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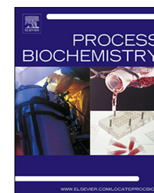

Anhydrobiosis in yeast: Glutathione overproduction improves resistance to dehydration of a recombinant *Ogataea (Hansenula) polymorpha* strain

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ABSTRACT

We show for the first time that a recombinant strain of yeast *Ogataea (Hansenula) polymorpha* is at least as tolerant to dehydration-rehydration treatment as the wild type strain. It is believed that this unusual characteristic of this recombinant yeast strain is linked with its ability to overproduce glutathione. Based on plasma membrane permeability analysis, we hypothesise that glutathione, in addition to its powerful antioxidative protective effects on membrane lipids, may also protect membrane proteins and/or nucleic acids. The combination of yeast cell dehydration with immobilisation and subsequent preliminary slow rehydration in water vapour gave good results in terms of recombinant strain cell viability in the dry state. Correspondingly, this recombinant strain can be used efficiently in the industrial production of glutathione.

1. Introduction

It is well known that efficient biotechnological production of many important compounds necessary for public use is linked with the development of corresponding genetically engineered strains. One such compound is glutathione, which has a tripeptide structure (γ-glutamyl-cysteinyl-glycine). Glutathione is an abundant and important antioxidant that prevents eukaryotic cells from damage by reactive oxygen species through detoxification. It also protects cells from heavy metal and xenobiotic damage by detoxification. In addition to this function, it is involved in redox signalling, regulation of gene expression, and exhibits enzymatic activity [1]. Recently, new functions of glutathione have been described and of particular interest are those connected to cellular iron homeostasis [1]. Problems with glutathione synthesis or uptake may lead to a wide range of pathologies in humans and animals including diabetes mellitus, neurodegenerative disorders, HIV, cancer, and aging [2]. Glutathione deficiency in microbial cells is also linked with serious consequences. For example, mutants of the yeast *Saccharomyces cerevisiae*, which cannot synthesise glutathione, are generally non-viable if they are not supplied with extracellular glutathione [3]. Therefore, it is clear that glutathione may have diverse applications in medicine and various areas of industry. Currently, it is widely used in the pharmaceutical, cosmetic, and food industries [2]. It is used for various purposes, such as the stabilisation of the taste of beer and wine, as a dough modifier, aging-delaying effects, skin protection, inhibition of melanin production, as a skin whitener, dietary supplement, liver protection, and treatment of eye diseases [2,4,5]. More than 200 tons of pure glutathione were produced annually worldwide at the beginning of this decade [6]. To optimise biotechnological production of glutathione, different approaches have been applied, including media optimisation, metabolic engineering, bioprocess engineering and, of course, the selection and development of more efficient strains of microorganisms that produce this compound. In the last case, genetically engineered strains that overproduce glutathione have been developed. However, new efficient strains, which are producers of various compounds, obtained with the use of genetic engineering usually do not survive the application of standard methods of long-term storage, such as dehydration and transfer into an anhydrobiotic state. Therefore, for each such new strain it is necessary to identify appropriate ways to increase resistance to the different treatments [7–9].

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The primary goal of this research was to identify approaches to increase the resistance to dehydration of a genetically engineered strain of *Ogataea* (*Hansenula*) *polymorpha* that exhibits increased production of glutathione.

2. Material and methods

2.1. Microorganisms, media, and growth conditions

The following *O. polymorpha* strains were used in this study: the DL-1 *leu2* wild-type strain and the strain mcGSH2/MET4(pGAP) which construction was described recently [10]. The reference strain evaluated is the strain transformed with the empty expression cassettes.

Yeast cells of both strains were cultivated in YPD medium (10 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose) in flasks at 180 rpm until mid-stationary phase (42 h). Because these strains of yeast *O. polymorpha* are thermotolerant, the cultivation of both strains was performed at two different temperatures, 30 °C and 37 °C.

In additional experiments, two genetically related strains of *O. polymorpha*, which are able to produce ethanol using xylose, were used: the wild-type strain *O. polymorpha* NCYC 495 leu 1-1 and the recombinant strain *O. polymorpha* 2ETOH/XYL1nn/XYL2/XYL3/BrPA/Δcat8/DAS1/TAL2. Both of these strains were obtained from the collection of microorganisms of the Institute of Cell Biology, National Academy of Sciences of Ukraine. These strains were cultivated in YPD or YPX (in which glucose was substituted with xylose) in flasks at 180 rpm at 30 °C until the mid-stationary phase (42 h).

2.2. Dehydration-rehydration of yeast biomass, and determination of the water remaining in the cells and viability

Yeast biomass was compressed using centrifugation, passed through a metal sieve (pore diameter of 1 mm), and subjected to convective dehydration in the oven at 30 °C until the amount of water remaining in the cells had decreased to 8–10% (0.085–0.100 g water/g dry weight). As shown earlier, this amount of water in yeast cells corresponds to the state of anhydrobiosis, in which metabolism is reversibly suspended [11–13]. The amount of water remaining in native and dehydrated samples was determined by drying to a constant weight at 105 °C. To determine the viability of dry yeast cells, fluorescence microscopy and fluorochrome primuline were used [14]. Two rehydration methods of dry yeast cells were applied in this study. Fast rehydration of dry yeast cells was performed by placing them in a tube containing distilled water (5 ml) for 10 min. Slow gradual rehydration of dried yeast was accomplished by incubation in water vapour in a chamber (over distilled water) at 37 °C for 2 h. Following gradual rehydration with water vapour, rapid rehydration of partially rehydrated yeast cells was performed by placing cells directly into distilled water (5 ml) for 10 min.

2.3. Determination of plasma membrane permeability

Changes in plasma membrane permeability were determined by calculating the total dry weight loss of cells following dehydration-rehydration [15,16]. To do this, dry and rehydrated cells were dried to a constant weight at 105 °C and the difference between the dry weights was determined as a percentage of the initial total dry weight of the dehydrated yeast cells.

2.4. Pre-treatment to improve resistance to dehydration

For pre-treatment of yeast cells directed at improving their resistance to dehydration, yeast biomass after cultivation was incubated in xylitol or NaCl solutions with increased osmotic pressure (0.5 and 1 M) for 3 h at 180 rpm at 30 °C. Cells were then compressed and subjected to dehydration as described above.

2.5. Immobilisation of yeast

Yeast cell immobilisation on hydroxylapatite particles was achieved using a previously described method that includes dehydration [17]. Briefly, this method consists of yeast incubation with hydroxylapatite particles on a rotary shaker at 30 °C, a short resting period that is necessary to allow the yeast cells to sediment on the surface of the hydroxylapatite particles, incubation of the obtained samples in distilled water to remove unattached cells, and finally, dehydration of the immobilised yeast at 30 °C.

2.6. Statistical analysis

At least five independent experiments were performed in triplicate for this study. Results were expressed as the mean ± standard deviation. A Student's *t*-test was used for comparisons between the two groups. $P < 0.05$ was defined as the threshold of statistical significance.

3. Results and discussion

3.1. Resistance to dehydration and permeability of the plasma membrane of the wild-type and recombinant strains cells of O. polymorpha

This study was initiated with experiments performed to determine the resistance to dehydration of the wild-type and recombinant glutathione-overproducing strains. Determination of the viability of dry yeast cells gave us unexpected results (Table 1).

As can be seen from Table 1, following growth at 37 °C, the strains were less resistant to dehydration compared with cells grown at a lower temperature despite being thermotolerant. Moreover, there was no difference in the resistance of the parental and recombinant strains. This is an unusual situation taking into account the conclusions of previous studies of the high sensitivity to dehydration-rehydration of recombinant strains of various microorganisms. Such results earlier we received only with recombinant strain *Saccharomyces cerevisiae* S600 which synthesized high amounts of xylitol [18]. Therefore, it was necessary to determine if this unusual resistance of recombinant glutathione-overproducing strain to dehydration is also characteristic of other recombinant strains of *O. polymorpha*. To do this, we verified the resistance to dehydration of two other genetically-related strains (wild-type and recombinant) of the same species, which are used for the

Table 1
Viabilities and plasma membrane permeabilities of dehydrated cells of glutathione producing yeast strains *Ogataea (Hansenula) polymorpha* grown at different temperatures after their rehydration. Media used in the experiments: YPD – 10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose. Cultivation in flasks at 180 rpm until mid-stationary phase (42 h). Five independent experiments were performed in triplicate. * $P < 0{,}05$ indicates a statistically significant difference between viability results of yeast grown at 30 °C and 37 °C after fast and slow rehydration.

	Viability, %		Losses of intracellular substances, %	
	Fast rehydration	Slow rehydration	Fast rehydration	Slow rehydration
Wild strain, 30 °C	37 ± 8	82 ± 4	28 ± 1	19 ± 3
Recombinant strain, 30 °C	37 ± 8	96 ± 2	26 ± 0.5	15 ± 2
Wild strain, 37 °C	24 ± 4 *	58 ± 2 *	30 ± 0.5	22 ± 1
Recombinant strain, 37 °C	26 ± 6 *	61 ± 3 *	26 ± 1	20 ± 1

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