*-T_{RPL15A}	This study
pUC18-*TbFRDg-R*	P_{TDH3}- *TbFRDg-R*-T_{CYC1}	This study
pUC18-*An*DCT-02	P_{ENO2}-*An*DCT-*02*-T_{DIT1}	This study

Table S2. Primers used in this study.

Purpose	Primer number	Sequence (5'-3')	Description
Exchange of the *CAN1* target sequence in the gRNA coding sequence of p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX by a 20 nt sequence targeting YPRCτ3 region	460	CCCGCGCGTTGGCCGATTCAT	Primer for Gibson Assembly - amplification of *SUP4* terminator and gRNA structural component with a 21 bp overhang homologous to the adjacent PvuII-linearized p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX backbone
	597	GCTCTAAAACATACAGCGTTACCAATATGGGATCATTTA TCTTTCACTGCGGAGAAG	Primer for Gibson Assembly - amplification of *SUP4* terminator and gRNA structural component containing the new 20 bp YPRCτ3 target sequence with a 35 bp overhang homologous to the adjacent SNR52 promoter of gRNA cassette
	596	ATAAATGATCCCATATTGGTAACGCTGTATGTTTTAGAG CTAGAAATAGCAAGTTAAAATAAGGC	Primer for Gibson Assembly - amplification of SNR52 promoter containing the new 20 bp YPRCτ3 target sequence with a 35 bp overhang homologous to the adjacent gRNA structural component
	463	GTCGACCTGCAGCGTACGAAGCTTCAG	Primer for Gibson Assembly - amplification of SNR52 promoter with a 16 bp overhang homologous to the adjacent PvuII-linearized p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX backbone
Construction of the expression cassette of *S. cerevisiae MDH3* (P_{PGK1}-*MDH3*-T_{IDP1}) in pUC18	311	GCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAG AGGATCCGAAGTACCTTCAAAGAATGGGGTCT	Primer for Gibson Assembly - amplification of *PGK1* promoter from *S. cerevisiae* Sc288c DNA containing a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
	726	AAGATGGCAACCTTAACCATTGTTTTATATTTGTTGTAA AAAGTAGATAATTACTTCCTTGATGATCTG	Primer for Gibson Assembly - amplification of *PGK1* promoter from *S. cerevisiae* Sc288c containing a 30 bp overhang homologous to the adjacent *MDH3* open reading frame
	727	TTATCTACTTTTTACAACAAATATAAAACAATGGTTAAG GTTGCCATCTTAGGTG	Primer for Gibson Assembly - amplification of *MDH3* from pGBS415FUM-3 containing a 30 bp overhang homologous to the adjacent *PGK1* promoter
	728	AAAGTGGTAGATTGGGCTACGTAAATTCGATTAACTGT CCAAGATGAAAGACTTACCC	Primer for Gibson Assembly - amplification of *MDH3* from pGBS415FUM-3 with a 30 bp overhang homologous to the adjacent *IDP1* terminator
	729	AAGGGTAAGTCTTTCATCTTGGACAGTTAATCGAATTTA CGTAGCCCAATC	Primer Gibson Assembly- amplification of *IDP1* terminator from *S. cerevisiae* Sc288c DNA with a 30 bp overhang homologous to the adjacent *MDH3* open reading frame
	576	GCTATGACCATGATTACGAATTCGAGCTCGGTACCCGG GGATGGTAATGATCCGAACTTGG	Primer Gibson Assembly - for amplification of *IDP1* terminator from *S. cerevisiae* Sc288c DNA with a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
Construction of the expression cassette of *R. oryzae fumR* (P_{TEF1}-*RfumR*-T_{RPL15A}) in pUC18	410	TGCAGGTCGACTCTAGAGCATAGCTTCAAAATGTTTCTA CTCCTTTTTTACTCTTCC	Primer for Gibson Assembly - amplification of *TEF1* promoter from *S. cerevisiae* Sc288c DNA containing a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
	730	GCAAAGCAGCAGAAGCAGAGGACATTTAGATTAGATT GCTATGCTTTCTTTCTAATGAGC	Primer for Gibson Assembly - amplification of *TEF1* promoter from *S. cerevisiae* Sc288c containing a 30 bp overhang homologous to the adjacent *RofumR* open reading frame
	731	TTAGAAAGAAAGCATAGCAATCTAATCTAAATGTCCTCT GCTTCTGCTG	Primer for Gibson Assembly - amplification of *RoFUMR* from pGBS415FUM-3 containing a 30 bp overhang homologous to the adjacent *TEF1* promoter

	732	ATAAAATTATATTTTCCATCAACCAGCTTATTAATCCTTG GCAGAAATCATGTCC	Primer for Gibson Assembly - amplification of *RoFUMR* from pGBS415FUM-3 with a 30 bp overhang homologous to the adjacent *RPL15A* terminator
	733	CCTGAGGACATGATTTCTGCCAAGGATTAATAAGCTGG TTGATGGAAAATATAATTTTATTGGGC	Primer Gibson Assembly- amplification of *RPL15A* terminator from *S. cerevisiae* Sc288c DNA with a 30 bp overhang homologous to the adjacent *RoFUMR* open reading frame
	354	GAAACAGCTATGACCATGATTACGAATTCGAGCTCGGT ACCCGGGGGAAAAACGGGAAGAAAAGGAAAGAAAAA AAATAC	Primer Gibson Assembly - for amplification of *RPL15A* terminator from *S. cerevisiae* Sc288c DNA with a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
Construction of the expression cassette of *T. brucei TbFRDg-R* (P_{TDH3}-*TbFRDg-R*-T_{CYC1}) in pUC18	571	GCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAG AGTCGAGTTTATCATTATCAATACTGC	Primer for Gibson Assembly - amplification of *TDH3* promoter from *S. cerevisiae* Sc288c DNA containing a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
	721	ACAATGGAAGCAGAAGATCTACCATCAACCATCCGTCG AAACTAAGTTCTTG	Primer for Gibson Assembly - amplification of *TDH3* promoter from *S. cerevisiae* Sc288c containing a 30 bp overhang homologous to the adjacent *TbFRDg-R* open reading frame
	722	TAAAACACCAAGAACTTAGTTTCGACGGATGGTTGATG GTAGATCTTCTGC	Primer for Gibson Assembly - amplification of *TbFRDg-R* from pGBS414PPK-3 containing a 30 bp overhang homologous to the adjacent *TDH3* promoter
	723	AATGTAAGCGTGACATAACTAATTACATGATTAACTTCC AGATGGTTCAGTTTCG	Primer for Gibson Assembly - amplification of *TbFRDg-R* from pGBS414PPK-3 with a 30 bp overhang homologous to the adjacent *CYC1* terminator
	724	GTTGACGAAACTGAACCATCTGGAAGTTAATCATGTAA TTAGTTATGTCACGC	Primer Gibson Assembly- amplification of *CYC1* terminator from *S. cerevisiae* Sc288c DNA with a 30 bp overhang homologous to the adjacent *TbFRDg-R* open reading frame
	725	AGCTATGACCATGATTACGAATTCGAGCTCGGTACCCG GGGCAAATTAAAGCCTTCGAG	Primer Gibson Assembly - for amplification of *CYC1* terminator from *S. cerevisiae* Sc288c DNA with a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
Construction of the expression cassette of *A. niger* DCT-02 (P_{ENO2}-*An*DCT-02-T_{DIT1}) in pUC18	716	GCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAG AGGTGTCGACGCTGCGGGTA	Primer for Gibson Assembly - amplification of *ENO2* promoter from *S. cerevisiae* Sc288c DNA containing a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
	717	AGAACCTGGCAAAGAAGTTTCAACGTTCATCATTATTAT TGTATGTTATAGTATTAGTTGCTTGGTG	Primer for Gibson Assembly - amplification of *ENO2* promoter from *S. cerevisiae* Sc288c containing a 30 bp overhang homologous to the adjacent *AnDCT02* open reading frame
	718	CAACTAATACTATAACATACAATAATAATGATGAACGTT GAAACTTCTTTGCCAG	Primer for Gibson Assembly - amplification of *An*DCT-02 from pGBS416DCT-2 containing a 30 bp overhang homologous to the adjacent *ENO2* promoter
	719	AAGGTAGACCAATGTAGCGCTCTTACTTTATTATTCAGA AACATCTTCATCTTGACCTG	Primer for Gibson Assembly - amplification of *An*DCT-02 from pGBS416DCT-2 with a 30 bp overhang homologous to the adjacent *DIT1* terminator
	720	CCAGGTCAAGATGAAGATGTTTCTGAATAATAAAGTAA GAGCGCTACATTGGTCT	Primer Gibson Assembly- amplification of *DIT1* terminator from *S. cerevisiae* Sc288c DNA with a 30 bp overhang homologous to the adjacent *AnDCT-02* open reading frame
	309	GAAACAGCTATGACCATGATTACGAATTCGAGCTCGGT ACCCGGGTTACTCCGCAACGCTTTTCTGAACG	Primer Gibson Assembly - for amplification of *DIT1* terminator from *S. cerevisiae* Sc288c DNA with a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone

Genomic integrations of expression cassettes employing the CRISPR-Cas9 system	690	CTGCACATAATTGAAATAAGGATGTAGTTCAACTTCTATGAATGCTCGGCGATACGATATGGATCCGAAGTACCTTCAAAGAATG	Primer for amplification of *MDH3* expression cassette with an overhang homologous to a sequence upstream of the YGLCτ3 coding sequence in chromosome VII
	770	AAAAAGGAGTAGAAACATTTTGAAGCTATGGATGGTAATGATCCGAACTTGG	Primer for amplification of *MDH3* expression cassette with an overhang homologous to the adjacent *TEF1* promoter of the *RoFUMR* expression cassette
	771	AAGGTTCCCCAAGTTCGGATCATTACCATCCATAGCTTCAAAATGTTTCTACTC	Primer for amplification of *RoFUMR* expression cassette with an overhang homologous to the adjacent *IDP1* terminator of the *MDH3* expression cassette
	590	GAAATGGCAGTATTGATAATGATAAACTCGAGGAAAAACGGGAAGAAAAGG	Primer for amplification of *RoFUMR* expression cassette with an overhang homologous to the adjacent *TDH3* promoter of the *TbFRDg-R* expression cassette
	591	TCTTTCCTTTTCTTCCCGTTTTTCCTCGAGTTTATCATTATCAATACTGC	Primer for amplification of *TbFRDg-R* expression cassette with an overhang homologous to the adjacent *RPL15A* terminator of the *RoFUMR* expression cassette
	772	TTCCGTCTCTGGCTGAAGGCTCATTTCCATGATGGGGTCACAATTATTATCGCACGCAAATTAAAGCCTTCGAGC	Primer for amplification of *TbFRDg-R* expression cassette with an overhang homologous to a sequence downstream of the YGLCτ3 coding sequence in chromosome VII
	871	TATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAAGTGTCGACGCTGCGGGTATAG	Primer for amplification of *An*DCT-02 expression cassette with an overhang homologous to a sequence upstream of the YPRCτ3 coding sequence in chromosome XVI
	872	TTACAATCTAGTCGCAAAAACAAGTACAGTGCTGACGTCCCATCTTACTCCGCAACGCTTTTCTGAACG	Primer for amplification of *An*DCT-02 expression cassette with an overhang homologous to a sequence downstream of the YPRCτ3 coding sequence in chromosome XVI
	991	TATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAAgatccGAAGTACCTTCAAAGAATG	Primer for amplification of *MDH3* expression cassette with an overhang homologous to a sequence upstream of the YPRCτ3 coding sequence in chromosome XVI
	992	TATAGAGTAAAGAACCCTTTCTATACCCGCAGCGTCGACACGCAAATTAAAGCCTTCGAG	Primer for amplification of *TbFRDg-R* expression cassette with an overhang homologous to the adjacent *ENO2* promoter of the *An*DCT-02 expression cassette
	993	GAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCGTGTCGACGCTGCGGGTATAG	Primer for amplification of *An*DCT-02 expression cassette with an overhang homologous to the adjacent terminator *CYC1* of the *TbFRDg-R* expression cassette
Deletion of *SDH1* gene	1243	AGAAAGAAAAAAATCCAATTTCATAGTACGAAGAAGAACGAGAATAAAGCCAGCTGAAGCTTCGTACGC	For amplification of phleomycin deletion cassette from pUG66 with a sequence complementary to the flanking regions of the genomic integration site at their 5' end and a sequence complementary to the loxP sites on pUG66 at their 3' end.
	1244	AAAGAAGAGTATGATATTCTTTTCCGTAAAATACAATGAGGTTCAAACGCATAGGCCACTAGTGGATCTG	
Deletion of *ICL1* gene	1189	AACAATTGAGAGAAAACTCTTAGCATAACATAACAAAAAGTCAACGAAAACCAGCTGAAGCTTCGTACGC	
	1190	ATATACTTGTCAGGAAATGCCGGCAGTTCTAATGGTTAATCCTTGTCCGCATAGGCCACTAGTGGATCTG	

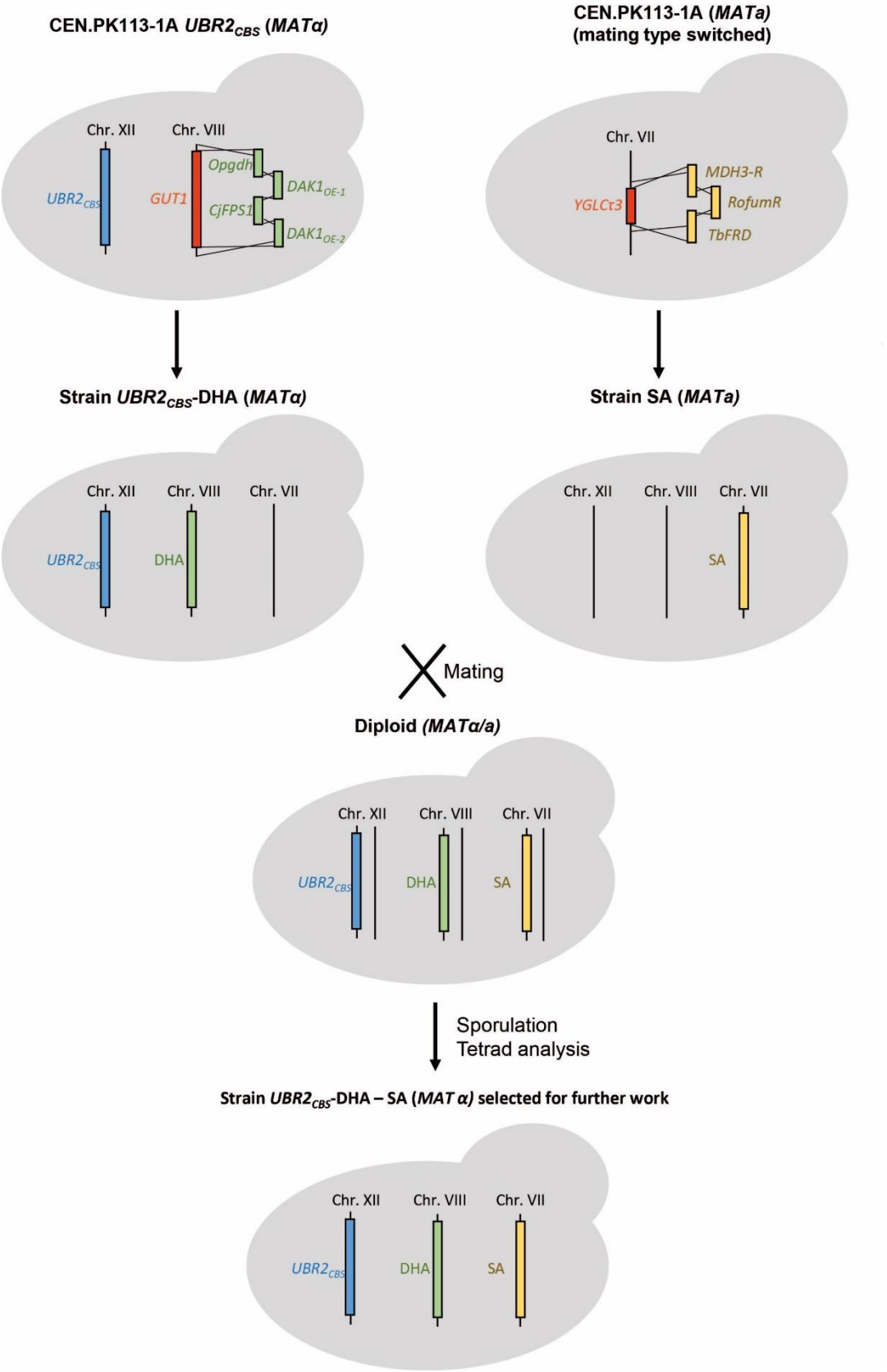

Fig. S1. Mating strategy used for construction of strain *UBR2*$_{CBS}$-DHA-SA.

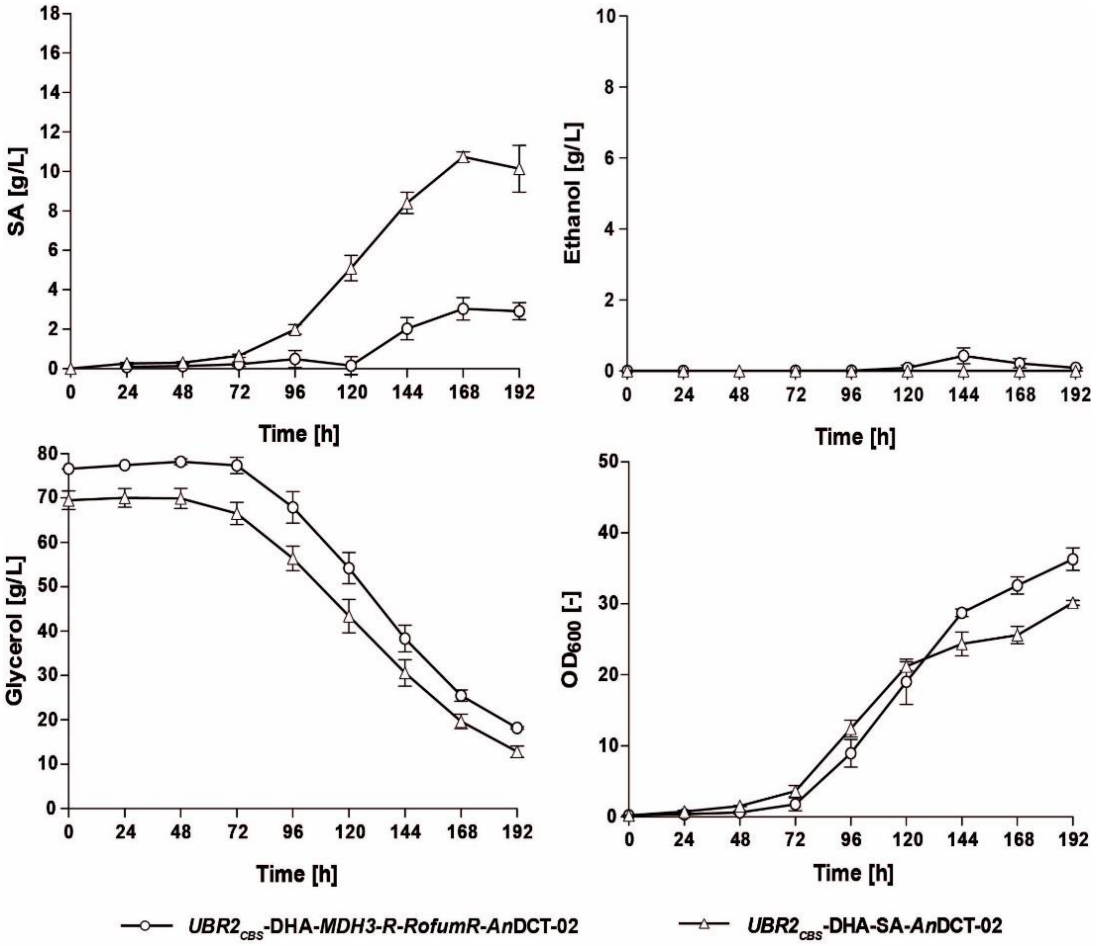

Fig. S2. Impact of removing the last enzymatic step of the `SA module` (heterologous fumarate reductase, *TbFRDg-R*) from the *S. cerevisiae* strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 on the production of SA, ethanol and biomass as well as consumption of glycerol. Cultivations were performed in 500 mL shake flasks cultures containing 100 mL of synthetic medium containing 6% (v/v) glycerol as the sole carbon source and culture supernatants were analysed by HPLC. Growth was recorded by optical density measurements at 600 nm. Mean values and standard deviations were determined from at least three biological replicates.

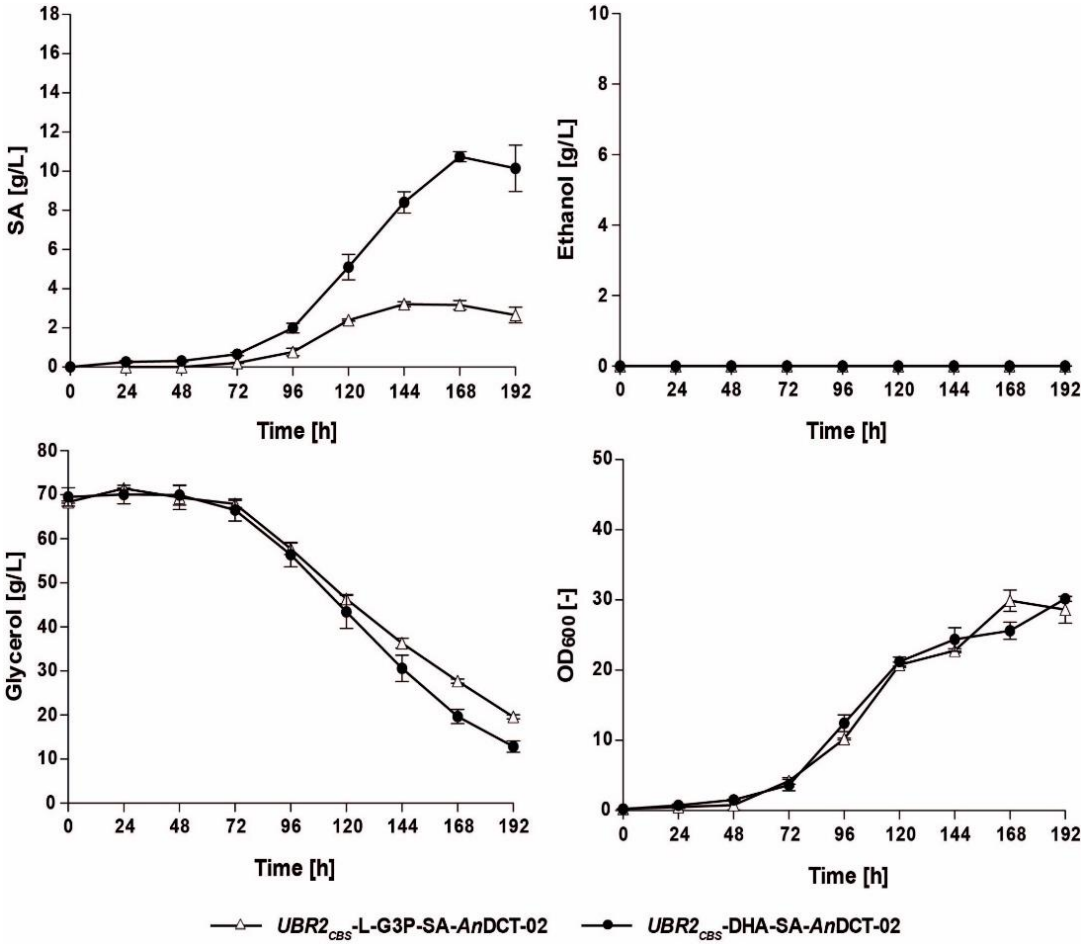

Fig. S3. Comparison of the L-G3P pathway (strain *UBR2*$_{CBS}$-L-G3P-SA-*An*DCT-02) and the DHA pathway (strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02) on the fermentation performance and product formation in *S. cerevisiae* in which the reductive SA production pathway and the *An*DCT-02 transporter was established. Cultivations were performed in 500 mL shake flasks cultures containing 100 mL of synthetic medium containing 6% (v/v) glycerol as the sole carbon source and culture supernatants were analysed by HPLC for production of SA, ethanol, and the consumption of glycerol. Growth was recorded by optical density measurements at 600 nm. Mean values and standard deviations were determined from at least three biological replicates.

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3.6 Manuscript IV

Understanding ethanol formation in a *Saccharomyces cerevisiae* strain engineered for succinic acid production from glycerol

Joeline Xiberras[1], Mathias Klein[1], and Elke Nevoigt[1*]

[1] Department of Life Sciences and Chemistry, Jacobs University Bremen gGmbH,

Campus Ring 1, 28759 Bremen, Germany

* Corresponding author:

E-mail: e.nevoigt@jacobs-university.de

Short title: Ethanol formation from glycerol

Abstract

A previous study reported succinic acid (SA) production in *S. cerevisiae* using glycerol as sole carbon source (Xiberras et al., submitted). However, SA was not only produced via the implemented cytosolic reductive branch of the TCA cycle ('SA module'), but also via endogenous pathways. This study attempted to increase flux via the 'SA module' by overexpressing either *PYC1, PYC2* or *PCK1* to increase the flux from either pyruvate or phosphoenolpyruvate to oxaloacetate. These genetic modifications resulted in a severe reduction in SA production and an onset of ethanol formation as well as improved biomass formation and glycerol consumption. In particular, the strain with *PYC2* overexpression showed a significantly faster biomass accumulation as well as specific glycerol consumption rate (enhanced glycolytic flux), and it generated up to ~ 13 g/L of ethanol during the first 72 h of cultivation. Even though a number of hypothesis have been discussed, the exact route by which ethanol is being formed in the engineered strains remains to be elucidated in the future.

KEYWORDS: Glycerol, NADH, Succinic acid, Metabolic engineering, Ethanol

1. Introduction

Concerns on environmental problems and climate change is urging human society to move away from chemical processes dependent on fossil resources and focus on the development of sustainable bio-based production. SA is considered as one of the most promising bio-based platform chemicals because it can be converted into many useful chemicals including tetrahydrofuran, 1,4-butanediol and gamma-butyrolactone (Ahn et al., 2016). SA is an intermediate of the central carbon catabolism and several commercial processes based on renewable resources and wild-type or engineered microbes as production hosts were already implemented. However some companies stopped the biobased production of SA since it is not competitive anymore with the SA generated from petroleum-derived maleic anhydride due to the drastic decrease in price of petroleum in the last few years (McCoy, 2019). Still, academic research on microbial SA production is important in a long-term perspective in the view of foreseeable depletion in fossil resources.

The yeast *Saccharomyces cerevisiae* has become one of the most intensively applied cell factories in industrial biotechnology, and it has been among those host microorganisms which have been engineered to produce a wide range of products including SA. Although high SA titres and yields have been achieved in bacteria (Ahn et al., 2016), *S. cerevisiae* is a preferred host for organic acid production such as SA due to its robustness against bacteriophage infection and high tolerance towards low pH. Production of SA at low pH facilitates the downstream processing since the production of unwanted by-products (such as gypsum) is avoided at low pH as discussed by Jansen and van Gulik (2014).

SA can be produced in *S. cerevisiae* via three routes, i.e. the glyoxylate cycle, the oxidative and the reductive branch of the TCA cycle (Raab & Lang, 2011). All these routes

have already been engineered by different authors in the past with the goal to produce SA from glucose (Ito et al., 2014; Raab et al., 2010; Yan et al., 2014). The reductive branch of the TCA cycle leads to the highest maximum theoretical yield on glucose (1.12 g/g glucose) because it involves net fixation of carbon dioxide which is in contrast to the other two routes (Beauprez et al., 2010). Using the cytosolic reductive branch of the TCA cycle, glycerol is an even better substrate for SA production because the metabolization of one carbon equivalent of glycerol generates additional reducing power on the way to DHAP (Fig. 1). Assuming that the additional reducing equivalents can be used for SA formation via the cytosolic reductive branch of the TCA cycle, a fermentative redox-neutral pathway could be established that results in a maximum theoretical yield of 1.28 g/g glycerol.

Glycerol is an abundant substrate because it is an inevitable by-product of the biodiesel industry (Burk & Van Dien, 2016; Yazdani & Gonzalez, 2007). Recently, our research group attempted to construct a *S. cerevisiae* strain that can ferment glycerol to SA (Xiberras et al., submitted) by starting with a CEN.PK-derivative able to utilize glycerol via the so-called DHA pathway and the Fps1 from *Cyberlindera jadinii* (Klein et al., 2016). This strain was used to genetically implement the genes required for both the cytosolic reductive branch of the TCA cycle (referred to as 'SA module') and for the transport of SA out of the cell (*An*DCT-02) as shown in Fig. 1. The 'SA module' consisted of the overexpression of the endogenous peroxisomal malate dehydrogenase retargeted to the cytosol (*MDH3-R*), the heterologous expression of cytosolic fumarase (Ro*fumR*) from *Rhizopus oryzae* and glycosomal fumarate reductase retargeted to the cytosol (*TbFRDg-R*) from *Trypanosoma brucei*. The resulting strain has been referred to as *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02. The *UBR2*$_{CBS}$ in the name results from the fact that this strain (in addition

to the mentioned rational modifications) was reverse engineered by replacing the *UBR2* allele in CEN.PK113-1A by the *UBR2* allele from the strain CBS 6412-13A. This modification has been demonstrated to be crucial to establish growth of CEN.PK strains in synthetic glycerol medium (Swinnen et al., 2016) and further improve growth of a CEN.PK strain equipped with the DHA pathway (Klein et al., 2016). This strain together with the modules DHA (DHA pathway plus *CjFPS1*), `SA module´ and the transporter *An*DCT-02 produced up to 10.7 g/L of SA corresponding to a yield of 0.22 ± 0.01 g/g glycerol (Xiberras et al., submitted).

Although production capacities of *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 can be considered promising, the data of Xiberras et al. (submitted) indicated that the glyoxylate and the TCA cycle significantly contributed to SA production in this strain and that more metabolic engineering is required to push the carbon flux into the 'SA module'. We hypothesized that increasing the carbon flux from C3 (pyruvate or PEP) to C4 (oxaloacetate) could be one possibility to achieve this. Our hypothesis has been based on the fact that a previous study using glucose as the carbon source has shown that overexpression of *PYC2*, the gene encoding one of the two isoenzymes of pyruvate carboxylase (Fig. 1), resulted in a higher production of SA in a *S. cerevisiae* strain bearing a cytosolic reductive branch of the TCA cycle (similar to our 'SA module') (Yan et al., 2014). This encouraged us to test the overexpression of *PYC1* and *PYC2* in our strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02. An alternative route to increase the flux from C3 to C4 is to overexpress the *S. cerevisiae PCK1* gene encoding phosphoenolpyruvate carboxykinase which reversibly converts the PEP to oxaloacetate. Even though this enzyme is assumed to have its major function in gluconeogenesis, i.e. the conversion of oxaloacetate to PEP (De Torrontegui et al., 1966), a previous study has shown that the overexpression of

S. cerevisiae PCK1 in a *Yarrowia lipolytica* strain engineered for SA production resulted in higher production of SA from glycerol (Cui et al., 2017).

Besides demonstrating the impact of *PYC1*, *PYC2* and *PCK1* overexpression in our strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02, the remaining part of the present study was undertaken to better understand why all three modifications resulted in a remarkable decrease in SA production and an increase in ethanol production. The goal was to obtain an insight in the central carbon fluxes active in these strains utilizing glycerol as the sole source of carbon. This knowledge could help in designing future metabolic engineering strategies with the goal to force the majority of carbon through the carbon dioxide fixing cytosolic `SA module'.

2. Materials & Methods

2.1. Strains and maintenance

The strains used in this study are listed in Table 1. Yeast cells were routinely grown on solid YPD medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 15 g/L agAgar plates were cultivated in a static incubator at 30 °C. Media were supplemented with phleomycin (20 mg/L), hygromycin B (300 mg/L) or nourseothricin (100 mg/L) for selection purposes when needed. *E. coli* DH5α was used for plasmid construction and isolation, and cells were routinely grown in lysogeny broth (LB) containing 10 g/L NaCl, 5 g/L yeast extract, 10 g/L peptone and adjusted to a pH of 7.5 with 2 M NaOH (Bertani, 1951). For selection and maintenance of plasmid containing cells, 100 mg/L ampicillin was added.

Table 4. *S. cerevisiae* strains used in this study.

Strain/modules	Genotype and genome modifications	Source or reference
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg-R*-T_{CYC1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}	Xiberras et al. (submitted)
***UBR2$_{CBS}$*-DHA-SA**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg-R*-T_{CYC1};	Xiberras et al. (submitted)
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC1*$_{OE}$**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg-R*-T_{CYC1}; YPRCτ3::P_{TDH3}-*PYC1*-T_{ADH1}-P_{ENO2}-*AnDCT-02*-T_{DIT1}	This study
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg-R*-T_{CYC1}; YPRCτ3::P_{TDH3}-*PYC2*-T_{CYC1}-P_{ENO2}-*AnDCT-02*-T_{DIT1}	This study
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PCK1*$_{OE}$**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg-R*-T_{CYC1}; YPRCτ3::P_{JEN1}-*PCK1*-T_{CYC1}-P_{ENO2}-*AnDCT-02*-T_{DIT1}	This study
***UBR2$_{CBS}$*-DHA**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}	Klein et al. (2016)
***UBR2$_{CBS}$*-DHA-*PYC2*$_{OE}$**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}-; YPRCτ3::P_{TDH3}-*PYC2*-T_{CYC1}	This study
***UBR2$_{CBS}$*-DHA-*An*DCT-02-*PYC2*$_{OE}$**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}-P_{TDH3}-*PYC2*-T_{CYC1}	This study
UBR2$_{CBS}$*-DHA-*MDH3-R	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1};	This study
***UBR2$_{CBS}$*-DHA-*MDH3-R-An*DCT-02-*PYC2*$_{OE}$**	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}-P_{TDH3}-*PYC2*-T_{CYC1};	This study
UBR2$_{CBS}$*-DHA-*MDH3-R-RofumR	*MAT*α; *ubr2::UBR2$_{CBS\ 6412\text{-}13A}$*; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-	This study

	T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A};	
***UBR2*$_{CBS}$-DHA-*MDH3-R-RofumR-An*DCT-02-*PYC2*$_{OE}$**	*MAT*α; *ubr2::UBR2*$_{CBS\ 6412\text{-}13A}$; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}- P_{TEF1}-*RofumR*-T_{RPL15A}; YPRCτ3::P_{ENO2}-*AnDCT-02*-T_{DIT1}-P_{TDH3}-*PYC2*-T_{CYC1};	This study
***UBR2*$_{CBS}$-DHA-SA-*PYC2*$_{OE}$**	*MAT*α; *ubr2::UBR2*$_{CBS\ 6412\text{-}13A}$; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}; YPRCτ3:: P_{TDH3}-*PYC2*-T_{CYC1}	This study
***UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ *mae1*Δ**	*MAT*α; *ubr2::UBR2*$_{CBS\ 6412\text{-}13A}$; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}; YPRCτ3::P_{TDH3}-*PYC2*-T_{CYC1}-P_{ENO2}-*AnDCT-02*-T_{DIT1}; *mae1*::*loxP-ble-loxP*;	This study
***UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ *ach1*Δ**	*MAT*α; *ubr2::UBR2*$_{CBS\ 6412\text{-}13A}$; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}; YPRCτ3::P_{TDH3}-*PYC2*-T_{CYC1}-P_{ENO2}-*AnDCT-02*-T_{DIT1}; *ach1*::*loxP-cloNAT-loxP*;	This study
***UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ *icl1*Δ**	*MAT*α; *ubr2::UBR2*$_{CBS\ 6412\text{-}13A}$; *gut1*::P_{TEF1}-*Opgdh*-T_{CYC1}-P_{ACT1}-*DAK1*-T_{TPS1}-P_{PGK1}-*CjFPS1*-T_{RPL15A}-P_{TDH3}-*DAK1*-T_{IDP1}; YGLCτ3::P_{PGK1}-*MDH3*-*R*-T_{IDP1}-P_{TEF1}-*RofumR*-T_{RPL15A}-P_{TDH3}-*TbFRDg*-*R*-T_{CYC1}; YPRCτ3::P_{TDH3}-*PYC2*-T_{CYC1}-P_{ENO2}-*AnDCT-02*-T_{DIT1}; *icl1*::*loxP-cloNAT-loxP*;	This study

2.2. General molecular biology techniques

Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Frankfurt am Main, Germany) was used for amplification of PCR products required for genetic manipulation and sequencing purposes. The correct integration of all expression and disruption cassettes was verified by diagnostic PCR using OneTaq Quick-load DNA polymerase (NEB). The conditions used for PCR were adapted to the guidelines of the respective manufacturer. A modified protocol from Hoffman and Winston (1987) was used for isolation of genomic DNA. In detail, single colonies obtained after transformations were re-streaked on respective agar plates. Approximately 50 mg of cells from these plates were suspended in 200 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) in 1.5 mL micro

centrifuge tube. Consequently, 300 mg of acid-washed glass beads (diameter of 0.425–0.6 mm) and 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) were added. The tubes were vortexed at maximum speed for 2 min and centrifuged at 13000 g for 10 min. The aqueous phase (1 µL) was used as template in 20 µL PCR reactions. PCR products were purified by using the GeneJET PCR Purification Kit (Thermo Fischer Scientific). Transformation of *S. cerevisiae* with plasmids as well as expression cassettes was performed according to the lithium acetate method described by Gietz et al. (1995).

2.3. Plasmids used in this study

All the plasmids used within this study are shown in Table S1. The codon-optimized coding sequence for *S. cerevisiae PYC2* was kindly provided by Royal DSM N.V. (Delft, The Netherlands) for construction of pUC18-*PYC2*. The cassette for expression of *PYC2* was under the control of the *TDH3* promoter and the *ADH1* terminator and was assembled in pUC18 using Gibson isothermal assembly (Gibson et al., 2009). All primers used for amplification of the promoter, coding sequence and terminator are listed in Table S2. The promoter and terminator were amplified from genomic DNA isolated from the *S. cerevisiae* strain Sc288C. The coding sequence of *PYC2* was amplified from plasmid pGBS415FUM-3 (Table S1). One-step isothermal DNA assembly reactions contained 15 µL of the reagent-enzyme mix as described by Gibson et al. (2009), 0.05 pmol of BamHI linearized pUC18 and 3-fold excess of the inserts (promoter, coding sequence and the terminator (each 0.1 pmol) in a final volume of 20 µL. Reaction mixtures were incubated at 50 °C for 1 h and subsequently 5 µL of the reaction were directly used for transformation of *E. coli* DH5α. The resulting vector was named pUC18-*PYC2* (Table S1).

2.4. *S. cerevisiae* strain construction

2.4.1. General strategies for genomic integrations by employing the CRISPR-Cas9 system

For genomic integrations the long terminal repeats YGLCτ3 and YPRCτ3 on chromosomes VII and XVI, respectively, were used (Flagfeldt et al., 2009). Expression of Cas9 in the desired strain was achieved by transformation with the plasmid p414-TEF1p-Cas9-CYC1t-nat1 (Table S1). Co-transformation of the *S. cerevisiae* strain expressing the Cas9 endonuclease with the respective expression cassette(s) or DNA fragments (promoters, coding sequence and terminators) and the respective vector for gRNA expression (either p426-SNR52p-gRNA.YGLCτ3-SUP4t-hphMX or p426-SNR52p-gRNA.YPRCτ3-SUP4t-hphMX, Table S1) resulted in assembly and integration of all expression cassettes/DNA fragments at the target locus. The positive transformants were selected on YPD agar containing both nourseothricin and hygromycin B. The two vectors (Cas9 and gRNA) were subsequently removed from the resulting clone by serial transfers in YPD medium lacking the respective antibiotics yielding the desired strain. Successively, all integrated expression cassettes were verified by diagnostic PCR and verified by Sanger sequencing.

Strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC1*$_{OE}$

The expression cassette for *An*DCT-02 was amplified from plasmid pUC18-*An*DCT-02 using primer pair 875/872 while the expression cassette for *PYC1* was *de novo* assembled. The cassette for expression of *PYC1* was under the control of the *TDH3* promoter and the *ADH1* terminator. The promoter and terminator were amplified from genomic DNA isolated from the *S. cerevisiae* strain Sc288C using primer pair 773/1108 and 1098/874 , respectively, while the coding sequence of *PYC1* was amplified from

genomic DNA isolated from the *S. cerevisiae* strain CEN.PK113-1A using primer pair 1096/1099. The primers contained 5′-extensions generating 40–60 bp sequences homologous to regions directly upstream and downstream of the inserted double strand break at the integration site or to the respective adjacent expression cassette (in case several cassettes were assembled at the same locus). This applies for all primers used for genomic integrations. The expression cassette for *An*DCT-02 and the three DNA fragments for the expression cassette of *PYC1* were integrated at the YPRCτ3 locus of strain *UBR2*$_{CBS}$-DHA-SA by employing the CRISPR-Cas9 system, yielding strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC1*$_{OE}$.

Strain *UBR2*$_{CBS}$-DHA-*MDH3-R*

The expression cassette for *MDH3-R* was amplified from plasmid pUC18-*MDH3-R* (Table S1) using primer pair 690/297 (Table S2). The expression cassette was integrated at the YGLCτ3 locus of strain *UBR2*$_{CBS}$-DHA by employing the CRISPR-Cas9 system, yielding strain *UBR2*$_{CBS}$-DHA-*MDH3-R*.

Strain *UBR2*$_{CBS}$-DHA-*MDH3-R-RofumR*

The two expression cassettes for *MDH3-R* and *RofumR* were amplified from plasmids pUC18-*MDH3-R* and pUC18-*RofumR* (Table S1) using primer pairs 690/770 and 771/609, respectively (Table S2). The expression cassettes were integrated at the YGLCτ3 locus of strain *UBR2*$_{CBS}$-DHA by employing the by employing the CRISPR-Cas9 system, yielding strain *UBR2*$_{CBS}$-DHA-*MDH3-R-RofumR*.

Strains *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$, *UBR2$_{CBS}$*-DHA-*An*DCT-02-*PYC2*$_{OE}$, *UBR2$_{CBS}$*-DHA-*MDH3-R-An*DCT-02-*PYC2*$_{OE}$ and *UBR2$_{CBS}$*-DHA-*MDH3-R-RofumR-An*DCT-02-*PYC2*$_{OE}$

The expression cassette for *An*DCT-02 and *PYC2* were amplified from plasmids pUC18-*An*DCT-02 and pUC18-*PYC*2 (Table S1) using primer pairs 873/874 and 875/872 respectively (Table S2). Afterwards, they were integrated at the YPRCτ3 locus of strains *UBR2$_{CBS}$*-DHA-SA, *UBR2$_{CBS}$*-DHA, *UBR2$_{CBS}$*-DHA-*MDH3-R* and *UBR2$_{CBS}$*-DHA-*MDH3-R-RofumR* (Table 1), by employing the CRISPR-Cas9 system, yielding strains *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$, *UBR2$_{CBS}$*-DHA-*An*DCT-02-*PYC2*$_{OE}$, *UBR2$_{CBS}$*-DHA-*MDH3-R-An*DCT-02-*PYC2*$_{OE}$ and *UBR2$_{CBS}$*-DHA-*MDH3-R-RofumR-An*DCT-02-*PYC2*$_{OE}$.

Strains *UBR2$_{CBS}$*-DHA-SA-*PYC2*$_{OE}$ and *UBR2$_{CBS}$*-DHA-*PYC2*$_{OE}$

The expression cassette for *PYC2* was amplified from plasmid pUC18-*PYC*2 (Table S1) using primer pair 873/876 (Table S2). Subsequently, the expression cassette was integrated at the YPRCτ3 locus of strain *UBR2$_{CBS}$*-DHA-SA and *UBR2$_{CBS}$*-DHA (Table 1), by employing the CRISPR-Cas9 system, yielding strains *UBR2$_{CBS}$*-DHA-SA-*PYC2*$_{OE}$ and *UBR2$_{CBS}$*-DHA-*PYC2*$_{OE}$.

Strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PCK1*$_{OE}$

The expression cassette for *An*DCT-02 was amplified from plasmid pUC18-*An*DCT-02 using primer pair 993/872 while the expression cassette for *PCK1* was *de novo* assembled, similar to the expression cassette of *PYC1.* The cassette for expression of *PCK1* was under the control of the *JEN1* promoter and the *CYC1* terminator. The promoter was amplified from genomic DNA isolate from the *S. cerevisiae* strain CEN.PK113-1A *GUT1$_{JL1}$ UBR2$_{JL1}$* (Ho et al., 2017) using primer pair 1088/1089 while the terminator was amplified from genomic DNA isolated from the *S. cerevisiae* strain Sc88C

using primer pair 1092/992, respectively. The coding sequence of *PYC2* was amplified from *S. cerevisiae* strain CEN.PK113-1A using primer pair 1090/1091. The expression cassette for *An*DCT-02 and the three DNA fragments for the expression cassette of *PCK1* were integrated at the YPRCτ3 locus of strain *UBR2*$_{CBS}$-DHA-SA by employing the CRISPR-Cas9 system, yielding strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PCK1*$_{OE}$.

2.4.2. Gene deletions

Deletions of either *MAE1*, *ACH1* or *ICL1* were obtained using a disruption cassette consisting of either the phleomycin (*ble*^{r}) or nourseothricin (*cloNAT*) resistance marker flanked by regions complementary to the upstream and downstream regions of the respective gene to be deleted. The deletion cassettes were amplified from plasmid pUG66 (Table S1) using primers 1232 and 1233 (Table S2) for *MAE1* deletion or from plasmid pUG74 (Table S1) using primer pairs 1313/1314 and 1189/1190 for *ACH1* or *ICL1* deletion, respectively (Table S2). The primers contained at their 5' terminal end a 60-bp sequence complementary to the region immediately upstream or downstream of the start or stop codon of the gene to be deleted.

2.5. Media and cultivation conditions used for characterization of the constructed strains

All media and cultivation conditions were the same as used in the study by Xiberras et al. (submitted). In detail, all pre-cultures were cultured in synthetic medium-containing 20 g/L glucose and ammonium sulfate as the carbon and nitrogen source, respectively. The experiments for assessing SA and ethanol formation in shake-flask batch cultivation were performed in synthetic medium-containing 60 mL/L glycerol as the sole carbon source with urea as the nitrogen source. The synthetic medium was prepared according to Verduyn et al. (1992), 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 15 mg/L EDTA, 4.5 mg/L

$ZnSO_4.7H_2O$, 0.84 mg/L $MnCl_2.2H_2O$, 0.3 mg/L $CoCl_2.6H_2O$, 0.3 mg/L $CuSO_4.5H_2O$, 0.4 mg/L $NaMoO_4.2H_2O$, 4.5 mg/L $CaCl_2.2H_2O$, 3 mg/L $FeSO_4.7H_2O$, 1 mg/L H_3BO_3, and 0.1 mg/L KI. Filter sterilized vitamins were added after heat sterilization of the medium. Final vitamin concentrations were: 0.05 mg/L D-(+)-biotin, 1 mg/L D-pantothenic acid hemicalcium salt, 1 mg/L nicotinic acid, 25 mg/L myo-inositol, 1 mg/L thiamine chloride hydrochloride, 1 mg/L pyridoxine hydrochloride, and 0.2 mg/L 4-aminobenzoic acid. In case urea was used as the nitrogen source (in main culture media containing glycerol), an appropriate aliquot of a stock solution was added after autoclaving to obtain a final concentration of 2.8 g/L while 5 g/L ammonium sulfate was added (in pre-culture media containing glucose as carbon source) before heat sterilization. The pH of the synthetic glucose medium was adjusted to 6.5 with 4 M KOH, while that of synthetic glycerol medium was adjusted to 4.0 with 2 M H_3PO_4.

For pre-cultivation, cells were taken from a single colony to inoculate 3 mL of the synthetic glucose medium in a 10 mL glass tube and were afterwards incubated at orbital shaking of 200 rpm and 30 °C for 16 h. The pre-culture was used to inoculate 10 mL of the same medium in a 100 mL Erlenmeyer flask adjusting an OD_{600} of 0.2. This culture, hereafter referred to as intermediate culture, was cultivated at the same conditions for 48 h. The appropriate culture volume from the intermediate culture (in order to later adjust an OD_{600} of 0.2 in 100 mL of synthetic glycerol medium) was centrifuged at 800 g for 5 min and the supernatant discarded. The cell pellet was then washed once by re-suspending the cells in synthetic glycerol medium. The cell suspension was centrifuged again and re-suspended in 100 mL of the same medium in a 500 mL Erlenmeyer flask. The main cultures were incubated at orbital shaking of 200 rpm and 30 °C and samples for OD_{600} determination and HPLC analysis were taken at regular time intervals. In case

growth rates of the respective strains were measured, two aliquots (750 µL each) of the main culture were transferred to separate wells of a white Krystal TM 24-well clear bottom microplate (Porvair Sciences, Leatherhead, United Kingdom) and cultivated in the Growth Profiler 1152 (Enzyscreen, Haarlem, The Netherlands) at 30 °C and orbital shaking at 200 rpm. The growth profiler took a scan of the plate every 40 min. These scans were used to calculate the density of the cultures expressed as green values (G-values). Subsequently, the G-values were expressed as OD_{600} values (referred to as OD_{600} equivalents) using the following calibration curve: OD_{600} equivalent = $6.1761\ 10^{-8}$ * (*G-value*) $^{3.4784}$.

2.6. Metabolite analysis

Culture supernatants (samples of 1 mL) were first filtered through 0.2 µm Minisart RC membrane filters (Sartorius, Göttingen, Germany) and if required stored at −20 °C until analysis. The concentrations of SA, glycerol and ethanol in culture media were determined using a Waters HPLC system (Eschborn, Germany) consisting of a binary pump system (Waters 1525), injector system (Waters 2707), the Waters column heater module WAT038040, a refractive index (RI) detector (Waters 2414) and a dual wavelength absorbance detector (Waters 2487). The samples were injected onto an Aminex HPX-87H cation exchange column (Biorad, München, Germany) coupled to a Micro-guard® column (Biorad) and eluted with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and a column temperature of 45 °C. Volumes of 20 µL of sample were used for injection. SA was detected using the dual wavelength absorbance detector (Waters 2487) while ethanol and glycerol were analysed with the RI detector (Waters 2414). The retention time for SA was 11.2 min, for glycerol 13.5 min and for ethanol 22.7 min. Data were processed and analyzed using the Breeze 2 software (Waters).

3. Results

3.1. Overexpression of *PYC1*, *PYC2* or *PCK1* in strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02 unexpectedly reduced SA production from glycerol and resulted in significant ethanol formation

The overexpression of either *PYC1*, *PYC2* or *PCK1* (Fig. 1) was achieved by integrating a respective expression cassette with the *TDH3* promoter (for *PYC1* and *PYC2*) or the *JEN1* promoter (for *PCK1*) and the *ADH1* terminator (for *PYC1* and *PYC2*) or *CYC1* terminator (for *PCK1*) in the baseline strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02 (see materials and methods). Afterwards, we analysed the impact of these genetic modifications on the strains' physiology in shake flask batch cultures. To our huge surprise, all three modifications individually led to a severe reduction in SA production (Fig. 2). In fact, the SA titre dropped from the 10.7 g/L produced in baseline strain after 168 h of cultivation to only about 2 g/L. Interestingly, the overexpression of all three tested genes (*PYC1*, *PYC2* or *PCK1*) improved growth compared to the control strain, and this was accompanied by an increased glycerol consumption (Fig. 2). Among the three tested strains, the strain with *PYC2$_{OE}$* showed by far the fastest growth and glycerol consumption as well as highest ethanol production (Fig. 2). In fact, the sole *PYC2$_{OE}$* in the baseline strain improved the μ_{max} (measured in the growth Profiler) from ~0.18 to ~0.28 h^{-1}. The specific glycerol consumption rate for the strain with *PYC2$_{OE}$* was 0.37 ± 0.07 g/g.h after 40 h (time point at which the highest glycerol consumption was detected), which was remarkably higher compared to that of strains with *PYC1$_{OE}$* (0.08 ± 0.01 g/g.h after 87 h, the time point at which the highest glycerol consumption was measured) or *PCK1$_{OE}$* (0.09± 0.01 g/g.h after 90 h, the time point at which the highest glycerol consumption was measured). Interestingly, the specific glycerol consumption

rate for the baseline strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 was in the same range as the latter two strains, i.e. 0.07 ± 0.01 g/g.h after 135 h (time point at which the highest glycerol consumption was measured, Table S3). The strain with *PYC2*$_{OE}$ accumulated up to ~13 g/L of ethanol during the first 72 h of cultivation. This was in contrast to the baseline strain where no ethanol was detected. A peak of up to ~6 g/L was detected after 120 h in the strains with either *PYC1*$_{OE}$ or *PCK1*$_{OE}$ (Fig. 2).

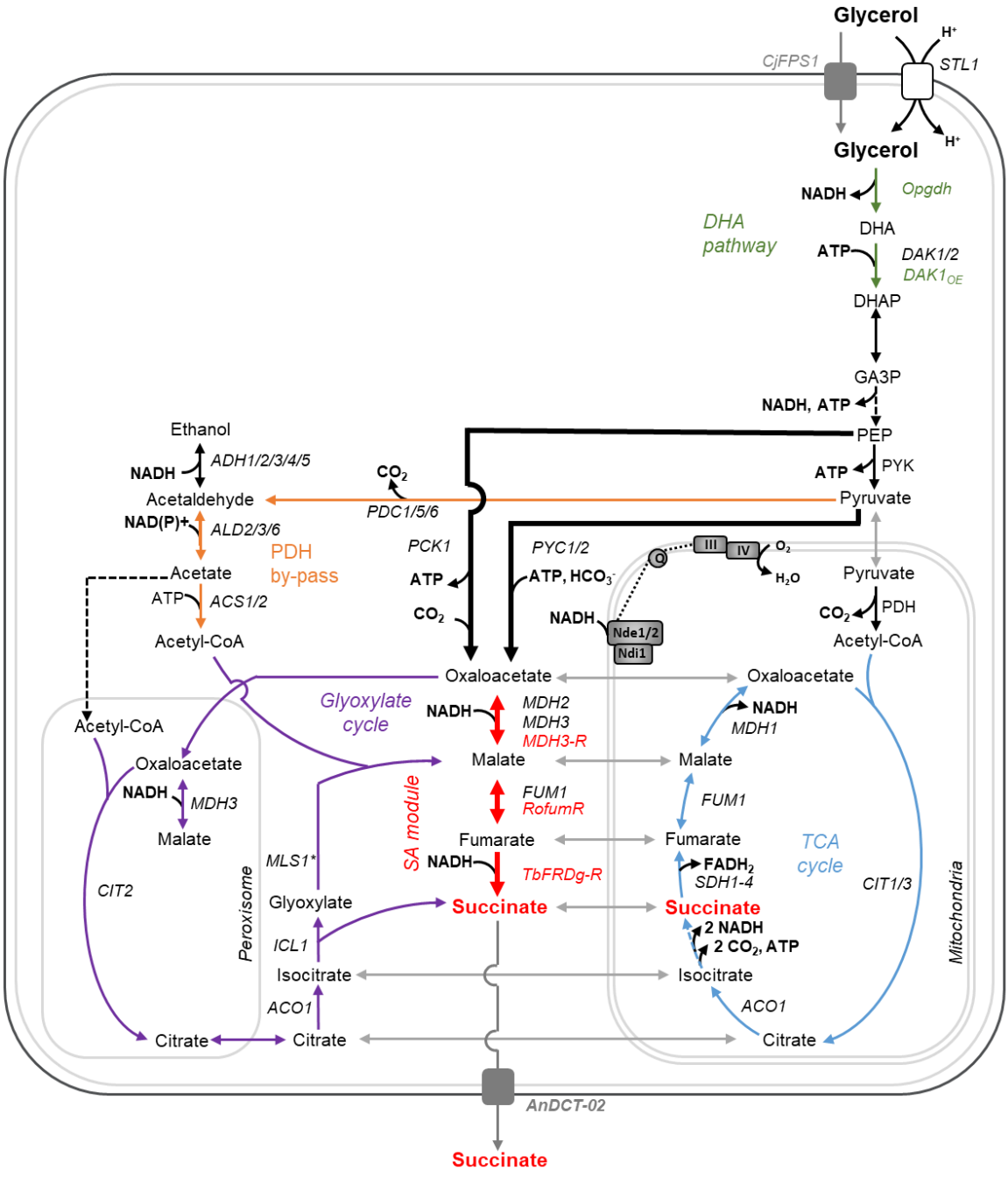

Fig. 1. Metabolic pathways leading to SA formation in *S. cerevisiae* and the genetic modifications conducted for attempting to overproduce SA through the cytosolic reductive branch of the TCA cycle ('SA module'). *MLS1**The exact localization of Mls1 on glycerol is not known as discussed by Xiberras et al. (2019). Thick black arrows indicate overexpression of either *PCK1*, *PYC1*, or *PYC2*. Abbreviations: DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde-3-phosphate,PEP: phosphoenolpyruvate, *STL1*: glycerol/H^+ symporter, *DAK1/2*: dihydroxyacetone kinase, *PYC1/2*: pyruvate carboxylase, *PCK1*: phosphoenolpyruvate carboxykinase, PYK: pyruvate kinase, *MDH1/MDH2/MDH3*: malate dehydrogenase, *FUM1*: fumarase, PDH: pyruvate dehydrogenase complex, *CIT1/2/3*: citrate synthase, *ACO1*: aconitase, *SDH1-4*: subunits of succinate dehydrogenase, *ICL1*: isocitrate lyase, *MLS1*: malate synthase, *ACS1/2*: acetyl-coA synthetase, *PDC1/5/6*: pyruvate decarboxylase, *ADH1/2/4/5*: alcohol dehydrogenase, *ALD2/3/6*: aldehyde dehydrogenase, *CjFPS:* glycerol facilitator from *Cyberlindnera jadinii*; *Opgdh:* glycerol dehydrogenase from *Ogatea polymorpha, MDH3-R*: peroxisomal malate dehydrogenase targeted to the cytosol, *RofumR: fumarase* from *R. oryzae, TbFRDg-R:* glycosomal fumarate reductase from *T. brucei* retargeted to the cytosol, *An*DCT-*02:* dicarboxylic acid transporter from *A. niger.* Nde1/2: mitochondrial external NADH dehydrogenase, Ndi1: NADH:ubiquinone oxidoreductase, Q: ubiquinone, III: complex III, IV: complex IV.

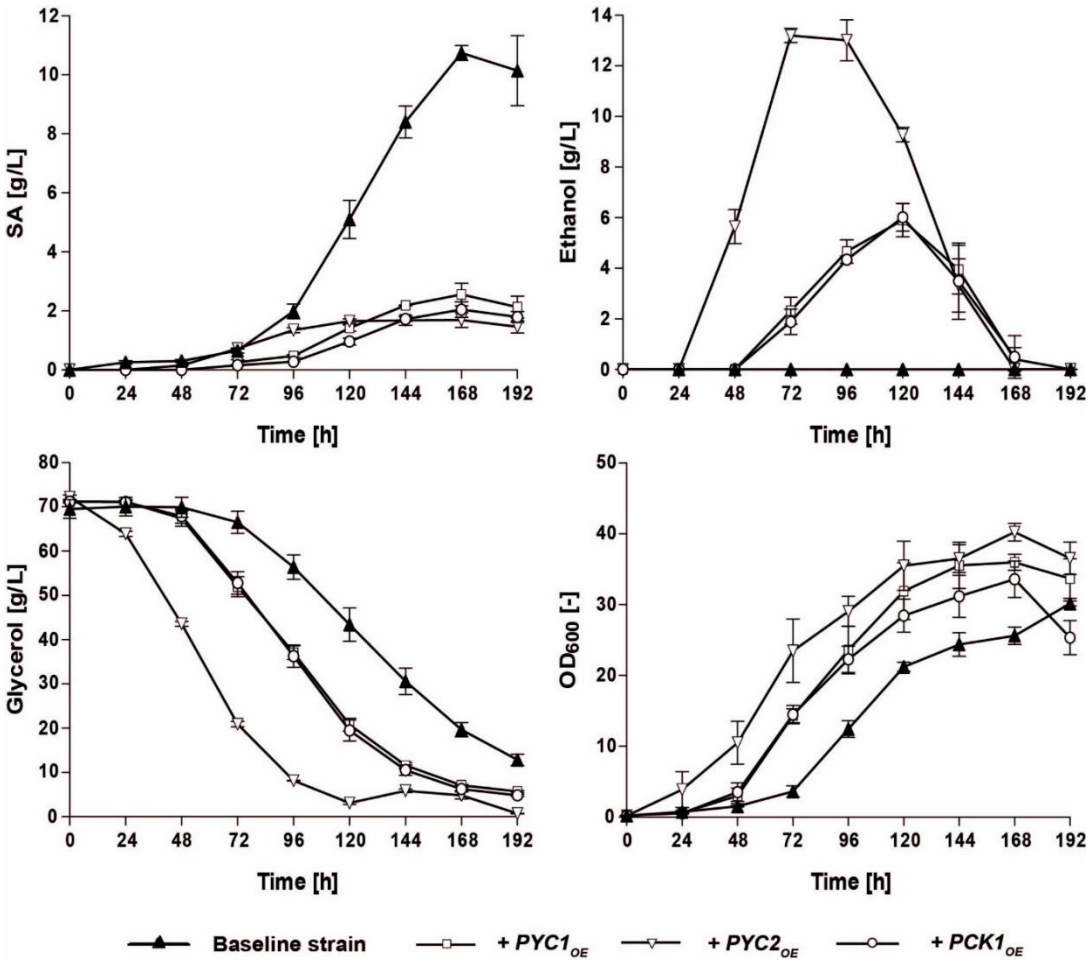

Fig. 2. Effect of overexpressing either *PYC1*, *PYC2* or *PCK1* on SA production, ethanol formation, growth and glycerol consumption in the baseline strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 previously engineered by Xiberras et al. (submitted) for the production of succinic acid from glycerol (strain name; modifications are explained in the introduction). Mean values and standard deviations were determined from at least three biological replicates. Abbreviations: *PYC1*/*PYC2*, the genes encoding the two isoenzymes of pyruvate carboxylase; *PCK1*; gene encoding phosphoenolpyruvate carboxykinase.

3.2. Dissecting the prerequisites for the extraordinary high glycolytic flux in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$

Starting from the results shown in Fig. 2., it was interesting to check whether the overexpression of *PYC2* in the absence of an 'SA module' and the transporter *An*DCT-02 also leads to fast growth and glycerol consumption as well as remarkable ethanol production. To investigate this, we established the same *PYC2* overexpression cassette in a control strain without 'SA module' and *An*DCT-02, i.e. in strain *UBR2*$_{CBS}$-DHA constructed by Klein et al., (2006), at position 21. This resulted in strain *UBR2*$_{CBS}$-DHA-*PYC2*$_{OE}$. It becomes clear by comparing the fermentation data of this strain *UBR2*$_{CBS}$-

DHA-*PYC2*$_{OE}$ (shown in pink in Fig. 3A) with the data obtained with strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ (shown in grey in Fig. 3B), that most likely the sole overexpression of *PYC2* did not result in the surprising phenotype of the latter strain. In contrast, the sole *PYC2*$_{OE}$ rather reduced growth and glycerol consumption compared to strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$. We next questioned the impact of *An*DCT-02 expression (in the absence of the 'SA module') and therefore, we established the cassettes for *An*DCT-02 and *PYC2*$_{OE}$ expression at position 21 in strain *UBR2*$_{CBS}$-DHA resulting in strain *UBR2*$_{CBS}$-DHA-*An*DCT-02-*PYC2*$_{OE}$. The results of the glycerol fermentation of this strain (shown in green in Fig. 3A) indicate that the additional expression of *An*DCT-02 interestingly improved the growth and glycerol consumption whereas the ethanol and SA production did not vary in comparison to the respective strain *UBR2*$_{CBS}$-DHA-*PYC2*$_{OE}$ without *An*DCT-02 expression (shown in pink in Fig. 3A). However, the strain *UBR2*$_{CBS}$-DHA-*An*DCT-02-*PYC2*$_{OE}$ (shown in green in Fig. 3A) exhibited slower growth and glycerol consumption (the specific glycerol consumption rate was similar to strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02, see Table S3) when compared to the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT02-*PYC2*$_{OE}$ bearing the 'SA module' (shown in grey, Fig. 3B). Therefore, it seems that the presence of both the 'SA module' and the *An*DCT-02 transporter are causative for the fast growth and glycerol consumption as well as the high ethanol production of strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT02-*PYC2*$_{OE}$.

As described in the introduction, the 'SA module' is composed of three genes, *MDH3-R*, *RofumR* and *TbFRDg-R*. Next, we wanted to scrutinize whether the entire 'SA module' (all three enzymes) is required for the physiological effects or whether the sole presence of the first enzyme (or the two first enzymes) have similar impacts. To investigate this, we overexpressed *MDH3-R* (encoding the first enzyme of the 'SA module'), individually

and in combination with the second enzyme of the 'SA module' *RofumR,* in the strain *UBR2$_{CBS}$*-DHA- *An*DCT-02- *PYC2$_{OE}$* resulting in strains *UBR2$_{CBS}$*-DHA-*MDH3-R-An*DCT-02-*PYC2$_{OE}$*- (shown in blue in Fig. 3A) and *UBR2$_{CBS}$*-DHA-*MDH3-R-RofumR-An*DCT-02-*PYC2$_{OE}$* (shown in brown in Fig. 3A). Notably, these two strains did not produce any ethanol and generated up to ~4 g/L of SA after 144 hrs of cultivation (Fig. 3A). Both strains also behaved very similar with regard to growth and glycerol consumption. Both measures in these two strains were lower compared to strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2$_{OE}$* (shown in grey in Fig. 3B). It therefore seems that, besides the transporter *An*DCT-02, the entire 'SA module' is required for the extraordinary strong glycolytic flux in the latter strain.

In order to further scrutinize the role of the transporter *An*DCT-02 for the fast growth and glycerol consumption, we wanted to test a strain with all modifications of strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2$_{OE}$* but without the transporter *An*DCT-02. For this purpose, we established the 'SA module' in the strain *UBR2$_{CBS}$*-DHA-*PYC2$_{OE}$* resulting in strain *UBR2$_{CBS}$*-DHA-SA-*PYC2$_{OE}$* (see materials and methods, shown in red in Fig. 3B). Results in Fig. 3B (shown in red) demonstrate that the lack of the transporter *An*DCT-02 indeed exhibits significantly slower growth and glycerol consumption in comparison to the strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2$_{OE}$* (shown in grey, in Fig. 3B) supporting the role of the transporter as an important prerequisite for the fast glycerol consumption rate in the latter strain. When compared to the SA-overproducing baseline strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02 (shown in black, Fig. 3B), the respective strain without the transporter *An*DCT-02 (shown in red, Fig. 3B) led to significant ethanol production (6 g/L after 120 h of cultivation) (Fig. 3B), even though this maximum level was only about half of the maximum concentration achieved by the strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-

PYC2$_{OE}$ (shown in grey, Fig. 3B). Still, the strain without the transporter *An*DCT-02 (shown in red, Fig. 3B) did not produce more SA; the level remained at ~ 2 g/L.

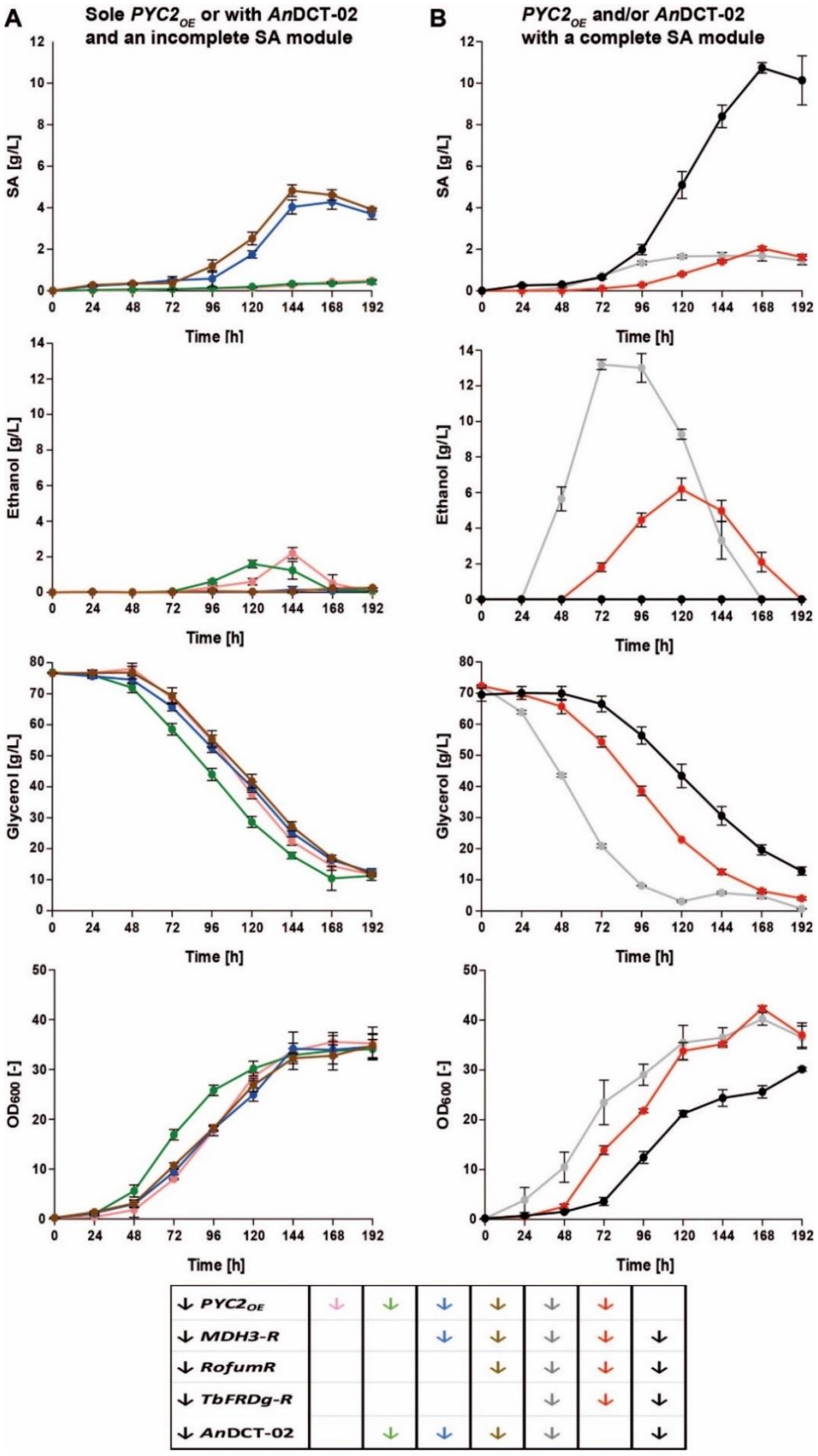

Fig. 3. Scrutinization of the prerequisites for the extraordinary high glycolytic flux and ethanol formation in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ by expressing different combinations of *An*DCT-02 and/or *PYC2*$_{OE}$ and genes of the 'SA module' (*MDH3-R*, *RofumR*, *Tb*FRDg-*R*). The impact of these genetic modifications on SA production, ethanol formation, glycerol consumption and growth was measured. Mean values and standard deviations were determined from at least three biological replicates.

3.3. Deletion of *MAE1* strongly affects physiology of strain *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2$_{OE}$* and considerably reduced ethanol production

A hypothesis emerging from the results obtained with the *PYC2$_{OE}$* in our SA-producing baseline strain (*UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2$_{OE}$*) was that the SA produced via the 'SA module' could be converted to ethanol instead of being exported out of the cell by *An*DCT-02. We speculated that the cytosolic SA produced via the 'SA module' could enter the mitochondria via the mitochondrial transporter Dic1 and be consequently converted to malic acid in the mitochondrial matrix by Sdh1-4 and Fum1 (Fig. 4). Afterwards, malic acid could be converted to pyruvate and carbon dioxide via the reversible reaction catalysed by malic enzyme encoded by *MAE1* and known to be located in the mitochondria in *S. cerevisiae* (Boles et al., 1998). Subsequently, the pyruvate could leave the mitochondria via the mitochondrial pyruvate carrier (Mpc1/3), and could eventually be converted to ethanol in the cytosol via the pyruvate decarboxylase (Pdc1/5/6) and aldehyde dehydrogenase (Adh1/3/4/5) as shown in Fig. 4. Another possibility could be that mitochondrial pyruvate is first converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDH). Consequently, acetyl-CoA hydrolase encoded by *ACH1* converts acetyl-CoA to acetate. Two possible further routes are conceivable for acetate. First, acetate could leave the mitochondria and be converted to acetaldehyde via the nonacetylating acetaldehyde dehydrogenase (Ald6) and finally to ethanol via Adh1/3/4/5 as described in Henningsen et al. (2015) (Fig. 4). A second scenario is that acetate is converted to acetaldehyde via Ald4/5, and this is consequently transported out of the mitochondria, and converted to ethanol via Adh1/3/4/5. We hypothesized further that an active malic enzyme would be a pre-requisite for all the aforementioned possibilities (Fig. 4), even though we were aware of the fact that it is

still unclear whether Mae1 is active on glycerol as recently reviewed by Xiberras et al. (2019). We deleted *MAE1* in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ and the impact of this deletion on the physiology of the strain was investigated. Interestingly, *MAE1* deletion indeed led to a remarkable reduction in ethanol formation (from ~13 g/L to ~1.9 g/L) (Fig. 5) and growth and glycerol consumption were significantly reduced (Fig. 5). Notably, *MAE1* deletion did not have a visible impact on SA production. In fact, the SA titre marginally increased at the end of the fermentation (from ~1.7 g/L to ~2.2 g/L after 168 h of cultivation) (Fig. 5).

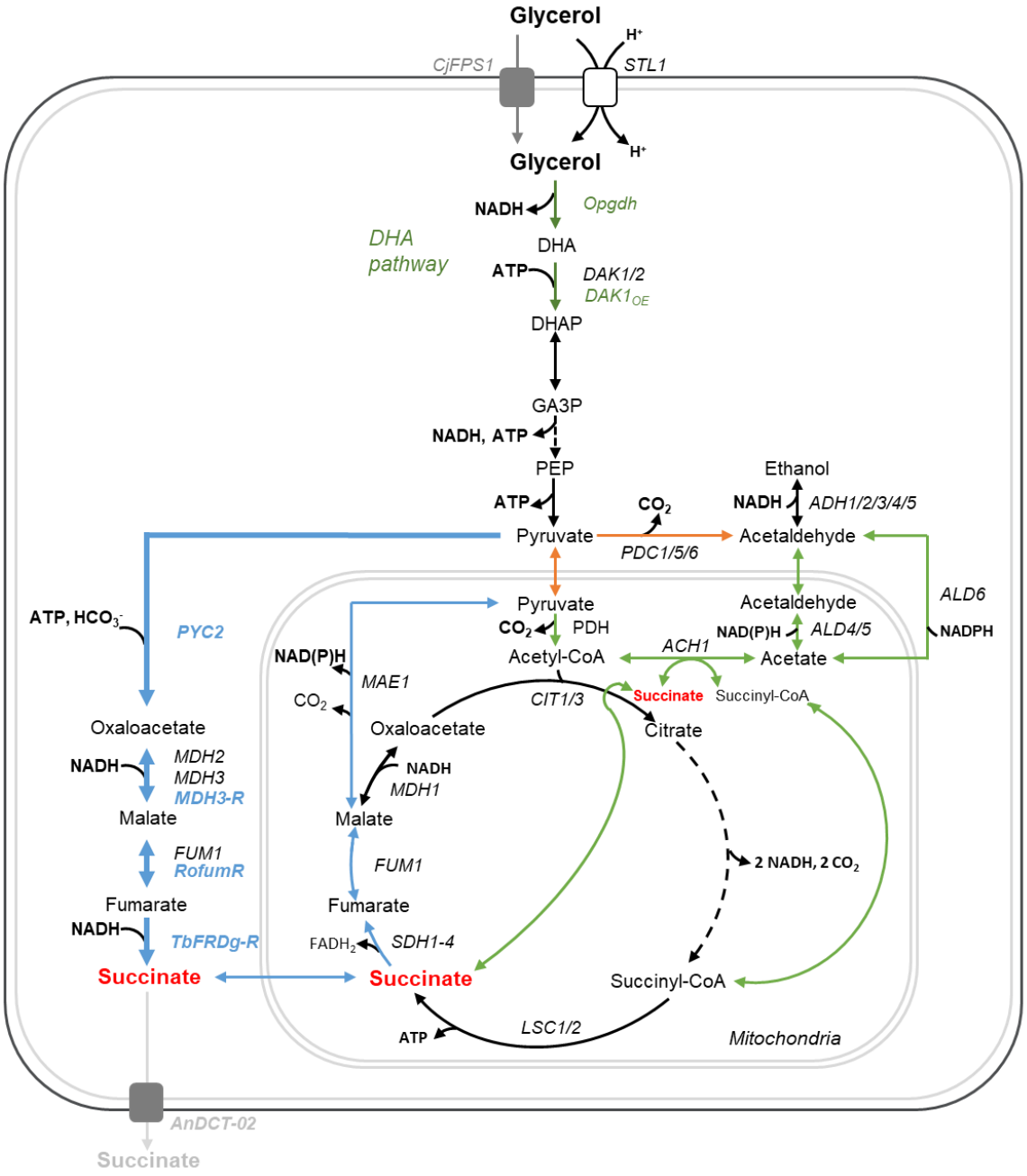

Fig. 4. Possible routes for conversion of SA produced via the 'SA module' to ethanol. Thick blue arrows indicate (over)expression of the *PYC2* and 'SA module' (*MDH3-R*, *RofumR* and *TbFRDg-R*). Abbreviations: *CjFPS:* glycerol facilitator from *Cyberlindnera jadinii*, *STL1*: glycerol/H^+ symporter, *Opgdh:* glycerol dehydrogenase from *Ogatea polymorpha,* DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde-3-phosphate, *DAK1/2*: dihydroxyacetone kinase, *MDH1*/*MDH2*/*MDH3*: malate dehydrogenase, *CIT1/3*: citrate synthase, *LSC1:* succinyl-CoA ligase, *SDH1-4*: subunits of succinate dehydrogenase, *FUM1*: fumarase, *MAE1*: malic enzyme, PDH: pyruvate dehydrogenase complex, *ACH1*, acetyl-CoA hydrolase, *ALD4/5/6*: aldehyde dehydrogenase, *ADH1/2/3/4/5*: alcohol dehydrogenase, *PDC1/5/6*: pyruvate decarboxylase, *PYC2*: pyruvate carboxylase, *MDH3-R*: peroxisomal malate dehydrogenase targeted to the cytosol, *RofumR: fumarase* from *R. oryzae, TbFRDg-R:* glycosomal fumarate reductase from *T. brucei* retargeted to the cytosol, *An*DCT-*02:* dicarboxylic acid transporter from *A. niger.*

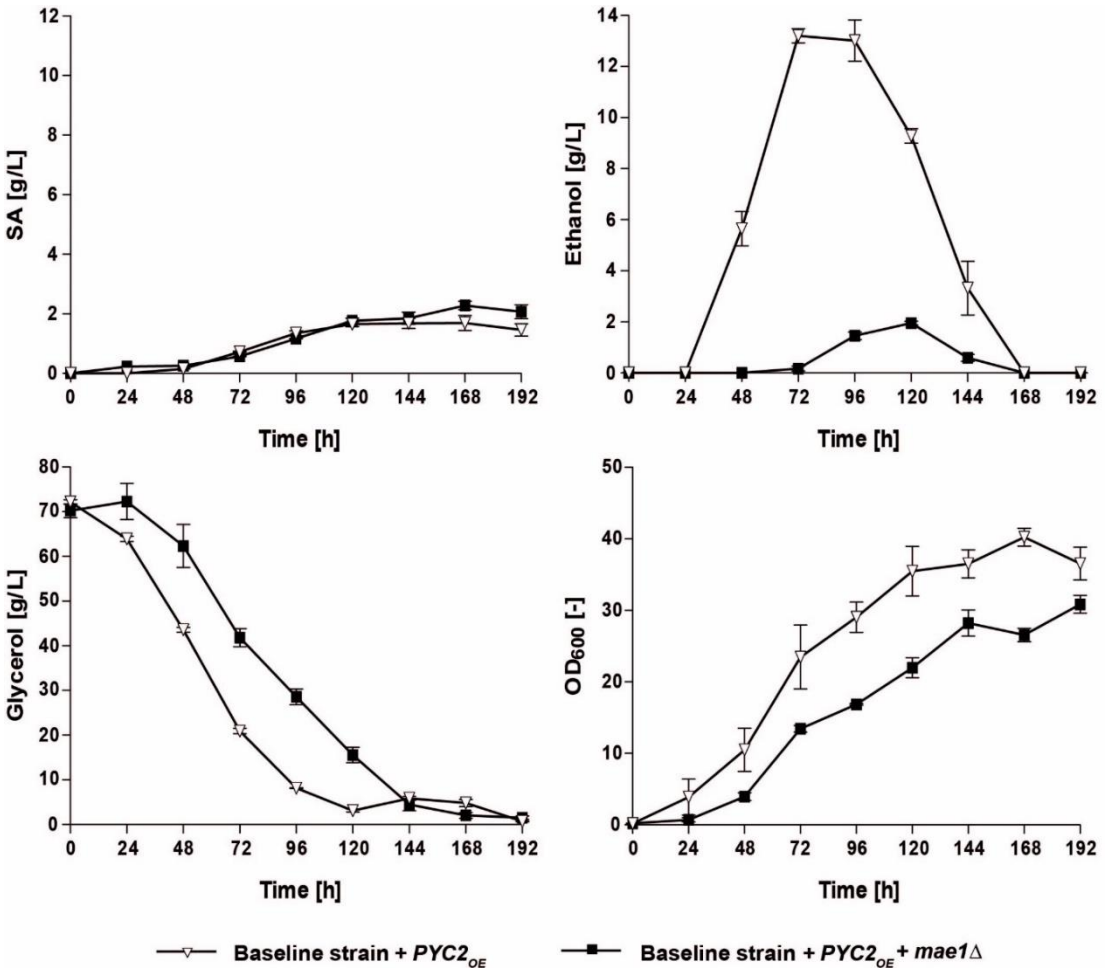

Fig. 5. Impact of *MAE1* deletion on SA production, ethanol formation, glycerol consumption and growth in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ (referred to as baseline strain + *PYC2*$_{OE}$). Mean values and standard deviations were determined from at least three biological replicates.

3.4. Deletion of *ACH1* compromised growth on glycerol

Mae1 seems to be important for ethanol production in the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$. We therefore questioned whether the pyruvate produced via Mae1 then goes via the Ach1 route and consequently ethanol is produced as discussed in section 3.3 and shown in Fig. 4. Interestingly, Cui et al. (2017) have shown that Ach1 was responsible for high acetate accumulation in a *Yarrowia lipolitica shd5*Δ mutant strain on glycerol. However, the exact role of Ach1 in *S. cerevisiae* growing on glycerol is not known. To investigate whether Ach1 is important in *S. cerevisiae* growing on glycerol and if it contributes to ethanol production, we deleted *ACH1* in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$. Remarkably, this deletion led to cessation of growth and glycerol

consumption after 100 h of cultivation (Fig. 6) as well as caused a reduction in ethanol and SA production (Fig. 6). However, the twelve biological replicates harbouring *ACH1* deletion behaved very differently in terms of ethanol production, growth and glycerol consumption as demonstrated by the high standard deviation (Fig. 6). Interestingly, a closer look at the individual curves of the twelve biological replicates indicate that in the first two fermentations, the biological replicates seem to split into two phenotypes (Fig. S1). In the third fermentation, the biological replicates still behaved differently but did not show two phenotypes as observed in fermentations 1 and 2 (Fig. S1).

It was interesting to investigate whether the cessation of growth upon deletion of *ACH1* was linked with the genetic modifications meant for SA production (i.e. 'SA module', $PYC2_{OE}$ and *An*DCT-02) in strain $UBR2_{CBS}$-DHA-SA-*An*DCT-02-$PYC2_{OE}$ or whether Ach1 also plays an important role in the central carbon catabolism of *S. cerevisiae* growing on glycerol. To investigate this, we deleted *ACH1* in strain $UBR2_{CBS}$-DHA. Interestingly, deletion of *ACH1* slowed growth and glycerol consumption (Fig. 7). In addition, this deletion did not have a major impact on SA while we still cannot conclude whether it has an impact on ethanol production in the respective strain due to the high standard deviation (Fig. 7).

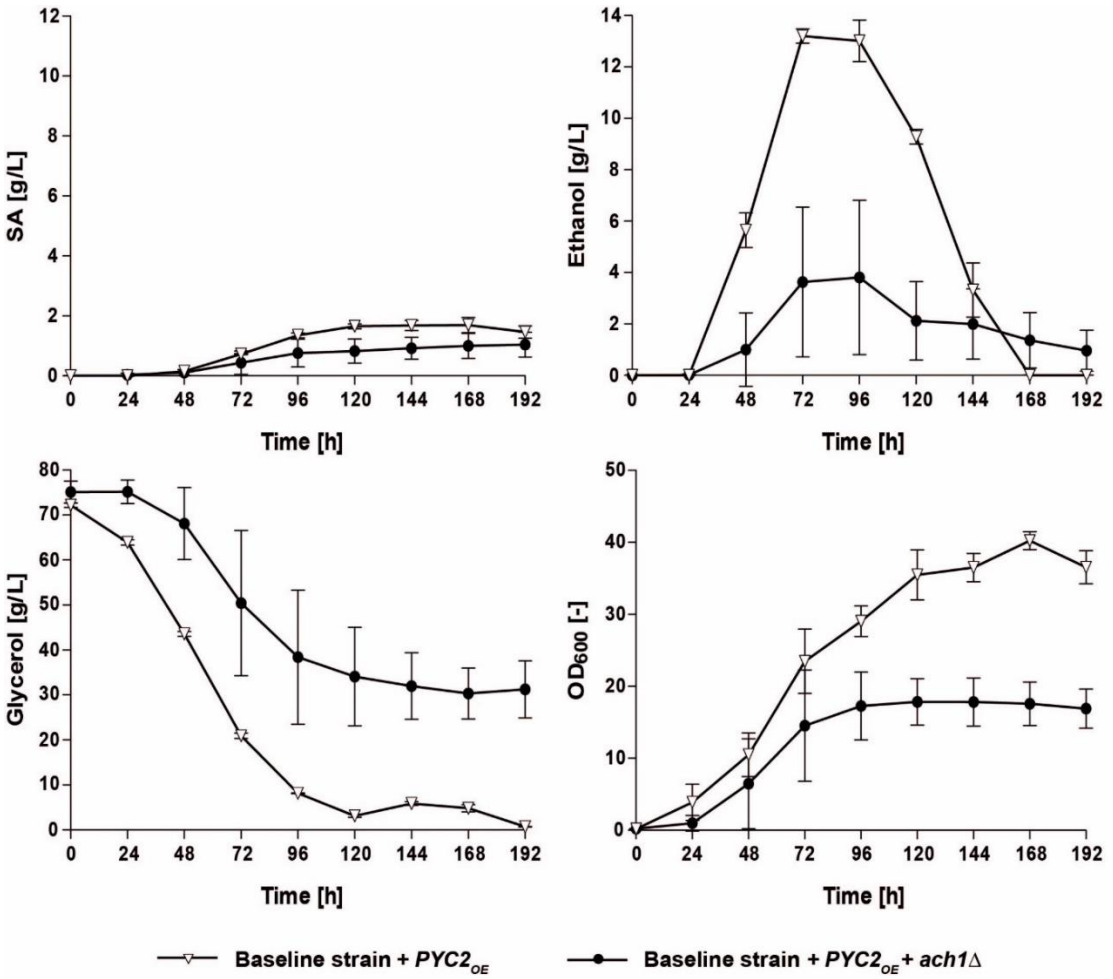

Fig. 6. Effect of deleting *ACH1* on SA production, ethanol formation, glycerol consumption and biomass formation in the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ (referred to as baseline strain + *PYC2*$_{OE}$). Mean values and standard deviations for strain the strain with the respective deletion were determined from twelve biological replicates.

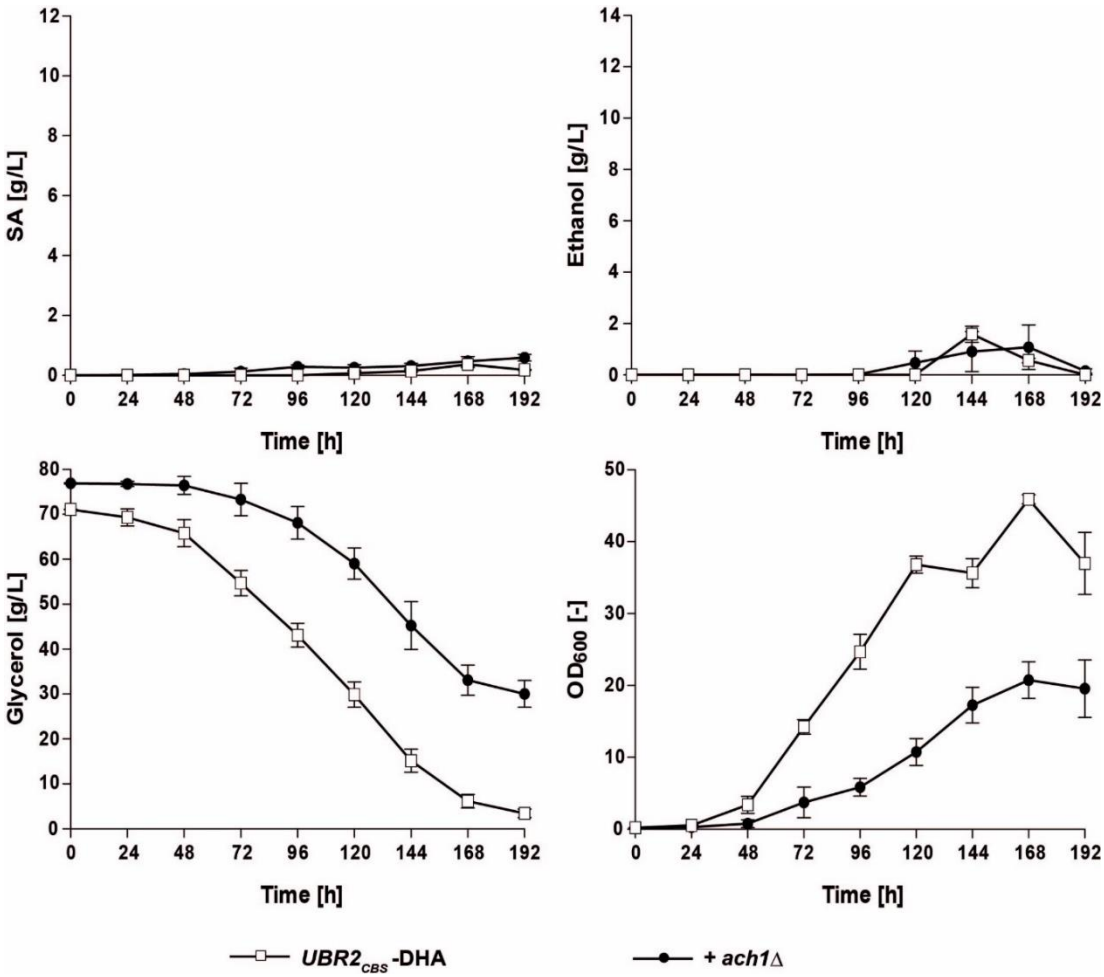

Fig. 7. Effect of deleting *ACH1* on SA production, ethanol formation, glycerol consumption and biomass formation in strain *UBR2*$_{CBS}$-DHA. Mean values and standard deviations for strain the strain with the respective deletion were determined from at least three biological replicates.

3.5. Abolishment of the glyoxylate cycle led to reduction in growth and glycerol consumption

We have previously shown that the glyoxylate cycle significantly contributes to SA production in the baseline strain of this study, *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (Xiberras et al., submitted). Interestingly, abolishment of the glyoxylate cycle in the latter strain led to an improvement in growth and glycerol consumption as well as an onset of ethanol production (Xiberras et al., submitted). We therefore asked whether abolishment of the glyoxylate cycle in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ will also cause an improvement in growth and glycerol consumption as well as increase in ethanol production. To investigate this, we abolished the glyoxylate cycle in strain *UBR2*$_{CBS}$-DHA-

SA-*An*DCT-02-*PYC2*$_{OE}$ by deleting *ICL1* encoding the key enzyme isocitrate lyase (Fig. 1). Remarkably, this deletion reduced ethanol production from ~13 g/L to 1.7 g/L after 96 hrs (Fig. 8). The maximum ethanol titre measured was of 4 g/L after 120 h of cultivation. Later, the ethanol titre decreased. In addition, deletion of *ICL1* reduced the growth of the respective strain, which coincided with decreased glycerol consumption while its impact on SA production was marginal (Fig. 8).

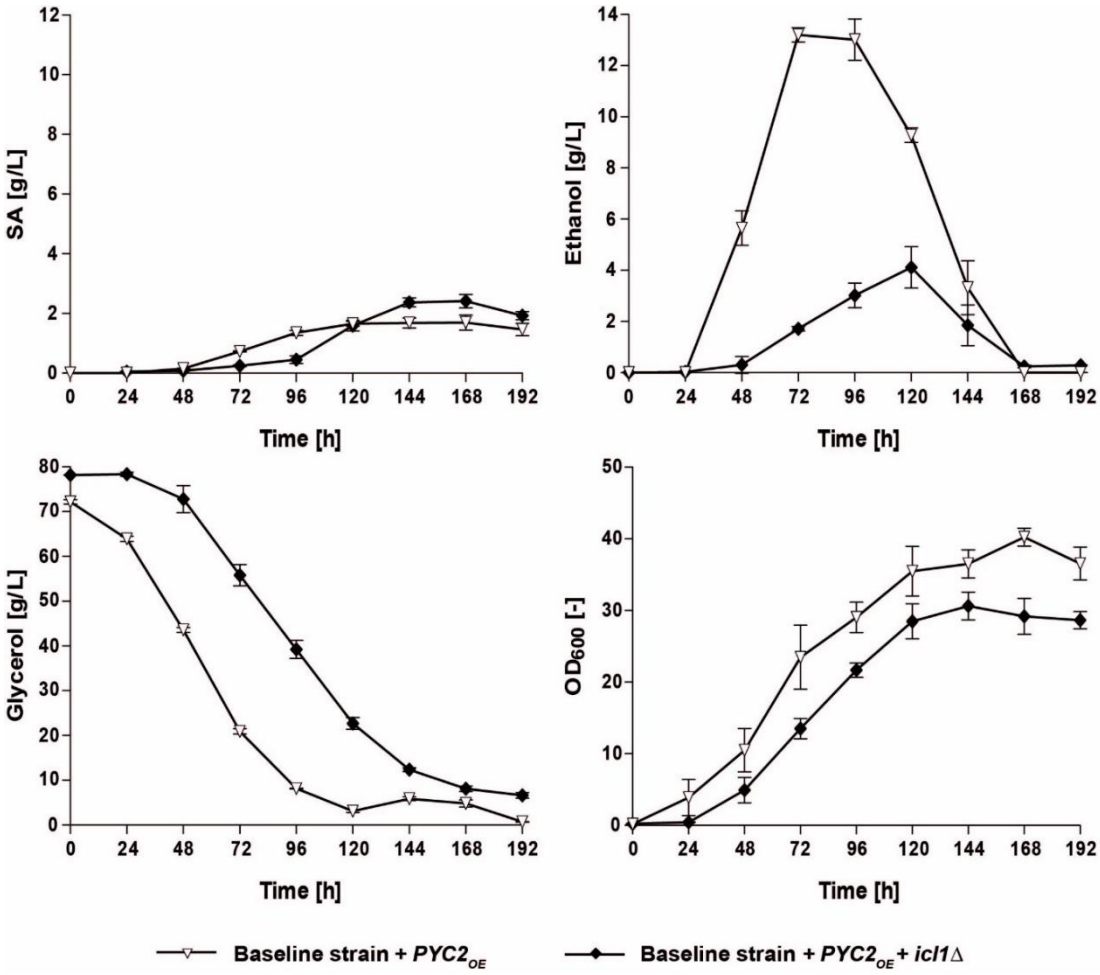

Fig. 8. Effect of the *ICL1* deletion on SA production, ethanol formation, glycerol consumption and growth in the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ (referred to as baseline strain + *PYC2*$_{OE}$). Mean values and standard deviations were determined from at least three biological replicates.

4. Discussion

The goal of this study was to increase the metabolic flux via the 'SA module' that has been previously integrated in an engineered *S. cerevisiae* strain able to utilize glycerol

via the DHA pathway (Xiberras et al., submitted). This was supposed to result in a redox-neutral, carbon dioxide assimilating SA production from glycerol. Unexpectedly, the first conducted engineering step in the envisaged direction, namely the overexpression of either *PYC1*, *PYC2,* or *PCK1* in the baseline strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 caused an onset of ethanol formation and considerably decreased SA production. This is in clear contrast to the study performed by Yan et al. (2014) who demonstrated a nearly 3-fold increase in SA production by the overexpression of *PYC2* in their engineered strain also carrying the genetic modifications for the establishment of the cytosolic reductive branch of the TCA cycle (similar to the 'SA module' in this study).

It is worth mentioning that the strain carrying the overexpression of *PYC2* caused a considerably higher ethanol production, as well as faster growth and glycerol consumption in comparison to *PYC1* (and *PCK1*) overexpression (Fig. 2). As the expression for the two tested genes *PYC1* and *PYC2* was under the control of the same promoter (*TDH3* promoter), it seems that the two isoenzymes of pyruvate carboxylase have different specific activities and/or are differently regulated at the posttranscriptional level. It would be interesting to measure the specific *in vitro* enzyme activities of the two isoenzymes of pyruvate carboxylase in the overexpression strains in order to better understand the reasons for the differences.

Interestingly, the strain with overexpression of *PCK1* behaved very similar to the strain harbouring the *PYC1* overexpression (Fig. 2). It has been shown that *PCK1* is mainly a gluconeogenetic enzyme (De Torrontegui et al., 1966) in *S. cerevisiae* and that it can convert PEP to oxaloacetate (Fig. 1) only in the presence of carbon dioxide sparging (Zelle et al., 2010). Still, Cui et al. (2017) showed that overexpression of *PCK1* from *S. cerevisiae* in a *Y. lipolytica* strain carrying deletion of *SDH5*, which encodes for one of

the sub-units of succinate dehydrogenase, improved SA production in shake flasks batch cultivations. The authors suggested that overexpression of *PCK1* led to an increase in the conversion of PEP to oxaloacetate, and afterwards, oxaloacetate was used for production of SA via the oxidative branch of the TCA cycle. It cannot be excluded that in our study, *PCK1* is converting PEP to oxaloacetate (Fig. 2) even in the absence of carbon dioxide sparging. Still, the question remains why neither the *PCK1* overexpression nor the overexpression of *PYC1* or *PYC2* led to increased SA formation in our study at all.

The overexpression of either *PYC1*, *PYC2* and *PCK1* in the baseline strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (Fig. 2) as well as the (over)expression of *An*DCT-02 and *PYC2* in the control strain without the 'SA module', i.e. *UBR2*$_{CBS}$-DHA-*An*DCT-02 (Fig. 3), led to faster volumetric consumption of glycerol. We therefore questioned whether the glycerol consumption is faster due to faster biomass accumulation, an enhanced glycolytic flux (specific glycerol consumption rate) or both. For this reason, we calculated the specific glycerol consumption rates. The maximum rates for the aforementioned strains are shown In Table S3. It becomes visible that the baseline strain and the baseline strain with either overexpression of *PYC1* and *PCK1* as well as strain *UBR2*$_{CBS}$-DHA-*An*DCT-02 had similar specific glycerol consumption rates. Among these three strains, only the strains carrying the overexpression of *PYC1* and *PCK1* led to ethanol formation. Therefore, it seems that the faster volumetric glycerol consumption in these strains was rather due to a faster biomass accumulation and not due to a faster glycolytic flux. In contrary to the latter three strains, the strain carrying overexpression of *PYC2* showed a five-fold higher specific glycerol consumption rate compared to the baseline strain (Table S3). The *PYC2* overexpressing strain also showed the fastest biomass accumulation and the highest ethanol titre (Fig. 2). It seems that there is a connection

between fast biomass accumulation, faster glycolytic flux and strong ethanol formation in this strain.

As the strain with *PYC2* overexpression (strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$) showed the surprisingly high glycolytic flux and ethanol production (Table S1), the objective of the current study shifted towards scrutinizing the metabolic route by which ethanol was produced and to understand the reasons for the significantly improved growth and glycerol consumption in the latter strain. Results show that the fast glycolytic flux in the aforementioned strain is not due to a single genetic modification, but is a result of the synergistic action of the (over)expression of the (entire) 'SA module', the transporter *An*DCT-02 and *PYC2* (Fig. 3). The results lead to different assumptions. The fact that all the genes of the 'SA module' were necessary, highly suggests that there is indeed a significant metabolic flux via the 'SA module' but this did not lead to SA accumulation, but to ethanol formation. Obviously, the SA was not exported out of the cell via *An*DCT-02 as expected. We therefore questioned how the SA produced via the 'SA module' can end up in ethanol.

When depicting all potential metabolic routes of how SA might be converted to ethanol in the engineered *S. cerevisiae* strain (shown in Fig. 4 and mentioned in the results section), it became obvious that they all rely on an active Mae1. This enzyme converts MA into pyruvate and carbon dioxide (ΔG^0 - 8 KJ/mol, (Zelle et al., 2011)), has been comprehensively characterized during growth of *S. cerevisiae* on ethanol. The enzyme seems to be localized in the mitochondria during growth on ethanol (Boles et al., 1998) and uses $NADP^+$ as a cofactor. It has been previously shown that malic enzyme provides the pyruvate required for biosynthesis in pyruvate kinase-negative strains growing on ethanol (Boles et al., 1998). So far, there is no experimental indication that malic enzyme

is active and important for growth on glycerol. In fact, the deletion of *MAE1* in a reversed engineered *S. cerevisiae* strain growing on glycerol did not have an impact on growth (data not shown). Apart from this, a separate study focusing on the yeast mitochondrial proteome did not detect Mae1 during growth on complex medium containing glycerol (Ohlmeier et al., 2004). Therefore, the data shown in Fig. 5 indicate for the first time that Mae1 plays a role in *S. cerevisiae* during growth on glycerol even though the functionality seems to depend on the genetic modifications present in the strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$. Our results do not deliver a proof yet, but strongly support the hypothesis that a significant portion of the carbon metabolism in the engineered strain occurs via malic enzyme.

One of the routes through which the mitochondrial pyruvate (produced by Mae1) could end up in ethanol is by leaving the mitochondria via the mitochondrial pyruvate carrier (encoded by *MPC1/3*), and eventually be converted to ethanol in the cytosol via pyruvate decarboxylase (encoded by *PDC1/5/6*) and alcohol dehydrogenase (encoded by *ADH1/3/4/5)* as shown in Fig. 4. This route would require that pyruvate can exit the mitochondria. A previous study has shown that a pyruvate-kinase negative strain can grow on ethanol as long as Mae1 is active (Boles et al., 1998). This suggests that Mae1 (localized in the mitochondria) can fulfil the cytosolic requirement for pyruvate after export from mitochondria. However, it remains puzzling to understand why the carbon flux uses the complicated detour via the mitochondria in strains overexpressing *PYC2* plus 'SA module' and AnDCT-02 and why pyruvate resulting from glycolysis does not directly enter alcoholic fermentation (see below).

It is still not clear whether the pyruvate generated via Mae1 is converted to ethanol via Ach1 (Fig. 4). This enzyme transfers the CoA from acetyl-CoA (generated via the PDH

complex) to SA with the formation of acetate and succinyl-CoA (Chen et al., 2015).The only information about the function of Ach1 during growth of *S. cerevisiae* on glycerol originates from the study of Ohlmeier et al. (2004), which has shown Ach1 expression is strongly induced after shifting the *S. cerevisiae* cells for fermentative (complex medium containing glucose) to respiratory growth (complex medium containing glycerol). The same study suggested that the role of Ach1 is linked with regulating the acetyl-CoA pool. Chen et al. (2015) showed that Ach1 shuttles mitochondrial acetyl units for cytosolic C2 provision in a *S. cerevisiae* mutant which cannot produce cytosolic acetyl-CoA via the PDH by-pass during growth on glucose (Fig. 1). It seems that Ach1 is very important during growth of *S. cerevisiae* on glycerol since its deletion had a severe impact on growth in a strain without (over)expression of 'SA module', *PYC2* and AnDCT-02 (strain $UBR2_{CBS}$-DHA) (Fig.7). Therefore, it seems that Ach1 is not only important upon (over)expression of the 'SA module', *PYC2* and *An*DCT-02 (Fig. 6). Although the biological replicates for the strain $UBR2_{CBS}$-DHA-SA-*An*DCT-02-$PYC2_{OE}$ with *ACH1* deletion produced less ethanol, it can be that this was caused by a reduction in biomass and not as a result of directly affecting the carbon flux via the proposed Ach1 route.

It is still not known why the (twelve) different biological replicates for the strain $UBR2_{CBS}$-DHA-SA-*An*DCT-02-$PYC2_{OE}$ with *ACH1* deletion behaved remarkably differently from each other with regard to the physiology (Fig. S1). The biological replicates were cultivated for 192 h in shake flasks batch cultivations (3.3 h generation time, 58 cell generations), thereby the chance of mutations occurring during the short fermentation is very low. In fact, the mutation rate for *S. cerevisiae* tend to be $\sim 10^{-9} - 10^{-10}$ per base pair per generation under most growth conditions (Drake et al., 1998). Apart from mutations, stochasticity effects, i.e. noise or random fluctuations in gene expression,

could also explain the phenotypic variability observed between the different biological replicates (Raser & O'Shea, 2004; Raser & O'Shea, 2005). However, this has still be to experimentally proven. Another explanation for the observed variability in the biological replicates could be a genetic instability arising as a consequence of the gene deletion, i.e. the deletion of *ACH1* in case of the current study. This genetic instability could give rise to heterogeneous populations of cells. In fact, several studies have reported genetic instability after deletion of a particular gene (Alabrudzinska et al., 2011; Bhattacharya et al., 2018; Strome et al., 2008). For example, one study have found that disruption in the ergosterol biosynthesis regulation by deletion of individual genes such as *ERG2* can cause a number of phenotypic effects such as alteration in doubling times and response to stress agents (Bhattacharya et al., 2018).

In case Ach1 indeed plays a role in ethanol formation upon *PYC2* overexpression by converting acetyl-CoA to acetate, it is highly improbable that the resulting acetate is converted to ethanol via Ald6 and Adh1/3/4/5 in the cytosol (Fig. 4) because the conversion of acetate to acetaldehyde via Ald6 is not thermodynamically favourable (Henningsen et al., 2015). Alternatively, the formed acetate could be converted inside the mitochondria to acetaldehyde by Ald4/5, and consequently, acetaldehyde diffuses out of the mitochondria and is converted to ethanol via Ald1/3/4/5 in the cytosol (Fig. 4). Interestingly, the *ALD4* promoter was shown to be one of the promoters with the highest activity in *S. cerevisiae* during growth in synthetic glycerol medium (Ho et al., 2018). This is highly induced in the absence of glucose (Navarro-Avino et al., 1999). This data suggests that upregulation of *ALD4* expression is due to its absence of glucose rather than the presence of glycerol. Notably, we consider this route of ethanol formation via SA more likely than the routes via the pyruvate transport or via cytosolic

conversion of acetate to acetaldehyde since it could better explain the cumbersome detour of carbon flux starting from glycolytic pyruvate (see above).

It is still a question why *S. cerevisiae* re-routes the central carbon metabolism to convert SA to ethanol via the aforementioned routes upon overexpression of either *PYC1*, *PYC2* or *PCK1* at all. One explanation might be linked to the ATP levels in the cell. In fact, *PYC1* and *PYC2* need ATP for its function (Brewster et al., 1994) while *PCK1* generates ATP if it indeed converts PEP to oxaloacetate (De Torrontegui et al., 1966). The high amounts of SA produced in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 is supposed to further limit the ATP availability for the cell since export of SA by *An*DCT-02 also costs ATP because the cell needs to export the protons that originate from SA, to maintain intracellular pH and charge. Nevertheless, it seems that the strain has enough ATP for the export of SA. The situation changes upon overexpression of *PYC2* since *PYC2* could use the ATP which was previously used for export of SA. This might decrease the export of SA and therefore result in the observed reduction in SA in the extracellular medium. By converting it to ethanol, the cells would have an opportunity to reduce SA inside the cell. This hypothesis is supported by the fact that the sole overexpression of *PYC2* in combination with the 'SA module' (strain *UBR2*$_{CBS}$-DHA-SA-*PYC2*$_{OE}$, Fig. 3, shown in red) also resulted in the formation of high amounts of ethanol in comparison to the strain without *PYC2* overexpression (strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02, Fig. 3, shown in black). Upon further ATP limitation caused by the presence of *An*DCT-02 in combination with 'SA module' and *PYC2* overexpression (strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$, Fig. 3, shown in grey), the ethanol formation increased even further. This hypothesis might also explain why the abolishment of the glyoxylate cycle (Fig. 1) in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ results in reduction of ethanol production but has no effect on the detected

SA in the medium (Fig. 8). In line with this, it has been previously shown that the glyoxylate cycle contributes to SA production on glycerol in a strain without *PYC2* overexpression (strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02, Xiberras et al., submitted). Therefore, it is very plausible that the glyoxylate cycle is also involved in SA production in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$. The fact that the abolishment of the glyoxylate cycle did not reduce SA production in the extracellular medium could be because the SA produced via the glyoxylate cycle cannot be exported in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ due to the ATP limitation caused by *PYC2* overexpression (Fig. 1). Instead of being exported, the SA generated via the glyoxylate cycle is converted to ethanol via the possible routes shown in Fig. 4. As a result, abolishment of the glyoxylate cycle results in a decrease in SA production inside the cell and therefore, less ethanol is produced (Fig. 8) via the possible routes shown in Fig. 4.

In conclusion, this study provided novel insights into the central carbon fluxes active in a previously engineered CEN.PK113-1A strain for SA production from glycerol. Although the SA production was not increased as initially aimed, the knowledge gained from this study is essential for future work focusing on improving the production of the valuable chemical SA via the redox-neutral pathway from glycerol.

5. Funding

We thank the German Research Foundation (DFG) for funding of our research (NE 697/7-1).

6. Acknowledgements

We thank Solvejg Sevecke for technical support.

7. Conflict of interest

The authors declare no competing interest.

8. References

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9. Supplementary material

Table S1. Plasmids used in this study.

Plasmid	Relevant characteristic	Source or reference
pGBS415FUM-3	Codon-optimized coding sequence for pyruvate carboxylase from *S. cerevisiae* (*PYC2)*	DSM, The Netherlands
p414-TEF1p-Cas9-CYCt-nat1	*natMX4, P_{TEF1}-cas9-T_{CYC1}*	Klein et al. (2016)
p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX	2 µm, *hphMX, SNR52p-gRNA.*CAN1.Y-*SUP4t*	Klein et al. (2016)
p426-SNR52p-gRNA.YGLCτ3- SUP4t-hphMX	2 µm, *hphMX, SNR52p-gRNA.*YGLCτ3-*SUP4t*	Islam et al. (2017)
p426-SNR52p-gRNA.YPRCτ3-SUP4t-hphMX	2 µm, *hphMX, SNR52p-gRNA.* YPRCτ3-*SUP4t*	Xiberras et al. (submitted)
pUG66	*ble^r*	Gueldener et al. (2002)
pUC18	*E. coli* cloning vector	Yanisch-Perron et al. (1985)
pUC18-*MDH3-R*	*P_{PGK1}-MDH3*-R-*T_{IDP1}*	Xiberras et al. (submitted)
pUC18-*RofumR*	*P_{TEF1}-RofumR-T_{RPL15A}*	Xiberras et al. (submitted)
pUC18-*An*DCT-02	*P_{ENO2}-An*DCT-*02-T_{DIT1}*	Xiberras et al. (submitted)
pUC18-*PYC2*	*P_{TDH3}-PYC2-T_{CYC1}*	This study

Table S2. Primers used in this study.

Purpose	Primer number	Sequence (5'-3')	Description
Construction of the expression cassette of *S. cerevisiae PYC2* (P_{TDH3}-*PYC2*-T_{ADH1}) in pUC18	571	GCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAG AGTCGAGTTTATCATTATCAATACTGC	Primer for Gibson Assembly - amplification of *TDH3* promoter from *S. cerevisiae* Sc288c DNA containing a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
	760	AAGACCGGCCAATTTCTTGCTACTGCTCATATCCGTCGA AACTAAGTTCTTG	Primer for Gibson Assembly - amplification of *TDH3* promoter from *S. cerevisiae* Sc288c containing a 30 bp overhang homologous to the adjacent *PYC2* open reading frame
	761	AAACACCAAGAACTTAGTTTCGACGGATATGAGCAGTA GCAAGAAATTGG	Primer for Gibson Assembly - amplification of *PYC2* from pGBS415FUM-3 containing a 30 bp overhang homologous to the adjacent *TDH3* promoter
	762	AAAATCATAAATCATAAGAAATTCGCTTACTTTTTTTGG GATGGGGGTAG	Primer for Gibson Assembly - amplification of *PYC2* from pGBS415FUM-3 with a 30 bp overhang homologous to the adjacent *ADH1* terminator
	763	CCCCATCCCAAAAAAAGTAAGCGAATTTCTTATGATTTA TGATTTTTATTATTAAATAAG	Primer Gibson Assembly- amplification of *ADH1* terminator from *S. cerevisiae* Sc288c DNA with a 30 bp overhang homologous to the adjacent *PYC2* open reading frame
	764	AGCTATGACCATGATTACGAATTCGAGCTCGGTACCCG GGCCGGTAGAGGTGTGGTC	Primer Gibson Assembly - for amplification of *ADH1* terminator from *S. cerevisiae* Sc288c DNA with a 40 bp overhang homologous to the adjacent BamHI-linearized-pUC18 vector backbone
Construction of strain Strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC1*$_{OE}$	773	GTATTTTTAATCGTCCTTGTATGGAAGTATCAAAGGGGA CGTTCTTCACCTCCTTGGAATCGAGTTTATCATTATCAAT ACTGC	Primer for amplification of *TDH3* promoter from *S. cerevisiae* Sc288c DNA containing an overhang homologous to the adjacent YPRCτ3 coding sequence
	1108	TTCATTGAAATTAATCCAAG	Primer for amplification of *TDH3* promoter from *S. cerevisiae* Sc288c DNA containing an overhang homologous to the adjacent *PCK1* coding sequence
	1096	ATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAAGAACTT AGTTTCGACGGATATGTCGCAAAGAAAATTCGC	Primer for amplification of *PYC1* from *S. cerevisiae* CEN.PK113-1A DNA containing an overhang homologous to the adjacent *TDH3* promoter
	1099	ATAACTTATTTAATAATAAAAATCATAAATCATAAGAAA TTCGCTCATGCCTTAGTTTCAACAGGAAC	Primer for amplification of *PYC1* from *S. cerevisiae* CEN.PK113-1A DNA containing an overhang homologous to the adjacent *ADH1* terminator
	1098	GTTCTATTAGAAGACCAAGTTCCTGTTGAAACTAAGGC ATGAGCGAATTTCTTATGATTTATGATTTTTATTATTAAA TAAG	Primer for amplification of *ADH1* terminator from *S. cerevisiae* Sc288c DNA containing an overhang homologous to the adjacent *PCK1* coding sequence
	874	ATAGAGTAAAGAACCCTTTCTATACCCGCAGCGTCGAC ACCCGGTAGAGGTGTGGTC	Primer for amplification of *ADH1* terminator from *S. cerevisiae* Sc288c DNA containing an overhang homologous to the adjacent *An*DCT-02 expression cassette

	875	TATAGCATGAGGTCGCTCTTATTGACCACACCTCTACCG GGTGTCGACGCTGCGGGTATAG	Primer for amplification of *An*DCT-02 expression cassette with an overhang homologous to the adjacent *TEF1* promoter of the *PYC1* expression cassette
	872	TTACAATCTAGTCGCAAAAACAAGTACAGTGCTGACGT CCCATCTTACTCCGCAACGCTTTTCTGAACG	Primer for amplification of *An*DCT-*02* expression cassette with an overhang homologous to a sequence downstream of the YPRCτ3 coding sequence in chromosome VII
Construction of strain *UBR2$_{CBS}$*-DHA-*MDH3-R*	690	CTGCACATAATTGAAATAAGGATGTAGTTCAACTTCTAT GAATGCTCGGCGATACGATATGGATCCGAAGTACCTTC AAAGAATG	Primer for amplification of *MDH3-R* expression cassette with an overhang homologous to a sequence upstream of the YGLCτ3 coding sequence in chromosome VII
	297	AGTTTTTCCGTCTCTGGCTGAAGGCTCATTTCCATGATG GGGTCACAATTATTATCGCACGATGGTAATGATCCGAA CTT	Primer for amplification of *MDH3-R* expression cassette with an overhang homologous to a sequence downstream of the YGLCτ3 coding sequence in chromosome VII
Construction of strain *UBR2$_{CBS}$*-DHA-*MDH3-R-RofumR*	690	CTGCACATAATTGAAATAAGGATGTAGTTCAACTTCTAT GAATGCTCGGCGATACGATATGGATCCGAAGTACCTTC AAAGAATG	Primer for amplification of *MDH3-R* expression cassette with an overhang homologous to a sequence upstream of the YGLCτ3 coding sequence in chromosome VII
	770	AAAAAGGAGTAGAAACATTTTGAAGCTATGGATGGTAA TGATCCGAACTTGG	Primer for amplification of *MDH3-R* expression cassette with an overhang homologous to the adjacent *TEF1* promoter of the *RoFUMR* expression cassette
	771	AAGGTTCCCCAAGTTCGGATCATTACCATCCATAGCTTC AAAATGTTTCTACTC	Primer for amplification of *RoFUMR* expression cassette with an overhang homologous to the adjacent *IDP1* terminator of the *MDH3-R* expression cassette
	609	GAGTTTTTCCGTCTCTGGCTGAAGGCTCATTTCCATGAT GGGGTCACAATTATTATCGCACGGAAAAACGGGAAGA AAAGG	Primer for amplification of *RoFUMR* expression cassette with an overhang homologous to a sequence upstream of the YGLCτ3 coding sequence in chromosome VII
Construction of strains (1) *UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$, (2) *UBR2$_{CBS}$*-DHA-*An*DCT-02-*PYC2*$_{OE}$, (3) *UBR2$_{CBS}$*-DHA-*MDH3-R-An*DCT-02-*PYC2*$_{OE}$ and (4) *UBR2$_{CBS}$*-DHA-*MDH3-R-RofumR-An*DCT-02-*PYC2*$_{OE}$-	873	TATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGG AATCGAGTTTATCATTATCAATACTGC	Primer for amplification of *An*DCT-02 expression cassette with an overhang homologous to a sequence downstream of the YPRCτ3 coding sequence in chromosome XVI
	874	ATAGAGTAAAGAACCCTTTCTATACCCGCAGCGTCGAC ACCCGGTAGAGGTGTGGTC	Primer for amplification of *An*DCT-*02* expression cassette with an overhang homologous to the adjacent *ADH1* terminator of the *PYC2* expression cassette
	875	TATAGCATGAGGTCGCTCTTATTGACCACACCTCTACCG GGTGTCGACGCTGCGGGTATAG	Primer for amplification of *PYC2* expression cassette with an overhang homologous to a sequence upstream of the YPRCτ3 coding sequence in chromosome XVI
	872	TTACAATCTAGTCGCAAAAACAAGTACAGTGCTGACGT CCCATCTTACTCCGCAACGCTTTTCTGAACG	Primer for amplification of *PYC2* expression cassette with an overhang homologous to the adjacent *ENO2* promoter of the *AnDCT-02* expression cassette

Construction of strains *UBR2*$_{CBS}$-DHA-SA-*PYC2*$_{OE}$ and *UBR2*$_{CBS}$-DHA-*PYC2*$_{OE}$	873	TATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAATCGAGTTTATCATTATCAATACTGC	Primer for amplification of *PYC2* expression cassette with an overhang homologous to a sequence upstream of the YPRCτ3 coding sequence in chromosome XVI
	876	AATCTAGTCGCAAAAACAAGTACAGTGCTGACGTCCCATCCCGGTAGAGGTGTGGTC	Primer for amplification of *PYC2* expression cassette with an overhang homologous to a sequence downstream of the YPRCτ3 coding sequence in chromosome XVI
Construction of strain Strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PCK1*$_{OE}$	1088	GTATTTTTAATCGTCCTTGTATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAATTAGAACGATTAGATGCCGTC	Primer for amplification of *JEN1* promoter from *S. cerevisiae* CEN.PK113-1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ DNA containing an overhang homologous to the adjacent YPRCτ3 coding sequence
	1089	TTCAACTTCGGAAGTAGATCCTACTGTAGCATTCATTTTAGAAGGGGACATATTTTCAGTAGCAGTATTTAATCTCTTTG	Primer for amplification of *JEN1* promoter from *S. cerevisiae* CEN.PK113-1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ DNA containing an overhang homologous to the adjacent *PCK1* coding sequence
	1090	GTAACAGTTTCAAAAGTTTTTCCTCAAAGAGATTAAATACTGCTACTGAAAATATGTCCCCTTCTAAAATGAATGCTAC	Primer for amplification of *PCK1* from *S. cerevisiae* CEN.PK113-1A DNA containing an overhang homologous to the adjacent *TDH3* promoter
	1091	ATGTGGGGGGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGATTACTCGAATTGAGGACCAGCG	Primer for amplification of *PCK1* from *S. cerevisiae* CEN.PK113-1A DNA containing an overhang homologous to the adjacent *ADH1* terminator
	1092	GATGTATTAGCCGCTGGTCCTCAATTCGAGTAATCATGTAATTAGTTATGTCACGC	Primer for amplification of *ADH1* terminator from *S. cerevisiae* Sc288c DNA containing an overhang homologous to the adjacent *PCK1* coding sequence
	992	TATAGAGTAAAGAACCCTTTCTATACCCGCAGCGTCGACACGCAAATTAAAGCCTTCGAG	Primer for amplification of *ADH1* terminator from *S. cerevisiae* Sc288c DNA containing an overhang homologous to the adjacent *An*DCT-02 expression cassette
	993	GAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCGTGTCGACGCTGCGGGTATAG	Primer for amplification of *An*DCT-02 expression cassette with an overhang homologous to the adjacent *TEF1* promoter of the *PCK1* expression cassette
	872	TTACAATCTAGTCGCAAAAACAAGTACAGTGCTGACGTCCCATCTTACTCCGCAACGCTTTTCTGAACG	Primer for amplification of *An*DCT-*02* expression cassette with an overhang homologous to a sequence downstream of the YPRCτ3 coding sequence in chromosome VII
Deletion of *MAE1* gene	1232	TAACGAGTTTAGTGCACATAAATACCAAGACAAAAGGTAGAAATACGGTTCCAGCTGAAGCTTCGTACGC	
	1233	CTATATGGTTTTTTTTTTTTAAGTGCAGGCGTTGGTTATGCTTCGTCGCATAGGCCACTAGTGGATCTG	

Deletion of *ACH1* gene	1313	CGCTTGCTGGAGCAAATGTGTAATCAAGTTGCTGTGTATATATAGACGTTAGATGTGTTCCAGCTGAAGCTTCGTACGC	For amplification of phleomycin or nourseothricin deletion cassette from pUG66 or pUG74, respectively, with a sequence complementary to the flanking regions of the genomic integration site at their 5′ end and a sequence complementary to the loxP sites on pUG66/pUG74 at their 3′ end.
	1314	GTACTACCTGTGAATTAACTAGACGTCATGCGTATTGATTAACTCCCTCCATGAGCGCGCCGCATAGGCCACTAGTGGATCTG	
Deletion of *ICL1* gene	1189	AACAATTGAGAGAAAACTCTTAGCATAACATAACAAAAAGTCAACGAAAACCAGCTGAAGCTTCGTACGC	
	1190	ATATACTTGTCAGGAAATGCCGGCAGTTCTAATGGTTAATCCTTGTCCGCATAGGCCACTAGTGGATCTG	

Table S3. Maximum specific glycerol consumption rates (time point at which the highest glycerol consumption was measured) and maximum ethanol titres detected in the respective *S. cerevisiae* strain on glycerol.

Strain	Maximum Specific Glycerol Consumption Rate [g/g h]	Maximum Ethanol Concentration [g/L]
***UBR2$_{CBS}$*-DHA-SA-*An*DCT-02**	0.07 ± 0.01 (135 h)	0.00 ± 0.00
UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PCK1$_{OE}$	0.09 ± 0.00 (90 h)	6.01 ± 0.00 (120 h)
UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC1$_{OE}$	0.08 ± 0.01 (87 h)	5.90 ± 0.66 (120 h)
UBR2$_{CBS}$*-DHA-SA-*An*DCT-02-*PYC2$_{OE}$	0.37 ± 0.07 (40 h)	13.20 ± 0.28 (72 h)
UBR2$_{CBS}$*-DHA-*An*DCT-02-*PYC2$_{OE}$	0.11 ± 0.03 (51 h)	1.60 ± 0.21 (120 h)

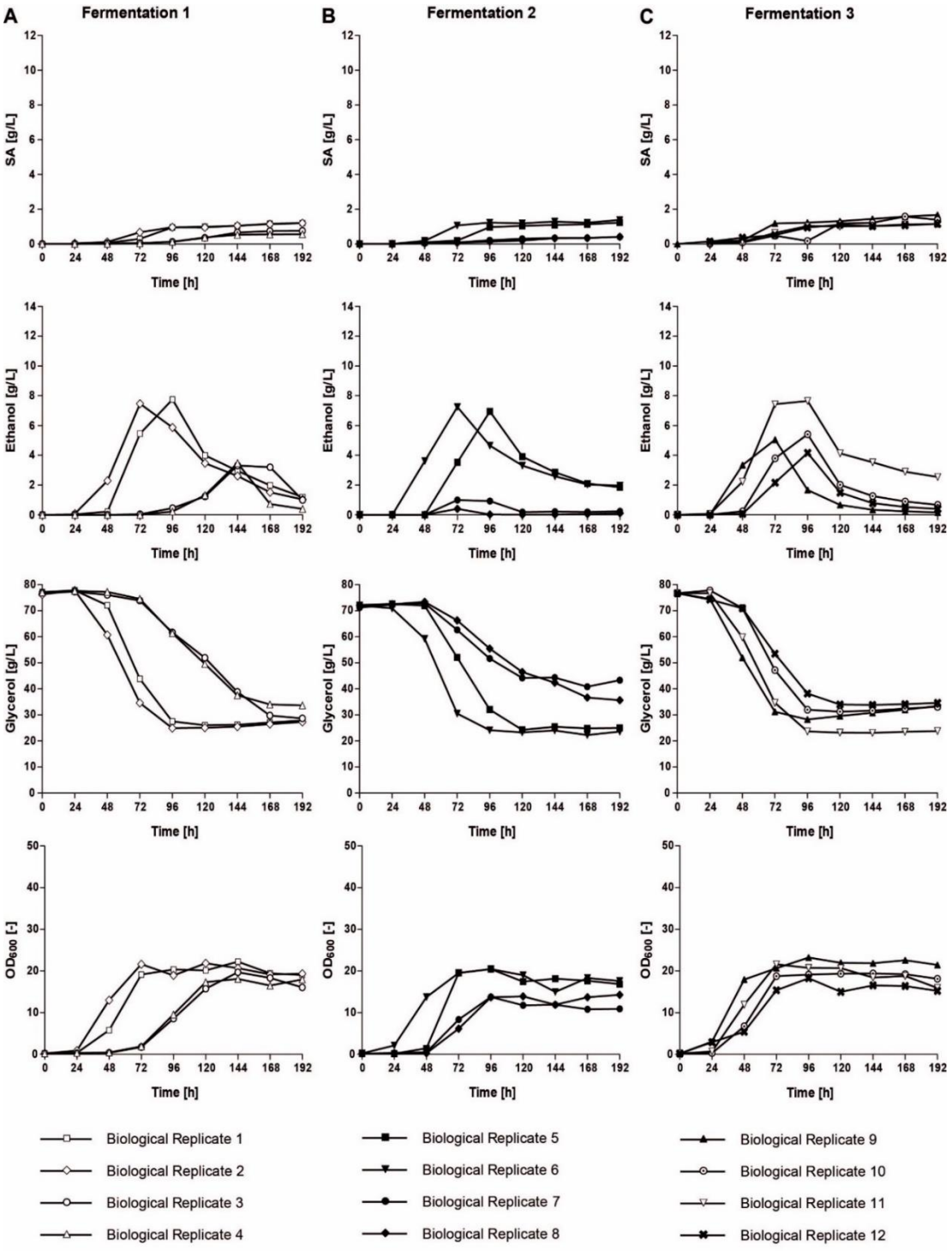

Fig. S1. Individual curves showing SA production, ethanol formation, glycerol consumption and biomass formation for the tested twelve biological replicates of strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02-*PYC2*$_{OE}$ *ach1*Δ (referred to as baseline strain + *PYC2*$_{OE}$ + *ach1*Δ in Fig. 6).

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Chapter 4. DISCUSSION

4.1 The need to collect novel experimental data using cells growing in synthetic glycerol medium

In **chapter 3.3**, the current knowledge regarding the central carbon catabolism of *S. cerevisiae* growing on glycerol has been scrutinized. It became clear that the actual *in vivo* fluxes on glycerol as the sole carbon source are still ambiguous. A number of uncertainties have been identified that particularly regarding the role of the glyoxylate cycle (discussed in **chapter 4.2**), the subcellular localization of the respective enzymes, the contributions of the mitochondrial transporters and the anaplerotic reactions active on glycerol (also discussed in **chapter 4.2**).

Omics studies are very useful in order to understand the cellular pathways and processes at a global and systems level (Snyder & Gallagher, 2009). However, genome-wide studies focusing on the central carbon catabolism of *S. cerevisiae* growing on glycerol have been rare. Roberts and Hudson (2006) have performed a transcriptome study on glycerol as the sole carbon source in direct comparison to ethanol; all experiments were conducted in batch cultivations using complex medium. Ethanol was included as a control in the latter study because it is catabolized in a fully respiratory manner similar to glycerol. Still, glycerol is a C3 carbon source while ethanol is a C2 compound and therefore, their central carbon catabolism is supposed to differ to some extent. Roberts and Hudson (2006) found a huge overlap between the genes upregulated on either glycerol or ethanol (both compared to glucose) but also identified a few differences in gene regulation between the two carbon sources. Our research group has previously shown that complex supplements have a strong impact on the growth of *S. cerevisiae* on glycerol (Swinnen et al., 2013). Henceforth, the results might be different in synthetic medium and it may be useful to perform a similar transcriptome

study medium as conducted by Robert and Hudson (2006) but using synthetic media. This requires the use of strain(s) able to grow in synthetic glycerol medium and such strains are available in our lab. The proposed study could provide a more precise picture of the similarities and differences between the gene regulation of these two carbon sources since any potential impact of the complex compounds present in yeast extract would be omitted. Comparing the results obtained from a transcriptome study in synthetic medium with that performed in complex medium (Roberts and Hudson, 2006) could also be used to investigate whether the addition of supplements in complex medium effects the expression levels of the different genes.

In principle, metabolic flux analysis would be a very useful omics method for solving the above questions since this approach allows the identification of the intracellular fluxes in the central metabolism (Wiechert, 2001). A ^{13}C flux analysis for *S. cerevisiae* growing on glycerol as the sole carbon source has not yet been performed and therefore, the *in vivo* fluxes under these conditions are not really known, but only hypotheses exist based on other non-fermentable carbon sources such as ethanol. In order to be able to perform a metabolic flux analysis on glycerol, a genome-scale model for *S. cerevisiae* growing on glycerol is required. Such a model exists for glucose but is not adapted for glycerol. To obtain such an adapted and verified model, our group is currently upgrading the genomic-scale metabolic model that is based from knowledge gained from glucose to growth on glycerol. Afterwards, the adapted model will be validated with new experimental data obtained from cells growing in synthetic glycerol medium. This work is currently ongoing as part of another project in our group in collaboration with two other partners. Once the metabolic model is finalized, it can be used to determine the metabolic fluxes based on ^{13}C labelling data as previously done

in other studies in glucose- containing media (Frick & Wittmann, 2005; Hayakawa et al., 2018). The data from the ^{13}C flux analysis on glycerol would provide for the first time experimental data about the actual active fluxes during growth of *S. cerevisiae* on glycerol, which could facilitate future metabolic engineering of *S. cerevisiae* on glycerol. In addition, the genome-scale model itself can be used to simulate the fluxes and identify the genetic modifications that lead to the optimization of product formation such as SA from glycerol as previously done in other studies (Bro et al., 2006; Choi et al., 2010; Otero et al., 2013).

Knowledge about the subcellular localization of a particular enzyme is essential to fully understand its function in the cell. A number of genome-wide studies have focused on determining the subcellular localization of proteins in *S. cerevisiae* on different carbon sources by using high-throughput methods of epitope-tagging and immunofluorescence analysis (Huh et al., 2003; Kumar et al., 2002; Yofe et al., 2016). However, glycerol was not among the carbon sources tested by these authors. Notably, the localization of an enzyme can vary depending on the carbon source as discussed in **chapter 3.3.** For example, it has been shown that one of the key enzymes of the glyoxylate cycle Mls1, localizes in the cytosol when cells grow on ethanol, but it is detected in the peroxisome during growth on oleate (Kunze et al., 2002; Kunze et al., 2006). Notably, no information is available with regard to the localization of Mls1 when the cells grow on glycerol. The example of Mls1 underlines the aforementioned necessity to obtain this lacking information in order to increase the accuracy of a metabolic model for growth of *S. cerevisiae* on glycerol (Thiele & Palsson, 2010). As recently reviewed by Nightingale et al. (2019), a number of techniques such as mass

spectroscopy, computational predictions and fluorescent microscopy methods can be used to elucidate protein localization.

In the context of metabolic engineering endeavours with the aim to produce C4 dicarboxylic acids (such as SA) via a fermentative pathway from glycerol, understanding of the mechanisms underlying the transport of the TCA and glyoxylate cycle intermediates, as well as of other organic solutes and inorganic ions between cytosol, the mitochondria and the peroxisome is also crucial. However, some of these mechanisms are still not fully understood and consequently, physiological characterization of some of the transporters is required before they can serve as targets for increasing dicarboxylic acid production on glycerol. For example, future metabolic engineering strategies aiming at increasing SA production via the fermentative pathway ('SA module') from glycerol in the cytosol could focus on deleting the mitochondrial transporters that transport SA from the cytosol into the mitochondria, i.e. *DIC1* and *SFC1* (**chapter 3.3**, Fig. 1). However, it is still not known whether a strain carrying deletions of *DIC1* and *SFC1* is able to grow on glycerol. Henceforth, it would be wise to first elucidate the role of these two mitochondrial transporters on glycerol before the aforementioned metabolic engineering strategy is envisaged. Apart from this, it is still unclear how the intermediates of the glyoxylate cycle are transported between the cytosol and the peroxisome (Antonenkov & Hiltunen, 2012). Such knowledge would be the basis for metabolic engineering strategies focusing on hindering that the cytosolic intermediates of the glyoxylate cycle, i.e. oxaloacetate and malate, enter the peroxisome (**chapter 3.6,** Fig 1). This would increase the concentration of oxaloacetate and malate in the cytosol, and eventually a higher concentration of these intermediates might be converted to SA

via the cytosolic reductive branch of the TCA cycle resulting in higher SA titre/yield (**chapter 3.6**, Fig. 1).

4.2 The higher metabolic flexibility of *S. cerevisiae* with regard to the active anaplerotic reactions during growth on glycerol

Anaplerotic reactions are responsible for the replenishment of TCA cycle intermediates during growth, and therefore play a vital role in the central carbon metabolism. In *S. cerevisiae,* pyruvate carboxylase and the glyoxylate cycle have been experimentally identified (using deletion mutants) to be the main anaplerotic routes during growth on glucose (C6) and on ethanol (C2), respectively (Brewster et al., 1994; Scholer & Schuller, 1993; Stucka et al., 1991). We realised that information is lacking which anaplerotic reaction(s) is/are most important when *S. cerevisiae* grows on glycerol. Such information is very helpful for re-routing fluxes of the central carbon metabolism toward valuable fermentation products because the operating route strongly influences net ATP yield and co-factor balance of the cell (Zelle et al., 2010b; Zelle et al., 2011).

The search for the main anaplerotic route during growth on glycerol is highly connected with the clarification of the role of the glyoxylate cycle on this carbon source. In fact, it has been previously suggested that the glyoxylate cycle is essential for growth on glycerol (Chen et al., 2012) similar to the situation on ethanol where this pathway is the only operating anaplerotic route (Scholer & Schuller, 1993). Notably, the key enzymes of the glyoxylate cycle (encoded by *ICL1* and *MLS1*) have been found to be upregulated on glycerol at both transcriptome and proteome level in the two studies available so far (Ohlmeier et al., 2004; Roberts & Hudson, 2006). During the course of this thesis, it has been found that abolishment of the glyoxylate cycle by deleting *ICL1* in a reversed engineered CEN.PK strain growing in synthetic medium glycerol did not have

any negative effect on growth (**chapter 3.4,** Fig. 2). Obviously, the glyoxylate cycle is dispensable for growth on glycerol. Nevertheless, it cannot be excluded that this cycle plays a role in the overall *in vivo* fluxes active in wild-type strains growing on glycerol. It would be interesting to perform ^{13}C metabolic flux analysis in the reversed engineered CEN.PK strain and compare the *in vivo* fluxes in a corresponding strain in which the glyoxylate cycle is abolished (*icl1* deletion mutant, strain constructed in **chapter 3.4**). Such a data set could also be very valuable for verifying the genome-scale metabolic model on glycerol under construction (**chapter 4.1**).

Although it is still unclear whether the glyoxylate cycle plays a role in the *in vivo* fluxes in the wild-type strains growing on glycerol, it definitely seems to contribute to the fluxes on glycerol in the presence of the 'SA module' and *An*DCT-02**.** According to the data shown in Fig. 2 in **chapter 3.5**, the glyoxylate cycle was obviously the main pathway through which SA was overproduced. The situation was different in the strains constructed for **chapter 3.6** which carry the overexpression of *PYC1, PYC2* or *PCK1* in addition to the 'SA module' and *An*DCT-02. Results show that abolishment of the glyoxylate cycle in the latter strains had only a marginal impact on SA production, but also led to a reduction in ethanol production, growth and glycerol consumption (**chapter 3.6**, Fig. 8). These results suggest that the metabolic fluxes for the aforementioned two genetic backgrounds are different but this hypothesis can only be confirmed via ^{13}C flux analysis.

The findings about the important anaplerotic routes on glycerol are very valuable for any metabolic engineering approach with the goal to valorise this carbon source. Particularly the fermentative production of compounds whose synthesis or export requires an increased ATP yield will highly profit from choosing the proper anaplerotic

route. In this thesis, the ATP-dependent pyruvate carboxylase has been overexpressed to increase the production of the intermediate oxaloacetate (**chapter 3.6**). Based on the findings about the anaplerotic reactions on glycerol shown in **chapter 3.4**, one can also consider to delete pyruvate carboxylase since it has been shown that pyruvate carboxylase negative strains are able to grow on glycerol. Pyruvate carboxylase can be replaced by the other anaplerotic reaction *PCK1*, which generates one ATP per carboxylation event. In this study, *PCK1* was overexpressed in combination with the 'SA module' and *An*DCT-02 but the pyruvate carboxylase was not deleted (**chapter 3.6**, Fig 2). It is still not clear whether *PCK1* converts PEP to oxaloacetate in the latter strain because it has been previously shown that this conversion is only possible upon carbon dioxide sparging (Zelle et al., 2010b). The stain was only tested in the absence of carbon dioxide sparging during the course of this study (**chapter 3.6**, Fig 2). With regard to deletion of pyruvate carboxylase, one has to consider that abolishment of pyruvate carboxylase activity is accompanied with significant reduction in growth on synthetic glycerol medium (**chapter 3.4**, Fig. 2). Therefore, one should evaluate whether the negative impact on growth will also influence product formation in terms of volumetric productivity (i.e. amount of product formed per volume and time) before the aforementioned approach to increase ATP yield is implemented.

4.3 SA production by *S. cerevisiae* from glycerol

As elaborated in the **chapter 1.5.3**, previous studies have focused on engineering *S. cerevisiae* for the production of SA from glucose (Table 1). Despite the discussed advantages of glycerol as mentioned in **chapter 1.2.1**, there have been no previous studies using *S. cerevisiae* as a host growing on glycerol for the production of SA. This is due to several reasons. The most important reason is that many industrial and

laboratory *S. cerevisiae* strains cannot grow on glycerol without the addition of medium supplements (as discussed in the **chapter 1.3.1**). In addition, the supplementation with substances such as yeast extract or peptone would not be economically viable in an industrial conversion of glycerol into products of relatively low market price such as SA. However, our group identified and characterized isolates of *S. cerevisiae* able to grow in pure synthetic glycerol medium (Swinnen et al., 2013). As these wild-type strains catabolize glycerol via the L-G3P pathway, we generated *S. cerevisiae* strains able to catabolize glycerol via the NADH-generating DHA pathway (Klein et al., 2016a) with the goal of providing a basis for production of SA from glycerol and carbon dioxide via the redox-neutral reductive pathway. Results of the current thesis verified that glycerol catabolism via the DHA pathway was indeed essential to achieve high SA titer and yield from glycerol (**chapter 3.5**, Fig. S3). Although the SA in **chapter 3.5** was not only produced via the initially envisaged redox-neutral pathway, the results show the potential of using *S. cerevisiae* for the production of SA from glycerol.

The 'SA module' established in the cytosol for SA production (**chapter 3.5)** was similar to the studies performed on glucose by Van De Graff et al. (2015) and Yan et al. (2014). In the latter two studies, the glyoxylate cycle was not considered to contribute to SA production, because the key enzymes are known to be repressed on glucose (Turcotte et al., 2010). This is in contrast to the current study, where the glyoxalyte cycle has been assumed to play a significant role in SA production from glycerol. Indeed, data suggests that the glyoxylate cycle and the reductive branch of the TCA cycle ('SA module') act together in a highly synergistic manner during SA production from glycerol in strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (**chapter 3.5**, Fig. 2), even though the exact contribution of the individual pathways is still unclear. A ^{13}C metabolic flux analysis

based on an adapted genome scale metabolic model on glycerol could be helpful to better quantify the fluxes. In fact, a previous study on glucose has performed ^{13}C metabolic flux analysis to unravel the pathways through which the dicarboxylic acid malic acid was produced (Zelle et al., 2008). The authors concluded that the malic acid was indeed produced via the envisaged pathway, i.e. the cytosolic reductive branch of the TCA cycle.

As expected, integration of *An*DCT-02 in the DHA pathway strain having the 'SA module' led to a remarkably higher production of SA (**chapter 3.5**, Fig. 2). This positive effect of the transporter is similar to what has been observed in the study conducted by Jansen et al. (2017) in glucose medium. According to Shah et al. (2016), this transporter can export SA and malic acid, but not fumaric acid. Still, the latter study thoroughly characterized the *An*DCT-02 solely in terms of its role in transporting fumaric acid. A detailed study focusing on characterization of *An*DCT-02 with regard to its affinity for export of different organic acids and whether this varies with pH as well as the exact mechanism of how this transporter exports organic acids and which metabolite(s) inhibit its function is still missing. Recently, Darbani et al. (2019) characterized another dicarboxylic acid transporter from the yeast *S. pombe*, encoded by *SpMAE1*, which has also been expressed to increase SA production in *S. cerevisiae* in separate studies as discussed in **chapter 1.5.3, Table 1**. Phylogenetic and protein motif analyses of *Sp*Mae1 showed that it belongs to the voltage-dependent slow-anion channel transporter (SLAC1) clade of transporters. In addition, the conserved phenylalanine residue F329 which closes the pore of *Sp*Mae1 is necessary for the activity of the transporter. It has been shown that it can transport SA, malic acid and fumaric acid in *S. cerevisiae* while not affecting growth on defined mineral media containing glucose as carbon source.

Such studies comprehensively characterizing membrane transporters are necessary to modulate the transport processes for improving secretion of the respective products (such as C4 dicarboxylic acids) as comprehensively reviewed by Borodina (2018).

Previous studies focusing on the production of dicarboxylic acids from glucose have characterized the strains using shake flasks containing calcium carbonate (Pines et al., 1996; Yan et al., 2014; Zelle et al., 2008). Higher concentrations of carbonate (and thus dissolved carbon dioxide) lead to an increase in the substrate concentration for pyruvate carboxylase, which favours the carboxylation of pyruvate to oxaloacetate, a crucial step towards production of SA via the 'SA module' (**chapter 3.5**, Fig.1). Due to this reason, addition of calcium carbonate in shake flasks can lead to higher titres/yield of dicarboxylic acids produced in *S. cerevisiae*. In line with this, we have also characterized the strains constructed in **chapter 3.5** and **3.6** in synthetic medium glycerol containing calcium carbonate (50 g/L), similar to Zelle et al. (2008). However, the strains did not grow under the envisaged condition (data not shown). It is recommended to test the strains in different concentrations of calcium carbonate before comprehensive conclusions can be made. Anyway, the use of shake flasks cultures is not optimal because the pH and oxygen transfer rate cannot be controlled (Zelle et al, 2010a). Therefore, future work will focus on characterization of the strains in pH-controlled bioreactors including carbon dioxide sparging to check whether we can further improve SA production from glycerol. Importantly, off gas analysis in bioreactors will allow the calculation of a precise carbon balance which might also help to understand the fluxes better and verify the metabolic model.

4.4 The potential and challenges of producing SA from glycerol via the redox-neutral pathway in *S. cerevisiae*

SA production via the redox-neutral pathway (i.e. glycerol catabolism via the DHA pathway and SA production via the 'SA module') leads to the highest theoretical product yield per glycerol consumed due to the fixation of additional carbon from carbon dioxide (via pyruvate carboxylase or an alternative route to produce oxaloacetate from C3 such as phosphoenolpyruvate carboxykinase (Beauprez et al., 2010)). This makes the use of this pathway very attractive for valorizing carbon dioxide as a carbon source. In fact, the development of markets and technologies that make use of carbon dioxide as a chemical feedstock have been promoted in order to limit the ongoing climate change (Zevenhoven et al., 2006). The work presented in the current thesis only achieved 34 % of the maximum theoretical SA yield so far (**chapter 3.5**) and it became obvious that the 'SA module' was used in a lesser extent than expected. Still, this redox-neutral, carbon dioxide-assimilating pathway has a great potential for the development of a sustainable SA production process. It would be interesting to computationally analyse the performance of the redox-neutral pathway for production of SA in *S. cerevisiae* from glycerol in terms of product yield, production rates and optimization potential by using a genome-scale model. This analysis would further improve the economic feasibility of the process. Such a study has recently been conducted by Liebal et al. (2018) to evaluate the several process variants for the conversion of C1 carbon substrates to SA in different organisms including *S. cerevisiae* (carbon dioxide fixation via the synthetic pathway crotonyl-coenzyme A (CoA)/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) pathway, (Schwander et al, 2016)). Simulations of this pathway by using the genome-scale model Yeast 7.5 have resulted in an overall SA yield of 64 %. However, this pathway, which

consist of 17 enzymes, from 9 different organisms has only been tested *in vitro* (Schwander et al. 2016). Therefore, this pathway has still to be successfully integrated in *S. cerevisiae* before it can be used for SA production.

The economic viability of SA production from glycerol depends not only on the strain performance but also on other factors. First of all, the production of SA via a bio-based process becomes competitive with the petrochemical-based production only if the price of crude oil price is high. In the last years, the price of crude oil decreased drastically due to oversupply of shale oil and gas (McCoy, 2019), and therefore the production of SA via a bio-based process is not competitive anymore with the petrochemical-based production. Henceforth, the future of bio-based SA production depends heavily on how the price of the crude oil develops in the coming years. Apart from this, one has to take into consideration the price of glycerol. Currently, glycerol is still abundant from the biodiesel industry, therefore it is still a cheap substrate, particularly if crude glycerol is used. A process for production of SA from crude glycerol has still to be adapted. Our lab is currently testing whether the *S. cerevisiae* strains capable of growing on synthetic glycerol medium are also able to grow on crude glycerol. Still, the future biodiesel industry faces a number of uncertainties due to changing politics and governmental priorities (Naylor & Higgins, 2017). Therefore, novel routes for direct or indirect production of glycerol from carbon dioxide should be explored to allow a sustainable supply of glycerol, and thereby increase the economic feasibility for production of SA and other products from this valuable carbon source.

Another challenge related to the production of SA from glycerol via the envisaged redox-neutral pathway is the fact that the overexpression of genes encoding for pyruvate carboxylase did not improve SA production on glycerol, but instead resulted

in an onset of ethanol formation (**chapter 3.6**, Fig. 2). This is in strong contrast to the studies by Yan et al. and Van De Graff et al. (2015) conducted on glucose, where an increase in the respective enzyme activities led to an increase in SA production via the cytosolic reductive branch of the TCA cycle (similar to the 'SA module' in this study). Our results obtained on glycerol compared to those resulting from glucose reflect the differences in the carbon metabolism for these two carbon sources as discussed in **chapter 3.3**. As elaborated in **chapter 3.6,** the exact mechanisms behind the metabolic shift towards ethanol are still not understood but the malic enzyme seems to be involved in redirecting the carbon from SA to ethanol production (**chapter 3.6,** Fig. 5). This observation has been concluded from the facts that i) the presence of a complete 'SA module' seems to be essential for the phenotype (**chapter 3.6**, Fig. 3) and ii) the deletion of *MAE1* reduced ethanol production but did not increase SA formation (**chapter 3.6**, Fig. 5). It is still an open question of how ethanol is formed in strains with *PYC2* overexpression and also what happens to the carbon in the strain that carries the *MAE1* deletion. One should consider that abolishment of the glyoxylate cycle in a strain with *PYC2* overexpression also led to reduction in ethanol production and reduction in growth and glycerol consumption (in the respective strain without deletion of *MAE1*, **chapter 3.6**, Fig. 8). However, the abolishment of the glyoxylate cycle in strain without *PYC2* overexpression, strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (**chapter 3.5**, Fig. 2B) showed an opposite effect, i.e. caused an off-set of ethanol production and increased growth and glycerol consumption. These results highlight once again the complexity of the central carbon metabolism in *S. cerevisiae* during growth on glycerol. It would be interesting to characterize these strains under controlled conditions and perform an off-gas analysis to better understand the fluxes as discussed in **chapter 4.3**. In addition, ^{13}C

metabolic flux analysis could also provide valuable data about the *in vivo* fluxes leading to ethanol production on glycerol in the strains constructed in **chapter 3.6**. The data from experiments in bioreactors and metabolic flux analysis together with metabolomics data could also help in the identification of the factors hindering SA production via the redox-neutral pathway.

In general, cultivation under micro-aerobic conditions (or even anaerobic conditions) is envisaged in order to achieve SA production from glycerol through the redox-neutral pathway. This seems to be necessary to avoid cytosolic NAD^+-regeneration via respiratory mechanisms. For example, it has been recently shown that the external mitochondrial NADH dehydrogenase encoded by *NDE1* (**chapter 3.6**, Fig. 1) has a major function for regenerating cytosolic NAD^+ during growth on glycerol in both the glycerol-utilizing wild-type strain CBS 6412-13A and the corresponding engineered strain CBS DHA in which glycerol is catabolized by the DHA pathway (Asskamp et al., 2019a). In addition, the so called L-glycerol 3-phosphate (L-G3P) shuttle could also be involved in cytosolic NAD^+-regeneration in *S. cerevisiae* (Overkamp et al., 2000) even though Asskamp et al. (2019a) showed that this shuttle cannot take the role of of the external NADH dehydrogenase Nde1. In this shuttle the cytosolic NAD^+-dependent L-glycerol-3 phosphate dehydrogenase (encoded by *GPD1* and *GPD2*) transfers the electrons from the cytosolic NADH to DHAP forming L-G3P. Afterwards, L-G3P crosses the outer mitochondrial membrane and is consequently oxidized by the mitochondrial FAD-dependent L-glycerol-3-phosphate dehydrogenase (encoded by *GUT2*) which transfers the electrons via the $FADH_2$ to complex II of the respiratory chain (Bakker et al., 2001). In principle, other mitochondrial redox shuttles including the malate-aspartate, the malate-oxaloacetate shuttle or the ethanol-acetaldehyde shuttle could

also transport NADH and NAD^+ across the mitochondrial membrane but their significance towards redox balancing in *S. cerevisiae* is still unclear (Bakker et al., 2001). The maximum oxygen transfer rates in shake flasks can be reduced by decreasing the surface area to volume ratio of the medium (Barge et al., 2014). Asskamp et al. (2019b) have shown that a filling volume of 100 mL of synthetic glycerol medium in 500 mL shake flasks led to a reduction in oxygen availability in shake flasks. This cultivation condition was assumed to favour the utilization of the additional NADH gained from glycerol catabolism for SA production via the 'SA module' by limiting the cytosolic NAD^+-regeneration. Therefore, in **chapter 3.5** and **3.6,** micro-aerobic conditions were achieved by characterizing the strains in 500 mL shake flasks containing 100 mL synthetic glycerol medium. In order to test whether further reduction in oxygen transfer rate in shake flasks has a positive effect on SA titre, strain *UBR2*$_{CBS}$-DHA-SA-*An*DCT-02 (**chapter 3.5**) was characterized in 500 mL shake flasks containing a filling volume of either 150 mL or 200 mL. This indeed led to further increase in SA production (up to 15 g/L in shake flasks with a filling volume of 200 mL) and resulted in an onset of ethanol production (up to 6 g/L in shake flasks with a filling volume of 200 mL, data not shown).

In theory, cultivation of the strain envisaged for SA production via the redox-neutral pathway under strictly anaerobic conditions is assumed to be ideal in terms of achieving high product yield (loss of carbon via carbon dioxide produced in the TCA cycle would be minimal), but is only possible if the sum of pathway engineering allows redox balancing and net ATP generation. With regard to the redox balancing, in addition to the redox-neutral pathway from glycerol to the product SA, a redox sink is required for the NADH formed during biomass formation. Interestingly, certain bacteria such as *Citrobacter freundii* and *Klebsiella pneumoniae* (both belong to the *Enterobacteriaceae*

family) are able to anaerobically ferment glycerol due to their ability to produce the highly reduced product 1,3-propanediol (1,3-PDO) as reviewed by Yazdani & Gonzalez (2007). An active 1,3-PDO pathway is required by these organisms because the incorporation of carbon from glycerol into cell biomass generates an excess of reducing equivalents. As the conversion of glycerol into 1,3-PDO results in the net consumption of reducing equivalents, this pathway is necessary to achieve redox-balance in the absence of electron acceptors. Therefore, in order to achieve SA production through the redox-neutral pathway from glycerol under anaerobic conditions in *S. cerevisiae*, a heterologous pathway for 1,3-PDO formation should be implemented to reoxidize the NADH formed during biomass synthesis. Our group is currently testing whether a heterologous pathway for 1,3-PDO formation is active in *S. cerevisiae* growing on glycerol. With regard to net ATP generation, the findings about the anaplerotic routes active on glycerol could provide a solid basis to increase ATP yield during growth on glycerol as discussed in **chapter 4.2**. Still, the improved ATP yield gained from metabolic engineering the anaplerotic reactions might not be sufficient to allow anaerobic production of SA from glycerol. In fact, the major disadvantage associated with production of SA at low pH is the futile cycling of the produced organic acid (Jansen & Van Gulik, 2014). SA is a weak acid, and therefore at low pH, such as 4.0, a major part of the formed SA is present in the lipophilic un-dissociated form in the medium (after it has been exported out of the cytoplasm). This undissociated form of SA can permeate back into the cells by passive diffusion across the cell membrane. Afterwards, the acid will dissociate into protons and the counter ion and the subsequent export of both the counter-ion (in case of this study by *An*DCT-02) and the related protons will require energy in the form of ATP. In fact, the passive diffusion of the undissociated acid into

the cytoplasm and active export of the ions create an ATP-dissipating futile cycle, which might require more ATP than the additional ATP gained by the modified anaplerotic reaction (Shah et al., 2016). Consequently, the success for anaerobic production of SA from glycerol at low pH relies on the availability of energetically neutral SA exporter or alternatively, on finding new routes for increasing the ATP yield.

4.5 Concluding remarks

In this thesis, a review of the state-of-the-art regarding the *S. cerevisiae* growing on glycerol has been presented (**chapter 3.3**), which highlights the importance of gaining more knowledge about the carbon catabolism during growth of *S. cerevisiae* on this carbon source. In line with this, this thesis presents novel findings regarding the anaplerotic reactions active during growth of *S. cerevisiae* on glycerol (**chapter 3.4**). This brought the understanding of the central carbon catabolism on this carbon source a step forward. In fact, the knowledge gained from investigating the anaplerotic reactions proved to be essential for engineering *S. cerevisiae* for SA production from glycerol (**chapter 3.5**). The data in **chapter 3.5** show for the first time SA production from glycerol in *S. cerevisiae.* Although the SA in **chapter 3.5** was not produced via the initially envisaged pathway, i.e. 'SA module', the experimental data in **chapter 3.6** provides a solid basis for future work focusing on further metabolic engineering to channel the carbon flux through the desired pathway for achieving redox-neutral production of SA.

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