Evolutionary engineering of a glycerol-3-phosphate dehydrogenase-negative, acetate-reducing \textit{Saccharomyces cerevisiae} strain enables anaerobic growth at high glucose concentrations

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Summary

Glycerol production by \textit{Saccharomyces cerevisiae}, which is required for redox-cofactor balancing in anaerobic cultures, causes yield reduction in industrial bioethanol production. Recently, glycerol formation in anaerobic \textit{S. cerevisiae} cultures was eliminated by expressing \textit{Escherichia coli} (acyetylated) acetaldehyde dehydrogenase (encoded by \textit{mhpF}) and simultaneously deleting the \textit{GPD1} and \textit{GPD2} genes encoding glycerol-3-phosphate dehydrogenase, thus coupling NADH reoxidation to reduction of acetate to ethanol. Gpd– strains are, however, sensitive to high sugar concentrations, which complicates industrial implementation of this metabolic engineering concept. In this study, laboratory evolution was used to improve osmotolerance of a Gpd– \textit{mhpF}-expressing \textit{S. cerevisiae} strain. Serial batch cultivation at increasing osmotic pressure enabled isolation of an evolved strain that grew anaerobically at 1 M glucose, at a specific growth rate of 0.12 h\(^{-1}\). The evolved strain produced glycerol at low concentrations (0.64 ± 0.33 g l\(^{-1}\)). However, these glycerol concentrations were below 10% of those observed with a Gpd+ reference strain. Consequently, the ethanol yield on sugar increased from 79% of the theoretical maximum in the reference strain to 92% for the evolved strains. Genetic analysis indicated that osmotolerance under aerobic conditions required a single dominant chromosomal mutation, and one further mutation in the plasmid-borne \textit{mhpF} gene for anaerobic growth.

Introduction

Bioethanol production with \textit{Saccharomyces cerevisiae} is the single largest fermentation process in industrial biotechnology with an annual global product volume of ca. 8.6 × 10\(^{10}\) l (Renewable Fuels Association, 2012). This puts \textit{S. cerevisiae} at the centre of a global research effort to improve its productivity, robustness under process conditions, substrate range and product yield (van Maris et al., 2006). Anaerobic fermentation of sugars to ethanol and CO\(_2\) is a redox-neutral process. However, in cultures of \textit{S. cerevisiae}, an ‘excess’ of NADH is generated from biosynthetic reactions such as oxidative decarboxylations in amino acid and lipid synthesis (van Dijken and Scheffers, 1986; Bakker et al., 2001). In anaerobic yeast cultures, this ‘excess’ NADH is reoxidized through glycerol formation via NADH-dependent reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (G3P), which is subsequently dephosphorylated to glycerol. Glycerol production has been estimated to account for a loss of 4% of the consumed sugar in industrial ethanol production (Nissen et al., 2000). Under tightly controlled laboratory growth conditions, where biomass yields are typically higher than in industrial yeast fermentation processes, this percentage can be as high as 10% (Verduyn et al., 1990; Nissen et al., 2000; Guadalupe Medina et al., 2010). Elimination of glycerol formation via metabolic engineering strategies has therefore attracted significant interest (Nissen et al., 2000; Nevoigt, 2008; Guadalupe Medina et al., 2010; Pagliardini et al., 2010; Jain et al., 2011).

In \textit{S. cerevisiae}, deletion of the \textit{GPD1} and \textit{GPD2} genes encoding NAD+-dependent G3P dehydrogenase (EC 1.1.1.8) eliminates glycerol formation (Björkqvist et al., 1997). However, such a double deletion also completely blocks growth under anaerobic conditions unless an external electron acceptor for NADH reoxidation, such as acetoin or acetaldehyde, is provided (Scheffers, 1966;
Ansell et al., 1997; Björkqvist et al., 1997). We recently proposed a metabolic engineering strategy for eliminating glycerol production in anaerobic S. cerevisiae cultures that is based on the use of acetic acid as electron acceptor (Guadalupe Medina et al., 2010). Acetic acid is a common inhibitor present in plant biomass hydrolysates (Palmqvist and Hahn-Hägerdal, 2000). This strategy encompasses expression of a NAD⁺-dependent (acetylating) acetaldehyde dehydrogenase (EC 1.2.1.10) mhpF gene (EMBL: CAAT0751) from Escherichia coli in a gpd1Δ gpd2Δ (Gpd⁻) S. cerevisiae strain. After activation of acetaldehyde dehydrogenase (S. cerevisiae acetyl-coenzyme A synthetase (van den Berg et al., 1996), the resulting acetyl-coenzyme A can be reduced to ethanol by the combined activity of the NAD⁺-dependent (acetylating) acetaldehyde dehydrogenase and yeast alcohol dehydrogenases. Anaerobic growth of the resulting engineered yeast strain on glucose was coupled to acetate reduction (Guadalupe Medina et al., 2010). Although the growth rate was reduced, glycerol production was eliminated, and the ethanol yield increased by 13% relative to that of a GPD1 GPD2 (Gpd⁺) reference strain (Guadalupe Medina et al., 2010).

Glycerol formation is not only crucial for redox balancing in anaerobic cultures of wild-type S. cerevisiae but, as its main compatible solute, is also required for osmotolerance. As a consequence, Gpd⁻ S. cerevisiae strains are sensitive to high osmotic pressures (Ansell et al., 1997). Industrial ethanol production use high sugar concentrations at the start of fermentation processes, which makes osmotolerance of the yeast strains an essential attribute (Albertyn et al., 1994; Blomberg and Adler, 1989; Nevoigt and Stahl, 1997). The response of S. cerevisiae to high osmolarity is regulated by the high-osmolarity glycerol pathway and involves not only intracellular glycerol accumulation but also regulation of other stress-related genes (Hohmann, 2002). The osmosensitivity of Gpd⁺ strains of S. cerevisiae (Ansell et al., 1997) can be partly alleviated by introduction of sorbitol-6-P-dehydrogenase and mannitol-1-P-dehydrogenase encoding genes (Shen et al., 1999). In such engineered strains, mannitol or sorbitol act as alternative compatible solutes, although growth rates are lower than in wild-type strains (Shen et al., 1999). An alternative strategy that does not completely eliminate glycerol formation is the replacement of the GPD1 or GPD2 promoters by lower-strength constitutive promoters (Hubmann et al., 2011).

Evolutionary engineering is a powerful approach to select for strain variants/mutants with (improved) industrially relevant traits. In evolutionary engineering, regimes for prolonged cultivation are designed such that a selective advantage is conferred to spontaneous mutants that express the trait of interest (Sauer, 2001; Oud et al., 2012). Evolutionary engineering not only generates strains with industrially relevant phenotypes, but subsequent analysis of molecular mechanisms responsible for their improved performance also enables reverse engineering of these traits into non-evolved strains (Sauer, 2001; Oud et al., 2012).

The goal of the present study was to investigate whether evolutionary engineering enables the isolation of osmotolerant mutants of Gpd⁻ S. cerevisiae expressing an E. coli (acyetylating) acetaldehyde dehydrogenase. The ability of these strains to grow anaerobically with acetic acid as electron acceptor makes it possible to specifically focus on anaerobic evolutionary engineering experiments for improvement of osmotolerance. To this end, sequential batch cultivation of the engineered strains was performed under anaerobic conditions and at high sugar concentrations that are relevant for industrial cultivation (Laluce, 1991; Jones et al., 1994; Bai et al., 2008). After prolonged cultivation under selective conditions, which involved glucose concentrations of up to 1 M, single-colony-derived isolates were characterized in anaerobic bioreactors.

Results

Evolutionary engineering for improved osmotolerance

The ability of S. cerevisiae IMZ160 (gpd1Δ gpd2Δ mhpF) to grow at industrially relevant osmotic pressures was assessed with spot assays on synthetic medium plates containing 0.1, 0.5 and 1.0 M glucose. In line with previous research on Gpd⁺ strains (Ansell et al., 1997), growth of strain IMZ160 was severely inhibited at 0.5 M glucose, both under aerobic and anaerobic conditions, and completely abolished at 1.0 M glucose. Growth of the Gpd⁺ reference strain S. cerevisiae IME076 was not inhibited at these glucose concentrations (Fig. 1).

Evolutionary engineering for improved osmotolerance was initiated in shake flask cultures with 20 g l⁻¹ glucose as the carbon source supplemented with sorbitol to increase osmolarity. At an initial concentration of 1.0 M sorbitol, the Gpd⁻ strain IMZ160 showed a specific growth rate of 0.06 ± 0.00 h⁻¹, as compared with 0.37 ± 0.00 h⁻¹ for the Gpd⁺ reference strain IME076. After 4 serial transfers at 1.0 M sorbitol, 8 transfers at 1.5 M sorbitol and 16 transfers at 2.0 M sorbitol, the cultures showed a maximum specific growth rate of 0.18 ± 0.01 h⁻¹ at 1.0 M sorbitol and 0.15 ± 0.01 h⁻¹ at 2.0 M sorbitol. To achieve anaerobic growth at 1.0 M glucose, a shake flask culture adapted for growth in 2.0 M sorbitol was used as inoculum for an anaerobic bioreactor batch culture at low osmotic pressure in synthetic media with 20 g l⁻¹ glucose and 2 g l⁻¹ acetate. After 10 days, an anaerobic specific growth rate of 0.12 h⁻¹ ± 0.00 h⁻¹ was observed. Subsequently, anaerobic sequential batch cultivation was performed on synthetic media supplemented with 2 g l⁻¹ acetic acid and...
with 1.0 M glucose as source of carbon and to increase osmolarity. Starting with an initial anaerobic specific growth rate of 0.05 h\(^{-1}\), the sequential batch culture showed a continuously increasing specific growth rate until, after 187 sequential batch cultures, a specific growth rate of 0.13 ± 0.01 h\(^{-1}\) was measured as the average of the last 10 sequential batches. Glycerol, which was not detected during the initial cycles, was detected in culture supernatants later in the evolution experiments, albeit at much lower levels than in cultures of the Gpd\(^{+}\) reference strain grown under identical conditions (data not shown).

After 187 sequential batch cultures, individual single colony isolates were obtained, whose growth rates were analysed in anaerobic batch cultures on synthetic glucose supplemented with 1.0 M glucose and 2 g l\(^{-1}\) acetate. A single-colony isolate that exhibited the highest maximum specific growth rate of 0.12 ± 0.00 h\(^{-1}\) and the lowest final extracellular glycerol concentration of 0.64 ± 0.33 g l\(^{-1}\) was named IMZ333 (evolved gpd1Δ gpd2Δ mhpF). Spot assay experiments under aerobic and anaerobic conditions at 0.1, 0.5 and 1.0 M glucose confirmed that, in contrast to the ancestral Gpd\(^{-}\) strain *S. cerevisiae* IMZ160, the evolved strain IMZ333 was able to grow at a concentration of 1 M glucose, albeit slower than the Gpd\(^{+}\) reference strain IME076 (Fig. 1).

**Growth and product formation in anaerobic batch cultures at high glucose concentrations**

To quantitatively characterize *S. cerevisiae* IMZ333 (evolved gpd1Δ gpd2Δ mhpF), this strain was grown in anaerobic bioreactors on synthetic medium supplemented with 2 g l\(^{-1}\) acetic acid. At a glucose concentration of 20 g l\(^{-1}\), the specific growth rate of strain IMZ333...
was 0.21 ± 0.01 h⁻¹, which is significantly higher than that of the ancestral Gpd⁻ strain IMZ160 (0.13 ± 0.01 h⁻¹), but still lower than the Gpd⁺ reference strain IME076 (0.32 ± 0.01 h⁻¹). Under these conditions, no glycerol formation was observed for either the Gpd⁻ parental (IMZ160) or evolved strain (IMZ333), whereas the Gpd⁺ reference strain produced up to 1.75 ± 0.20 g l⁻¹ glycerol.

At an initial glucose concentration of 1.0 M glucose, the evolved strain IMZ333 grew with a specific growth rate of 0.12 ± 0.01 h⁻¹ and consumed all sugar in approximately 1 week (Fig. 2A). Under identical conditions, its ancestral strain IMZ160 did not grow during a 10 day incubation, while the Gpd⁺ reference strain grew at 0.24 ± 0.01 h⁻¹ and consumed all sugar within 1 day (Fig. 2B).

Growth of the evolved Gpd⁻ strain IMZ333 was clearly coupled to the use of acetic acid as electron acceptor to reoxidize the excess NADH generated during growth (Fig. 2A). During the growth phase, no glycerol was formed by the Gpd⁻ strain. Upon depletion of acetic acid, growth stopped and glucose consumption slowed down. Increasing the acetic acid concentration resulted in a continuation of glucose consumption and in drastically shortened fermentation times (Fig. 2C). During the growth phase, no glycerol was formed by the Gpd⁻ strain. Upon depletion of acetic acid, growth stopped and glucose consumption slowed down. Increasing the acetic acid concentration resulted in a continuation of glucose consumption and in drastically shortened fermentation times (Fig. 2C). When all glucose was consumed, low amounts of glycerol, up to 0.64 ± 0.33 g l⁻¹ at 182 h, appeared in the supernatant of cultures of the evolved Gpd⁻ strain IMZ333 (Fig. 2A). However, the glycerol concentration in these cultures remained at least 10-fold lower than those observed in cultures of the Gpd⁻ reference strain (7.4 ± 0.37 g l⁻¹ at 19.5 h) (Fig. 2B). The concentration of glycerol measured at the end of the batch cultures supplemented with 3 g l⁻¹ acetate (0.53 ± 0.02 g l⁻¹ at 93.6 h) was lower than the glycerol concentration measured when 2 g l⁻¹ acetate was used (Fig. 2C). Enzyme activity assays in cell extracts of strain IMZ333 confirmed that G3P dehydrogenase activity remained below the detection level of 0.002 μmol min⁻¹ (mg protein)⁻¹. The activity of (acetylating) acetaldehyde dehydrogenase in IMZ333 was 0.011 ± 0.005 μmol mg protein⁻¹ min⁻¹, which is not significantly different from the value previously observed for the non-evolved strain IMZ132 [0.020 ± 0.004 μmol mg protein⁻¹ min⁻¹ (Guadalupe Medina et al., 2010)].

The ultimate goal of eliminating glycerol formation in anaerobic yeast cultures is to increase the ethanol yield on sugar. In the nitrogen-sparged anaerobic bioreactors, a significant amount of ethanol is lost through evaporation. Because ethanol loss via evaporation is time dependent, it will be higher for cultures with a lower specific growth rate (Guadalupe Medina et al., 2010). After correction for ethanol evaporation, the apparent ethanol yield on glucose of the evolved strain (IMZ333; 1.77 ± 0.09 mol mol⁻¹) was 11% higher than that of the Gpd⁻ reference strain (IME076; 1.59 ± 0.02 mol mol⁻¹) in cultures grown on 1 M glucose and 2 g l⁻¹ acetic acid. At 3 g l⁻¹ acetic acid, the apparent ethanol yield of the evolved strain (IMZ333) was 1.84 ± 0.01 mol mol⁻¹, which represents 92% of the theoretical ethanol yield on glucose.
Mating and sporulation, followed by analysis of segregants, is a powerful approach to investigate the number and nature of mutation(s) in evolved haploid yeast strains (Swinnen et al., 2012). To further investigate the evolved osmotolerant genotype, the evolved strain IMZ333 was mated with its osmosensitive ancestral strain IMK006, after transformation with a selection marker and mating-type switching of the latter. Reliable conclusions from crossing and segregation experiments can only be made when the causal mutation(s) reside on the chromosomes rather than on plasmids and when the evolved strain contains the same plasmids as the ancestral strain, thereby avoiding random segregation of different plasmids in the spores. The fact that the acetylation acetaldehyde dehydrogenase activity in the evolved strain (IMZ333) did not increase during the evolution (see above) makes it unlikely that the copy number of the plasmid changed significantly. To assess whether or not there are different versions of the multicopy plasmid present within IMZ333, a subset of 10 plasmids were isolated from IMZ333 and characterized by restriction analysis with XmnI. This indicated that there were at least two types of plasmids in this strain: one that had lost the XmnI restriction site in the TDH3 promoter upstream of the mhpF gene and a second that matched the restriction pattern of the original pUDE043 plasmid. These plasmids were named pUDE043ev1 and pUDE043ev2 respectively. Reinserting these two plasmids and the original pUDE043 plasmid into a plasmid-free ancestral strain IMX031 and in the plasmid-cured evolved strain IMS343 indicated that causal mutations for aerobic osmotolerance were chromosomal because only the evolved strain, transformed with either of the three plasmids, was able to grow aerobically on 1 M glucose plates. Further analysis showed that only the evolved strain with the reintroduced pUDE043ev2 was able to grow anaerobically, albeit at a lower specific growth rate of \(0.07 \pm 0.01\) h\(^{-1}\), than the original evolved IMZ333 strain (Fig. 3). This observation indicated that anaerobic growth of the evolved strain on 1 M glucose required chromosomal as well as (a) plasmid-borne mutation(s). Sequencing of the mhpF gene on this plasmid revealed a point mutation at base pair position 112 of the open reading frame, resulting in an amino acid change (D38N).

To prevent interference of plasmids in the backcross analysis, the backcross was performed with a plasmid-free ancestral (IMK527) and a plasmid-cured evolved strain (IMS343), and the osmotolerance was tested under aerobic conditions only. The resulting diploid IMD011 was able to grow on a 1 M glucose plate, indicating that the causal mutation(s) conferring aerobic osmotolerance was (were) dominant. Sporulation of this diploid strain revealed a 2:2 segregation of growth on 1 M glucose plates in 19 out of 19 tetrads, indicating that aerobic osmotolerance in the evolved strain is caused by a single mutation (assuming that there are no linked mutations). Also the mutation(s) enabling anaerobic osmotolerance was (were) dominant because the diploid IMD012, resulting from a cross between the ancestral strain IMK527 with the plasmid-containing evolved strain IMZ333, was able to grow anaerobically on 1 M glucose. Sporulation of this diploid strain yielded very few viable spores and no complete tetrads on non-selective medium, which precluded an accurate analysis of the number of causal mutations underlying anaerobic osmotolerance.

**Discussion**

We recently proposed a metabolic engineering strategy that enables the use of acetic acid as an electron acceptor for reoxidation of the excess NADH generated in biosynthetic reactions by *S. cerevisiae* and thereby obviates the need for glycerol production for redox balancing (Guadalupe Medina et al., 2010). This strategy, which enables increased ethanol yields on sugar, is especially attractive for conversion of lignocellulosic feedstocks, in which acetic acid is invariably present and inhibits yeast fermentation performance. However, the deletion of the GDP1 and GDP2 genes encoding G3P dehydrogenase proposed by Guadalupe Medina and colleagues (2010) renders *S. cerevisiae* osmosensitive ([Ansell et al., 1997; Guadalupe Medina et al., 2010]; Fig. 1). The present study provides a proof of principle that evolutionary engi-
neering can be successfully applied to enable growth of acetate-reducing, *gpd1Δ gpd2Δ* strains to levels that are compatible with industrial bioethanol production. Although further increases in rate are definitely required, this represents an important step towards industrial implementation of an acetate-reducing *gpd1Δ gpd2Δ* *S. cerevisiae* strain with (acytlyating) acetaldehyde dehydrogenase.

The evolved osmotolerant strain (IMZ333) was not only able to grow at 1 M glucose, but converted this sugar to ethanol at increased yields relative to a *GPD1 GPD2* reference strain (11 and 15% increases in cultures grown at 2 and 3 g l\(^{-1}\) acetic acid respectively). Part of the increased ethanol yield arises from the elimination of glycerol formation (80 mM in the reference strain), which frees up additional glucose that can be converted to ethanol. A further increase of the ethanol yield can be attributed to the slower growth and longer duration of the fermentation, which increases the fraction of sugar that is converted to ethanol by increasing the cellular maintenance energy requirement (Verduyn *et al.*, 1990; Boender *et al.*, 2009). Finally, additional ethanol is produced during the reduction of acetic acid, which when all acetic acid is consumed leads to maximal increases of 33 and 55 mM for the fermentations containing 2 and 3 g l\(^{-1}\) respectively. The complete consumption of acetate in the cultures grown at 2 g l\(^{-1}\) acetate (Fig. 2) illustrates that the (acytlyating) acetaldehyde dehydrogenase strategy not only increases ethanol yields, but also enables the detoxification of significant amounts of inhibiting acetic acid. This detoxifying effect may be particularly relevant when high initial acetate concentrations are prevented by gradual feeding, for example by simultaneous saccharification and fermentation or by fed-batch feeding of hydrolysates to yeast fermentation processes (Taherzadeh *et al.*, 2001; Rudolf *et al.*, 2005).

Even though both genes encoding G3P dehydrogenase were deleted and the enzymatic activity of G3P dehydrogenase was confirmed to be below the detection limit, low concentrations of glycerol were observed upon cessation of growth of the evolved strain IMZ333. Glycerolipids are essential for the growth of *S. cerevisiae* and are formed by acylation of G3P (Racenis *et al.*, 1992). However, glycerolipids can also be obtained by acylation of dihydroxyacetone phosphate (DHAP) by the same G3P/DHAP acyltransferase, producing acyl-DHAP, which is later reduced to acyl-G3P by 1-acyldihydroxyacetone-phosphate reductase (EC 1.1.1.101), encoded by *AYR1* (Athenstaedt and Daum, 2000). Through this route, Gpd\(^{-}\) *S. cerevisiae* strains are able to form glycerolipids and grow. The low concentration of glycerol that was observed in the evolved Gpd\(^{-}\) strain at the end of the fermentation might be formed by deacylation of the glycerolipids and subsequently released when growth stopped and/or cells lysed.

Analysis of the evolved strain IMZ333 indicated that few mutations were required to increase osmotolerance in *gpd1Δ gpd2Δ* acetate-reducing *S. cerevisiae* strain. Detailed analysis of the molecular basis of improved osmotolerance in evolved acetate-reducing strains by whole genome resequencing, thereby enabling its reverse engineering into industrial strains, will require additional, independent evolution experiments to facilitate the separation of causal and random mutations (Oud *et al.*, 2012). Such experiments should also reveal whether evolutionary engineering always leads to the low-level *GPD1/ GPD2* independent glycerol formation found in strain IMZ333, or that alternative pathways, for example involving trehalose or proline as alternative compatible solutes (Hounsa *et al.*, 1998; Takagi, 2008; Mahmud *et al.*, 2009), can also contribute to evolution of increased osmotolerance in these strain backgrounds.

Although analysis of the molecular basis of improved tolerance was outside its scope, the present study provides valuable information for experimental design towards this goal. Firstly, our results indicate that mutations which confer osmotolerance under aerobic conditions are not necessarily sufficient to enable growth at high glucose concentrations in anaerobic cultures. In view of the envisaged application of strains in anaerobic bioethanol processes, future experiments should therefore preferably be performed under anaerobic conditions from the start of the evolution. Secondly, our results indicate that mutations on the mphF expression plasmid contributed to anaerobic osmotolerance. To facilitate the application of classical genetics and whole genome resequencing, it is therefore preferable to perform future evolution experiments with engineered strains in which the mphF expression cassette has been integrated into the *S. cerevisiae* genome. Moreover, the identified point mutation indicates that mutagenesis of mphF and/or expression of other (acytlyating) acetaldehyde dehydrogenase genes may contribute to osmotolerance in anaerobic cultures.

**Experimental procedures**

**Strain construction and maintenance**

All *S. cerevisiae* strains in this study (Table 1) originate from the CEN.PK family (van Dijken *et al.*, 2000; Entian and Kötter, 2007; Nijkamp *et al.*, 2012). Stock cultures and precultures were grown as described previously (Guadalupe Medina *et al.*, 2010). *S. cerevisiae* IMK006, obtained by removing the KanMX marker from the *gpd1 Δaspergillus* strain RWB0094 (Guadalupe Medina *et al.*, 2010) by expression of Cre recombinase (Güldener *et al.*, 1996), was transformed with the *LEU2*-bearing integrative vector pRS405, which was linearized with BstEII (NEB, Ipswich, MA, USA), yielding the leucine-prototrophic strain IMX031. Transformation of strain IMX031 with the *URA3*-bearing mphF-expression plasmid
pUDE43 (Guadalupe Medina et al., 2010) yielded the prototrophic, Gpd° mhpF-expressing strain IMZ160. Plasmid(s) were isolated from S. cerevisiae IMZ333 with the Sigma GenElute plasmid miniprep kit (Sigma-Aldrich Chemie Gmbh, Munich, Germany) according to manufacturer's instructions. Plasmids were transformed into E. coli One Shot TOP10 Z-competent cells (Invitrogen, Paisley, UK), and transformants were selected on Luria-Bertani medium plates containing ampicillin (100 mg l⁻¹). Restriction analysis of isolated plasmids was performed with XmnI (Fermentas, Sankt Leon-Rot, Germany). Plasmid sequencing was performed by BaseClear (Leiden, the Netherlands).

Shake flask cultivation

Shake flask cultivation was performed as described previously (Guadalupe Medina et al., 2010) using synthetic media (Verduyn et al., 1990). All shake flasks cultures were grown at 30°C in an Innova incubator shaker (New Brunswick, NJ, USA) at 200 r.p.m. For serial shake flask cultivation, synthetic media with urea as the nitrogen source were used (Verduyn et al., 1990). Glucose and sorbitol were autoclaved separately at 110°C and sterile, 10-fold concentrated synthetic medium was added afterwards. Three parallel evolution experiments were performed by serial transfer in aerobic shake flasks. One millilitre from a shake flask preculture of IMZ160 was used to inoculate a first shake flask containing 1 M sorbitol. Serial transfer was performed with 1 ml inocula from shake flasks that had become turbid and reached cell density (OD₆₀₀) higher than 1. At the end of the evolution experiment, a sample of the evolving population was stored at −80°C.

Anaerobic shake flask cultures for inoculum or characterization were incubated in a BactronX anaerobic chamber (Shell Lab, Cornelius, OR, USA) at 30°C and 200 r.p.m. (Heidolph Unimax 2010 shaker; Heidolph, Schwabach, Germany).

Table 1. Saccharomyces cerevisiae strains used in this study.

| Strain      | Relevant genotype/description                                      | Source/reference                      |
|-------------|-------------------------------------------------------------------|---------------------------------------|
| IME076      | MATa ura3 LEU2 GPD1 GPD2 p426_GPD(pTDH3::CYC1t URA3 2µ)           | Guadalupe Medina et al. 2010          |
| RWB0094     | MATa ura3 leu2 gpd1::loxP-KanMX-loxP gpd2::hphMX4                | BIRD Engineering, Rotterdam,          |
|             |                                                                   | Guadalupe Medina et al. 2010          |
| IMK006      | MATa ura3 leu2 gpd1::loxP gpd2::::hphMX4                         | This study                            |
| IMX031      | MATa ura3 leu2::LEU2[pRS405] gpd1::loxP gpd2::hphMX4             | This study                            |
| IMZ132      | MATa ura3 leu2 gpd1::loxP gpd2::hphMX4                           | Guadalupe Medina et al. 2010          |
| IMZ160      | (pTDH3::mhpF(E. coli)::CYC1t URA3 2µ) YEpplac181(LEU2)           | This study                            |
| IMZ380      | IMZ160 evolved for anaerobic growth at 1 M glucose                | This study                            |
| IMS343      | IMZ333 cured of plasmid                                          | This study                            |
| IMZ381      | IMZ333 with pUDE43ev1(pTDH3:: mhpF(E. coli)::CYC1t URA3 2µ evolved) | This study                            |
| IMJ004      | IMZ333 with pUDE43ev2(pTDH3:: mhpF(E. coli)::CYC1t URA3 2µ evolved) | This study                            |
| IMJ005      | IMZ333 with pUDE43ev1(pTDH3:: mhpF(E. coli)::CYC1t URA3 2µ evolved) | This study                            |
| IMJ006      | IMZ333 with pUDE43ev2(pTDH3:: mhpF(E. coli)::CYC1t URA3 2µ evolved) | This study                            |
| IMJ009      | IMZ333 with p426_GPD(pTDH3::CYC1t URA3 2µ)                       | This study                            |
| IMK527      | MATα ura3::loxP-KanMX-loxP leu2 gpd1::loxP gpd2::hphMX4           | This study                            |
| IMD011      | Diploid strain resulting from IMS343·IMK527                      | This study                            |
| IMD012      | Diploid strain resulting from IMZ333·IMK527                      | This study                            |

Sequential batch reactors (SBR) and physiological characterization in batch bioreactors

Anaerobic bioreactor batch cultures, off-gas and metabolite analysis, enzymatic glycerol determination, optical density readings, determination of dry weight and enzymatic activity measurements for NAD−-dependent (acetylating) acetaldehyde dehydrogenase and G3P dehydrogenase were performed as described previously (Guadalupe Medina et al., 2010). All fermentations were carried out at least in duplicate. To correct for ethanol evaporation during cultivation in nitrogen-sparged bioreactors, evaporation kinetics were analysed as described previously (Guadalupe Medina et al., 2010).

Sequencing batch reactors were operated as described previously (Wisselink et al., 2009). The medium vessels were prepared by autoclaving 18 l of demineralized water containing glucose (1.11 M), and subsequently adding 2 l of 10-fold
concentrated synthetic media containing acetic acid (20 g l\(^{-1}\)), antifoam [0.2 g l\(^{-1}\), Emulsion C (Sigma-Aldrich, Zwijndrecht, the Netherlands)], ergosterol (0.1 g l\(^{-1}\)) and Tween 80 (4.2 g l\(^{-1}\)). The pH of the 10-fold concentrated synthetic medium containing acetic acid was adjusted to pH 4.8 with potassium hydroxide before autoclaving. A control routine was programmed in MFCs/win 3.0 (Sartorius AG, Göttingen, Germany) to initiate the switch to a new batch cycle. Fermenters were automatically emptied, leaving ca. 1.5 ml of remaining culture volume, and refilled when the CO\(_2\)% in the off gas reached 1.2%. When growth accelerated after the first three cycles, this threshold was gradually increased to 3.2% CO\(_2\).

Single colony isolates were obtained by streaking a sample taken from the SBR on synthetic media agar plates (1% w/v) containing 1 M glucose as carbon source, 2 g l\(^{-1}\) acetic acid and anaerobic growth factors. The plates were placed under anaerobic environment in a BactronX anaerobic chamber (Shell Lab) and kept at 30°C. After two transfers of single isolates to fresh agar plates, one colony was inoculated in 1 M glucose synthetic media for stock and named IMZ333.

Before characterization in bioreactors, the evolved strains were precultured anaerobically in synthetic media agar plates (1% w/v) and anaerobic growth factors. The plates were placed under anaerobic environment in a BactronX anaerobic chamber (Shell Lab) and kept at 30°C. After two transfers of single isolates to fresh agar plates, one colony was inoculated in 1 M glucose synthetic media for stock and named IMZ333. Before characterization in bioreactors, the evolved strains IMZ333 and IMJ006 were precultured anaerobically in synthetic media shake flasks with 1 M glucose as carbon source.

**Spot assays to test osmotolerance**

Growth under high osmotic stress was assessed by spotting 5 μl of serial dilution of 10\(^6\), 10\(^5\), 10\(^4\) cells ml\(^{-1}\) of exponentially growing cultures onto 0.1, 0.5 and/or 1 M glucose synthetic media agar plates (1% w/v). Overnight shake flasks cultures grown as described before (Guadalupe Medina et al., 2010) were used to measure cell density using a Z-Coulter Counter (Beckman Coulter, Brea, CA, USA) and prepare appropriate dilutions in sterile demineralized water. Agar plates were prepared by autoclaving separately glucose and agar from concentrated synthetic media, and mixed thoroughly before pouring the plates. After spotting, all plates were incubated at 30°C under anaerobic and aerobic conditions for 7 days, and pictures were taken at 3 and 7 days.

**Backcrossing and sporulation**

To enable crossing, MAT\(_a\) strain IMK006 was transformed with the marker gene KanMX using primer pairs for the ura3 locus (URA3-KanMXF TTCTTAAACCAACTGCACAGAACAAAAA CTCGCAGGAACGAAGATAAATCCAGCTGAAGCTTGTA CGC and URA3-KanMXR AGCTCTAATTTGAGGTATTATG ATACATGCATTACTTATAATACAGGGTTTACCG CCGCAT) before the mating was switched by transforming plasmid pHO (Herskowitz and Jensen, 1991; Sugawara and Haber, 2012) into this strain. The resulting diploid strain was sporulated yielding a MAT\(_a\) strain IMK527. Sporulation was performed as described by Bahalul and colleagues (2010). Strains were inoculated in YP medium with 10 g l\(^{-1}\) acetate as carbon source. After incubation at 30°C for 24 h, cultures were washed and resuspended in sporulation medium (20 g l\(^{-1}\) potassium acetate). After 48 h at 30°C, spore formation was checked microscopically. Prior to dissection, a culture sample (1 ml) was incubated with 2 μl zymolyase (1000 U ml\(^{-1}\)) in a 200 μl 0.5 M sorbitol solution at 37°C for 10 min. Tetrad dissection on YP media plates with 20 g l\(^{-1}\) glucose was performed with a dissection microscope (Singer MSM System 300, Singer Instruments, Somerset, UK). Plates were incubated at 30°C. IMK527 (MAT\(_a\)) was crossed with haploid strains IMS343 (MAT\(_a\)) and IMZ333 (MAT\(_a\)) by streaking cultures over each other on selective synthetic medium agar plates containing G418 (100 mg l\(^{-1}\)) on which only diploids could grow. For auxotrophic diploids, uracil (20 g l\(^{-1}\)) was added to the medium. The resulting diploids were re-streaked, and single colonies were isolated, yielding strain IMD011 (IMS343\(\times\)IMK527) and IMD012 (IMZ333\(\times\)IMK527). Sporulation and tetrad dissection of IMD011 and IMD012 were performed as described above. Dissected spores were replica plated on 1 M glucose synthetic medium agar plates to score for osmotolerant segregants.

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**Conflict of interest**

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**References**

Albertyn, J., Hohmann, S., Thevelein, J.M., and Prior, B.A. (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol Cell Biol* 14: 4135–4144.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M., and Adler, L. (1997) The two isoenzymes for yeast NAD\(^{+}\)-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. *EMBO J* 16: 2179–2187.

Athenstaedt, K., and Daum, G. (2000) 1-Acylhydroxyacetone-phosphate reductase (Ayr1p) of the yeast *Saccharomyces cerevisiae* have distinct roles in osmoadaptation. *J Biol Chem* 275: 235–240.

Bahalul, M., Kaneti, G., and Kashi, Y. (2010) Ether-zymolyase ascospore isolation procedure: an efficient protocol for ascospores isolation in *Saccharomyces cerevisiae* yeast. *Yeast* 27: 999–1003.

Bai, F.W., Anderson, W.A., and Moo-Young, M. (2008) Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol Adv* 26: 89–105.

Bakker, B.M., Overkamp, K.M., van Maris, A.J.A., Köter, P., Luttik, M.A.H., Van Dijken, J.P., and Pronk, J.T. (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 25: 15–37.

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van den Berg, M.A., de Jong-Gubbels, P., Kortland, C.J., Van Dijken, J., Pronk, J.T., and Steensma, H.Y. (1996) The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. *J Biol Chem* 271: 28953–28959.

Björkqvist, S., Ansell, R., Adler, L., and Lidén, G. (1997) Physiological response to anaerobiosis of glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 63: 128–132.

Blomberg, A., and Adler, L. (1989) Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD\(^+\)) in acquired osmotolerance of *Saccharomyces cerevisiae*. *J Bacteriol* 171: 1087–1092.

Boender, L.G.M., de Hulster, E.A.F., van Maris, A.J.A., van den Berg, M.A., de Jong-Gubbels, P., and Kortland, C.J., *et al.* (2011) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* 72: 379–412.

van Dijken, J., Pronk, J.T., and Steensma, H.Y. (1996) The phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. *J Bacteriol* 174: 5702–5710.

van Dijken, J., Bauer, J., Brambilla, L., Duboc, P., Francois, J.M., Gancedo, C., *et al.* (2000) An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb Technol* 26: 706–714.

Entian, K.D., and Kötter, P. (2007) Yeast Genetic Strain and Plasmid Collections. In *Methods in Microbiology: Yeast Gene Analysis*, 2nd edn. Stansfield, I., and Stark, M.J.R. (eds). Amsterdam: Academic Press, pp. 629–666.

Guadalupe Medina, V., Almering, M.J.H., van Maris, A.J.A., and Pronk, J.T. (2010) Elimination of glycerol production in *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. *Microb Cell Fact* 9: 36.

Herskovitz, I., and Jensen, R.E. (1991) Putting the HO gene to work: practical uses for mating-type switching. In *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, Guthrie, C., and Fink, G.R. (eds). Amsterdam: Academic Press, pp. 132–146.

Hohmann, S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* 66: 300–372.

Hounsa, C.G., Brandt, E.V., Thevelein, J., Hohmann, S., and Prior, B.A. (1998) Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. *Microbiology* 144: 671–680.

Hubmann, G., Guillouet, S., and Nevoigt, E. (2011) Gpd1 and Gpd2 fine-tuning for sustainable reduction of glycerol formation in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 77: 5857–5867.

Jain, V., Divol, B., Prior, B., and Bauer, F. (2011) Elimination of glycerol and replacement with alternative products in ethanol fermentation by *Saccharomyces cerevisiae*. *J Ind Microbiol Biotechnol* 38: 1427–1435.

Jones, A.M., Thomas, K.C., and Ingledew, W.M. (1994) Ethanol production of blackstrap molasses and sugar cane juice using very high gravity technology. *J Agric Food Chem* 42: 1242–1246.

Laluce, C. (1991) Current aspects of fuel ethanol production in Brazil. *Crit Rev Biotechnol* 11: 149–161.

Nevoigt, E., and Stahl, U. (1997) Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 21: 231–241.

Oud, B., van Maris, A.J.A., Daran, J.M., and Pronk, J.T. (2012) Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. *FEMS Yeast Res* 12: 183–196.

Pagliardini, J., Hubmann, G., Bideaux, C., Alfenore, S., Nevoigt, E., and Guillouet, S. (2010) Quantitative evaluation of yeast’s requirement for glycerol formation in very high ethanol performance fed-batch process. *Microb Cell Fact* 9: 36.

Palmqvist, E., and Hahn-Hägerdal, B. (2000) Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioreour Technol* 74: 17–24.

Prasad, S., and Moore, J.W. (2006) High throughput screening for metabolic phenotypes. *Appl Environ Microbiol* 72: 44–53.
Scheffers, W.A. (1966) Stimulation of fermentation in yeasts by acetoin and oxygen. Nature 210: 533–534.
Shen, B., Hohmann, S., Jensen, R.G., and Bohnert, H. (1999) Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol 121: 45–52.
Sugawara, N., and Haber, J. (2012) Monitoring DNA recombination initiated by HO Endonuclease. In DNA Repair Protocols. Bjergbæk, L. (ed.). New York, NY, USA: Humana Press, pp. 349–370.
Swinnen, S., Thevelein, J.M., and Nevoigt, E. (2012) Genetic mapping of quantitative phenotypic traits in Saccharomyces cerevisiae. FEMS Yeast Res 12: 215–227.
Taherzadeh, M., Millati, R., and Niklasson, C. (2001) Continuous cultivation of dilute-acid hydrolysates to ethanol by immobilized Saccharomyces cerevisiae. Appl Biochem Biotechnol 95: 45–57.
Takagi, H. (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. Appl Microbiol Biotechnol 81: 211–223.
Verduyn, C., Postma, E., Scheffers, W.A., and van Dijken, J.P. (1990) Physiology of Saccharomyces cerevisiae in anaerobic glucose-limited chemostat cultures. J Gen Microbiol 136: 395–403.
Wisselink, H.W., Toirkens, M.J., Wu, Q., Pronk, J.T., and van Maris, A.J.A. (2009) Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered Saccharomyces cerevisiae strains. Appl Environ Microbiol 75: 907–914.