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# Improvement of natural isolates of *Saccharomyces cerevisiae* strains for synthesis of a chiral building block using classic genetics

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**Abstract** The asymmetric bio-reduction of 4-chloro-acetoacetic-acid-ethyl-ester to the pharmaceutical building block (*S*)-4-chloro-3-hydroxybutanoate-ethyl-ester requires the utilization of an enantioselective robust biocatalyst. Some of the natural *Saccharomyces cerevisiae* strains, isolated from Mount Carmel National Park in Israel, were characterized as resistant to environmental stress. Nevertheless, these strains showed relatively low enantiomeric-excess (ee), while a laboratory strain, Y103, exhibited a selectivity of 98% ee. The enantioselective lab strain was crossed with the multi-stress resistant environmental isolate (93% ee) followed by backcross with Y103, to subsequently obtain a haploid offspring of backcross-1, exhibiting both high multi-stress resistance and high enantioselectivity (98% ee). Introducing osmotic (1 M NaCl), oxidative (0.6 mM $H_2O_2$) and thermal stress (44°C) to growing cultures of the enantioselective parent, resulted in a decrease of 24–32% in specific activity, while the enantioselectivity of the stress-resistant parent decreased by 4–12% ee. Unlike its original parental strains, the new strain maintained constant specific activity and enantioselectivity when introduced to the various stress factors. This work shows that the classic introgression method, can serve as a viable approach for creating a robust enantioselective biocatalyst, designed for industrial production of chiral compounds.

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## Introduction

(*S*)-4-chloro-3-hydroxybutanoate ethyl ester [(*S*)-CHB] is an important chiral building block used in the synthesis of many enantio-pure pharmaceuticals. For example, it is a key precursor for the production of cholesterol-lowering drugs such as Atorvastatin (an HMG–CoA reductase inhibitor) and can also be converted into 1,4-dihydropyridine-type β -blocker (Yasohara et al. 1999; Stewart 2001; Yang et al. 2004). Among the various routes to produce (*S*)-CHB, asymmetric reduction of 4-chloro-acetoacetic acid ethyl ester (CAAE) using microorganisms is an attractive method, due to its low costs, mild reaction conditions, high yields, and high enantioselectivity. This reaction is performed by enzymes with a reducing activity such as Gcy1p, Ypr1p, Ara1p, Gre3p, Gre2p, and Ygl157wp (Johanson et al. 2005). Most of these enzymes are grouped into two distinct protein superfamilies: aldo-keto reductase (AKR) and the short chain dehydrogenase/reductase (SDR) superfamilies and require the cofactor NADH for their activity (Katz et al. 2003; Kroutil et al. 2004). Applying whole cells in this reaction is favorable over using isolated enzymes due to the ability to spontaneously regenerate NADH in vivo (Houng et al. 2003; Yang et al. 2004). Recycling the co-factor requires the presence of a co-substrate, usually glucose, and is performed by glucose dehydrogenase (GDH; Katz et al. 2003).

Having a broad substrate acceptance and being inexpensive, readily available, fast growing, and easy to use, places *Saccharomyces cerevisiae* among the most popular biocatalysts for asymmetric reduction (Kaluzna et al. 2004; Yang et al. 2004; Johanson et al. 2005). However, previous studies using non-modified *S. cerevisiae* for (*S*)-CHB production reported poor enantioselectivity with a maximum enantiomeric excess (ee) of 92% (Yasohara et al. 1999) when the minimum value required for industrial use is over 95% ee (Houng et al. 2003). This non-satisfactory value is mostly due to the presence of nearly fifty open reading frames to hypothetical reductases with conflicting streoselectivities (Stewart 2000). The simplest solution to this problem is screening for strains lacking competing reductases (Kaluzna et al. 2004). Other approaches for overcoming this drawback include: process engineering (Shimizu et al. 1990; Yasohara et al. 1999; Chen et al. 2002; Houng et al. 2007), metabolic engineering (Bertau 2002; Yang et al. 2004; Vitinius et al. 2005), and genetic engineering (Kataoka et al. 1997; Rodriguez et al. 1999, 2000, 2001; Katz et al. 2003; Ema et al. 2005; Johanson et al. 2005). Unfortunately, these attempts to improve enantioselectivity are either partially successful or suffer from high complexity.

In addition to high enantioselectivity, nearly all biocatalytic industrial applications require a robust biocatalyst, capable of adjusting to extreme environmental conditions (Attfield 1997; Houng et al. 2003; Cakar et al. 2005; Hirasawa et al. 2006). This complex phenotype is difficult to improve because many of the involved components and their interactions are unidentified (Cakar et al. 2005; Serra et al. 2005).

Novel approaches like genetic knockout, overexpression, and mutagenesis can be used for maintaining both enantioselectivity and multi-stress resistance traits in one strain. Nevertheless, traditional genetic crossing and selection technique appears to be preferable in the case of combining such complex characteristics. First, since both desirable traits involve multiple genes and complex interactions, recombinant DNA approaches are expected to be complicated and inefficient for improving both traits. Second, mutational selection requires the analysis of numerous mutants, whereas an introgression strategy requires screening of much less offspring (Naumov et al. 2006) for fewer generations (Riva et al. 1982; Stepanova et al. 2001). Third, genetic mating is a natural process among yeast (de Barros Lopes et al. 2002; Gonzalez et al. 2006). Thus, an additional advantage of the classic introgression strategy, in contrast to recombinant DNA technology, is the higher likelihood of public acceptance as a 'natural' procedure.

A previous study described the isolation and characterization of natural *S. cerevisiae* strains, from Mount Carmel National Park, Haifa, Israel (Katz-Ezov et al. 2006). Some of the progeny of these natural isolates showed a multi-stress resistance phenotype. Here we describe the identification of a laboratory enantioselective strain and a multi-stress resistant progeny of a natural isolate, and their successful introgression in an attempt to develop an industrially oriented biocatalyst. This classic introgression strategy proved to be an efficient method for combining complex multi-component traits in a single strain.

## Experimental procedures

*Chemicals* (*S*)- and (*R*)-4-chloro-3-hydroxybutanoic acid ethyl ester (CHB) and 4-chloro-acetoacetic acid ethyl ester (CAAE) were purchased from Aldrich Chemical Co. (Sigma-Aldrich, Rehovot, Israel). Ethanol, sodium chloride and hydrogen peroxide were obtained from Frutarom Industries LTD (Haifa, Israel). *tert*-Butyl benzene was obtained from Riedel-de-Haen (Seelze, Germany). Ethyl acetate was purchased from Gadot Biochemical Industries LTD (Haifa, Israel). All materials used were of the highest purity available and were used without further purification.

*Yeast strains and growth conditions* The yeast strains used in this study include natural isolates, designated #1 (tetraploid) and #9 (diploid), that were isolated from "Evolution Canyon" at Mount Carmel National Park in Haifa, Israel and their offspring: Ye1-301, Ye1-302, Ye1-303, Ye1-304 (diploids), and Ye9-531, Ye9-532, Ye9-533, Ye9-534, Ye9-503, Ye9-511, Ye9-517, Ye9-518, Ye9-521, Ye9-540, Ye9-519, Ye9-612 (haploids; Katz-Ezov et al. 2006). The laboratory strain Y103 (*Mat* a, Lys1) was employed in this study, as the acceptor parent in the backcross introgression program, with Ye9–519 (*Mat* α) as a donor. The progeny of the backcross introgression program includes: C13 (F1), C103, C136, and C141 (F1 offspring), C23, C24, and C29 (BC1), C202, C207, C220, C238, C242, C251, C268, and C274 (BC1 offspring) and C36 (BC2). All strains were maintained as glycerol stock solutions at −80°C in the collection of the Faculty of Biotechnology and Food Engineering at Technion, Haifa, Israel. The yeasts were routinely cultivated at 30°C with shaking at 250 rpm on an orbital shaker incubator in YPD rich medium (1% Yeast Extract, 2% Bacto-peptone, 2% glucose). Solid medium was supplemented with 2% agar.

*Screening for thermotolerance* Thermotolerance was chosen as the first phenotype to be screened. A fast, easy-to-use screening method was developed, including two steps: (a) 72 h growth at 40°C on solid YPD medium, and (b) growth kinetics at 40°C. Overnight cells were diluted to 0.1–0.2 $OD_{600}$ and were grown at 40°C with maximum

shaking (level 4) in a multiplate reader Synergy HT (BioTek Instruments, VT, USA) for 24 h. Strains that grew on solid medium at 40°C and showed high growth rate and final cell concentration in the second step, were characterized as thermotolerant and were later examined for high enantioselectivity and additional stress resistance abilities.

*Stress resistance tests* Stress resistance evaluation was conducted by diluting overnight cells to 0.1–0.2 $OD_{600}$, and growth at 30°C with maximum shaking (level 4) in a multiplate reader Synergy HT. $OD_{600}$ was measured at 15 min intervals until reaching 0.5. Stress factors (sodium chloride 1 M, hydrogen peroxide 1 mM and temperature shift to 44°C) were added to the exponentially grown cells and growth continued until reaching a stationary phase. Typical growth curves of three representative strains in response to hydrogen peroxide addition are presented in Fig. 1. The response to the stress factors was quantified by three parameters derived from the growth curves: lag time (hours), change in growth rate (%), and decrease in cell concentration (%; presented in Table 1). Lag time was the time interval between the addition of the stress factor and the beginning of the secondary logarithmic growth (represented by the solid vertical line in Fig. 1). The change in growth rate was calculated as the ratio between the slopes of the exponential phase before and after the addition of the stress factor. Two to three independent experiments were performed to characterize each strain.

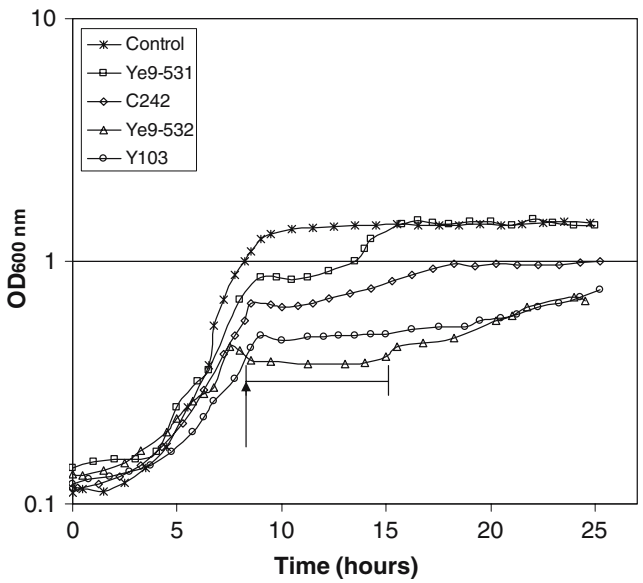


**Fig. 1** Effect of 1 mM $H_2O_2$ on growth of selected *S. cerevisiae* strains. $H_2O_2$ was added during mid log phase (*arrow*). Ye9-531 (*open square*) and Ye9-532 (*open triangle*) represent typical stress resistant and non-resistant offspring of the natural isolate #9, respectively. Y103 (*open circle*) represents a non-resistant laboratory strain. C242 (*open diamond*) is the haploid BC1 offspring of the enantioselective strain, Y103, and the multi-stress resistant strain, Ye9-519. Ye9-531 grown at optimal conditions (without $H_2O_2$) serves as a control (*asterisk*). These growth curves were used for assessing the response resulting from stress factors addition by measurements of three parameters: lag time (hours), change of growth rate (%) and decrease in cell concentration (%)

*Biotransformation for CAAE reduction* Yeast strains were grown in YPD medium at 30°C and 250 rpm into early stationary phase (approximately 24 h). They were then centrifuged at 11,000 g for 10 min at 30°C and re-suspended in phosphate buffer (pH 7, 0.1 M) containing 5% (*w*/*v*) glucose to a cell concentration of 7 $OD_{600}$. The biotransformation reaction was performed in test tubes under aerobic conditions at 25°C with vigorous shaking (600 rpm in a Vibramax 100 shaker, Heidolph, Germany). Two milliliters of concentrated cell suspension were contacted with substrate (CAAE) at a concentration of 30 mM (from a 2-M stock solution in ethanol). The negative control was devoid of substrate. The reaction was stopped periodically, by extraction with 2 ml of ethyl acetate. The organic phase was analyzed by gas chromatography (GC). The ee value was measured at full conversion (24 h). The conversion rate was calculated as the ratio between the product concentration and the initial substrate concentration using *t*-butyl benzene as an internal standard. Specific activity was calculated as the ratio of the conversion rate and the total protein content determined by the Bradford analysis method, with bovine serum albumin as a calibration standard (Bio-Rad Protein Assay Kit; Bradford 1976). Two to three independent experiments were performed to characterize each strain.

Evaluation of biotransformation performance following exposure to stress conditions was carried out by growing cell cultures to mid-log phase ($OD_{600}$=1) and adding either 1 M NaCl, 0.6 mM $H_2O_2$ or incubating at 40°C. After 3 h of reaction with the stress factor, the cells were harvested, and the biotransformation was performed as described previously.

*Analytical methods* Conversion of CAAE to CHB was determined using an HP-5-MS 5% phenyl methyl siloxane capillary column (Agilent Technologies, 30 m×0.323 mm×0.25 μm) with a Hewlett-Packard 6890N gas chromatograph and an HP-5975 mass spectra detector (Agilent Technologies, Santa Clara, CA, USA). The temperature was programmed as follows: $T_1$=70°C, 1 min; d$T$/d$t$=6°C/min; $T_2$=150°C, split ratio of 1:10. Under these conditions, the retention times were: $R_F$=5.3 min for *tert*-butyl benzene, $R_F$=7.6 min for CAAE, $R_F$=8.2 min for CHB.

(*S*)- and (*R*)-CHB were determined using a gamma cyclodextrin trifluoroacetyl capillary 30 m×0.25 mm column (Chiraldex™ G-TA, Astec, Bellefonte, PA, USA) with a Hewlett-Packard 6890N gas chromatograph equipped with a flame ionization detector (Agilent Technologies).

**Table 1** Determination of stress resistance parameters in different *S. cerevisiae* strains

| Stress condition[a] | Strain[b] | Decrease in growth rate (%) | Lag time (hours) | Decrease in cell concentration (%) |
|---|---|---|---|---|
| Osmotic (NaCl 1 M) | Ye9–531 | 67.5±0.5 | 2.5±0.3 | 28.0±1.0 |
| | Ye9–532 | 87.0±1.4 | 4.9±0.3 | 46.7±3.8 |
| | Y103 | 92.5±0.5 | 6.3±0.3 | 62.5±0.5 |
| | C242 | 86.5±0.5 | 3.3±0.3 | 49.5±1.5 |
| Oxidative ($H_2O_2$ 1 mM) | Ye9–531 | 50.5±0.5 | 3.1±0.4 | 4.5±0.5 |
| | Ye9–532 | 66.0±3.0 | 7.9±0.4 | 43.5±4.5 |
| | Y103 | 76.0±1.0 | 10.0±0.5 | 45.5±1.5 |
| | C242 | 59.3±2.1 | 3.7±0.3 | 23.0±3.3 |
| Thermal (44°C) | Ye9–531 | ** | ** | 20.7±1.7 |
| | Ye9–532 | ** | ** | 44.3±1.9 |
| | Y103 | ** | ** | 54.7±1.9 |
| | C242 | ** | ** | 33.0±1.4 |

All values represented in the table are mean values±standard deviations based on two to three independent experiments.
[a] The stress factors were introduced during mid log phase.
[b] Ye9-531 and Ye9-532 represent typical resistant and non-resistant offspring of the natural isolate #9, respectively. Y103 represents a non-resistant laboratory strain. C242 is the haploid BC1 offspring of enantioselective strain Y103 and multi-stress resistant strain Ye9-519.
** The response profile to the thermal stress did not include lag time or change in growth rate but only in cell concentration.

The temperature was isocratic at 110°C, and the split ratio was 1:9. Under these conditions, the retention times were: $R_F$=11.31 min for CAAE, $R_F$=12.13 min for (*R*)-CHB, $R_F$=12.81 min for (*S*)-CHB.

*Mating-type tests* Mating-type tests were performed as described by Sherman (2002). The tested strain was mixed both with the *Mat* a and the *Mat* α tester strains separately on a YPD plate and incubated at 30°C overnight. These cultures were then replica plated on an SPO plate (0.1% Bacto-yeast extract, 1% potassium acetate, 0.05% dextrose, 2% Bacto-agar), along with the original strain. Sporulation was detected microscopically after 24 and 48 h of incubation at room temperature.

*Genetic crosses* Overnight-grown strains of opposite mating type were mixed on a YPD plate, and then incubated for 10 h at 30°C. Zygotes (dumbbell-shaped cells) were then isolated with a Nikon Eclipse 50i micromanipulator.

*Tetrad dissection* A Nikon Eclipse 50i micromanipulator equipped with a glass needle was used to isolate spores from asci. The ascus walls were removed using 0.25 mg/ml Zymolase T100 (Sigma-Aldrich) in 1 M sorbitol.

## Results

*Screening for enantioselective S. cerevisiae strains in the asymmetric reduction of CAAE to (S)-CHB* A panel of 19 *S. cerevisiae* strains was examined for the ability to reduce CAAE to (S)-CHB in a whole-cell reduction system, in the presence of glucose as a co-substrate (Fig. 2). The panel included two of the natural strains that were isolated from Mount Carmel, Israel (Katz-Ezov et al. 2006), designated #1 and #9 and their progeny, as well as a laboratory strain, Y103. The natural isolates and their offspring show high genetic and phenotypic diversity, including different ploidy levels and different stress-resistance. Most of the tested strains yielded over 85% ee, but the highest value of 98% was obtained with the laboratory strain, Y103. This strain was also the most active, showing a specific activity of 1.88 μmol/min/mg protein. Being the most active and selective strain in the panel, Y103 was chosen as the parental strain for the genetic breeding program discussed further.

*Screening for multi-stress resistant S. cerevisiae strains* The multi-stress resistance trait is the ability to adjust to various stress conditions such as osmotic pressure, oxidative environment, non-optimal temperatures, etc. Offspring of the natural *S. cerevisiae* isolates #1 and #9 were screened for this desirable phenotype. The offspring of isolate #1 showed poor resistance ability in the initial screening phase and therefore were not further evaluated in this study. Subsequently, resistance of strain #9 and its progeny to induced osmotic, oxidative, and thermal stress was evaluated. A summary of the response of three representative strains to osmotic, oxidative, and thermal stress conditions is depicted in Table 1. Presented are a resistant strain, Ye9-531, a non-resistant strain, Ye9-532 (both offspring of the natural isolate #9), and the enantioselective laboratory strain Y103. Although similar in profile (Fig. 1), the extent of the response to induced stress, varied among the tested

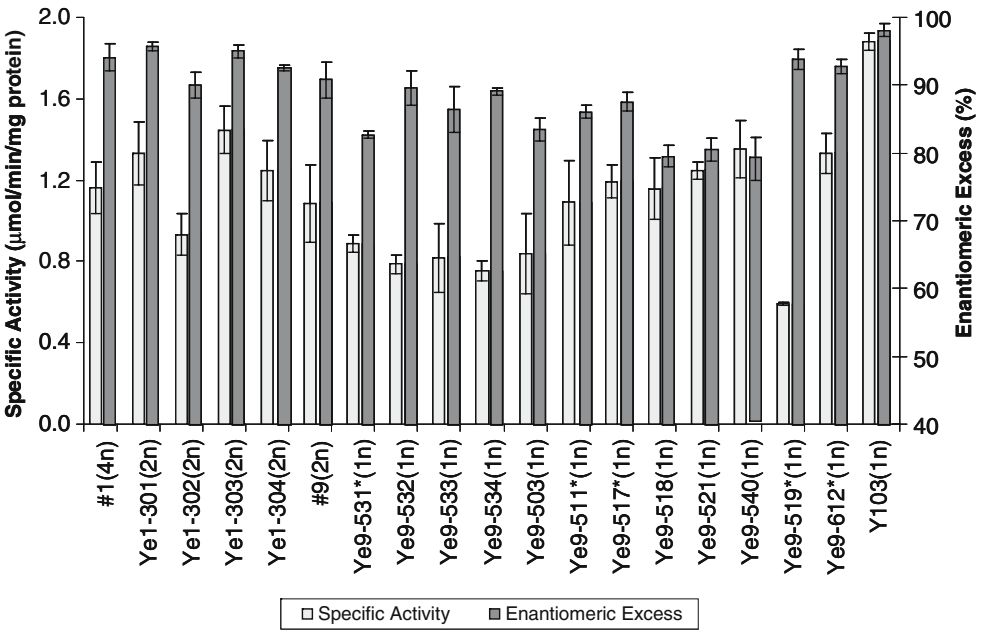


**Fig. 2** Screening for efficient *S. cerevisiae* strains in the asymmetric reduction of CAAE to (*S*)-CHB. Two parameters were measured: specific activity (left *Y*-axis) and enantioselectivity (right *Y*-axis). The yeast panel included the natural isolates (#1, #9) and their offspring, as well as a laboratory strain Y103. Ploidy level is given in *brackets*. Among the offspring, some showed a multi-stress resistance phenotype (indicated with an *asterisk*). Biotransformations were performed at 25°C with 30 mM substrate, 5% glucose in phosphate buffer (pH 7, 0.1 M), using cell concentrations of 7 $OD_{600\ nm}$

strains. Generally, the offspring of #9 showed higher tolerance towards the different stress factors, compared to the laboratory strain. For example, the lag duration caused by addition of 1 M NaCl lasted approximately 5 h amongst the #9 offspring, while Y103 lagged for approximately 6 h before re-entering logarithmic growth. Even so, the ability to tolerate stress conditions varied among the natural isolates progeny with some being more tolerant than others. Comparing the effect of NaCl on Ye9-531 (2.5 h of lag phase, 67.5% decrease in growth rate and 28.0% decrease in cell concentration) and Ye9-532 (4.9 h of lag phase, 87.0% decrease in growth rate and 46.7% decrease in cell concentration), both offspring of #9, indicates that Ye9-531 has higher osmo-resistance ability. Similarly, Ye9-531 performed better than Ye9-532 under oxidative and thermal stress conditions. Hence, the strains that showed high resistance to all three stress factors (i.e. Ye9-531) were characterized as multi-stress resistant.

*Comparing the biotransformation performance of an enantioselective laboratory strain and a multi-stress resistant strain in response to stress factors* Y103 was chosen for further examination due to its high enantioselectivity, and Ye9-531 was chosen for being multi-stress resistant. The specific activity of strains Y103 and Ye9-531, following three different stress treatments is presented in Fig. 3a and b. While the specific activity of Ye9-531 remained constant in response to stress conditions, Y103 showed ca. 30% lower activity. The effect of the exposure to stress on the enantioselectivity of the tested strains was of opposite trend (Fig. 3d and e). While Y103 showed invariable ee values of about 98%, Ye9-531 exhibited a 4–12% decline in ee value, following the addition of stress factors.

*Backcross introgression program: attaining a strain that is both multi-stress resistant and enantioselective* Having an enantioselective recipient strain and a set of multi-stress resistant progeny of a natural isolate, backcross introgression was performed in an attempt to create a unique strain that is applicable to the industrial production of (*S*)-CHB. After crossing Y103 and Ye9-519, the resulting F1 diploid, C13, showed 95.7% ee. This value is significantly higher than the multi-stress resistant parental strain Ye9-519 (93.3% ee; Fig. 4). C13 underwent sporulation and tetrad dissection and 42 offspring were screened for thermotolerance and mating type. The best performing *Mat* α offspring, C136, (94.4% ee) was chosen for backcrossing with Y103. C29 (96.3% ee), was selected for sporulation and tetrad dissection. Eighty-three haploid offspring were tested and C242 demonstrated the best performance, with enantioselectivity (98.2% ee) as high as the parental strain Y103. Additional backcross of C242 with Y103 formed the BC2 diploid, C36. Its enantioselectivity (97.7%) was not significantly different than its parental strains, as expected (Fig. 4).

The improvement in performance of the newly developed strain, C242, under osmotic, oxidative, and thermal stress is presented in Table 1. In comparison with Y103, addition of hydrogen peroxide caused lag duration of only 3.7 h vs 10 h. The decrease in growth rate and cell concentration of C242 (59.3 and 23.0%, respectively) are also significantly lower than Y103 (76.0 and 45.5%). The performance of C242 in response to stress is generally better than the non-resistant Ye9-532, (33.0% in comparison to a 44.3% decrease in cell concentration in response to raised temperature) and somewhat lower than the resistant Ye9-531 (20.7% decrease in cell concentration in response to raised temperature).

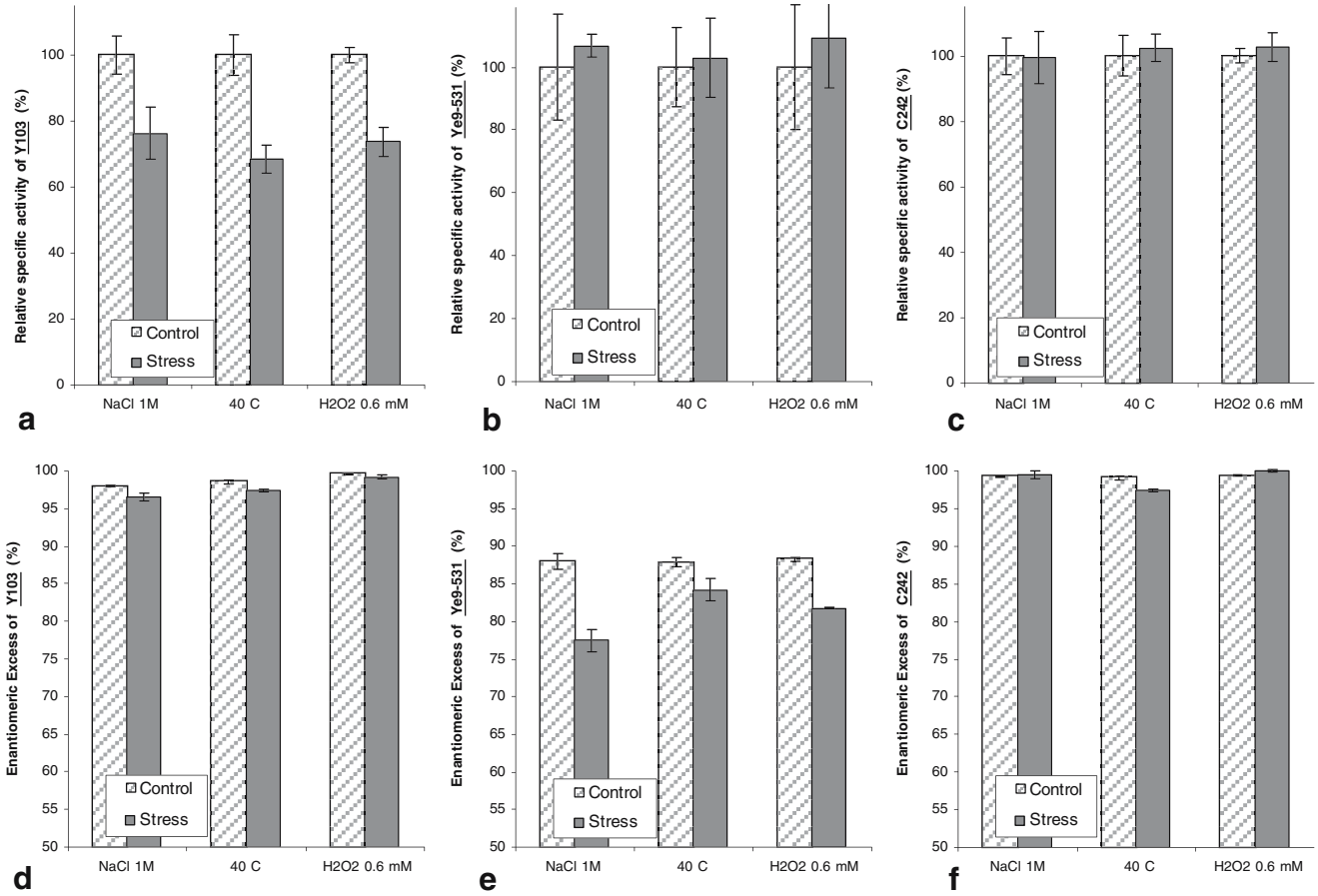


**Fig. 3** Effect of stress factors on the biotransformation of CAAE to (*S*)-CHB. Y103 was selected for its high enantioselectivity in producing (*S*)-CHB. Ye9-531 was selected for its high tolerance to various stress factors. C242 is the BC1 progeny of strains Y103 and Ye9-519. The stress factors were applied at mid-log phase for 3 h, and the biotransformation was subsequently performed at 25°C. The specific activity of strains Y103, Ye9-531 and C242 (**a**, **b**, and **c**, respectively) is presented as the relative value compared to a control culture taken as 100%. The enantioselectivity of Y103, Ye9-531 and C242 (**d**, **e**, and **f**, respectively) is expressed as % enantiomeric excess at full conversion. Results represent an average of at least two independent experiments for each strain

As seen in Fig. 3c and f, the specific activity, as well as enantioselectivity, of C242 remain constant regardless the addition of various stress types.

## Discussion

*Screening S. cerevisiae strains for the selective asymmetric reduction of CAAE to (S)-CHB* Asymmetric reduction of CAAE is a simple and efficient method for producing the pharmaceutical building block, (*S*)-CHB. 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl ruthenium complex was reported as a good chemical catalyst, yielding 97% ee, but it required elevated hydrogen pressure (Yasohara et al. 1999). In an attempt to develop more environmental-friendly procedures, the use of *S. cerevisiae* as a biocatalyst was widely studied. Despite its many advantages, the non-satisfactory 92% ee (Yasohara et al. 1999) prevents it from being industrially applied. Numerous methods were developed to increase the enantioselectivity such as medium engineering (Shimizu et al. 1998), substrate engineering (Nakamura et al. 2003), and addition of inhibitors (Yang et al. 2004). More recent studies focused on genetic improvements (Rodriguez et al. 1999, 2000). For example, Ema et al. (2005) reported a 97% ee with whole-cell system of a reductase (encoded by the *S. cerevisiae Gre2* gene) over-expressed in *E. coli*, and a 98% ee with the purified enzyme. In our screening experiments (Fig. 2), we found a highly selective strain (Y103), naturally yielding 98% ee without any genetic improvements. To our knowledge, this is the highest ee ever reported with a non-modified *S. cerevisiae* strain as a whole-cell production system of (*S*)-CHB.

*Screening for multi-stress resistant S. cerevisiae strains* In biocatalytic processes, the biocatalyst encounters a variety of environmental stresses such as thermal, osmotic or oxidative stress, that lead to low cell viability and activity and therefore, low production yields. Circumventing the stress-causing factors by cooling or lowering the substrate

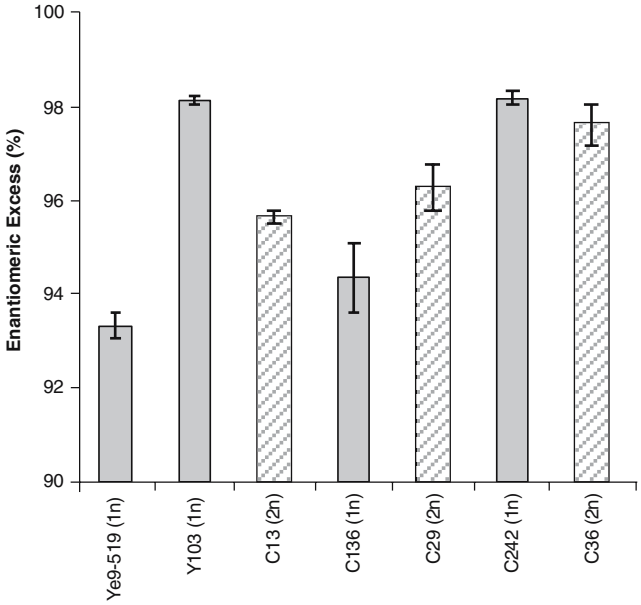


**Fig. 4** Enantioselectivity of the yeast strains studied. Parents (Y103, Ye9-519), diploid F1 (C13), BC1 diploid (C29), BC2 diploid (C36), C13's haploid offspring (C136), and C29's haploid offspring (C242) in the reduction of CAAE to (*S*)- and (*R*)-CHB. Each value represents an average of at least two independent experiments

concentration may be a considerable economical drawback. For this reason, screening for multi-stress resistant biocatalysts is of a great industrial interest (Wei et al. 2007). Previous works compared the response to stress factors of laboratory *S. cerevisiae* strains and industrial strains, resulting in favor of the latter (Smits and Brul 2005; Hirasawa et al. 2006). Originating from the Mount Carmel habitat, the *S. cerevisiae* isolates underwent natural selection, allowing only the most robust and highly adaptive to survive in this stressful and changing environment. Hence, the *S. cerevisiae* isolates and their offspring, were expected to show higher resistance ability to various stress types as was indeed obtained experimentally (Table 1). Y103 was much inferior in stress resistance ability in comparison with the natural isolates. As the natural isolates' population was genotypically and phenotypically diverse (Katz-Ezov et al. 2006), some of its members showed better resistance than others (see Ye9-531 and Ye9-532 in Table 1). Interestingly, strains that were characterized as thermotolerant in the initial screening phase also showed high tolerance to osmotic and oxidative stress. This indicates that thermotolerance holds a strong positive correlation with other stress resistance abilities.

Strains that demonstrated an improved resistance to the tested stress-factors, were designated "multi-stress resistant". This multi-resistance phenotype was previously documented (Trollmo et al. 1988; Cakar et al. 2005; Smits and Brul 2005; Du and Takagi 2007) and may be explained by response mechanisms that are common to different stress types (Attfield 1997; Lewis et al. 1997; Cakar et al. 2005). Despite their obvious advantage as biocatalysts, the newly discovered multi-stress resistant strains presented poor enantioselectivity in the asymmetric reduction of CAAE (Fig. 2). In an attempt to generate a *S. cerevisiae* strain that is both multi-stress resistant and enantioselective, introgression was performed using Ye9-519 as the multi-stress resistant donor parent and Y103 as the enantioselective recipient parent.

*Comparing the biotransformation performance of an enantioselective laboratory strain and a multi-stress resistant strain in response to stress factors* The role of reductases in the transformation of CAAE to CHB is well documented (Katz et al. 2003; Kroutil et al. 2004; Johanson et al. 2005). Additionally, studies have shown that some reductase-encoding genes are up-regulated as a result of stress conditions (Blomberg 2000; Ford and Ellis 2002; Hirasawa et al. 2006). For example, *Gre2*, that encodes an NADPH-dependent reductase, is up-regulated 24.4-fold after the addition of 1 M NaCl (Hirasawa et al. 2006). Similarly, the levels of *Ypr1p*, that encodes a 2-methylbutyraldehyde reductase, are rapidly induced by osmotic and oxidative stress (Ford and Ellis 2002).

Under the three stress conditions evaluated in this work, the enantioselectivity of the stress-tolerant strain Ye9-531, decreased (Fig. 3e), while the enantioselectivity of Y103 remained constantly high (Fig. 3d). This finding suggests that the Ye9-531 stress-resistance mechanisms involve a change in the reductase composition. The fact that the enantioselectivity of the non-resistant Y103 remained constant at all stress conditions supports this assumption. While the ee value of Y103 was consistent when grown under different stress conditions, the specific activity decreased by approximately 30%, whereas the multi-stress resistant Ye9-531 was unaffected by these stress factors (Fig. 3a and b). It may be speculated that Y103 reductase composition does not change under the stress conditions, in consistency with the role of reductases in stress resistance mechanism (Blomberg 2000; Ford and Ellis 2002; Hirasawa et al. 2006). Another explanation for the difference in results of the stress-resistant strain vs the lab strain may be not only the different reductase composition, but also the changes in NADH and /or NADPH levels in the cell. It has been shown that stress factors like calorie restriction result in decreased levels of NADH (Lin et al. 2004). Silva-Graca et al. found that some dehydrogenases favor NADPH over NADH under saline stress in *Candida versatilis* (Silva-Graca et al. 2003). And finally, it has been reported recently by Singh et al. (2007) that oxidative stress evokes a metabolic adaptation that favors increased NADPH synthesis and decreased NADH production in *Pseudomonas fluorescens*.

Our activity measurements substantiate the results obtained on the genetic level and indicate that the reductases expression level and composition in the yeast cell may change in response to stress factors. This does not rule out possible changes in the co-factor level.

*Backcross introgression program: attaining a strain that is both multi-stress resistant and enantioselective* Introgression is a method used for achieving a number of traits determined by different genes in one genome (Naumov et al. 2006). Combining two desirable traits in one strain without the need to identify the involved genes makes introgression a viable approach for improving industrial yeasts over the 'one factor at a time' recombinant DNA methods (Benitez et al. 1996). For example, Stepanova et al. (2001) used a series of backcrosses to introduce lysine overproduction mutations into industrial *S. cerevisiae* strains.

Industrial production of (*S*)-CHB for pharmaceutical use requires an enantioselective biocatalyst, which is also tolerant to highly stressed environments. In the screening experiments described in this work, we obtained these two desirable phenotypes in separate strains. As the two traits are controlled each by multiple genes, we used backcross introgression between the two strains. Indeed, we obtained a haploid offspring of BC1, C242, which was not only as enantioselective as Y103 (Fig. 4) but also had improved stress tolerance over the parent (Table 1). The stress tolerance characteristics of strain C242 were somewhat lower than those of other natural isolates such as Ye9-531, as evaluated by the three quantitative parameters chosen in this study (Table 1). The intermediary stress-resistance of the enantioselective offspring can be explained by the complex genetic character of the resistance phenotype. Choosing the enantioselective strain as the recipient parent for backcrossing increased the chance of having offspring with similar reductase combination but accordingly decreased the chance of maintaining all of the resistance components similar to those of the multi-stress resistant parent. The constant enantioselectivity (Fig. 3f) and specific activity (Fig. 3c) under various stress types indicate that C242 can efficiently adjust to various stress environments while maintaining high enantioselectivity and with no effect on its activity.

In conclusion, genetic introgression allowed the successful generation of a multi-stress tolerant, enantioselective *S. cerevisiae* variant, specifically designed for producing chiral compounds. In his review, Attfield (1997) predicted that improvement of industrial oriented *S. cerevisiae* robustness is most likely to be achieved through recombinant DNA technology. Still, 10 years later, classic breeding methods such as genetic introgression seem evermore relevant.

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