Understanding and engineering the cytosolic redox balance of *Saccharomyces cerevisiae* during glycerol catabolism

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SUMMARY

The valorization of carbon-rich industrial waste-streams is a promising avenue to overcome the current dependence on fossil resources for the production of certain chemicals. One of these waste-streams is (crude) glycerol, derived from the still increasing biodiesel industry. Glycerol is an attractive substrate for industrial biotechnological applications aiming at the microbial production of small reduced compounds. It has a higher degree of reduction compared to sugars, which allows higher theoretical yields of reduced molecules, such as carboxylic acids and certain alcohols.

Several microorganisms are able to catabolize glycerol, including the *Saccharomyces cerevisiae* strain CBS 6412-13A. *S. cerevisiae* is routinely employed in industrial biotechnology as a platform organism for the production of various molecules. It is well-characterized in terms of physiology and genetics, it is easy to genetically engineer and robust towards harsh process conditions, such as low pH values. However, in order to utilize glycerol's degree of reduction for *S. cerevisiae*-based synthesis of fermentation products, metabolic engineering is required. The native FAD-dependent glycerol catabolic pathway is coupled to the respiratory chain and therefore transfers electrons to oxygen. The replacement of this native L-glycerol 3-phosphate (L-G3P) pathway by the artificial NADH-delivering dihydroxyacetone (DHA) pathway is supposed to provide glycerol-derived electrons for the cytosolic formation of fermentation products in the cytosol. Glycerol catabolism via the DHA pathway provides, in contrast to the L-G3P pathway, one mol NADH per mol of glycerol converted to dihydroxyacetone phosphate. For the production of reduced compounds, it is required that NADH remains cytosolically available. However, *S. cerevisiae* has potent respiratory mechanisms that oxidize cytosolic NADH and transfer the electrons to oxygen, as cytosolic redox balance is a prerequisite for life. The external NADH dehydrogenases (Nde1/2) and the L-G3P shuttle (composed of Gpd1/2 and Gut2), are known to contribute to cytosolic NAD^+ regeneration during growth with ethanol, galactose or low glucose concentrations.

The overall objective of this thesis was to generate a better understanding of the mechanisms that maintain cytosolic redox balance in *S. cerevisiae* catabolizing glycerol. Therefore, the contribution of the mentioned mechanisms was tested in the glycerol-utilizing wild-type strain CBS 6412-13A and the engineered strain in which glycerol is catabolized via the DHA pathway. In both strains *GPD1/2, GUT2* as well as *NDE1/2* were deleted, separately and in combination and the mutant's growth with glycerol as sole carbon source was recorded. In particular, the *nde1*Δ mutants showed significantly reduced growth while the *nde1*Δ *nde2*Δ double deletion abolished growth. Furthermore, it was demonstrated that deleting *NDE1* significantly increased titer and yield of the fermentation product 1,2-propanediol in a previously engineered *S. cerevisiae* strain under certain conditions.

S. cerevisiae's major native fermentative mechanism to oxidize cytosolic NADH is alcoholic fermentation. When growing with glucose levels above 1 g L^{-1} baker's yeast performs alcoholic fermentation, in order to maintain redox balance, even when oxygen is available (so-called Crabtree effect). However, glycerol was generally considered to be a 'non-fermentable' carbon source for *S. cerevisiae*. The current work demonstrates that at least *S. cerevisiae* strains catabolizing glycerol via the DHA pathway are able to perform alcoholic fermentation. Increasing the uptake of glycerol through the expression of a heterologous aquaglyceroporin, seems to increase the rate of cytosolic NADH formation and the glycolytic flux towards pyruvate, which cumulates in overflow metabolism towards ethanol (8.5 g L^{-1}). By reducing the oxygen availability in shake flask cultures, the ethanol titer of the respective strain was almost doubled to 15.7 g L^{-1} with a yield of 0.34 g g^{-1}, which corresponds to 69 % of the maximum theoretical yield.

Optimal production of small reduced compounds from glycerol by *S. cerevisiae* requires that the fermentation product pathway is redox balanced, and that electrons are transferred to the desired product instead of oxygen. Therefore, fermentative processes should ideally be conducted under anaerobic conditions. However, even if the pathway from substrate to the target fermentation product is redox-balanced, a surplus of NADH is always generated by the

cells during the formation of biomass. In order to balance the NAD^+/NADH ratio under anaerobic conditions, an additional pathway as redox sink is required. The expression of a heterologous pathway from *Clostridium butyricum* for the formation of 1,3-propanediol, was supposed to oxidize surplus NADH, but failed to be functional in its current form as demonstrated in a suitable model strain.

Altogether, the results of this thesis provide important insights in redox metabolism of *S. cerevisiae* catabolizing glycerol and might facilitate the production of valuable small reduced compounds from this attractive feedstock in the future.

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CHAPTER 1: INTRODUCTION

1.1. Glycerol as a promising substrate for the production of fermentation products in industrial biotechnology

1.1.1. The need of microbial bio-processes based on renewable resources

Currently, humankind is still dependent on fossil hydrocarbons for transportation as well as for the production of indispensable daily life products. The environmental impacts of the excessive use of fossil resources are apparent by the ongoing climate change. Carbon dioxide released from fossil resources is a major contributor to global warming (Höök and Tang, 2013; IPCC Report 2018; Fernandez-Martinez *et al*. 2019). Hence, the development of alternative carbon-neutral processes for the production of transportation fuels and platform chemicals is of public, political and economic interest. Such an alternative route is the application of microbial-based processes based on renewable carbon sources (biomass) (Demirbas, 2001; Henry, 2010; Scott and Buchard, 2019). The valorization of biomass requires the efficient conversion of the various constituting carbon compounds to the target products. Microorganisms are well-able to utilize a wide range of components of biomass as a carbon substrate and their metabolic activities offer the opportunity to synthesize a large portfolio of different compounds (Lee *et al*. 2019). This opens the avenue to produce sustainable, eco-friendly chemicals via industrial biotechnological processes.

Although biomass related processes provide highly attractive alternatives to processes based on fossil resources, a fact that always has to be considered is that biomass supply most often competes with the production of food and feed for men and animals. Therefore, especially the valorization of carbon rich waste streams derived from other industrial processes relying on biomass, is appealing for microbial biotechnology (Willke and Vorlop, 2004; Burk and van Dien, 2016; Zeng, 2019). These waste streams are displayed by agricultural-industry by-products, such as citrus peels, sugar beet or olive pulp, whey or sawdust. In addition, also wastes from chemical processing of biomass, like biomass hydrolysates or crude glycerol derived from

transesterification of triglycerides during biodiesel production, can be exploited as feedstocks (extensively review by Koutinas *et al*. 2014).

1.1.2. Availability of Glycerol

Glycerol is omnipresent in living organisms. It serves as the molecular skeleton of all phospholipids and triacylglycerols (Nelson and Cox, 2013). Those lipids are the constituents of all cell membranes as well as fat tissues of higher organisms. Certain organisms such as yeasts or algae produce and accumulate glycerol *per se* as a protectant against various kinds of environmental stresses (Ben-Amotz *et al*., 1982). Moreover, glycerol is also produced in small amounts as an electron sink in some yeast species (particularly under anaerobic conditions) (see **section 1.2.2**).

Nowadays, human society contributes to a large extent to the production and release of glycerol (Ma and Hanna, 1999; Luo *et al.* 2016). Chemical synthesis accounted over decades for about 25 % of the global demand, while 75 % were met by soap manufacturing. Saponification of fatty acids/ triglycerides results in formation of an alcohol (glycerol) and carboxylates (Ciriminna *et al*. 2014). Since 2003, the contribution of chemical synthesis to total glycerol production has decreased to insignificant 0.25 %. The reason for this dramatic reduction was the worldwide increased production of biodiesel. Biodiesel is produced from plant and animal fats and oils by a transesterification process and in total up to 10 % of the resulting products consists of crude glycerol that is often considered a waste stream (Dharmadi *et al*. 2006; Mattam *et al*. 2013; Ardi *et al*. 2015) (Figure 1.1). Particularly, the European Union drove biodiesel production by the directive 2003/30/EC “On the promotion of the use of biofuels […] for transport” (European Parliament), demanding all members to increase the proportion of transportation fuels derived from renewable resources to 5.75 %. This directive was replaced in 2009, thereby aggravating the requests for each country by setting national targets for the proportions of energy (including biofuels) derived from renewable sources (European

Parliament, directive 2009/28/EC). This led to excess supply of foremost crude glycerol (Quispe *et al*. 2013).

As crude glycerol is directly precipitated from the biodiesel producing processes, it is still contaminated with methanol, water, salts, ash, free fatty acids (FFA), and fatty acid methyl esters (FAME) (Dasari 2007; Hu *et al*. 2012; Yang *et al.* 2012; Valerio *et al*. 2015). In order to valorize it for example for pharmaceutic or food applications, costly purification is required, which makes its use economically less favorable (Ardi *et al*. 2015). In 2018, 99.7 % pure glycerol had a market value of around 0.50 \$ lb^{-1} while crude glycerol (for further refining) costed only 0.18 \$ lb^{-1} (HB International SAS, 2018).

Today, almost two-third of the worldwide glycerol production results from biodiesel output (Vivek *et al*. 2017). Hence, the glycerol market is completely dependent on the booming biodiesel economy. This means that to date the glycerol prices are tightly coupled to the biodiesel production. However, public and political discussions about transportation fuels from fossil resources, especially diesel, remain diverse. The future of diesel as gasoline and thus the future of biodiesel might be less bright after the so-called 'Dieselgate' in 2015 (Brand, 2016; Naylor and Higgins, 2017). Therefore, the current political discussion will definitely also affect the glycerol market.

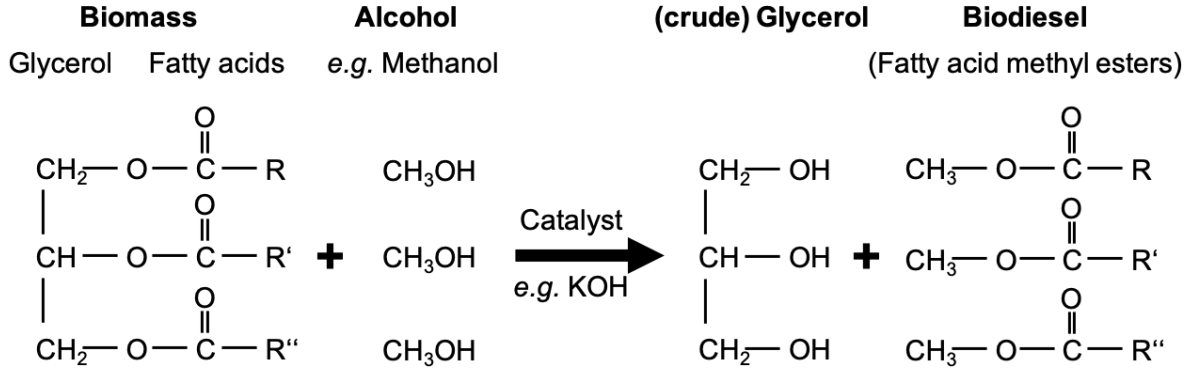

Figure 1.1: Biodiesel and glycerol formation from vegetable oils and animal fats (biomass). During the transesterification of plant oils or animal fats up to 10% (v/v) glycerol is formed.

1.1.3. Glycerol is a promising feedstock for microbial bio-processes

Apart from the aspect of current excess supply, glycerol has one important advantage in terms of its chemical properties. When compared to hexoses, the usual feedstock for industrial bioconversions, glycerol has a greater degree of reduction (DOR) per carbon than e.g. glucose (DOR 4.7 versus 4.0), meaning more electrons per carbon can be released during its (complete) oxidation (Villadsen *et al.* 2011). Taking the catabolic pathways of glucose and glycerol into account, the conversion of glycerol to pyruvate (via glycolysis) generates twice the amount of reducing equivalents related to carbon equivalents as compared to glucose (Yazdani and Gonzalez, 2007). Provided that the respective electron carriers are not oxidized via respiration, the oxidation of glycerol alone or as co-substrate theoretically provides the opportunity to obtain fermentation products with a high degree of reduction (such as succinate or propionate) at an improved theoretical yield compared to their synthesis from glucose (Figure 1.2) (Johnson and Taconi 2007; da Silva *et al*. 2009; Clomburg and Gonzalez 2013; de Bont *et al.* 2018).

To date several industrial scale applications for the production of chemicals based on glycerol as the (intermediate-) substrate have been developed. The most famous commercial product, derived from chemicals (1,3-propanediol) partially produced by glycerol catabolizing microorganisms, is the fibre Sorona® by DuPont (Laffend *et al.* 1996; Chotani *et al*. 2000; Emptage *et al.* 2003). Besides, the production of 1,3-propanediol (1,3-PDO), dihydroxyacetone and 2,3-butanediol by microorganisms growing on glycerol has reached commercial scale (Chen and Liu, 2016; Luo *et al.* 2016). Potentially, other small molecules such as 1,2-propanediol (1,2-PDO), organic acids, ethanol, hydrogen, or amino acids could also be produced using appropriately engineered organisms (Wendisch *et al.* 2011; Almeida *et al*. 2012; Viana *et al*. 2014; He *et al*. 2017).

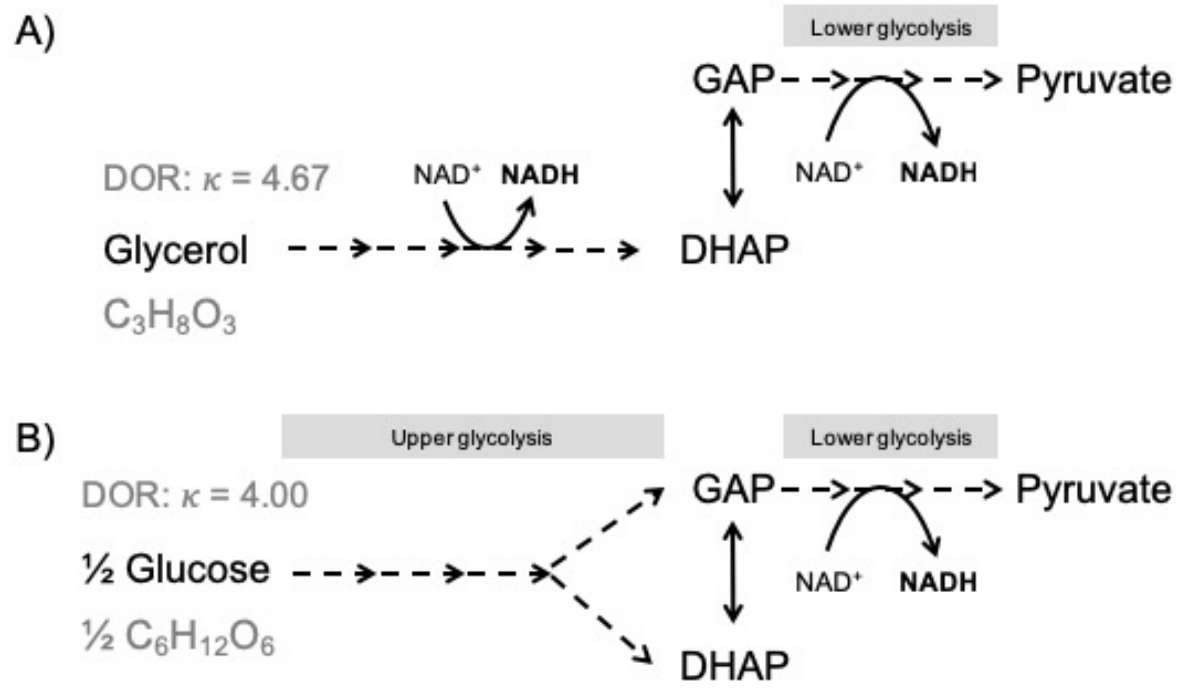

Figure 1.2: Overview of the advantage of glycerol over glucose in terms of the number of reduced redox cofactors (NADH) formed during their catabolism towards pyruvate via glycolysis. A) Per 1 mol glycerol (= 1 C_3-equivalent) 2 mol NADH can be produced. B) Starting from 1/2 mol glucose (= 1 C_3-equivalent) as substrate only 1 mol NADH is gained during glycolysis. Abbreviations: DHAP – dihydroxyacetone phosphate; DOR [κ]: degree of reduction; GAP – glyceraldehyde 3-phosphate.

1.1.4. Microbial glycerol catabolism

A great variety of bacteria and fungi is able to use glycerol as carbon (co-)substrate. It is usually catabolized via one of two principle oxidative pathways, both resulting in the formation of the central glycolytic intermediate dihydroxyacetone phosphate (DHAP) (Gancedo *et al*. 1968; Fakas *et al*. 2009; Yazdani and Gonzalez, 2007). On the one hand, glycerol can be first oxidized to dihydroxyacetone (DHA), followed by phosphorylation to DHAP. On the other hand, glycerol can be first phosphorylated to L-glycerol 3-phosphate (L-G3P) which is subsequently reduced to DHAP (Figure 1.3).

Among fungi, in particular yeasts from the genera *Pichia*, *Saccharomyces, Schizosaccharomyces* and *Yarrowia* can naturally catabolize glycerol (Gancedo *et al*. 1986; Kurtzmann *et al*. 2011; Merico *et al*. 2011; Swinnen *et al*. 2013). However, glycerol-based

growth is highly variable among yeasts. Certain species (e.g. *Yarrowia lipolytica* or *Cyberlindnera jadinii*) are efficient in utilizing glycerol and reach growth rates comparable to those observable when growing on glucose, while others (e.g. *S. cerevisiae*) show comparably poor growth (comprehensively reviewed by Klein *et al.* 2017). Similarly, the ability to ferment glycerol varies between yeast species. *Pachysolen tannophilus* for example was shown to be able to produce ethanol from glycerol (Liu *et al*. 2012), while glycerol is regarded as a 'non-fermentable' carbon source for (wild-type) *S. cerevisiae* (Schüller, 2003; Xiberras *et al*. 2019).

Prominent examples for prokaryotes able to utilize glycerol come from the families of *Enterobacteriaceae*, *Clostridiaceae*, *Lactobacillaceae* and *Pseudomonaceae*. Although glycerol is usually metabolized in a respiratory manner, some of these bacteria are even able to anaerobically ferment glycerol (Bouvet *et al.* 1995; Gonzalez *et al*. 2008; da Silva *et al.* 2009). A prerequisite (besides the formation of a product that is more oxidized than glycerol) is the availability of a pathway that can serve as an electron sink leading to the generation of a reduced (by)product. As the DOR of glycerol is greater than that of cell biomass (DOR 4.3; average biomass formula: $CH_{1.9}O_{0.5}N_{0.2}$ according to Villadsen *et al.* 2011), the formation of biomass results in surplus redox equivalents (*i.e.* NAD(P)H) (van Dijken and Scheffers, 1986; Villadsen *et al*. 2011). Therefore, depending on the organism, fermentative glycerol oxidation is connected to the formation of 1,3- or 1,2-propanediol (1,3-/1,2-PDO) (Nissen *et al.* 1997; Biebl *et al.* 1999; Clomburg and Gonzalez, 2013).

The substrate for the 1,3-PDO pathway is glycerol. The first enzyme in the two-step pathway towards 1,3-PDO is a glycerol dehydratase (GDHt), converting glycerol to 3-hydroxypropionaldehyde (3-HPA), the second is an NADH-dependent 1,3-PDO oxidoreductase (PDOR), which catalyzes the reduction of 3-HPA to 1,3-PDO (Figure 1.3). In contrast, the pathway(s) for fermentative 1,2-PDO production start(s) from DHAP (Figure 1.3). In a first step, a methylglyoxal synthase forms methylglyoxal (MG). Dependent on the organism MG is subsequently either reduced by an aldo-keto reductase to acetol or by a glycerol dehydrogenase to R/S-lactaldehyde. The final step is a predominantly NADH-dependent

reduction of the respective pathway intermediate to 1,2-PDO, catalyzed by alcohol dehydrogenases (Murata *et al.*, 1985; Jez *et al.*, 1997; Sulzenbacher *et al.*, 2004; Ko *et al.*, 2005; Chang *et al.*, 2007).

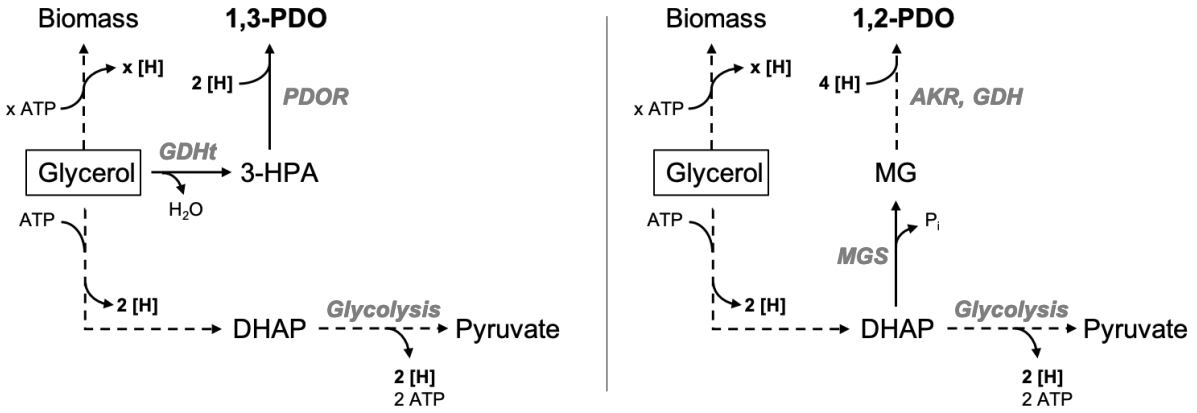

Figure 1.3: Exemplary presentation of bacterial glycerol fermentation (adapted from Clomburg and Gonzalez, 2013)**.** Depending on the species, bacteria either form 1,3-propanediol (1,3-PDO) or 1,2-propanediol (1,2-PDO) in order to oxidize surplus redox equivalents [H] formed during biomass formation under anoxic conditions. On the left side, generalized glycerol catabolism plus formation of 1,3-PDO is shown (e.g. in *K. pneumoniae* or *C. butyricum*), while on the right glycerol catabolism plus formation of 1,2-PDO (e.g. in *E. coli*) is displayed. Pyruvate is further catabolized to H_2, CO_2, ethanol or formate depending on the species. Abbreviations: 3-HPA – 3-hydroxypropionaldehyde; AKR – Aldo-Keto reductase; DHAP – dihydroxyacetone phosphate; GDH – glycerol dehydrogenase; GDHt – glycerol dehydratase; MG – methylglyoxal; MGS – methylglyoxal synthase; PDOR – 1,3-PDO oxidoreductase.

1.2. The yeast *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* is one of the most widely studied model organisms in science (Karathia *et al.* 2011). Before becoming important in research, *S. cerevisiae* has already been used for ages in beer brewing, wine making and as leavening agent during baking (Barnett, 2003; Legras *et al*. 2007). The latter application earns the organism its common name “baker’s yeast”. Nowadays, broad knowledge about the physiology and genetics is available with regard to this species. Being unicellular, *S. cerevisiae* is an easy-to-handle eukaryotic organism for fundamental research (Botstein and Fink, 2011). Basic cellular mechanisms such as replication machinery, cell division and recombination, as well as metabolic cascades related to (human) diseases are highly conserved between *S. cerevisiae* and higher eukaryotes (Petranovic *et al*. 2010). *S. cerevisiae* was the first eukaryotic organism whose genome was entirely sequenced (Goffeau *et al.* 1996), an advent that even further facilitated its use as a model system in research. As a model organism, there are plenty of molecular biological tools available for engineering *S. cerevisiae* e.g. transformation methods, expression vectors and a range of genetic selection markers (Gietz and Woods, 2001; Akada *et al.* 2002; Nevoigt, 2008; Lian *et al.* 2018). Also, the CRISPR/Cas9 system was recently adapted for its use in this yeast (DiCarlo *et al.* 2013; Jakočiūnas *et al*. 2015; Stovicek *et al*. 2015). The latter complements *S. cerevisiae’*s naturally excellent homologous recombination abilities (Paques and Haber, 1999), with regard to precise manipulation of the genome, even allowing the deletion of several genes in a single step (Wijsman *et al*. 2019) or the one-step integration of whole metabolic pathways (Horwitz *et al.* 2015; Tsai *et al.* 2015; Klein *et al*. 2016b; Stovicek *et al*. 2017).

S. cerevisiae did not only become a model organism in science but also a popular production host in novel biotechnological applications. In fact, baker’s yeast has become a platform organism for industrial bioprocesses (Nielsen *et al.* 2013). *S. cerevisiae* is classified as “generally recognized as safe” (GRAS) by the U.S. Food and Drug Administration, facilitating its handling in research as well as expanding its field of industrial applications. Its usage in

industry has also been supported by the short generation time, possibility of achieving high cell density cultures and its obvious immunity to bacteriophage infections as well as natural robustness to extreme conditions, such as high sugar and ethanol concentrations and tolerance towards low pH-values (Yin *et al*. 2007; Nevoigt 2008; Bertozzi Silva and Sauvageau, 2014).

S. cerevisiae naturally produces certain metabolites, such as ethanol, succinate or pyruvate, that are of interest to be industrially exploited (Hong and Nielsen, 2012; Costenoble *et al*. 2011). Via metabolic engineering approaches they can be overproduced and even secreted. In addition, non-native compounds can be synthesized. Biofuels (Turner *et al*. 2016), Bulk chemicals, like organic acids (Raab *et al*. 2010) as well as fine chemicals (Dai *et al*. 2015), therapeutic proteins (Kim *et al*. 2015) and even vaccines (Bill, 2014) are nowadays produced by genetically engineered *S. cerevisiae* strains (Borodina and Nielsen, 2014). Most recent achievements with (potential) industrial scale applications are the production of the antimalarial drug artemisinin (Westfall *et al*. 2012; Paddon *et al*. 2013), human milk oligosaccharides (Liu *et al*. 2018), synthesis of opioids or cannabinoids (Galanie *et al*. 2015; Luo *et al*. 2019), and dopamine (DeLoache *et al*. 2015).

Along with the platform character of *S. cerevisiae* in industrial biotechnology, it is worth mentioning that the natural substrate spectrum of *S. cerevisiae* is rather narrow. Numerous attempts have already been undertaken to engineer *S. cerevisiae* in a way to overcome its substrate limitations (Ostegaard *et al*. 2000; Çakar *et al.* 2012; Buschke *et al*. 2013; Klein *et al*. 2016; Lane *et al*. 2018). In fact, it was shown that *S. cerevisiae* can even be genetically engineered for exploitation of relevant polysaccharides and the component sugars, present in more complex sources such as lignocellulose or pectin-rich wastes (Nevoigt, 2008; Wendisch *et al.* 2011; Sandström *et al.* 2014; Biz *et al*. 2016).

1.2.1. Carbon metabolism in *S. cerevisiae*

General background

Saccharomyces cerevisiae is a chemoorganoheterotrophic organism. It metabolizes the carbon substrates in order to meet three major demands: biomass formation, energy production (in the form of ATP), and redox potential maintenance (Lagunas, 1986). Unlike most other eukaryotic organisms, bakers' yeast is a facultative anaerobic organism. It is well able to catabolize different carbon sources in a respiratory, a respiro-fermentative or a fermentative manner (Flores *et al*. 2000; Merico *et al.* 2007). The preferred natural carbon sources are hexose sugars, and in particular glucose. In addition, also some disaccharides or C2 compounds such as ethanol or acetic acid can be utilized (Rodrigues *et al.* 2006). However, wild-type *S. cerevisiae* shows natively no growth with carbon sources such as pentose sugars or sugar acids present in plant biomass.

Glucose catabolism

Glucose catabolism to pyruvate via glycolysis delivers the energy required for growth. During glycolysis, glucose is first activated and subsequently oxidized in several steps. In the end, the cells yield two molecules pyruvate, two molecules of NADH and two net ATP. Pyruvate can be further catabolized via two different ways. When excess glucose is available, *S. cerevisiae* shows a metabolic abnormality as it ferments pyruvate to ethanol and CO_2 (and glycerol) not only in the absence of oxygen, but even when oxygen is available, although it is energetically less favorable than respiring it. This phenomenon is called the 'Crabtree-effect' (Crabtree, 1929). It describes the behavior of several yeasts to switch to ethanol fermentation as soon as glucose levels in the medium are above a certain threshold (*i.e.* approximately 1 g L^{-1} for *S. cerevisiae*). This abnormality in aerobic carbon metabolism can be divided into a short-term and a long-term Crabtree-effect (Pronk *et al.* 1996). The short-term effect is characterized as immediate onset of alcoholic fermentation (within seconds) in response of *S. cerevisiae* growing with low glucose concentrations, to a glucose pulse. It is caused by a saturated respiratory metabolism, leading to overflow of NADH and pyruvate. During the long-term effect,

respiratory enzymes are additionally repressed by glucose, which reduces ATP generation by oxidative phosphorylation (Trumbly, 1992; Carlson, 1999; Hedbacker and Carlson, 2008; Kayikci and Nielsen, 2015).

The NADH formed during glycolysis needs to be oxidized. This is maintained by diverting carbon from pyruvate to acetaldehyde and using the latter as the final electron acceptor (de Deken, 1966; Käppeli, 1986; Dashko *et al*. 2014). This process, called alcoholic fermentation, proceeds in the cytosol via decarboxylation of pyruvate to acetaldehyde and CO_2. The responsible enzyme, pyruvate decarboxylase (PDC), is encoded by the structural genes *PDC1/5/6* (Hohmann and Cederberg, 1990; Hohmann, 1991). The following reaction from acetaldehyde to ethanol oxidizing NADH is mainly catalyzed by the alcohol dehydrogenase encoded by *ADH1,* whose expression is induced by glucose (Ciriacy *et al*. 1997). Cells with a deletion of *ADH1* produce significantly lower amounts of ethanol (Denis *et al*. 1983). As the conversion of glucose to ethanol is redox neutral, *S. cerevisiae* additionally forms glycerol to keep redox balance during the Crabtree-effect as well as under anaerobic conditions (see **section 1.2.2**).

As a direct consequence of the Crabtree-effect, the respiro-fermentative glucose catabolism is characterized by the so-called ‘diauxic shift’. The diauxic shift is a growth arrest when the glucose level drops below a certain threshold, during which the metabolism switches from glucose to ethanol and glycerol as carbon source. This switch causes metabolic adaptations, *i.e.* derepression of the respiratory chain and induction of several enzymes, which leads to a lag phase (Haarasilta and Oura, 1975; Wills, 1990; Haurie *et al*. 2001; Roberts and Hudson, 2006; DeRisi *et al*. 2007). The originally glucose-derived carbon (present in the form of ethanol and glycerol) is fully respired to CO_2 and H_2O (see below) (Fiechter *et al*. 1981).

The second way pyruvate is catabolized, is fully respiratory. Only when low glucose concentrations are available, as maintained in fed-batch approaches or chemostats, *S. cerevisiae* completely respires this carbon source (Marres *et al*. 1991; Larsson *et al.* 1993;

Luttik *et al*. 1998; Bakker *et al*. 2001). Under these conditions, pyruvate is transported into the mitochondria. Inside the mitochondria pyruvate is activated to acetyl-CoA by the pyruvate dehydrogenase complex (PDH) (Kresze and Ronft, 1981), which is finally completely oxidized into CO_2 and H_2O by the TCA cycle and the respiratory chain.

Catabolism of purely respiratory ('non-fermentable') carbon sources: ethanol and glycerol

In contrast to glucose, other carbon sources are purely respiratory (e.g. ethanol and glycerol) for *S. cerevisiae* (Schüller, 2003; Rodrigues *et al*. 2006; Turcotte *et al.* 2010; Wendisch *et al.* 2011). In the cytosol, Adh2 is mainly responsible for oxidizing ethanol to acetaldehyde. Acetaldehyde is afterwards further oxidized to acetate by an aldehyde dehydrogenase. Acetate is converted to Acetyl-CoA, which is required as substrate for the glyoxylate cycle. The products of the glyoxylate cycle are then respired via the tricarboxylic acid (TCA) cycle. Parts of malate and oxaloacetate derived from the TCA cycle are subsequently channeled into gluconeogenesis (Beier *et al*. 1985; Young and Pilgrim, 1985). As a consequence, the cells require anaplerotic reactions in order to replenish the TCA cycle (de Jong-Gubbels *et al*. 1998; Blank and Sauer, 2004; Zelle *et al*. 2010).

Besides glycerol synthesized by the cell itself, *S. cerevisiae* is well able to take it up actively from its surrounding. However, it is not a preferred carbon source for *S. cerevisiae,* which can be attributed to rather inefficient utilization (Gancedo *et al.* 1968). First glycerol is taken up by the cell via active transport using a glycerol/H^+ symporter encoded by *STL1* (Ferreira *et al.* 2005). A deletion of *STL1* abolishes growth on glycerol (Luyten *et al*. 1995; Swinnen *et al.* 2013). Glycerol is natively catabolized via the L-G3P pathway, where glycerol is first phosphorylated to L-G3P by the glycerol kinase (GK) encoded by *GUT1*. L-G3P is subsequently oxidized to DHAP by the mitochondrial L-G3P dehydrogenase (mtGPD) encoded by *GUT2* (Rønnow and Kielland-Brandt, 1993; Sprague and Cronan, 1977; Pavlik *et al*. 1993; Swinnen *et al*. 2013). The mtGPD is located at the outer surface of the inner mitochondrial membrane, with its catalytic site facing the intermembrane space. The electrons

from L-G3P are transferred via $FADH_2$ (FAD is bound to Gut2) to the quinone pool of the respiratory chain (Påhlman *et al*. 2001b). Carbon derived from glycerol enters glycolysis at the level of the central glycolytic intermediate DHAP. As glycerol is a C3-carbon compound, only half the number of molecules compared to glucose (C6) are further metabolized to pyruvate during lower glycolysis, yielding only one molecule of NADH as well as one net ATP per molecule of glycerol.

1.2.2. Redox metabolism in *S. cerevisiae*

The viability of cells depends on the availability of redox cofactors that accept electrons. Around 200 redox reactions require either $NAD(P)^+$ or NAD(P)H (Förster *et al*. 2003). Especially the availability of NAD^+ is required for continuous carbon oxidation. If one state – oxidized or reduced – cannot be regenerated, the metabolism will halt. The NAD^+/NADH couple cannot be generated easily *de novo* by cells in sufficient amounts within a short time (Panozzo *et al.* 2002; Rongvaux *et al.* 2003; Pollak *et al.* 2007). Therefore, NADH has to be immediately re-oxidized in order to keep a balanced state of NAD^+ and NADH. NAD^+ is either regenerated by respiration, transferring electrons carried by NADH to oxygen as the terminal electron acceptor, or by fermentation, which displays a reduction of a metabolic carbon intermediate (e.g. acetaldehyde is reduced to ethanol). An important fact to be considered with regard to redox balance is the compartmentation of eukaryotic cells. A direct exchange between mitochondrial and cytosolic NADH is impossible, which results in two separate pools of redox equivalents. Particularly the inner mitochondrial membrane is impermeable for NADH, whereas porins in the outer membrane allow its diffusion (von Jagow and Klingenberg, 1970). In contrast, NAD^+ can be at least transported from the cytosol (where the *de novo* synthesis of NAD^+ is located) into the mitochondria by unidirectional import. The respective transport proteins in *S. cerevisiae* are encoded by *NDT1* and *NDT2* (Todisco *et al.* 2006). The described

compartmentation requires that each molecule of NAD^+ reduced to NADH in the cytosol has to be re-oxidized, in order to maintain redox balance.

Maintenance of redox balance by respiration

When oxygen is available, electrons captured in the form of intracellular carriers such as NADH are in almost all eukaryotic organisms exclusively transferred to the respiratory chain, where they finally reduce O_2. *S. cerevisiae* follows this general mechanism with all other carbon substrates except glucose (excess) (see **section 1.2.1**). The compartmentation of eukaryotic cells requires special mechanisms in order to couple the respiratory chain to reduced electron carriers generated outside the mitochondria. When considering the different mechanisms for maintenance of redox equivalent balance, it has to be kept in mind that most data have been obtained almost exclusively from experiments conducted with *S. cerevisiae* cells grown on glucose or ethanol as a carbon source so far. Moreover, it is not completely clear to which extent these mechanisms exactly contribute to cytosolic or mitochondrial NADH re-oxidation (Bakker *et al*. 2001; Rigoulet *et al*. 2004).

Baker's yeast expresses similar to plants a specific NADH dehydrogenase (Luttik *et al*. 1998). It is localized at the outer surface of the inner mitochondrial membrane and is therefore called the 'external mitochondrial NADH dehydrogenase'. It is encoded by the isogenes *NDE1* and *NDE2,* whereas *NDE1* is the isoform, which is physiologically more important (Luttik *et al*. 1998; Small and McAlister-Henn, 1998). These enzymes oxidize cytosolic NADH directly, thus regenerating cytosolic NAD^+. The electrons are transferred to the quinone pool of the respiratory chain. It was shown that both isogenes are upregulated after the diauxic shift as well as in cells growing exclusively with ethanol or under glucose-limited conditions indicating their importance for respiratory redox metabolism (DeRisi *et al*. 1997; ter Linde *et al*. 1999).

Along with the NADH dehydrogenases, several shuttle mechanisms are discussed to contribute to indirect transfer of electrons from cytosolic NADH into the mitochondria (Bakker *et al*. 2001). The fundamental functionality of all these shuttles is that cytosolic compounds are reduced in dependency of NADH. The reduced compound subsequently crosses the inner

mitochondrial membrane by transport or diffusion, or it remains in the cytosol and is oxidized by an enzyme, that couples electron transfer to the respiratory chain. In case of the shuttles where the reduced intermediate enters the mitochondria, the catabolite is oxidized in the mitochondrial matrix, thereby reducing mitochondrial NAD^+. The resulting mitochondrial NADH is oxidized by the activity of a NADH:ubiquinone oxidoreductase encoded by *NDI1* (Figure 1.4) (de Vries and Grivell, 1988; Marres *et al*. 1991). This enzyme, referred to as the internal NADH dehydrogenase, replaces in yeast the multi-subunit complex I of the respiratory (Nosek and Fukuhara, 1994). It catalyzes the transfer of two electrons to the respiratory chain and does not pump protons in contrast to complex I of other eukaryotes (de Vries and Grivell 1988; Marres *et al*. 1991).

The most often described shuttle mechanism in *S. cerevisiae*, is the L-G3P shuttle (Larsson *et al.* 1998; Overkamp *et al*. 2000) (Figure 1.4). In this shuttle DHAP is reduced to L-G3P via the cytosolic NAD-dependent L-G3P dehydrogenase (ctGPD) encoded by *GPD1* and *GPD2* (Albertyn *et al*. 1994b; Eriksson *et al*. 1995). L-G3P is then oxidized to DHAP by the FAD-dependent mtGPD (Gut2) which eventually transfers the electrons to ubiquinone in the respiratory chain. However, the L-G3P shuttle was never found to be solely responsible for redox balance maintenance. It was shown to be involved in NADH re-oxidation together with the external NADH dehydrogenase in glucose-limited chemostat cultures (Overkamp *et al*. 2000; Påhlman *et al*. 2001b). Both mechanisms also have kinetic interactions as single deletions of *NDE1* and *NDE2* or *GUT2* result in increased activity of the residual enzymes (Påhlman *et al*. 2002).

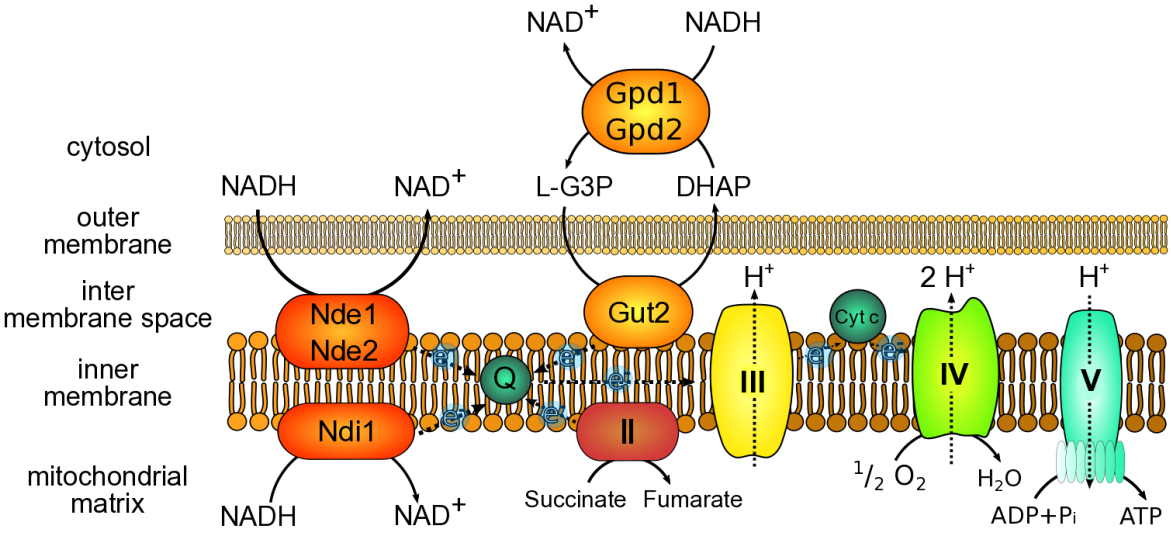

Figure 1.4: Respiratory chain and mechanisms involved in maintenance of redox balance in *S. cerevisiae*. e^- - electrons; Q - quinone pool of the respiratory chain; complex; Cyt c - cytochrome c; Gpd1/Gpd2 - cytosolic glycerol 3-phosphate dehydrogenase; Gut2 - mitochondrial glycerol-3-phosphate dehydrogenase; Nde1/Nde2 - external NADH dehydrogenases; Ndi1 - internal NADH dehydrogenase; II/ III/ IV/ V - respiratory complexes II - V; L-G3P - L-Glycerol 3-phosphate; DHAP – dihydroxyacetone phosphate.

Maintenance of redox balance without oxygen available

When excess glucose is available, it is fermented to ethanol (compare to **section 1.2.1**). This is a redox neutral process, meaning that the two molecules of NADH formed during the conversion of glucose into two molecules pyruvate (glycolysis) are re-oxidized during the formation of two molecules of ethanol. However, as NADH is not exclusively formed during glycolysis, but also generated during anabolic processes, such as amino acid synthesis, a surplus of NADH is present. During growth on glucose without oxygen available, this additional NADH can neither be re-oxidized via ethanol formation, nor via respiration, which is why cells form glycerol (van Dijken and Scheffers, 1986; Jouhten and Pentiliiä, 2014). In fact, Björkqvist *et al.* (1997) as well as Hubmann *et al.* (2011) showed in two independent studies that the abolishment of glycerol formation results in strains incapable to grow under anaerobic conditions, indicating that the generation of glycerol is indispensable for maintaining cellular redox balance.

Glycerol formation starts from DHAP, the central intermediate of the glycolysis. The ctGPD, also involved in the L-G3P shuttle when oxygen is available, catalyzes the NADH-dependent reduction of DHAP to L-G3P. This step is the crucial one for NAD^+ regeneration under anoxic conditions. Anaerobically growing *S. cerevisiae* cells are dependent on activity mainly of the isoenzyme Gpd2 (while Gpd1 is rather responsible for osmotic stress adaptation) (Ansell *et al*. 1997). L-G3P is subsequently dephosphorylated to glycerol by L-G3P phosphatases encoded by *GPP1* and *GPP2* (Figure 1.5) (Larsson *et al*. 1993; Norbeck *et al*. 1996). While Gpp1 was shown to be upregulated under anoxic conditions and to be definitely involved in glycerol formation, the exact contribution of Gpp2 is not clarified (ter Linde *et al*. 1999; Påhlman *et al*. 2001a). In addition to Gpp1/2 also other, yet unidentified, phosphatases seem to be able to also catalyze the latter reaction (Nguyen and Nevoigt, 2009).

Mitochondria are also involved in carbon and free-energy metabolism under anaerobic conditions, although not to the same extent as under aerobic conditions. Some NADH is still generated in the mitochondria by irreplaceable assimilatory reactions (oxaloacetate and 2-oxoglutarate formation) (Nissen *et al*. 1997; Visser *et al*. 2004). The only way to rebalance this mitochondrial NADH is to connect its oxidation to glycerol formation. Therefore, especially one redox shuttle, the ethanol-acetaldehyde shuttle is used to indirectly transport electrons into the cytosol (Bakker *et al*. 2000).

The alcohol dehydrogenases Adh3 (mitochondrial) and Adh1/2 (cytosolic) are involved in the reversible reaction of acetaldehyde to ethanol and vice versa (Albertyn *et al*. 1994a; Nissen *et al*. 1997; Bakker *et al*. 2000). Acetaldehyde is reduced in the mitochondrial matrix by Adh3, thereby oxidizing one molecule of NADH. The formed ethanol diffuses into the cytosol and gets oxidized to acetaldehyde with concomitant reduction of NAD^+ to NADH. The generated acetaldehyde diffuses back into the mitochondria and the cycle begins again. The resulting molecule of cytosolic NADH is re-oxidized afterwards via glycerol formation (see above).

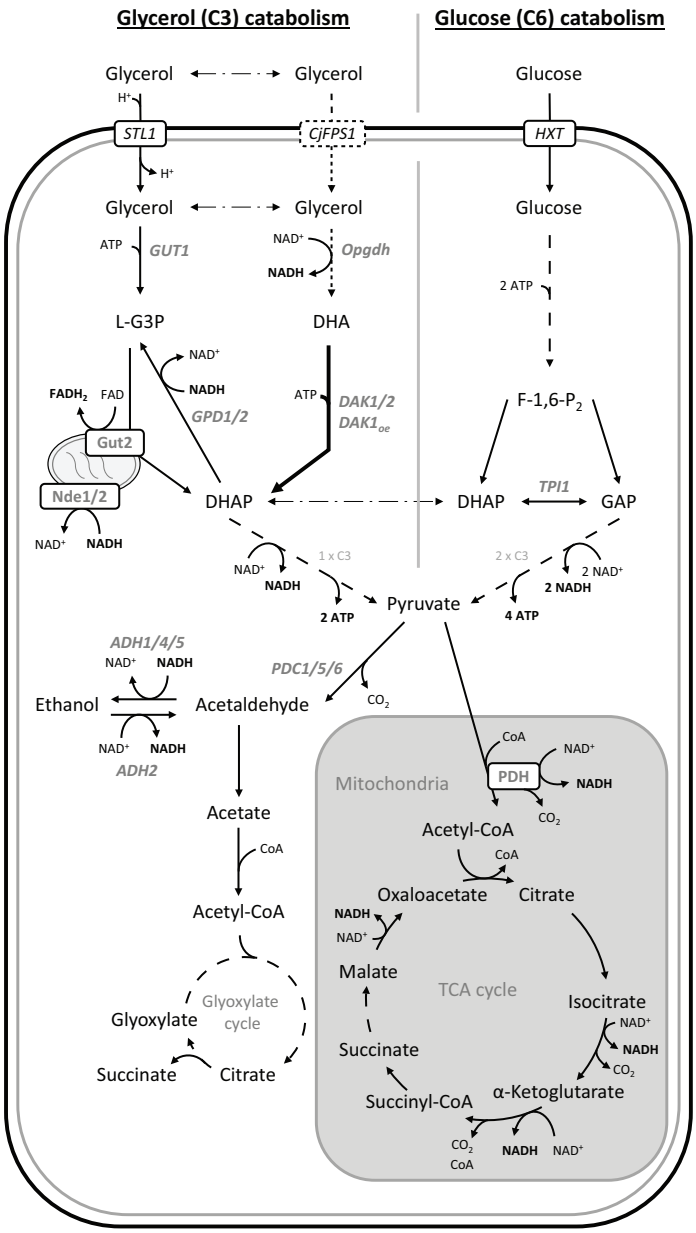

Figure 1.5: Simplified scheme of the central metabolic pathways in *S. cerevisiae* (natural and modified pathway for glycerol catabolism) during growth on glycerol in comparison to growth on glucose. Glycerol catabolism via Gut1 and Gut2 reflects the native L-G3P pathway. Glycerol catabolism via the heterologously expressed *Opgdh* and the overexpressed *DAK1/2 DAK1oe* reflects the synthetic DHA pathway; Dashed arrows represent several reactions. Dotted arrows show heterologously expressed enzymes; the bold arrow indicates an enzyme activity caused by overexpression of an endogenous gene. Dashed/dotted arrows do not stand for reactions. Abbreviations: *ADH1/4/5, ADH2* - alcohol dehydrogenases; *CjFPS1* – aquaglyceroporin from *C. jadinii*; *DAK1/DAK2 DAK1oe* – dihydroxyacetone kinase, with DAK1 overexpressed; DHA - dihydroxyacetone; DHAP – dihydroxyacetone phosphate; F-1,6-P_2 fructose-1,6-bisphosphate; GAP – glyceraldehyde 3-phosphate; *GPD1/GPD2* – cytosolic glycerol dehydrogenase; *GUT1* – glycerol kinase; *GUT2* – mitochondrial glycerol dehydrogenase; *HXT* – hexose transporters mediating glucose uptake; L-G3P – L-glycerol 3-phosphate; Nde1/2 – external mitochondrial NADH dehydrogenases; *Opgdh* – glycerol dehydrogenase from *O. parapolymorpha*; *PDC1/5/6* – pyruvate decarboxylase; PDH – pyruvate dehydrogenase complex; *STL1* – glycerol/H^+ symporter; *TPI1* – triosephosphate isomerase.

1.2.3. Glycerol as feedstock for *S. cerevisiae*

1.2.3.1. Variability of growth with glycerol among the species *S. cerevisiae*

The knowledge regarding the glycerol catabolism in *S. cerevisiae* nearly exclusively originates from studying laboratory strains in the presence of supplements (Swinnen *et al*. 2013; Klein *et al.* 2017; Xiberras *et al*. 2019). When compared to several other yeast species, able to grow on glycerol with a maximum specific growth rate (μ_{max}) of up to 0.4 h^{-1} (Lages *et al*. 1999; Mattanovich *et al*. 2009; Workman *et al*. 2013; Klein *et al*. 2016b), *S. cerevisiae* only shows poor growth (if at all) (Swinnen *et al.* 2013; Klein *et al.* 2016b). Particularly in synthetic glycerol medium without supplementation of amino acids and nucleic bases (CSM), several laboratory and industrial strains, such as CEN.PK113-1A, W303-1A, Ethanol Red and S288c do not grow at all.

Swinnen *et al*. (2013) tested 52 prototrophic *S. cerevisiae* strains for their ability to utilize glycerol as the sole carbon source in synthetic medium without supplements. Among the selected strains, a haploid segregant of the strain CBS 6412 (CBS 6412-13A) exhibiting a maximal growth rate of around 0.13 h^{-1}, was chosen for further investigation, as it displayed the best ratio between growth rate and the duration of lag phase after transfer from the same synthetic medium containing glucose as the carbon source. A genetic mapping approach revealed three genetically altered loci (*UBR2*, *GUT1, SSK1*) to contribute to the glycerol positive phenotype of CBS 6412-13A in comparison to the well-established laboratory strain CEN.PK113-1A (Swinnen *et al*. 2013; Swinnen *et al*. 2016).

Although the molecular basis for the differences between *S. cerevisiae* and other yeasts in terms of glycerol utilization are not unraveled, the glycerol uptake seems to be one factor (Gancedo *et al.*1968; Klein *et al.* 2016a). In fact, other yeast species, such as *Cyberlindnera jadinii* (former names *Candida utilis*, *Pichia jadinii*) have, compared to *S. cerevisiae*, much higher glycerol uptake rates (Gancedo *et al.* 1968). While *S. cerevisiae* was shown to be dependent on the glycerol/H^+ symporter Stl1 (Swinnen *et al*. 2013; Ferreira *et al*. 2005) some

other species take up glycerol also with the help of proteins, that are supposed to belong to the family of aquaglyceroporins (Lucas *et al.* 1990; Oliveira *et al*. 2003; Sabir *et al.* 2016). In the past, such a protein encoded by *FPS1* was also assumed to play a role in glycerol uptake in *S. cerevisiae,* but later studies revealed that it is mainly involved in the control of releasing intracellularly accumulated glycerol during osmoregulation (when cells grow on glucose) (Luyten *et al.* 1995; Tamás *et al.* 1999).

Klein *et al*. (2016a) expressed several Fps homologues from various yeast species with superior glycerol utilization in the moderately growing *S. cerevisiae* strain CBS 6412-13A and showed that their expression could improve growth rates and reduce lag phases after a shift from glucose to glycerol containing synthetic medium. The μ_{max} were improved from 0.13 h^{-1} up to almost 0.18 h^{-1} with expression of Fps1 of all tested homologues in addition to the endogenous Stl1 and in mutants lacking Stl1.

1.2.3.2. Metabolic engineering to allow fermentation of glycerol by *S. cerevisiae*

As elaborated in **section 1.1.2** and **1.1.3** it is attractive to apply glycerol as carbon substrate and *S. cerevisiae* as platform for the production of reduced small molecules. In order to use glycerol's reduction state with *S. cerevisiae* it requires metabolic adjustments. The native L-G3P pathway (see **section 1.2.1**) for glycerol catabolism, does not provide extra reducing equivalents for the formation of fermentation products, because only $FADH_2$ (FAD is bound to Gut2) is formed and electrons are directly transferred to the quinone pool of the respiratory chain. Therefore, no cytosolic redox equivalents are generated. An alternative to overcome the shortage of cytosolic reducing equivalents is the reductive glycerol catabolic pathway (from now on called 'DHA pathway'). Dissimilation of glycerol via an (engineered) DHA pathway provides net NADH in contrast to the L-G3P pathway in *S. cerevisiae*. This alternative oxidative glycerol catabolic pathway has been shown to be the main glycerol utilization pathway in *Schizosaccharomyces pombe* (May *et al.* 1982; Gancedo *et al*. 1986; Matsuzawa *et al.* 2010) and *Ogataea parapolymorpha* (Tani and Yamada, 1987a; 1987b). Glycerol is oxidized by a

NAD^+-dependent glycerol dehydrogenase (GDH) to DHA, which afterwards undergoes phosphorylation to DHAP, catalyzed by a DHA kinase (DAK). A DHA pathway was also proposed to exist naturally in *S. cerevisiae.* While a functional DAK encoded by the two isogenes *DAK1* and *DAK2* is also present in *S. cerevisiae*, (Sprague and Cronan, 1977; Norbeck and Blomberg, 1997; Molin *et al.* 2003) no *in vitro* GDH activities could be measured (Norbeck and Blomberg, 1997; Ford and Ellis, 2002; Nguyen and Nevoigt, 2009). Recent studies in our lab showed that *S. cerevisiae* is indeed solely dependent on the L-G3P pathway for glycerol catabolism (Swinnen *et al*. 2013; Klein *et al*. 2016b).

Klein *et al*. (2016b) recently replaced the L-G3P pathway in the glycerol catabolizing strain CBS 6412-13A by a DHA pathway. A GDH from the naturally on glycerol growing yeast *O. parapolymorpha* (*Opgdh*) was heterologously expressed, allowing oxidation of glycerol to DHA. The overexpression of endogenous *DAK1* was additionally necessary for growth (Klein *et al*. 2016b). The latter modification was shown to allow sufficient flux towards DHAP and in addition prevented accumulation of high amounts of the toxic intermediate DHA. The additional deletion of *GUT1* (Figure 1.5), guaranteed the carbon flux, starting from glycerol, exclusively via the DHA pathway (Klein *et al*. 2016b). The replacement of the glycerol catabolizing pathway and additional expression of *PtFPS2* allowed the strain CBS 6412-13A to grow with a μ_{max} of about 0.10 h^{-1}. Further increased Dak1 activity, achieved by introduction of additional expression cassettes for Dak1 resulted in μ_{max} of 0.12 h^{-1}.

Interestingly, the replacement of the L-G3P pathway by the DHA pathway (Figure 1.5), enabled growth in strains of the CEN.PK and Ethanol Red family, natively not growing on glycerol (Klein *et al*. 2016b). Additionally, Klein *et al*. (2016b) and Ho *et al*. (2018) recently showed the possibility to even improve glycerol growth further (μ_{max} up to 0.26 h^{-1} in an engineered CEN.PK derivative), by optimizing the DHA pathway.

1.3. Production of reduced small molecules from glycerol using *S. cerevisiae*

1.3.1. Production of 1,2-propanediol as a model

A major advantage of the DHA pathway for the production of fermentation products is that the redox equivalents are directly accessible in the cytosol. Consequently, the cytosolic production of certain reduced compounds from glycerol, would become redox neutral, allowing for theoretical higher yields of such compounds.

The proposed advantage of the DHA pathway for the production of small reduced compounds from glycerol was recently confirmed. A pathway for the production of 1,2-PDO (DOR 5.3) has been established in the CBS 6412-13A strain (Islam *et al*. 2017). 1,2-PDO finds application in production of food and personal care goods, as cooling and deicing agent and as building block for unsaturated polyester resins. Its current global market volume is about one billion US-dollar (Biddy *et al*., 2016; Chen and Liu, 2016).

Islam *et al*. (2017) showed that the expression of a methylglyoxal (MG) synthase (encoded by *Ecmgs*A) and a glycerol dehydrogenase (encoded by *EcgldA*) from *E. coli* resulted in formation of 1,2-PDO in a strain expressing the DHA pathway and the aquaglyceroporin Fps1 from *C. jadinii*. This pathway allows DHAP to be converted to MG by the heterologous MG synthase. The subsequent fate of MG is not yet clear. The proposed way is via reduction to acetol/hydroxyacetone by an unknown enzyme and another reduction catalyzed by *EcgldA* to 1,2-PDO. In addition to the expression of the heterologous enzymes, the native triosephosphate isomerase (encoded by *TPI1*) was downregulated to allow the carbon flow into the 1,2-PDO pathway to compete with lower glycolysis. This approach resulted in the highest reported titer for 1,2-PDO production in *S. cerevisiae* so far (4.3 g L^{-1} in complex medium) as well as in the unintended formation of up to 20 g L^{-1} ethanol (in synthetic medium with urea as nitrogen source). However, yield and titer of 1,2-PDO are not yet high enough to compete with neither chemical production nor the quantities achieved using bacteria and glycerol as feedstock (*E. coli* 5.62 g L^{-1}) (Clomburg and Gonzalez, 2011; Lee *et al*. 2016).

1.3.2. Requirements for anaerobic fermentation of glycerol by *S. cerevisiae*

Glycerol's advantage as substrate for industrial biotechnological processes of providing additional redox equivalents only applies, when the electrons are not transferred to oxygen during respiration. Therefore, anaerobic fermentation of glycerol is desirable. The native glycerol catabolic pathway in *S. cerevisiae,* however*,* is coupled to the respiratory chain (see **section 1.2.1**). In contrast, the DHA pathway decouples glycerol breakdown from respiration and provides cytosolic NADH, thus allowing anaerobic processes (**section 1.2.3**).

Nevertheless, anaerobic fermentation of glycerol requires further metabolic engineering of *S. cerevisiae*. Without oxygen available net ATP production exclusively via substrate level phosphorylation and cellular redox balance have to be ensured (Bakker *et al*. 2001; Rodrigues *et al*. 2006). Provided, that enough ATP is generated and the pathway for the product of interest is redox neutral (e.g. the 1,2-PDO pathway), the cells would still need a possibility to oxidize surplus NADH, generated during biomass formation. As described in **section 1.2.2**, *S. cerevisiae* produces natively glycerol as redox sink for this purpose. When growing on glycerol (synonymous with already high intracellular glycerol concentrations) this redox sink is thermodynamically unfeasible for the cells. In order to artificially balance the NAD^+/NADH ratio, there are two general possibilities: i) addition of a co-substrate to the media, which is then reduced (Björqkvist *et al*. 1997; Gonzalez *et al*. 2000; Wahlbom and Hahn-Hägerdal, 2002) or ii) the implementation of a heterologous product pathway, that requires reduced cofactors (Guo *et al*. 2019). Both possibilities display the opportunity for the organism to use a metabolite as terminal electron acceptor. Co-substrates as alternative electron acceptors are for example acetaldehyde, acetoin or pyruvate. All three can be taken up by *S. cerevisiae* and become reduced by native reactions to ethanol/CO_2 or 2,3-butanediol. For the application of an entire pathway as redox sink, the native bacterial option during glycerol fermentation (**section 1.1.4**) to produce 1,3-PDO is attractive. In fact, the latter is particularly attractive, as its substrate is also glycerol, it oxidizes one molecule of NADH, it does not require ATP consumption and on top, but of minor relevance, 1,3-PDO itself is a valuable platform chemical (Biddy *et al*., 2016).

CHAPTER 2: OBJECTIVES AND OUTLINE

Glycerol is a very appealing substrate for biotechnological applications for several reasons. Especially its high degree of reduction makes it an ideal substrate for the microbial production of reduced small molecules (fermentation products). *S. cerevisiae* is a popular platform organism for industrial biotechnology, but most commonly used strains do only grow on glycerol as a carbon source if complex supplements such as yeast extract are added to the medium. A previous screening conducted in our lab, identified the *S. cerevisiae* isolate CBS 6412 which even grows in synthetic glycerol medium (Swinnen *et al*. 2013). A segregant of this strain (CBS 6412-13A) exhibiting a growth rate of 0.12 h^{-1} was used as a basis for the current study. Wild-type *S. cerevisiae* strains including CBS 6412-13A use the so-called L-G3P pathway for glycerol catabolism, which is directly coupled to respiration via the FAD-dependent enzyme Gut2. However, the advantage of glycerol's reduction state only becomes available for the production of fermentation products, if the electrons are not eventually transferred to oxygen. In order to make the electrons available in the form of cytosolic NADH, the replacement of the native L-G3P pathway by the so-called DHA pathway was recently established (Klein *et al.* 2016b). Still, no fermentation products were detected in the respective DHA pathway derivative of the *S. cerevisiae* strain CBS 6412-13A suggesting that all electrons from cytososlic NADH eventually enter the respiratory chain. The current work can be allocated to the long-term goal of fermenting glycerol (preferably under microaerobic or even anaerobic conditions). The study aims at a better understanding of the fate of electrons during growth of *S. cerevisiae* on glycerol and how these electrons can be directed into the formation of fermentation products such as the native fermentation product ethanol and/or the heterologous product 1,2-propanediol.

Chapter 3.3 focuses on the identification of the mechanisms which are responsible for the maintenance of the re-oxidation of cytosolic NADH in the wild-type *Saccharomyces cerevisiae* strain CBS 6412-13A and its DHA pathway derivative during growth with glycerol as the sole carbon source in synthetic medium. In fact, the electrons from cytosolic NADH must eventually enter the respiratory chain under these conditions since no fermentation products were

detected in the respective strains. Two major respiratory routes are known from previous studies in carbon sources other than glycerol, *i.e.* the external NADH dehydrogenases and the so-called L-G3P shuttle. Therefore, mutants with deletions of genes encoding for crucial enzymes involved in these mechanisms were analyzed with regard to impacts on glycerol growth performance in synthetic glycerol medium. After identifying the most important mechanism, it will be tested how its abolishment affects the production of 1,2-propanediol (as a model reduced small molecule which is in demand for industry) from glycerol in a respectively engineered DHA pathway derivative of CBS 6412-13A.

The native fermentation product of *S. cerevisiae* formed during growth on fermentable carbon sources such as glucose is ethanol. However, glycerol is generally believed to be a non-fermentable carbon source for *S. cerevisiae.* **Chapter 3.4** scrutinizes whether this also holds for the *S. cerevisiae* CBS 6412-13A derivative which uses glycerol exclusively via the DHA pathway. In fact, this strain is supposed to form more cytosolic NADH (per mol glycerol consumed) than the wild-type strain using the native L-G3P pathway. Ethanol production of the DHA pathway derivative will be tested with and without the presence of the heterologous *C. jadinii* Fps1. The latter genetic modification has previously been demonstrated to accelerate glycerol uptake in *S. cerevisiae*. This might increase the proposed overflow metabolism towards alcoholic fermentation. and presumably further increasing the rate of cytosolic NADH formation. The effect decreased oxygen availability on ethanol formation will be tested as well.

The yield of fermentation products is supposed to be maximal under anoxic conditions. In order to achieve anaerobic glycerol fermentation, it requires a redox neutral product pathway from glycerol to the fermentation product(s). As the formation of biomass also results in a surplus of NADH (Villadsen *et al.* 2011), an additional redox sink is required. Certain bacteria which are naturally able to ferment glycerol under anaerobic conditions, oxidize this extra NADH by forming 1,3-propanediol (1,3-PDO) via a two-step pathway. In **Chapter 4** the expression of a bacterial 1,3-PDO pathway in *S. cerevisiae* will be tested. The used model *S. cerevisiae* strain (a *gpd1*Δ *gpd2*Δ double deletion strain) is supposed to be only capable of growing on a mixture

of glucose and glycerol under anoxic conditions if the heterologous 1,3-PDO pathway from *Clostridium butyricum* is functional. Furthermore, expression of the clostridial 1,3-PDO pathway will be tested in strains from the background CBS 6412-13A catabolizing glycerol either via the L-G3P or the engineered DHA pathway.

Chapter 5 discusses the results gathered throughout the course of this PhD thesis. In addition, potential future research is evaluated.

CHAPTER 3: MANUSCRIPTS

3.1. List of manuscripts

I. **Aßkamp, M.R.**, Klein, M. and E. Nevoigt. Involvement of the external mitochondrial NADH dehydrogenase Nde1 in glycerol metabolism by wild-type and engineered *Saccharomyces cerevisiae* strains. FEMS Yeast Research 19, foz026.

II. **Aßkamp, M.R.**, Klein, M. and E. Nevoigt. *Submitted*. *Saccharomyces cerevisiae* exhibiting a modified route for uptake and catabolism of glycerol forms significant amounts of ethanol from this carbon source considered as 'non-fermentable'. Biotechnology for Biofuels.

3.2. Statement of the author's contribution to the manuscripts

Title of Thesis: Understanding and engineering the cytosolic redox balance of Saccharomyces cerevisiae during glycerol catabolism

Discipline/ in Field: Biochemical Engineering

Chapter	Contribution	Please provide the following information about the article (if applicable)				
		Title	**Co-authors**	**Journal**	**Status (submitted, accepted, published)**	**Date of submission/acceptance**
3.3	I participated in the design of the study, performed the all the molecular genetic engineering and the growth as well as the HPLC analysis. I analyzed the data and wrote the draft of the manuscript.	Involvement of the external mitochondrial NADH dehydrogenase Nde1 in glycerol metabolism by wild-type and engineered *Saccharomyces cerevisiae* strains	Klein, Mathias Nevoigt, Elke	FEMS Yeast Research	published	20.03.2019
3.4	I participated in the design of the study, performed the molecular genetic engineering, the all the batch cultivations and the HPLC analysis. I analyzed the data and wrote a draft of the manuscript.	*Saccharomyces cerevisiae* exhibiting a modified route for uptake and catabolism of glycerol forms significant amounts of ethanol from this carbon source considered as 'non-fermentable'	Klein, Mathias Nevoigt, Elke	Biotechnology for Biofuels	submitted	10.07.2019

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.

Signature Place, Date

3.3. Manuscript I

Essentially as published in FEMS Yeast Research 19, foz026

Involvement of the external mitochondrial NADH dehydrogenase Nde1 in glycerol metabolism by wild-type and engineered *Saccharomyces cerevisiae* strains

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Short title: Fate of cytosolic NADH in *S. cerevisiae* on glycerol

One sentence summary: During growth of baker's yeast on glycerol, a significant portion of cytosolic NADH is re-oxidized by the respiratory chain via the external NADH dehydrogenase isoenzyme Nde1.

Abstract

Glycerol is an attractive substrate for microbial fermentations due to its higher degree of reduction compared to glucose. The replacement of the native FAD-dependent glycerol catabolic pathway in *Saccharomyces cerevisiae* by an artificial NADH-delivering dihydroxyacetone (DHA) pathway is supposed to facilitate the capturing of electrons in fermentation products. This requires that the electrons from the cytosolic NADH are not exclusively transferred to oxygen. However, the external NADH dehydrogenases (Nde1/2) and the L-glycerol 3-phosphate shuttle (composed of Gpd1/2 and Gut2), both coupled to the respiratory chain, are known to contribute to cytosolic NAD^+ regeneration during growth on non-fermentable carbon sources. In order to evaluate the role of these mechanisms during growth on glycerol, we deleted *GPD1/2, GUT2* as well as *NDE1/2,* separately and in combinations 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. Particularly, the *nde1*Δ mutants showed a significant reduction in growth rate and the *nde1*Δ *nde2*Δ double deletion mutants did not grow at all in synthetic glycerol medium. The current work also demonstrates a positive impact of deleting *NDE1* on the production of the fermentation product 1,2-propanediol in an accordingly engineered *S. cerevisiae* strain.

Keywords: glycerol, *Saccharomyces cerevisiae*, NADH, redox balance, 1,2-propanediol

Introduction

The yeast *Saccharomyces cerevisiae* is a popular microorganism for industrial bioprocesses (Nielsen *et al.* 2013). Several bulk chemicals as well as fine chemicals, biofuels, organic acids, therapeutic proteins and even vaccines are nowadays produced by engineered strains of this organism (Nevoigt 2008; Raab *et al.* 2010; Bill 2014; Dai *et al.* 2015; Kim, Yoo and Kang 2015). The industrial use of baker's yeast is favored by its robustness in industrial settings and the ease of genetic engineering (Borodina and Nielsen 2014; Chao, Yuan and Zhao 2015; David and Siewers 2015). Still, the natural substrate spectrum of baker's yeast is rather narrow (Rodrigues, Ludovico and Leao 2006) and there has already been a lot of effort to engineer this organism for the utilization of carbon sources other than hexose sugars (Nevoigt 2008; Hong and Nielsen 2012; Hasunuma, Ishii and Kondo 2015).

Glycerol is one of the compounds that derives from plant biomass and cannot be very efficiently utilized by the yeast *S. cerevisiae*. It is, however, an interesting carbon source due to several reasons; i) it has been generated as a by-product of biodiesel industry in huge amounts (Yazdani and Gonzalez 2007; Mattam *et al.* 2013), ii) it does not exert a Crabtree effect and iii) it has a higher degree of reduction per carbon atom compared to sugars. Due to the latter fact, the use of glycerol as a substrate allows higher maximum theoretical yields if a reduced small molecule (*i.e.* fermentation products) is the target product (Dharmadi, Murarka and Gonzalez 2006; Murarka *et al.* 2008; Zhang *et al.* 2013; Luo *et al.* 2016). Moreover, this molecule may also be a valuable co-substrate in case a compound with a low degree of reduction such as acetate is the substrate (de Bont *et al.* 2018).

Most of the commonly employed *S. cerevisiae* strains do not grow in synthetic glycerol medium unless growth-supporting supplements (amino acids and nucleic bases) are added to the medium (Merico *et al.* 2011; Swinnen *et al.* 2013; Klein *et al.* 2016b). However, a screening of about 50 strains previously demonstrated that a few natural *S. cerevisiae* isolates are able to grow in under such conditions (Swinnen *et al.* 2013). Among the tested strains, CBS 6412

exhibited a relatively good growth performance. A haploid segregant (CBS 6412-13A) showed a maximum specific growth rate (μ_{max}) of around 0.13 h^{-1} and the genetic determinants for superior glycerol utilization were characterized in more detail (Swinnen *et al.* 2013; Swinnen *et al.* 2016).

In wild-type cells of *S. cerevisiae,* glycerol enters the cell via the glycerol/H^+-symporter encoded by *STL1* (Ferreira *et al.* 2005; Swinnen *et al.* 2013) (Figure 3.3.1). It is then catabolized via the L-glycerol 3-phosphate (L-G3P) pathway, comprised of a glycerol kinase encoded by *GUT1* and an L-G3P dehydrogenase encoded by *GUT2*. The latter enzyme channels the electrons via the tightly bound $FADH_2$ directly to the respiratory chain (Figure 3.3.1) (Sprague and Cronan 1977; Rønnow and Kielland-Brandt 1993; Klein *et al*. 2017). From a biotechnological point of view, this is a major disadvantage since the higher reduction state of glycerol can only be harnessed for the production of fermentation products when the electrons are captured in the form of soluble cytosolic NAD(P)H. Therefore, Klein *et al.* (2016a) replaced the native glycerol catabolic pathway by the NAD-dependent DHA pathway (Figure 3.3.1). Using 1,2-propanediol (1,2-PDO) as a model product, this glycerol pathway replacement has been confirmed to be crucial for the onset of fermentative metabolism of glycerol (Islam *et al.* 2017). Nevertheless, the existence of metabolic routes transferring electrons from cytosolic NADH to the respiratory chain are assumed to compete with the production of small molecules as long as oxygen is available.

Our knowledge about endogenous pathways for cytosolic NADH re-oxidation in wild-type *S. cerevisiae* exclusively originates from studies on glucose, galactose or ethanol and is comprehensively reviewed by Bakker *et al.* (2001). During growth on glucose (above a certain threshold concentration), cytosolic NADH is well-known to be oxidized via alcoholic fermentation in *S. cerevisiae* even in the presence of oxygen (Crabtree 1929; de Deken 1966). Studies in media containing the non-fermentable carbon source ethanol have revealed two alternative mechanisms to be mainly involved in the regeneration of cytosolic NAD^+ in

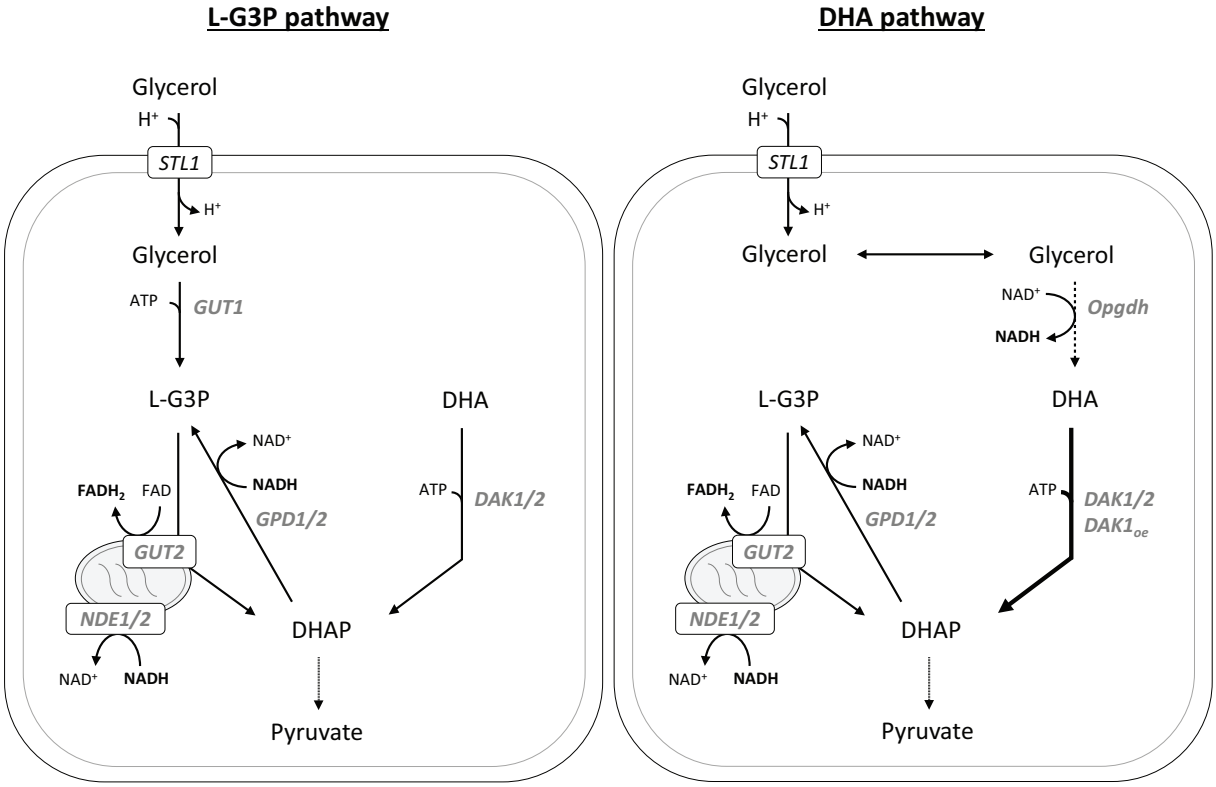

Figure 3.3.1. Endogenous (L-G3P) and artificial (DHA) pathway for glycerol catabolism in the *S. cerevisiae* strains CBS 6412-13A (wild type) and CBS DHA (engineered), respectively. Dashed arrows – heterologous expression; bold arrow – overexpression of an endogenous gene. *Genes*: *GUT1* – glycerol kinase, *GUT2* –membrane-bound FAD-dependent L-glycerol 3-phosphate dehydrogenase, *GPD1*/*GPD2* – cytosolic NAD^+-dependent L-glycerol 3-phosphate dehydrogenase, *NDE1*/*NDE2* – external NADH dehydrogenase, *STL1* – glycerol/H^+ symporter, *DAK1*/*DAK2* – DHA kinase ($DAK1_{oe}$ – *DAK1* overexpression), *CjFPS1* –heterologous glycerol facilitator from *C. jadinii. Abbreviations*: L-G3P – L-glycerol-3-phosphate, DHA – dihydroxyacetone, DHAP – dihydroxyacetone phosphate.

S. cerevisiae (Bakker *et al.* 2001). Both routes eventually transfer the electrons to the respiratory chain. The first of the two options is the so-called 'external NADH dehydrogenase', an enzyme that is characteristic for fungi and plants and that is encoded by the isogenes *NDE1* and *NDE2* in *S. cerevisiae* (Figure 3.3.1). The two isoenzymes are localized at the outer surface of the inner mitochondrial membrane, directly oxidize cytosolic NADH and shuttle the electrons to the quinone pool of the respiratory chain (Luttik *et al.* 1998; Small and McAlister-Henn 1998). Deletion experiments revealed that the protein encoded by *NDE1* is the major contributor to cytosolic redox balance when glucose is fully respired (*i.e.* at concentrations < 1 g L^{-1}) or when ethanol is used whose catabolism is exclusively respiratory as well

(Overkamp *et al.* 2000; Påhlman *et al.* 2001). The second mechanism is the L-G3P shuttle. In this shuttle, a cytosolic NAD^+-dependent L-G3P dehydrogenase, encoded by *GPD1* first transfers the electrons from cytosolic NADH to DHAP forming L-G3P (Figure 3.3.1). The latter crosses the outer mitochondrial membrane and is subsequently oxidized by the described mitochondrial, FAD-dependent L-G3P dehydrogenase (Larsson *et al.* 1998; Bakker *et al.* 2001). Notably, the exact quantitative contribution of this shuttle to the cytosolic redox balance is still a matter of discussion (Pa¨hlman *et al.* 2002; Rigoulet *et al.* 2004; Rodrigues, Ludovico and Leao 2006).

The goal of the current work was to determine to which extent the external NADH dehydrogenase and the L-G3P shuttle contribute to the maintenance of cytosolic redox balance in *S. cerevisiae* solely utilizing glycerol as the carbon source. We analyzed the contribution of these mechanisms in a wild-type background, *i.e.* a strain catabolizing glycerol via the L-G3P pathway (CBS 6412-13A), and in a strain expressing the NAD-dependent DHA pathway (CBS DHA) (Figure 3.3.1). Moreover, we applied the obtained fundamental knowledge and tested whether the titer and yield of the model target product 1,2-PDO achieved by Islam *et al.* (2017) can be improved by deleting *NDE1* in an appropriately engineered strain.

MATERIALS & METHODS

Strains, plasmids, medium composition and general cultivation conditions

All *S. cerevisiae* strains used in this study are listed in Table S1 (Supporting Information). *Saccharomyces cerevisiae* strains were pre-grown on solid YPD medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 15 g L^{-1} agar. The strain CBS DHA PDO as well as the corresponding *nde1*Δ mutant were pre-grown on the same medium but containing 60 mL L^{-1} glycerol instead of glucose. This was necessary due to the down-regulation of triose phosphate isomerase activity in these two strains (Islam *et al.* 2017). Cultures in liquid medium were grown on an orbital shaker at 200 rpm and 30°C, cultures on solid medium were incubated in a static incubator at 30°C. If required, the media were supplemented with 200 mg L^{-1} G418 or 20 mg L^{-1} phleomycin. *E. coli* DH5α was used for plasmid main-tenance and isolation. Plasmid carrying strains were routinely grown at 250 rpm and 37°C in lysogeny broth (10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl, pH 7.0) containing 100 mg L^{-1} ampicillin for selection. Plasmids were isolated by using the GeneJET^{TM} Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

All Growth Profiler experiments for the determination of μ_{max} for the different *S. cerevisiae* strains/mutants were performed in synthetic medium (SMG) according to Verduyn *et al.* (1992) containing: 5 g L^{-1} $(NH4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 15 mg L^{-1} EDTA, 4.5 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.84 mg L^{-1} $MnCl_2 \cdot 2H_2O$, 0.3 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 0.3 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.4 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 4.5 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 3 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 1 mg L^{-1} H_3BO_3 , and 0.1 mg L^{-1} KI. After autoclaving of this basal medium, a filter sterilized vitamin solution was added. The concentrations of vitamins in the final medium were 0.05 mg L^{-1} D-(+)-biotin, 1 mg L^{-1} D-pantothenic acid hemicalcium salt, 1 mg L^{-1} nicotinic acid, 25 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine chloride hydrochloride, 1 mg L^{-1} pyridoxine hydrochloride, and 0.2 mg L^{-1} 4-aminobenzoic acid. The carbon source added to the medium was either 20 g L^{-1} glucose or 60 mL L^{-1} glycerol. The pH of the glucose-containing medium

was adjusted to 6.5 with 4 M KOH, and the pH of the glycerol-containing medium to 4.0 with 2 M H_3PO_4, respectively. We chose pH 4 as standard for analyzing growth of *S. cerevisiae* strains in unbuffered SMG since we previously found that low pH values are particularly advantageous for glycerol utilization of the natural isolate CBS 6412–13A in synthetic medium (Swinnen *et al.* 2013). The media used for the cultivations for recording glycerol consumption and 1,2-PDO production were SMG_{buff} and YG. SMG_{buff} has exactly the same composition as SMG, but 20.5 g L^{-1} potassium hydrogen phthalate ($C_8H_5KO_4$) was added and the pH was set to 5 using 2 M H_3PO_4 (Islam *et al.* 2017). The used YG medium was the same used by Islam *et al.* (2017) and contained 10 g L^{-1} yeast extract in addition to 60 mL L^{-1} glycerol.

General molecular biology techniques

All primers used within the present study are listed in Table S2 (Supporting Information). Preparative PCRs for cloning were performed using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Frankfurt am Main, Germany). PCR conditions were adapted according to the guidelines of the manufacturer. PCR products were purified by using the GeneJET™ PCR Purification Kit (Thermo Fisher Scientific). Transformation of *S. cerevisiae* with expression as well as deletion cassettes was performed according to the lithium acetate method described by Gietz *et al.* (1995).

Genetic modifications of *S. cerevisiae*

In order to replace the glycerol catabolic pathway (DHA pathway replacing the endogenous L-G3P pathway), the strain CBS 6412–13A *DAK1$_{oe}$ gut1*Δ was used as a basis (Table S1, Supporting Information) (Klein *et al.* 2016a). The cassette of *Ogataea parapolymorpha gdh (Opgdh*; under the control of *S. cerevisiae TEF1* promoter and *CYC1* terminator) was integrated via a two-step seamless integration procedure (as described by Akada *et al.* (2002) into the long terminal repeat (LTR) *YPRCτ3* on chromosome XVI (Flagfeldt *et al.* 2009). First, the *GALp-GIN11M86* growth inhibitory sequence (amplified from plasmid pGG119 with primers 300 and 155 (Table S2, Supporting Information) and the phleomycin resistance marker

(amplified from plasmid pUG66 with primers 171 and 301) were assembled and integrated at the target locus by homologous recombination. The entire *GALp-GIN11M86/ble* cassette was afterwards replaced by integration of the *Opgdh* expression cassette (amplified with primers 416 and 417 from plasmid p41bleTEF-Opgdh (Klein *et al.* 2016a). Counter-selection was performed on solid synthetic medium containing 2 % (w/v) galactose as the sole carbon source inducing the growth inhibitory sequence *GIN11M86*.

For gene disruption, deletion cassettes, containing either *kanMX4* or *ble* resistance markers, were amplified from pUG6 and pUG66, respectively (Gueldener *et al.* 2002; Table S3, Supporting Information) using the primers listed in Table S2 (Supporting Information). The cassettes were then used to transform *S. cerevisiae* cells in order to delete the respective genes either individually or in combination. Double deletions were achieved by consecutive transformations of the respective deletion cassettes.

For the construction of the strain CBS DHA PDO, the strain PDO_{min}-FPS-$TPI1_{down}$ $DAK1_{oe}$-*gut1*Δ from Islam *et al.* (2017) was taken as a basis to assemble and integrate the expression cassettes for *Opgdh* and the second copy of *EcmgsA* into the LTR *YPRCτ3* in analogy to the procedure described above and by Islam *et al.* (2017).

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

Proof of the correct integration of all disruption cassettes was performed by diagnostic PCR using One*Taq* Quick-Load DNA Polymerase (NEB) according to the manufacturer's guidelines. PCR primers were designed to bind upstream and downstream of the genomic integration sites as well as within the integrated deletion cassette (Table S2, Supporting Information). Single colonies obtained after transformations were re-streaked on respective agar plates. According to a protocol modified from Hoffman and Winston (1987), about 50 mg of cells from these plates were re-suspended in 200 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Afterwards, 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. From the aqueous phase, 1 µL was used as template in 20 µL PCR reactions.

Quantitative analysis of *S. cerevisiae* growth using the Growth Profiler

Quantitative analysis of *S. cerevisiae* growth in defined media with glycerol was carried out according to Swinnen *et al.* (2013). A pre-culture of 4 mL synthetic glucose medium in 10 mL glass tube was inoculated with cells taken from a single cell colony and incubated shaking at 200 rpm and 30°C overnight. The preculture was used to inoculate 4 mL of fresh synthetic glucose medium (here referred to as intermediate culture) to an optical density (OD_{600}) of 0.2 (corresponding to 4.0×10^6 cells mL^{-1}). This culture was subsequently incubated under the same conditions as described above for 48 h. The cells from 1 mL of the intermediate culture were washed once in 1 mL SMG. Afterwards the cells were pelleted and resuspended in 1 mL of SMG. The appropriate volume of this cell suspension was taken to obtain a final culture of 4 mL with an OD600 of 0.2. Finally, aliquots of 750 µL were transferred to a well of a White Krystal™ 24-well clear bottom microplate (Porvair Sciences, Leatherhead, United Kingdom) and cultivated in the Growth Profiler 1152 (Enzyscreen, Haarlem, The Netherlands) at 30°C with orbital shaking at 200 rpm. The Growth Profiler took a scan of the plates every 40 min. These scans were then used to determine the density of the culture in each well expressed as green values (*G*-values). The *G*-values were subsequently converted to OD_{600} values (referred to here as OD_{600} equivalent) using the following calibration curve:

$$OD600\ \text{equivalent} = 6.1761 * 10^{-8} * G - \text{value}^{3.4784}.$$

Characterization of *S. cerevisiae* in shake flask batch cultivation

Pre- and intermediate cultivations in liquid medium were conducted in the same medium as the one used for the main culture. First, 4 mL of the respective medium in a 10 mL glass tube were inoculated with cells from a single colony and cultivated while shaking for 24 h at 30°C. This pre-culture was used to inoculate 15 mL of the same medium in a 100 mL Erlenmeyer flask adjusting at an initial OD_{600} of 0.2. This intermediate culture was incubated at the same conditions for another 24 h (YG) or 48 h (SMG_{buff}). The filling volume of the flasks was 50 mL in 500 mL Erlenmeyer flasks. Samples for OD_{600} and pH determination as well as for HPLC analysis were taken at regular time intervals.

Analytical methods

A volume of 1.2 mL culture supernatant was filtered through 0.2 µm Minisart RC membrane filters (Sartorius, Göttingen, Germany), analyzed immediately or stored at -20°C. The concentrations of glycerol, 1,2-PDO and ethanol in the samples were determined using a Waters HPLC system (Eschborn, Germany) consisting of a binary pump system (Waters 1525), injector system (Waters 2707) and a Waters column heater module WAT038040 and a refractive index detector (Waters 2414). 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^{-1} and a column temperature of 45°C, while the internal heater was set to 30°C. A volume of 20 µL of each sample was injected. Under these conditions, the retention times were 13.5 min for glycerol, 17.3 min for 1,2-PDO and 22.7 min for ethanol. Data were processed and analyzed using the Breeze 2 software (Waters).

RESULTS

Importance of the external NADH dehydrogenases and the L-G3P shuttle for growth of *S. cerevisiae* in synthetic glycerol medium

As mentioned in the introduction, the *S. cerevisiae* wild-type strain CBS 6412-13A is able to grow in synthetic glycerol medium (Swinnen *et al.* 2013). To construct an isogenic strain that exclusively uses the DHA pathway for glycerol catabolism, the strain was equipped with a heterologous NAD-dependent glycerol dehydrogenase (*Opgdh*) and the endogenous *DAK1* was overexpressed as described in Material and Methods. In addition, *GUT1* (encoding the endogenous glycerol kinase) was deleted for the abolishment of the native L-G3P pathway. The resulting strain (CBS DHA) was able to grow with a μ_{max} of 0.10 ± 0.002 h^{-1} in synthetic glycerol medium. This value was only slightly lower than that of the corresponding wild-type strain CBS 6412-13A which showed in the same experiment a μ_{max} of 0.12 ± 0.011 h^{-1} (Figure 3.3.2). Thus, two strains with a similar µ*max* on glycerol, one carrying the L-G3P pathway and the other one the DHA pathway, were available for studying the contributions of the L-G3P shuttle and the external NADH dehydrogenases for re-oxidation of cytosolic NADH.

The deletion of *GUT2* abolished growth on glycerol in the wild-type strain CBS 6412–13A (Figure 3.3.2) confirming the well-established key role of Gut2 for the native L-G3P pathway (Sprague and Cronan 1977; Rønnow and Kielland-Brandt 1993; Swinnen *et al.* 2013; Klein *et al.* 2016a). In clear contrast, no significant influence on the growth performance was detected after deleting *GUT2* in the strain CBS DHA. This is in accordance with the assumption that glycerol catabolism in this strain solely depends on the artificial DHA pathway. The latter result obtained in the strain CBS DHA also indicates that the L-G3P shuttle, in which Gut2 plays a crucial role (Figure 3.3.1), is not significantly involved in the re-oxidation of cytosolic NADH.

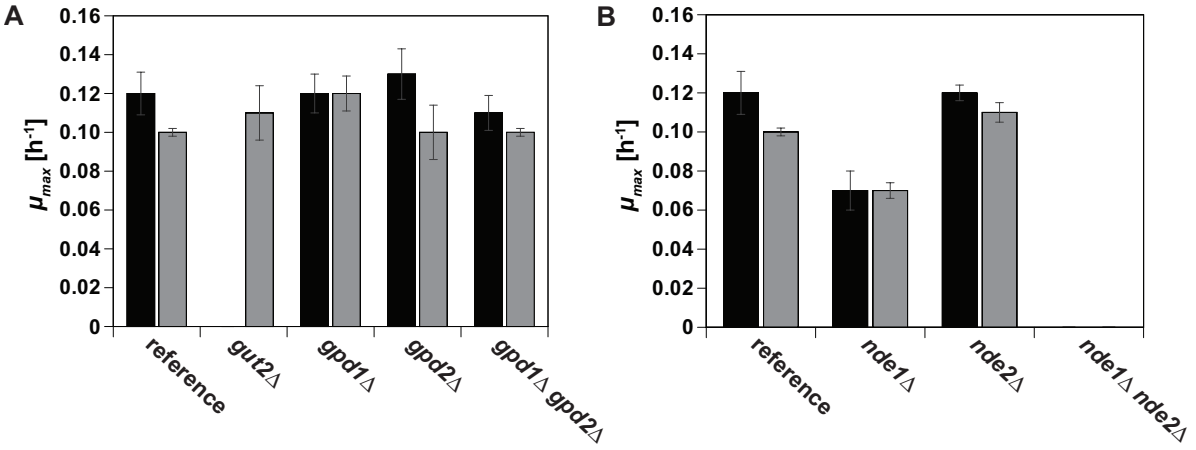

Figure 3.3.2. Maximum specific growth rates (μ_{max}) of *S. cerevisiae* strains carrying deletions of genes encoding for (iso)enzymes known to contribute to (A) the L-G3P shuttle and (B) the external NADH dehydrogenase activity. Black bars represent the wild-type strain CBS 6412-13A, while grey bars show the strain CBS DHA in which the native FAD-dependent (L-G3P) pathway for glycerol catabolism was replaced by the NAD^+-dependent (DHA) pathway. Mean values and standard deviations from at least three independent experiments are shown. Cells were gown in synthetic glycerol medium (SMG) (6% glycerol v/v).

The contribution of the L-G3P shuttle was also tested by single and double deletions of *GPD1* and *GPD2* encoding cytosolic glycerol 3-phosphate dehydrogenase, the second important enzyme involved in the shuttle (Figure 3.3.1). Although *GPD1* and *GPD2* exhibit high sequence homology, the isoenzymes have different reported physiological roles and regulation. While Gpd1 is crucial when the cells suffer from osmotic stress, Gpd2 predominantly maintains cytosolic redox balance when cells grow on glucose under anaerobic conditions (Albertyn, Hohmann and Prior 1994; Pronk, Steensma and Van Dijken 1996; Ansell *et al.* 1997; Björkqvist *et al.* 1997; Rep *et al.* 2000). None of the deletions resulted in a significant reduction of μ_{max} in the wild-type strain CBS 6412–13A or in the strain CBS DHA (Figure 3.3.2). Obviously, the L-G3P shuttle does not significantly contribute to the re-oxidation of cytosolic NADH during utilization of glycerol independent of the pathway, which is in use.

In contrast, specific growth rates on glycerol were reduced by 30% and 42% upon deletion of *NDE1* in strains CBS 6412–13A and CBS DHA, respectively (Figure 3.3.2). Deletion of *NDE2*, however, did not affect growth rates on glycerol. The results indicate that Nde1 mainly

contributes to the re-oxidation of cytosolic NADH when *S. cerevisiae* grows on glycerol regardless of the catabolic pathway used. These results obtained in glycerol medium are similar to those described for *nde1*Δ and *nde2*Δ mutant strains growing on ethanol or galactose. Luttik *et al.* (1998) showed that the deletion of *NDE1* led to a reduction of μ_{max} by 22% and 43%, respectively, while the deletion of *NDE2* did not affect growth.

The isogene *NDE1* was shown to be upregulated when *S. cerevisiae* cells grow on glycerol as the sole source of carbon in comparison to cells exponentially growing on excess glucose (Ohlmeier *et al.* 2004; Roberts and Hudson 2006). In these studies, carbon source dependent regulatory differences at the transcript and protein level, respectively, were analyzed using batch cultivations in complex medium. Our own transcriptome study conducted by Ho *et al.* (2017) recently also confirmed this observation in synthetic glycerol medium. The upregulation of *NDE1* transcription might indicate its importance for growth on glycerol and thereby explain why the deletion of *NDE1* in the current study was more severe than that of *NDE2*.

In contrast to the sole deletion of *NDE1*, the combined deletions of *NDE1* and *NDE2* completely abolished growth on glycerol in both genetic backgrounds (Figure 3.3.2). These results differ from the study of Luttik *et al.* (1998) who did not observe any further reduction of μ_{max} in shake flask cultures with glucose, ethanol or galactose when *NDE2* was deleted in the *nde1*Δ mutant background. The different results might indicate that Nde2 has a more prominent role on glycerol compared to other non-fermentable carbon sources.

With regard to the *nde1*Δ *nde2*Δ double deletion mutant, it is interesting to note that its metabolism has been shown to be completely respiratory in glucose-limited chemostat cultures at low dilution rates (Luttik *et al.* 1998; Bakker *et al.* 2001). This result has been interpreted as a proof that other potentially active respiratory mechanisms such as the L-G3P shuttle or the ethanol-acetaldehyde shuttle can take over the role of the external NADH dehydrogenases. The situation in synthetic glycerol medium in batch cultures seems to be different, since our

results obtained here with the *nde1*Δ *nde2*Δ strains suggest that these shuttles were unable to compensate the lacking NADH dehydrogenase activity.

Although the L-G3P shuttle does not seem to play a major role for cytosolic redox balancing in the strains and conditions used here, we could not rule out that there is a certain contribution of the L-G3P shuttle to NAD^+-regeneration in *nde1*Δ mutants. To check this, we deleted *GPD1* and *GPD2* (separately and in combination) in the *nde1*Δ mutants of both strains. The respective single deletions did not significantly alter the strains' growth behavior in synthetic glycerol medium (Figure 3.3.3). However, complete abolishment of GPD activity (triple deletion *nde1*Δ *gpd1*Δ *gpd2*Δ) reduced the μ_{max} in CBS DHA from 0.07 ± 0.004 h^{-1} to 0.04 ± 0.008 h^{-1}, while no additional effect was detectable in the CBS 6412-13A background (Figure 3.3.3). Interestingly, the deletion of *GUT2* in the *nde1*Δ mutant of strain CBS DHA also reduced the growth rate to 0.05 ± 0.004 h^{-1}, which is virtually the same level as measured for the *nde1*Δ *gpd1*Δ *gpd2*Δ triple deletion strain. The results indicate that the L-G3P shuttle indeed seems to be involved to some extent in the *nde1*Δ deletion mutant of strain CBS DHA *i.e*. the strain in which glycerol catabolism depends on the NADH-generating DHA pathway.

In order to exclude pleiotropic effects of the mutations, we also recorded the μ_{max} of all constructed strains in synthetic glucose medium. In accordance to the literature (for a comprehensive review see Bakker *et al.* 2001), there was no significant impact of the mutations on the growth performance in glucose-containing medium (Figure S1).

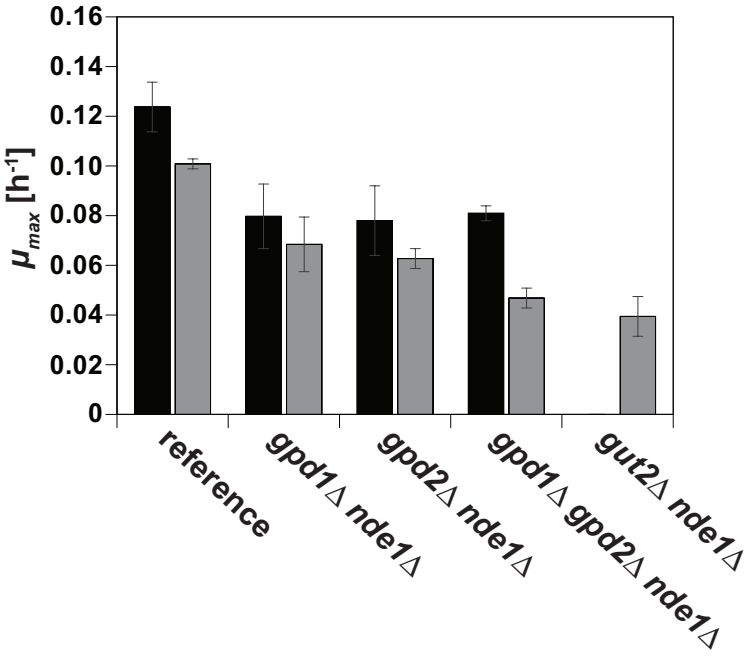

Figure 3.3.3. Maximum specific growth rates (μ_{max}) of *S. cerevisiae* strains in which the deletion of *NDE1* was combined with additional deletions of (iso)genes whose gene products contribute to the L-G3P shuttle. Black bars represent μ_{max} of deletion strains in the CBS 6412-13A, grey bars in the CBS DHA background. Mean values and standard deviations from at least three independent experiments are shown. Cells were gown in synthetic glycerol medium (SMG) (6% glycerol v/v).

Deletion of *NDE1* increased 1,2-propanediol yield from glycerol in an accordingly engineered strain grown in YG medium

As mentioned above, we have previously generated engineered CBS DHA derivatives for the production of 1,2-PDO from glycerol and observed 1,2-PDO concentrations in the range of 3-4 g L^{-1} (Islam *et al.* 2017). One important motivation for the current study was to identify respiratory routes for cytosolic NAD^+ regeneration with the goal to identify targets for genetic modifications that could improve 1,2-PDO formation from glycerol in *S. cerevisiae.* Based on the results obtained in the current study, *NDE1* deletion was considered a promising target in this context. To test this hypothesis, a strain able to produce 1,2-PDO from glycerol published by Islam *et al.* (2017) was used as a baseline strain and referred to as CBS DHA PDO. Besides the DHA pathway and a heterologous pathway for 1,2-PDO production, the strain contained a heterologous glycerol facilitator and a genetic modification for down-regulating the expression

of *TPI1* for increased supply of DHAP (Figure 3.3.4). These modifications were shown to be necessary to manifest significant 1,2-PDO production (Islam *et al.* 2017).

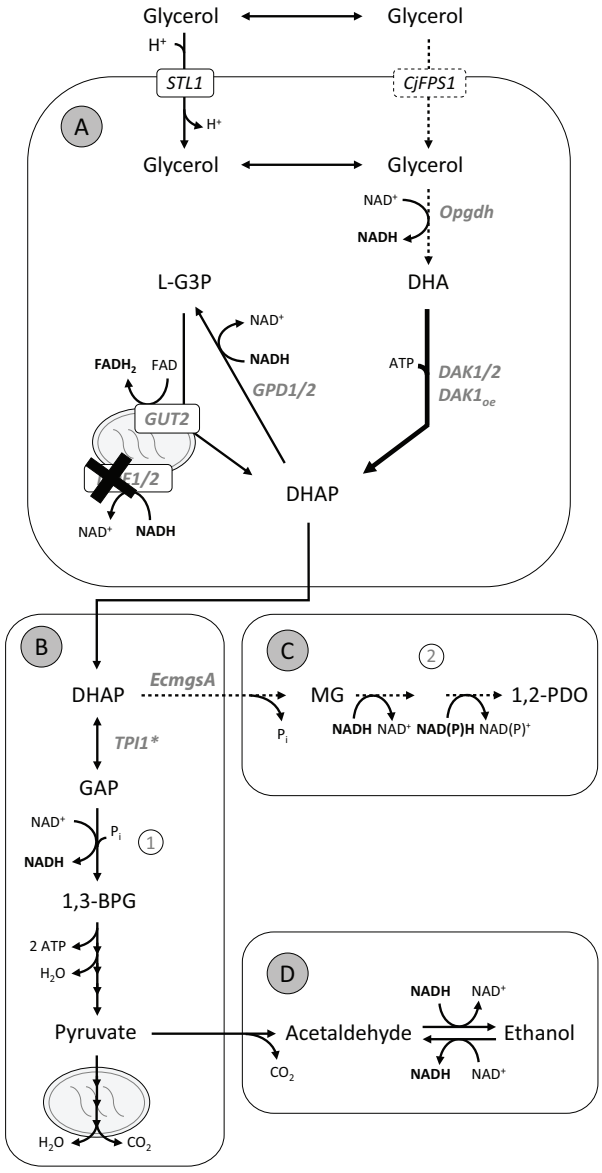

Figure 3.3.4. Pathways relevant in the strain CBS DHA PDO engineered for the production of 1,2-PDO from glycerol. The respective 1,2-PDO producing strain has been used in order to test the impact of deleting *NDE1*. A) DHA pathway, B) lower glycolysis, C) synthetic 1,2-PDO pathway consisting of *E. coli mgsA* and *gldA* (*EcmgsA* and *EcgldA)*, D) native ethanol formation and consumption. *) *TPI1* is down-regulated in CBS DHA PDO. 1) GAP dehydrogenase encoded by *TDH1*/*2*/*3*. 2) one reduction reaction is NADH-dependent due to specificity of the used glycerol dehydrogenase, while the co-factor specificity/specificities of the endogenous enzyme(s) catalyzing the second reduction step is/are unknown. Abbreviation: L-G3P – L-glycerol-3-phosphate, DHA – dihydroxyacetone, DHAP – dihydroxyacetone phosphate, GAP – glyceraldehyde-3-phosphate, 1,3-BPG – 1,3-bisphosphoglycerate, MG – methylglyoxal, 1,2-PDO – 1,2-propanediol.

After constructing an isogenic *nde1*Δ mutant of strain CBS DHA PDO, the two strains were analyzed in shake flask experiments with glycerol as the sole source of carbon. Both a buffered synthetic medium (SMG_{buff}) and a complex medium (YG) were used in parallel. These media were selected since they resulted in the highest titers of 1,2-PDO in our previous study (Islam *et al.* 2017).

When evaluating the impact of the *NDE1* deletion on the performance of strain CBS DHA PDO (Figure 3.3.5), three major observations become obvious. First, biomass formation and glycerol consumption were significantly slower in the *nde1*Δ mutant in both media (Figure 3.3.5). Moreover, the specific glycerol consumption rates in both media (Table 3.1) imply that the glycolytic flux in the *nde1*Δ mutant was significantly reduced compared to the reference strain. Second, the deletion of *NDE1* almost completely abolished ethanol production in both media. Third, the deletion of *NDE1* only resulted in an increased 1,2-PDO titer in YG medium (4.9 ± 0.06 vs. 2.8 ± 0.76 g L^{-1}) but not in the buffered synthetic medium.

While the first result could be expected, the second and the third observation were rather surprising. The fact that NAD^+ cannot be regenerated via Nde1 in the *nde1*Δ mutant led to the general assumption that fermentative routes such as 1,2-PDO and/or ethanol formation might potentially take over the re-oxidation of cytosolic NADH. This assumption proved to be correct in YG medium, but it remains unclear why this was not the case in the synthetic medium. It seems that so far unknown regulatory mechanisms varying in the two media have resulted in different *in vivo* fluxes to 1,2-PDO. In fact, the specific 1,2-PDO production rates in both strains (CBS DHA PDO and CBS DHA PDO *nde1*Δ) were three- and four-fold higher, respectively, in YG medium compared to the corresponding values in the medium SMG_{buff} (Table 3.1).

The highest maximum 1,2-PDO yield observed with the *nde1*Δ deletion mutant in YG medium was almost 0.2 g g^{-1}. This yield was 2-fold higher than the 0.09 g g^{-1} obtained for CBS DHA PDO. This result provides a proof of concept that the production of a reduced small molecule from glycerol can be improved by reducing the activity of the external NADH

dehydrogenases. Nevertheless, it remains unclear why the improvement was not visible in synthetic medium, a finding which future studies have to take into account.

Another important question, which deserves a more comprehensive investigation, is ethanol formation from glycerol. Although glycerol is considered a non-fermentable carbon source, ethanol formation could, in theory, also contribute to NADH re-oxidation, particularly if glycerol is catabolized via the DHA pathway, which may result in excess NADH in the cytosol. However, among all strains constructed in our lab, only the strain CBS DHA PDO showed significant production of ethanol in the current study (e.g. up to 8 g L^{-1} in SMG_{buff}), a result which confirms the data obtained by Islam *et al.* (2017). It is important to consider that the strain CBS DHA PDO has several additional genetic modifications when compared to the strain CBS DHA. It is very likely that the respective modifications contribute to the ethanol production of strain CBS DHA PDO. We are currently dissecting the genetic and environmental conditions that cause ethanol formation from glycerol.

Table 3.1. Rates of glycerol consumption and 1,2-PDO production in a *S. cerevisiae* strain engineered for 1,2-PDO production and a corresponding *nde1*Δ deletion mutant. Abbreviation: CDW - cell dry weight.

Medium	SMG_{buff}		YG	
Strain	CBS DHA PDO	CBS DHA PDO *nde1*Δ	CBS DHA PDO	CBS DHA PDO *nde1*Δ
Maximum volumetric glycerol consumption rate [g L^{-1} h^{-1}]	1.599 (24-48 h)	0.879 (48-72 h)	0.711 (24-48 h)	0.318 (24-48 h)
*Specific glycerol consumption rate [g g_{CDW}^{-1} h^{-1}]	0.280	0.136	0.308	0.170
*Volumetric 1,2-PDO production rate [g L^{-1} h^{-1}]	0.068	0.065	0.082	0.076
*Specific 1,2-PDO production rate [g g_{CDW}^{-1} h^{-1}]	0.012	0.010	0.036	0.041

*The respective rates were calculated for the time interval in which the maximal volumetric glycerol consumption rate was achieved. The rates have been calculated from a curve with the best fit to the data points shown in Figure 3.3.5.

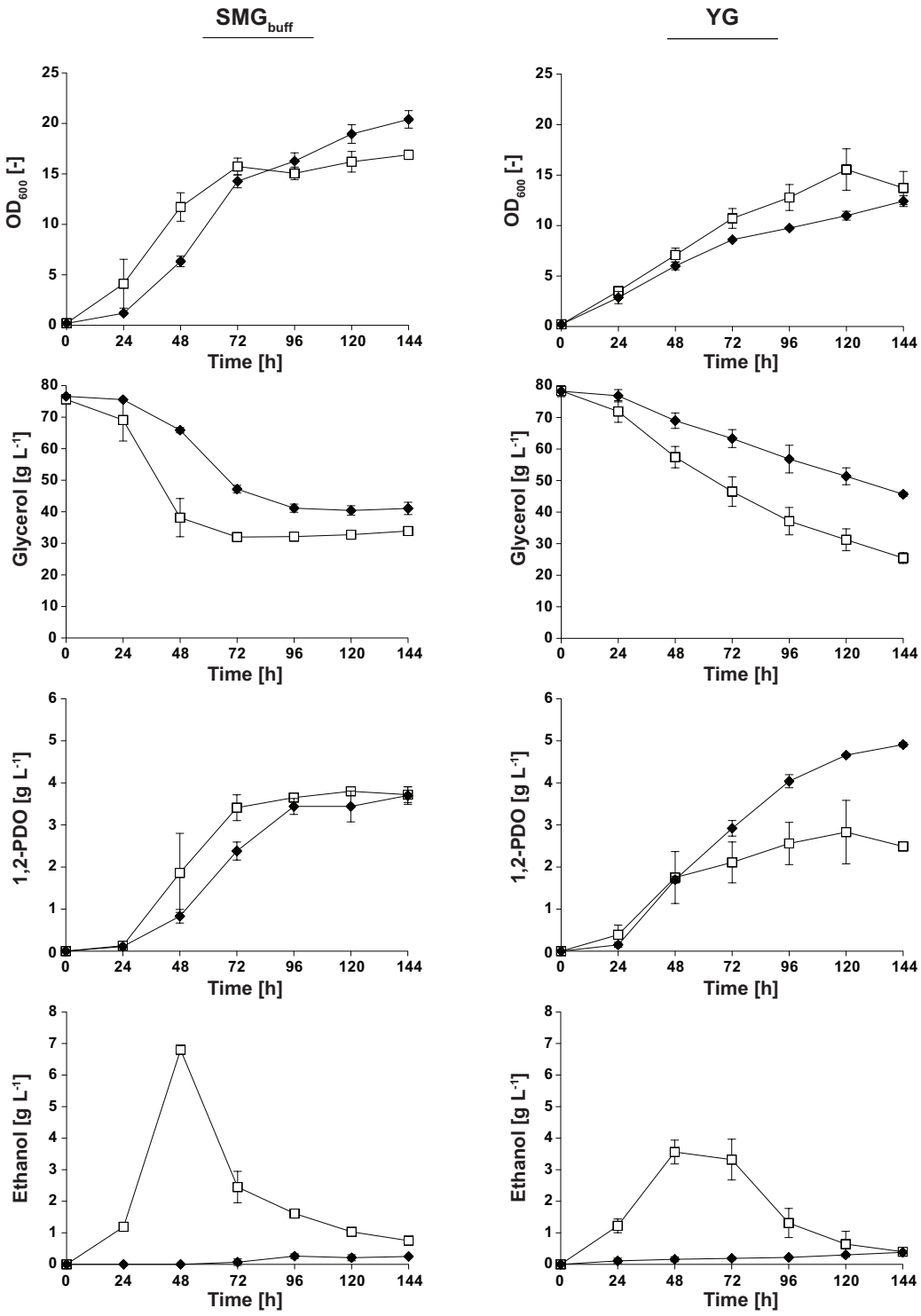

Figure 3.3.5. Effect of deleting *NDE1* on 1,2-PDO formation in the *S. cerevisiae* strain DHA PDO engineered for the production of 1,2-propanediol in synthetic medium SMG_{buff} and complex medium YG. Filled symbols represent the strain CBS DHA PDO *nde1*Δ, while open symbols refer to the reference strain CBS DHA PDO**.** Shake flask experiments were conducted with 50 mL filling volume. Glycerol (6% v/v) was used as sole carbon source. Mean values and standard deviations from three independent experiments are shown.

CONCLUSION

The current study conducted in synthetic glycerol medium confirmed results of other authors who showed that Nde1 is important for re-oxidizing cytosolic NADH when *S. cerevisiae* grows in a fully respiratory manner. In contrast to other non-fermentable carbon sources, Nde2 seems to play a more prominent role on glycerol. Moreover, the L-G3P and other shuttle mechanisms only play minor roles when glycerol is the sole carbon source. Based on these fundamental research results, a reduction of external NADH dehydrogenase activity was suggested as a promising route to redirect the electrons from cytosolic NADH towards the production of fermentation products when *S. cerevisiae* strains utilize glycerol via the DHA pathway. Although the promising results with regard to the model product 1,2-PDO delivered a proof of concept, the current study confronted us with a number of unexpected results and open questions with regard to the fermentation of glycerol. Further work will focus on understanding the influence of medium composition and genetic modifications on the metabolic flux distribution at the DHAP branch point as well as on dissecting the prerequisites for ethanol formation from glycerol.

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Conflict of interest

The authors declare no competing interest.

Supplementary Material

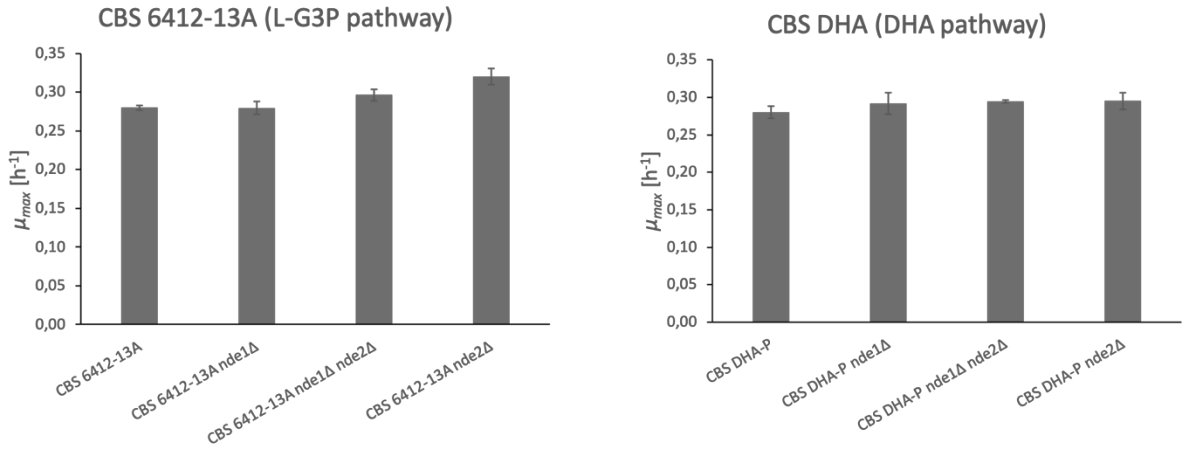

Figure S1: Maximum specific growth rates (μ_{max}) of *S. cerevisiae* strains carrying deletions of genes encoding for (iso)enzymes of the external NADH dehydrogenase activity on glucose. Both the wild-type strain CBS 6412-13A and the isogenic derivative CBS DHA in which the native FAD-dependent (L-G3P) pathway for glycerol catabolism was replaced by the NAD^+-dependent (DHA) pathway were studied. Mean values and standard deviations from at least two independent experiments are shown. Cells were gown in synthetic glucose medium (SMD) (2% glucose w/v).

Table S1: *S. cerevisiae* strains used in this study.

Strain / Modules	Genome modifications	Source or reference
CBS 6412-13A (CBS)	wild type	Swinnen *et al.* (2013)
CBS 6412-13A *gut2*Δ	*gut2::loxP-ble-loxP*	Swinnen *et al.* (2013)
CBS 6412-13A *nde1*Δ	*nde1::loxP-kanMX4-loxP*	This study
CBS 6412-13A *nde2*Δ	*nde2::loxP-ble-loxP*	This study
CBS 6412-13A *nde1*Δ*nde2*Δ	*nde1::loxP-kanMX4-loxP; nde2::loxP-ble-loxP*	This study
CBS 6412-13A *gpd1*Δ	*gpd1::loxP-kanMX4-loxP*	This study
CBS 6412-13A *gpd2*Δ	*gpd2::loxP-ble-loxP*	This study
CBS 6412-13A *gpd1*Δ*gpd2*Δ	*gpd1::loxP-kanMX4-loxP; gpd2::loxP-ble-loxP*	This study

CBS 6412-13A *gpd1*Δ*nde1*Δ	*gpd1::loxP-kanMX4-loxP; nde1::loxP-ble-loxP*	This study
CBS 6412-13A *gpd2*Δ*nde1*Δ	*gpd2::loxP-ble-loxP; nde1::loxP-KanMX4-loxP*	This study
CBS 6412-13A *gpd1*Δ *gpd2*Δ *nde1*Δ	*gpd1::loxP; gpd2::loxP; nde1::loxP-KanMx-loxP*	This study
CBS 6412-13A *gut2*Δ *nde1*Δ	*gut2::loxP-ble-loxP; nde1::loxP-KanMx-loxP*	This study
CBS 6412-13A *DAK1$_{OE}$ gut1*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *gut1::loxP-kanMX4-loxP*	Klein *et al.* (2016a)
CBS 6412-13A *DAK1$_{OE}$ Opgdh gut1*Δ (CBS DHA)	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP-kanMX4-loxP*	This study
CBS DHA	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP*	This study
CBS DHA *nde1*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1:: loxP; nde1::loxP-kanMX4-loxP*	This study
CBS DHA *nde2*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; nde2::loxP-ble-loxP*	This study
CBS DHA *nde1*Δ *nde2*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; nde1::loxP-kanMX4-loxP; nde2::loxP-ble-loxP*	This study
CBS DHA *gpd1*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gpd1::loxP-kanMX4-loxP*	This study
CBS DHA *gpd2*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gpd2::loxP-ble-loxP*	This study
CBS DHA *gpd1*Δ *gpd2*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gpd1::loxP-kanMX4-loxP; gpd2::loxP-ble-loxP*	This study
CBS DHA *gut2*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gut2::loxP-ble-loxP*	This study
CBS DHA *gpd1*Δ *nde1*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gpd1::loxP-ble-loxP; nde1::loxP-kanMX4-loxP*	This study
CBS DHA *gpd2*Δ *nde1*Δ	*YPRC*Δ*15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gpd2::loxP-ble-loxP; nde1*Δ*::loxP-kanMX4-loxP*	This study

CBS DHA *gut2Δ nde1Δ*	*YPRCΔ15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gut2::loxP-ble-loxP; nde1::loxP-kanMX4-loxP*	This study
CBS DHA *gpd1Δ* gpd2Δ *nde1Δ*	*YPRCΔ15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; gut2::loxP; nde1::loxP-kanMX4-loxP; nde2::loxP-ble-loxP*	This study
PDO_{min}-FPS-$TPI1_{down}DAK1_{oe}$-*gut1Δ*	*YGLCτ3::TEF1p-EcmgsA-CYC1t:TDH3p-EcgldA-IDP1t:PGK1p-CjFPS1-RPL15At; tpi1p::TEFmut2p-TPI1; YPRCΔ15::ACT1p-DAK1-TPS1t*; *gut1::loxP-kanMX4-loxP*	Islam *et al.* (2017)
CBS DHA PDO	*YGLCτ3::TEF1p-EcmgsA-CYC1t:TDH3p-EcgldA-IDP1t:PGK1p-CjFPS1-RPL15At; tpi1p::TEFmut2p-TPI1; YPRCΔ15::ACT1p-DAK1-TPS1t*; *YPRCτ3::TDH3p-EcmgsA-IDP1t:TEF1p-Opgdh-CYC1t*; *gut1::loxP-kanMX4-loxP;*	This study
CBS DHA PDO *nde1Δ*	*YGLCτ3::TEF1p-EcmgsA-CYC1t:TDH3p-EcgldA-IDP1t:PGK1p-CjFPS1-RPL15At; tpi1p::TEFmut2p-TPI1; YPRCΔ15::ACT1p-DAK1-TPS1t*; *YPRCτ3::TDH3p-EcmgsA-IDP1t:TEF1p-Opgdh-CYC1t*; *gut1::loxP-kanMX4-loxP; nde1::loxP-ble-loxP*	This study

Table S2. PCR primers used in this study. 5' overhangs used for generating overlapping PCR products to regions in the *S. cerevisiae* genome (for *in vivo* homologous recombination) are underlined.

Primer No.	Name	Sequence 5' – 3'
Primers for the integration of the GALp-GIN11M86/kanMX4 *cassette at the YGLCτ3 locus on chromosome VII*		
155	half-GIN-YAP-rv	GGTACCAGGAAATGAAAGCG
171	YAP1-lox-fwd	GGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGCAGCTGAAGCTTCGTACGC
175	HAA1-GIN/YAP F5	AGCTTCTTTCCACGAAAGAAATAGTGTAAGTTAGAGGTACATCGGAACCCTAAAGGGAGC
176	HAA1-GIN/YAP R5	ATTAAGAAGCAAATACCTTCCTGTTGCTTGATTTGCCCTGGCATAGGCCACTAGTGGATCTG
Primers for the integration of the GALp-GIN11M86/ble *cassette at the YPRCτ3 locus on chromosome XVI (besides 155 and 171)*		
300	Pos 21 GIN F7	GTATTTTTAATCGTCCTTGTATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAAAAATCGGAACCCTAAAGGGAGC
301	Pos 21 loxP R7	GATTAAATAAAGAATGATTTACAATCTAGTCGCAAAAACAAGTACAGTGCTGACGTCCCATCGCATAGGCCACTAGTGGATCTG

Primers for the deletion of GUT2, GPD1, GPD2, NDE1 and NDE2		
115	gut2-loxP-fw	GTCTAAAGCAAGGACTCTCCCTCCCTTATCTTGACCGTGCTATTGCAGCTGAAGCTTCGTACGC
116	gut2-loxP-rv	TTGCAAAATGGCGTCACTGTATGGGCCCGTGGCATTGACCACACAGCATAGGCCACTAGTGGATCTG
545	GPD1_del fw	CATCAAATCTATCCAACCTAATTCGCACGTAGACTGGCTTGGTATCAGCTGAAGCTTCGTACGC
546	GPD1_del rv	CGACGTCCTTGCCCTCGCCTCTGAAATCCTTTGGAATGTGGTAAGGCATAGGCCACTAGTGGATCTG
786	GPD2 loxP f	TTCAATTCTCTTTCCCTTTCCTTTTCCTTCGCTCCCCTCAGCTGAAGCTTCGTACGC
787	GPD2 loxP r	CAATTTCAAAATCTTATGATCTACATCCTTGCCATCACCTGCATAGGCCACTAGTGGATCTG
790	NDE2 loxP f	GAAATCTTACACTGCGGTATTCAAGAAATGGTTTGTCAGAGGTTTCCAGCTGAAGCTTCGTACGC
791	NDE2 loxP r	GTAACGCGAATCTTCCATGACCAATAAAGGGAAATACTGGTTATAGCATAGGCCACTAGTGGATCTG
794	NDE1 loxP f	ATGATTAGACAATCATTAATGAAAACAGTGTGGGCTAACTCCTCCCAGCTGAAGCTTCGTACGC
795	NDE1 loxP r	AAAGTTGAAGTTTGTTATCTATCAACAAACCACGTCTTGAGTCCTGCATAGGCCACTAGTGGATCTG
Primers for the integration of the Opgdh and EcmgsA_2 expression cassettes into the YPRCτ3 locus on chromosome XVI		
416	P21 M13-rev F	TCTCAGGGTTCATGATCATAATAAATTGCGCATTCGCAAGGCGGTAGTATTATAATGGGGCAGGAAACAGCTATGACCATG
417	P21 M13-fwd R	CAAATTCTTCCTACTGTTATATATGTTCGAATTATCTTTTAAATTGTTGCTAAAAAAGTTTGTAAAACGACGGCCAGT
421	recom T-CYC1 R	CATGGCAATTCCCGGGGATCGCAAATTAAAGCCTTCGAGC
422	recom P-TDH3 F	GATCCCCGGGAATTGCCATGTCGAGTTTATCATTATCAATACTG

Table S3. Plasmids used in this study.

Plasmid	Relevant characteristics	Source or reference
pUG6	*loxP-kanMX4-loxP* disruption cassette	Euroscarf
pUG66	*loxP-ble-loxP* disruption cassette	Euroscarf
pGG119	*GALp-GIN11M86* growth inhibitory sequence	Akada *et al.* (2002)
pNatCre	*CEN6/ARSH4, natMX4, GAL1p-Cre-CYC1t*	Steensma and Ter Linde (2001)
pUC18-*mgsA*	*TDH3p-EcmgsA$_2$-IDP1t*	Islam *et al.* (2017)
p41bleTEF-*Opgdh*	*CEN6/ARSH4, ble, TEF1p-Opgdh-CYC1t*	Klein *et al.* (2016a)

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3.4. Manuscript II

Essentially as submitted to Biotechnology for Biofuels

Saccharomyces cerevisiae exhibiting a modified route for uptake and catabolism of glycerol forms significant amounts of ethanol from this carbon source considered as 'non-fermentable'

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Short title: Ethanol formation from glycerol by *S. cerevisiae*

Keywords: glycerol, ethanol, *S. cerevisiae*, fermentation, dihydroxyacetone pathway, Fps1/aquaglyceroporin, redox balance, overflow metabolism, NADH

Abstract

Background: Due to its inevitable formation during biodiesel production and its relatively high degree of reduction, glycerol is an attractive carbon source for microbial fermentation processes. Glycerol can be fermented by certain bacterial species but is catabolized in a fully respiratory manner by the eukaryotic platform organism *Saccharomyces cerevisiae*. We previously engineered *S. cerevisiae* strains in order to favor fermentative metabolism of glycerol by replacing the native FAD-dependent glycerol catabolic pathway with the NAD-dependent 'DHA pathway'. In addition, a heterologous aquaglyceroporin (Fps1 homolog) was expressed to facilitate glycerol uptake. The formation of *S. cerevisiae*'s natural fermentation product ethanol has occasionally been observed in certain engineered strains and growth conditions, but a respective in-depth study was missing.

Results: A strain solely exhibiting the glycerol catabolic pathway replacement produced ethanol at concentrations close to the detection limit. The expression of the heterologous aquaglyceroporin caused significant ethanol production (8.5 g L^{-1} from 51.5 g L^{-1} glycerol consumed) in a strain catabolizing glycerol via the DHA pathway but not in the wild-type background. A reduction of oxygen availability in the shake flask cultures further increased the ethanol titer up to 15.7 g L^{-1} (from 45 g L^{-1} glycerol consumed).

Conclusion: The increased yield of cytosolic NADH caused by the glycerol catabolic pathway replacement seems to be a minimal requirement for the occurrence of alcoholic fermentation in *S. cerevisiae* growing in synthetic glycerol medium. The remarkable metabolic switch to ethanol formation in the DHA pathway strain with the heterologous aquaglyceroporin supports the assumption of a much stronger influx of glycerol accompanied by an increased rate of cytosolic NADH production via the DHA pathway. The fact that a reduction of oxygen supply increases ethanol production in DHA pathway strains is in line with the hypothesis that a major part of glycerol in normal shake flask cultures still enters the catabolism in a respiratory manner.

Background

Glycerol has been considered a substrate for biotechnological processes since it is a major by-product of the transesterification process during the production of biodiesel and can be used by many microorganisms as the sole source of carbon (Mattam et al., 2013). A feature that makes glycerol particularly interesting for the fermentative production of reduced small molecules is its comparably high degree of reduction per carbon (da Silva et al., 2009; Wendisch et al., 2011) which generally allows higher maximum theoretical product yields in comparison to sugars (Clomburg and Gonzalez, 2013; Yazdani and Gonzalez, 2007).

The yeast *Saccharomyces cerevisiae* has increasingly been employed as a production host in industrial biotechnology (Bill, 2014; Nevoigt, 2008; Nielsen et al., 2013; Paddon et al., 2013; van Maris et al., 2006). Apart from equipping this yeast with novel product formation pathways, extensive metabolic engineering incentives have been initiated in recent years in order to extend the organism's range of used carbon sources with the goal to establish *S. cerevisiae* as a platform organism for processes based on renewable feedstocks (Borodina and Nielsen, 2014; d'Espaux et al., 2017; Edwards and Doran-Peterson, 2012; Garcia Sanchez et al., 2010).

Most strains of the species *S. cerevisiae* only seem to be able to grow on glycerol as the sole source of carbon if complex supplements such as yeast extract or amino acid mixtures are added to the medium (Barnett et al., 2000; Juszczyk and Rmyowicz, 2009; Merico et al., 2011). In a previous study, 52 *S. cerevisiae* isolates were screened for their ability to utilize glycerol in synthetic medium without any of these supplements and confirmed that many strains indeed do not grow at all under these conditions (Swinnen et al., 2013). Still, several strains showed growth, and the strain CBS 6412 was one of the best performing strains. A haploid segregant of this strain (CBS 6412-13A) was studied in more detail. It exhibited a maximum specific growth rate (μ_{max}) of about 0.13 h^{-1} under the conditions used (Swinnen et al., 2013).

In *S. cerevisiae* wild-type cells, the uptake of glycerol is conducted by a glycerol/H^+ symporter encoded by *STL1* (Ferreira et al., 2005) before it is catabolized via the L-glycerol 3-phosphate (L-G3P) pathway (Sprague and Cronan, 1977; Swinnen et al., 2013). In this pathway, a glycerol kinase (encoded by *GUT1*) and an FAD-dependent L-G3P dehydrogenase (encoded by *GUT2*) catalyze the conversion of glycerol to dihydroxyacetone phosphate via the intermediate L-G3P (Pavlik et al., 1993; Rønnow and Kielland-Brandt, 1993).

It is obvious that the native L-G3P pathway of *S. cerevisiae* is not optimal to exploit the substrate's attractive reducing power (for the production of reduced compounds) since part of the electrons are eventually channeled via $FADH_2$ and the mitochondrial respiratory chain to oxygen. In fact, no fermentation products have ever been reported in cultivations using *S. cerevisiae* wild-type strains growing on glycerol. This also holds for engineered or evolved strains growing with higher μ_{max}. For example, Ochoa-Estopier et al. (2011) evolved strains for improved growth in synthetic glycerol medium (μ_{max} of 0.20 h^{-1}) but did not detect any fermentation products even when tested under microaerobic conditions. Moreover, the CBS 6412-13A derivative, in which a heterologous aquaglyceroporin (Fps1 homolog) was expressed, achieved a μ_{max} of 0.18 h^{-1} but did not form any ethanol (Klein et al., 2016b).

To save the electrons in the form of cytosolic NADH and make them available for fermentative routes, the native glycerol catabolic pathway was previously replaced by the NAD^+-dependent 'DHA pathway' (Klein et al., 2016a). In the respective study, the strains were equipped with the heterologous gene *CjFPS1* encoding an aquaglyceroporin from *Cyberlindnera jadinii*. Notably, all three tested *S. cerevisiae* strains engineered in this way were able to utilize glycerol, some of them even better than their wild-type counterparts (Klein et al., 2016a). However, no ethanol formation was detected in these strains under the employed conditions.

Another previously conducted study, aimed at the production of 1,2-propanediol (1,2-PDO) from glycerol (Islam et al., 2017). Here, the strain CBS 6412-13A was equipped with the DHA pathway, the heterologous aquaglyceroporin encoded by *CjFPS1* and a heterologous pathway

for 1,2-PDO production. In addition, the promoter of the endogenous *TPI1* gene was replaced by a weaker one with the goal to force the metabolic flux into 1,2-PDO production. The same synthetic medium as described in Klein et al. (2016a) was used but 100 mM potassium hydrogen phthalate were added as a buffer in order to reduce medium acidification and thus to achieve higher glycerol consumption and biomass production in the shake flask cultures. Surprisingly, apart from 3.6 g L^{-1} 1,2-PDO, the constructed strain produced a considerable amount of ethanol (~8 g L^{-1}) (Islam et al., 2017). However, the experimental data did not allow a precise conclusion which genetic modifications were crucial for the metabolic switch towards ethanol formation.

The initial goal of the current study was to scrutinize whether the mere replacement of the L-G3P by the DHA pathway can cause a partial fermentative metabolism of glycerol in shake flasks as soon as buffered synthetic medium is used. Moreover, the effect of an increased glycerol influx into a cell (by the expression of *CjFPS1*) utilizing the DHA pathway, was tested. This was supposed to be accompanied by an increased rate of cytosolic NADH generation. Finally, the effect of oxygen limitation on ethanol formation from glycerol was tested since this reduces the re-oxidation of cytosolic NADH via respiratory routes.

Results

Increased cytosolic NADH generation by the DHA pathway was a minimal requirement for ethanol production from glycerol

In order to re-evaluate whether the glycerol catabolic pathway replacement (using the NADH-generating DHA pathway instead of the native L-G3P pathway) is a minimum requirement for alcoholic fermentation on glycerol, the strain CBS DHA was used. The latter strain (DHA pathway strain) is an engineered derivative of the wild-type strain CBS 6412-13A and was recently constructed by Aßkamp et al. (2019). In this strain, the glycerol catabolic pathway replacement was achieved by the genomic integration of cassettes for the expression of a glycerol dehydrogenase from *Ogataea parapolymorpha* (OpGDH) and the overexpression of endogenous dihydroxyacetone kinase (Dak1) as well as deletion of *GUT1* (to abolish the L-G3P pathway) (Klein et al., 2016a; Aßkamp et al., 2019).

The strain was characterized in buffered synthetic glycerol medium (SMG_{buff}). By the addition of a buffering component (100 mM potassium hydrogen phthalate) to the SMG medium and adjusting an initial pH of 5, it was previously shown that the cells in the shake flask cultivations reached significantly higher densities (Islam et al., 2017). The use of buffered medium was able to slow down the medium acidification which can be observed when *S. cerevisiae* grows with ammonium sulfate as the nitrogen source. Without buffering, the pH value in batch cultivations rapidly decreases to values below 2.6 accompanied by rather low final optical densities (<10).

As visible in Figure 3.4.1, the strain CBS DHA produced virtually no ethanol even though buffered medium was used. However, it must be emphasized that the HPLC chromatogram always showed a small peak for ethanol which was in contrast to cultivations in unbuffered SMG (Additional File 2). It can be concluded that glycerol metabolism via the DHA pathway can induce some ethanol production but cannot explain at all the high ethanol titers observed in the study of Islam et al. (2017).

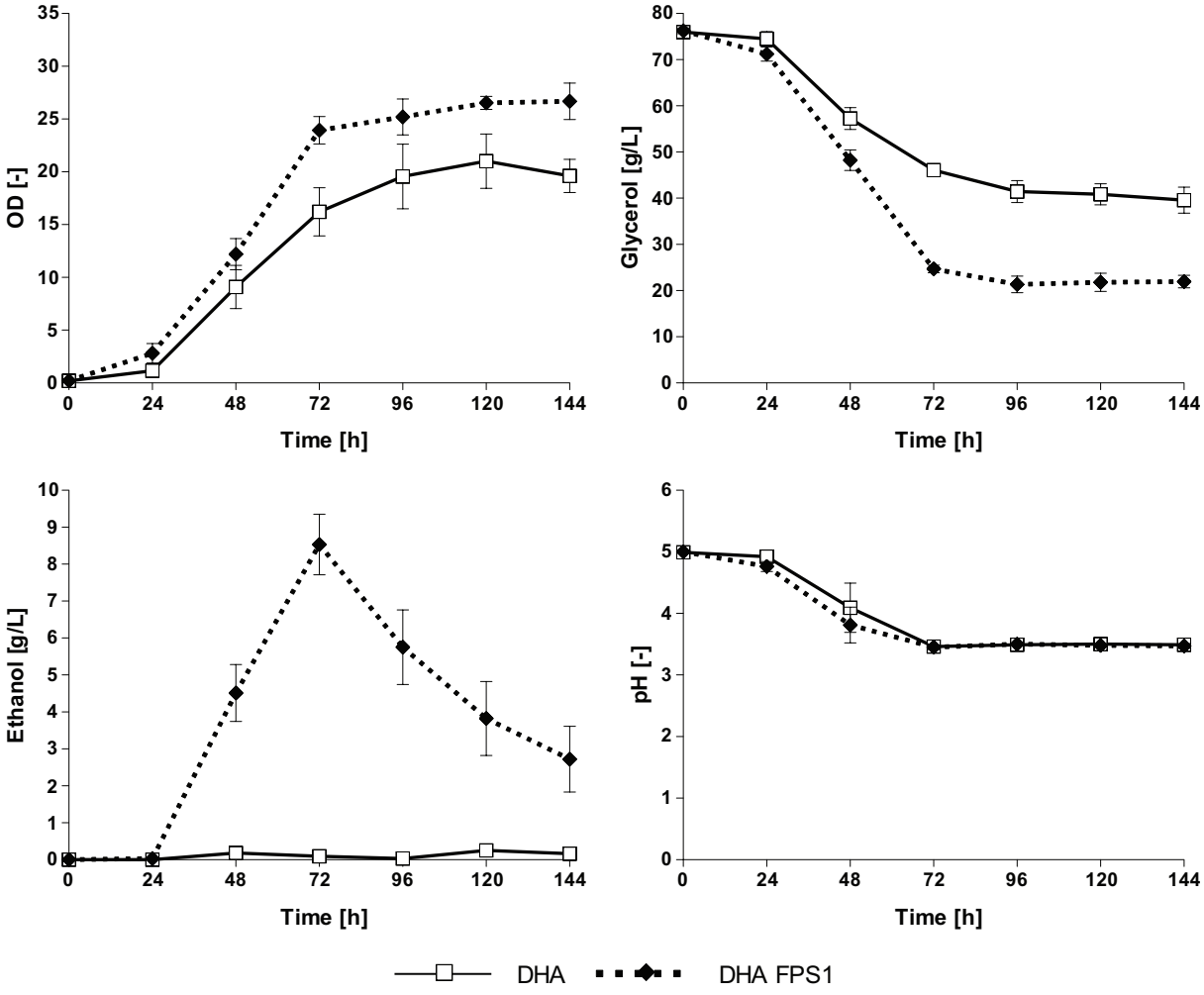

Figure 3.4.1. Fermentation performance of the glycerol-utilizing *S. cerevisiae* strain CBS 6412-13A catabolizing glycerol via the 'DHA pathway' (CBS DHA) and in an isogenic strain additionally expressing *C. jadinii FPS1* (CBS DHA FPS1). The shake flask experiments were conducted in synthetic media (SMG_{buff}) Cells were grown in 500 mL Erlenmeyer flasks with 50 mL filling volume. Glycerol (6% v/v) was used as the sole carbon source. Mean values and standard deviations from three independent experiments are shown.

Enhanced glycerol uptake significantly accelerated the overflow metabolism

The impact of an enhanced glycerol uptake (due to the presence of *CjFPS1*) on ethanol formation from glycerol in the DHA pathway strain was tested. Therefore, the respective expression cassette was integrated in the genome of the strain CBS DHA generating strain CBS DHA FPS1 (see Material and Methods). Compared to the strain CBS DHA, a remarkable increase in ethanol production was detected in strain CBS DHA FPS1. The maximum ethanol concentration of ~8.5 g L^{-1} was detected after 72 h of cultivation (Figure 3.4.1). Similar results

were obtained when the strain was cultivated in synthetic glycerol medium containing urea as the nitrogen source (data not shown).

The high ethanol concentration obtained for strain CBS DHA FPS1 encouraged us to also test a CBS 6412-13A wild-type derivative solely carrying the *CjFPS1* expression cassette in SMG_{buff}. However, no ethanol was detected in supernatants of the respective cultivations. Taken together, the results suggest that the DHA pathway is an essential/minimal requirement for the observed overflow metabolism with the 'non-fermentable' carbon source glycerol but an increased glycerol influx capacity can significantly accelerate this phenomenon.

The strain CBS DHA FPS1 showed a shorter lag phase compared to CBS DHA, reached a higher total biomass accumulation, consumed glycerol at a higher volumetric rate and consumed around 20 g L^{-1} more glycerol than strain CBS DHA (Figure 3.4.1).

Notably, the ethanol titer measured in the cultivations with strain CBS DHA FPS1 decreased after reaching a maximum. This phenomenon has also previously been described by Islam et al. (2017) in the strains engineered for 1,2-PDO production. The observed decrease in ethanol concentration in the current study seems to be predominantly caused by evaporation of ethanol from the medium (Additional File 3), although a certain degree of ethanol utilization cannot be excluded.

Oxygen limitation strongly increases ethanol formation from glycerol in strain CBS DHA FPS1

The fermentation of glycerol into ethanol described in the previous paragraphs was obtained in shake flasks, *i.e.* under aerobic conditions. Still, it might be that the increasing cell densities during the course of fermentation have caused a constant reduction in oxygen transfer rates which might have caused ethanol formation due to limitation of respiration. Thus, the question arose whether an additional reduction of the maximum oxygen transfer rate (OTR_{max}) would alter the cells' metabolism even further towards fermentation, *i.e.* even higher ethanol formation. A straightforward possibility to reduce OTR_{max} in shake flasks is the reduction of the

surface area to volume ratio of the medium (Barge et al., 2014). Thus, the volume of SMG_{buff} used for cultivation in the 500 mL shake flasks was stepwise increased. Both strains (CBS DHA and CBS DHA FPS1) were tested in 50 mL, 75 mL, 100 mL, 125 mL and 200 mL of cultivation medium (Figure 3.4.2).

The results show that reducing the oxygen availability indeed increased the maximum ethanol titer (Figure 3.4.2). Interestingly, the reduced oxygen availability also resulted in significant ethanol production in the strain CBS DHA which only formed trace amounts of ethanol when a filling volume of 50 mL was used. The highest ethanol titer for each strain was detected with the highest filling volume (200 mL) and reached a value of 5.0 ± 0.1 g L^{-1} for strain CBS DHA and 15.7 ± 0.1 g L^{-1} for strain CBS DHA FPS1. The highest ethanol yield (0.344 g g^{-1}) was obtained after 96 h cultivation for strain CBS DHA FPS1.

The limited oxygen availability had a strong impact on growth of both tested strains. The rate of biomass formation clearly decreased with increasing filling volume (Figure 3.4.2). It was therefore interesting to recognize that the initial volumetric glycerol consumption and ethanol production rates were relatively independent of oxygen availability for each of the strain. The only exception was the 200 mL culture. Here, both glycerol consumption and ethanol production rates were significantly lower compared to the other tested volumes for both strains. Another interesting finding is that the consumption of glycerol in the 50 mL culture stopped at a higher remaining substrate concentration compared to the higher volumes (75 – 125 mL). In the latter three cultures, the total amount of consumed glycerol increased with the culture volume. It seems that the reduced access to oxygen provides an advantage for the fitness of the cells. Future experiments will have to show whether this could be connected to the reduced production of reactive oxygen species potentially in combination with other types of stress (such as low pH) during the later phase of the cultivation.

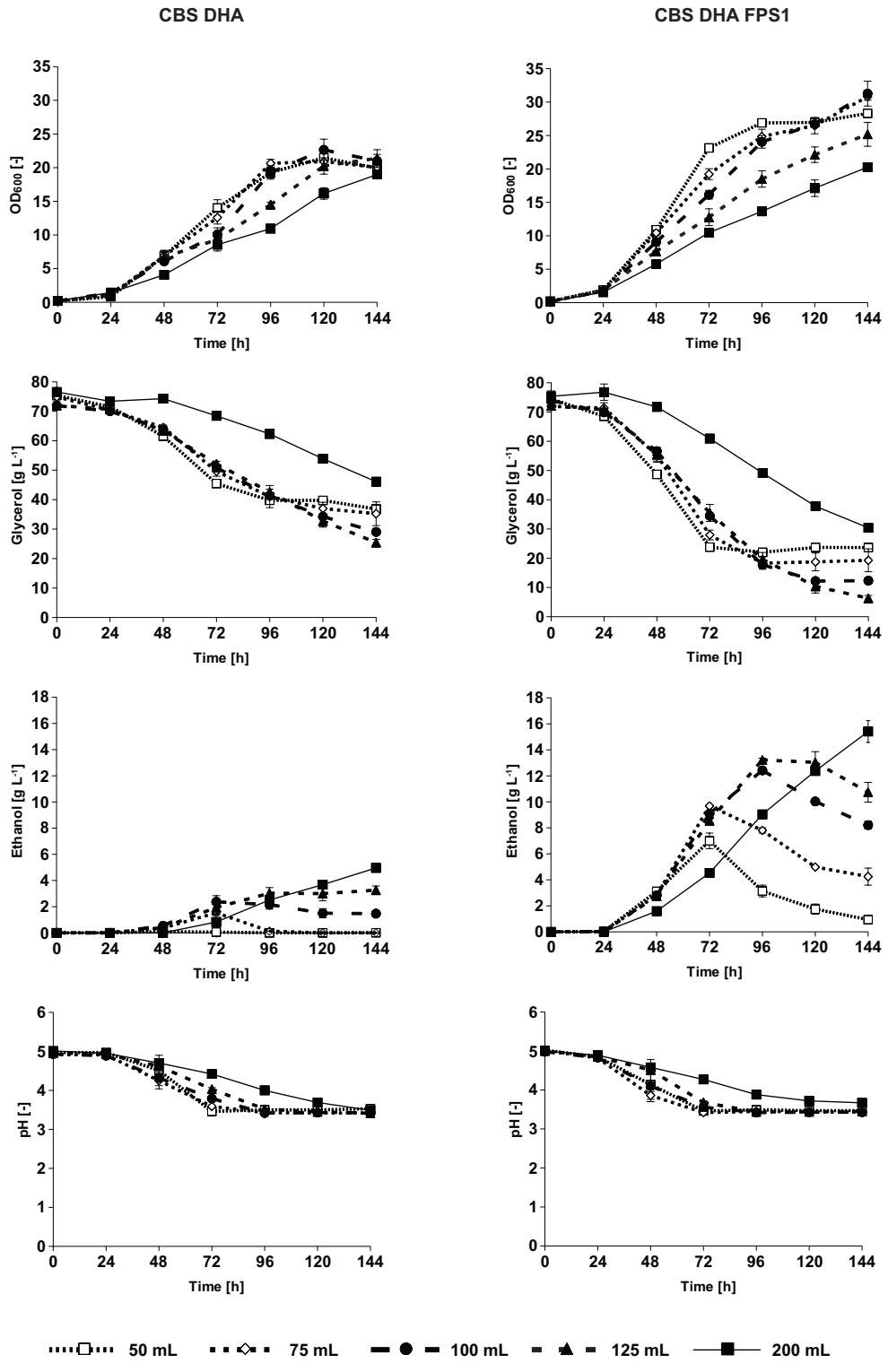

Figure 3.4.2. Effect of increased culture volumes (limited oxygen availability) on ethanol formation in the *S. cerevisiae* strains CBS DHA and CBS DHA FPS1 in the synthetic medium SMG_{buff}. Experiments were conducted with 50 mL, 75 mL, 100 mL, 125 mL and 200 mL filling volume in 500 mL Erlenmeyer shake flasks. Glycerol (6% v/v) was used as sole carbon source. Mean values and standard deviations from three independent experiments are shown.

Discussion

As described in the introduction, it has been generally accepted that *S. cerevisiae* catabolizes glycerol solely in a respiratory manner (Schüller, 2003; Turcotte et al., 2010). This seems to hold for wild-type strains and derivatives thereof, which catabolize glycerol via the L-G3P pathway. The current study demonstrates that the extra cytosolic NADH (per glycerol consumed) generated via the synthetic DHA pathway is indeed crucial for a partial shift to fermentative metabolism. However, a remarkable production of ethanol in a similarly high range as previously observed by Islam et al. (2017) could not be obtained without expressing the *FPS1* gene from *C. jadinii* in parallel to the glycerol catabolic pathway replacement. The *CjFPS1* expression is assumed to have significantly increased the rate of glycerol uptake and cytosolic NADH generation in the DHA pathway strain thereby strongly increasing the need for the observed overflow metabolism.

The occurrence of alcoholic fermentation during aerobic growth in glucose-containing medium has been intensively studied in *S. cerevisiae* and is generally referred to as the “Crabtree effect” (Crabtree, 1929). So far, this effect has been reported to occur when cells from a glucose-limited chemostat cultivation were subjected to a short glucose pulse (short-term Crabtree effect) or when cells grow in batch cultivation in excess glucose (long-term Crabtree effect) (Pronk et al., 1996). Studying the molecular basis for the regulation occurring during the Crabtree effect is challenging since it cannot be easily decoupled from the presence of glucose. Therefore, it is difficult to dissect between direct regulatory effects of glucose and indirect regulatory mechanisms triggered e.g. by changes in the level of NADH or other intracellular metabolites. Moreover, glucose present at a concentration exceeding a certain threshold level is well known to cause huge changes in global gene expression (at the level of transcription), a phenomenon that is also known as glucose repression (Carlson, 1999; Kayikci and Nielsen, 2015). With regard to the long-term Crabtree effect, the contributions of changed protein concentrations caused by the glucose repression and of metabolic regulation of enzymes caused by changes in metabolite levels is highly interwoven. The fact that ethanol

production can now be observed even in the absence of glucose (*i.e.* when cells catabolize glycerol via the DHA pathway) provides a promising future opportunity to further dissect the molecular reasons of the Crabtree effect in *S. cerevisiae*.

One theory for explaining the Crabtree effect is applicable even in the absence of glucose repression. This theory assumes that an increased rate of glycolysis together with a limitation in respiratory capacity leads to an imbalanced NADH/NAD^+ ratio and pyruvate accumulation (Dai et al., 2018; Hagman and Piškur, 2015; Vemuri et al., 2007; van Urk et al., 1990; Pronk et al., 1996). The quick response during the short-term Crabtree effect (also known as metabolic overflow) seems to be regulated by a rapid change of enzymatic activities in response to NADH overflow. When a *S. cerevisiae* strain grows on glycerol and, most importantly, uses the DHA pathway, the higher yield of cytosolic NADH generated per carbon consumed (together with its accelerated formation due to increased glycerol influx by CjFps1 activity) is supposed to be significantly increased compared to a strain using the L-G3P pathway and to cause significant ethanol formation from the 'non-fermentable' carbon source glycerol. This effect might have been triggered by the same 'NADH overflow' accompanied by the respective regulatory processes as described during the short-term Crabtree effect on glucose. In this context it is interesting to note that Hagman and Piškur (2015) suggest overflow metabolism to be the fundamental mechanism behind the long- and short-term Crabtree effects and to be evolutionary much older than glucose repression of respiration. None of the strains that use the native L-G3P pathway for glycerol catabolism was capable to produce ethanol from glycerol (even with additional expression of an *FPS1* homolog and cultivation under oxygen-limited conditions). These results seem to confirm that the NADH-generating glycerol oxidation step was indeed crucial for the switch to alcoholic fermentation when DHA pathway strains grow on glycerol.

In the current study, it has also been shown that lower oxygen transfer rates resulted in a further increase in ethanol formation in both strains CBS DHA and CBS DHA FPS1. This is in line with the fact that the capacity of any respiratory mechanism for re-oxidizing cytosolic

NADH such as the external NADH dehydrogenases or the L-G3P shuttle must become limiting as soon as the availability of the final electron acceptor is strongly decreased. Under oxygen limited conditions, re-oxidation of cytosolic NADH via fermentation is the only possible route to maintain redox balance and to sustain carbon flux through glycolysis. Otherwise, the cells have to respond with a reduction in growth rate. Interestingly, the latter has been observed in an *nde1*Δ deletion mutant of strain CBS DHA supposed to have reduced activity of the external NADH dehydrogenase when it was cultivated in synthetic glycerol medium (Aßkamp et al., 2019). This mutant did not produce any ethanol even if buffered medium was used (data not shown). It is still not clear, why the strain CBS DHA can (at least partly) switch to alcoholic fermentation when oxygen availability is strongly reduced but not if a major route for respiratory re-oxidation of cytosolic NADH is abolished by deleting *NDE1*. It might be that the abolishment of Nde1 activity is too severe or causes pleiotropic effects when the mutant grows in glycerol medium.

Ethanol production from glycerol in engineered strains of *S. cerevisiae* has already been reported by Yu et al. (2010). The genetic modifications considered causative for ethanol formation were the overexpression of *GCY1* in combination with the overexpression of the endogenous dihydroxyacetone kinase. As *GCY1* is assumed to encode an NADP-dependent glyceraldehyde reductase (Blomberg, 2000; Hur and Wilson, 2001; Norbeck and Blomberg, 1997), the results of Yu et al. (2010) are counterintuitive. Moreover, these authors used a medium containing amino acids and nucleic bases. The presence of these supplements has previously been shown to significantly affect glycerol utilization by wild-type *S. cerevisiae* strains (Merico et al., 2011; Swinnen et al., 2013) and might thus have also affected the production of ethanol.

The current study demonstrated for the first time alcoholic fermentation of glycerol by *S. cerevisiae* in pure synthetic medium. The highest titer obtained here was ~15 g L^{-1} ethanol by growing the strain CBS DHA FPS under oxygen-limited conditions. Taking the consumed glycerol into account, this corresponds to an ethanol yield of 0.34 g per g glycerol which is 69%

of the maximum theoretical yield. The actual yield is supposed to be even higher since it has to be assumed, that part of the ethanol has evaporated.

Conclusions

Genetic modifications leading to an increased yield and production rate of cytosolic NADH induce a metabolic overflow similar to the Crabtree effect in *S. cerevisiae* even if the cells grow on glycerol, a carbon source considered as non-fermentable for this species so far. The replacement of the L-G3P pathway by the DHA pathway for glycerol utilization seems to be a minimal requirement for ethanol formation in synthetic glycerol medium. The obtained results might provide interesting future opportunities to study the Crabtree effect uncoupled from glucose repression and for the valorization of glycerol-containing feedstocks.

Materials & Methods

Strains, medium composition and general cultivation conditions

All *S. cerevisiae* cells (Additional File 1) were routinely grown on solid (15 g L^{-1} agar) or in liquid YPD medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose. Liquid cultures were incubated in an orbital shaker at 200 rpm and 30 °C. Plasmid carrying *E. coli* DH5α strains were grown at 250 rpm and 37°C in lysogeny broth (LB) (10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl, pH 7.0) containing 100 mg L^{-1} ampicillin for selection purposes.

Media for analyzing yeast growth and fermentation behavior with glycerol as the sole carbon source

The synthetic media were based on the medium described by Verduyn et al. (1992) containing: 5 g L^{-1} $(NH_4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 15 mg L^{-1} EDTA, 4.5 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.84 mg L^{-1} $MnCl_2 \cdot 2H_2O$, 0.3 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 0.3 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.4 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 4.5 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 3 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 1 mg L^{-1} H_3BO_3, and 0.1 mg L^{-1} KI. As carbon source 60 mL L^{-1} glycerol was added to the medium. In order to buffer the medium, 20.5 g L^{-1} potassium hydrogen phthalate ($C_8H_5KO_4$) was added (SMG_{buff}). After autoclaving of this basal medium, a filter sterilized vitamin solution was added. The final concentrations of vitamins in the medium were 0.05 mg L^{-1} D-(+)-biotin, 1 mg L^{-1} D-pantothenic acid hemicalcium salt, 1 mg L^{-1} nicotinic acid, 25 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine chloride hydrochloride, 1 mg L^{-1} pyridoxine hydrochloride, and 0.2 mg L^{-1} 4-aminobenzoic acid. The pH was adjusted to 5.0 using 2 M H_3PO_4.

Construction of strain CBS DHA FPS1

Plasmids (Additional File 1) were isolated from *E. coli* overnight cultures using the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The *CjFPS1* expression cassette was amplified from the plasmid pUC18-*PGK1p-CjFPS1* (Islam et al., 2017) with Phusion® High-Fidelity DNA Polymerase (NEB, Frankfurt am Main, Germany)

according to the manufacturer's. The GeneJET^{TM} PCR Purification Kit (Thermo Fisher Scientific) was afterwards used to purify the PCR product. Transformation of *S. cerevisiae* was performed using the lithium acetate method described by Gietz et al. (1995). The expression cassette was integrated in the genome of strain CBS DHA (Aßkamp et al., 2019) at position YGLCτ3 on chromosome VII using the CRISPR/Cas9 method described by DiCarlo et al. (2013) and adapted by Klein et al. (2016a). First, the strain was transformed with p414-TEF1p-Cas9-CYC1t-nat1 for expression of Cas9. In a second step, the resulting strain was transformed with the p426-SNR52p-gRNA.YGLCτ3-SUP4t-hphMX (Islam et al., 2017) for gRNA expression and the PCR amplified expression cassette for *CjFPS1*. The latter contained PCR generated regions upstream of the used *PGK1* promoter and downstream of the *RPL15A* terminator that were homologous to sequences on either side of the introduced double strand break at the target site for integration by homologous recombination. Correct integration of the cassette was verified by diagnostic PCR. Single colonies obtained after transformation were re-streaked. Cells from these plates were used for the isolation of genomic DNA using a protocol adapted from Hoffman and Winston (1987) and described in Swinnen et al. (2013) in more detail. Diagnostic PCRs were performed using One*Taq* Quick-Load DNA Polymerase (NEB). The primers (Additional File 1) bind upstream and downstream of the targeted genomic integration site and within the integrated *CjFPS1* expression cassette, respectively.

Characterization of *S. cerevisiae* in shake flask batch cultivation

Cells from single colonies were transferred to 5 mL of SMG_{buff} in a 10 mL glass tube and cultures incubated by shaking at 200 rpm and 30°C for 48 h. From this pre-culture, a 15 mL intermediate culture using the same medium in a 100 mL Erlenmeyer flask was inoculated adjusting an OD_{600} of 0.2. This culture was grown under the same conditions for another 48 h. For experiments aiming at limiting the oxygen transfer by increased filling volumes, two 15 mL intermediate cultures in 100 mL Erlenmeyer flasks were inoculated from the same pre-culture and combined before preparing the respective main culture. For main culture preparation, an appropriate culture volume (in order to adjust to an OD_{600} of 0.2 in the main culture) was

centrifuged at 800 g for 5 min and the supernatant was discarded. The cell pellet was then resuspended in fresh SMG_{buff} in a 500 mL Erlenmeyer flask. The final cultures had a volume of 50 mL, while additional filling volumes of 75 mL, 100 mL, 125 mL and 200 mL culture medium were used for the experiment analyzing the influence of oxygen limitation. Samples of 1.2 mL were taken every 24 h for OD_{600} and pH determination as well as for HPLC analysis.

Quantification of Ethanol and Glycerol via HPLC

The samples (1.2 mL culture supernatant) were filtered through 0.2 µm Minisart RC membrane filters (Sartorius, Göttingen, Germany) and stored at -20 °C until analysis. Detection and quantification of glycerol and ethanol was performed using a Waters HPLC system (Eschborn, Germany) consisting of a binary pump (Waters 1525), an injector system (Waters 2707) and a Waters column heater module WAT038040 and a refractive index detector (Waters 2414). An Aminex HPX-87H cation exchange column (Biorad, München, Germany) coupled to a Micro-guard® cation exchange column (Biorad) was used for chromatography. As a solvent, 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1} was used. The column was kept at 45 °C. A sample volume of 20 µL was injected. Under these conditions, the retention times were about 13.5 min for glycerol and 22.7 min for ethanol. Data were analyzed using the Breeze 2 software (Waters).

Additional files

Additional file 1: Lists of all *S. cerevisiae* strains, Primers and Plasmids used in this study.
Additional file 2: Growth, glycerol consumption and ethanol formation of a wild-type CBS 6412-13A strain with additional expression of an aquaglyceroporin from *C. jadinii* (*CjFPS1*).
Additional file 3: Evaporation of ethanol in SMG_{buff} and time-course of ethanol concentration detectable in supernatants of cultivations with the *S. cerevisiae* strain CBS DHA FPS1 in the same medium.

Abbreviations

DHA: Dihydroxyacetone; HPLC: High pressure liquid chromatography; L-G3P: L-glycerol 3-phosphate, LB: lysogeny broth; OD_{600}: Optical density determined at a wavelength of 600 nm; OTR_{max}: maximum oxygen transfer rate; PCR: Polymerase chain reaction; μ_{max}: maximum specific growth rate; SMG: synthetic glycerol medium; SMG_{buff}: SMG plus 100 mM potassium hydrogen phthalate; YPD: yeast extract peptone dextrose.

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Competing interests

The authors declare no competing interest.

Authors' contributions

MRA carried out the experiments. MRA, MK, and EN wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Additional file 1

Table S1: Strains used in this study.

Strain / Modules	Genome modifications	Source or reference
CBS 6412-13A	-	Swinnen *et al.* (2013)
CBS 6412-13A *CjFPS1*	*YGLCτ3:: PGK1p-CjFPS1-RPL15At:loxP-ble-loxP*	This study
CBS DHA-P	*YPRCΔ15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP*	Aßkamp *et al.* (2019)
CBS DHA-P *CjFPS1*	*YPRCΔ15::ACT1p-DAK1-TPS1t*; *YPRC τ3::TEF1p-Opgdh-CYC1t; gut1::loxP; YGLCτ3::PGK1p-CjFPS1-RPL15At:loxP-ble-loxP*	This study

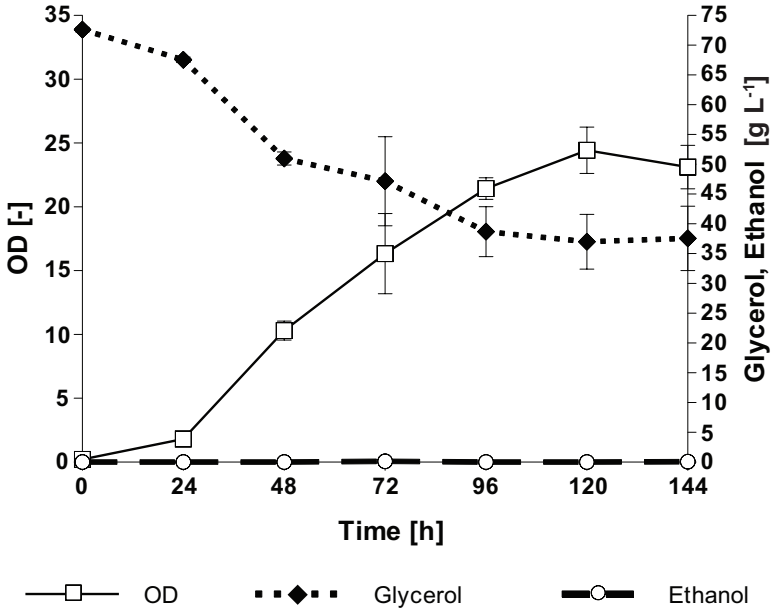
Table S2: Plasmids used in this study.

Plasmid	Relevant characteristics	Source or reference
p426-SNR52p-gRNA-YGLCτ3-SUP4t-hphMX	gRNA targeting *YGLCτ3; hygromycin B* resistance	Klein *et al*. (2016a)
pUC18-PGK1p-*CjFPS1*	*CjFPS1* under *PGK1*p and *RPL15At*	Islam *et al*. (2017)
414-TEF1p-Cas9-CYC1t-nat1	Cas9 from *S. pyogenes*; nourseothricin resistance	Klein *et al*. (2016a)

Table S3: PCR primers used in this study. 5' overhangs used for generating overlapping PCR products to regions in the *S. cerevisiae* genome (for *in vivo* homologous recombination) are underlined. (Primer Numbers according to the AG Nevoigt Primer collection).

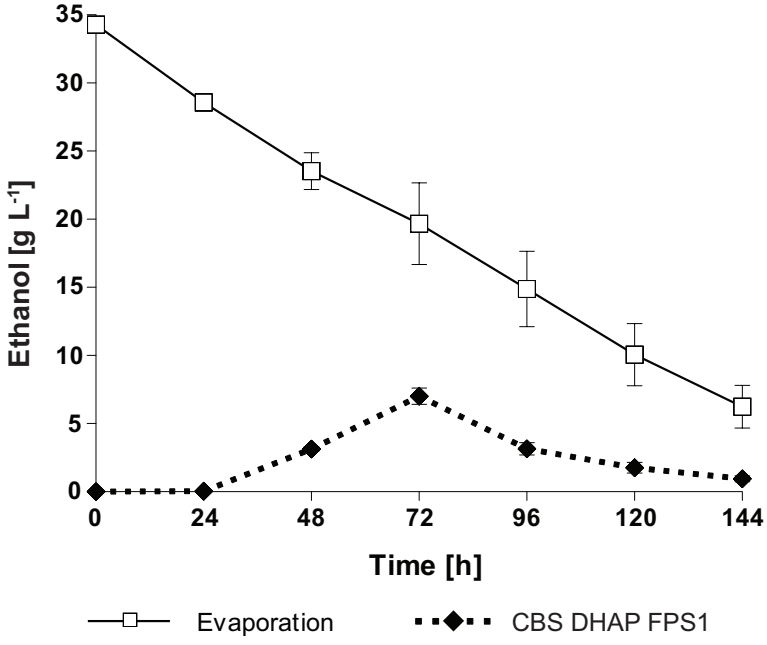
Primer No.	Name	Sequence 5' – 3'
Primers for the integration of the CjFPS1 expression cassettes into the YGLCτ3 locus on chromosome VII		
390	CHRVII-PGK1p-F	CGCGGTAAATTCTTAAGGCCATATTTCAGGTAGGAACCATCGT CAACAATTGCTACAACGGAAGTACCTTCAAAGAATGGGGTCTT
392	RPL15At-loxP-F	ATTAAGGGTTGTCGACCTGCAGCGTACGAAGCTTCAGCT GGGGAAAAACGGGAAGAAAAGGAAAGA
Primers for the verification of the CjFPS1 expression cassettes into the YGLCτ3 locus on chromosome VII		
179	HAA1 OE check F1	AGCGTTCGTTCTATGCCTCT
181	HAA1 OE check R1	AAGAACCAGAATGGCAGGAC
359	PjFPS1-VER 2	GACCCTTGGTGTTCAAGTA
708	PjFPS1-F-qPCR	GCTGTTCTGCGGGTATCTCT

Additional file 2



Growth, glycerol consumption and ethanol formation of a wild-type CBS 6412-13A strain with additional expression of an aquaglyceroporin from *C. jadinii* (*CjFPS1*). Cells were grown in 50 mL SMG_{buff} in a 500 mL shake flask. Glycerol (6% v/v) was used as sole carbon source. Mean values and standard deviations from three independent replicates are shown.

Additional file 3



Ethanol (dissolved in SMG_{buff}) evaporation in 500 mL shake flasks with 50 mL filling volume and ethanol formation by CBS DHA FPS1 grown under the same conditions.

CHAPTER 4: 1,3-PROPANEDIOL AS A REDOX SINK

4.1. Background

Establishment of a 1,3-propanediol pathway as redox sink for biomass-derived NADH to provide a prerequisite for anaerobic fermentation of glycerol *by S. cerevisiae*

It is well accepted that the formation of chemicals by microbial fermentation is most efficient if the production process can be performed anaerobically (Cueto-Rojas *et al*. 2015). The superordinate objective under which the current study is placed, is the anaerobic formation of fermentation products (with maximum theoretical yields) from glycerol by engineered *S. cerevisiae*. Any anaerobic conversion of a substrate into a product of interest requires a redox neutral pathway from the substrate to this product. In addition, the cells require a possibility to oxidize the surplus of redox equivalents (e.g. NADH) formed during the formation of biomass in order to maintain redox balance. Another prerequisite for efficient formation of products as well as to sustain growth without oxygen, is the availability of net ATP produced by substrate level phosphorylation. This thesis focused on the first requirement, i.e. the establishment of a redox sink for additional NADH.

When glucose is catabolized under anoxic conditions, the glycerol formation pathway functions as a redox sink for NADH formed during biomass formation (see **Chapter 1.2.2**). With glycerol as substrate, the establishment of another (heterologous) pathway is required to fulfill this task. Ideally, such an redox sink should not require ATP, as the generation of net ATP under anaerobic conditions is lower compared to aerobic conditions and is therefore another issue to be kept in mind. As suggested in the German Research Foundation (DFG) proposal [NE-697/7-1], a pathway for the formation of 1,3-propanediol (1,3-PDO) was designated to meet these requirements and we embarked on integrating the respective pathway as redox sink in strains able to catabolize glycerol.

In bacteria, 1,3-PDO is formed from glycerol by the activity of two enzymes (compare **Chapter 1.1.4**). With regard to the first enzymatic step of the 1,3-PDO pathway, two types of bacterial GDHts are known: one is cobalamin (better known as vitamin B_{12}) dependent, the

other one is independent of this cofactor (Liu *et al*., 2016; Feliks and Ullmann, 2012). In most of the studied glycerol-fermenting prokaryotes, the GDHt is dependent on vitamin B_{12}, including the human pathogenic bacterium *Klebsiella pneumoniae* as the best studied one (Biebl *et al.* 1999; Nakamura and Whited, 2003; Lee *et al.* 2015a). The genes encoding for the two enzymes involved in the formation of 1,3-PDO in this organism are encoded by the complex *dha* operon (Tong *et al.* 1991). Additionally, the *dha* operon encodes for the glycerol dissimilation pathway and a facilitator for glycerol uptake. The GDHt (first enzyme of the 1,3-PDO pathway) is encoded by three ORFs, which encode for three subunits of the enzyme (Tobimatsu *et al.* 1996; Toraya, 2000). The PDOR (second enzyme of the pathway) is encoded by a single gene *dhaT.* Along with the genes encoding for the proteins responsible for GDHt and the PDOR enzyme activities, two ORFs of the *dha* operon encode for a reactivation factor of the GDHt (GDHt RA). This reactivation factor is required since the dehydratase undergoes suicide inactivation by its substrate glycerol (Mori *et al.* 1997; Celińska, 2010).

A GDHt which is independent of vitamin B_{12} has only been identified in *Clostridiaceae* species (Raynaud *et al.* 2003; Saxena *et al.* 2009). *Clostridium butyricum* is the best described 1,3-PDO producing bacterium among them. In contrast to the seven *K. pneumoniae* genes contributing to the formation of the active enzymes for 1,3-PDO synthesis, the respective operon in *Clostridium butyricum* is only composed of three genes (Raynaud *et al.* 2003). The GDHt is encoded by *dhaB1* and *dhaB2* (the latter encodes for the reactivation factor GDHt RA) and the oxidoreductase is encoded by *dhaT*.

The goal of the current study was the establishment of a bacterial 1,3-PDO pathway in *S. cerevisiae* as a redox sink to provide a prerequisite for anoxic growth on glycerol. As *S. cerevisiae* is incapable of synthesizing vitamin B_{12} (Martens *et al*. 2002), the enzymes of the 1,3-PDO pathway from *C. butyricum* were selected to be expressed.

4.2. Materials and Methods

Strains, Plasmids, Media

All *S. cerevisiae* strains used in this part of the study are listed in Table 4.1. The cells were routinely grown on solid (1.5 % (w/v) agar) or in liquid YPD medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose. Liquid cultures were incubated in an orbital shaker at 200 rpm and 30°C. Plasmid carrying *E. coli* DH5α strains were grown at 250 rpm and 37 °C in lysogeny broth (LB) (10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl, pH 7.0) containing 100 mg L^{-1} ampicillin for selection purposes. The GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for plasmid isolation. All plasmids used in this study are shown in Table 4.2. For analyzing the different strains with regard to growth or product formation, synthetic media containing either glucose (SMD) or glycerol (SMG) were prepared (see **Chapter 3.3**). The codon-optimized genes from *C. butyricum* were synthesized by Invitrogen (Carlsbad, CA). The respective sequences are listed in the appendix.

Table 4.1: Strains utilized, constructed and analyzed in this study.

Strain	Genome modifications	Source or reference
S288c	-	Mortimer and Johnston, 1986
CEN.PK113-7D	-	van Dijken *et al.* 2000
CE1.01.04.02	*gpd1::loxP; gpd2::loxP*	Hubmann *et al.* 2011
CE1.01.04.02 1,3-PDO	*gpd1::loxP; gpd2::loxP*; *YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}* (#102)	This study
CE1.01.04.02 1,3-PDO *FPS1*	*gpd1::loxP; gpd2::loxP; YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}; YORWΔ22::P_{PGK1}-CjFPS1-T_{RPL15A}-loxP-ble-loxP* (#188)	This study
CEN.PK113-7D FPS1	*YORWΔ22::P_{PGK1}-CjFPS1-T_{RPL15A}-loxP-ble-loxP* (#205)	This study

CE1.01.04.02 FPS1	*gpd1::loxP; gpd2::loxP; YORWΔ22::P_{PGK1}-CjFPS1-T_{RPL15A}-loxP-ble-loxP* (#207)	This study
CEN.PK113-7D FPS1 1,3-PDO	*YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}; YORWΔ22::P_{PGK1}-CjFPS1-T_{RPL15A}-loxP-ble-loxP* (#213)	This study
CE1.01.04.02 dhaT	*gpd1::loxP; gpd2::loxP; YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}* (#262)	This study
CE1.01.04.02 dhaB1-B2	*gpd1::loxP; gpd2::loxP; YGLCτ3::P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}* (#266)	This study
CBS 6412-13A	-	Swinnen *et al.* 2013
CBS DHA	*gut1::loxP; YPRCτ3::P_{TEF1}-gdh-T_{CYC1}; YPRCΔ15::P_{ACT1}-DAK1-T_{TPS1}*	Aßkamp *et al.* 2019
CBS nde1	*nde1::loxP*	Aßkamp *et al.* 2019
CBS DHA nde1	*nde1::loxP; gut1::loxP; YPRCτ3::P_{TEF1}-gdh-T_{CYC1}; YPRCΔ15::P_{ACT1}-DAK1-T_{TPS1}*	Aßkamp *et al.* 2019
CBS DHA nde1Δ dhaT	*gut1::loxP; YPRCτ3::P_{TEF1}-gdh-T_{CYC1}; YPRCΔ15::P_{ACT1}-DAK1-T_{TPS1}; nde1::loxP; YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}* (#264)	This study
CBS DHA nde1Δ dhaB1-B2	*gut1::loxP; YPRCτ3::P_{TEF1}-gdh-T_{CYC1}; YPRCΔ15::P_{ACT1}-DAK1-T_{TPS1}; nde1::loxP; YGLCτ3::P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}* (#268)	This study
CBS DHA 1,3-PDO	*gut1::loxP-KanMx-LoxP; YPRCτ3::P_{TEF1}-gdh-T_{CYC1}; YPRCΔ15::P_{ACT1}-DAK1-T_{TPS1}; YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}* (#59)	This study
CBS 1,3-PDO	*YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}* (#60)	This study
CBS DHA nde1Δ 1,3-PDO	*gut1::loxP; nde1::loxP; YPRCτ3::P_{TEF1}-gdh-T_{CYC1}; YPRCΔ15::P_{ACT1}-DAK1-T_{TPS1}; YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1} (#181)*	This study
CBS nde1Δ 1,3-PDO	*nde1::loxP; YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1} (#183)*	This study
CBS 1,3-PDO FPS1	*YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}; YORWΔ22::P_{PGK1}-CjFPS1-T_{RPL15A}-loxP-ble-loxP (#190)*	This study
CBS DHA 1,3-PDO FPS1	*gut1::loxP; nde1::loxP; YPRCτ3::P_{TEF1}-gdh-T_{CYC1}; YPRCΔ15::P_{ACT1}-DAK1-T_{TPS1}; YGLCτ3::P_{TEF1}-dhaT-T_{CYC1}:P_{PGK1}-dhaB1-T_{DIT1}:P_{ACT1}-dhaB2-T_{TPS1}; YORWΔ22::P_{PGK1}-CjFPS1-T_{RPL15A}-loxP-ble-loxP (#192)*	This study

Table 4.2: Plasmids used and constructed in this study.

Plasmid	Relevant characteristics	Source or reference
pUG66	*loxP-ble-loxP* disruption cassette	Euroscarf
pUG6	*loxP-natMX4-loxP* disruption cassette	Euroscarf
p414-TEF1p-Cas9-CYC1t-nat1	*CEN6/ARSH4, natMX4,* P_{TEF1}*-Cas9-*T_{CYC1}	Klein *et al.* 2016
p426-SNR52p-gRNA YGLCт3-SUP4t-hphMX	2 µm, *hphMX4,* P_{SNR52}*-gRNA.YGLCт3-*T_{SUP4}	Islam *et al.* 2017
pUC18-*dhaB2*	P_{ACT1}*-dhaB2-*T_{TPS1}	This study
pUC18-*CjFPS1*	P_{PGK1}*-CjFPS1-*T_{RPL15A}	Klein *et al.* 2016
pMAT-dhaB1_DHAt		This study/ Invitrogen
pMAT-dhaB2_DHAt_RA		This study/ Invitrogen
pMAT-dhaT_PDOR		This study/ Invitrogen

Quantitative analysis of *S. cerevisiae* growth

Growth of liquid cultures in SMG and SMD was recorded using the Growth profiler as described by Aßkamp *et al.* (2019).

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

Isolation of genomic DNA as well as PCR amplification was performed as described by Aßkamp *et al.* (2019).

General molecular biology techniques

Amplification of the expression cassettes for *dhaB2* and *CjFPS1* as well as the coding sequences of *dhaB1* and *dhaT* from plasmids was performed using the primers listed in Table 4.3 with Phusion® High-Fidelity DNA Polymerase (NEB, Frankfurt am Main, Germany) at an annealing Temperature of 60°C. Same conditions were applied for amplification of the respective promoter and terminator sequences from genomic DNA, isolated from the well-characterized *S. cerevisiae* strain S288c. PCR products were purified using the GeneJET™ PCR Purification Kit (Thermo Fisher Scientific).

Table 4.3. PCR primers used in this study. 3' binding sites are highlighted in bold. Primer numbers according to the Nevoigt AG Primer List.

Primer No.	amplifying	Sequence 5' – 3'
Primers for the integration of the entire pathway at the YGLCτ3 locus on chromosome VII		
624	dhaB1	ACAACAAATATAAAACA**ATGATCTCCAAGGGTTTCTCTACTCAAAC**
625	dhaB1	AGCGCTCTTACTTTA**TCACATAACGTGTTCGGTTCTGGC**
608	PGK1p	GTTTTGGGACGCTCGAAGGCTTTAATTTGC**GAAGTACCTTCAAAGAATGGGGTC**
623	PGK1p	ACCCTTGGAGATCAT**TGTTTTATATTTGTTGTAAAAAGTAGATAATTACTTCCTTG**
344	DIT1t	GAAAATAGAGGCCGGGTGACAGAGAAATATTG**TTACTCCGCAACGCTTTTCTGAACG**
626	DIT1t	GAACACGTTATGTGA**TAAAGTAAGAGCGCTACATTGGTCTACC**
737	dhaT	TAGCAATCTAATCTAAG**ATGAGAATGTACGATTACTTGGTCCCA**
738	dhaT	ACATAACTAATTACATGA**TTAGTAGGCAGCCTTAAAAATGTTAATGATGTCC**
257	TEF1p	CTGCACATAATTGAAATAAGGATGTAGTTCAACTTCTATGAATGCTCGGC GATACGATATG**ATAGCTTCAAAATGTTTCTACT**
736	TEF1p	GTAATCGTACATTCTCAT**CTTAGATTAGATTGCTATGCTTTCTTTCTAATGAGCA**
607	CYC1t	AGATAAGACCCCATTCTTTGAAGGTACTTC**GCAAATTAAAGCCTTCGAGC**

739	CYC1t	TTAAGGCTGCCTACTAA**TCATGTAATTAGTTATGTCACGCTTACGTTCAC**
345	dhaB2-cas	GCGGGCGTTCAGAAAAGCGTTGCGGAGTAA**CAATATTTCTCTGTCACC CGGCCTC**
827	dhaB2-cas	AGTTTTTCCGTCTCTGGCTGAAGGCTCATTTCCATGATGGGGTCACAATT ATTATCGCAC**GATAAATTATGGCTTTTTTTTCTTGA**
Primers for construction of the dhaB2 expression cassette in pUC18		
630	dhaB2	CGAAAATTTACTGAATT**ATGTCCAAAGAAATCAAGGGTGTCTTG**
631	dhaB2	TGCATCGGGTTCA**TCATTCGGCACCAATTGTACATGGAATACC**
628	ACT1p	GCATGCCTGCAGGTCGACTCTAGAG**CAATATTTCTCTGTCACCCGGCC**
629	ACT1p	CTTGATTTCTTTGGACAT**AATTCAGTAAATTTTCGATCTTGGGAAGAAA AAAGC**
632	IDP1t	GGTGCCGAATGA**TGAACCCGATGCAAATGAGACG**
633	IDP1t	ATTACGAATTCGAGCTCGGTACCCGGG**GATAAATTATGGCTTTTTTTTCT TGATTTCCTTTTTCTTTTTATTCA**
Primers for the integration of dhaB1-dhaB2 at the YGLCτ3 locus on chromosome VII		
624	dhaB1	ACAACAAATATAAAACA**ATGATCTCCAAGGGTTTCTCTACTCAAAC**
625	dhaB1	AGCGCTCTTACTTTA**TCACATAACGTGTTCGGTTCTGGC**
859	PGK1p	CTGCACATAATTGAAATAAGGATGTAGTTCAACTTCTATGAATGCTCGGC GATACGATATG**GAAGTACCTTCAAAGAATGG**
623	PGK1p	ACCCTTGGAGATCAT**TGTTTTATATTTGTTGTAAAAAGTAGATAATTACT TCCTTG**
344	DIT1t	GAAAATAGAGGCCGGGTGACAGAGAAATATTG**TTACTCCGCAACGCTTT TCTGAACG**
626	DIT1t	GAACACGTTATGTGA**TAAAGTAAGAGCGCTACATTGGTCTACC**
345	dhaB2-cas	GCGGGCGTTCAGAAAAGCGTTGCGGAGTAA**CAATATTTCTCTGTCACC CGGCCTC**
827	dhaB2-cas	AGTTTTTCCGTCTCTGGCTGAAGGCTCATTTCCATGATGGGGTCACAATT ATTATCGCAC**GATAAATTATGGCTTTTTTTTCTTGA**
Primers for the integration of dhaT at the YGLCτ3 locus on chromosome VII		
737	dhaT	TAGCAATCTAATCTAAG**ATGAGAATGTACGATTACTTGGTCCCA**
738	dhaT	ACATAACTAATTACATGA**TTAGTAGGCAGCCTTAAAAATGTTAATGATGT CC**
257	TEF1p	CTGCACATAATTGAAATAAGGATGTAGTTCAACTTCTATGAATGCTCGGC GATACGATATG**ATAGCTTCAAAATGTTTCTACT**
736	TEF1p	GTAATCGTACATTCTCAT**CTTAGATTAGATTGCTATGCTTTCTTTCTAAT GAGCA**

258	CYC1t	GAGTTTTTCCGTCTCTGGCTGAAGGCTCATTTCCATGATGGGGTCACAA TTATTATCGCAC**GGCCGCAAATTAAAGCCTTCGAGCGT**
739	CYC1t	TTAAGGCTGCCTACTAA**TCATGTAATTAGTTATGTCACGCTTACGTTCAC**
Primers for the integration of CjFPS1 at the YGLCτ3 locus on chromosome VII		
1187	CjFPS1-cas	GGATAAGTAACAGCCCGCGAATCAAGCTGGTAATCCGTTTTGACAGCTG GTTGAAG**TACCTTCAAAGAATGGGGTCTT**
392	CjFPS1-cas	ATTAAGGGTTGTCGACCTGCAGCGTACGAAGCTTCAGCTGGGGAAA**AA CGGGAAGAAAAGGAAAGA**
666	*bleMX*	CAGTAATTAGCGGATGATAGTTGGTCCCTATTCCGATAATCTTAGCAGA GTGAATAG**GCATAGGCCACTAGTGGATCTG**
391	*bleMX*	TTCTTGTATTTTTTTTCTTTCCTTTTCTTCCCGTTTTTC**CCCAGCTGAAGC TTCGTACGC**

Construction of expression cassettes via Gibson Assembly®

The expression cassettes were constructed and integrated into a pUC18-plasmid, in order to facilitate amplification of the entire cassette. Primers for construction of the expression cassettes were designed to bind with 20 bp at the beginning/end of the coding sequences of the respective fragment. In addition, the primers were designed to create a minimum of 40 bp 5′ extensions homologous to the adjacent fragment.

The method described by (Gibson *et al.* 2009) was used for one-step isothermal DNA assembly reactions of expression cassettes in pUC18. For calculation of volumes of fragments needed for the assembly mix, the Promega (Madison, WI, USA) biomath-calculator for “dsDNA: µg to pmol” was used (http://www.promega.de/resources/tools/biomath-calculators/; 19.01.2019).

In general, the reaction mixtures contained of 15 µL Gibson Assembly mix (Table 4.4), 0.05 pmol of plasmid, linearized with *BamHI* (NEB) and additionally treated with alkaline phosphatase (NEB) to prevent relegation, and 0.15 pmol of each fragment, to be inserted, all together not exceeding a volume of 5.2 µL. Reaction mixtures were incubated at 50°C for 1 h in a thermocycler. Afterwards *E. coli* cells were transformed with the mixture, containing

assembled plasmids. Sequencing, of the constructed plasmids, was performed by Eurofins Scientific (Luxemburg) using the universal M13 primers.

Table 4.4: Gibson Assembly master mix (1.33x). Stored in aliquots of 15 µL at -20°C for at most one year. Enzymes purchased from NEB.

Compound	Volume [µL]
5x ISO buffer (Table 5)	100
T5 exonuclease (10 U μL^{-1})	0.20
Phusion pol. (2 U μL^{-1})	6.25
Taq ligase (40 U μL^{-1})	50
ddH_2O	218.55

Table 4.5: 5x ISO (isothermal) buffer. Stored in aliquots of 100 µL at -20°C.

Compound	Volume [mL]
TRIS-HCl pH 7.5 (1 M)	1.50
$MgCl_2$ (2 M)	0.075
dNTPs (100 mM)	0.03
1,4-Dithiothreitol (DTT) (1 M)	0.15
NAD^+(50 mM)	0.30
PEG-8000	0.75 g
ddH_2O	Add to 3 mL

Integration of expression cassettes into the genome of *S. cerevisiae*

Transformations into *S. cerevisiae* were performed using the lithium acetate method described by Gietz *et al.* (1995). CRISPR/Cas9 mediated integration followed in general the protocol described by Klein *et al*. (2016a). For plasmid-based expression of the Cas9 endonuclease, the plasmid p414-TEF1p-Cas9-CYC1t-nat1 was transferred in a first step. Selection was performed using YPD plates supplemented with 100 mg L^{-1} nourseothricin. In a second step, the gRNA-plasmid p426-SNR52p-gRNA YGLCт3-SUP4t-hphMX and the expression-cassette, respectively the single components (promoter, CDS, terminator), encoding for the 1,3-PDO pathway, were transferred together. Afterwards selection was performed on YPD plates

containing nourseothricin and hygromycin B (100 mg L^{-1}/ 300 mg L^{-1}). Integration of *CjFPS1* into position *YORWΔ22* (Flagfeldt *et al.* 2009) plus a phleomycin resistance marker cassette framed by *loxP*-sides*,* was not CRISPR/Cas9 guided, but relied on conventional in vivo homologous recombination in *S. cerevisiae* alone*.* Selection was conducted on YPD plates containing 30 mg L^{-1} phleomycin. Correct integration of the expression cassettes was verified by diagnostic PCR using One*Taq* Quick-Load DNA Polymerase (NEB) and the primer pairs listed in (Table 4.3).

Detection of 1,3-PDO, 3-HPA and Glycerol via HPLC

Substrate glycerol and the (intermediate-) product 3-HPA or 1,3-PDO were analyzed with system and method described by Aßkamp *et al.* (2019). Under these conditions, glycerol was measured after 13.5 min, while the retention time for 1,3-PDO was 17.9 min. Due to a missing standard for 3-HPA we relied on information from Lindlbauer *et al*. (2017), who observed a peak with a similar system in the RI detector, which overlapped with the peak of 1,3-PDO. Their solution was to use the UV detector at 210 nm to detect and separate 3-HPA from 1,3-PDO. Therefore, 3-HPA measurement was performed according to the latter authors and the occurrence of a peak around 15.5 min was investigated.

Serial drop dilution assay (Spot-test)

For evaluating growth under anaerobic conditions, synthetic medium (Aßkamp *et al.* 2019) with a pH of 6.5 containing 2,5 % (w/v) agar was prepared in three different variations: with 2 % (w/v) glucose as sole carbon source (SMD), 6 % (v/v) glycerol (SMG) or 2 % (w/v) glucose plus 6 % (v/v) glycerol (SMDG). The agar was washed five times with 2 L of deionized water, to reduce any residues of other nutrient sources to a minimum. Cells from a single cell colony (from an YPD-plate) were incubated overnight in 3 mL SMD. From this pre-culture, 3 mL fresh SMD were inoculated with an initial OD_{600} of 0.2. The cells were incubated again for 24 h. Afterwards the cells were washed twice with 3 mL of 0.85 % NaCl before the OD_{600} was measured again. From these cultures, an appropriate number of cells to achieve an OD_{600} of 1

in 3 mL was diluted with 0.85 % NaCl to generate the starting culture. From the latter culture, serial dilutions from 10^{0} to 10^{-4} in 100 µL were prepared (in a 96-well plate). Three microliter per dilution were spotted onto the respective plates. The plates were inoculated for 120 h at 30°C in a static incubator. For evaluating anoxic growth, the plates were placed in an Oxoid anaerobic jar (Thermo Scientific, Waltham, MA). Exclusion of oxygen was established by adding an AnaeroGen™ 2.5L Pack (Thermo Scientific).

4.3. Results

4.3.1. Expression of the 1,3-PDO pathway from *C. butyricum* and Fps1 from *C. jadinii* in a *gpd1*Δ *gpd2*Δ mutant

For a proof of concept of a functional redox sink, necessary to allow anoxic glycerol fermentation in *S. cerevisiae*, the 1,3-PDO pathway from *C. butyricum* was expressed in several strains (Table 4.1). The enzymes from *C. butyricum* were assumed to be sensitive to oxygen (because of their origin from an obligate anaerobe bacterium). Therefore, a strategy was used in which the establishment of an active 1,3-PDO pathway was supposed to restore anaerobic growth. As a background strain for this strategy, the strain CE1.01.04.02, previously constructed by Hubmann *et al.* (2011), was used (Figure 4.1). This strain is a derivative of the well characterized strain CEN.PK113-7D and carries deletions of *GPD1* and *GPD2* to abolish the native glycerol formation pathway, which is the natural redox sink for biomass-related NADH when *S. cerevisiae* anaerobically grows with glucose (see **Chapter 1.2.2**). The absence of this redox sink causes an imbalanced NAD^+/NADH ratio, which disables the organism to grow on glucose under anaerobic conditions (Hubmann *et al.* 2011).

The successful implementation of an alternative redox sink (*i.e.* 1,3-PDO pathway) was supposed to restore anoxic growth on a medium containing glucose as carbon source and which is supplemented with glycerol. In order to be active as redox sink, the 1,3-PDO pathway requires intracellular glycerol as the initial reaction is the dehydration of glycerol to 3-HPA (see Figure 4.1). Naturally, *S. cerevisiae* takes up glycerol via the native glycerol/H^+ symporter Stl1. However, Stl1 is repressed and inactivated by glucose (Lages and Lucas, 1997), thus glycerol uptake was not ensured for cells without modified glycerol uptake in the presence of glucose. In a previous study of our laboratory, the aquaglyceroporin Fps1 from *Cyberlindnera jadinii* (encoded by *CjFPS1*) was expressed in *S. cerevisiae* (Klein *et al.* 2016b). The results demonstrated that this modification improved growth on glycerol. We used a constitutive promoter active in glucose and glycerol to ensure transcription in a mixture of glucose and glycerol. To the best of our knowledge, there is no report about glucose inactivation of *CjFPS1.*

Therefore, *CjFPS1* was assumed to be active in the test conditions, and it was expressed in parallel to the genes encoding for the 1,3-PDO pathway enzymes resulting in the strain CE1.01.04.02 1,3-PDO FPS1.

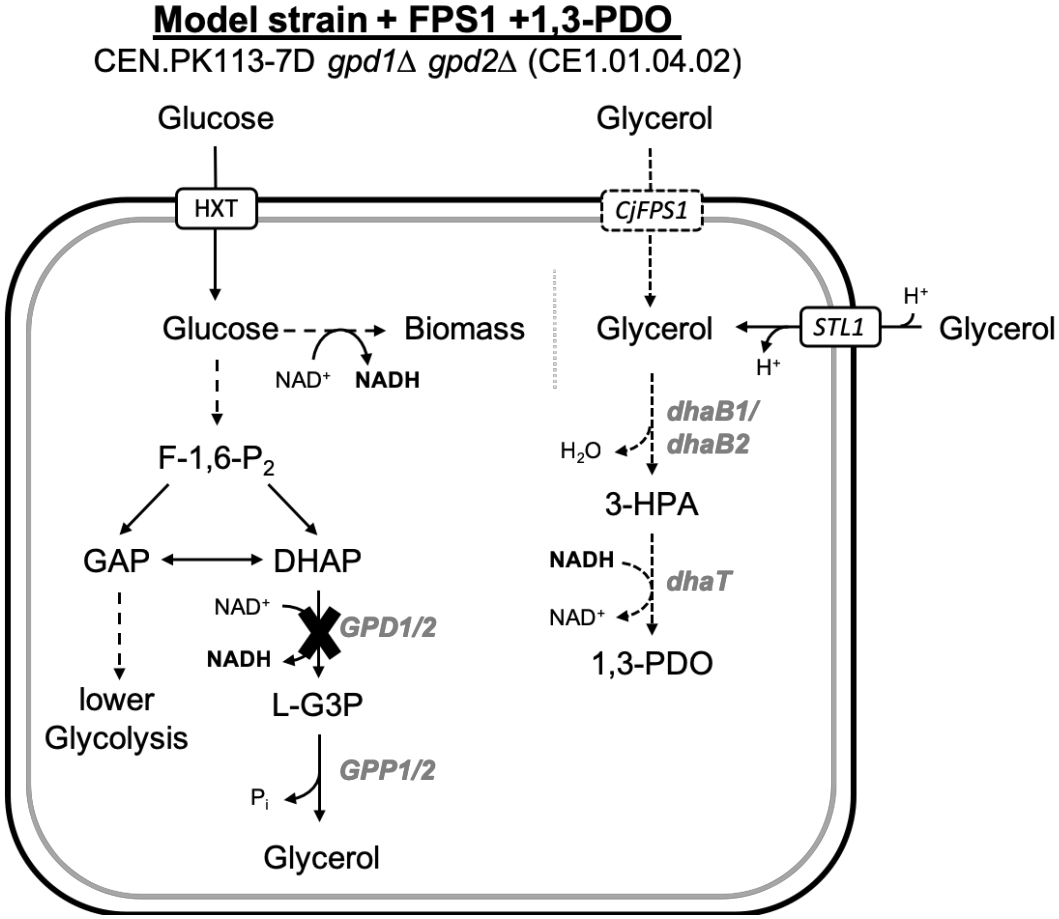


Figure 4.1: Overview of the genetic modifications of the model strain CE1.01.04.02 with expression of *CjFPS1* and the 1,3-PDO pathway from *C. butyricum* as a redox sink. As 'model strain' a *gpd1*Δ *gpd2*Δ mutant of the strain CEN.PK113-7D was selected. Abbreviations: *CjFPS1* – aquaglyceroporin from *C. jadinii; dhaB1/dhaB2/dhaT*– genes encoding for the glycerol dehydratase, its reactivator and the 1,3-PDO oxidoreductase from *C. butyricum*; F-1,6-P_2 – fructose-1,6-bisphosphate; GAP – glyceraldehyde 3-phosphate; *GPD1/GPD2* – cytosolic glycerol dehydrogenase; *GPP1/GPP2* – L-G3P phosphatase; *HXT* – hexose transporters mediating glucose uptake; *STL1* – glycerol/H^+ symporter; 1,3-PDO – 1,3-propanediol; 3-HPA – 3-hydroxypropionaldehyde. Dashed arrows indicate several reactions. Dotted arrows indicate reactions catalyzed by heterologously expressed enzymes. Black cross indicates deletion of Gpd1/2.

Codon-optimized sequences (see Appendix) of the clostridial genes, encoding the enzymes for the 1,3-PDO pathway, were used to design expression cassettes for genomic integration into the *S. cerevisiae* model strain. The sequences were placed under the control of commonly

used *S. cerevisiae* promoters and terminators. The glycerol dehydratase was placed under control of the *PGK1* promoter and the *DIT1* terminator (P_{PGK1}-*dhaB1*-T_{DIT1}). The expression cassette of the dehydratase reactivator consisted of the *ACT1* promoter and the *TPS1* terminator (P_{ACT1}-*dhaB2*-T_{TPS1}), while the expression of the 1,3-PDO dehydrogenase was regulated by the strong constitutive *TEF1* promoter and the *CYC1* terminator (P_{TEF1}-*dhaT*-T_{CYC1}). Strong expression of PDOR was assumed to be necessary in order to exclude, that insufficient expression of this enzyme leads to accumulation of the toxic intermediate 3-HPA. At the time the expression cassettes were designed, no studies showing the strength of *S. cerevisiae* promoters in the presence of glycerol were available. Therefore, the promotor P_{TEF1} known to be constitutively strong expressed (at least in glucose medium) was selected for expression of the PDOR. A recent study conducted in our lab, confirmed, that P_{TEF1} is also in glycerol containing medium among the strongest promoters (Ho *et al.* 2018).

The fully equipped model strain CE1.01.04.02 1,3-PDO FPS1 was incubated in a serial drop dilution assay on synthetic medium containing glucose plus glycerol (SMDG) or either glucose (SMD) or glycerol (SMG) (the latter two media were used as control conditions). The plates were incubated aerobically for 48 h or in anaerobic jars for either 48 h or 96 h. Derivatives of CEN.PK113 are unable to grow in synthetic glycerol medium even in the presence of oxygen (Swinnen *et al.* 2013; Swinnen *et al.* 2016; Ho *et al.* 2017). This is confirmed by the data shown for SMG medium (Figure 4.2). On SMD and SMDG all strains grew under aerobic conditions which was also in line with the expectations. Moreover, the tested strains did not show significantly different growth phenotypes in these two media in the presence of oxygen (Figure 4.2). Under anaerobic conditions, only the strains with *GPD1 GPD2* wild-type alleles, *i.e.* strains CEN.PK113-7D and CEN.PK113-7D FPS1, were able to grow on SMD and SMDG since they have an intact native redox sink for the biomass-derived NADH. In contrast, all strains with the background of CE1.01.04.02 (*gpd1*Δ *gpd2*Δ) only showed very weak growth under these conditions. Thus, the expression of the 1,3-PDO pathway genes did not result in additional growth-supporting effect.

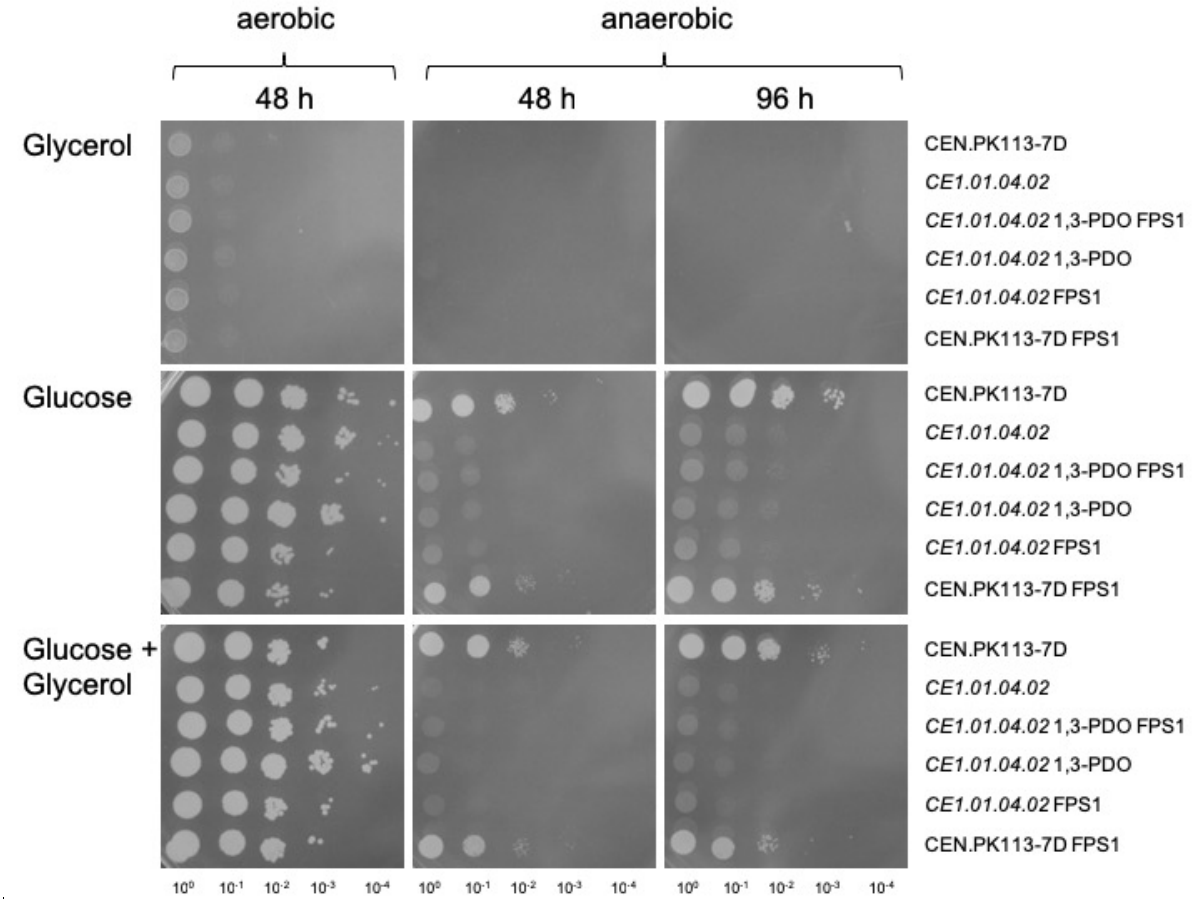


Figure 4.2: Serial drop dilution assay of the model strain CE1.01.04.02 (*gpd1*Δ *gpd2*Δ) expressing the 1,3-PDO pathway and respective control strains. 3 µl cell suspension from different serial dilutions (10^0 – 10^{-4}) were spotted onto three different solid synthetic media. The plates were incubated aerobically for 48 h or anaerobically for 48 h respectively 96 h. The figure shows one representative set out of triplicates. Abbreviations: 1,3-PDO – expression of GDHt, GDHt RA and PDOR; FPS1 – expression of the aquaglyceroporin *FPS1* from *C. jadinii*.

4.3.2. Towards the establishment of a 1,3-PDO pathway in *S. cerevisiae* strains able to utilize glycerol as carbon source

The intracellular availability of glycerol is a prerequisite for the clostridial 1,3-PDO pathway to work as a redox sink in the selected model strain used in **section 4.3.1**. The results described in this section raised doubts about the functionality of the aquaglyceroporin Fps1 under the applied conditions. Another plausible explanation for the fact that the genes expressed in the used model strain did not restore growth in the medium containing glucose and glycerol could be an accumulation of the toxic intermediate 3-HPA due to imbalanced in vivo enzyme activities. Therefore, we launched a second approach and expressed the same expression cassettes for the 1,3-pathway in *S. cerevisiae* strains which naturally take up and grow in synthetic medium with glycerol as the sole source of carbon in order to directly provide the precursor for the 1,3-formation. The original plan was to later subject the resulting strains to adaptive laboratory evolution in decreasing oxygen concentrations. For this purpose, the 1,3-PDO pathway was introduced into different strains such as the wild-type strain CBS 6412-13A (Swinnen *et al.* 2013), its derivative CBS DHA (Aßkamp *et al.* 2019), both with and without *CjFPS1*, as well as isogenic strains (the strains without *CjFPS1*) with a deletion of *NDE1*, encoding for the external NADH dehydrogenase (Figure 4.3). The latter mutants showed a significantly reduced growth rate on glycerol compared to their reference strains with the *NDE1* wild-type allele (described in **Chapter 3.3)**. We wanted to check whether an in vivo activity of the 1,3-PDO pathway can rescue the growth of the *nde1*Δ mutant, particularly the DHA pathway derivative, since the latter is assumed to have an increased rate of cytosolic NADH generation.

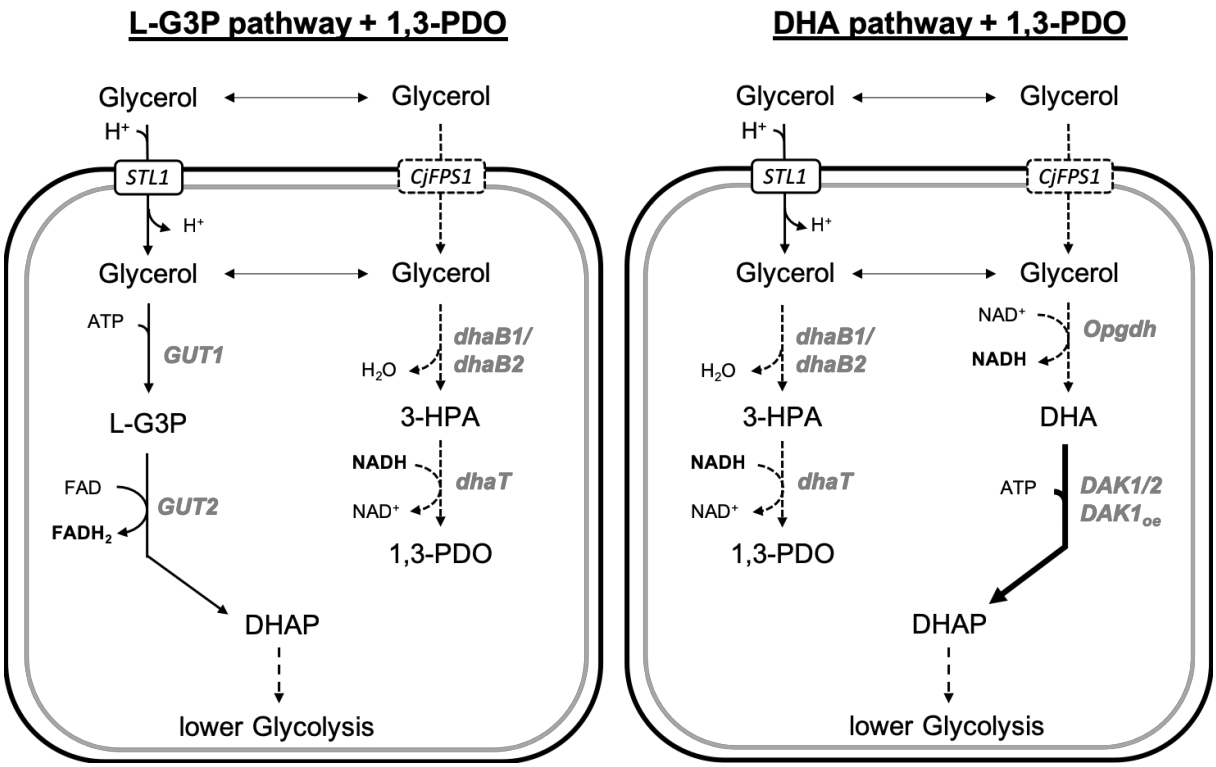


Figure 4.3: Overview of the genetic modifications of the *S. cerevisiae* strains from the background CBS 6412-13A catabolizing glycerol via the L-G3P or the DHA pathway with the 1,3-PDO pathway from *C. butyricum* expressed. 'L-G3P pathway' as well as 'DHA pathway' indicate strains with the background CBS 6412-13A with native respectively engineered glycerol catabolism. Abbreviations: *CjFPS1* – aquaglyceroporin from *C. jadinii; DAK1/DAK2 DAK1oe* – dihydroxyacetone kinase/ DAK1 overexpressed; *dhaB1/dhaB2/dhaT*– genes encoding for the glycerol dehydratase, its reactivator and the 1,3-PDO oxidoreductase from *C. butyricum*. DHA - dihydroxyacetone; DHAP – dihydroxyacetone phosphate; *GUT1* – glycerol kinase; *GUT2* – mitochondrial glycerol dehydrogenase; L-G3P – L-glycerol 3-phosphate; *Opgdh* – glycerol dehydrogenase from *O. parapolymorpha*; *STL1* – glycerol/H^+ symporter; *TPI1* – triosephosphate isomerase; 1,3-PDO – 1,3-propanediol; 3-HPA – 3-hydroxypropionaldehyde. Dashed arrows indicate several reactions. Dotted arrows indicate reactions catalyzed by heterologously expressed enzymes. Bold arrow shows overexpressed DAK.

The maximum specific growth rates (μ_{max}) of all strains expressing the 1,3-PDO pathway, either catabolizing glycerol via the L-G3P pathway (CBS 6412-13A) or the DHA pathway (CBS DHA), were analyzed in the Growth Profiler under aerobic conditions. The μ_{max} were recorded in synthetic medium with glycerol (SMG). Interestingly, the expression of the genes related to the 1,3-PDO pathway abolished growth in SMG in all strains but the *nde1*Δ mutants (Table 4.6). While the CBS *nde1*Δ mutant expressing the 1,3-PDO pathway genes reached a μ_{max} of 0.055 ± 0.033 h^{-1} (the high standard deviation resulting from high variability of growth between

several transformants, ranging from no growth at all to maximum μ_{max} of 0.065 h^{-1}), the CBS DHA *nde1*Δ mutants showed a μ_{max} of 0.032 ± 0.03 h^{-1} (transformants showed either μ_{max} of 0.032 h^{-1} or no growth).

All strains expressing the 1,3-PDO pathway were also tested on glucose and neither strain showed a growth impairment suggesting that the phenotype was caused by the presence of glycerol in the medium and or its metabolization. The strains expressing the 1,3-PDO pathway grew in SMD with a μ_{max} between 0.17 h^{-1} and 0.23 h^{-1}, which corresponded with the wild-type behavior (data not shown). The growth impairment of the strains depending on the availability of glycerol as carbon source but not in glucose containing medium supported the hypothesis, that the heterologous glycerol dehydratase could be active and that imbalanced activities of GDHt and PDOR might have caused an accumulation of the toxic intermediate 3-HPA. In order to confirm this hypothesis, the glycerol dehydratase (together with its reactivation factor) and the 1,3-PDO oxidoreductase, were expressed separately in the strain CBS DHA *nde1*Δ. This strain was first tested since it did not show a complete growth impairment after expression of the 1,3-PDO pathway among the tested CBS DHA background strains. Preliminary results show that sole expression of the PDOR recovered and even improved the strains growth in SMG in comparison to the parental strain CBS DHA (μ_{max} 0.151 ± 0.006 h^{-1}), while the strain that only expressed the genes for the first enzyme of the pathway (GDHt and the GDHt RA) did not grow at all (data not shown), supporting our hypothesis of an imbalanced pathway.

The strains which expressed the 1,3-PDO pathway and showed growth in SMG (CBS *nde1*Δ, CBS DHA *nde1*Δ, CBS DHA *nde1*Δ *dhaT*) were also tested once for 1,3-PDO formation in shake flask cultivations in synthetic glycerol medium. The procedure was similar to the approach described by Lindlbauer *et al*. (2017) who produced 3-HPA the intermediate of the 1,3-PDO pathway via a biotransformation using *Lactobacillus diolivorans*. The cells were pre-grown in synthetic glucose medium in order to produce biomass. After 72 h the cells were shifted to glycerol containing medium. However, this whole cell biotransformation of glycerol to the desired product 1,3-PDO was not successful. Neither 1,3-PDO nor 3-HPA could be

detected in none of the strains by the applied HPLC method (data not shown). Still, the fact that the growth impairment of the strains expressing the 1,3-PDO pathway is correlated to the availability of glycerol, indicates a certain activity of the heterologous pathway.

Table 4.6 Maximum specific growth rates (μ_{max}) of *S. cerevisiae* strains catabolizing glycerol via the native L-G3P pathway (CBS) or the DHA pathway (CBS DHA) with or without the expression of the 1,3-PDO pathway ('redox sink'). Mean values and standard deviations from at least three independent experiments are shown. Cells were gown in synthetic glycerol medium (SMG) (6% glycerol w/v). -±- - no growth; *FPS1* - expression of the aquaglyceroporin *FPS1* from *C. jadinii*; *nde1*Δ - deletion of NADH dehydrogenase encoded by *NDE1*; reference – background strains.

Strain		μ_{max} with 1,3- PDO pathway enzymes [h^{-1}]	μ_{max} without 1,3-PDO pathway enzymes [h^{-1}]
CBS	reference	- ± -	0.124 ± 0.011
	*nde1*Δ	0.055 ± 0.033	0.072 ± 0.010
	CjFPS1	- ± -	0.169 ± 0.003
CBS DHA	reference	- ± -	0.101 ± 0.002
	*nde1*Δ	0.032 ± 0.030	0.070 ± 0.004
	CjFPS1	0.011 ± 0.009	0.114 ± 0.012

4.4. Discussion

In **Chapter 4**, the expression of a 1,3-PDO pathway from *Clostridium butyricum* was tested as a redox sink, meant to allow (anaerobic) glycerol fermentation in future *S. cerevisiae*-based processes. The heterologous pathway was expressed in *S. cerevisiae* strains catabolizing glycerol via the L-G3P or the DHA pathway as well as in a model strain (*gpd1*Δ *gpd2*Δ mutant), which can only grow anaerobically on glucose in the presence of a functional redox sink. As glycerol is required as substrate for the 1,3-PDO pathway, the medium for the latter strain was supplemented with glycerol. In addition, this strain had to be equipped with a glycerol uptake mechanism (the aquaglyceroporin Fps1 from *C. jadinii*), as mentioned in the introduction.

The applied serial drop dilution assay did not show the assumed recovery of anoxic growth of the model *S. cerevisiae* strain CE1.01.04.02 1,3-PDO FPS1 in a medium containing glucose and glycerol (**Chapter 4.3.1**). For the highest concentrations (10^0 and 10^{-1}), however, colony spots were visible on SMD and SMDG in contrast to SMG. This displays minimum initial growth, caused by an artefact of the method. As the plates were aerobically prepared and stored, some oxygen was probably still dissolved in the medium, after they were inoculated in the anaerobic jar. This residual oxygen presumably enabled the cells to start growing catabolizing glucose for a short period.

One probable explanation, why the genetic modifications (CjFps1 plus expression of the 1,3-PDO pathway) in the model strain did not lead to the recovery of anaerobic growth on glucose and glycerol, could be that glycerol uptake via CjFps1 was inactive in the presence of glucose. Its functionality has indeed not yet been unambiguously proven in presence of both glycerol and glucose. Another possible explanation is that the activities of the GDHt and the PDOR enzymes are not balanced, leading to the accumulation of the intermediate 3-hydroxypropionaldehyde (3-HPA). This intermediate is inhibiting glycerol fermentation and is even toxic to cells (Barbirato *et al.* 1996; Talarico *et al.* 1988). Indeed, the overexpression of the *dha* operon (encoding for the enzymes catalyzing 1,3-PDO formation) in *K. pneumoniae*

led to similar negative effects on growth (Zheng *et al.* 2006). The authors postulated 3-HPA accumulation to be the reason behind this phenotype. Later, it was shown by Hao *et al*. (2008), that indeed the overexpression of the PDOR alone decreases 3-HPA levels and improves growth rates of the respective bacterial strains without further modifications.

The results obtained after expression of the 1,3-PDO pathway enzymes in the glycerol catabolizing strains CBS 6412-13A and CBS DHA corroborate the assumption of toxic 3-HPA accumulation. In fact, growth of strains expressing the respective genes was abolished when glycerol was available (in SMG and SMDG), while the μ_{max} of the respective strains remained unaffected with glucose as sole carbon source (in SMD). These results seem to indicate that at least the glycerol dehydratase, catalyzing the reaction from glycerol to 3-HPA, is active *in vivo*.

In order to further test the hypothesis of an unbalanced pathway, we separately expressed the essential genes for each of the two enzymes. The experiments were carried out in the strain CBS DHA *nde1*Δ as explained in the results section. The sole expression of the GDHt (and its reactivator encoded *dhaB2*) indeed caused growth impairment on glycerol. In contrast, sole expression of the PDOR (second enzyme of the pathway) in the same strain background had no negative effect on the μ_{max} of the strain, but even showed a higher growth rate in synthetic glycerol medium when compared to both the strain CBS DHA *nde1*Δ and its parental strain CBS DHA (compare to **Chapter 3.2**). In particular, the observed increased growth indicates activity of the PDOR. The growth-supporting effect of the PDOR expression on the strain CBS DHA *nde1*Δ suggests that the sole expression of the oxidoreductase is already sufficient as a redox sink. A remaining question is which metabolite serves as electron acceptor in a respective reaction.

To avoid an accumulation of 3-HPA, a stronger expression of *dhaT* (PDOR) and a weaker expression of *dhaB1* (GDHt) might be worth testing. This could be achieved by changing the promoters by a stronger respectively a weaker one. The originally chosen commonly used

promoters (P_{PGK1}-*dhaB1* and P_{TEF1}-*dhaT*) were originally selected to reduce the risk of 3-HPA accumulation. The promotor P_{TEF1} is commonly used for strong constitutive expression in metabolic engineering approaches using *S. cerevisiae* (Alper *et al.* 2005; Hubmann *et al.* 2014). The recent study from Ho *et al.* (2018), who selected and characterized 25 native *S. cerevisiae* promoters for their applicability as promoters in glycerol based metabolic engineering approaches, also confirmed that P_{TEF1} allows stronger expression than P_{PGK1}. However, most probably the difference in terms of strength is not big enough between those two. According to the results from Ho *et al.* (2018), promoters like P_{ENO2} or P_{ACT1} should be chosen for a weak expression on glycerol, while strong expression is enabled by P_{ALD4} or P_{ADH2}.

Further work should also include the expression of only the GDHt and GDHt RA in the strain CEN.PK113-7D FPS1. This modification should impair anaerobic growth of this strain in an experimental setup as described in **Chapter 4.3.1**. Further strains that are required to build a solid line of argumentation (that were not constructed in this study due to time constraints) are strains from the backgrounds CBS 6412-13A and CBS DHA with sole expression of GDHt and GDHt RA or PDOR. Moreover, it is definitely interesting to also solely express the PDOR in the *nde1*Δ *nde2*Δ double deletion mutants described in **Chapter 3.3**. If the PDOR is indeed able to catalyze an unspecific, but NADH-dependent reaction, those strains, suffering from redox cofactor imbalance, should be enabled to grow again on glycerol.

Notably, PDOR has high sequence identity with alcohol dehydrogenases from other bacteria (Daniel *et al.* 1995; Bairoch, 1991; Raynaud *et al*. 2003) and to a NADP-dependent broad-substrate range aldehyde reductase (encoded by *yqhD*) from *E. coli*, an organism that naturally does not produce 1,3-PDO, but 1,2-PDO (Jarboe *et al.* 2011; Sulzenbacher *et al.* 2004; Emptage *et al.* 2003).

A Nucleotide BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of PDOR from *C. butyricum* also revealed 46% identity with the alcohol dehydrogenase Adh4 of *S. cerevisiae* (data not shown). Therefore, it would be interesting to analyze the strain CBS DHA *nde1*Δ with

the PDOR expressed in shake flasks with buffered synthetic glycerol medium (similar to conditions applied in **Chapter 3.4**), with regard to ethanol formation. In case the PDOR has indeed similar activity to Adh4, an increased ethanol titer could support this hypothesis.

The fact that both clostridial enzymes GDHt and PDOR seem to show *in vivo* activity under aerobic conditions is a remarkable result. While the enzymes catalyzing 1,3-PDO formation in *K. pneumoniae* are known to be functional in the presence of oxygen (Slininger and Bothast, 1985; Zhang *et al.* 2006; Rao *et al.* 2008; Celinska *et al.* 2010; Ma *et al.* 2010), it was expected that they are highly oxygen sensitive, since they are derived from the obligate anaerobic bacterium *C. butyricum* (Raynaud *et al*. 2003). Both enzymes, GDHt and PDOR, belong to the family of glycyl radical enzymes, like the pyruvate formate lyase (Raynaud *et al.* 2003; Selmer *et al.* 2005; Zhang *et al*. 2001), which are inactivated by molecular oxygen (Sawers and Watson 1998; Buckel and Golding, 1999; Frey, 2001). To the best of my knowledge, no reports about GDHt and PDOR from *C. butyricum* (putatively) active under aerobic conditions are available. Further experiments should therefore also include measurement of the enzyme activities, e.g. as comprehensively described by Ahrens *et al.* (1998).

The strain background might have also influenced the results shown in **Chapter 4.3.1 and 4.3.2**. There are obviously differences between the two strain backgrounds CEN.PK and CBS 6412-13A with regard to the native ability to consume glycerol and also further yet unidentified genomic differences (Swinnen *et al*. 2016). Furthermore, the CBS strains engineered in **Chapter 4.3.2** catabolized glycerol also via the DHA-pathway. Therefore, expression of the 1,3-PDO pathway in CEN.PK strains as constructed by Klein *et al*. (2016a) or Ho *et al.* (2018) and simultaneous deletion of *GPD1* and *GPD2* should be tested for anoxic glycerol fermentation. In addition, the strains constructed in **Chapter 4.3.2** display a promising baseline for an adaptive laboratory evolution (ALE) under microaerobic conditions. An ALE experiment could first of all lead to a decrease of GDHt activity, resulting in a more balanced 1,3-PDO pathway and secondly anoxic fermentation of glycerol seems to be achievable.

Another redox sink, *i.e*. alternative to the 1,3-PDO pathway, could be the 1,2-PDO pathway as described in **Chapter 3.3.** 1,2-PDO formation origins from DHAP, which means that this pathway requires one molecule of ATP for the conversion of glycerol to DHAP. The 1,2-PDO pathway requires two molecules of NAD(P)H and could therefore also fulfill the requirements of a redox sink for surplus NADH under anoxic conditions, generated during biomass formation. Even more interesting than expressing the entire pathway, could be the sole expression of the glycerol dehydrogenase (compare to **Chapter 3.3**) encoded by *EcgldA.* It was shown, that the enzyme has a wide substrate spectrum, including glycerol and methylglyoxal (MG) (St. Martin *et al.* 1977; Tang *et al.* 1979; Truniger and Boos, 1994). It is known, that MG is naturally formed through the spontaneous non-enzymatic breakdown of triose phosphates (such as DHAP or GAP) (Richard, 1993; Martins *et al.* 2001). As MG only accounts for up to 0.3 % of the total glycolytic flux (Penninckxs *et al*. 1983), its conversion to 1,2-PDO by *EcgldA* could hypothetically be just sufficient enough to oxidize the surplus NADH formed during biomass formation under anoxic conditions.

4.5. Appendix

Sequence of glycerol dehydratase (encoded by *dhaB1)* from *Clostridium butyricum* 2CR371.5 - codon optimized for *S. cerevisiae.*

ATGATCTCCAAGGGTTTCTCTACTCAAACCGAAAGAATCAACATTTTGAAGGCCCAAATT
TTGAACGCTAAGCCATGTGTTGAATCCGAAAGAGCTATTTTGATCACCGAATCTTTCAAG
CAAACTGAAGGTCAACCAGCCATTTTGAGAAGAGCTTTGGCTTTGAAACACATCTTGGA
AAACATTCCAATCACCATCAGAGATCAAGAATTGATCGTTGGTTCCTTGACCAAAGAACC
TAGATCTTCTCAAGTTTTCCCAGAATTCTCTAACAAGTGGTTGCAAGATGAATTGGACAG
ATTGAACAAGAGAACTGGTGATGCCTTCCAAATCTCCGAAGAATCTAAAGAAAAGTTGAA
GGACGTTTTCGAATACTGGAACGGTAAGACTACTTCTGAATTGGCTACTTCTTACATGAC
CGAAGAAACTAGAGAAGCCGTTAACTGTGATGTTTTCACTGTTGGTAACTACTACTACAA
CGGTGTTGGTCATGTTTCTGTTGATTACGGTAAGGTTTTGAGAGTTGGTTTCAACGGTAT
TATCAACGAAGCCAAAGAACAATTGGAAAAGAACAGATCCATCGACCCAGACTTCATCA
AGAAAGAAAAATTCTTGAACTCCGTCATCATCTCTTGCGAAGCTGCTATTACTTACGTTA
ACAGATACGCTAAGAAGGCCAAAGAAATTGCTGATAACACTTCCGATGCTAAGAGAAAG
GCTGAATTGAACGAAATTGCCAAGATCTGCTCTAAGGTTTCTGGTGAAGGTGCTAAGTC
TTTTTACGAAGCTTGTCAATTATTCTGGTTCATCCATGCCATCATCAACATCGAATCTAAC
GGTCATTCTATTTCCCCAGCCAGATTCGATCAATATATGTACCCATATTACGAAAACGAC
AAGAACATCACCGATAAGTTTGCCCAAGAATTGATTGATTGCATCTGGATCAAGTTGAAC
GACATCAACAAGGTTAGAGACGAAATTTCTACTAAGCACTTTGGTGGTTACCCAATGTAC
CAAAATTTGATTGTCGGTGGTCAAAACTCCGAAGGTAAAGATGCTACAAACAAGGTTTC
CTACATGGCTTTGGAAGCTGCTGTTCATGTTAAGTTGCCACAACCATCTTTGTCCGTTAG
AATTTGGAACAAGACTCCAGACGAATTCTTGTTGAGAGCTGCAGAATTGACCAGAGAAG
GTTTGGGTTTGCCAGCTTATTACAACGATGAAGTTATTATCCCAGCCTTGGTTTCTAGAG
GTTTGACTTTAGAAGATGCCAGAGACTATGGTATTATCGGTTGTGTTGAACCACAAAAGC
CAGGTAAGACTGAAGGTTGGCATGATTCTGCTTTTTTCAACTTGGCTAGAATCGTCGAAT
TGACTATCAACTCTGGTTTCGATAAGAACAAGCAAATTGGTCCAAAGACCCAAAACTTCG
AAGAAATGAAGTCCTTCGACGAATTCATGAAGGCTTACAAAGCTCAAATGGAATACTTCG
TCAAACATATGTGTTGCGCCGATAACTGCATTGATATTGCTCATGCTGAAAGAGCACCAT
TGCCATTTTTATCCTCTATGGTTGATAACTGTATCGGTAAGGGTAAATCATTGCAAGACG
GTGGTGCTGAATACAATTTTTCTGGTCCTCAAGGTGTTGGTGTTGCTAATATTGGTGATT
CTTTAGTTGCCGTCAAAAAGATCGTCTTTGACGAAAACAAGATCACCCCATCCGAATTGA
AGAAAACCTTGAACAACGACTTCAAGAACTCTGAAGAAATCCAAGCCTTGTTGAAGAAT
GCTCCAAAGTTCGGTAACGATATCGATGAAGTAGATAACTTGGCAAGAGAAGGTGCTTT
GGTTTACTGTAGAGAAGTTAACAAGTACACCAATCCAAGAGGTGGTAATTTTCAACCAG
GTTTGTATCCATCCTCCATCAATGTTTACTTCGGTTCTTTGACTGGTGCTACTCCAGATG
GTAGAAAATCTGGTCAACCATTGGCTGATGGTGTTTCTCCATCAAGAGGTTGTGATGTTA
GTGGTCCAACTGCTGCTTGTAATTCTGTTTCTAAGTTGGATCACTTCATTGCCTCTAACG
GTACTTTGTTTAATCAAAAGTTCCACCCATCTGCCTTGAAGGGTGATAATGGTTTGATGA
ACTTGTCCTCCTTGATCAGATCTTACTTCGATCAAAAGGGTTTCCACGTACAATTCAACG
TTATCGACAAGAAGATTTTGTTGGCTGCTCAAAAGAACCCAGAAAAGTACCAAGACTTGA
TAGTTAGAGTTGCTGGTTACTCCGCTCAATTCATTTCATTGGATAAGTCCATCCAAAACG
ACATTATTGCCAGAACCGAACACGTTATGTGA

Sequence of glycerol dehydratase reactivation factor (encoded by *dhaB2*) from *C. butyricum* 2CR371.5 - codon optimized for *S. cerevisiae.*

ATGTCCAAAGAAATCAAGGGTGTCTTGTTCAATATCCAAAAGTTCTCCTTGCATGATGGT
CCAGGTATTAGAACTATCGTTTTCTTCAAGGGTTGCTCCATGTCTTGTTTGTGGTGTTCT
AATCCAGAATCCCAAGATATCAAGCCACAAGTCATGTTCAACAAGAACTTGTGTACTAAG
TGTGGTAGATGCAAGTCCCAATGTAAATCTGCTGCTATCGATATGAACTCCGAATACAG
AATCGATAAGTCTAAGTGTACCGAATGTACCAAGTGTGTTGATAACTGTTTGTCTGGTGC
CTTGGTTATTGAAGGTAGAAACTACTCTGTTGAAGATGTCATCAAAGAATTGAAGAAGGA
CTCCGTTCAATATAGAAGATCCAACGGTGGTATTACTTTGTCAGGTGGTGAAGTTTTGTT
GCAACCAGATTTCGCTGTTGAATTATTGAAAGAATGCAAGTCTTACGGTTGGCATACTGC
TATTGAAACTGCTATGTACGTCAACTCTGAATCCGTTAAGAAGGTTATCCCTTACATTGA
TTTGGCCATGATCGACATCAAGTCTATGAACGACGAAATCCATAGAAAGTTCACCGGTG
TTTCCAACGAAATCATCTTGCAAAACATCAAGTTGTCCGACGAATTGGCTAAAGAAATTA
TCATCAGAATTCCAGTCATCGAAGGTTTCAATGCTGACTTGCAATCTATTGGTGCTATTG
CCCAATTCTCTAAGTCTTTGACTAACTTGAAGAGAATCGACTTGTTGCCATACCATAACT
ACGGTGAAAACAAGTACCAAGCCATCGGTAGAGAATACTCATTGAAAGAATTGAAATCC
CCATCCAAGGACAAGATGGAAAGATTGAAAGCCTTGGTTGAAATCATGGGTATTCCATG
TACAATTGGTGCCGAATGA

Sequence of 1,3-propanediol oxidoreductase (encoded by *dhaT*) from *C. butyricum* 2CR371.5 - codon optimized for *S. cerevisiae.*

ATGAGAATGTACGATTACTTGGTCCCATCCGTTAATTTCATGGGTGCTAATTCTGTTTCC
GTTGTTGGTGAAAGATGCAAGATTTTGGGTGGTAAAAAGGCTTTGATCGTTACCGATAA
GTTCTTGAAGGATATGGAAGGTGGTGCTGTTGAATTGACTGTTAAGTACTTGAAAGAAG
CCGGTTTGGACGTTGTTTATTACGATGGTGTTGAACCTAACCCAAAGGATGTTAACGTTA
TCGAAGGTTTGAAGATTTTCAAAGAAGAAAACTGCGACATGATCGTCACTGTTGGTGGT
GGTTCTTCTCATGATTGTGGTAAAGGTATTGGTATTGCTGCTACTCATGAAGGTGACTTG
TATGATTATGCTGGTATCGAAACTTTGGTCAATCCATTGCCACCAATAGTTGCTGTTAAC
ACTACTGCTGGTACTGCTTCTGAATTGACAAGACATTGTGTTTTGACCAACACCAAGAAG
AAGATCAAGTTCGTTATCGTCAGTTGGAGAAACTTGCCATTGGTTTCTATTAACGACCCA
ATGTTGATGGTTAAGAAACCAGCAGGTTTGACTGCTGCTACAGGTATGGATGCTTTGAC
TCATGCTATTGAAGCTTACGTTTCTAAGGATGCTAACCCAGTTACTGATGCTTCTGCTAT
TCAAGCCATTAAGTTGATCTCCCAAAACTTGAGACAAGCTGTTGCTTTGGGTGAAAATTT
GGAAGCTAGAGAAAACATGGCTTACGCATCTTTGTTGGCTGGTATGGCTTTTAACAATG
CTAACTTGGGTTATGTCCATGCTATGGCTCATCAATTGGGTGGTTTATATGATATGCCAC
ATGGTGTTGCTAACGCTATGTTGTTGCCACATGTTGAAAGATACAATATGTTGTCCAACC
CAAAGAAGTTCGCTGATATTGCTGAATTCATGGGTGAAAACATCTCCGGTTTGTCTGTTA
TGGAAGCTGCTGAAAAAGCTATTAACGCCATGTTCAGATTGTCCGAAGATGTTGGTATT
CCAAAGTCTTTGAAAGAAATGGGTGTCAAGCAAGAAGATTTCGAACATATGGCTGAATT
GGCCTTGTTGGATGGTAATGCATTTTCTAATCCAAGAAAGGGTAACGCCAAGGACATCA
TTAACATTTTTAAGGCTGCCTACTAA

CHAPTER 5: DISCUSSION AND OUTLOOK

5.1. Mechanisms important for oxidation of cytosolic NADH in the wild-type *S. cerevisiae* strain CBS 6412-13A growing on glycerol

Maintenance of a balanced cellular NAD^+/NADH ratio is a prerequisite for growth in all organisms (Villadsen *et al.* 2011). In eukaryotic cells, the cellular pool of the redox cofactors NAD^+/NADH is divided into two: a mitochondrial and a cytosolic pool. As described in the introduction, the reason for this separation is, that the inner mitochondrial membrane is impermeable for NADH. This entails, that mechanisms have been evolved, that allow electrons bound to cytosolic NADH, to be translocated indirectly into the mitochondria, where they can be transferred to oxygen via complex I of the respiratory chain.

The current study is the first focusing on cytosolic redox balance of a *S. cerevisiae* wild-type strain able to grow in synthetic glycerol media. Previous studies regarding glycerol growth, were mostly conducted in complex media and with strains, that do not grow in synthetic media at all but require supplements such as amino acids and nucleic bases (Barnett *et al.* 2000; Juszczyk and Rmyowicz, 2009; Merico *et al.* 2011; Swinnen *et al.* 2013).

In the **Chapter 1.2.2**, two mechanisms transferring electrons from cytosolic NADH to the respiratory chain in *S. cerevisiae* were described: the external mitochondrial NADH dehydrogenases Nde1 and Nde2, and the L-G3P shuttle, consisting of the activity of Gpd1/Gpd2 and Gut2. In **Chapter 3.3**, the contribution of these mechanisms in maintaining cytosolic redox balance with glycerol as sole carbon source was examined in the *S. cerevisiae* segregant CBS 6412-13A, which naturally grows on glycerol. The growth performance of mutant strains with deletions of crucial genes involved in the respective mechanism was tested in synthetic glycerol medium. The abolishment of key enzyme activities involved in the L-G3P shuttle (*i.e.* cytosolic glycerol 3-phosphate dehydrogenase encoded by *GPD1/2* or mitochondrial glycerol 3-phosphate dehydrogenase encoded by *GUT2*) had no significant effect on the maximum specific growth rate (μ_{max}) in synthetic glycerol medium. In this regard, the data obtained in this thesis match the effects of the respective gene deletions on growth

rates observed on other carbon sources (e.g. limited glucose, galactose or ethanol) (Larsson *et al.* 1998; Overkamp *et al.* 2000). Similarly, the effects of *NDE1* deletion (reduced growth) or *NDE2* deletion (no effect on μ_{max}) were comparable to previously published results obtained with respective mutants on the other carbon sources. However, the results obtained in the current study also revealed that a double deletion of both *NDE1* and *NDE2* completely abolished growth in the background of the tested strains under the applied conditions. This is in contrast to observations made on other carbon sources, where the growth of *nde1*Δ *nde2*Δ mutant was reduced but not abolished. The result of the current study that the double deletion mutant was unable to grow on glycerol suggests a superordinate role of the external NADH dehydrogenases for maintenance of cytosolic redox balance particularly during glycerol metabolism. Interestingly, some transcriptome and proteome studies are available for *S. cerevisiae* cells growing with glycerol as the sole carbon source in complex media. These studies include data regarding the external NADH dehydrogenase and the L-G3P shuttle (Ho et al. 2018; Ohlmeier *et al.* 2004; Roberts and Hudson, 2006). The authors showed, that transcription levels of *NDE1* and *NDE2* were significantly upregulated during growth in glycerol compared to excess glucose as carbon source. In contrast, the transcript levels of the genes *GPD1* and *GPD2* were not affected or even slightly lower with glycerol as substrate, while transcript levels of *GUT2* were higher in glycerol medium compared to glucose medium (*GUT2* expression is repressed by glucose) (Roberts and Hudson, 2006; Valadi et al. 2004). The upregulation of the *NDE1/2* expression in glycerol could support the idea of a superordinate role of the external NADH dehydrogenases over the L-G3P shuttle (and other potentially acting mechanisms) for maintenance of cytosolic redox balance during glycerol metabolism.

5.2. Ethanol formation in CBS 6412-13A derivatives catabolizing glycerol via the NAD-dependent heterologous DHA pathway

Alcoholic fermentation is well known to be the native mechanism of *S. cerevisiae* growing with glucose even under aerobic conditions in order to oxidize cytosolic NADH. However, wild-type *S. cerevisiae* cells do not produce any ethanol from glycerol. This has been shown in **Chapter 3.4** and the result matches the observations made in several previous studies (Klein *et al*. 2016; Ochoa-Estopier *et al.* 2011; Swinnen *et al.* 2013).

Taking solely the stoichiometry of the breakdown of two molecules of glycerol (2 x C3) via the L-G3P pathway into account, alcoholic fermentation from glycerol is as redox neutral as it is from glucose (1 x C6). In excess glucose, the two molecules of NADH formed during the oxidation of GAP to 1,3-bisphosphoglycerate (per 2 x C3 molecules) are mainly re-oxidized via ethanol formation even under aerobic conditions. The question emerges why alcoholic fermentation does not occur on glycerol as well. One explanation might be, that the genes encoding for enzymes involved in the formation of ethanol, such as the cytosolic alcohol dehydrogenase Adh1 or the pyruvate decarboxylases (Pdc1/5/6), are not strongly expressed on non-fermentable carbon sources in comparison to glucose grown cells (Roberts and Hudson, 2006). Thus, it seems that the flux from pyruvate goes to the TCA cycle and oxidative phosphorylation, rather than to ethanol formation. In fact, enzymes involved in respiration show higher expression levels on non-fermentable carbon sources compared to excess glucose as comprehensively reviewed by Xiberras *et al.* (2019). At the same time, enzymes catalyzing the oxidation of ethanol are upregulated (e.g. Adh2) (Daran-Lapujade *et al*. 2004; Ho *et al*. 2018; Ohlmeier *et al.* 2004). Considering all this, one can conclude that the metabolism of *S. cerevisiae* with glycerol as sole carbon source is naturally programmed for consumption rather than production of ethanol.

A major reason for ethanol formation from excess glucose seems to be an overflow of cytosolic NADH (Vemuri *et al.* 2007). With glycerol as a carbon source, this overflow might be less strong since the enzymes responsible for the oxidation of cytosolic NADH via respiration, such as Nde1/Nde2 are upregulated on glycerol (Ho *et al*. 2018; Roberts and Hudson, 2006). Indeed, in wild-type cells no ethanol formation was detectable. However, the formation of ethanol, shown in the current study in engineered *S. cerevisiae* strains, which display the DHA pathway, suggest that alcoholic fermentation on glycerol is also an overflow mechanism in response to extra cytosolic NADH. In the DHA pathway, the electrons derived from glycerol are transferred to free cytosolic redox cofactors (NAD^+) and not to FAD, covalently bound to Gut2 (see **Chapter 1.2.1**), which results in the reduction of one additional molecule of cytosolic NAD^+ per molecule of glycerol consumed. This leads to the total formation of 2 mol cytosolic NADH per mol glycerol consumed (from glycerol to pyruvate), while the wild-type only produces 1 mol cytosolic NADH per mol glycerol consumed. It seems that this excess of cytosolic NADH in the strain CBS DHA, triggers an overflow reaction from pyruvate towards ethanol formation, to regenerate cytosolic NAD^+. The additional expression of a heterologous aquaglyceroporin (CjFps1) in the strain CBS DHA resulted in even higher ethanol titers (**Chapter 3.4**). Improved glycerol uptake seems to increase the flux through the DHA pathway (or it at least increases the flux via the glycerol dehydrogenase). This leads to a significantly higher rate of cytosolic NADH generation which can explain the elevated ethanol titers caused by the Fps1 expression.

To answer the question whether the expression of CjFps1 can also lead to an onset of alcoholic fermentation in the wild-type background by increasing the rate of cytosolic NADH generation, the respective control experiment was conducted in **Chapter 3.4**. While the growth rate was significantly increased (from about 0.12 h^{-1} to 0.18 h^{-1}), no ethanol at all was detected. Obviously, the increased glycerol uptake, increases the glycolytic flux, indicated by the increased growth, but does not increase the rate of cytosolic NADH generation to an extent, which triggers overflow towards alcoholic fermentation.

The fact that the DHA pathway can cause ethanol formation from the 'non-fermentable' carbon source glycerol, raises the question whether the NADH overflow alone can cause ethanol formation. Hou *et al*. (2010) showed that overexpression of the native formate dehydrogenase (FDH) and co-feeding of formate, leads to a significant increase of ethanol in glucose-grown cells due to the additional cytosolic NADH production. The FDH catalyzes the NAD-dependent oxidation of formate to CO_2. The authors showed, that even at low dilution rates in glucose-limited cultures, where normally no ethanol is formed, overexpression of FDH caused alcoholic fermentation (Hou *et al*. 2010; Zhang *et al*. 2009). It would therefore be interesting to test whether the overexpression of FDH plus co-feeding of formate can also induce alcoholic fermentation in the strain CBS 6412-13A during growth on the 'non-fermentable' carbon source glycerol.

Another possibility to check whether the high production of cytosolic NADH in CBS DHA is indeed the trigger for ethanol formation would be the expression of an NADH oxidase (Nox) (e.g. from *Lactococcus lactis*) in the DHA pathway strains. Nox directly oxidizes NADH to NAD^+ and transfers the electrons to molecular oxygen, forming H_2O. Accordingly, the expression of Nox should result in a reduction of NADH and thus reduction of overflow metabolism similar to studies conducted on glucose (Hou *et al.* 2014; Kim *et al*. 2016; Vemuri *et al*. 2007).

In **Chapter 1.2.2**, the ethanol-acetaldehyde shuttle was mentioned as a redox mechanism. The functionality of this shuttle bases on the ability of ethanol (formed from acetaldehyde and cytosolic NADH) to diffuse into the mitochondria, where it can be oxidized back to acetaldehyde by the NAD-dependent alcohol dehydrogenase encoded by *ADH3.* The generated mitochondrial NADH could subsequently be oxidized by the internal mitochondrial NADH dehydrogenase, encoded by *NDI1,* which transfers electrons directly to the respiratory chain (Bakker *et al.* 2001, 2000). The resulting molecule of acetaldehyde can diffuse back to the cytosol and become reduced again. In glucose-limited chemostat cultures it was shown that this shuttle contributes to respiratory growth in mutants lacking the mitochondrial NADH dehydrogenase (encoded by *NDI1*) (Bakker *et al*. 2000). The formation of ethanol in the strain

CBS DHA could theoretically allow a contribution of this shuttle to cytosolic redox balance. Any contribution of the ethanol-acetaldehyde shuttle could be tested by recording the μ_{max} of *nde1*Δ *adh3*Δ respectively *nde1*Δ *ndi1*Δ mutants of the strain CBS DHA (provided that *adh3*Δ and *ndi1*Δ single mutants grow with glycerol). Although, we cannot exclude a contribution of the ethanol-acetaldehyde shuttle until the respective experiments are conducted, it was already shown that it could not compensate for the lack of the NADH dehydrogenase activity **(Chapter 3.3**).

5.3. Influence of the cultivation conditions and the strain background on ethanol formation from glycerol

Glycerol is generally considered a non-fermentable carbon source for *S. cerevisiae* (Schüller, 2003; Turcotte *et al*. 2010). The term 'non-fermentable' refers to the fact that the native glycerol catabolic pathway is dependent on a functional respiratory chain (Hampsey, 1997), as well as the fact, that no fermentation products have been reported so far from wild-type strains catabolizing glycerol in synthetic medium (see **Chapter 1.2**). The results displayed in **Chapter 3.4** confirmed this observation for the wild-type *S. cerevisiae* strain CBS 6412-13A, which catabolizes glycerol via the L-G3P pathway. The study revealed, that the establishment of the DHA pathway was the prerequisite for alcoholic fermentation and that improved uptake due to the expression of the aquaglyceroporin CjFps1 accelerated it. However, the ethanol titers detected in the cultivations with these strains were also significantly influenced by the cultivation conditions which will be discussed in the following.

As it was best visible for the strain CBS DHA FPS1, two different external factors were shown in the current study to increase alcoholic fermentation of DHA pathway strains: i) the application of buffered synthetic glycerol medium and ii) limited oxygen availability. When the synthetic medium was buffered by adding 100 mM potassium dihydrogen phosphate (to avoid strong medium acidification) and the pH adjusted to 5 (SMG_{buff}), the strain CBS DHA FPS1

produced up to 8.5 g L^{-1} ethanol as shown in **Chapter 3.3**. In contrast, almost no ethanol was detectable when growing the same strain in synthetic medium with a pH of 4 (SMG) (data not shown). A plausible reason for this could be the poor glycerol consumption (<10 g L after 120 h) and growth performance (maximum OD was ca. 8 after 120 h) in the unbuffered medium SMG (data not shown) in comparison to SMG_{buff}, where much higher optical densities were achieved (OD ~25) and almost 50 g L^{-1} glycerol were consumed in the same period of time. If solely the stoichiometry is considered, this would allow for an about 5-fold higher concentration of the fermentation product ethanol. Still, it cannot be excluded that a reduced oxygen availability caused by the higher cell concentration might have contributed to the significant difference in ethanol production between buffered and unbuffered medium.

The absence of a buffering compound was most probably the reason why no ethanol was detected in the experiments with DHA pathway strains described in **Chapter 3.3** as well as in the previous study conducted by Klein *et al.* (2016). In fact, the respective experiments were all conducted in unbuffered SMG and early cessation of biomass formation did not allow fast and complete glycerol consumption accompanied by the lack of ethanol formation. In addition, it might be that Klein *et al.* (2016) just missed the perfect time point at which ethanol formation was visible or the low amount of ethanol formed, evaporated during the sample preparation for HPLC analysis. The influence of buffering the medium on fermentation of glycerol was also shown in another study conducted in our lab even though it aimed at the production of 1,2-PDO rather than ethanol (Islam *et al.* 2017). In this study, no fermentation products were detected in SMG, while 1,2-PDO (up to 3.5 g L^{-1}) as well as ethanol (around 8 g L^{-1}) were detectable in SMG_{buff}. The study of Islam *et al.* (2017) tested a strain that derived from the same strain background (CBS 6412-13A) as the strains used in the current study. Moreover, the strains carried the same genetic modifications for establishing the DHA pathway and expressed also the aquaglyceroporin CjFps1. The only difference was the presence of an additional heterologous pathway for 1,2-PDO in the study of Islam *et al.* (2017). Notably, the strain tested by Islam *et al.* (2017) produced around 8 g L^{-1} ethanol beside the 1,2-PDO. This is almost the

same ethanol concentration as the one detected in CBS DHA FPS1 in the current study (see **Chapter 3.4**). This indicates that the ethanol in the study of Islam *et al.* (2017) was mainly caused by the glycerol catabolic pathway replacement together with expression of CjFps1 and not due to moonlight activity of the established enzyme activities involved in the 1,2-PDO pathway.

The second external factor to support alcoholic fermentation in the strains CBS DHA and CBS DHA FPS1 found in this study was the oxygen availability **(Chapter 3.4)**. The increase of the volume to surface ratio by increasing the culture volume in 500 mL shake flasks, resulted in higher maximum ethanol titer detected in culture supernatants. This indicates, that the native respiratory mechanisms oxidizing cytosolic NADH are not sufficient enough under these conditions to maintain redox balance, which increases the metabolic overflow into alcoholic fermentation.

Ethanol is very volatile and evaporates easily (approx. 0.2 g L^{-1} h^{-1} in 50 mL in a 500 mL shake flask). In fact, all ethanol titers recorded in **Chapter 3.4** have certainly been higher in reality since evaporation has resulted in the detection of reduced titers. Therefore, the maximum yield of 0.344 g g^{-1} is probably also underestimated and the real yield is already much closer to the maximum theoretical yield of 0.5 g g^{-1}. This might even open the opportunity to think about the application of glycerol as (co)substrate for the production of ethanol.

Recent unpublished results of our group suggest that the genetic background of the strain is another important factor when considering alcoholic fermentation from glycerol. A strain constructed by Ho *et al.* (2018) catabolizing glycerol via an improved DHA pathway and being a derivative form the strain CEN.PK113-1A, produced only 2.5 g L^{-1} ethanol in SMG_{buff}, while an appropriately engineered strain with the CBS 6412-13A background formed around 10 g L^{-1} (data not shown). It seems that the two background strains generally show significant differences in carbon catabolism. The strain CEN.PK113-1A seems to be more efficient in

oxidizing cytosolic NADH via respiratory mechanisms and does not require NADH oxidation via ethanol formation to the same extent as CBS 6412-13A. This difference seems to allow the CEN.PK derivatives to sustain better biomass formation. Throughout the course of this thesis, engineered CEN.PK113-1A strains reached always higher optical densities compared to similarly engineered CBS 6412-13A derivatives, while the latter showed higher ethanol titers (data not shown). Interestingly, Klein *et al.* (2016) also found that a CEN.PK113-1A derivative with a functional DHA pathway reached a μ_{max} that was more than two times higher than that of an analogously engineered CBS 6412-13A strain (0.26 h^{-1} vs. 0.12 h^{-1}).

An indirect indication on differences with regard to alcoholic fermentation between the strains CEN.PK113-1A and CBS 6412-13A could theoretically be displayed by the strains critical dilution rate (μ_{crit}) on glucose. The μ_{crit} is the growth rate after which *S. cerevisiae* starts aerobic fermentation under controlled conditions. For CEN.PK113-7D (a close relative of CEN.PK113-1A) μ_{crit} was measured to be 0.41 h^{-1} (van Hoek *et al.* 2000), which is slightly higher compared to other *S. cerevisiae* strains (van Dijken *et al.* 2000). Unfortunately, for CBS 6412-13A μ_{crit} was not determined so far. However, the parent strain CBS 6412 was isolated from a sake brewery (CBS-KNAW Fungal Biodiversity Centre, The Netherlands). Therefore, the assumption is likely that this strain has been selected for its high ethanol production.

5.4. Limiting respiratory capacity: *nde1*Δ vs limited O_2

In **Chapter 3.3** and **3.4** it was demonstrated, that exclusively respiratory mechanisms are involved in the maintenance of cytosolic redox balance (NAD^+/NADH) when the wild-type *S. cerevisiae* derivative CBS 6412-13A grows in synthetic glycerol medium. On the opposite, strains expressing the DHA pathway instead of the native L-G3P pathway oxidize cytosolic NADH via respiratory mechanisms and also via alcoholic fermentation.

In **Chapter 3.4,** the respiratory capacity of wild-type and DHA pathway strains was further limited by restricting oxygen (increasing the culturing volume), which enhanced alcoholic fermentation from glycerol in DHA pathway strains, but not in the wild-type. In **Chapter 3.3,** a limited respiratory capacity was simulated by metabolic engineering *i.e.* by deleting *NDE1* (and *NDE2*). The respective deletion mutants showed reduced μ_{max} (abolishment of the activity of Nde1) or no growth (abolishment of the activities of Nde1 and Nde2) independently of the glycerol catabolic pathway used. Surprisingly, the lack of these enzymes, oxidizing cytosolic NADH, did not increase ethanol formation in CBS DHA derivatives grown in a volume of 50 mL SMG_{buff} in 500 mL shake flasks (data not shown). Instead, deletion of *NDE1* significantly reduced the detectable ethanol in the CBS DHA derivatives (data not shown). The question arises, why the deletion mutants cannot re-oxidize the surplus NADH via (increased) ethanol formation.

First, it could be, that the deletion of *NDE1/2* caused unidentified pleiotropic effects. It was shown by other authors, that Nde1, Nde2, Gut2, Dld1 (D-Lactate dehydrogenase), and Cyb2 (L-lactate cytochrome-c oxidoreductase) are associated in a supramolecular complex in the inner mitochondrial membrane (Grandier-Vazeille *et al.* 2001). Deletions of one or two enzymes in this complex could have resulted in rearrangements in the inner mitochondrial membrane, which caused further regulatory or deleterious effects. However, it should be noted that all *nde1*Δ or *nde1*Δ *nde2*Δ mutants generated in the current study did not show impaired growth in synthetic medium with glucose excess, which is in accordance to previously reported

results testing the respective mutants in other strain backgrounds (Luttik *et al.* 1998). As growth of *S. cerevisiae* with excess glucose is fermentative, respiration does not play a role and pleiotropic effects might only become visible on glycerol as the sole carbon source where the external NADH dehydrogenases seem to play a major role for maintenance of cytosolic redox balance.

Secondly, it might be that the cells are indeed highly dependent on the functional external mitochondrial NADH dehydrogenases when growing on glycerol and their abolishment is too restrictive. Although the cytosolic NADH in the strains expressing the DHA pathway can partly be oxidized by ethanol formation, the direct transfer of a number of electrons to the respiratory chain might be necessary to sustain sufficient ATP formation via oxidative phosphorylation. In line with this, one would expect that further oxygen restriction would also abolish growth even with functional NADH dehydrogenases in the DHA pathway strains. This implies that anoxic glycerol consumption might not be possible. Interestingly it was shown by Kwast *et al.* (2002), that especially transcription of *NDE1* is among the highest upregulated ones upon oxygen depletion (in galactose medium). It would therefore be interesting to perform a transcriptome study under aerobic and microaerobic (oxygen-limited) conditions and to analyze whether major transcriptomic changes are also induced in synthetic glycerol medium by the limitation of oxygen. It is well known from glucose-grown cells that *S. cerevisiae* can sense the oxygen availability and responds to lower oxygen levels with metabolic adaptations (Kwast *et al.* 1998; Zitomer and Lowry, 1992). Limited respiratory capacity has been shown to trigger redistribution of carbon fluxes through respiratory and fermentative pathways with glucose as carbon source (da Costa *et al.* 2018; Frick and Wittmann, 2005; Jouhten *et al.* 2008). For example, the carbon fluxes in the TCA cycle were shown to be lower under oxygen limitation (Jouhten *et al*. 2008). In line with this, genes encoding for enzymes involved in the TCA cycle are downregulated at limited oxygen availability (Wiebe *et al.* 2008). It seems likely that a similar regulation by oxygen also happens with other carbon sources including glycerol.

5.5. Outlook

The results described in this thesis as well as the data previously published by our group, provide a basis for the production of value-added compounds from glycerol using *Saccharomyces cerevisiae* as the platform organism. However, there are still a number of open questions regarding the metabolic fluxes and their regulation in cells that grow solely with glycerol as carbon source. A flux analysis with a wild-type strain able to grow in synthetic glycerol medium (e.g. CBS 6412-13A) and a strain catabolizing glycerol via a DHA pathway (e.g. CBS DHA) is definitely missing. The existing knowledge gaps regarding metabolic fluxes on glycerol need to be closed in order to reach the goal of the production of valuable compounds from glycerol. The results from this thesis should therefore be considered to be implemented in a future metabolic model of *S. cerevisiae*'s glycerol catabolism.

The glycerol catabolism via the DHA pathway makes glycerol's reducing power available in the form of one extra molecule of cytosolic NADH per glycerol consumed. However, as described in **Chapter 3.3,** *S. cerevisiae* has strong native mechanisms to oxidize cytosolic NADH when it grows on glycerol. This became obvious since the DHA pathway strains are able to grow with relatively high growth rates and do not produce ethanol. Thus, electrons derived from cytosolic NADH must be transferred to the respiratory chain by natural mechanisms. The external NADH dehydrogenases are the most powerful enzymes in this regard as shown in **Chapter 3.3**. The deletion of Nde1 was able to improve yield and titer of 1,2-PDO production at least in YG medium. Interestingly, a previous study by Lee *et al.* (2015b) showed that a deletion of the external NADH dehydrogenases also improved lactic acid formation in engineered *S. cerevisiae* strains on glucose. However, the abolishment of this mechanism in glycerol-based approaches seems to be too drastic for the metabolism of the cell as indicated by the severe growth impairment in the respective mutant strains. A defined regulation of the external NADH dehydrogenase Nde1 for minimum necessary activity might therefore be attractive. Another possibility to prevent the external NADH dehydrogenases from re-oxidizing cytosolic NADH would be the total omission of oxygen in such fermentations.

The results described in this thesis indicate that it might be possible to engineer *S. cerevisiae* strains which catabolize glycerol anaerobically. A promising approach for the establishment of an anoxic process could be adaptive laboratory evolution (ALE). ALE is a powerful tool even though the genetic alterations are difficult to control (Dragosits and Mattanovich, 2013). The parameters have to be chosen carefully in order to evolve the right features. Evolution of anoxic growth seems to require straightforward settings. By reducing the oxygen availability from generation to generation, it could be possible to achieve anoxic glycerol utilization. The strain CBS DHA PDO (**Chapter 3.3**) would be a promising baseline strain. This strain has all prerequisites for anoxic growth with regard to redox balance. The production of 1,2-PDO is redox neutral from glycerol, when glycerol is catabolized via the DHA pathway. The formation of ethanol can cover re-oxidation of surplus NADH formed during biomass formation. Only the question regarding the formation of net ATP without involvement of the respiratory chain is unsolved. An ALE experiment might reveal a 'natural' solution for this problem.

The most abundant form of glycerol to be valorized via microbial biotechnological processes is crude glycerol (see **Chapter 1.1.2**). Despite *S. cerevisiae* is quite tolerant against e.g. methanol or weak carboxylic acids, the overall tolerance against the mixture of impurities in crude glycerol can be improved by metabolic engineering and ALE (Winkler and Kao, 2014). Çakar *et al.* (2012) as well as Deparis *et al.* (2017) extensively reviewed possibilities to increase the tolerance of *S. cerevisiae* strains over industrially relevant stress factors, including impurities of crude glycerol by evolutionary engineering. Therefore, an ALE approach with increasing concentrations of crude glycerol, could lead to increased tolerance over crude glycerol of strains constructed in the current study.

Another idea, in order to increase product yield from glycerol, is the reduction of carbon loss by the formation of by-products (e.g. ethanol, acetate) or as CO_2. The deletion of pathways leading to by-products is common practice (Guadalupe Medina *et al*. 2010; Nielsen *et al.* 2013; Pagliardini *et al.* 2013; Papapetridis *et al.* 2018). Reduction of CO_2 release can be achieved by deletion of the pyruvate decarboxylase catalyzing the reaction of pyruvate to acetaldehyde

(Hohmann, 1991). Another yet not comprehensively studied engineering attempt could be the reduction of carbon loss during the TCA cycle activity. The two consecutive reactions of isocitrate to α-ketoglutarate catalyzed by isocitrate dehydrogenase and the reaction of α-ketoglutarate to succinyl-CoA catalyzed by the α-ketoglutarate dehydrogenase complex (KGD) result in CO_2 formation. A suitable target for interrupting the TCA cycle could be the α-ketoglutarate dehydrogenase complex encoded by *KGD1* and *KGD2* (Repetto and Tzagoloff, 1990, 1989). It is well-known that the TCA cycle in anaerobic bacteria is naturally branched into two branches due to a lack of this complex (Madigan *et al*. 2012; Nelson and Cox, 2013; Thauer *et al.* 1977). On the one branch, acetyl-CoA is converted to α-ketoglutarate. The other branch is built by the reversible reactions of malate dehydrogenase, fumarase, succinate dehydrogenase and succinyl-CoA synthase, from oxaloacetate to succinyl-CoA. Both 'end products', α-ketoglutarate and succinyl-CoA, serve as substrates for biosynthetic processes. So far, the role of the KGD in *S. cerevisiae's* glycerol catabolism was not scrutinized in detail. However, a recent study revealed in an ALE experiment aiming at establishing glycerol growth in synthetic medium with the *S. cerevisiae* strain CEN.PK113-7D, that the flux via the α-ketoglutarate dehydrogenase encoded by *KGD1* was reduced in the evolved strain during glycerol catabolism (Strucko *et al.* 2018). The authors show additionally that the reduced flux through the KGD was predicted by a metabolic model. On the contrary, older studies found *KGD1/2* to be upregulated with non-fermentable carbon sources (DeRisi *et al.* 1997; Ohlmeier *et al.* 2004). A simple deletion experiment in a comparably well-characterized strain like the strain CBS DHA (**Chapter 3.3**), would give indications, whether the deletion of *KGD1/KGD2* affects the growth behavior of this strain on glycerol. Furthermore, in case there would be no significant effects on the growth rate, it would certainly be interesting, to analyze a yet hypothetical KGD-negative mutant strain (e.g. CBS DHA *kgd1*Δ *kgd2*Δ), for the formation of ethanol in synthetic glycerol medium. An increased ethanol titer in comparison to CBS DHA would already be informative, whether the proposed branching of the

TCA-cycle could in fact be a promising approach to increase product formation from (crude) glycerol.

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