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ORIGINAL PAPER

Generation of an evolved *Saccharomyces cerevisiae* strain with a high freeze tolerance and an improved ability to grow on glycerol

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Abstract Glycerol is a residue generated during biodiesel production and represents around 10% of the total product output. Biodiesel production is currently having a significant impact on glycerol price, leading to an increased interest in the use of glycerol as a cheap substrate for fermentation processes. We have analysed the growth kinetics of two wild-type strains of *Saccharomyces cerevisiae* grown on synthetic media containing glycerol as the sole carbon and energy source. Both strains were initially unable to grow when cultivated under these conditions, and an unusually long lag phase was necessary prior to the appearance of slow-growing cells. Following the application of an "evolutionary engineering" approach, we obtained *S. cerevisiae* strains with an improved ability to grow on glycerol. We report here the isolation of an evolved strain that exhibits a reduction of the lag phase, a threefold increase of the specific growth rate and a higher glycerol consumption rate compared to wild-type strains. The evolved strain has retained its fermentative activity, producing ethanol at the same rate and yield as the wild type. Interestingly, the yeast biomass obtained by cultivating the evolved strain on synthetic glycerol-based media also showed a high viability after prolonged storage at −20°C. The strategy adopted in our study could be easily applied to obtain *S. cerevisiae* strains with new industrially relevant traits, such as an improved ability to use cheap substrates and high resistance to freeze and thaw procedures.

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Introduction

The transesterification of vegetable oils and animal fats used to produce biodiesel leads to glycerol production, which is estimated to be around 10% of the total product output worldwide [22]. The increasing production of alternative fuels is therefore expected to produce large amounts of glycerol, with the output eventually greatly outpacing demand. Biodiesel production has already had a significant impact on the price of glycerol. However, glycerol has the potential to be an "alternative" feedstock for sugar-based processes, such as fermentations [4, 11, 16]. Glycerol can be utilised aerobically by yeasts, but the pathways involved may differ in different species. In *Saccharomyces cerevisiae*, as well as in other more halotolerant yeasts, glycerol has been found to be taken up by both an active and a facilitated transport system as well as by passive diffusion, and the genes encoding the putative glycerol transporters, *STL1* and *FPS1*, have been characterised [5, 15, 18]. In budding yeast (see Fig. 1), the canonical metabolic pathway for glycerol utilisation proceeds via glycerol kinase, encoded by the *GUT1* gene [23], and mitochondrial glycerol-3-phosphate dehydrogenase, encoded by *GUT2* [25]. Another pathway for glycerol utilisation has been found in *Schizosaccharomyces pombe* [6], *Debaryomyces hansenii* [1], *Zygosaccharomyces rouxii* [31] and in some *Candida* and *Pichia* species [8, 29]. This alternative pathway requires the oxidation of glycerol to dihydroxyacetone, catalysed by a glycerol dehydrogenase, followed by its phosphorylation, catalysed by

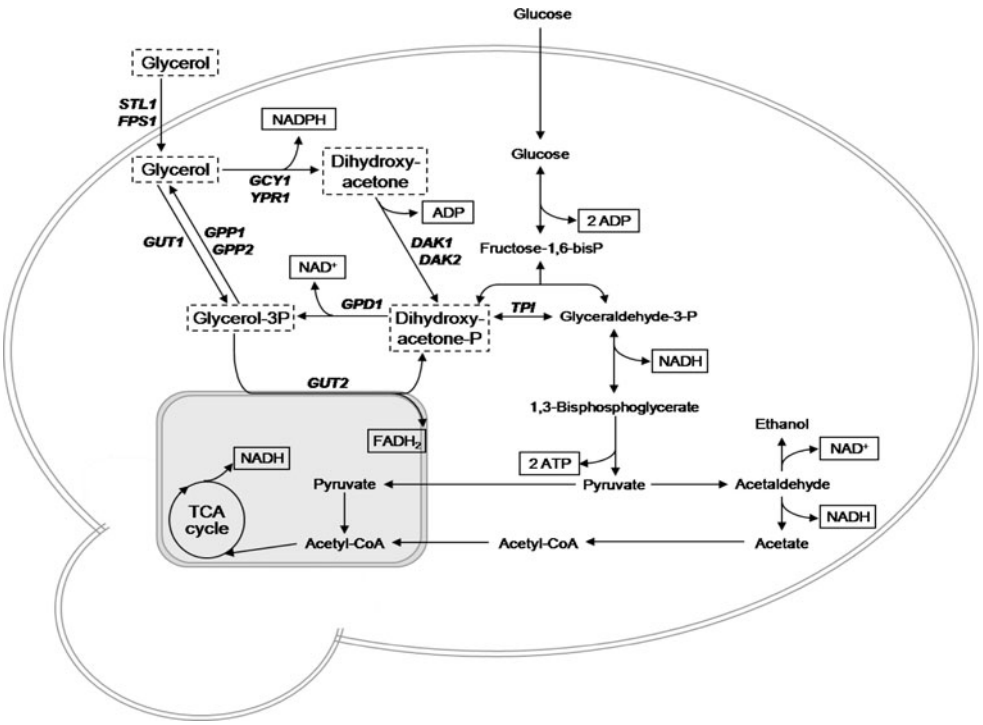

Fig. 1 A scheme of the pathways involved in glucose and glycerol assimilation under aerobic conditions underlining the role of energy (ADP/ATP) and redox balance (NAD^+/NADH, FAD^+/FADH). *Dotted frames* highlight the main intermediates in glycerol metabolism

dihydroxyacetone kinase, to dihydroxyacetone phosphate (DHAP). This alternative pathway is also present in *S. cerevisiae* (Fig. 1), but its role in glycerol metabolism is still unclear.

Glycerol also plays other fundamental roles in different aspects of yeast cell life. During hyperosmotic stress, *S. cerevisiae*, as well as other yeast, increase the production and intracellular accumulation of glycerol in order to achieve an osmotic balance with the environment [3, 9]. Glycerol is a well-known intracellular cryoprotective agent that accumulates in *S. cerevisiae* cells in response to temperature downshifts [21].

Glycerol metabolism plays an additional role in the process of reoxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) to the oxidised form (NAD^+). Under anaerobic conditions, glycerol formation is the only way for a cell to restore the cytosolic and mitochondrial redox balance that has been upset by the excess of NADH generated during the biosynthesis of amino acids and organic acids, since mitochondrial activity is limited by oxygen availability and ethanol production is a redox neutral process [30]. Under aerobic conditions, the cytoplasmic NADH can be oxidised by the external NADH-dehydrogenases (Nde1p/Nde2p) located on the outer side of the inner membrane of the mitochondria [14, 19] and via shuttle systems, like the glycerol-3-phosphate shuttle (Fig. 1). In this system, NADH is oxidised by the cytosolic glycerol-3-phosphate dehydrogenase (Gpd) during the catalysis of the reduction of dihydoxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). G3P subsequently delivers its electrons to the respiratory chain via FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p) [13], and DHAP reappears.

In this study we focused on the ability of *S. cerevisiae* to utilise glycerol as the sole carbon and energy source. We analysed the main growth parameters of wild-type strains under various conditions of growth on synthetic glycerol-based media. An evolved strain with an improved ability to grow on glycerol was isolated and further characterised. Interestingly, this mutant exhibited an increased resistance to freeze and thaw stress, a phenotypic trait of relevant interest in industrial applications.

Materials and methods

Yeast strains and growth conditions

The *S. cerevisiae* strains used in this work were the haploid strains CEN.PK 113-7D (CBS8340) (*MAT*a *MAL2-8*^{c} *SUC2*) and W303-1A (*MAT*a *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11*). These strains were routinely grown on YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose) in shake-flask culture at 30°C and 200 rpm in a rotary shaker. For experiments on glycerol-containing media, nutrient-rich glycerol medium (YPG; 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 10 g l^{-1} glycerol) was used for the pre-inoculum. Cells were grown overnight, collected, washed and used to inoculate cultures on synthetic YNBG medium containing 6.7 g l^{-1} yeast

nitrogen base (YNB) without amino acids (Difco, Detroit, MI), 10 g l^{-1} glycerol and supplements when required (50 mg l^{-1} amino acids and uracil, 100 mg/l^{-1} adenine). When specified in the text, 0.5 g l^{-1}peptone, 1 g l^{-1}glutamic acid or 6 g l^{-1} acetoin were also added. Cell growth was monitored by measuring the optical density at 450 nm (OD_{450}). Parallel samples varied by about 3–5%.

Isolation and characterisation of an improved strain

The evolution of non-mutagenised cultures was performed by serial transfers of shake-flask cultures of CEN.PK 113-7D strain on YNBG at 30°C. To enrich the culture for potential mutant cells**,** the culture was allowed to grow for several generations. Cells in the stationary phase for approximately 24 h were collected and inoculated into fresh medium at an initial OD of 0.05–0.1 and allowed to grow again to the stationary phase. After a total of 111 generations (50 days), 30 colonies were isolated by plating for single clones on solid glucose medium (YPD) and then cultivating the colonies for a further 50 generations in liquid YPD. The colonies were tested again for growth on YNBG, after a pre-inoculum on YPG, and the best performing cultures were stored at −80°C in 20% glycerol.

The evolved strain was further characterised by generating diploids by crossing either the wild-type strain CEN.PK 113-7D, used as a control, or the evolved strain MG16C with the haploid wild-type strain of the opposite mating type, CEN.PK 111-61A (*MAT α ura3 his3 leu2*). The strains were mixed on YPD plates, and zygotes were manually isolated using a Nikon Eclipse E400 micromanipulator (Nikon, Japan). The isolated diploids were tested for the improved ability to grow on glycerol, as described above. Diploids were also induced to sporulate, and tetrad analysis was performed according to standard procedures [27]. In total, 28 tetrads were analysed. Spores were allowed to germinate on YPD plates at 30°C for 4–5 days.

Batch cultivation

Aerobic batch cultivations were performed in a Biostat-Q-system (B-Braun; Sartorius BBI Systems, Bethlehem, PA) with a working volume of 0.8 l. An air flow of 1 l min^{-1} and a stirrer speed of 1,000 rpm maintained a dissolved oxygen concentration >50% of air saturation. The temperature was kept at 30°C, and the pH was maintained at pH 5 by the automatic addition of 2 M KOH. Pre-inoculum and batch fermentation were performed on the same YNB synthetic medium as described above, except that the carbon source was glucose at a concentration of 20 g l^{-1} (YNBD) in the batch culture. Batch experiments were performed in duplicates.

Anaerobic batch cultivations were performed in the same YNB synthetic medium as described above supplemented with ergosterol (10 mg l^{-1}) and Tween 80 (420 mg l^{-1}). The bioreactor was continuously flushed with N_2 (containing <3 ppm O_2) at a flow rate of 0.2 l min^{-1} per litre of medium, and a stirring rate of 500 rpm was maintained. In order to minimise the diffusion of oxygen into the bioreactor, Norprene tubing (Cole-Palmer, Milan, Italy) was used throughout the experimental setup.

Analysis of extracellular metabolites

The concentrations of glucose, ethanol, glycerol and acetate were determined in culture supernatants obtained after having removed the cells by centrifugation. R-biopharm enzymatic kits (Roche, R-Biopharm Italia, Milan, Italy; cat. no. 10716251, 10176290, 10148270 and 10148261, respectively) were used for the assays. All samples were analysed in triplicate, and the values of standard deviation (SD) obtained varied between 1 and 2%.

Preparation of cell extracts and enzyme assays

The cell extracts and enzyme assays were prepared by collecting cells from cultures at the mid-exponential growth phase. The protein concentration of cell extracts was determined using the Bio-Rad no. 500-002 kit (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard. The cell extraction procedure and enzyme assays for dihydroxyacetone kinase 1 and 2 (Dak1p/Dak2p) were performed according to the method of Gancedo et al. [6]. The addition of 4 mM dihydroxyacetone started the reaction. For glycerol kinase (Gut1p), the cell extract procedure and enzyme assays were performed as described by Pavlik et al. [23]. The addition of 1.5 mM NAD^+ started the reaction. For glycerol-3-phosphate dehydrogenase (Gpdp), the cell extracts were prepared and desalted essentially as described by André et al. [2] with the exception that cells were disrupted by agitation with glass beads on a vortex (alternating 1 min in ice and 1 min on vortex; repeated five times). The assay was performed in TrED buffer (10 mM triethanolamine, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5), 1 mM $MgCl_2$ and 0.1 mM NADH. The reaction was started by the addition of dihydroxyacetone phosphate 0.67 mM.

A unit (U) of enzyme activity was defined as 1 μmol of substrate transformed per minute using an extinction coefficient for NADH of 6.22 l/mmol/cm. Enzyme assays were performed in triplicate and the mean SD calculated. Standard deviations varied between 2.5 and 6%.

Freeze and thaw stress tolerance test

Cells were cultivated on YNBG (30 g l^{-1} glycerol) or on YNBD (20 g l^{-1} glucose) medium. At the end of the exponential growth phase, the cells were collected by centrifugation and the pellets stored in tubes at −20°C or resuspended in PBS and plated on YPD to determine the number of colony-forming units (CFU) prior to freezing. Frozen cells were thawed by resuspending them in PBS at the same optical density as before freezing and incubated for an additional 30 min at 30°C. The diluted aliquots were then spread on YPD plates to determine the number of colony-forming units after freezing. The percentage of survival for each strain was expressed relative to the initial viability before freezing, set as 100%.

Results

Growth of *S. cerevisiae* strains on glycerol as the sole carbon and energy source

In order to investigate in detail the ability of *S. cerevisiae* to utilise glycerol as the sole carbon and energy source, we analysed the behaviour of two laboratory strains of *S. cerevisiae*, CEN.PK 113-7D and W303-1A, during growth in synthetic glycerol-containing medium (YNBG). When cells were inoculated in YNBG, growth started after an unusually long lag phase that lasted from 3 to 5 weeks, as shown in Fig. 2. This long lag phase occurred despite the cells having been pre-cultured on nutrient-rich glycerol media YPG, to induce the expression of the genes required for glycerol utilisation, which are known to be under glucose repression [28]. After this phase, the cells started to grow at a specific growth rate of 0.060 h^{-1} (CEN.PK 113-7D) or 0.008 h^{-1} (W303-1A). The addition of 0.05% peptone to the YNBG abolished the long lag phase and promoted higher specific growth rates (0.110 h^{-1} for CEN.PK 113-7D and 0.036 h^{-1} for W303) (Fig. 2). One possible explanation for this action is that the presence of low concentrations of peptone may avoid a surplus of NADH being generated as a result of amino acid biosynthesis. To test this hypothesis, cells were cultivated in YNBG medium supplemented with amino acids. Similarly to the effect of peptone, even the presence of the sole amino acid glutamate promoted the growth of the yeast strains in glycerol-containing medium (Fig. 2). Acetoin is known to act as a specific NADH redox-sink, and in *S. cerevisiae* it is transformed to 2,3-butanediol by a NADH-dependent enzyme [7]. The addition of acetoin to the YNBG medium also resulted in growth starting without any lag phase (Fig. 2), but it arrested early when the acetoin was completely depleted. Thus, inefficient NADH reoxidation is likely to contribute to the long lag phase observed on synthetic glycerol-containing medium.

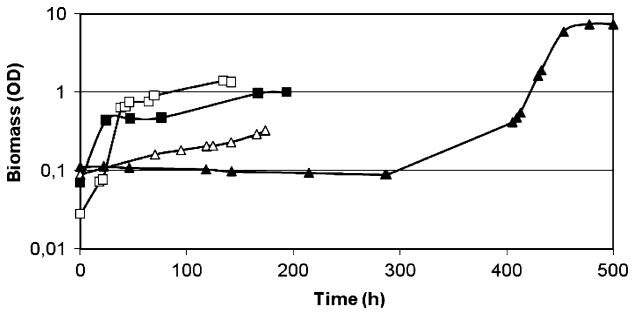

Fig. 2 Growth profiles of *Saccharomyces cerevisiae* wild-type strain CEN.PK 113-7D on synthetic glycerol-containing medium (YNBG). The results of a representative experiment are shown. *Filled triangle* Growth profile on YNBG medium; *other symbols* growth on YNBG medium supplemented with 1 g l^{-1} glutamate (*open triangle*), 6 g l^{-1} acetoin (*filled square*), 0.5 g l^{-1} peptone (*open square*). Cells from a pre-culture on YPG (glucose medium) were collected, washed and inoculated at *time zero*

The unusually long lag phase suggested us that slow-growing cells arose spontaneously in the cell population after prolonged cultivation in YNBG. This prompted us to repeat the procedure in order to isolate faster growing cells.

Generation of mutants with improved growth on glycerol

We adopted an "evolutionary engineering" approach to obtain a strain with an improved ability to use glycerol as the carbon source. Serial transfers of batch cultures of CEN.PK 113-7D strain in YNBG medium were performed. Following inoculation, the culture was allowed to grow until the stationary phase in order to enrich the culture of potential spontaneous mutant cells. The cells were then collected, diluted and inoculated into fresh medium. This procedure was operated several times in order to allow the population to evolve an improved ability to grow on glycerol. Analysis of the growth kinetics of the cultures (Fig. 3) revealed that after several generations in glycerol synthetic medium, the cell population growth rate gradually increased from 0.063 h^{-1} after 41 generations to 0.15 h^{-1} after 111 generations. Colonies were isolated by plating on glucose-based solid medium after a total of 111 generations of growth in glycerol-containing medium and cultivated for further 50 generations on the glucose-based medium. Some of the colonies which were subsequently cultivated again on the glycerol synthetic medium exhibited an improved ability to grow on glycerol, demonstrating that this phenotypic trait had been stably maintained, with the cells behaving as mutants. In particular, the mutant labelled MG16C did not display any lag phase when cultivated in synthetic glycerol medium (Fig. 3), grew at a

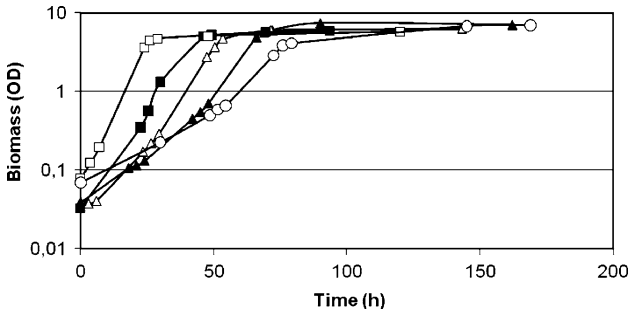

Fig. 3 Growth kinetics of the cultures obtained by re-inoculation on synthetic glycerol medium: the culture after 8 generations (*open circle*), after 41 generations (*filled triangle*), after 73 generations (*open triangle*), after 96 generations (*filled square*). The kinetics of the evolved mutant strain MG16C (*open square*) on glycerol medium represents data on one single clone isolated after 111 generations on glycerol medium and further 50 generations on glucose medium

threefold faster specific growth rate than the wild-type on glycerol (Table 1) and exhibited a twofold higher specific rate of glycerol consumption (7.34 vs. 3.44 mmol g dry weight h^{-1}, respectively). Surprisingly, the MG16C mutant strain showed a less efficient ability to grow on galactose, but it maintained the same growth rate as the wild type on ethanol (Table 1).

In an attempt to further characterise the evolved strain, we crossed MG16C mutant cells with the haploid wild-type strain of the opposite mating-type. When the isolated diploids were cultivated in synthetic glycerol medium, they showed the same growth parameters as the wild-type diploid or haploid strains (long lag phase and reduced growth rate), indicating that the mutation(s) had a recessive character. Diploids were induced to sporulate, and tetrad analysis was performed. Whereas all of the spores from the control diploid germinated, only 24% of the spores from the MG16C derived-diploid gave rise to visible colonies, while the remaining spores arrested after a few cell divisions. This is an indication that more than one mutation is present in the strain. In addition, half of the grown spores exhibited the phenotypic traits typical of MG16C, indicating that a limited set of mutations are responsible for the phenotype.

Characterisation of the fermentative activity of the evolved strain MG16C

The fermentative metabolism pathway of MG16C was studied in more detail in aerobic and anaerobic batch cultures in synthetic glucose-containing media (YNBD). Under aerobic conditions, the two strains showed little differences in terms of growth rate and ethanol yield as well as in the specific rate of glucose consumption and ethanol production (Table 2). However, the MG16C strain produced glycerol at a reduced rate and yield than the parental strain (Table 2). A higher acetic acid production was observed in MG16C cultures.

Under strict anaerobic conditions, the MG16C strain exhibited a reduced growth rate and a reduced rate of glucose consumption compared to the wild-type parental strain, but ethanol yield was slightly higher (Table 2). In

Table 1 Specific growth rates on various carbon sources of *Saccharomyces cerevisiae* wild-type strain CEN. PK 113-7D and of the evolved strain mutant strain MG16C

Strain	Specific rate of growth (h^{-1})			
	Non-fermentable carbon sources		Fermentable carbon sources	
	Glycerol	Ethanol	Glucose	Galactose
Wild type	0.06	0.15	0.38	0.25
MG16C	0.17	0.15	0.31	0.17

Table 2 Growth parameters and production of metabolites of the *S. cerevisiae* parental strain (CEN.PK 113-7D) and evolved mutant strain (MG16C) in batch cultures in synthetic glucose medium (YNBD) under controlled aerobic and anaerobic conditions

Strain	Specific growth rate (h^{-1})	Yield (g g^{-1} glucose)				q (mmol g^{-1}dry weight h^{-1})			
		Biomass	Ethanol	Glycerol	Acetate	Glucose	Ethanol	Glycerol	Acetate
Aerobic conditions									
Wild type	0.38	0.115	0.382	0.057	0.005	13.26	21.87	1.98	<0.10
MG16C	0.31	0.087	0.365	0.024	0.010	17.90	27.61	0.85	0.45
Anaerobic conditions									
Wild type	0.35	0.081	0.390	0.074	0.005	26.85	35.39	4.17	<0.10
MG16C	0.20	0.068	0.419	0.072	0.003	14.58	27.03	2.40	<0.10

Table 3 Activities of enzymes involved in glycerol metabolism in the wild-type strain CEN.PK 113-7D and in the evolved strain MG16C

Strain	Enzyme activity (mU/mg total proteins)		
	Gpdp on glucose	Dak1p/Dak2p on glycerol	Gut1p on glycerol
Wild type	38.2 ± 1.3	15 ± 2.5	43 ± 5.1
MIN16C	1.4 ± 0.1	16 ± 1.0	39 ± 7.7

Gpdp Glycerol-3-phosphate dehydrogenase, *Gut1p* glycerol kinase, *Dak1p/Dak2p*, dihydroxyacetone kinase 1 and 2

contrast to the aerobic condition, the same yield of glycerol was attained by both strains, but the production rate by MG16C was lower, as already observed under aerobic conditions (Table 2).

Activity of enzymes involved in the different pathways for glycerol utilisation

To investigate the role of the different pathways involved in glycerol utilisation in the mutant strain, we determined the activity of the dihydroxyacetone kinases Dak1p/Dak2p and the glycerol kinase Gut1p (see Fig. 1). In MG16C cells growing on synthetic glycerol medium, the specific activities of both Dak1p/Dak2p and Gut1p were similar to those detected in the wild-type cells (Table 3), thus indicating that in the mutant the contribution of these enzymes to glycerol metabolism was unaffected. In contrast, the assay of Gpdp activity revealed that this enzyme was less active in the mutant than in the wild type.

Response to freeze and thaw stress

It has been reported that *S. cerevisiae* wild-type cells cultured under rich glycerol-based media show higher tolerance to freezing than cells cultured on glucose-based media [10]. Nevertheless, the requirement of rich media to achieve growth and the low growth rate make the process of biomass production on glycerol-based media unsuitable at the industrial level. The increased ability of the evolved strain MG16C to grow on synthetic glycerol-based media led us to test if the yeast biomass cultured in this way also showed an increased resistance to freeze and thaw stress.

MG16C cells were collected at the end of their growth on synthetic glycerol-containing media (YNBG) and stored in tubes at −20°C for 30 days. The number of colony-forming units was determined before and after freezing (as described in the "Materials and methods"). After thawing, MG16C cells showed 80% viability, indicating a high resistance to freezing. As expected, the viability of the wild-type parental strain cultivated on glucose-based synthetic media (YNBD) was only 23%. Thus, the improved ability of the mutant strain to grow on glycerol in synthetic media resulted in the production of yeast biomass with high resistance to freeze and thaw stress.

Discussion

The approach termed "evolutionary engineering" has been applied successfully to a number of industrial processes as a rational natural selection method alternative to metabolic engineering [12, 26]. In this report, we describe the selection and characterisation of an evolved *S. cerevisiae* strain with an improved ability to grow on glycerol as the sole carbon source through the use of a simple evolution strategy consisting of sequential batch cultivations on synthetic glycerol-based media. After a total of 111 generations on glycerol and 50 on glucose, this ability remained stable in cells of the mutant MG16C clone and was retained after storage at −80°C. This means that the new strain MG16C behaved like a mutant. MG16C showed a threefold higher specific growth rate than the wild-type strain on synthetic glycerol-based media (Table 1).

In *S. cerevisiae,* glycerol utilisation proceeds mainly via glycerol kinase (Gut1p) and mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p) (Fig. 1). In fact, the inability of *gut1* and *gut2* mutants to grow on glycerol [18, 23] indicates that the activity of the alternative pathway, through dihydroxyacetone, is not sufficient to allow the utilisation of glycerol as a sole carbon source. Gut2p has also been shown to play an important role in the redox metabolism, being part of the shuttle system for the re-oxidation of cytosolic NADH, together with Gpdp (Fig. 1). In addition, Gut2p has been demonstrated to work in a complex together with Nde1p/Nde2p and its activity seems to be strongly inhibited by the activity of these external NADH dehydrogenases [20, 24]. This fact implicates that the catalytic role of Gut2p could be insufficient in promoting growth on glycerol under specific conditions, like those occurring during growth on synthetic medium, in which the synthesis of amino acids results in an excess of NADH production. A reduced activity of Gut2p would consequently result in a low flux toward DHAP production. A reduced/re-balanced NADH production could then

alleviate this inhibition and enable more efficient growth, as we observed on media containing peptone, glutamic acid or acetoin.

The activity of triose phosphate isomerase (Tpi1p, Fig. 1) is essential for glycerol utilisation, allowing DHAP to enter the lower part of the glycolysis pathway as well as the gluconeogenesis pathway. A strong competition between two enzymes, Gpd1p and Tpi1p, may occur in *S. cerevisiae* growing on glycerol, given that the K_M (concentration of substrate that leads to half-maximal velocity) of Gpd1p and Tpi1p for DHAP is very similar (0.50 and 0.68 mM, respectively). Gpd1p activity can then drain the DHAP produced by Gut2p back to G3P, giving rise in vivo to a futile cycle between these two compounds and, in this way, limit the flux of DHAP toward the glycolysis/gluconeogenesis pathway. Interestingly, our analysis of the key enzymatic activities in the MG16C mutant demonstrated a reduced activity of Gpd1p (Table 3). On the other hand, we did not detect any increase in the activity of the enzymes involved in the glycerol utilisation pathways (Table 3). In conclusion, the improved growth on glycerol of strain MG16C might be associated to a reduced activity of Gpd1p which could decrease the futile cycle and conversely increase the flux of DHAP into the glycolysis/gluconeogenesis pathways, thereby promoting growth on the sole carbon source glycerol. Nevertheless, mutations affecting other genes or regulatory regions could work in synergy in determining the "improved growth on glycerol" phenotypic trait. In fact, the simple knock-out of the *GPD1* gene in the CEN.PK 113-7D background did not result in any improved phenotype (data not shown). The complete understanding of the genetic traits that are specifically required to "evolve" the ability to grow more efficiently on glycerol would require a genome-wide analysis, as by a single-nucleotide polymorphism chip or transcriptome analyses, and a further study of the cause–effect relations. This would lead to the identification of targets that could be used to perform a strategy of strain improvement by inverse metabolic engineering, as it has been effectively employed for *S. cerevisiae* strains in an increasing number of approaches [17].

The MG16C strain also exhibited an high resistance to freeze and thaw stress after prolonged storage at −20°C when cultivated on glycerol-based synthetic media. This trait cannot be ascribed specifically to the mutant phenotype because it has been previously observed in *S. cerevisiae* wild-type cells cultivated on rich glycerol-based media [10]. However, the improved ability of the mutant to grow in glycerol-based media makes the process for the production of yeast biomass more industrially suitable. We suggest that this improved growth in glycerol-based media combined with the improved freeze tolerance are industrially relevant features, such as in the case of producing baker's yeast for frozen dough. The mutant genotype did not affect the fermentative ability of the strain in terms of ethanol production (Table 2), furthermore supporting its potential useful application.

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