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# A modular metabolic engineering approach for the production of 1,2-propanediol from glycerol by *Saccharomyces cerevisiae*

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## KEYWORDS

Yeast, *Saccharomyces cerevisiae*, glycerol, 1,2-propanediol, dihydroxyacetone, triosephosphate  
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1,2-propanediol production from glycerol in *Saccharomyces cerevisiae*

**ABSTRACT**

Compared to sugars, a major advantage of using glycerol as a feedstock for industrial bioprocesses is the fact that this molecule is more reduced than sugars. A compound whose biotechnological production might greatly profit from the substrate's higher reducing power is 1,2-propanediol (1,2-PDO). Here we present a novel metabolic engineering approach to produce 1,2-PDO from glycerol in *S. cerevisiae*. Apart from implementing the heterologous methylglyoxal (MG) pathway for 1,2-PDO formation from dihydroxyacetone phosphate (DHAP) and expressing a heterologous glycerol facilitator, the employed genetic modifications included the replacement of the native FAD-dependent glycerol catabolic pathway by the 'DHA pathway' for delivery of cytosolic NADH and the reduction of triosephosphate isomerase (TPI) activity for increased precursor (DHAP) supply. The choice of the medium had a crucial impact on both the strength of the metabolic switch towards fermentation in general (as indicated by the production of ethanol and 1,2-PDO) and on the ratio at which these two fermentation products were formed. For example, virtually no 1,2-PDO but only ethanol was formed in synthetic glycerol medium with urea as the nitrogen source. When nutrient-limited complex YG medium was used, significant amounts of 1,2-PDO were formed and it became obvious that the concerted supply of NADH and DHAP are essential for boosting 1,2-PDO production. Additionally, optimizing the flux into the MG pathway improved 1,2-PDO formation at the expense of ethanol. Cultivation of the best-performing strain in YG medium and a controlled bioreactor set-up resulted in a maximum titer of  $> 4 \text{ g L}^{-1}$  1,2-PDO which, to the best of our knowledge, has been the highest titer of 1,2-PDO obtained in yeast so far. Surprisingly, significant 1,2-PDO production was also obtained in synthetic glycerol medium after changing the nitrogen source towards ammonium sulfate and adding a buffer.

## 1. INTRODUCTION

A major challenge for the production of chemicals by industrial biotechnology is the fact that the commonly used sugar-based substrates inevitably result in low carbon yields which can be attributed to the high oxidation state (O:C ratio) of sugars relative to many products (Burk and Van Dien, 2016). Although glycerol is still less reduced than hydrocarbons derived from fossil resources, the degree of reduction (4.667) is higher than that of common sugars such as glucose (4.000) and xylose (4.000) (Yazdani and Gonzalez, 2008). Therefore, the utilization of glycerol as a substrate or co-substrate in the fermentative production of chemicals can lead to higher maximum theoretical product yields of compounds with a relatively high degree of reduction as well as of molecules whose biochemical production from sugars is not redox-neutral but requires additional reducing equivalents such as 1,2-propandiol or succinic acid (Murarka et al., 2008; Yazdani and Gonzalez, 2007; Zhang et al., 2013).

Glycerol is an unavoidable by-product of the biodiesel industry since transesterification of vegetable oils or animal fats generates app. 10% (w/w) of glycerol (Karinen and Krause, 2006). As a result of the growing demand for renewable transportation fuels, the production of biodiesel steadily increased during the last decades accompanied by the accumulation of huge amounts of crude glycerol (Mattam et al., 2013). In principle, this crude glycerol has to be considered as a waste stream since it still contains various residual components from the transesterification process such as methanol, NaOH, carry-over fats/oils as well as low amounts of proteins, minerals and sulfur compounds (Thompson and He, 2006). The utilization of excess crude glycerol as a feedstock for the microbial production of various value-added chemicals in industrial bioprocesses has been considered as a promising approach for its valorization (da Silva et al., 2009; Johnson and Taconi, 2007; Yang et al., 2012).

The yeast *Saccharomyces cerevisiae* has become a favorite platform organism for the production of various chemical compounds by means of metabolic engineering. In fact, re-routing the carbon flux in this organism allowed the commercial production of advanced biofuels (e.g. isoprenoids, isobutanol),

bulk chemicals (*e.g.* succinic acid) as well as pharmaceuticals and nutraceuticals (Borodina and Nielsen, 2014; Hong and Nielsen, 2012; Nevoigt, 2008). Major factors driving the increasing metabolic engineering incentives in *S. cerevisiae* are its robustness in industrial setups and its easy accessibility for genetic engineering including the tremendous developments with regard to DNA assembly, chromosomal engineering and genome editing as comprehensively reviewed by Chao et al. (2015) and David and Siewers (2015).

Most of the commonly employed industrial and laboratory strains of *S. cerevisiae* utilize glycerol only in the presence of complex medium supplements such as amino acids and nucleic bases but do not grow in synthetic medium containing glycerol as the sole carbon source (Merico et al., 2011; Swinnen et al., 2013). Therefore, glycerol has been rather neglected as a feedstock for *S. cerevisiae*-based bioprocesses. Still, certain wild-type *S. cerevisiae* isolates including the strain CBS 6412 are able to moderately grow in synthetic glycerol medium and a haploid segregant of this strain (CBS 6412-13A) showed a maximum specific growth rate ( $\mu_{\max}$ ) of  $\sim 0.13 \text{ h}^{-1}$  (Swinnen et al., 2013). The genetic determinants underlying the latter segregant's positive phenotype with regard to growth on glycerol in comparison to the non-growing popular laboratory strain CEN.PK113-1A have been unraveled recently (Swinnen et al., 2016). Moreover, the maximum specific growth rate of CBS 6412-13A in synthetic glycerol medium has been even further improved up to  $\sim 0.18 \text{ h}^{-1}$  by the expression of a glycerol facilitator (Klein et al., 2016b). Several facilitators originating from various yeast species that naturally show superior growth in glycerol medium as compared to *S. cerevisiae* (such as *Cyberlindnera jadinii*, *Yarrowia lipolytica*, *Pachysolen tannophilus* and *Komagataella phaffii* syn. *Pichia pastoris*) had a similar positive effect on glycerol utilization.

Wild-type *S. cerevisiae* strains exclusively utilize the so-called G3P (L-glycerol-3-phosphate) pathway for catabolizing glycerol as recently reviewed by Klein et al. (2016c). This pathway transfers the electrons derived from G3P oxidation directly via  $\text{FADH}_2$  to the cell's mitochondrial respiratory chain thereby 'squandering' the substrate's aforementioned superior reducing power. In fact, the respective electrons have to be available in the form of cytosolic NADH in order to support the

formation of reduced fermentation products. Recently, we replaced the endogenous G3P glycerol catabolic pathway by the so-called DHA pathway which transfers the electrons to cytosolic NAD<sup>+</sup> thereby delivering NADH for fermentative pathways (Klein et al., 2016a; Nevoigt, 2017). *S. cerevisiae* strains engineered in this way show similar or even better glycerol utilization as compared to the corresponding wild-type strains offering the possibility to employ the pathway replacement approach for the production of reduced chemicals from glycerol in *S. cerevisiae*.

1,2-propanediol (1,2-PDO, propylene glycol) is one example for a reduced compound (degree of reduction per Cmol: 5.333), whose biotechnological production may greatly profit from using glycerol as a feedstock. 1,2-PDO is an important commodity chemical applied in food, drug, and cosmetic industries. It is frequently used for the preparation of unsaturated polyester resins, for the production of biodegradable plastics, as a solvent in photographic industries, as an aircraft de-icing fluid and due to its lower toxicity as an environmentally friendly substitute for ethylene glycol in automotive antifreeze (Berrios-Rivera et al., 2003; Niimi et al., 2011; Shelley, 2007). A wide range of bacteria is naturally able to produce 1,2-PDO by fermentation, such as *Thermoanaerobacterium thermosaccharolyticum* (Cameron and Cooney, 1986), *Clostridium sphenoides* (Tran-Din and Gottschalk, 1985), *Bacteroides ruminicola* (Turner and Robertson, 1979), *Klebsiella pneumoniae* (Badia et al., 1985) and *E. coli* (Boronat and Aguilar, 1979; Boronat and Aguilar, 1981; Gonzalez et al., 2008; Murarka et al., 2008). Interestingly, there has also been a very early report about some aerobic 1,2-PDO production by several non-conventional yeast species (Suzuki and Onishi, 1968) and small amounts have been detected in several industrial *S. cerevisiae* strains (Dowd et al., 1994). However, one has to note that a full metabolic pathway for the production of 1,2-PDO has never been identified in yeast. In addition, the respective cultivations were performed in undefined media which may contain one or several of the pathway intermediates. These intermediates could have been converted to 1,2-PDO by endogenous, non-specific oxidoreductase activities (Bennett and San, 2001).

Two main natural biochemical pathways leading to 1,2-PDO production have been discovered in bacteria. A widely spread pathway, which exists for example in *E. coli* and *Salmonella typhimurium*, depends on the fermentation of the deoxy hexose sugars L-rhamnose and L-fucose (Badia et al., 1985; Bennett and San, 2001; Boronat and Aguilar, 1981). A second natural route identified in *T. thermosaccharolyticum* and *C. sphenoides* converts the glycolytic carbon intermediate dihydroxyacetone phosphate (DHAP) by a methylglyoxal synthase (MGS) to methylglyoxal (MG) followed by two subsequent NAD(P)H dependent reductions catalyzed by aldo-keto reductases (AKRs) to 1,2-PDO (see Figure 1) (Bennett and San, 2001; Cameron and Cooney, 1986; Tran-Din and Gottschalk, 1985). 1,2-PDO formation by wild-type *E. coli* had been originally assumed to be restricted to the fermentation of the deoxy hexose sugars as mentioned above. However, more recent studies demonstrated that the so-called 'methylglyoxal pathway' is also used in *E. coli* to anaerobically ferment glycerol where 1,2-PDO production is used as a redox sink in order to cope with the surplus cytosolic NADH derived from biomass formation (Gonzalez et al., 2008; Murarka et al., 2008). As the latter pathway allows for the production of 1,2-PDO from inexpensive sugars or glycerol it has been the preferred route in the context of rational engineering of microorganisms for 1,2-PDO production. So far, the bacterium *E. coli* has been the mainly employed chassis in numerous engineering studies aiming at the fermentative production of 1,2-PDO using either glucose (Altaras and Cameron, 1999; Altaras and Cameron, 2000; Berrios-Rivera et al., 2003; Cameron et al., 1998; Huang et al., 1999; Jain et al., 2015a; Jain et al., 2015b; Lee et al., 2016) or glycerol (Clomburg and Gonzalez, 2011) as the sole carbon source. The 'methylglyoxal pathway' was also used to produce 1,2-PDO from glucose in engineered cells of *Corynebacterium glutamicum* (Niimi et al., 2011; Siebert and Wendisch, 2015) and from CO<sub>2</sub> by metabolic engineering of the cyanobacterium *Synechococcus elongatus* (Li and Liao, 2013). Eukaryotic microorganisms previously engineered for 1,2-PDO production include *S. cerevisiae* (Jeon et al., 2009; Jung et al., 2008; Jung et al., 2011; Lee and DaSilva, 2006) and *K. phaffii* (*P. pastoris*) (Barbier et al., 2011). Notably, the highest titers of 1,2-PDO such as

5.62 g/L for *E. coli* (Clomburg and Gonzalez, 2011) and 2.19 g/L for *S. cerevisiae* (Jung et al., 2011) were achieved when using glycerol as the carbon source.

Here, we present a novel metabolic engineering study for the production of 1,2-PDO from glycerol in the yeast *S. cerevisiae*. The current work was initiated since we assumed that our previously reported glycerol catabolic pathway replacement (Klein et al., 2016a) might be crucial for providing sufficient amounts of cytosolic NADH for the production of reduced chemicals such as 1,2-PDO. Apart from obtaining the highest 1,2-PDO titer produced in yeast so far, we also got surprising new insights into the physiology of glycerol metabolism in our engineered strains including unexpected effects caused by the medium composition.

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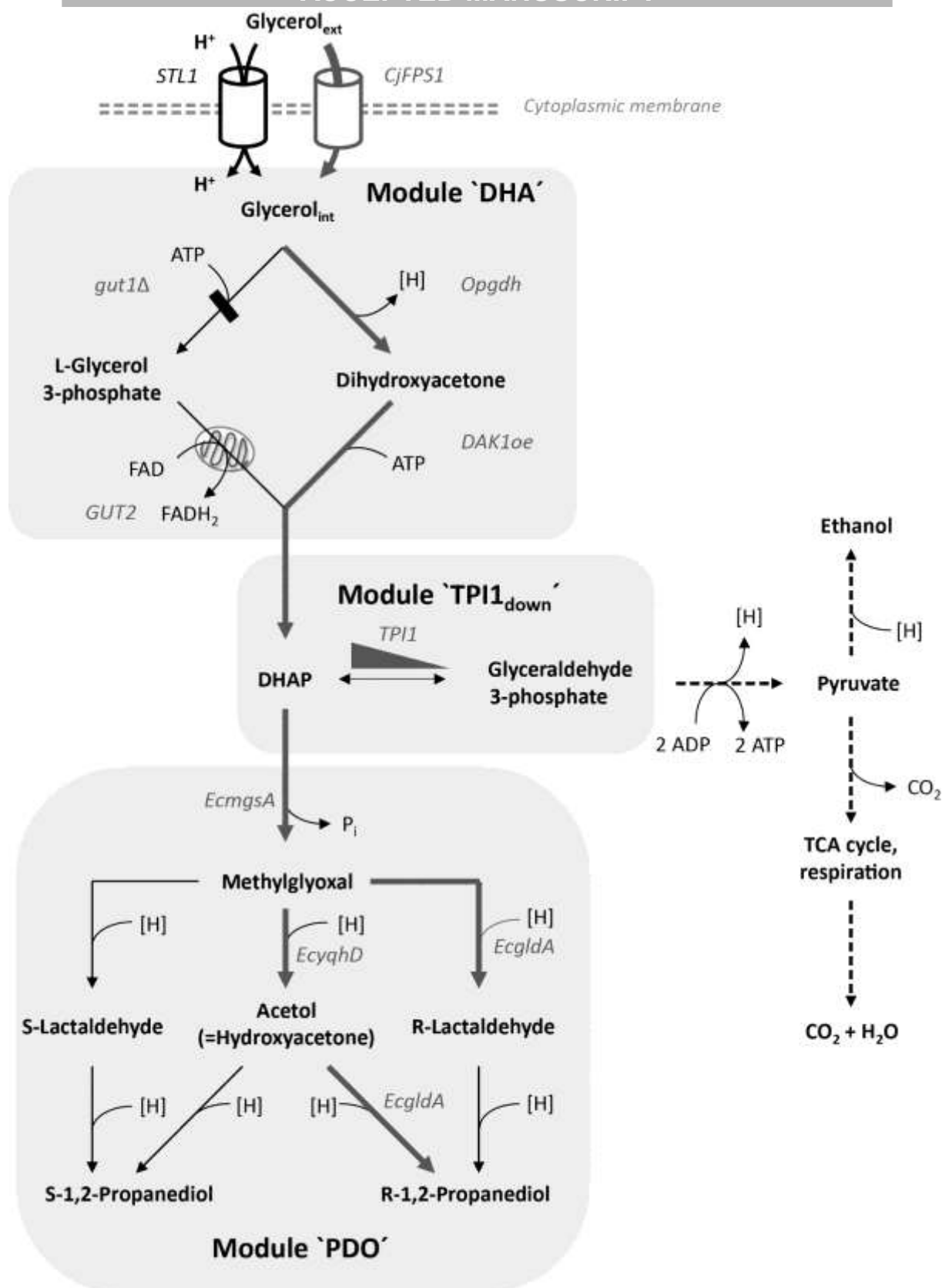


Figure 1 (2 column fitting image). Metabolic engineering for 1,2-propanediol (1,2-PDO) production from glycerol in *S. cerevisiae*. Module 'PDO': 'methylglyoxal pathway' converting dihydroxyacetone phosphate (DHAP) via methylglyoxal to 1,2-PDO by expressing *E. coli* methylglyoxal synthase (encoded by *EcmgsA*), *E. coli*

aldehyde reductase (encoded by *EcyqhD*) and *E. coli* glycerol dehydrogenase (encoded by *EcglDA*). Module 'DHA': replacement of the native FAD-dependent glycerol catabolic pathway via L-glycerol-3-phosphate by the NAD<sup>+</sup>-dependent 'DHA pathway' by deletion of the gene encoding glycerol kinase (*GUT1*), expression of a glycerol dehydrogenase from *O. parapolyomorpha* (*Opgdh*) and overexpression of endogenous dihydroxyacetone kinase (encoded by *DAK1*). Module 'TPI1<sub>down</sub>': downregulation of the expression of *TPI1* encoding triosephosphate isomerase by replacement of the gene's native promoter by the weak *TEF1* promoter variant *TEFmut2* (Nevoigt et al., 2006). All constructed *S. cerevisiae* strains express the glycerol facilitator from *C. jadinii* encoded by *CjFPS1* in addition to the endogenous glycerol/H<sup>+</sup> symporter encoded by *STL1*. The latter modification has been considered to be useful as it resulted in a significantly improved glycerol uptake in the genetic background of CBS 6412-13A (Klein et al., 2016b). Bold grey arrows highlight the carbon flux from glycerol to 1,2-PDO as it is assumed based on the published knowledge regarding the *in vivo* and/or *in vitro* activities of transport and enzymes as explained in the main text. Still, it has to be considered that the heterologous enzymes encoded by *EcglDA*, *EcyqhD* and *Opgdh* have relatively broad substrate specificities and might also contribute to other reduction/oxidation reactions in addition those allocated to the gene names in this Figure. Moreover, unspecific/uncharacterized endogenous enzyme activities in *S. cerevisiae* might also contribute to the different potential reactions from methylglyoxal to 1,2-PDO.

## 2. MATERIALS AND METHODS

### 2.1 Strains and their maintenance

All *S. cerevisiae* strains used in this study were based on CBS 6412-13A (Swinnen et al., 2013) and are listed in Table 1. The yeast strains were routinely grown on solid YPD medium containing 10 g L<sup>-1</sup> yeast extract, 20 g L<sup>-1</sup> peptone, 20 g L<sup>-1</sup> glucose, and 15 g L<sup>-1</sup> agar. All strains, whose native *TPI1* promoter had been replaced by the engineered *TEF1* promoter variant *TEFmut2*, were grown on solid YP glycerol medium (YPG) containing 30 g L<sup>-1</sup> glycerol as the carbon source. If required, solid medium was supplemented with G418 (200 mg L<sup>-1</sup>), phleomycin (20 mg L<sup>-1</sup>), nourseothricin (100 mg L<sup>-1</sup>) or hygromycin B (300 mg L<sup>-1</sup>). *E. coli* DH5 $\alpha$  was used for plasmid construction and isolation, and cells were routinely grown in lysogeny broth (LB) (10 g L<sup>-1</sup> peptone, 5 g L<sup>-1</sup> yeast extract and 10 g L<sup>-1</sup> NaCl, pH 7.0). For selection and maintenance of plasmid containing cells, 100 mg L<sup>-1</sup> ampicillin was added.

### 2.2 General molecular biology techniques

All primers used within the present study are listed in Table S1 (supplementary information). Preparative PCRs for cloning were performed using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Frankfurt am Main, Germany) or AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). PCR conditions were adapted to the guidelines of the respective manufacturer. Restriction enzymes, FastAP alkaline phosphatase and T4 DNA ligase were obtained from Thermo Fisher Scientific and used according to the manufacturer's instructions. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and DNA fragments obtained after restriction were excised and purified using the QIAquick Gel Purification Kit (Qiagen). Plasmids were isolated from *E. coli* DH5α overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen). Transformation of *S. cerevisiae* with plasmids as well as with linear DNA fragments for genomic integration was performed according to the lithium acetate method described by Gietz et al. (1995).

**Table 1:** Strains used in this study.

| Strain / Modules   | Genome modifications   | Plasmid  | Source or reference   |
|--|--|--|-----------------------|
| CBS 6412-13A   | -  | -  | Swinnen et al. (2013) |
| CBS 6412-13A <i>CjFPS1</i>                                     | <i>YGLCt3::TEF1p-CjFPS1-CYC1t</i>  | -  | Klein et al. (2016b)  |
| CBS 6412-13A <i>DAK1<sub>OE</sub></i><br><i>gut1Δ</i>          | <i>YPRCΔ15::ACT1p-DAK1-TPS1t</i> ;<br><i>gut1::loxP-kanMX4-loxP</i>  | -  | Klein et al. (2016a)  |
| FPS  | <i>YGLCt3::TEF1p-CjFPS1-CYC1t</i>  | p41ble-E-E   | This study            |
| PDO-FPS  | <i>YGLCt3::TEF1p-EcmgsA-CYC1t:TDH3p-EcglDA-IDP1t-PGK1p-CjFPS1-RPL15At</i>  | p41ble-E-yqhD<br>( <i>ACT1p-EcyqhD-TPS1t</i> )                                 | This study            |
| PDO-FPS-DHA  | <i>YGLCt3::TEF1p-EcmgsA-CYC1t:TDH3p-EcglDA-IDP1t-PGK1p-CjFPS1-RPL15At</i> ; <i>YPRCΔ15::ACT1p-DAK1-TPS1t</i> ; <i>gut1::loxP-kanMX4-loxP</i>   | p41ble-gdh-yqhD<br>( <i>TEF1p-Opgdh-CYC1t</i> ;<br><i>ACT1p-EcyqhD-TPS1t</i> ) | This study            |
| PDO-FPS-TPI1 <sub>down</sub>                                   | <i>YGLCt3::TEF1p-EcmgsA-CYC1t:TDH3p-EcglDA-IDP1t-PGK1p-CjFPS1-RPL15At</i> ; <i>tpi1p::TEFmut2p-TPI1</i>  | p41ble-E-yqhD<br>( <i>ACT1p-EcyqhD-TPS1t</i> )                                 | This study            |
| PDO-FPS-TPI1 <sub>down</sub> -DHA                              | <i>YGLCt3::TEF1p-EcmgsA-CYC1t:TDH3p-EcglDA-IDP1t-PGK1p-CjFPS1-RPL15At</i> ; <i>tpi1p::TEFmut2p-TPI1</i> ;<br><i>YPRCΔ15::ACT1p-DAK1-TPS1t</i> ; <i>gut1::loxP-kanMX4-loxP</i>  | p41ble-gdh-yqhD<br>( <i>TEF1p-Opgdh-CYC1t</i> ;<br><i>ACT1p-EcyqhD-TPS1t</i> ) | This study            |
| PDO-FPS-TPI1 <sub>down</sub> -DHA<br><i>EcmgsA<sub>2</sub></i> | <i>YGLCt3::TEF1p-EcmgsA-CYC1t:TDH3p-EcglDA-IDP1t-PGK1p-CjFPS1-RPL15At</i> ; <i>tpi1p::TEFmut2p-TPI1</i> ;<br><i>YPRCΔ15::ACT1p-DAK1-TPS1t</i> ; <i>gut1::loxP-kanMX4-loxP</i> ; <i>YPRCt3::TDH3p-EcmgsA-IDP1t:TEF1p-Opgdh-CYC1t</i> ; <i>YORWΔ22::ACT1p-EcyqhD-TPS1t</i> | -  | This study            |

### 2.3 Construction of plasmids and expression cassettes

All constructed plasmids are listed in Table S2 (supplementary information). Codon-optimized coding sequences for *E. coli mgsA*, *gldA* and *yqhD* were obtained via GeneArt™ gene synthesis from Thermo Fisher Scientific (Waltham, MA, USA). The plasmids for either the sole expression of *E. coli yqhD* (*EcyqhD*) under the control of the *ACT1* promoter and the *TPS1* terminator or its expression in combination with the *O. parapolyomorpha gdh* (*Opgdh*), under the control of the *TEF1* promoter and the *CYC1* terminator, were obtained by using Gibson isothermal assembly (Gibson et al., 2009). The assembly was performed in the previously constructed *S. cerevisiae* expression vectors p41bleTEF or p41bleTEF-*Opgdh* (Klein et al., 2016a). The *ACT1* promoter and the *TPS1* terminator sequences were amplified from genomic DNA isolated from the *S. cerevisiae* strain S288c using primers 531 and 532, and 533 and 534, respectively (see Table S1). The coding sequence of *E. coli yqhD* was amplified from pMA-T-*yqhD* (Table S2) using primers 535 and 536, and the template vector was subsequently removed by digestion with *DpnI*. The assembly reactions contained 15 µL of the reagent-enzyme mix, 0.05 pmol of *KpnI*-linearized and dephosphorylated p41bleTEF or p41bleTEF-*Opgdh* and 3-fold molar excess of the inserts (promoter, coding sequence and terminator each 0.15 pmol) in a final volume of 20 µL. Reaction mixtures were incubated at 50°C for 1 h. *E. coli* DH5α cells were transformed with 5 µL of the reaction and the resulting vectors named p41ble-E-*yqhD* and p41ble-*gdh-yqhD* where “E” specifies an empty expression cassette consisting solely of the *TEF1* promoter and *CYC1* terminator sequences (Table S2). The empty vector control (carrying two empty expression cassettes) was constructed accordingly by assembling only the *ACT1* promoter and the *TPS1* terminator in p41bleTEF. The primers used for amplification were 531 and 538, and 533 and 537, respectively. The resulting ‘empty’ vector was named p41ble-E-E and used for the construction of control strains.

The expression cassettes for *C. jadinii FPS1* (*CjFPS1*) and *S. cerevisiae DAK1* (*ScDAK1*) for genomic integration were obtained by PCR amplification from the plasmids pUC18-*CjFPS1* and pUC18-*DAK1* (Table S2). Construction of these plasmids is described in Klein et al. (2016a). One of the two expression cassettes constructed for integrating *E. coli mgsA* into the genome (*EcmgsA* under the

control of the *S. cerevisiae* *TEF1* promoter and the *CYC1* terminator) was obtained by cloning the respective coding sequence into the multiple cloning site of p416TEF-KanMX (Table S2). For this purpose the *EcmgsA* coding sequence was amplified from pMA-T-*mgs* (Table S2) using primers 276 and 277. The cloning via *Bam*HI and *Eco*RI yielded the plasmid p416TEF-*mgsA*-KanMX. The second *EcmgsA* expression cassette (*EcmgsA*<sub>2</sub> under the control of the *TDH3* promoter and the *IDP1* terminator) and the cassette for expression of *E. coli gldA* (*EcglDA*) (also under the control of the *TDH3* promoter and the *IDP1* terminator) were both assembled in pUC18 using Gibson isothermal assembly (Gibson et al., 2009). Promoters and terminators were amplified from genomic DNA isolated from the *S. cerevisiae* strain S288c using primer combinations 413/419 and 415/328 for the expression cassette of *EcmgsA*<sub>2</sub>, and 324/323 and 327/328 for the expression cassette of *EcglDA*. The coding sequences for *EcmgsA* and *EcglDA* were amplified from the plasmids pMA-T-*mgs* and pGA-*gldA* using primer combinations 420/414 and 325/326, respectively. Both template vectors were removed after PCR by digestion with *Dpn*I. The target vector pUC18 was linearized using *Bam*HI and subsequently dephosphorylated to avoid self-ligation. The further steps for assembly and transformation were the same as described above and the resulting plasmids named pUC18-*gldA* and pUC18-*mgsA*. All constructed plasmids were verified by Sanger sequencing (GATC Biotech, Konstanz, Germany).

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

For CRISPR-Cas9 mediated genome editing, the plasmids p414-TEF1p-Cas9-CYC1t-nat1 and p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX (Table S2) were used. Construction of the respective plasmids has been reported by Klein et al. (2016a). The specific 20 nt sequence at the 5'-end of the gRNA targeting the *CAN1* gene was exchanged in p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX by a 20 nt sequence targeting the YGLCt3 region on chromosome VII (Flagfeldt et al., 2009) by generating two overlapping PCR products of the gRNA expression cassette and their subsequent assembly in the same vector backbone according to Gibson et al. (2009). The target sequence within the YGLCt3 locus was selected after analysis of the respective genomic region using the CRISPRdirect online tool

developed by Naito et al. (2015). The two overlapping fragments of the gRNA expression cassette were obtained by PCR amplification from p426-SNR52p-gRNA.CAN1.Y-SUP4t (Table S2) using primers 460 and 464, and 465 and 463, respectively (Table S1), where the 5'-termini of primers 464 and 465 generated flanking sequences at the ends of the two PCR products which overlapped with the YGLCt3 target sequence for genomic integration. The primers 460 and 463 contained 5'-extensions resulting in overlaps with *PvuII*-linearized p426-SNR52p-gRNA.CAN1.Y-SUP4t-hphMX. The accordingly digested vector was gel-purified and Gibson isothermal assembly was performed as described above (section 2.3) yielding p426-SNR52p-gRNA.YGLCt3-SUP4t-hphMX.

### 2.5 *S. cerevisiae* strain construction

A detailed scheme for the construction of engineered CBS 6412-13A derivatives (Table 1) is shown in Figure S1 (supplementary information). As the expression of *Ecyqhd* and *Opgdh* in all constructed strains except PDO-FPS-TPI1<sub>down</sub>-DHA *EcmgsA*<sub>2</sub> was plasmid-based, the final step for completion was always a transformation with p41ble-E-*yqhd*, p41ble-*gdh-yqhd* or p41ble-E-E.

#### *Strains PDO-FPS and FPS*

As mentioned in section 2.4 the long terminal repeat (LTR) YGLCt3 on chromosome VII (Flagfeldt et al., 2009) was selected as a target site for the genomic integration of the expression cassettes for *EcmgsA* (under the control of the *TEF1* promoter and the *CYC1* terminator) and *EcglidA* (under the control of the *TDH3* promoter and the *IDP1* terminator) in CBS 6412-13A. The seamless integration was achieved in two steps. First, a counter-selectable, galactose-inducible growth inhibitory sequence (referred to as *GALp-GIN11M86*) and the *kanMX4* selectable marker were assembled and integrated at the target site by homologous recombination. The *GALp-GIN11M86* cassette was amplified from plasmid pGG119 (Akada et al., 2002) using primers 175 and 155. The *kanMX4* expression cassette was amplified from plasmid pUG6 (Gueldener et al., 1996) using primers 171 and 176. The PCR primers were modified at their 5'-ends generating homologous regions of ~40-60 bp at the ends of the PCR products for assembly and integration at the target locus. In the second step, the

two aforementioned expression cassettes *EcmgsA* and *EcglDA* were assembled and integrated by homologous recombination at the target locus replacing the entire *GALp-GIN11M86/kanMX4* cassette. The respective expression cassettes were amplified from the previously constructed plasmids p416TEF*mgsA*-KanMX (primers 257 and 302) and pUC18-*gldA* (primers 303 and 297), respectively. Primer pairs were designed to generate PCR products whose flanking sequences were homologous to the chromosomal regions upstream and downstream of the previously integrated *GALp-GIN11M86/kanMX4* cassette. After transformation, cells were plated on solid synthetic medium containing 2% (w/v) galactose as the sole carbon source in order to induce the growth inhibitory sequence *GIN11M86* for counter-selection. The resulting intermediate strain was named PDO<sub>min</sub> (Figure S1). Subsequently, an expression cassette for *CjFPS1* (under the control of the *PGK1* promoter and the *RPL15A* terminator) was integrated together with a phleomycin resistance marker (*ble*) downstream of *EcmgsA* and *EcglDA*. The *CjFPS1* cassette was amplified with primers 390 and 392 from pUC18-*CjFPS1*, while the *ble* cassette was amplified from pUG66 (Gueldener et al., 2002) using primers 391 and 393. The 5'-termini of the employed primers were designed to allow assembly and integration of the generated PCR products into the genome of strain PDO<sub>min</sub>, immediately downstream of the previously integrated expression cassettes for *EcmgsA* and *EcglDA*, and the resulting strain was named PDO<sub>min</sub>-FPS. The phleomycin resistance cassette was subsequently removed from this intermediate strain by using Cre-loxP-mediated recombination according to Steensma and Ter Linde (2001) using the Cre-recombinase expressing plasmid pNatCre (Table S2). Finally the resulting strain was transformed with p41ble-E-*yqhD* to yield the strain PDO-FPS. In order to generate a control strain without an implemented pathway for 1,2-PDO production the previously constructed strain CBS 6412-13A *CjFPS1* (Klein et al., 2016b) was transformed with the 'empty' vector p41ble-E-E (section 2.3; Table S2).

#### *Strain PDO-FPS-DHA*

In order to combine the modules for the 1,2-PDO pathway and the glycerol catabolic pathway replacement (DHA pathway replacing the endogenous G3P pathway) in an *FPS1* expressing strain of

CBS 6412-13A, the previously generated strain CBS 6412-13A *DAK1<sub>OE</sub> gut1Δ* was used as a basis (Table 1) (Klein et al., 2016a). First, the expression cassettes for *EcmgsA*, *EcglDA* and *CjFPS1* were integrated into the YGLCt3 region of this strain by employing the CRISPR-Cas9 system. For this purpose, the expression of Cas9 in the strain CBS 6412-13A *DAK1<sub>OE</sub> gut1Δ* was achieved by transformation with the plasmid p414-TEF1p-Cas9-CYC1t-nat1 (Table S2). The three expression cassettes were PCR-amplified from the plasmids p416TEF*mgsA*-KanMX, pUC18-*gldA* and pUC18-*CjFPS1* (see section 2.3 and Table S2) using primers pairs 257/302, 303/552, and 550/551, respectively. These primers contained 5'-extensions generating 40-60 bp sequences homologous to regions directly upstream and downstream of the YGLCt3 integration locus or to the respective adjacent expression cassette (Figure S1). Co-transformation of the expression cassettes and plasmid p426-SNR52p-gRNA.YGLCt3-SUP4t-hphMX into the *S. cerevisiae* strain expressing the Cas9 endonuclease resulted in assembly and integration of all three expression cassettes at the target locus. Positive transformants were selected on YPD agar containing both nourseothricin and hygromycin B as described in section 2.1. Both plasmids were subsequently removed from the resulting clone by serial transfers in YPD medium lacking the respective antibiotics. In order to complete both modules (1,2-'PDO' and 'DHA'), the resulting intermediate strain was transformed with p41ble-*gdh-yqhD* (see section 2.3) yielding strain PDO-FPS-DHA.

#### *Strains PDO-FPS-TPI1<sub>down</sub> and PDO-FPS-TPI1<sub>down</sub>-DHA*

In order to downregulate *TPI1* expression, the gene's native promoter was replaced by a promoter replacement cassette containing the weak *TEF1* promoter variant *TEFmut2p* (Nevoigt et al., 2006). The replacement cassette consisting of the *kanMX4* selectable marker and the *TEFmut2* promoter variant was amplified from the plasmid p416-TEFmut2-yECitrine (Table S2) using primers 426 and 427. The primers contained 5'-extensions of 60 bp homologous to sequences directly upstream and downstream of the endogenous *TPI1* promoter. Strain PDO<sub>min</sub>-FPS (Figure S1) was transformed with the amplified cassette leading to the replacement of the endogenous *TPI1* promoter by the engineered promoter variant *TEFmut2*. Transformants were selected on medium containing glycerol



as the carbon source. Both dominant markers (the *ble* cassette downstream of *CjFPS1* and the *kanMX4* cassette upstream of *TPI1*) in the intermediate strain PDO<sub>min</sub>-FPS-TPI1<sub>down</sub> were subsequently rescued by Cre-loxP mediated recombination as described for strain PDO<sub>min</sub>-FPS (see above). The resulting strain was transformed with plasmid p41ble-E-*yqhD* yielding strain PDO-FPS-TPI1<sub>down</sub>. The previous strain (PDO<sub>min</sub>-FPS-TPI1<sub>down</sub> after marker removal) also served as the basis for the construction of strain PDO-FPS-TPI1<sub>down</sub>-DHA (Figure S1). First, the cassette for *ScDAK1* overexpression (*ACT1* promoter and *TPS1* terminator) was integrated via the two-step seamless integration procedure employing the counter-selectable, galactose-inducible growth inhibitory sequence *GALp-GIN11M86* as described above. The LTR YPRCΔ15 on chromosome XVI (Flagfeldt et al., 2009) served as a target site and the *GALp-GIN11M86* growth inhibitory sequence (amplified from plasmid pGG119 with primers 298 and 155) was first integrated at this position together with the phleomycin resistance marker (amplified from plasmid pUG66 with primers 171 and 299) (Table S2). The *ScDAK1* expression cassette was amplified from plasmid pUC18-*DAK1* using primers 388 and 389 and subsequently integrated at the target locus by homologous recombination replacing the entire *GALp-GIN11M86/ble* cassette. Afterwards, the endogenous *GUT1* coding sequence was disrupted by integration of the *kanMX4* cassette amplified from plasmid pUG6 using primers 111 and 112 yielding the intermediate strain PDO<sub>min</sub>-FPS-TPI1<sub>down</sub> *DAK1*<sub>OE</sub> *gut1*Δ. Finally, the resulting intermediate strain was transformed with plasmid p41ble-*gdh-yqhD* to finalize the construction of the strain PDO-FPS-TPI1<sub>down</sub>-DHA.

#### Strain PDO-FPS-TPI1<sub>down</sub>-DHA *EcmgsA*<sub>2</sub>

In order to implement a second expression cassette for *mgsA* into the strain and, at the same time, obtain a CBS 6412-13A derivative that carries all above-described genetic modifications for 1,2-PDO production in the genome, the strain PDO-FPS-TPI1<sub>down</sub>-DHA *EcmgsA*<sub>2</sub> was generated (Figure S1). The aforementioned intermediate strain PDO<sub>min</sub>-FPS-TPI1<sub>down</sub> *DAK1*<sub>OE</sub> *gut1*Δ was used as a starting point for the integration of the second expression cassette for *EcmgsA* (*EcmgsA*<sub>2</sub>, coding sequence under the control of the *TDH3* promoter and the *IDP1* terminator). Similarly to the expression cassettes of

*EcmgsA*, *EcglDA* and *ScDAK1*, the cassette was integrated together with that of *Opgdh* into the genome via the two-step seamless integration procedure described above using the LTR YPRCt3 on chromosome XVI as a target site (Flagfeldt et al., 2009). First, the *GALp-GIN11M86* growth inhibitory sequence (amplified from plasmid pGG119 with primers 300 and 155) 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 whole *Galp-GIN11M86/ble* cassette was subsequently replaced by assembly and integration of the *EcmgsA<sub>2</sub>* and *Opgdh* expression cassettes (amplified with primers 418 and 422 from plasmid pUC18-*mgsA* and with primers 416 and 421 from plasmid p41bleTEF-*Opgdh*). Subsequently, the above-described expression cassette for *E. coli yqhD* was integrated along with the phleomycin resistance marker at the YORWΔ22 locus (Flagfeldt et al., 2009) yielding strain PDO-FPS-TPI1down-DHA *EcmgsA<sub>2</sub>* (see Figure S1). The *EcyqhD* cassette was amplified with primers 663 and 664 from plasmid p41ble-E-*yqhD*, while the *ble* cassette was amplified from plasmid pUG66 (Gueldener et al., 2002) using primers 665 and 666. The 5'-termini of the employed primers were designed to allow assembly and integration of the generated PCR products at the target site in the genome.

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

Correct integrations of all expression and disruption cassettes were verified by diagnostic PCR using TaKaRa Ex Taq Polymerase and buffer according to the manufacturer's guidelines (Merck KGaA, Darmstadt, Germany). For this purpose, single colonies obtained after transformations were re-streaked on respective agar plates. Approximately 50 mg of cells from these plates were suspended in 200 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Subsequently, 300 mg of acid-washed glass beads (diameter of 0.425 to 0.6 mm) and 200 μL of phenol:chloroform:isoamyl alcohol (25:24:1) were added. The tubes were then vortexed at maximum speed for 2 min and centrifuged at 15,700 g for 10 min according to a protocol modified from Hoffman and Winston (1987). The aqueous phase (1 μL) was used as template in 20 μL PCR reactions. PCR primers were designed to bind upstream and downstream of the genomic integration sites as well as within the integrated expression/deletion

cassette. For analyzing integrations of multiple expression cassettes, additional primers were designed to produce amplicons covering the junctions between the individual integrated expression cassettes. After each integration step, the presence of all previously integrated expression cassettes was verified.

## 2.7 Media and cultivation conditions for the production of 1,2-PDO from glycerol

Media used for shake flask batch cultivations assessing 1,2-PDO production were synthetic or complex media containing 60 mL L<sup>-1</sup> glycerol as the sole carbon source. The synthetic medium according to Verduyn et al. (1992) contained 3 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 15 mg L<sup>-1</sup> EDTA, 4.5 mg L<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.84 mg L<sup>-1</sup> MnCl<sub>2</sub>·2H<sub>2</sub>O, 0.3 mg L<sup>-1</sup> CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.3 mg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.4 mg L<sup>-1</sup> NaMoO<sub>4</sub>·2H<sub>2</sub>O, 4.5 mg L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 3 mg L<sup>-1</sup> FeSO<sub>4</sub>·7H<sub>2</sub>O, 1 mg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, and 0.1 mg L<sup>-1</sup> KI. In case ammonium sulfate was used as the nitrogen source 5 g L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added. After heat sterilization, an appropriate aliquot of vitamin stock solution was added. The final concentrations of vitamins were 0.05 mg L<sup>-1</sup> D-(+)-biotin, 1 mg L<sup>-1</sup> D-pantothenic acid hemicalcium salt, 1 mg L<sup>-1</sup> nicotinic acid, 25 mg L<sup>-1</sup> myo-inositol, 1 mg L<sup>-1</sup> thiamine chloride hydrochloride, 1 mg L<sup>-1</sup> pyridoxine hydrochloride, and 0.2 mg L<sup>-1</sup> 4-aminobenzoic acid. In case urea was used as the nitrogen source, an appropriate aliquot of a stock solution was added after heat sterilization to obtain a final concentration of 2.8 g L<sup>-1</sup>. For buffering the synthetic medium, 100 mM of potassium hydrogen phthalate was added to the medium prior autoclaving. The pH of the synthetic glycerol medium was adjusted to 4.0 (for non-buffered medium) and to 5.0 (for medium buffered with potassium hydrogen phthalate) with 2 M H<sub>3</sub>PO<sub>4</sub>. A pH of 4-5 was previously determined to be optimal for the growth on glycerol of wild-type CBS 6412-13A (unpublished results). The complex media used were YPG<sub>6%</sub> containing 10 g L<sup>-1</sup> yeast extract, 20 g L<sup>-1</sup> peptone and 60 mL L<sup>-1</sup> glycerol or YG containing only 10 g L<sup>-1</sup> yeast extract in addition to 60 mL L<sup>-1</sup> glycerol. The pH of the complex media was adjusted to 6.0 with 2 M H<sub>3</sub>PO<sub>4</sub>. Except for the strain PDO-FPS-TPI1<sub>down</sub>-DHA-*EcmgsA*<sub>2</sub>, all liquid media were supplemented with 30 mg L<sup>-1</sup> phleomycin for plasmid maintenance.

For pre-cultivation, cells from a single colony were generally used to inoculate 3 mL of the respective medium in a 10 mL glass tube and incubated at orbital shaking of 200 rpm and 30°C for 24 (complex glycerol medium) to 48 h (synthetic glycerol medium). The pre-culture was used to inoculate 10 mL of the same medium in a 100 mL Erlenmeyer flask adjusting an  $OD_{600}$  of 0.2. This intermediate culture was cultivated at the same conditions until an  $OD_{600}$  of ~2.0 was reached. The appropriate culture volume (in order to later adjust an  $OD_{600}$  of 0.2 in 50 mL of medium) was centrifuged at 800 g for 5 min and the supernatant discarded. The cell pellet was then resuspended in 50 mL of the respective glycerol-containing medium in a 500 mL Erlenmeyer flask. The main cultures were incubated at orbital shaking of 200 rpm and 30°C and samples for  $OD_{600}$  determination and HPLC analysis were taken at regular time intervals.

## 2.8 Cultivations in controlled bioreactors

All reactor-based cultivations were carried out using Sartorius 1 L bioreactors (Sartorius, Stedim Biotech, Göttingen, Germany) with equivalent working volumes and equipped with 2 Rushton six-blade disc turbines. The pH electrode (Mettler Toledo, OH, USA) was calibrated according to manufacturer's standard procedures and pH was controlled by automatic addition of 2 M NaOH and  $H_2SO_4$ . The bioreactor was sparged with sterile atmospheric air and the temperature was maintained at 30°C throughout the cultivations. Culture conditions were: pH 5, stirring rate: 800 rpm and air flow: 1 volume of air per volume of liquid per minute (vvm). Samples were taken across the exponential growth phase to monitor growth ( $OD_{600}$ ) as well as for HPLC analysis for metabolite profiling.

## 2.9 Analytical methods

Samples of culture supernatants were first filtered through 0.2  $\mu$ m Minisart RC membrane filters (Sartorius) and stored at -20°C until analysis. The concentrations of glycerol, 1,2-PDO and ethanol in culture media were determined using a Waters HPLC system (Eschborn, Germany) equipped with two consecutive Aminex HPX-87H cation-exchange columns (Biorad, München, Germany) using

5 mM H<sub>2</sub>SO<sub>4</sub> as the mobile phase at a flow rate of 0.45 mL min<sup>-1</sup> and a column temperature of 45°C. Volumes of 20 µL of sample were used for injection. All components were detected using the Waters 2414 Refractive Index detector. Under these conditions, the retention times were 35.6 min for glycerol, 45.4 min for 1,2-PDO and 57.7 min for ethanol. For quantification, standards were run in appropriate concentrations (i.e. between 1 g L<sup>-1</sup> and 100 g L<sup>-1</sup> for glycerol, 0.075 g L<sup>-1</sup> and 5 g L<sup>-1</sup> for 1,2-PDO, and 0.394 g L<sup>-1</sup> and 39.37 g L<sup>-1</sup> for ethanol). The obtained raw data were processed using the Breeze 2 software (Waters).

### 3. RESULTS

#### 3.1 Modular metabolic engineering of *S. cerevisiae* for the production of 1,2-PDO from glycerol

All metabolic engineering steps in this study were conducted in the genetic background of our previously described and intensively studied haploid meiotic segregant CBS 6412-13A originating from the glycerol-utilizing wild-type *S. cerevisiae* strain CBS 6412 (Swinnen et al., 2016). This strain exhibiting a  $\mu_{\max}$  of 0.13 h<sup>-1</sup> in synthetic glycerol medium was the best-performing glycerol-utilizing *S. cerevisiae* strain available to us at the time when the current study has been initiated. As demonstrated in our previous study, the strain CBS 6412-13A solely utilizes the native FAD-dependent G3P pathway for glycerol catabolism (Swinnen et al., 2013).

As *S. cerevisiae* does not produce 1,2-PDO naturally, the first engineering module implemented during strain construction was the expression of the so-called 'methylglyoxal pathway' (see section 1 and Figure 1). The key enzyme of this pathway is methylglyoxal synthase (MGS) and several previous studies have shown that even the sole expression of *E. coli mgsA* in *S. cerevisiae* resulted in the production of small amounts of 1,2-PDO (Hoffman, 1999; Jeon et al., 2009; Lee and DaSilva, 2006). Nevertheless, the additional expression of *E. coli gldA* encoding a glycerol dehydrogenase was demonstrated to increase 1,2-PDO production in *S. cerevisiae* (Hoffman, 1999). The *E. coli* aldehyde reductase encoded by *yqhD* was also considered here to be a useful enzyme for converting methylglyoxal to 1,2-PDO. The enzyme was shown to reduce methylglyoxal *in vitro* (Jarboe, 2011; Lee

et al., 2010) and has been successfully used to improve 1,2-PDO formation in engineered *C. glutamicum* additionally expressing *E. coli mgsA* and *gldA* (Siebert and Wendisch, 2015). Therefore, we decided to use codon-optimized versions of the *E. coli* genes *mgsA*, *yqhD* and *gldA* (referred to as *EcmgsA*, *EcyqhD* and *EcglDA*, respectively) in the current study and placed them between different *S. cerevisiae* promoters and terminators (see Material and Methods) in order to establish a '1,2-PDO module' in yeast. All engineered strains harboring this module (referred to as 'PDO') carried head-to-tail genomic integrations of *S. cerevisiae* expression cassettes for *EcmgsA* and *EcglDA*, while the expression cassette for *EcyqhD* was expressed from a *S. cerevisiae* low-copy expression vector (see section 2.5 and Table 1).

While establishing the module 'PDO', a parallel study in our laboratory revealed that the expression of a heterologous glycerol facilitator significantly improves the growth of strain CBS 6412-13A in synthetic glycerol medium (Klein et al., 2016b). As we assumed that an improved glycerol uptake might also be beneficial for 1,2-PDO production from glycerol, we decided to integrate an expression cassette bearing the coding sequence of *FPS1* from *Cyberlindnera jadinii* (referred to here as *CjFPS1*) into the genome of all strains engineered for 1,2-PDO production. Accordingly, the term 'FPS' was included in the respective strain names (see section 2.5 and Table 1). The strain CBS 6412-13A expressing *CjFPS1* was used as the negative reference throughout this study but *CjFPS1* expression was not considered here as a separate module.

A second module addressed the co-factor supply for 1,2-PDO formation. The three step conversion from DHAP to 1,2-PDO includes two enzymatic reactions that require reducing equivalents in the form of cytosolic NAD(P)H (Figure 1). As mentioned in the introduction (section 1), the native FAD-dependent glycerol catabolic pathway (up to DHAP) in *S. cerevisiae* does not deliver cytosolic reducing equivalents. However, the pathway can be functionally replaced by an NAD<sup>+</sup>-dependent 'DHA pathway' as recently demonstrated by Klein et al. (2016a). The respective genetic modifications included the plasmid-based heterologous expression of glycerol dehydrogenase from *Ogataea parapolymorpha* (*Opgdh*), the overexpression of endogenous dihydroxyacetone kinase (*DAK1*) and

the deletion of endogenous glycerol kinase (*GUT1*). These modifications are collectively referred to as module 'DHA' in the respective strain names (Table 1). This module has been supposed to strongly increase cytosolic NADH availability for 1,2-PDO production since the FAD-dependent native pathway for glycerol catabolism has been completely abolished.

An additional module addressed the need for increased availability of DHAP, the metabolic precursor for the 'methylglyoxal pathway'. The introduction of the heterologous 1,2-PDO pathway creates a metabolic branch point at which MGS must compete with the endogenous glycolytic enzyme triosephosphate isomerase (TPI) (encoded by *TPI1*) for their common substrate DHAP. Jung et al. (2008) showed that the deletion of the *TPI1* gene in *S. cerevisiae* bearing a heterologous 1,2-PDO pathway resulted in increased 1,2-PDO production in a medium containing a mix of glucose, galactose and ethanol as carbon sources. However, the complete abolishment of TPI activity is not feasible when glycerol serves as the sole source of carbon since this enzyme is essential to channel the carbon flux from the substrate glycerol into the central carbon metabolism (i.e. both glycolysis and gluconeogenesis). Therefore, a strategy for downregulating *TPI1* expression (without fully abolishing it) via promoter replacement was employed. In a previous study, a collection of mutant promoters with gradually decreasing strengths (based on the constitutive *TEF1* promoter) had been generated (Alper et al., 2005). Nevoigt et al. (2006) used this collection to develop sets of promoter replacement cassettes allowing the exchange of any promoter in the *S. cerevisiae* genome with a *TEF1* promoter variant of defined strength. Two weak promoters from this collection (*TEFmut7* and *TEFmut2*) have initially been tested in the current study (Figure S2 and the respective supplementary information), and we eventually selected the weakest promoter (*TEFmut2*) in order to replace the native *TPI1* promoter. The respective genetic modification is referred to here as module 'TPI<sub>1down</sub>' (Table 1). The impact of the promoter replacement on growth of the corresponding intermediate strain in synthetic medium containing different carbon sources is shown in Figure S2 (supplementary information). These results are an indirect proof for the fact that the promoter replacement (using the *TEFmut2* promoter) indeed resulted in decreased TPI activity.

### 3.2 The choice of cultivation medium strongly affected 1,2-PDO production of the engineered strains

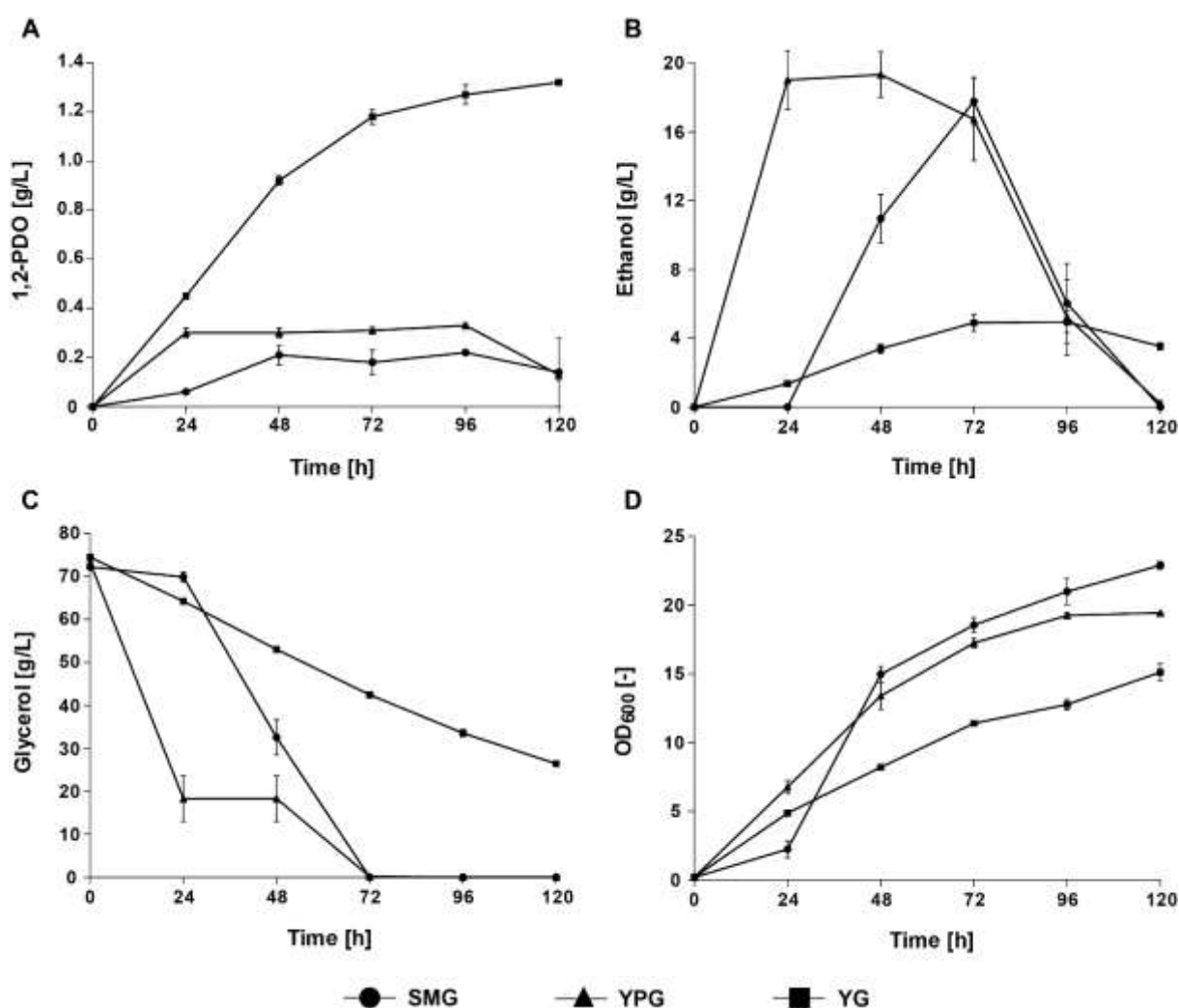
Our initial intention was to use synthetic glycerol medium for studying the impacts of the different engineering modules on 1,2-PDO production. We decided to use urea as the source of nitrogen in our synthetic medium as it, in contrast to ammonium sulfate, does not lead to medium acidification and is therefore known to result in much higher final optical densities compared to synthetic medium with ammonium sulfate. In the urea-containing medium, the strain PDO-FPS-TPI1<sub>down</sub>-DHA produced less than 0.2 g L<sup>-1</sup> after 120 h of cultivation in synthetic glycerol medium (Figure 2A). Instead, significant concentrations of ethanol indicating an onset of fermentative metabolism were detected in the first half of the cultivation with a peak of up to 18 g L<sup>-1</sup> after 72 h. Later, the ethanol concentration decreased (Figure 2B).

In a previous study, Jung et al. (2011) successfully demonstrated the production of 1,2-PDO in engineered *S. cerevisiae* when using a complex medium (YPG containing 10 g L<sup>-1</sup> yeast extract, 20 g L<sup>-1</sup> peptone, 10 g L<sup>-1</sup> glycerol and 1 g L<sup>-1</sup> galactose). We therefore tested our advanced strain PDO-FPS-TPI1<sub>down</sub>-DHA in YPG<sub>6%</sub> medium (10 g L<sup>-1</sup> yeast extract, 20 g L<sup>-1</sup> peptone and 60 mL L<sup>-1</sup> glycerol). However, only a slight improvement of 1,2-PDO production could be observed compared to synthetic medium with urea. The highest detected titer was ~0.3 g L<sup>-1</sup> after 96 h of cultivation. Notably, the switch to YPG<sub>6%</sub> medium resulted in faster growth, glycerol consumption as well as ethanol production (Figure 2). Under these conditions, the strain accumulated up to 18 g L<sup>-1</sup> ethanol already during the first 24 h of cultivation (Figure 2B).

Surprisingly, omitting peptone from our YPG<sub>6%</sub> medium remarkably increased 1,2-PDO production from ~0.3 g L<sup>-1</sup> to ~1.3 g L<sup>-1</sup> where most of the product accumulated during the first 48 h (Figure 2A). The plausible nutrient limitation caused a much slower growth and is also reflected by a slower rate of glycerol consumption. Surprisingly, much less ethanol was produced in the absence of peptone (Figure 2B). On the basis of these results, we decided to first characterize the influence of the



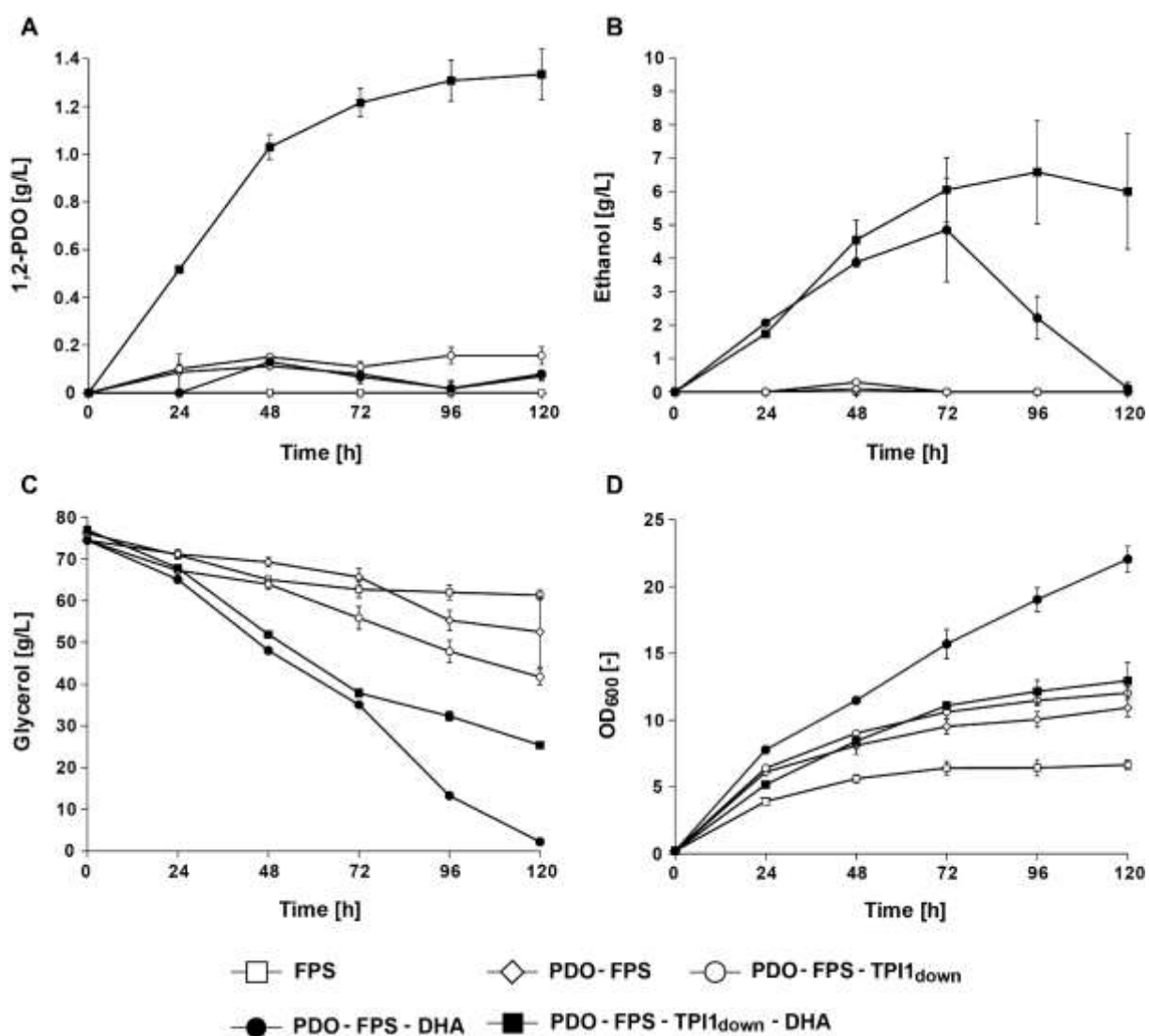
different metabolic engineering modules on 1,2-PDO production in YG<sub>6%</sub> medium and dedicated separate experiments scrutinizing the medium effects on 1,2-PDO production.



**Figure 2 (2 column fitting image).** Physiological characterization of strain PDO-FPS-TPI1<sub>down</sub>-DHA in three different cultivation media containing 60 mL L<sup>-1</sup> glycerol as the sole carbon source. The strain carried all engineering modules as described in section 3.1. Cultivations were performed in 50 mL shake flask cultures and culture supernatants were analyzed by HPLC for the production of 1,2-propanediol (1,2-PDO) (A), ethanol (B) and the consumption of glycerol (C). Growth was recorded by determining the optical density of the cultures at a wavelength of 600 nm (OD<sub>600</sub>) (D). The different media used were SMG (synthetic defined glycerol medium containing urea as the nitrogen source), YPG (complex glycerol medium containing 1% yeast extract and 2% peptone) and YG (complex glycerol medium containing 1% yeast extract). All mean values and standard deviations are derived from at least three biological replicates.

### 3.3 The combination of modules DHA and TPI1<sub>down</sub> was crucial to achieve the highest concentration of 1,2-PDO in YG<sub>6%</sub> medium

As mentioned in section 3.2, YG<sub>6%</sub> medium was used in order to characterize all constructed strains and dissect the impacts of the individual modules and their combinations on 1,2-PDO production and the cells' physiology (Figure 3).



**Figure 3 (2 column fitting image).** Physiological characterization of *S. cerevisiae* strains (derivatives of CBS 6412-13A) engineered for 1,2-PDO production from glycerol in YG medium containing 60 mL L<sup>-1</sup> glycerol. Besides an expression cassette for the glycerol facilitator encoded by *CjFPS1* ('FPS'), the analyzed strains carried the engineering module 'PDO' alone or in combination with the modules 'DHA' and 'TPI1<sub>down</sub>' as described in section 3.1. Cultivations were performed in 50 mL shake flask cultures and culture supernatants were analyzed by HPLC for the production of 1,2-PDO (A), ethanol (B) and the consumption of glycerol (C).

Growth was recorded by optical density measurements at 600 nm (D). Mean values and standard deviations were determined from at least three biological replicates.

Compared to the control strain FPS, all strains bearing the '1,2-PDO module' produced at least small amounts of 1,2-PDO (Figure 3 A). Interestingly, neither the sole downregulation of *TPI1* expression (module 'TPI1<sub>down</sub>') nor the sole glycerol catabolic pathway replacement (module 'DHA') in the genetic background of the strain PDO-FPS resulted in a significant improvement of the 1,2-PDO titer. Only the combination of these two metabolic engineering modules with the '1,2-PDO module' considerably improved 1,2-PDO production from less than 0.2 g L<sup>-1</sup> to ~1.3 g L<sup>-1</sup> when measured after 120 h of cultivation. The results demonstrate that both above-mentioned modules were essential for 1,2-PDO production from glycerol.

The glycerol catabolic pathway replacement in strain PDO-FPS improved the strain's glycerol consumption (Figure 3C, strains PDO-FPS-DHA and PDO-FPS-TPI1<sub>down</sub>-DHA) indicating that the DHA pathway allows a better flux from glycerol to DHAP compared to the G3P pathway present in the wild-type strain. In strain PDO-FPS-DHA, the increased glycerol consumption coincides with a faster growth, while the presence of the module 'TPI1<sub>down</sub>' seems to abrogate the growth supporting effect of module 'DHA' as visible in the growth curve for strain PDO-FPS-TPI1<sub>down</sub>-DHA (Figure 3D).

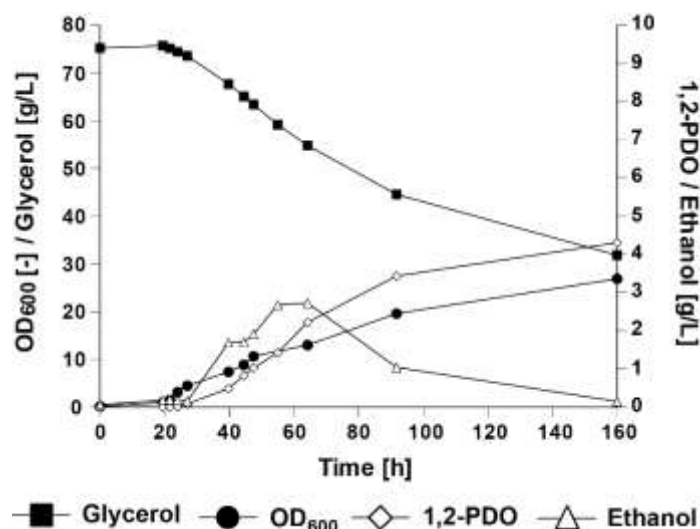
Our data clearly show that the genetic manipulation mainly responsible for the production of ethanol observed in YG<sub>6%</sub> medium is the replacement of the endogenous glycerol catabolic pathway by the NADH-generating DHA pathway (Figure 3B). A switch to fermentative metabolism (visible by ethanol formation) is plausible when considering the excess of cytosolic NADH generated by the latter pathway. Again, the ethanol production was particularly high in the first half of the cultivation period, while its concentration declined during the second part of the cultivation (Figure 3B). During the second phase of cultivation, glycerol was still present and its concentration continued to decrease. Interestingly, the superior growth of strain PDO-FPS-DHA coincided with a strong decrease in ethanol concentration while the respective reduction in ethanol concentration during the second

cultivation half was very low in the strain with additional downregulation of *TPI1* expression (Figure 3B).

### 3.4 Implementation of a second expression cassette for *E. coli* MGS and analysis in controlled batch cultivation in bioreactors

In the previous section, it was shown that the modules for cofactor-supply and carbon flux re-direction ('DHA' and 'TPI1<sub>down</sub>', respectively) were essential genetic manipulations to increase the capacity of *S. cerevisiae* to produce 1,2-PDO from glycerol. The next question that arose was whether the flux into the 1,2-PDO pathway was rate-controlling for 1,2-PDO production in the strain PDO-FPS-TPI1<sub>down</sub>-DHA. At this stage, we decided to construct a strain that carries all genetic modifications within its genome (including the implementation of an additional expression cassette for *E. coli* *mgsA*) resulting in strain PDO-FPS-TPI1<sub>down</sub>-DHA *EcmgsA*<sub>2</sub>. This also facilitated the physiological characterization in larger volumes since no antibiotic had to be added anymore. When this strain was analyzed in shake flasks under the same conditions as used before, the additional *EcmgsA* expression cassette resulted in increased 1,2-PDO production (~2.5 g L<sup>-1</sup> after 140 h of cultivation) while the maximum ethanol titer decreased from ~6.6 g L<sup>-1</sup> to ~3.8 g L<sup>-1</sup> as shown in Figure S3 (supplementary information).

As a next step, the strain PDO-FPS-TPI1<sub>down</sub>-DHA *EcmgsA*<sub>2</sub> was characterized under controlled conditions in batch cultivations in bioreactors using the same medium (YG<sub>6%</sub>). In general, the strain's physiology was very similar to that observed in shake flask cultures. However, the controlled cultivation conditions allowed the cells to reach higher optical densities than in shake flasks. The maximum 1,2-PDO titer reached was ~4.3 g L<sup>-1</sup> (Figure 4). This has been the highest reported 1,2-PDO titer obtained by fermentations with engineered *S. cerevisiae*.



**Figure 4 (single column fitting image). Physiological characterization of strain PDO-FPS-TPI1<sub>down</sub>-DHA-EcmgsA<sub>2</sub> in batch cultivations in 1 L bioreactors using YG medium containing 60 mL L<sup>-1</sup> glycerol as the carbon source.** The analyzed strain carried genomic integrations of all engineering modules, i.e. besides the glycerol facilitator encoded by *CjFPS1* ('FPS'), the methylglyoxal pathway ('PDO') including a second expression cassette for *E. coli mgsA* (*EcmgsA<sub>2</sub>*), the glycerol catabolic pathway replacement ('DHA') and the replacement of the native *TPI1* promoter by the weak *TEF1* promoter variant *TEFmut2* ('TPI1<sub>down</sub>'). One representative out of three experiments is shown.

### 3.5 1,2-PDO production in buffered synthetic medium with ammonium sulfate as a carbon source

The rather surprising results concerning the influence of the medium composition on the observable product pattern encouraged us to conduct a number of experiments in order to gain further insight into this phenomenon. The results (data not shown) revealed that the impact of medium components is very complex and most probably even strain dependent. Further experiments have to be carried out in order to dissect and understand the observed medium effects. Among the already performed experiments regarding medium effects, there has been one that was set to test whether glycerol fermentation is also visible in synthetic glycerol medium with ammonium sulfate as the nitrogen source as soon as a buffer is added and thereby rapid medium acidification is avoided resulting in significantly higher optical densities. This question came up after we detected significant ethanol production in our strains with the 'DHA' module (sections 3.2 and 3.3), a fact that has not

been recognized in our previous study regarding DHA pathway strains (Klein et al., 2016a). However, the earlier study was performed using unbuffered synthetic medium with ammonium sulfate as the nitrogen source. Therefore, 100 mM potassium phthalate buffer was added to synthetic medium containing either ammonium sulfate or urea. This allowed a direct comparison of the two nitrogen sources. The respective experiment was performed with the above-mentioned strain PDO-FPS-TPI1<sub>down</sub>-DHA *EcmgsA*<sub>2</sub> carrying two expression cassettes for *EcmgsA* and in which all genetic modifications were stably integrated into the genome. To our huge surprise, the buffered ammonium sulfate containing medium led to a significant formation of 1,2-PDO and a comparably low ethanol production which was in clear contrast to the results obtained for the urea-containing medium (Figure 5). The remarkable difference with regard to the 1,2-PDO/ethanol ratio between the two nitrogen sources (Figure 5A and B) cannot simply be explained by the obtained culture density (Figure 5A). In fact, the used nitrogen source seemed to have a (so far unknown) impact on the carbon flux distribution in our engineered strains.

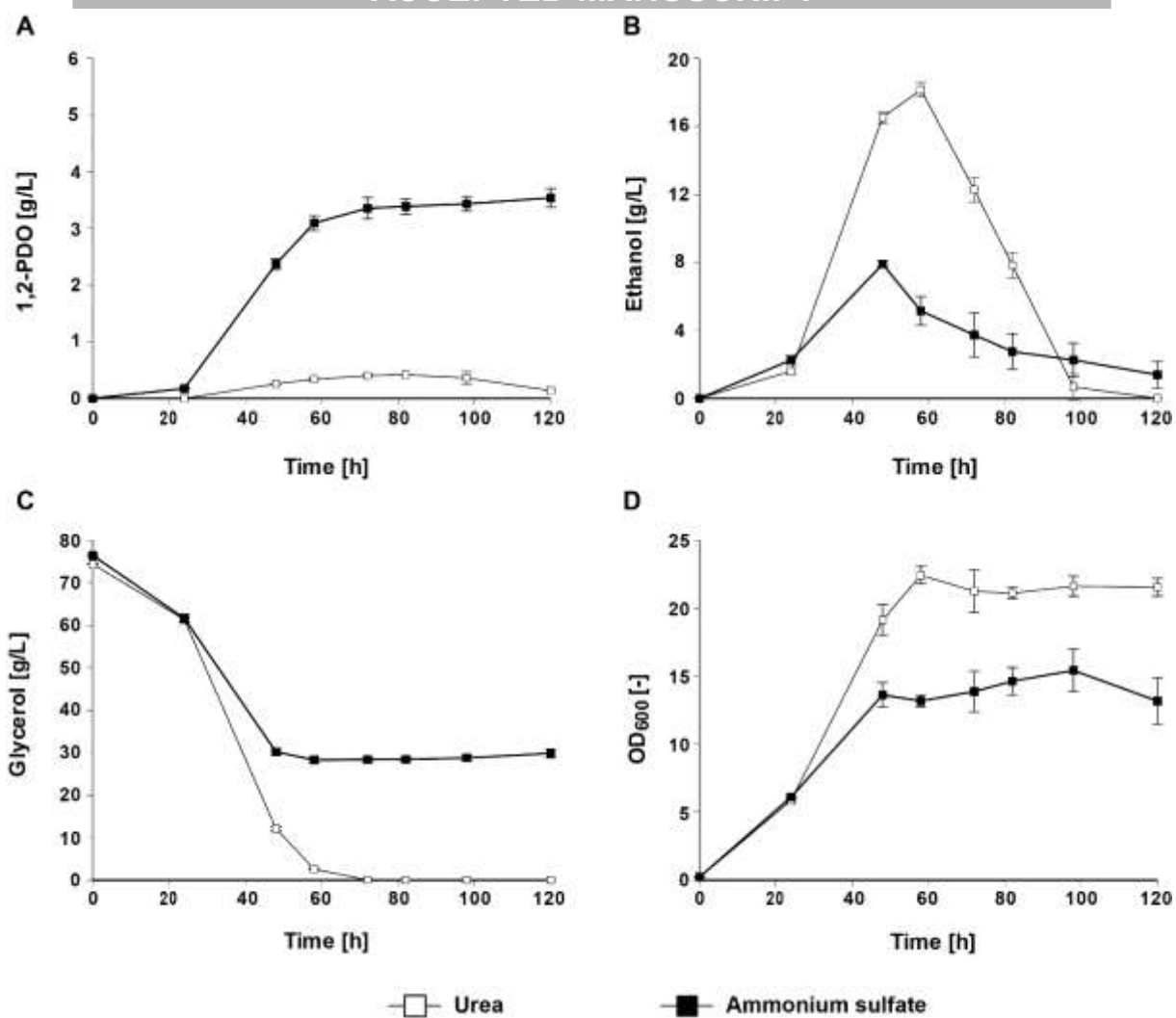


Figure 5 (2 column fitting image). Influence of the used nitrogen source on the physiology of the *S. cerevisiae* strain PDO-FPS-TPI1<sub>down</sub>-DHA-EcmgsA<sub>2</sub> engineered for 1,2-PDO production from glycerol in synthetic medium buffered with 100 mM potassium phthalate buffer. The initial pH of the media was set to 5.0. Cultivations were performed in 50 mL shake flask cultures and culture supernatants were analyzed by HPLC for the production of 1,2-PDO (A), ethanol (B) and the consumption of glycerol (C). Growth was recorded by optical density measurements at 600 nm (D). Mean values and standard deviations were determined from at least three biological replicates.

#### 4. Discussion

In this study, a novel metabolic engineering approach was used to produce 1,2-PDO in *S. cerevisiae* from glycerol as the sole carbon source. A previous attempt to establish 1,2-PDO production from glycerol in *S. cerevisiae* (equipped with the 'methylglyoxal pathway') focused on the overexpression of *GUT1* and *GUT2* which encode the enzymes for the native (FAD-dependent) glycerol catabolic pathway (Jung et al., 2011). However, the electrons stemming from glycerol oxidation through this pathway are eventually transferred to oxygen via the respiratory chain. In contrast, our current approach was based on the complete replacement of the endogenous G3P pathway by the NAD<sup>+</sup>-dependent DHA pathway according to Klein et al. (2016a). This replacement has been supposed to provide cytosolic reducing equivalents from glycerol and facilitate the formation of fermentation products such as 1,2-PDO. The current study demonstrates that the module 'DHA' indeed increased the formation of the target product at least when TPI expression was reduced in parallel thereby increasing the availability of the precursor DHAP. Notably, downregulation of TPI expression alone did not result in significant 1,2-PDO formation. These results underline the importance of providing sufficient concentrations of both pre-cursor and co-factor for the successful re-direction of metabolic fluxes. The fact that 1,2-PDO production in the presence of increased pre-cursor and co-factor could be even further increased by a second *mgsA* expression cassette demonstrates that the flux through methylglyoxal synthase has been the rate-controlling step in the respective genetic background and might be one important target for further strain improvement.

As already mentioned, the 1,2-PDO titer of > 4 g/l achieved in the current study has been the highest reported for *S. cerevisiae*. This titer obtained in the bioreactor experiment in YG medium corresponded to a maximum product yield of  $\sim 129 \pm 21$  mg per g glycerol consumed. Jung et al. (2011) reported a 1,2-PDO yield of 213 mg per g glycerol consumed. However, their experiments were performed in modified YEPD medium (yeast extract 1%, peptone 2%, glycerol 1% and galactose 0.1%). Although the galactose was only added for induction of gene expression in the latter study, its utilization as a carbon source may have falsified the calculated yields. Moreover, ingredients of the



added peptone might have also been used as minor carbon sources. These facts hamper a reliable comparison with our results.

Regarding the pathway from DHAP to 1,2-PDO, there are several possible metabolic routes which may result in either R- or S-1,2-PDO (Figure 1). However, we have not dissected in our analytics so far whether only one or both enantiomers were formed by our engineered *S. cerevisiae* strains. In addition, it has been difficult to draw a strong hypothesis on the actual carbon flux solely based on published knowledge. First, the genes *EcglcA* and *EcyqhD* encode for enzymes with relatively broad substrate specificities (Jarboe, 2011; St. Martin et al., 1977; Tang et al., 1979). Second, the gene *Opgdh* originally used for the module 'DHA' might also contribute to the reductive reactions from methylglyoxal to 1,2-PDO to a certain degree since its expression (instead of *EcglcA*) in a PDO<sub>min</sub> strain also increased 1,2-PDO formation as compared to the sole expression of *EcmgsA* (results not shown). Third, endogenous (probably rather unspecific) reductases of the yeast *S. cerevisiae* have also to be considered to be active in the respective chemical conversions as mentioned in section 1. Nevertheless, our 1,2-PDO producing strains formed a significant amount of an additional product as visible by a conspicuous peak in the HPLC (data not shown). HPLC analysis of the retention times of the pathway intermediates methylglyoxal, R-/S-lactaldehyde and acetol indicated that acetol might be the compound that was accumulated by our 1,2-PDO producer. The retention time of lactaldehyde under the employed conditions is close to that of glycerol and the latter was still present in relatively large concentrations in the medium at the end of the cultivation period in most of our experiments. Therefore, a peak for lactaldehyde is assumed to partially overlap with the peak for glycerol. At least, we did not recognize any shoulder for the glycerol peak in our chromatograms. Notably, other authors expressing the same *E. coli* genes for 1,2-PDO formation in *C. glutamicum* also reported significant acetol formation (Siebert and Wendisch, 2015), while Altaras and Cameron (1999) found R-lactaldehyde as an intermediate in an *E. coli* strain overexpressing *E. coli mgsA* and *gldA*. Future work including metabolomics will focus on a more comprehensive characterization of the carbon flow in our engineered strains.

Glycerol has been considered as a carbon source that is solely metabolized in a respiratory manner in *S. cerevisiae*. The full dependence of glycerol catabolism on respiration in wild-type *S. cerevisiae* strains can be explained by the fact that the electrons are directly transferred by Gut2 via FADH<sub>2</sub> to the mitochondrial respiratory chain. This also holds for strains which display higher growth rates (and glycolytic flux) in synthetic glycerol medium than the majority of wild-type *S. cerevisiae* strains. In fact, Ochoa-Estopier et al. (2011) comprehensively studied an evolved derivative of CBS 8066 (FL20) that exhibited a  $\mu_{\max}$  of 0.20 h<sup>-1</sup> in synthetic glycerol medium in a controlled bioreactor setup and no reduced fermentation products such as ethanol were detected. Similarly, no fermentation products were detected in shake-flask cultivations using our non-engineered wild-type *S. cerevisiae* strain CBS 6412-13A which is able to grow in synthetic glycerol medium with a  $\mu_{\max}$  of 0.13 h<sup>-1</sup> via the G3P pathway (data not shown).

In the view of the current study it is, however, important to note that the applied glycerol catabolic pathway replacement strategy rendered the strain's glycerol catabolism solely dependent on NAD<sup>+</sup>. This has been assumed to result in surplus cytosolic NADH and to facilitate the production of fermentation products in our strains such as 1,2-PDO (heterologous pathway) and/or ethanol (*S. cerevisiae*'s major native fermentation product). Indeed, our data indicates that cells, which depend on the DHA pathway for glycerol catabolism, at least partially ferment the glycerol, even though both the degree of fermentation and the ratio between 1,2-PDO and ethanol formation are strain- and medium dependent.

So far, we cannot dissect whether the surplus of cytosolic NADH caused the fermentative metabolism of glycerol right at the beginning of the cultivation or whether any kind of nutrient or oxygen limitation was an additional requirement for the metabolic switch. At least, one has to note that ethanol formation was also visible in the bioreactor experiment employing the YG<sub>6%</sub> medium where air was flushed with a rate of 1 vvm (stirring rate 800 rpm) into the culture. The underlying basis why 1,2-PDO is produced in significant amounts in YG<sub>6%</sub> medium (and in the buffered ammonium sulfate-containing synthetic medium) while only trace amounts of the target product

were formed in synthetic medium with urea as a nitrogen source and in YPG<sub>6%</sub> medium (where ethanol is the prominent fermentation product) has not been clarified yet. By unknown reasons, the engineered 1,2-PDO pathway can probably better compete with the endogenous ethanol formation pathway in the first two media.

It has been very intriguing that the concentration of ethanol in the medium started to decrease at a certain point in time. In general, the actual ethanol concentration could have been affected by three different processes which might, at least partly, have occurred in parallel: i) ethanol formation, ii) evaporation and iii) ethanol consumption. Evaporation was tested in a parallel experiment using medium without cells and an initial ethanol concentration of 25 g L<sup>-1</sup>. Under the applied conditions, the ethanol evaporated with a maximum rate of ~0.10 g L<sup>-1</sup> h<sup>-1</sup>. This rate was lower than observed for the decrease in ethanol concentration for the analyzed cultures which varied between 0.16 and 0.44 g L<sup>-1</sup> h<sup>-1</sup>. Although its impact can definitely not be neglected, evaporation can therefore not completely explain the observed decrease in ethanol concentration in our experiments. Thus, it can be concluded that ethanol might have been consumed together with glycerol and even further when glycerol was completely depleted (Figure 2). Assuming the actual occurrence of ethanol consumption by the cells, the observed decrease of the ethanol titer in the second cultivation period may have been caused by the fact that ethanol production by fermentation stopped (or at least slowed down) while respiratory ethanol utilization continued or even increased. A reduction in glycerol consumption rate at a later stage in the cultivation was also visible. Although we do not have a plausible explanation for this result, it might underpin our hypothesis with regard to a certain overflow metabolism at increased glycerol consumption rates. The lower glycerol consumption rate might have resulted in less NADH generation and the rate of respiration might have been sufficient to reoxidize the formed NADH explaining why the cells did not need fermentation at the later phase of cultivation. Nevertheless, we cannot exclude that regulatory mechanisms changed the rate of fermentation and/or respiration at this point in time and further work will be necessary to clarify this aspect.

The data presented in the current manuscript demonstrate that *S. cerevisiae* can be engineered to produce significant concentrations of 1,2-PDO ( $> 4 \text{ g L}^{-1}$  in a controlled bioreactor set-up). Our study shows that both metabolic precursor and co-factor supply are essential for target product formation. The work also demonstrates the crucial impact of the medium composition and the experimental conditions on the metabolic mode and, thus product titer and yield. We therefore believe that there is still a lot of room for further improving 1,2-PDO production in *S. cerevisiae*.

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### Highlights

- Highest reported 1,2-propanediol (1,2-PDO) titer for yeast ( $>4 \text{ g L}^{-1}$ ) from glycerol
- NADH-generating glycerol catabolic pathway crucial for 1,2-PDO formation
- Increased metabolic supply of precursor dihydroxyacetone phosphate essential
- Strong impact of medium composition on 1,2-PDO to ethanol ratio