Research Article

Enhanced Glutathione Production by Evolutionary Engineering of *Saccharomyces cerevisiae* Strains

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Abbreviations: **GSH**, glutathione; **MNNG**, N-methyl-N'-nitro-N-nitrosoguanidine; **CDW**, cell dry weight;

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Abstract

Glutathione is an important natural tripeptide mainly used because of its antioxidative properties. Commercial glutathione is microbially synthesized by yeasts and the growing demand requires the development of new production strains. An adaptive laboratory evolution strategy using acrolein as a selection agent was employed to obtain strains with an enhanced glutathione accumulation phenotype accompanied by an acrolein resistance phenotype. Two particularly interesting isolates were obtained: one with a high volumetric productivity for glutathione reaching 8.3 mg $_{glutathione}$/L h, which is twice as high as the volumetric productivity of its parental strain. This strain reached an elevated intracellular glutathione content of 3.9%. A second isolate with an even higher acrolein tolerance exhibited a lower volumetric productivity of 5.8 mg $_{glutathione}$/L h due to a growth phenotype. However, this evolved strain accumulated glutathione in 3.3-fold higher concentration compared to its parental strain and reached a particularly high glutathione content of almost 6%. The presented results demonstrate that acrolein is a powerful selection agent to obtain high glutathione accumulation strains in an adaptive laboratory evolution experiment.

1 Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) is a biologically important natural tripeptide that consists of the amino acids glutamic acid, cysteine and glycine. It is present in almost all eukaryotic cells and also in some prokaryotes. GSH serves many important physiological functions in cells mainly through its redox-active sulfhydryl group. It plays roles as a redox buffer, as an antioxidant and as a detoxifier of xenobiotics, metals and acetaldehydes [1–3]. Due to its antioxidative properties the applications in medicine, food and cosmetic industry led to an increased demand for glutathione in recent years. For example, glutathione is used as a supplement in various pharmaceuticals for the treatment of cancer, diabetes and HIV [1, 4]. In addition, glutathione enriched yeast extract is applied as food additive for the refinement of taste profiles [5]. In cosmetics, glutathione is typically used for skin whitening [6]. Presently the estimated annual production of pure crystalline GSH in the world exceeds 200 tons [5].

Even though glutathione can be produced by chemical or enzymatic synthesis, microbial synthesis is currently the major commercial method for glutathione production. The most commonly used microorganisms for glutathione production are the yeasts *Saccharomyces cerevisiae* and *Candida utilis* which have high glutathione contents (0.1-1 % cell dry weight) and have been accepted as food-processing microorganisms [1].

Many studies in the field of process optimization, genetic/metabolic engineering and classical screening strategies have been carried out to increase the glutathione content of *S. cerevisiae* [1, 7–9]. The mechanism for the selection was targeted to disrupt the feedback inhibition of glutathione on the gene *GSH1*, which catalyzes the first step in the glutathione biosynthesis [1, 10]. The most successful approaches were classical selection strategies, usually resulting in glutathione contents of 3-5 % [1, 11, 12]. Classical selection is based on repeated cycles of mutagenesis and screening for a given phenotype on plates [13]. The used physical or chemical mutagenesis methods included UV, X-radiation, γ-

radiation, and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment, while resistance to toxic compounds such as ethionine, 1,2,4-triazole, high zinc concentrations, sodium cyanide and sulfite was applied as selection regimen to isolate glutathione overproducing strains.

Acrolein is the simplest α, β-unsaturated aldehyde and highly toxic for cells. It was found that the toxicity is due to the binding of acrolein to sulfhydryl groups of proteins [14]. Glutathione has been demonstrated to play a central role in the cellular defense against acrolein toxicity. Vollenweider et al. [15] have observed that *E. coli* cultures supplemented with glutathione had an increased tolerance to acrolein. A similar effect was seen in chloroplasts, where the supplementation of glutathione suppressed the acrolein-induced inactivation of photosynthesis [16]. These observations indicate that resistance to acrolein is associated to glutathione overproduction. Consequently, we hypothesized that acrolein can be used for the selection of glutathione overproducing strains.

As classical selection strategies on plates have many drawbacks like small population size, accumulation of unfavorable mutations and unnatural environmental conditions, we focused on directed evolutionary engineering strategies for this selection scheme [13, 17]. Evolutionary engineering has been shown to be highly successful as can be seen from van Maris et al. [18], who observed a 2-fold increase in pyruvate production and from Cakar et al. [19], who isolated strains which exhibited a 62-fold increased resistance to ethanol.

In this work, we present a directed evolutionary engineering strategy to isolate glutathione overproducing yeast strains resistant to toxic levels of acrolein. Such glutathione overproducing strains were obtained by continuous evolution over 250 generations at gradually increasing acrolein stress levels.

2 Materials and methods

2.1 Strains, media and growth conditions

The diploid *S. cerevisiae* wild type strains CBS 7962 and A4 (collection Organobalance) and the glutathione overproducing mutant strain 572 were used as the parental strains. Strain 572 derived from A4 by conventional selection procedures using resistance against high zinc concentration as selection criteria. Supplementary Table 1 summarizes strains used in this paper. All strains were stored in 10 % (v/v) glycerol at -80°C.

Shake flask cultivations were performed in WMVIII medium [20] at 30°C and shaking with 220 rpm. WMVIII medium contained per liter: 22 g glucose monohydrate, 0.25 g $NH_4H_2PO_4$, 2.8 g NH_4Cl, 0.25 g $MgCl_2$ x $6H_2O$, 0.1 g $CaCl_2$ x $2H_2O$, 2.0 g KH_2PO_4, 0.55 g $MgSO_4$ x $7H_2O$, 0.075 g myo-Inositol, 10 g sodium glutamate monohydrate, 4 mL trace salts stock solution, 4 mL vitamin solution. Trace salts stock solution contained per 50 mL: 21.88 mg $ZnSO_4$ x $7H_2O$, 6.25 mg Fe_2SO_4 x $7H_2O$, 1.25 mg $CuSO_4$ x $5H_2O$, 1.25 mg $MnCl_2$ x $4H_2O$, 1.25 mg Na_2MoO_4 x $2H_2O$, 146.1 mg EDTA. Vitamin solution contained per 50 mL: 125 mg nicotinic acid, 312.5 mg pyridoxal hydrochloride, 125 mg thiamine hydrochloride, 31.25 mg biotin, 625 mg calcium panthotenate.

The cultures were inoculated at an initial OD_{600} of 0.05. Cell growth was monitored by determining the optical density at 600 nm and/or the cell dry weight (CDW).

2.2 Bioreactor cultivations

Cultivations were done in 1.2 L working volume bioreactors (DASGIP AG, Jülich, Germany). The reactors were sterilized by autoclaving at 121°C for 20 min. Five hundred milliliters WMVIII medium containing 1 mL/L antifoam were sterile filtered into the bioreactor and inoculated to an OD_{600} of 0.05 from an exponentially growing preculture. The dissolved oxygen was controlled at DO = 20 % with the stirrer speed (200-1200 rpm) and the aeration rate (6-40 L/h). The temperature was set to 30°C and the pH was controlled at 5 with 2 M NaOH. Inlet and outlet gases were followed with the off-gas sensor provided by the bioreactor system (DASGIP Off-Gas Analyzer GA4, DASGIP AG, Jülich, Germany).

Carbon balances were set up with the biomass, the produced metabolites and the CO_2. The carbon content of the biomass was assumed to be 48 % [21].

2.3 Determination of cell dry weight (CDW)

Two milliliters of culture were harvested and cells were washed once with 0.1 M HCl. The pellets were transferred to weighed glass tubes and dried at 105 °C for 48 hours. Each sample was analyzed in duplicate.

2.4 Determination of total glutathione content

Glutathione levels were measured by HPLC analysis (Shimadzu Corporation, Kyoto, Japan). The separation was achieved on an Ascentis Express RP-Amide column (150 mm × 3.0 mm; 2.7 µm; Supelco analytical, Bellefonte, PA, USA) equipped with guard column (5 mm × 3.0 mm; 2.7 µm; Supelco analytical, Bellefonte, PA, USA). The detection of total glutathione, which included reduced form (GSH) and oxidized form (GSSG), was obtained at 200 nm with a photodiode array detector (SPD-M20A, Shimadzu Corporation, Kyoto, Japan). The column was operated at 25°C, 0.4 mL/min flowrate and 25 mM NaH_2PO_4, pH=3 (H_3PO_4).

Five milliliters of sample were harvested and cells were washed with 0.9 % (w/v) NaCl. The cells were resuspended in 0.1 M H_3PO_4 and incubated for 3 min at 75°C. Subsequently, the samples were cooled down, centrifuged, filtrated and 5 µL of sample were injected for total glutathione analysis.

2.5 Determination of glucose and ethanol

The concentrations of glucose and ethanol in the culture were determined by HPLC analysis (Shimadzu Corporation, Kyoto, Japan) with a Phenomenex Rezex ROA column (300 mm × 7.8 mm) and a refraction index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan). The column was operated at 60°C temperature, 1 mL/min flow rate and 0.004 M H_2SO_4 as mobile phase. HPLC samples were prepared by mixing 900 µL sample

with 100 µL of 0.04 M H_2SO_4. The mixture was filtrated through 0.20 µm RC membrane filters and 10 µl were injected for analysis.

2.6 Spotting experiments

Strains were grown to exponential growth phase and washed once with distilled water. Five microliter of serial dilutions (10^6 - 10^0 cells/mL) were spotted onto WMVIII plates containing different acrolein concentrations. The plates were incubated at 30°C for 5 days.

2.7 Strain mutagenesis

Chemical mutagenesis was carried out using MNNG (N-methyl-N'-nitro-N-nitrosoguanidine). Conditions were used that resulted in the killing of ~ 95 % after the chemical treatment (110 µg/mL for strains A4 and CBS 7962, 80 µg/mL for strain 572).

Cells were grown in YPD medium to mid-exponential growth phase and washed twice with 50 mM KH_2PO_4 buffer at pH 7. The culture was diluted to a cell density of 5 x 10^7 cells/mL and divided into 1 mL aliquots. The aliquots were centrifuged to discard the supernatant. The cells were resuspended in 1 mL MNNG (freshly prepared) and treated for 30 min at 30 °C. After MNNG exposure an equal volume of 10 % sodium thiosulfate was added to inactivate the MNNG. Finally, the cells were washed twice with 1 mL of 50 mM KH_2PO_4 buffer and used directly as the starting population for the selection experiment or for the determination of survival rates.

In order to identify MNNG concentrations resulting in the killing of ~ 95 %, survival rates were determined. A defined cell density of strains 572, A4 and strain CBS 7962 was treated with MNNG at concentrations between 0 to 500 µg/mL. Subsequently, 100 µL of serial dilutions (10^{-3}-10^{-6}) of the MNNG treated cells were plated onto YPD plates and incubated at 30 °C for 4 days. Total survival was measured by scoring colony formation on YPD plates.

2.8 Single-colony analysis

The population was plated on WMVIII and incubated for 2 days at 30 °C. Twenty single colonies of each population were picked into 15 mL WMVIII medium each, cultivated at 30 °C for one day and analyzed with respect to glutathione concentration.

2.9 Evolutionary method

The evolutionary experiments were performed in shake flasks (Fig. 1). The selection started from *S. cerevisiae* strains A4, 572, CBS 7962 and the respective MNNG-mutagenized populations (in total six populations). Cells were inoculated in 15 mL WMVIII medium containing initially 0.2 mM acrolein at an OD_{600} of 0.5. As soon as the mid-exponential growth phase was reached (OD_{600} ~ 15-30), the cells were washed once with distilled water and transferred into fresh medium. At every transfer into fresh medium, the acrolein concentration was increased in 0.01 mM steps. An aliquot of the yeast cultures was stored at each round of selection. After approximately 100 generations (~ 20 cycles) the acrolein concentration had been increased from 0.2 to 0.4 mM acrolein. Subsequently, the evolved pools were analyzed at single colony level and the best evolved clones were isolated (see single colony analysis). These were subjected to a second round of directed evolution. Here selected isolates were grown in WMVIII medium supplemented initially with 0.42 mM acrolein and exposed to sequential repeated batches by increasing acrolein doses up to 1.34 mM. To accelerate the selection process the acrolein concentration was increased in 0.02 mM, 0.04 mM and 0.06 mM steps in parallel at every transfer. The culture able to grow at the highest acrolein concentration was used for the next passage and the others were discarded. The experiment was stopped after 150 additional generations. A single-colony analysis was performed again to isolate the best evolved strains from the second evolutionary experiment.

3 Results and Discussion

3.1 Acrolein is a potential selection agent to screen for glutathione accumulation strains

To test the hypothesis if glutathione plays a role in the resistance to acrolein in yeast cells, the growth behavior of the glutathione overproducing strain 572 and the corresponding parental strain A4 was compared at increasing acrolein concentrations. Both strains were cultivated in liquid medium supplemented with 0 mM, 0.01 mM, 0.1 mM, 0.4 mM, 0.8 mM and 1.2 mM acrolein. The final biomass (Supplementary Fig. 1A) and the glutathione level (Supplementary Fig. 1B) were measured after 24 hours for strain A4 and for strain 572 after 47 h. The different cultivation times result from the different growth rates of both strains. After the indicated cultivation period the strains reached the maximal biomass on media without acrolein (approximately 8 g/L). The toxicity of acrolein can be clearly observed: Acrolein concentrations of 0.1 mM decreased growth of strain A4 and no growth was seen for this strain at higher concentrations while strain 572 showed no growth reduction up to a concentration of 0.4 mM. At acrolein concentrations higher than 0.8 mM also strain 572 showed no growth. As expected, strain 572 shows higher glutathione concentrations than strain A4. The glutathione levels in both strains do not significantly change in the presence of acrolein. In previous experiments analyzing the stress response of yeast towards acrolein it was shown that a depletion of glutathione occurred after addition of acrolein to a culture after one hour [22]. However, our data suggests that yeast strains growing in the presence of acrolein are able to restore their intracellular glutathione levels to the levels obtained in media without acrolein and that the glutathione depletion effect [22] is only observed shortly after the addition of acrolein.

3.2 Different glutathione overproducing strains can be isolated following an adaptive laboratory evolution strategy.

As resistance to acrolein is associated with intracellular glutathione concentration, we hypothesized that acrolein can be a potential selection system for the isolation of glutathione overproducing strains.

Before starting the evolutionary experiments in shake flasks, an initial acrolein concentration had to be determined. This acrolein concentration was chosen to inhibit the growth of both strains when grown in liquid cultures. Based on the previous experiment (Supplementary Fig. 1), we decided to use 0.2 mM acrolein as the starting concentration for the evolutionary experiments. At this acrolein concentration the biomass formation of strain A4 was already negatively affected.

The selection process started from six populations, i.e. the *S. cerevisiae* strains A4, 572, CBS 7962 and their MNNG-mutagenized populations. The cells were exposed to MNNG mutagenesis to increase their genetic diversity before the selection procedure.

The populations were grown in WMVIII medium supplemented with 0.2 mM acrolein in repeated batches. At every transfer into fresh medium the acrolein concentration was increased in 0.01 mM steps. The progress of selection was monitored by measuring the glutathione content of the populations in continuous intervals (Fig. 2 A). After approximately 100 generations, the acrolein concentration had been increased from 0.2 to 0.4 mM and all populations were able to grow on this elevated acrolein concentration. Four out of six populations showed clearly elevated glutathione levels compared to their starting values, namely A4-100, 572m-100, 62-100 and 62m100. The populations A4-100 and 572m-100 showed the highest glutathione content of about 3 %. Two MNNG treated populations 572m-100 and 62m-100 evolved a higher glutathione accumulation phenotype although differences were first seen after 100 generations. It can be concluded that a mutagenesis with MNNG added only in the initial phase of the adaptive laboratory

evolution experiment does not lead to a faster adaptation compared to the non-mutagenized populations. Probably a continuous addition of the mutagen as shown for instance by Lee et al. would be more suitable to shorten the adaptation period [23]. No significant increase was found for the populations A4m-100 and 572-100. Interestingly, the population 572-100 showed a lower glutathione accumulation than the starting population but an increased growth rate (Fig. 2 B). In other experiments (data not shown), it was observed that strain 572 eventually loses the glutathione accumulation phenotype when cultivated in serial batches without acrolein. For both populations it seems that *Saccharomyces cerevisiae* has unsurprisingly alternative solutions to adapt to elevated acrolein concentrations besides a glutathione accumulation. One such alternative mechanism to defend against acrolein toxicity in *S. cerevisiae* was observed by Trotter et al. [22]. They demonstrated that the Old Yellow Enzyme (*OYE2*) helps to protect against acrolein stress and the overexpression of *OYE2* and *OYE3* (isoenzyme of *OYE2*) increases acrolein tolerance. *OYE2* is a NADPH dependent oxidoreductase, which reduces acrolein to the much less toxic product propionaldehyde.

In order to determine the fitness of the evolved populations after a selection time of 100 generations the growth behavior was analyzed (Fig. 2 B). The growth of population A4m-100 could not be determined because the culture showed strong flocculation phenotype. The population 572-100 showed the highest growth rate and at the same time its glutathione content was one of the lowest. In contrast the population 572m-100 had the lowest growth rate but the highest glutathione content of all evolved populations. This behavior indicates that growth is inversely correlated with intracellular glutathione accumulation.

Subsequently, the evolved pools showing the highest glutathione contents and best fitness characteristic, A4-100 and 62-100, were analyzed at single colony level. For isolation of single colonies the evolved populations were streaked out onto WMVIII agar plates.

Twenty colonies of each population were cultivated in WMVIII medium and analyzed regarding glutathione accumulation and final biomass as fitness criterion after 24 h of cultivation. The values of the final biomass were heterogeneous within the pools (data not shown). Supplementary Fig. 2 shows the glutathione content of 20 single colonies from the evolved pools A4-100 and 62-100, respectively.

Isolates from pool A4-100 had varying glutathione contents ranging from 2% up to 3.5 %, whereas isolates from pool 62-100 revealed a homogenous glutathione content of about 2.5 %. The glutathione analysis was repeated for the best four isolates from pool A4-100 with biological duplicates confirming the results from the previous screening. The best isolate number 19 had a glutathione content of up to 3.5 ± 0.1 % after 24 hours of cultivation and reached up to 3.9 ± 0.2 % after 40 hours. In summary, within 100 generations isolates with glutathione contents reaching up to 3.9% were obtained. Thus the best isolate from population A4-100 exhibited a 2.2-fold increased glutathione content and the best isolate from population 62-100 showed a 2.5-fold increased glutathione content. No obvious growth defect was observed for these isolates.

These best evolved isolates, A4-19 (from starting population A4) and 62-8 (from starting population 62) were used for a second evolutionary experiment on acrolein. The isolates were grown in WMVIII medium containing initially 0.4 mM acrolein and exposed to sequential serial passages by increasing acrolein doses. To accelerate the selection process the acrolein concentration was increased in 0.02, 0.04 and 0.06 mM steps in parallel at every transfer. The culture able to grow at the highest acrolein concentration was used for the next passage and the others were discarded. The glutathione content of both populations was measured in intervals. The progress of this evolutionary experiment is shown in Fig. 3.

After 150 additional generations, the acrolein concentration had been increased from 0.4 to 1.34 mM. The glutathione content of population A4-19 had significantly increased

whereas the glutathione accumulation of population 62-8 did not alter significantly over the 150 generations. These results demonstrate that acrolein can be used as a selection agent to obtain glutathione accumulation strains. However, the effectiveness of the strain selection using acrolein seems to be dependent on the initial starting strain. The pool 62-8 has evolved other mechanisms than the overproduction of glutathione to acquire resistance to elevated acrolein concentrations.

Subsequently, the pool A4-19 was analyzed at single colony level and an isolate (named A4-19-13) was obtained which reached a glutathione content of 5.9 ± 0.3 %. The glutathione content of this isolate was 3.3-fold higher than that of its parental strain A4. At the same time it was observed that all of the analyzed isolates from pool A4-19 exhibited a reduced growth rate. Strain A4-19-13 reached a specific growth rate of 0.17 h^{-1} whereas the parental strain A4 had a growth rate of 0.32 h^{-1}. Furthermore, the final biomass yield reached only 50 % of that of the initial population A4.

3.3 Bioreactor cultivations of selected isolates confirm the glutathione accumulation phenotype

The growth and glutathione accumulation properties of two particular evolved mutants A4-19 (3.9 % glutathione) and A4-19-13 were examined and compared to their parental strain A4 (1.8 % glutathione). Strain A4-19 was selected because this strain shows an elevated glutathione content of up to 3.9 % and nearly no reduction in growth rate and strain A4-19-13 was further characterized because of its very high glutathione content of up to 5.9 % but exhibited already a reduced growth phenotype. In shake flasks both isolates tolerated an acrolein concentration of 0.4 mM (strain A4-19) and 1.34 mM (strain A4-19-13). In order to confirm the acrolein tolerance of those strains they were spotted together with the parental strain A4 on plates with and without acrolein (Supplementary Fig. 3). As expected, both isolated strains tolerate higher acrolein concentrations as the parental strain and strain A4-19-13 shows a higher tolerance than A4-19.

Batch cultivations were carried out on WMVIII medium in the bioreactor. Based on the glutathione contents of the strains and the final biomass the product yields were calculated. Additionally, the volumetric productivity and the specific product formation rate were calculated over the whole batch cultivation process. Table 1 shows that strain A4-19 gave the highest total glutathione production of 0.32 g $_{glutathione}$/L (2.0 times higher than strain A4), the highest product yield of 15.7 mg $_{glutathione}$/g $_{glucose}$ and the highest volumetric productivity of 8.28 mg $_{glutathione}$/L h, whereas strain A4-19-13 has the highest glutathione content per CDW and the highest specific product formation rate of 1.27 mg $_{glutathione}$/g $_{CDW}$ h (2.6 times higher than strain A4). Figure 4 shows the growth kinetics and metabolite conversions of the strains.

The growth pattern, the glucose consumption as well as the ethanol formation and consumption characteristic of the glutathione overproducing strain A4-19 were nearly identical to that of its parental strain A4. Strain A4-19-13 showed a reduced growth rate and a delayed glucose consumption and ethanol formation/consumption behavior compared to its parental strain. Interestingly, the final biomass of strain A4-19-13 was half of its parental strain while glucose and ethanol were completely consumed. In addition, it was observed that the supernatant of strain A4-19-13 colored yellowish with cultivation time. Carbon balance calculation showed that after accounting for carbon, produced biomass, glucose, ethanol, acetate and CO_2, there was about 16 % of carbon missing. In contrast, a complete carbon recovery (100 ± 3 %) was observed for the parental strain A4. This can be explained by the assumption that strain A4-19-13 formed some other products, which can be responsible for the growth deficit, the yellowish coloration and the deviation in the carbon balance.

Another possible source for the growth deficit of strain A4-19-13 can be the very high intracellular glutathione content. It was already observed that evolved pools with the lowest growth rates had the highest glutathione contents. These observations point into

the direction that significantly increased glutathione levels can be toxic for the cells and lead to reduced growth. This assumption is in agreement with a study by Srikanth et al. [24]. They have shown that overexpression of the glutathione transporter *OPT1* (also known as *HGT1*) leads to an increased intracellular glutathione concentration in the presence of extracellular added glutathione and to glutathione dependent toxicity in the cells.

4 Conclusion

Evolutionary engineering using toxic acrolein as selection compound is a novel strategy for the isolation of glutathione overproducing strains of *S. cerevisiae*. A mutant with a particularly high glutathione content of almost 6 % [w/w] glutathione was evolved. This mutant accumulates glutathione in 3.3-fold higher concentrations than its parental strain. To our best knowledge, this is the second highest glutathione content reported. Li et al. [1] reported a glutathione content of 9.5 % in *S. cerevisiae*.

We provide further evidence that growth is inversely correlated with intracellular glutathione accumulation. As glutathione accumulates within the cells, the glutathione productivity is limited because of toxicity issues.

The effectiveness of strain selection using acrolein seems to be strain dependent. Mutants from different strain backgrounds had the same resistance to acrolein (1.34 mM) but significantly different glutathione contents. Unsurprisingly, alternative evolutionary strategies exist in nature. Conceivable are for instance strategies which lead to higher expression levels of *OYE2* and *OYE3* which are reported to increase the acrolein tolerance in yeasts.

Thus, acrolein selection is a powerful selection tool for glutathione producing cells, though strains with different genetic background might react differently in providing protection mechanisms to this toxic chemical.

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Conflict of interest

Anett Patzschke, Matthias G. Steiger, Diethard Mattanovich, Michael Sauer declare that they have no financial or commercial conflict of interest. Caterina Holz is an employee of Organobalance GmbH and Christine Lang owns stock in Organobalance GmbH.

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Table 1. Glutathione contents and production characteristics of the parental strain and selected evolved strains for the batch cultivations on WMVIII medium in the bioreactor of strains A4, A4-19 and A4-19-13.

Strain	Glutathione[a)] [% g_{CDW}^{-1}]	Total glutathione [$g_{glutathione}$ L^{-1}]	$Y_{P/S}$ [b)] [mg glutathione g glucose^{-1}]	$Q_{p\ average}$ [c)] [mg glutathione L^{-1} h^{-1}]	$q_{p\ average}$ [d)] [mg glutathione g_{CDW}^{-1} h^{-1}]
A4	1.8	0.16	7.9	4.17	0.49
A4-19	3.9	0.32	15.7	8.28	1.04
A4-19-13	5.9	0.27	13.5	5.83	1.27

[a)] Glutathione content measured at batch end

[b)] $Y_{P/S}$ [mg glutathione g glucose^{-1}] product yield

[c)] $Q_{p\ average}$ [mg glutathione L^{-1} h^{-1}] volumetric productivity

[d)] $q_{p\ average}$ [mg glutathione g $_{CDW}^{-1}$ h^{-1}] specific product formation rate

Figure legends

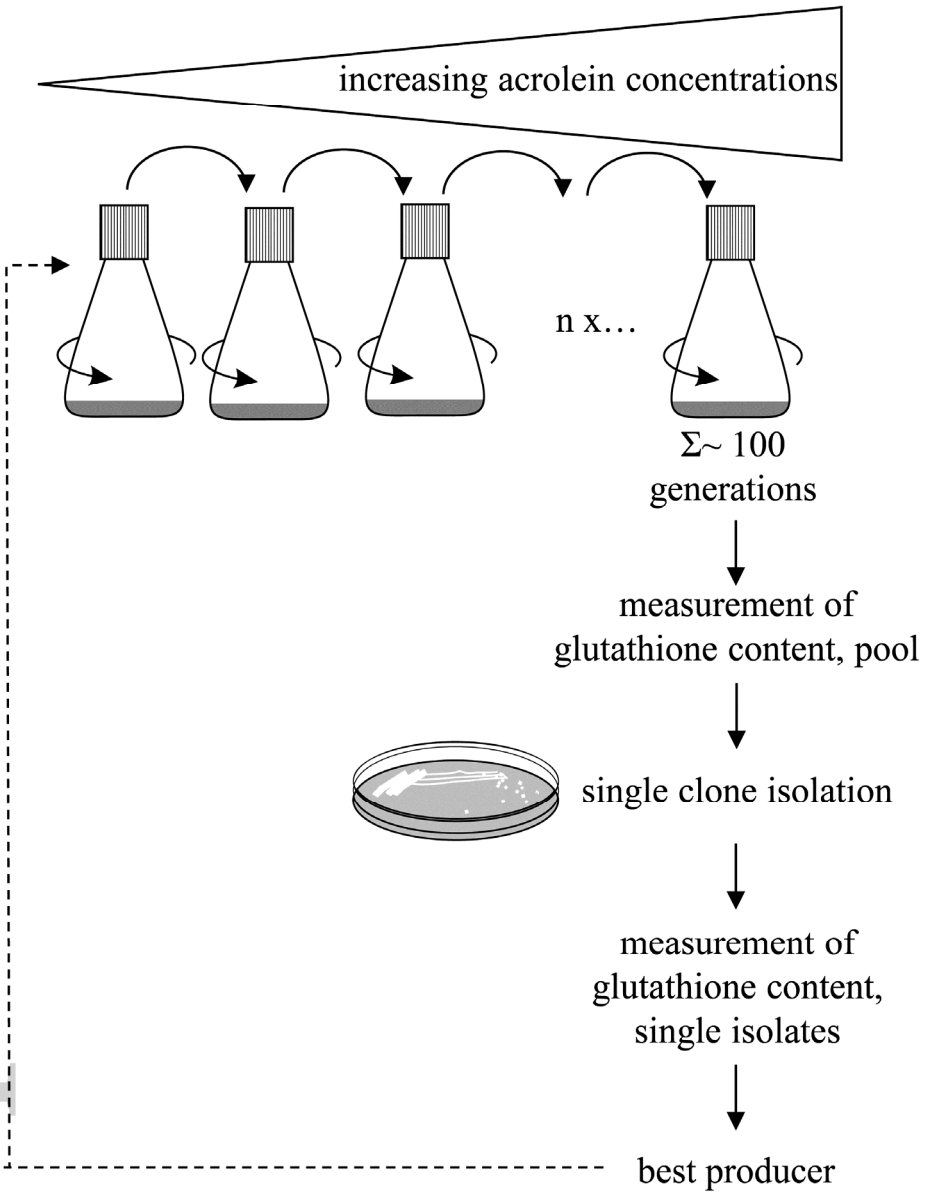

Figure 1. Schematic illustration of the directed evolutionary engineering method. The initial population was grown in WMVIII medium supplemented initially with 0.2 mM acrolein and subjected to repeated batchesby increasing acrolein doses. After approximately 100 generations (~ 20 cycles) the acrolein concentration had been increased to 0.4 mM. The population at the last passage was analyzed at single colony level. 20 colonies were analyzed with respect to glutathione content and final biomass concentration. The best evolved mutant was exposed to a second round of evolution. Additional 30 transfer cycles (150 generations) were carried out and the acrolein concentration had been increased from 0.42 mM to 1.34 mM.

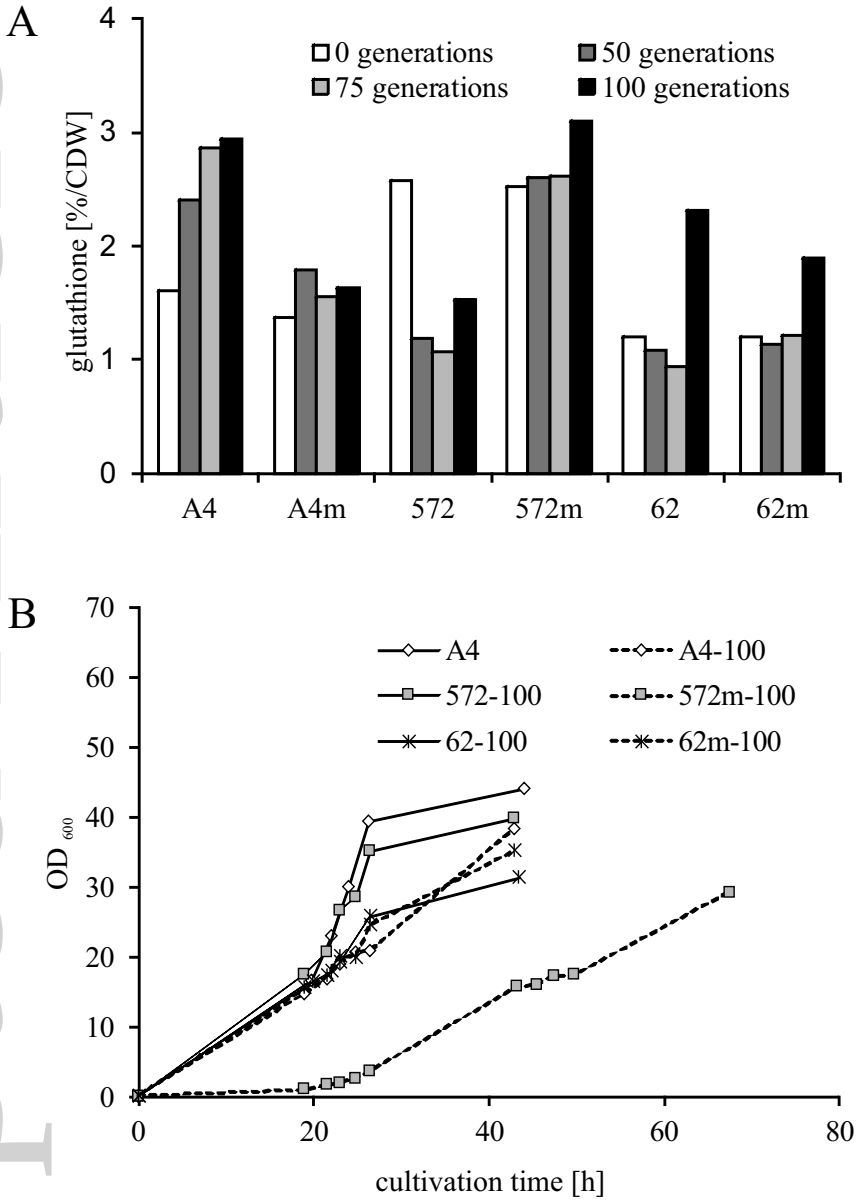

Figure 2. Progress of the selection of glutathione overproducing strains in shake flasks using the selection agent acrolein. (A) Glutathione was measured at the indicated generation times in technical duplicates after cultivation for 24 hours on WMVIII media. The selection started from strains A4, 572, CBS 7962 (abbr. 62) and their MNNG-mutagenized populations (A4m, 572m, 62m). The populations were grown in WMVIII medium supplemented initially with 0.2 mM acrolein and exposed to sequential serial passages by increasing acrolein doses. (B) Growth behavior of evolved populations (after a selection time of 100 generations) compared to the wild-type strain A4. OD_{600} was determined when grown on WMVIII medium in shake flasks in a single experiment.

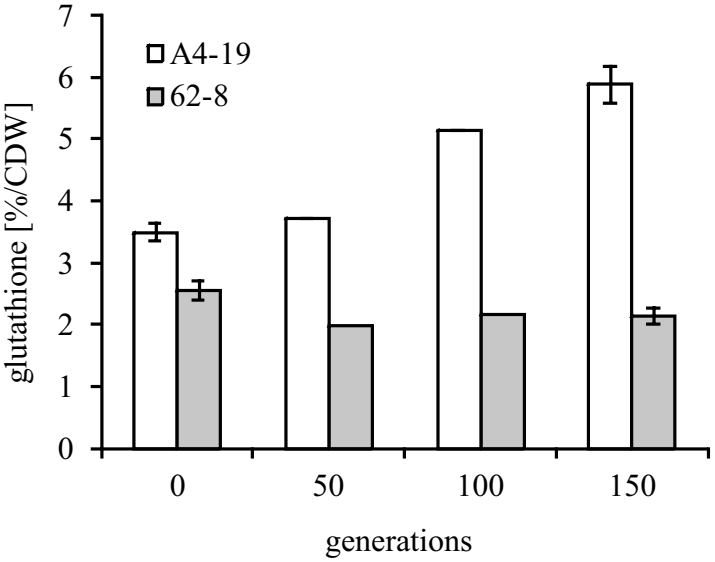

Figure 3. Progress of selection of glutathione overproducing strains in shake flasks using the selection agent acrolein. The selection started from the populations A4-19 and 62-8. The populations were grown in WMVIII medium supplemented initially with 0.4 mM acrolein and exposed to sequential serial passages by increasing acrolein doses up to 1.34 mM. Glutathione was measured at the indicated generation times in technical duplicates after 24 hours. At 0 and 150 generations the strains were grown in biological duplicates.

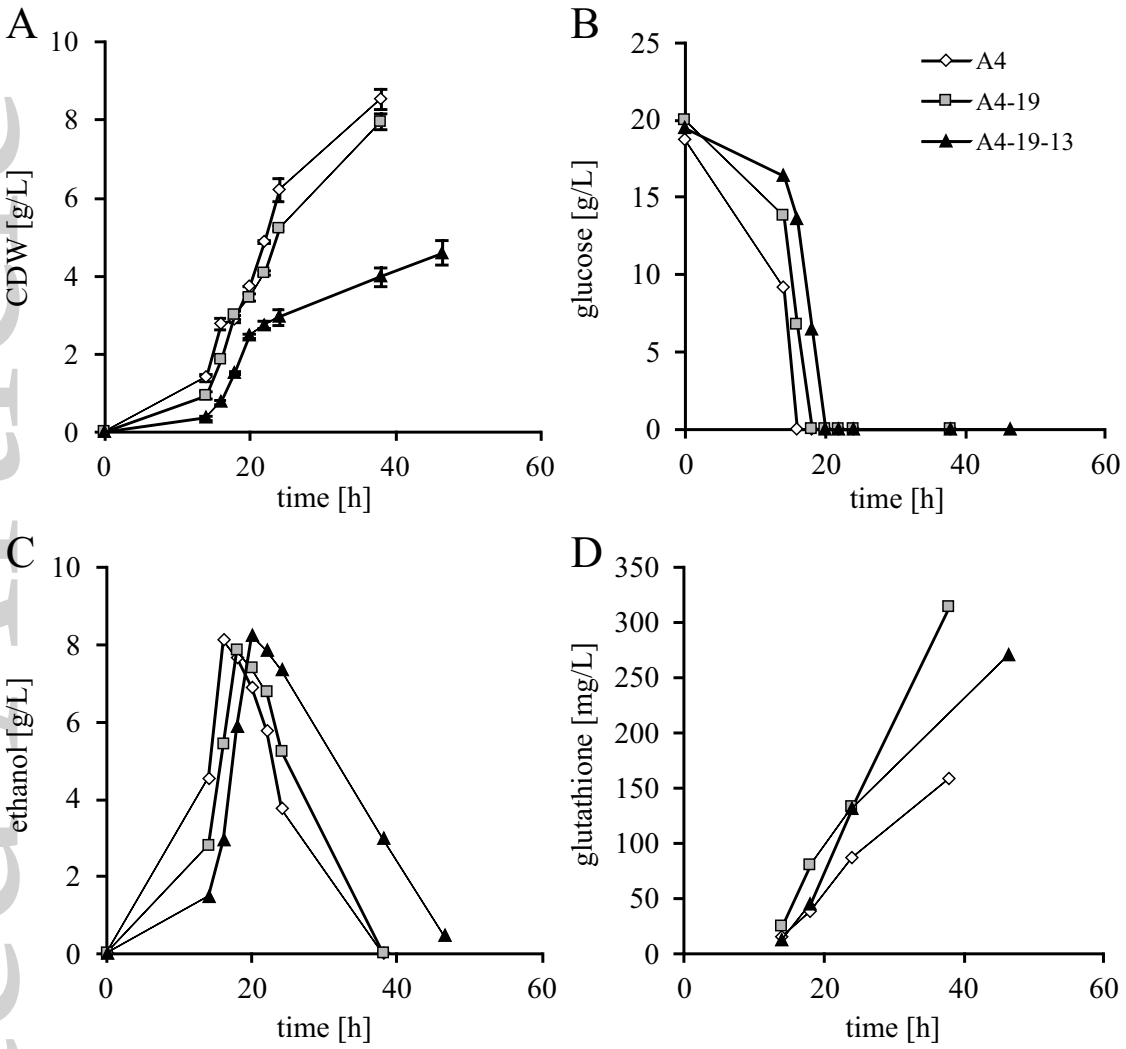

Figure 4. Cultivation characteristics of the glutathione overproducing mutant strains A4-19, A4-19-13 and their parental strain A4. Batch cultivations were carried out in controlled bioreactors. (A) Cell dry weight versus time from technical duplicates. (B) Glucose concentration versus time. (C) Ethanol concentration versus time. (D) Glutathione concentration versus time. The standard error of the measurements of glucose, ethanol and glutathione concentrations was less than 5%.