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ORIGINAL PAPER

Elimination of glycerol and replacement with alternative products in ethanol fermentation by *Saccharomyces cerevisiae*

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Abstract Glycerol is a major by-product of ethanol fermentation by *Saccharomyces cerevisiae* and typically 2–3% of the sugar fermented is converted to glycerol. Replacing the NAD^+-regenerating glycerol pathway in *S. cerevisiae* with alternative NADH reoxidation pathways may be useful to produce metabolites of biotechnological relevance. Under fermentative conditions yeast reoxidizes excess NADH through glycerol production which involves NADH-dependent glycerol-3-phosphate dehydrogenases (Gpd1p and Gpd2p). Deletion of these two genes limits fermentative activity under anaerobic conditions due to accumulation of NADH. We investigated the possibility of converting this excess NADH to NAD^+ by transforming a double mutant (*gpd1*Δ*gpd2*Δ) with alternative oxidoreductase genes that might restore the redox balance and produce either sorbitol or propane-1,2-diol. All of the modifications improved fermentative ability and/or growth of the double mutant strain in a self-generated anaerobic high sugar medium. However, these strain properties were not restored to the level of the parental wild-type strain. The results indicate an apparent partial NAD^+ regeneration ability and formation of significant amounts of the commodity chemicals like sorbitol or propane-1,2-diol. The ethanol yields were maintained between 46 and 48% of the sugar mixture. Other factors apart from the maintenance of the redox balance appeared to influence the growth and production of the alternative products by the genetically manipulated strains.

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Keywords Yeast · Redox imbalance · Glycerol · Sorbitol · Propane-1,2-diol · Ethanol

Introduction

Glycerol is a major by-product of ethanol fermentation (7–10% of the ethanol produced) by *Saccharomyces cerevisiae* and typically 2–3% of the sugar fermented is converted to glycerol [13, 34]. Recovery of glycerol from ethanol fermentations is currently not economically viable. Traditionally commercial glycerol was mainly produced as a by-product in soap and oleochemicals production, and more recently from the biodiesel industry which has resulted in a price collapse due to the excess of glycerol in the world market [11]. Elimination of glycerol from the ethanol fermentation process and possible replacement with other valuable products could have significant commercial advantages. In yeast, glycerol is produced in a two-step branch of the glycolytic pathway. The first reaction involves the catalysis of dihydroxyacetone phosphate to glycerol-3-phosphate by cytosolic NADH-dependent glycerol-3-phosphate dehydrogenases (Gpd) [13]. The two isogenes coding for Gpd in *S. cerevisiae* are the osmotically induced *GPD1* [2] and the anaerobically induced *GPD2* [3]. The second step involves dephosphorylation of glycerol-3-phosphate to yield glycerol [32]. Under osmotic stress conditions glycerol is accumulated within the cell and acts as an osmolyte [26]. A *gpd1*Δ*gpd2*Δ double mutant (DM) is unable to grow under strict anaerobic conditions because NAD^+ regeneration through glycerol production is no longer possible and intracellular NADH is accumulated, resulting in a redox imbalance [3]. In principle, this excess NADH should be available to drive other NADH-dependent reduction reactions. The introduction of

heterologous proteins which catalyze NADH-coupled reactions should allow the DM to restore its redox balance and support anaerobic growth.

The objective of this study was to investigate the manipulation of the *gpd1*Δ*gpd2*Δ strain by inserting a number of alternative pathways able to oxidize excess NADH by producing compounds such as sorbitol and propane-1,2-diol which can be derived from intermediates of glycolysis (Fig. 1). Sorbitol is the most important commercial polyol with applications in the food, pharmaceutical and cosmetic industries. Propane-1,2-diol (also known as propylene glycol or 1,2-propanediol) is mainly used for the production of plastics, as an industrial solvent and has applications in the food and pharmaceutical industries. The large market (>500,000 tonnes/annum) for both compounds is growing rapidly [1, 5, 21]. Current production of sorbitol is by hydrogenation of sugars, whereas propane-1,2-diol is derived from petroleum [1, 5]. The biotechnological route offers a more environmentally friendly and specific process and a number of studies on the use of recombinant bacteria and yeasts to produce sorbitol and propane-1,2-diol have been reported. In most of these studies, the authors attempted to redirect the metabolic flux only towards these products with varying degrees of success [1, 5]. Sorbitol production was achieved by expressing the *E. coli srlD* gene encoding for NADH/NADPH-dependent sorbitol-6-phosphate dehydrogenase [19, 31, 38] or by overexpressing the native *SOR1* gene encoding for NADH-dependent sorbitol dehydrogenase [36] (Fig. 1). Production of propane-1,2-diol was achieved by a three-step process with simultaneous expression of the heterologous *gldA* and *mgsA* genes encoding for glycerol dehydrogenase [39] and methylglyoxal synthase [35], respectively, and overexpression of the native *GRE3* gene encoding for aldose reductase [22] (Fig. 1).

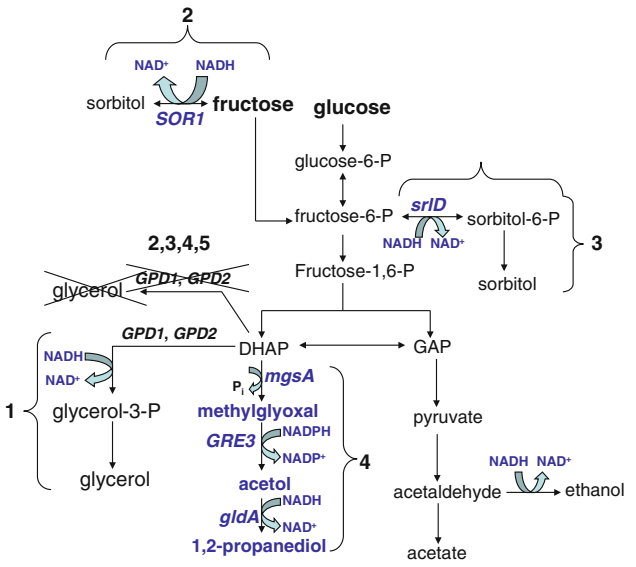

Fig. 1 Pathways of glucose and fructose metabolism in *S. cerevisiae* strains *1* WT, *2* DM(*SOR1*), *3* DM(*srlD*), *4* DM(*gldA*, *GRE3*, *mgsA*), *5* DM

Materials and methods

Yeast strains and genotypes

S. cerevisiae BY4742 wild-type (WT) and BY4742 *gpd1*Δ strains were obtained from the Euroscarf deletion library (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/yeast.html). The DM was created by deleting *GPD2* from the BY4742 *gpd1*Δ strain and was the recipient strain for various gene constructs. The yeast strains and their genotypes are as described in Table 1.

Strain construction, primers and PCR templates

Deletion of *GPD2* gene in the BY4742 *gpd1*Δ strain to obtain DM was carried out by using Ura3p as auxotrophic marker. Primers were designed to amplify the *URA3* gene from the Yep24 vector [33] containing the *URA3* gene with a 50-bp region homologous to the 5′ and 3′ parts of *GPD2* gene. The PCR product was then integrated into the BY4742 *gpd1*Δ strain to obtain DM. The primers used to delete *GPD2* gene are listed in Table 2.

The *srlD*, *gldA* and *mgsA* genes were amplified by using the *E. coli* DH5α genomic DNA, and *SOR1* and *GRE3* genes were amplified using the *S. cerevisiae* BY4742 genomic DNA. The 2 μ origin of replication gene was amplified by using the Yep24 vector. The primers used to amplify the genes are listed in Table 2.

DNA manipulations, construction of plasmids, and yeast and bacterial transformation

The *SOR1*, *srlD*, and *gldA* PCR products were cloned into the pDMPM multicopy shuttle vector [27] with an

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strains	Genotype	Source
WT	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	[9]
DM	BY4742; *gpd1*::*KanMx*; *gpd2*::*URA3*	This study
DM(*srlD*)	DM[PGK_P-*srlD*-PGK_T]	This study
DM(*SOR1*)	DM[PGK_P-*SOR1*-PGK_T]	This study
DM(*gldA*, *GRE3*, *mgsA*)	DM[PGK_P-*gldA*-PGK_T, PGK_P-*GRE3*-PGK_T, PGK_P-*mgsA*-PGK_T]	This study

Genetic modifications introduced on plasmids are indicated in square brackets

Table 2 Primers for gene deletion and amplification used in this study

Forward primer (5′–3′)	
Deletion of genes[a]	
GPD2	ATGCTTGCTGTCAGAAGATTAACAAGATACACATTCCTTA AGCGAACGCAATGTCGAAAGCTACATATAAGG
ALD6	ATGACTAAGCTACACTTTGACACTGCTGAACCAGTCAA GATCACACTTCCCTGCAGGTCGACGGATCCGG
Amplification of genes[b]	
SOR1	GAATTCATGTCTCAAAATAGTAACCCTGCAG
srlD	AGATCTATGAATCAGGTTGCCGTTGTCATCG
gldA	AGATCTATGCCGCATTTGGCACTACTCATCTC
mgsA	GAATTCATGGAACTGACGACTCGCACTTTACCTG
GRE3	GAATTCATGTCTTCACTGGTTACTCTTAATAACGG
2 μ origin of replication	GCCGGCATCCAATATCAAGGAAATGATAGC
Reverse primer (5′–3′)	
Deletion of genes[a]	
GPD2	CTATTCGTCATCGATGTCTAGCTCTTCAATCATCTCCG GTAGGTGTTCCATTAGTTTTGCTGGCCGCATCTTCT
ALD6	TTACAACTTAATTCTGACAGCTTTTACTTCAGTGTATGCA TGGTAGACTTTACGTAATGCTCAACCTTAAGCTGCTGC
Amplification of genes[b]	
SOR1	CTCGAGTCATTCAGGACCAAAGATAATAGTC
srlD	CTCGAGTCAGAACATCACCTGACCGCCG
gldA	CTCGAGTTATTCCCACTCTTGCAGGAAACGCT
mgsA	CTCGAGTTACTTCAGACGGTCCGCGAGATAACG
GRE3	CTCGAGTCAGGCAAAAGTGGGGAATTTAC
2 μ origin of replication	CAGCTGTTAACGAAGCATCTGTGCTTCA

[a] Underlined parts of the primers are the overhanging part of the gene to be deleted

[b] Underlined parts of the primers are the restriction sites

ampicillin resistance marker (amp^r) and a leucine auxotrophic marker (*LEU2*) and a modified multiple cloning site (MCS) flanked by the constitutive phosphoglycerate-kinase-1 gene promoter (*PGK1P*) and terminator (*PGK1T*) [14] derived from pHVX2 [43]. The *GRE3* gene was cloned into the pSTAH integrating vector [17] containing a histidine auxotrophic marker (*HIS3*), amp^r, and an MCS flanked by the *PGK1P* and *PGK1T* resulting in the pSTAH(*GRE3*) vector. To convert this vector into a multicopy shuttle vector, the pSTAH(*GRE3*) vector was digested with *Nae*I and *Pvu*II and the 5,215-bp band was ligated with the 2 μ yeast origin of replication gene (1,341 bp) with *Nae*I and *Pvu*II overhangs. To construct the pSTAL(*mgsA*) vector, the *mgsA* gene was cloned into pSTAH [17], restricted with *Ssp*I, followed by gel purification of the 1,318-bp band containing *mgsA* gene with *PGK1P* and *PGK1T* and finally ligation with the Yip5 vector (http://www.atcc.com) digested also with *Ssp*1 restriction enzyme. The resultant vector was digested with *Eco*RV to remove a 1,659-bp fragment containing a tetracycline resistance marker (tet^r) and the remaining fragment was self-ligated. The self-ligated vector was then digested with *Eco*RV and *Nsi*I and the 4,561-bp band was gel purified. This band was then ligated to the YdpK vector [6] digested with *Pvu*II and *Pst*I restriction enzymes. The 4,600-bp DNA obtained after digestion of the YdpK vector [6] with *Pvu*II and *Pst*I restriction enzymes contains the lysine auxotrophic marker (*LYS2*) gene having its native promoter and terminator. The resulting vector was digested with *Sma*I and *Pvu*II and the 8,544-bp band was ligated with the 2 μ yeast origin of replication gene (1,341 bp) with *Nae*I and *Pvu*II overhangs. The final vector thus obtained was pSTAL(*mgsA*) containing a *LYS2*, the 2 μ yeast origin of replication and amp^r. The pDMPM(*SOR1*) and pDMPM(*srlD*) plasmids were transformed into DM resulting in the DM(*SOR1*) and DM(*srlD*) strains, respectively. The pDMPM(*gldA*), pSTAH(*GRE3*) and pSTAL(*mgsA*) plasmids were transformed into DM to obtain the DM(*gldA*, *GRE3*, *mgsA*) strain. Prior to transformation, the genes were sequenced at the Central Sequencing Facility of Stellenbosch University. The

Table 3 Genotypes of the final plasmids used in this study

Plasmid	Genotype	Source
YIp5	pBR322 *URA3*	ATCC
pDMPM	pHVX2 multiple cloning sites	[27]
pSTAH	YIp5 *ura3::HIS3* PGK_P-PGK_T *bla*	[17]
pSTAH(*GRE3*)	pSTAH PGK_P-*GRE3*-PGK_T 2 μ	This study
pSTAL(*mgsA*)	YIp5 PGK_P-*mgsA*-PGK_T tet^r::*LYS2* 2 μ	This study
pDMPM(*SOR1*)	pDMPM PGK_P-*SOR1*-PGK_T	This study
pDMPM(*srlD*)	pDMPM PGK_P-*srlD*-PGK_T	This study
pDMPM(*gldA*)	pDMPM PGK_P-*gldA*-PGK_T	This study

ATCC American Type Culture Collection, Manassas, VA, USA

sequence for all the genes was 100% homologous to the sequence obtained from their GenBank accession number. The accession numbers for *SOR1*, *srlD*, *gldA*, *GRE3* and *mgsA* genes are P35497, P05707, P0A9S5, P38715 and P0A731, respectively. The genotypes of the final plasmids used in this study are listed in Table 3.

Subcloning in *E. coli* DH5α, yeast and bacterial transformations and isolation of genomic DNA from *E. coli* and *S. cerevisiae* was done using standard protocols [15, 18].

Medium and fermentation conditions

The fermentations were conducted fourfold without agitation in 250-ml Erlenmeyer flasks with a 100-ml working volume and fitted with a fermentation cap filled with sterile water to restrict air permeation. The initial aerobic conditions in the flask became anaerobic as O_2 in the headspace of the flask was metabolized and CO_2 was released, resulting in a self-generated anaerobic system. The shift from aerobic to anaerobic conditions was confirmed by adding methylene blue (2 mg/l) to the medium and monitoring the colour change from dark blue (aerobic conditions) to colourless (anaerobic conditions) [16]. The liquid medium for the growth of strains contained 10% total sugar (5% glucose and 5% fructose) and 6.7 g/l yeast nitrogen base (YNB) without amino acids. The medium was supplemented with the amino acids required for the growth of the auxotrophic strains. The amino acids supplied were uracil, leucine, lysine and histidine to final concentrations of 24, 72, 36 and 24 mg/l, respectively. The pH and temperature were maintained at 3.5 and 30°C respectively to mimic fermentation conditions in many industrial environments. The medium was inoculated at an initial absorbance (600 nm) of 0.5. The cultures were monitored for 20 days by measuring flask weight loss (for fermentation rate), sampling (for metabolite analysis and sugar consumption) and determining biomass (for growth) each alternate day after inoculation. The samples were centrifuged at 5,000 rpm for 10 min, the supernatants were filtered (0.2 μm membrane filter) and stored at 4°C until further analysis. Cells were resuspended in 1 ml water and centrifuged in 1.5-ml microcentrifuge tubes. The supernatant was removed and the preweighed microcentrifuge tubes containing cells were dried at 60°C overnight until constant weight.

Chemical analyses

Substrates consumed and metabolites formed were analyzed by using a Waters HPLC system equipped with an Aminex HPX-87H column (BioRad, USA), connected to a refractive index–ultraviolet detector (RID-6A, Shimadzu, Japan). A mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 45°C were used. Glucose, fructose, ethanol, sorbitol and glycerol were measured by using a refractive index detector. Propane-1,2-diol was extracted [24] and concentration determined by gas chromatography with a Nukol free fatty acid phase fused-silica capillary column coupled to a mass spectrometer (Agilent 6890 GC coupled to a Agilent 5975C MSD) [29].

Results

Expression of oxidoreductase genes partially restored sugar utilization and biomass production of DM

To assess the impact of the genetic modifications, self-generated anaerobic conditions in liquid YNB-based medium with high sugar content (100 g/l) were used to cultivate the strains. The WT strain consumed the highest amount of sugar among all the strains (Fig. 2a) and the DM the least. Sugar consumption by the genetically manipulated strains was in between that by the WT and the DM. Among the genetically manipulated strains, the DM(*SOR1*) strain consumed the highest amount of both glucose and fructose, whereas the DM consumed the least of both the sugars (Fig. 2b–c). DM(*srlD*) and DM(*gldA*, *GRE3*, *mgsA*) strains showed similar total sugar utilization, while relative glucose and fructose utilisation varied among these strains (Fig. 2a–c; Table 4). Glucose was the preferred carbon source in all strains (Fig. 2b), but the fructose consumption was higher in the DM(*SOR1*) compared with the other strains (Fig. 2c; Table 4). This probably reflects the conversion of fructose to sorbitol by the overexpressed NADH-dependent sorbitol dehydrogenase (Fig. 1). As expected, biomass production was the highest in the WT strain and lowest in the DM. Among the engineered strains, DM(*SOR1*), DM(*srlD*) and DM(*gldA*, *GRE3*, *mgsA*) showed increased biomass production compared with the DM (Fig. 2d; Table 4). The biomass concentration in the

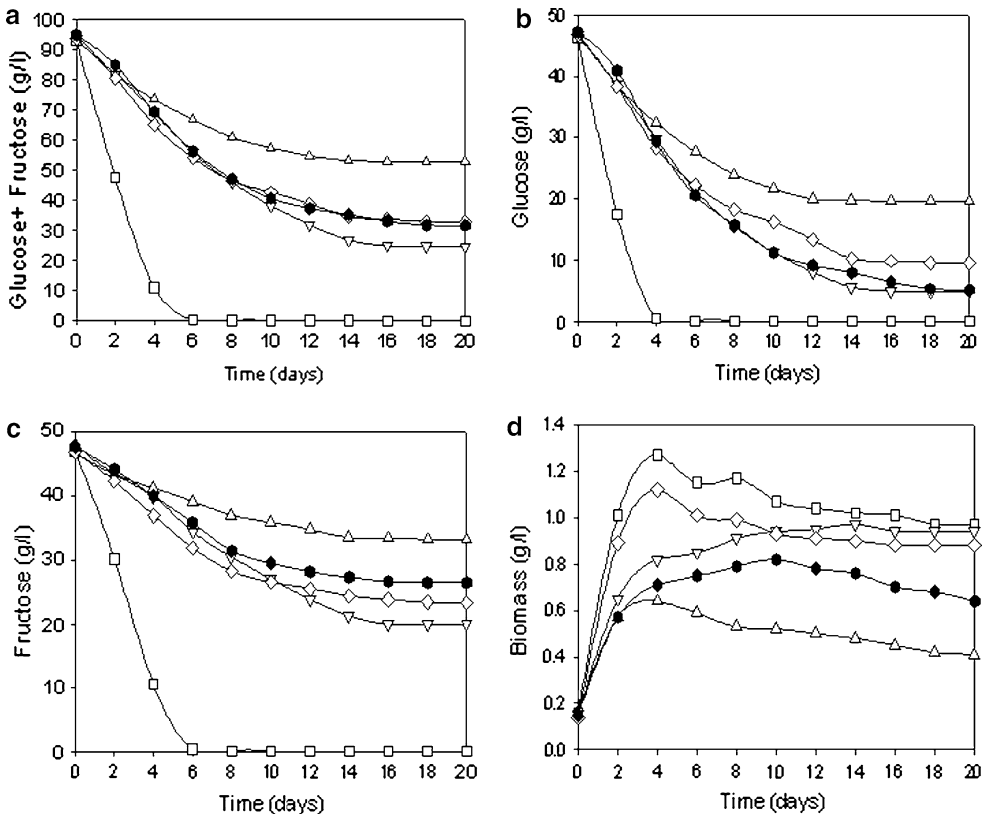

Fig. 2 Total sugar consumption (**a**), glucose consumption (**b**), fructose consumption (**c**) and biomass (dry weight) production (**d**) by *S. cerevisiae* WT (*squares*), DM (*up-pointing triangles*), DM(*SOR1*) (*down-pointing triangles*), DM(*srlD*) (*diamonds*) and DM(*gldA*, *GRE3*, *mgsA*) (*hexagons*) when cultivated in 5% glucose–5% fructose and 6.7 g/l YNB in a representative experiment

Table 4 Concentrations (g/l) of sugar consumed, biomass and metabolites formed by WT and genetically manipulated strains after 20 days of fermentation cultivated in 5% glucose–5% fructose and 6.7 g/l yeast nitrogen base under oxygen-limited fermentation

Primary metabolites	WT	DM	DM(*SOR1*)	DM(*srlD*)	DM(*gldA*, *mgsA*, *GRE3*)
Glucose consumed	50 ± 0	30.4 ± 0.81	45.23 ± 0.66	40.4 ± 0.55	44.88 ± 0.51
Fructose consumed	49.85 ± 0.07	17.01 ± 1.38	30.08 ± 1.14	26.6 ± 0.79	23.5 ± 1.06
Total sugar consumed	99.85 ± 0.07	47.41 ± 1.95	75.31 ± 1.58	67.01 ± 1.21	68.39 ± 1.28
Highest growth rate (h^{-1})	0.038	0.026	0.030	0.038	0.028
Biomass	0.97 ± 0.008	0.41 ± 0.03	0.94 ± 0.02	0.88 ± 0.03	0.58 ± 0.02
CO_2[a]	43.2 ± 2.21	21.9 ± 1.7	34.6 ± 1.98	29.5 ± 1.37	30.4 ± 1.91
Ethanol	45.17 ± 2.56	22.88 ± 1.68	36.31 ± 2.47	30.89 ± 2.34	31.78 ± 1.23
Glycerol	4.08 ± 0.09	0	0	0	0
Sorbitol	0	0	1.17 ± 0.13	4.39 ± 0.14	0
Propane-1,2-diol	0	0	0	0	1.68 ± 0.02
Total (yield g/g)[b]	0.94	0.95	0.97	0.98	0.94

Mean ± standard deviation of four independent cultivations

[a] Concentration of CO_2 is calculated using its molar yield

[b] Total yield for a strain is calculated by adding up the individual yield each metabolite

WT, DM and DM(*srlD*) strains decreased or remained constant after the 4th day (Fig. 2d). Anaerobic conditions prevailed in the flasks inoculated with these strains after this time as methylene blue dye was observed to change from blue (aerobic) to colourless (anaerobic) (data not shown). However, the DM(*SOR1*) strain continued to produce biomass until the 12th day and the DM(*gldA*, *GRE3*, *mgsA*) strain until the 10th day under anaerobic conditions (Fig. 2d) pointing to the relative efficiency of the heterologous pathways in maintaining growth conditions.

Metabolite molar yields and concentrations varied among the strains

During alcoholic fermentation, ethanol is the most important primary metabolite produced in terms of reoxidation of

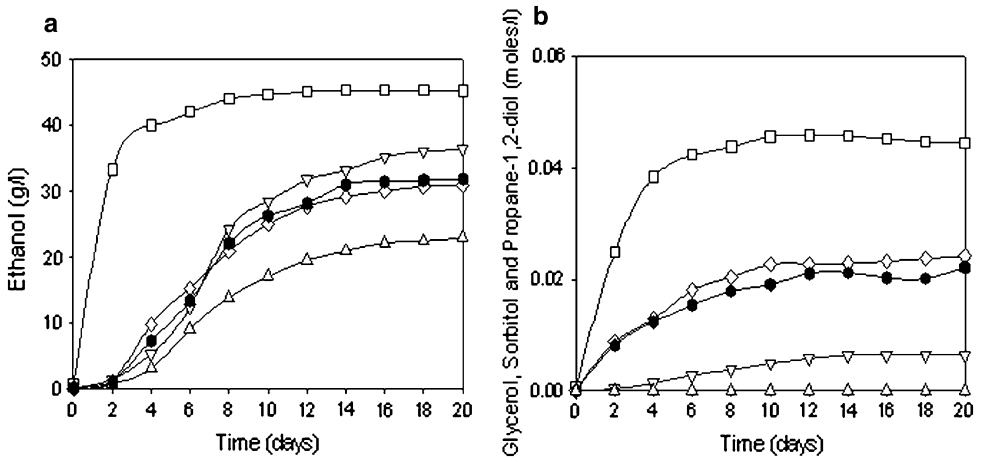

Fig. 3 Ethanol (**a**), glycerol, sorbitol and propane-1,2-diol (**b**), production by the *S. cerevisiae* WT (*squares*), DM (*up-pointing triangles*), DM(*SOR1*) (*down-pointing triangles*), DM(*srlD*) (*diamonds*), and DM(*gldA*, *GRE3*, *mgsA*) (*hexagons*) when cultivated in 5% glucose–5% fructose and 6.7 g/l YNB in a representative experiment. WT formed glycerol, DM(*SOR1*) and DM(*srlD*) formed sorbitol and DM(*gldA*, *GRE3*, *mgsA*) formed propane-1,2-diol, whereas DM formed no other metabolite in significant concentrations

Table 5 Yield (moles/mole sugar consumed) of metabolites formed by WT and genetically manipulated strains after 20 days of fermentation cultivated in 5% glucose–5% fructose and 6.7 g/l yeast nitrogen base under oxygen-limited fermentation

Primary metabolites	WT	DM	DM(*SOR1*)	DM(*srlD*)	DM(*gldA*, *mgsA*, *GRE3*)
Biomass	0.0711 ± 0.0006	0.0633 ± 0.0052	0.091 ± 0.002	0.096 ± 0.005	0.062 ± 0.002
CO_2^a	1.77 ± 0.10	1.89 ± 0.1995	1.88 ± 0.098	1.80 ± 0.17	1.82 ± 0.06
Ethanol	1.77 ± 0.10	1.89 ± 0.1995	1.88 ± 0.098	1.80 ± 0.17	1.82 ± 0.06
Glycerol	0.08 ± 0.002	0	0	0	0
Sorbitol	0	0	0.015 ± 0.001	0.065 ± 0.002	0
Propane-1,2-diol	0	0	0	0	0.047 ± 0.001

Mean ± standard deviation of four independent cultivations

[a] Molar yield of CO_2 is assumed to be same as molar yield of ethanol

excess NADH and redox balancing, followed by the production of glycerol or of the metabolites selected for its replacement. The WT strain produced the highest total amount of ethanol and DM the least (Fig. 3a; Table 4). Among the engineered strains, DM(*SOR1*) showed the highest final ethanol production capacity (Fig. 3a; Table 4). Around 95% of mass of sugars was recovered in the products (Table 4). Lower recovery than 100% could be due to loss of water and CO_2 from sugar respiration during the initial stage of growth when oxygen was available in the flask.

The molar yield (Table 5) showed a different pattern with the WT strain having the lowest ethanol molar yield, whereas all genetic manipulations led to an increase with DM and DM(*SOR1*) with a similar molar yield and DM(*srlD*) and DM(*gldA*, *GRE3*, *mgsA*) producing a slightly lower molar yield of ethanol. No metabolite replaced glycerol in significant concentrations in cultures of the DM strain (Table 4). Furthermore the DM(*srlD*) and DM(*gldA*, *GRE3*, *mgsA*) strains produced similar molar concentrations of sorbitol (24 ± 8 mM) and propane-1,2-diol (22 ± 2 mM) respectively, whereas DM(*SOR1*) produced a lower molar concentration of sorbitol (6 ± 1 mM) (Fig. 3b), in all cases significantly below the glycerol molar concentration (45 ± 1 mM) produced by the WT strain. In terms of yield, DM(*srlD*) strain showed the highest yield of sorbitol followed by yields of propane-1,2-diol and sorbitol by DM(*gldA*, *GRE3*, *mgsA*) and DM(*SOR1*) strains respectively (Table 5).

Discussion

This study shows that glycerol can be eliminated as a fermentation product and replaced by other products of biotechnological interest. The propane-1,2-diol concentration produced was greater than values reported in other studies where *S. cerevisiae* was used as the recombinant host but higher concentrations have been observed when bacteria are used. The glycerol-synthesizing pathway was not removed

in studies using *S. cerevisiae* and glycerol remained a significant co-product [5, 21]. Similar to our approach, genes encoding glycerol dehydrogenase and methylglyoxal synthase were inserted into *S. cerevisiae* but we added an aldose reductase to our strain which might have improved the conversion to propane-1,2-diol. Sorbitol production by recombinant *Lactobacillus* species encoding a sorbitol-6-phosphate dehydrogenase but not by *S. cerevisiae* has been reported. The conversion of glucose to sorbitol by *Lactobacillus plantarum* cell suspensions almost attained the theoretical maximum, whereas growing cells produced sorbitol at much lower levels similar to those found in our study [23].

The insertion of genes encoding oxidoreductases and other enzymes into the glycerol defective strain only partially restored yeast growth under fermentative conditions. Each genetic manipulation was constructed in order that excess NADH would be stoichiometrically converted to NAD^+. However, the metabolite concentrations formed varied considerably and this observation suggests that other factors also influence metabolism in these strains. The poor growth of DM under the fermentative conditions pointed to a reduced NAD^+ pool available for biomass synthesis due to the inability to reoxidize NADH to NAD^+ by alternative routes in this strain. The complete inhibition of growth of the DM in liquid medium under strict anaerobic conditions has been previously reported [8, 30]. This suggests that the fermentative conditions used in our experiments might still allow some reoxidation of NADH in spite of the methylene blue assay revealing anaerobic conditions in the medium. The partial growth recovery of the genetically manipulated strains compared with DM under these conditions indicates an improved regeneration of NAD^+ and functional replacement of glycerol by other metabolites. Nevertheless, the regeneration of NAD^+ through alternative enzymes was not as efficient as by Gpd1p and Gpd2p in WT. Several reasons may be advanced to explain this relative inefficiency of the heterologous pathways in ensuring optimal NAD^+ regeneration. Firstly, expression of the heterologous genes was not optimized in any way, because the aim of the study was only to assess general feasibility to eliminate glycerol and replace with other metabolites. Optimal and improved growth might be achieved by adjusting expression levels of the heterologous genes. Furthermore, the metabolites produced by the genetically manipulated strains might not be optimal for growth. Glycerol has proved to be an important product in maintaining optimal intracellular osmotic conditions in yeast [20, 41]. Sorbitol was shown to partially protect *S. cerevisiae* during salt stress in a glycerol defective mutant [37]. However, the degree of protection by sorbitol as osmolyte was less than observed with glycerol and suggests that glycerol may have specific functions that cannot be completely replaced by sorbitol.

The poorer growth by DM(*gldA*, *GRE3*, *mgsA*) strain compared with the sorbitol-producing strains might be due the inability of propane-1,2-diol to act as an osmolyte. To the best of our knowledge this molecule has not been reported to possess osmotic functions. Furthermore the inhibition of the growth in DM(*gldA*, *GRE3*, *mgsA*) strain could be due to the production of toxic methylglyoxal as an intermediate of the conversion of dihydroxyacetone phosphate to propane-1,2-diol [28]. However, the genetic manipulations constructed in this strain would lead to the generation of $NADP^+$ in addition to NAD^+. In yeast, NADPH is required for the anabolic synthesis of biomass [40]. The insertion of *S. cerevisiae GRE3* resulted in NADPH specific aldose reductase activity [22] converting methylglyoxal to acetol. This could have led to a reduced NADPH pool in the cell and thereby reduced biomass formation. Replacement of *GRE3* with an aldose reductase with co-activity for NADH and NADPH [42] might have improved biomass formation in this strain. Other redox shuttles might also function under fermentative conditions in *S. cerevisiae* [4] but it is unknown whether these mechanisms might influence the NADH/NAD^+ balances observed in these experiments.

The similar sugar consumption and biomass production by DM(*SOR1*) and DM(*srlD*) strains but lower levels of sorbitol production by the DM(*SOR1*) strain suggests regeneration of NAD^+ by some other means in this strain. This may occur through additional ethanol production because Sor1p has been described as having a non-specific Adh1p activity [12]. The higher yield and production of sorbitol for the strain DM(*srlD*) as compared with DM(*SOR1*) might therefore also be due to the low affinity of Sor1p for fructose [25] and the relative unavailability of free fructose inside the cell [7, 10] when compared with fructose-6-phosphate (Fig. 1).

This study has shown that glycerol can be eliminated from the ethanol fermentation process and replaced with other industrially important chemicals. However, none of the approaches described here were further optimized beyond the assessment of initial feasibility, suggesting that significantly improved results could be achieved by optimizing various aspects of the strategies such as alternative gene selection, improved gene expression levels and cultivation conditions.

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