J Ind Microbiol Biotechnol (2011) 38:1853–1859
DOI 10.1007/s10295-011-0974-z

ORIGINAL PAPER

Alcoholic fermentation by wild-type *Hansenula polymorpha* and *Saccharomyces cerevisiae* versus recombinant strains with an elevated level of intracellular glutathione

Dorota Grabek-Lejko · Olena O. Kurylenko · Vladimir A. Sibirny · Vira M. Ubiyvovk · Michel Penninckx · Andriy A. Sibirny

Received: 22 September 2010 / Accepted: 7 April 2011 / Published online: 29 April 2011

Abstract The ability of baker's yeast *Saccharomyces cerevisiae* and of the thermotolerant methylotrophic yeast *Hansenula polymorpha* to produce ethanol during alcoholic fermentation of glucose was compared between wild-type strains and recombinant strains possessing an elevated level of intracellular glutathione (GSH) due to overexpression of the first gene of GSH biosynthesis, gamma-glutamylcysteine synthetase, or of the central regulatory gene of sulfur metabolism, *MET4*. The analyzed strains of *H. polymorpha* with an elevated pool of intracellular GSH were found to accumulate almost twice as much ethanol as the wild-type strain during glucose fermentation, in contrast to *GSH1*-overexpressing *S. cerevisiae* strains, which also possessed an elevated pool of GSH. The ethanol tolerance of the GSH-overproducing strains was also determined. For this, the wild-type strain and transformants with an elevated GSH pool were compared for their viability upon exposure to exogenous ethanol. Unexpectedly, both *S. cerevisiae* and *H. polymorpha* transformants with a high GSH pool proved more sensitive to exogenous ethanol than the corresponding wild-type strains.

D. Grabek-Lejko · V. A. Sibirny · A. A. Sibirny
University of Rzeszow,
Cwiklinskiej 2, 35-601 Rzeszow, Poland

O. O. Kurylenko · V. M. Ubiyvovk · A. A. Sibirny (✉)
Institute of Cell Biology NAS of Ukraine,
Drahomanov Street, 14/16, Lviv 79005, Ukraine
e-mail: sibirny@cellbiol.lviv.ua

M. Penninckx
Laboratory of Microbial Physiology and Ecology,
Universite Libre de Bruxelles, 642 rue Engeland,
1180 Brussels, Belgium

Keywords Alcoholic fermentation · Yeasts · Glutathione · Ethanol tolerance · *Hansenula polymorpha* · *Saccharomyces cerevisiae*

Introduction

Glutathione (GSH) is an abundant nonprotein thiol present in most living cells, from microorganisms to humans [12]. It acts as the major cellular redox buffer, as its easy interconversions between reduced and oxidized forms maintain the cellular redox status [11]. In addition, glutathione plays pivotal roles in stress responses caused by nutrient starvation, heavy metals, xenobiotics or free radicals and is involved in sulfur storage, gene regulation, and cell signaling [2, 13, 17]. Ethanol produced during alcoholic fermentation is known to induce metabolic stress [1]. This, in turn, could impair ethanol productivity, but to our knowledge, the role of GSH as a major stress defense factor in ethanol production and ethanol tolerance has not been studied. Isolation of robust yeast strains resistant to metabolic stress and accumulating enhanced amounts of ethanol is an important biotechnological goal. Therefore, we investigated possible interrelationships between the intracellular GSH pool, ethanol production, and ethanol tolerance in baker's yeast *Saccharomyces cerevisiae* and the methylotrophic yeast *Hansenula polymorpha*. *S. cerevisiae* is used for industrial ethanol production [3, 27], whereas *H. polymorpha* is capable of high-temperature glucose, xylose, and cellobiose fermentation and glycerol conversion to ethanol [14, 18]. Enhancement of the intracellular GSH pool was achieved in both *S. cerevisiae* and *H. polymorpha* by overproduction of the enzyme catalyzing the first key enzyme of GSH biosynthesis: gamma-glutamylcysteine synthetase, encoded by the genes *GSH1*

in *S. cerevisiae* and *GSH2* in *H. polymorpha* [23]. On the other hand, the pool was raised in *H. polymorpha* by overexpression of *MET4*, the central regulatory gene of sulfur metabolism [21; personal communications with Prof. H.A. Kang, Korea Research Institute of Bioscience and Biotechnology, Daejeon]. In this article, we describe effects of GSH overexpression on ethanol synthesis and tolerance in *S. cerevisiae* and *H. polymorpha*.

Materials and methods

Strains and growth conditions

The *Hansenula polymorpha* DL-1 strains used were: wild type 356 (prototrophic), transformant *mcHpMET4* (DL-1 *leu2::mcMET4::LEU2*, *Δura3::URA3*, prototrophic), and transformant *mcHpGSH2* (DL-1 *Δura3*, *Δtrp1::URA3*, *leu2::mcGSH2CBS::LEU2*, *Δtrp1::TRP1*, prototrophic) with multicopy integration of *H. polymorpha MET4* and *GSH2* genes from the collections of Prof. A. Sibirny (Institute of Cell Biology NAS of Ukraine, Lviv, Ukraine) and Prof. H.A. Kang (Korea Research Institute of Bioscience and Biotechnology, Daejeon, 305–333, Korea).

The *Hansenula polymorpha* CBS4732 strains used were: wild type CBS4732 *ura3* (*leu2::LEU2 ura3*) and mutant *Δggt1 ura3* (*leu2 ggt1::LEU2 ura3*) [22].

The *Hansenula polymorpha* NCYC 495 strains used were: wild type NCYC 495 *leu1-1*, wild type NCYC 495 *ade11* (*leu 1-1:: LEU2 ade11*), and mutants vps34 *leu1-1* (*vps34/pdd1 leu1-1*) [9], *vps34mcHpGSH2* (*vps34/pdd1 leu1-1::mcHpGSH2::LEU2*), and *Δgsh2 ade11* (*leu 1-1 Δgsh2::LEU2 ade11*) from the laboratory collection of Prof A. Sibirny (Institute of Cell Biology NAS of Ukraine, Lviv, Ukraine).

The *Saccharomyces cerevisiae* wild-type strains used were: BY4742 (*MAT alpha his3 leu2 lys2 ura3*) and BY4742 carrying the YEp352 plasmid (*MAT alpha his3 leu2 lys2 ura3::URA3*). Transformants *mcGSH1* and *mcGSH1/AatII* (*MAT alpha his3 leu2 lys2 ura3::mcGSH1::URA3*) were obtained as described below.

In alcoholic fermentation studies, yeasts were grown in synthetic minimal medium consisting of Yeast Nitrogen Base (YNB) without amino acids (1.7 g/l), ammonium sulfate (5 g/l), a sugar (4% or 12% glucose; 12% xylose), with appropriate amino acids and nucleic bases. In the case of *Δgsh2* mutants defective in glutathione synthesis, the medium additionally contained 0.1 mM glutathione. *H. polymorpha* was grown at 37°C and *S. cerevisiae* at 28°C.

Xylose fermentation studies in *H. polymorpha* were carried out in 125-ml flasks containing 50 ml appropriate medium with 12% xylose. The cells were grown for 48, 72, and 96 h at 37°C on a rotary shaker (100 rpm, limited aeration).

Glucose fermentation studies in *H. polymorpha* were carried out: (1) by incubating high-cell-density cultures ($OD_{600} \approx 10.0$) in 100-ml flasks containing 40 ml YNB medium with high glucose concentration (12%) for 150 h on a rotary shaker at 100 rpm, and (2) by batch cultivation in an Applicon fermenter under limited aeration (working volume: 1 l YNB medium with 4% glucose), starting at $OD_{600} \approx 0.2$. In both cases, precultured cells were grown to mid-exponential phase in 200 ml medium at 200 rpm and 37°C, washed twice with sterile water, and then inoculated.

S. cerevisiae alcoholic fermentation was studied in batch flask and fermenter cultures. Glucose fermentation by *S. cerevisiae* under low aeration was studied by growing the cells (starting OD $\approx$ 2.0) in 100-ml flasks containing 40 ml of the indicated medium with 4% glucose on a rotary shaker at 100 rpm. Yeast inoculates were pregrown to mid-exponential phase in the same medium with shaking at 200 rpm. Cell biomass was determined turbidimetrically at 600 nm.

Batch cultivation under anaerobic conditions was performed in an Applicon fermenter (working volume: 1 l YNB medium with 4% glucose and appropriate amino acids) with nitrogen blowing through the culture medium. Yeast cells (starting $OD_{600} \approx 1.8–2.0$) were incubated in the fermenter for 3 days at 28°C. The cells used for inoculation were pregrown for 30 h at 28°C and under shaking at 200 rpm in 250-ml Erlenmeyer flasks containing 50 ml YNB medium with 1.5% glucose.

Construction of plasmids bearing the *S. cerevisiae GSH1* gene

The *GSH1* gene of *S. cerevisiae* (total DNA fragment size: 3,332 bp, comprising 924 bp upstream from the ATG codon, 2,037 bp of open reading frame, and 371 bp downstream from stop codon) was polymerase chain reaction (PCR)-amplified with primers IRAF'8 (5′-GCGAGCTC GTAAGCTTCGTTACTCA-3′) and IRAR'9 (5′-GCGAG CTCGTAAGCTTCGTTACTCA-3′), and genomic DNA of *S. cerevisiae* BY4742 as template. The amplified DNA fragment was digested with *Sac*I and *Xba*I endonucleases and cloned into corresponding sites of the YEp352 plasmid (*S. cerevisiae*/*Escherichia coli* shuttle vector, GenBank accession L14758, possessing *ScURA3* as selection marker). This yielded the recombinant construct YEp352-*ScGSH1*. The lithium acetate method was used to introduce YEp352-*ScGSH1* plasmid (circular or linearized with the *Aat*II endonuclease) into the *S. cerevisiae* BY4742 (*MAT alpha his3 leu2 lys2 ura3*) recipient strain. Transformants were selected on YNB medium without uracil. The presence of the

corresponding plasmids in the transformants was confirmed by diagnostic PCR. Standard DNA manipulations were performed as described in [15].

Analytical methods

The ethanol concentration accumulated in the medium during glucose fermentation was measured using the Alcotest alcohol oxidase/peroxidase-based enzymatic kit. The standard deviation of this method is SD = 0.006 [7]. Glucose was detected refractometrically with the Merck kit for glucose determination. Glutathione was measured by two methods: (1) cellular GSH content was determined in cell-free extract deproteinated with 5% trichloroacetic acid (TCA) and neutralized with 0.5 M NaOH according to the photometric method using Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) for thiol determination [8]. The cellular GSH concentration was expressed in mg/g dry biomass. (2) Total glutathione concentration (GSH + GSSG) was also measured in cell-free extracts by means of the standard recycling assay based on DTNB reduction in the presence of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) [4]. The extracts used were prepared by vortexing yeast cells in Eppendorf microtubes at 4°C for 20 min with 0.1 M potassium phosphate buffer pH 7.5 and glass beads (425–600 μm) in the ratio 1:1:1 (v/v/v) and then centrifuging the mixture for 20 min at 4°C and 20,000 × *g*. Protein was determined by the Lowry method. In this case, the glutathione level was expressed in nmoles (GSH + GSSG)/mg protein. Optical density was determined at 600 nm using a Spectronic Helios Gamma UV–Vis spectrophotometer.

Resistance to exogenous ethanol

For the spot test, *H. polymorpha* cells were grown overnight at 37°C under shaking (250 rpm) in 10-ml tubes, each containing 3 ml liquid YPD medium. The final density was about 10^8 cells/ml. The cultures were then diluted serially (1:10). Then 4-μl samples of cell suspension (starting OD: 2.0) were spotted onto YPX plates (1% yeast extract, 1% peptone, 1% xylose or glucose, 2% agar) supplemented with various concentrations of ethanol (4–10%). The plates were incubated at 37°C, and colony growth was recorded after 72 h. Ethanol evaporation from agar medium used for studying yeast ethanol tolerance at 37°C was experimentally measured and was equal near 20% after 3 days of plate incubation. Glutathione did not change ethanol evaporation.

The viability of *S. cerevisiae* and *H. polymorpha* yeast cells was assayed by fluorescence microscopy. These viability assays were performed as follows. Yeast cells were pregrown to late exponential phase at 37°C (*H. polymorpha*) or 28°C (*S. cerevisiae*) on YPG medium on a rotary shaker (150 rpm). These late-exponential-phase cells (1×10^8/ml) were then incubated for 1 h in 100 mM phosphate buffer pH 7.0 with different concentrations of ethanol. After incubation, the cells were centrifuged, washed twice with the same phosphate buffer, and stained simultaneously for 20 min in the dark at room temperature with two fluorescent dyes: 5 μg/ml propidium iodide (PI, 1 mg/ml stock solution) and 10 μg/ml fluorescein diacetate (FDA, 1 mg/ml stock solution, dissolved in acetone). FDA/PI fluorescence was examined at 480 nm excitation wavelength under an Olympus BX-51 microscope equipped with a DP-72 digital camera [30].

Results

To study how the GSH level might relate to alcohol fermentation, we tested various *H. polymorpha* strains: strains expressing either *GSH2* (a homolog of the *S. cerevisiae* gene *GSH1*, involved in GSH biosynthesis) or *MET4* (a gene involved in regulating sulfur metabolism) from a multicopy vector and mutants defective in genes involved in GSH synthesis and degradation (*Δgsh2*, *Δggt1*). In *S. cerevisiae*, we studied ethanolic fermentation in the wild-type strain and in recombinant strains with multicopy expression of the *GSH1* gene.

Glucose and xylose fermentation by *H. polymorpha* in shake flasks and fermenter cultures

Hansenula polymorpha strains overexpressing *GSH2* or *MET4* were found to accumulate ethanol to higher concentrations than the wild type. The latter accumulated about 1.5% ethanol at most, as opposed to 3% and 4.5%, respectively, for the strains overexpressing *MET4* or *GSH2* (Table 1). Higher ethanol accumulation was accompanied by enhanced glucose consumption by the corresponding strains (Table 1). Semi-anaerobic batch fermentations of these strains in a bioreactor yielded the same picture (Fig. 1). The strains overexpressing *GSH2* or *MET4* showed an elevated intracellular GSH level and accumulated ethanol very quickly, to a maximum level (reached after 30 h) three times as high as the level reached in similar cultures of the wild type. After 30 h of incubation, the ethanol level dropped very quickly to zero. This may be connected to total glucose utilization by the yeast cells. Exogenous GSH (0.1 mM) had no effect on ethanol synthesis from glucose in the *H. polymorpha* wild-type strain (data not shown). It is known that exogenous GSH is not transported into *H. polymorpha* wild-type cells to any significant extent, whereas it accumulates endogenously in *gsh1* and *gsh2* mutants [24].

Table 1 Alcoholic fermentation by wild-type, recombinant, and mutant *H. polymorpha* strains according to carbon substrate on day 2

Strain	Glucose, 12%		
	Biomass (OD_{600})	Ethanol (g/l)	Residual glucose (g/l)
DL-1 356 (wild type)	12	13	45
mcHpMET4	14	30	30
mcHpGSH2	13	45	20
NCYC495 *ade11* (wild type)	14	15	39
Δgsh2 ade11[a]	13	18	28
CBS4732 *ura3* (wild type)	13	18	30
Δggt1 ura3	12	15	38
NCYC495 *leu1-1* (wild type)[b]	13	17	45
vps34 leu1-1[b]	13	29	40
vps34 mcHpGSH2[b]	14	33	35

Growth at 100 rpm (starting OD ≈ 10) and 37°C ("Materials and methods")

[a] Mutant cells were incubated in the indicated medium with added 0.1 mM GSH

[b] Data are for day 2 of yeast cultivation

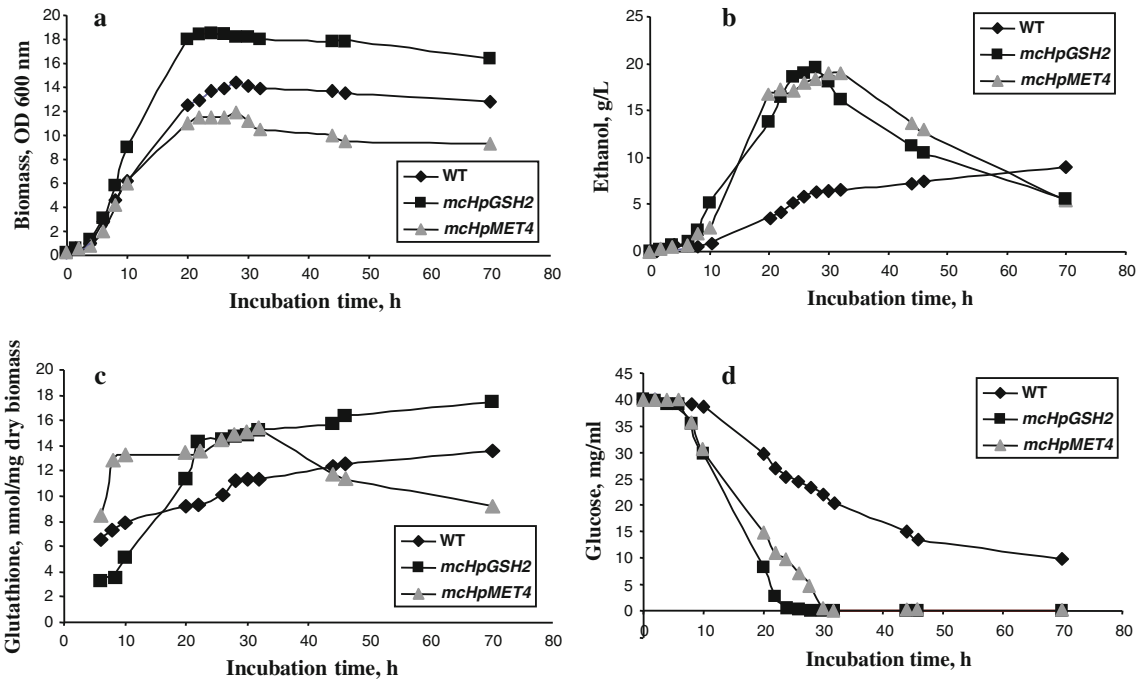

Fig. 1 Alcoholic fermentation by wild-type *H. polymorpha* DL-1 and by recombinant strains (*mcHpGSH2* and *mcHpMET4*) with an elevated level of glutathione. The strains were grown at 37°C on medium with 4% glucose under limiting aeration (batch fermenter cultures). Biomass (**a**), ethanol (**b**), and glutathione (**c**) accumulation and glucose consumption (**d**). *Values* are means of three independent determinations

We also observed increased ethanol accumulation in cultures of several other mutants, notably a *vps34* strain defective in autophagy [9] and the *vps34* strain over-expressing the *GSH2* gene (Table 1). Strains defective in glutathione synthesis (*Δgsh2*) [23] or GSH degradation (*Δggt1*) [22] did not differ from the parental wild-type strain as regards ethanol accumulation (Table 1). The *Δggt1* mutation did not lead to any change in the cellular glutathione pool, and the defect in GSH biosynthesis in the *Δgsh2* mutant was counterbalanced by GSH included in the growth medium (at 0.1 mM concentration) (data not shown).

When xylose was used as the fermentable carbon source, we observed no significant differences in ethanol

accumulation between the wild-type and recombinant strains; however, the level accumulated was considerably lower than when glucose was used (data not shown).

Glucose fermentation by *S. cerevisiae* strains in shake flasks and bioreactors

We next analyzed glucose fermentation by wild-type *S. cerevisiae* and by a recombinant strain overexpressing the *GSH1* gene. In the latter, the intracellular pool of GSH was elevated (Fig. 2). In contrast to the *H. polymorpha* strains with an elevated GSH pool, their *S. cerevisiae* counterpart did not differ from its parental wild-type strain as regards ethanol accumulation, either in shake-flask fermentations carried out under semi-anaerobic conditions or in strictly anaerobic bioreactor fermentations (data not shown). *S. cerevisiae* and *H. polymorpha* thus differ as regards the effect of the GSH pool on ethanol yield and productivity.

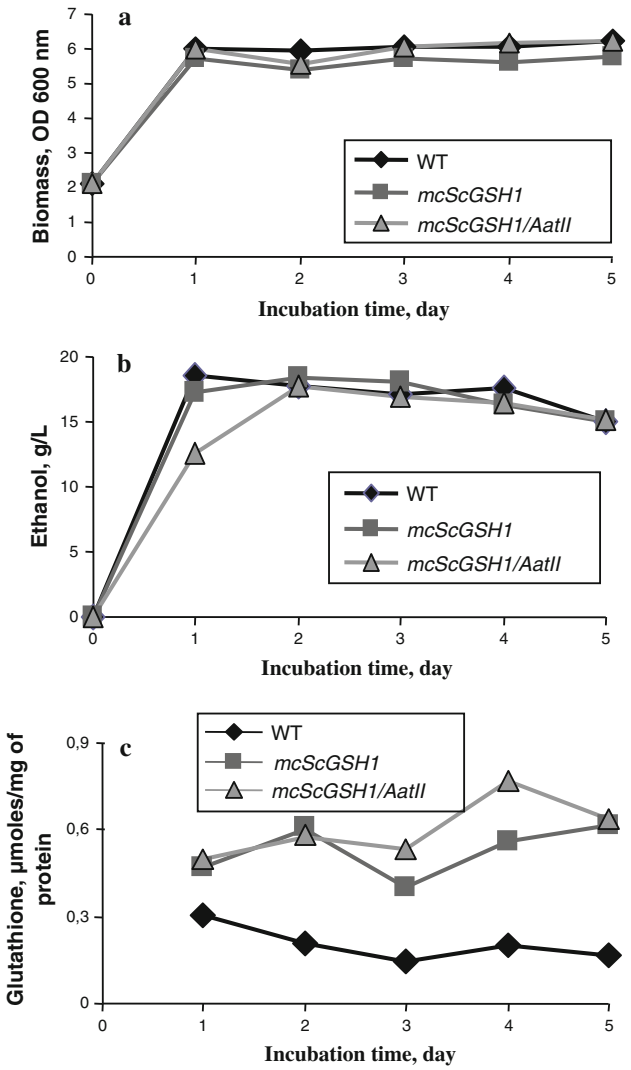

Fig. 2 Alcoholic fermentation by wild-type *S. cerevisiae* (*WT*) and by recombinant strains (*mcScGSH1* and *mcScGSH1/AatII*) with an elevated level of glutathione. The strains were grown in medium with 4% glucose at 100 rpm and 30°C. Biomass (**a**), ethanol (**b**), and glutathione (**c**) accumulation. *Values* are means of three independent determinations

Effect of GSH overproduction on yeast ethanol tolerance

We also studied the resistance of the above-mentioned strains to exogenous ethanol (Figs. 3, 4). Invariably, the *H. polymorpha* and *S. cerevisiae* strains with a higher glutathione pool appeared to be more susceptible to exogenous ethanol than the corresponding wild-type strains. Among the *H. polymorpha* strains, the most sensitive to ethanol was the *vps34/pdd1* strain, which could not grow on 5% ethanol. It is known that *S. cerevisiae* mutants with *VPS34* gene deletions are more sensitive to ethanol [19].

The *H. polymorpha* strains mc*MET4* and mc*GSH2* likewise proved significantly more sensitive than the wild type to exogenous ethanol on both glucose (data not shown) and xylose plates (Fig. 3). Viability assays performed on yeast cells stained with two fluorescent dyes (fluorescein diacetate and propidium iodide) confirmed the lesser ethanol tolerance of both *H. polymorpha* and *S. cerevisiae* strains overexpressing genes involved in glutathione biosynthesis (Fig. 4).

Discussion

We show here that GSH overproduction has a strong stimulatory effect on alcoholic fermentation of glucose in the thermotolerant yeast *H. polymorpha*, although it does not appear to alter ethanol production from xylose. This contrasts with the situation in *S. cerevisiae*, where the GSH pool seems not to affect alcoholic fermentation of glucose under either semi-anaerobic or strictly anaerobic conditions. Why these two yeasts behave differently in this respect is not known. *S. cerevisiae* and *H. polymorpha* are

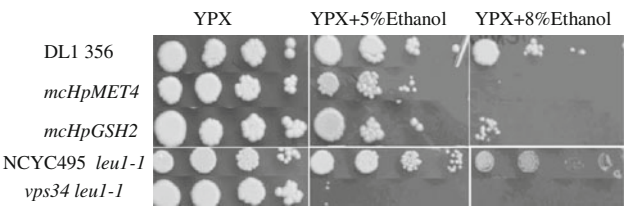

Fig. 3 Resistance of *H. polymorpha* wild-type strains DL-1 356 and NCYC495 *leu1-1*, recombinant strains *mcHpGSH2* and *mcHpMET4*, and the *vps34 leu1-1* mutant strain to exogenous ethanol on YPX medium at 37°C on day 3

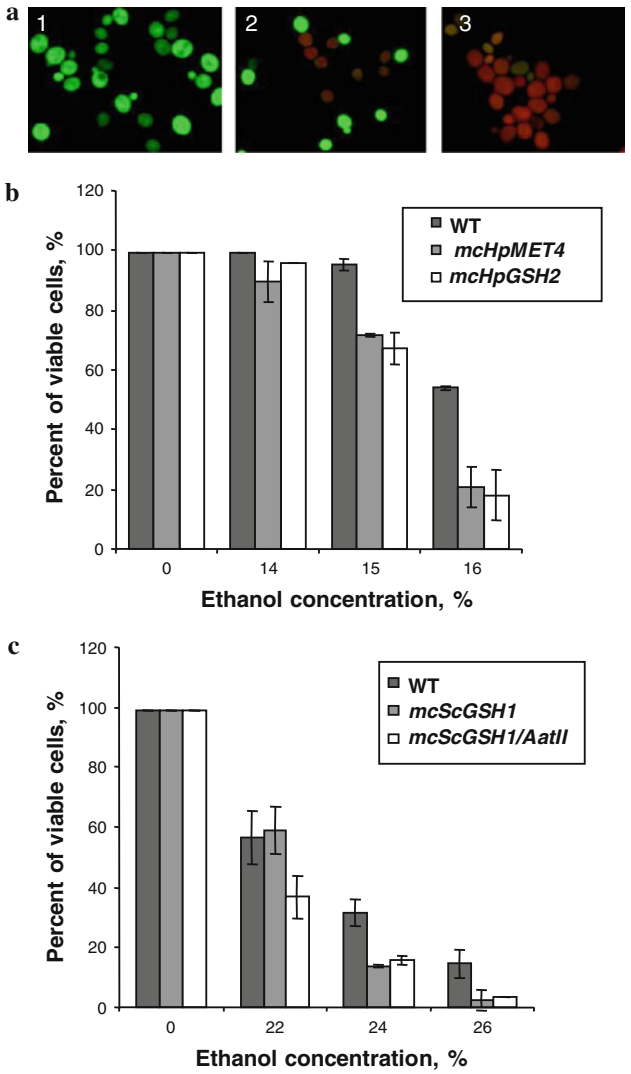

Fig. 4 Viability of yeast cells incubated at different ethanol concentrations and stained with two fluorescent dyes: fluorescein diacetate and propidium iodide. Viable cells are *light grey* (**a**-1, 2); dead cells are *dark grey* (**a**-2, 3). Viability of wild-type (WT) and recombinant strains (*mcHpMET4*, *mcHpGSH2*, *mcScGSH1*, and *mcScGSH1/AatII*) of *H. polymorpha* (**b**) and *S. cerevisiae* (**c**) after a 1-h exposure to ethanol as described under "Materials and methods"

different species by many criteria. *S. cerevisiae* is positive for the Crabtree effect and negative for the Kluyver effect, the reverse being true for *H. polymorpha* [6, 25, 28]. Furthermore, *S. cerevisiae* is a facultative anaerobe capable of growth under strict anaerobiosis, whereas *H. polymorpha* needs oxygen for growth (fermentation being most active, however, under oxygen limitation) [14, 26]. Although the specificities of these yeasts as regards the involvement of GSH in the regulation of alcoholic fermentation are not yet known, we suggest that the observed differences may relate to the nutritional differences just mentioned.

Our data showing decreased ethanol tolerance in GSH-overproducing strains of both *H. polymorpha* and *S. cerevisiae* are quite unexpected. It could be that, in the presence of ethanol, GSH at high concentration performs some of its functions incorrectly, e.g., fails to maintain the proper ratio between –SH groups and S–S bonds in proteins. Alternatively, GSH might enhance deleterious effects of ethanol on the cells. As ethanol can act on many targets [10, 20, 29], identifying its primary targets when the cellular GSH pool is high will require special investigations, which we plan to undertake. As both ethanol and GSH inhibit glycolytic enzymes [5, 16], one hypothesis worth exploring is that a high GSH level might increase ethanol susceptibility by potentiating inhibition of glycolysis and growth by ethanol. Finally, one may assume that GSH oversynthesis leads to impairment of vacuole protein sorting (affected in *vps34* mutants), which is known to decrease ethanol tolerance. This hypothesis is planned to be studied.

Acknowledgments This research was funded by a bilateral Polish–Wallonian cooperation grant "Interactions between glutathione and alcoholic fermentation in *Saccharomyces* and non-conventional yeasts" and by the National Academy of Science of Ukraine.

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