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Genetic stabilization of *Saccharomyces cerevisiae* oenological strains by using benomyl

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Summary. Wild-type oenological strains of *Saccharomyces cerevisiae* are usually aneuploid and heterozygotes; thus, when they are used as starters in must fermentation the resulting wine characteristics may vary from year to year. Treatment of a wild-type *S. cerevisiae* oenological strain with benomyl (methyl-l-butylcarbamoyl-2-benzimidazole carbamate), an antifungal agent shown to cause chromosome loss in yeasts, resulted in a stable starter strain in which the parental oenological traits were unchanged. The oenological *S. cerevisiae* strain was treated with benomyl in two different ways (A and B), and sporulation ability and spore viability were subsequently assayed. Treatment A resulted in both the highest numbers of tetrads and a reduction in DNA cell content, while treatment B increased spore viability. Fermentation assays were carried out with spore clones obtained from treatment A, and the concentrations of glycerol, lactic acid, acetic acid, and ethanol resulting from the treated strains were found to be similar to those of the parental strain. Benomyl treatment thus achieved stable, highly sporulating oenological *S. cerevisiae* strains of low ploidy, but preserved the desirable oenological properties of the parental strain. [**Int Microbiol** 2008; 11(2):127-132]

Key words: *Saccharomyces cerevisiae* · benomyl · ploidy · sporulation · wine production

Introduction

Evidence for spontaneous fermentation can be found as far back as the Miocene or even the Cretaceous, as shown by the detection of yeasts in amber samples from those geological periods [25]. Indeed, in many wine regions of the globe, wine is still elaborated through spontaneous must fermentations carried out by wild-type yeast strains either associated with the grape surface or present as cellar residents [14]. However, in modern times the tendency has been to use oenological strains of *Saccharomyces cerevisiae* as starters, as these yeast quickly initiate fermentation, owing to their efficient fermentative catabolism under selective pressure. Consequently, either wild-type or tailor-made yeast strains are employed as starters, with the added benefit that their use ensures standardized wine production over consecutive years [24].

The sensory properties of wines depend on the type of grape, but also on the yeast strain used. *Saccharomyces cerevisiae* modifies the aroma, flavor, mouth feel, color, and complexity of wine [24]. Winemakers have traditionally isolated new yeast strains in order to improve wine quality and the elaboration processes. Nevertheless, to improve specific oenological traits it may be necessary to genetically modify these strains. The improvement of a given trait by genetic means requires knowledge of the nature of the trait. As applied to yeasts, such knowledge may prove to be elusive since the oenological characteristics obtained with these microorganisms are often polygenic; for instance, at least five loci encoding quantitative traits are linked with genes involved in ethanol tolerance [9,11,15,24].

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Oenological *S. cerevisiae* wild-type strains, unlike their laboratory counterparts, are commonly prototrophic, homothallic, heterozygous diploids or aneuploids and, in some cases, polyploids. In addition they tend to have low sporulation frequencies and produce poorly viable spores [16,17]. In nature, polyploidy is advantageous because gene redundancy protects cells from deleterious mutations; however, it may also generate genetic instability [23]. Nevertheless, with respect to must fermentation, the gene dose, conferred by the ploidy level, and the heterozygosity of alleles of oenological strains can result in the loss of homogeneous wine quality [2,15,22].

Benomyl is an antifungal compound whose active component is methyl-l-butylcarbamoyl-2-benzimidazole carbamate (MBC). The derivative carbendazine binds to microtubules, hence interfering with functions such as cell division, intracellular transportation, and chromosome segregation. These effects, in turn, cause cells to remain arrested at the metaphase stage of mitosis [3]. In *S. cerevisiae*, benomyl induces mitotic chromosome loss at high and equal frequencies for all chromosomes, since it disrupts the structure or function of the mitotic spindle pole by altering tubulin polymerization. This is a random time-exposed process, such that the longer yeasts are exposed to MBC, the higher the probability of chromosome loss [26]. In the present study, a more stable oenological yeast starter was obtained by treating a wild-type *S. cerevisiae* strain with benomyl and then selecting the resulting offspring on the basis of improved sporulation ability and spore viability but without alterations in the parental oenological traits.

Materials and methods

Microorganisms and culture media. *Saccharomyces cerevisiae* strain 145, isolated in our laboratory [6], was used throughout the experiment. Starting from the wild-type, three consecutive cycles of meiosis were accomplished and different strains were isolated (Table 1).

The vegetative growth of *S. cerevisiae* was carried out in yeast extract, peptone, and dextrose (YPD) medium (10 g yeast extract/l, 20 g peptone/l, and 20 g glucose/l) at a temperature of 30ºC; the same medium was employed for presporulation. Benomyl (100 μg/ml) was added as needed from a stock solution (10 mg/ml) prepared in dimethyl sulfoxide (DMSO) and incubated at 23ºC. Sporulation was accomplished in SPOI medium (10 g potassium acetate, 1 g yeast extract/l, 0.5 g glucose/l) at 23ºC. Auxotrophy analyses were carried out in synthetic defined (SD) medium (6.7 g yeast extract without amino acids/l, 20 g dextrose/l, 20 g Bacto-agar, supplemented accordingly with the required amino acid/l), and growth was evaluated at 30ºC. The media were solidified by the addition of 2% agar.

Collection of meiotic products. First, meiosis was induced in a suspension of *S. cerevisiae* strain 145 cells. The spores from this first meiosis were allowed to grow and sporulate. Subsequently, the spores from the second meiosis were micromanipulated, grown, and sporulated for a third time. Sporulation was accomplished by keeping the vegetative cells in presporulation medium for 1 day, followed by 7 days in SPOI medium for meiotic division. The numbers of asci were then counted, and, after a brief treatment (15 min) with 0.5 μg lyticase (Sigma-Aldrich)/ml at 30°C, 20 asci from each meiosis were dissected with a micromanipulator (Nikon SE).

Table 1. Origin of the strains and the meiotic cycle number from which they were derived

Strain[a]	Origin	Cycle number
MMY2[b]	Genetic type	0
145	Wild-type	0
1451A	Treatment A	1
14511A	Treatment A	2
145111A,145112A, 145113A,145114A, 145211A, 145212A, 145213A, 145214A	Treatment A	3
1451B	Treatment B	1
14511B	Treatment B	2
145111B	Treatment B	3
1451	Without benomyl	1
14511	Without benomyl	2
145111, 145112, 145113, 145114, 145211, 145212, 145213, 145214	Without benomyl	3

[a]The source of the strains was this study, except for MMY2 genetic type, received from Richard Bailey, Solar Energy Research Institute, and the 145 wild type from the authors laboratory.
[b]MATa ura3-52 Cyhr.

Study of the influence of benomyl. Benomyl treatment was performed in two ways. In treatment A, spores were gathered by allowing the yeast to proceed through three consecutive mitosis-meiosis cycles. In all cases, presporulation medium for mitotic divisions was supplemented with benomyl. In treatment B, the effect of benomyl either on each division independently or on all three rounds cumulatively was determined by once again allowing the yeast to undergo three consecutive cycles of mitosis and meiosis in the absence of the drug, but spore clones from each meiosis were then kept in presporulation medium containing benomyl and then allowed to sporulate. Benomyl-free cultures in which the same manipulations were carried out served as controls.

Cellular DNA quantification. Yeast strains were grown to stationary phase in YPD medium. DNA from 10^9 cells was extracted with perchloric acid [20], and quantified colorimetrically with diphenylamine reagent [4].

Mutagenesis and auxotrophy studies. Three strains of *S. cerevisiae* were obtained from each cycle of meiosis, carried out according to treatment A. Then, together with the wild-type strain, these strains were mutagenized with ethyl methane sulfonate (EMS), as described by Lawrence [13]. After EMS treatment, the cells were serially diluted, placed on Petri dishes containing YPD medium, and kept at 30ºC for at least 2 days. Replicas were made on SD medium supplemented with a mixture of all amino acids, except that for which auxotrophy was assayed. The cultures were incubated at 30ºC for 3 days after which the number of colony-forming units obtained in the absence of the appropriate amino acid was counted. The study was done for three amino acids: arginine, glutamic acid, tyrosine, plus uracil.

Fermentations. Fermentations at laboratory scale were accomplished in 125-ml capped flasks with an outlet connected to a valve. The containers were filled with 100 ml of Mencía musts from Ribera Sacra (Orense, northwestern Spain, 2005 harvest). All fermentations, except the control without starter culture, were conducted in sterilized musts. The starters were the four meiotic products derived from the third meiosis of two asci from treatment A. The four spores from untreated asci and the original culture of strain 145 were also employed as starters.

Starters were prepared in 100 ml of YPD medium and grown at 30ºC until the population density reached 10^7 cells/ml (ca. 48 h). The cells were then harvested by centrifugation at 5000 ×*g* for 20 min at 4ºC. The pellet was resuspended in must at a density of 10^6 cells/ml and fermented at 25ºC. Periodically, the containers were weighed to determine the CO_2 curve, in order to note the end of the fermentation process. Triplicate controls were made, as for evaluating the evaporation index, with uninoculated tyndalized musts. Once the fermentations had finished, the wine was centrifuged at 5000 ×*g* for 20 min at 4ºC and subsequently filtered through 0.22-µm filters (Millipore). All fermentations were carried out in triplicate. The ethanol, lactic acid, acetic acid, and glycerol contents of the wines were measured using commercial kits (Boehringer-Mannheim).

Statistical analyses. The data were analyzed statistically by Student's t test, variance analysis by ANOVA, and the chi square test (χ^2, SPSS 12.05).

Results

Effect of benomyl on sporulation and spore viability. Approximately 50% of wild-type strain 145 cells underwent sporulation (Table 2). Of the resulting asci, 22% contained four spores and 50% contained two; the rest contained three spores. In the second meiosis, the yield of asci was 76% but decreased by 10% in the third meiosis. The same result was obtained when the cells were treated with benomyl according to treatment B. Under treatment A, however, the ability of the cells to ascosporulate increased steadily from the first to the third meiosis, in which ca. 17% more asci were obtained than in the first.

In all cases, the percentage of tetrads increased, that of dyads decreased, and that of triads remained the same. The effect on sporulation and tetrad production differed significantly between treatments A and B in all cases, except in the first meiosis. Treatment B did not differ from the control with respect to tetrad production in the first meiosis. In the third meiosis, although the highest sporulation index was obtained with untreated cells, the highest four-spore-bearing asci were obtained with treatment A. In addition, spore viability increased with successive cycles of meiosis when the cells were treated with benomyl (Table 2). The spore viability of the control strains was similar in second and third cycles of meiosis. The results of spore viability for the different treatments were statistically significant. Although treatment B allowed the recovery of the highest number of viable spores, treatment A produced the highest levels of sporulation as well as complete tetrad numbers. Accordingly, treatment A was chosen to study auxotrophy and fermentation.

Study of auxotrophies. Strains that had been subjected to treatment A (145, 1451A, 14511A, 145111A) were mutagenized with EMS. The frequencies of auxotrophs increased according to the meiosis cycle (Table 3). Significant differences in the number of auxotrophs were observed between the products of each meiotic cycle and also when compared with either the wild-type strain 145 or strain 145111A, from the third meiosis.

Cellular DNA quantification. The DNA content was measured in cells from strains corresponding to spore clones of four asci, of which, two were subjected to treatment A and the other two not treated. The DNA content of a haploid control, strain MMY2, was 24 mg/10^9 cells. This figure is similar to the 20 mg/10^9 cells established for haploid *S. cerevisiae* [1].

Table 2. Sporulation and viability of spore clones isolated in each treatment

		Asci spore number (%)				
Meiosis	Treatment	2	3	4	Total spores (%)	Tetrad spore viability (%)[a]
	Control	51.3	26.5	22.1	52.4	39.0
1	A	40.8	36.9	22.0	40.0	45.0
	B	40.8	36.9	22.0	40.0	45.0
	Control	49.2	22.7	28.1	76.0	78.0
2	A	35.0	31.3	33.3	47.0	75.0
	B	30.3	35.7	34.1	53.7	80.0
	Control	41.9	23.2	34.7	65.5	74.0
3	A	24.1	33.9	42.0	56.8	83.0
	B	27.0	33.3	40.0	42.0	95.0

[a]Percent asci with four viable spores.

Table 3. Auxotrophs resulting from benomyl treatment in each of cycle of meiosis

	Number of colonies with auxotrophy					
Strain	arg	tyr	glu	ura	Total auxotrophs	Total no. of colonies examined
145	0	0	0	0	0	182
1451A	1	0	0	0	1	123
14511A	4	0	1	0	5	64
145111A	9	3	4	3	19	166

Chi square test:(χ^2) =31.650; $P = 0.05$; gl = 3.
No auxotrophies were obtained for the meiotic products that were not treated with EMS.

The DNA content of the analyzed strains was greater than that of the haploid. Thus, wild-type strain 145 contained 64 mg/10^9 cells, whereas the value was lower in strains subjected to treatment A, and the DNA content of control untreated strains was variable, depending on the respective ascus. Thus, in one ascus the DNA content of spore clone strains was higher than in the wild-type, whereas in another it was similar to that of the wild-type in three of the spore clones and lower in one (Table 4).

Analysis of oenological properties. When treated and untreated strains were employed as starters, fermentations began after 24 h; by contrast, in the absence of starter it took almost 3 days. In all cases, fermentation was completed in about 12 days. Analyses of the different oenological parameters showed that all of them fell within the normal range. However, in the fermentations of untreated strains, lactic acid values were off-range as were acetic acid levels in fermentations carried out by strains 145112, 145113, and 145212 (Fig. 1B,C).

The oenological characteristics were found to vary according to the procedure employed as well as with the different asci and even with the four spore clones of a single ascus. Thus, the amounts of ethanol and acetic acid varied for the ascus meiotic products and for the two asci subjected to treatment A. Also, differences were found in the glycerol contents resulting from spore clones of untreated ascus 2 (Fig. 1A). Based on a comparison of the oenological characteristics of treated and untreated strains, it was concluded that only in the case of ethanol were no apparent significant differences (Fig. 1D).

Discussion

The wild-type *S. cerevisiae* genotype is more variable than that of laboratory strains so that, over time, the useful properties of industrial yeast may become altered [18]. Genetic improvement of wild-type yeast strains offers a way to obtain more genetically stable offspring with improved technological

Table 4. DNA content of strains with and without benomyl treatment

Control		Treatment A	
Strain	DNA content (μg/10^9cells)	Strain	DNA content (μg/10^9cells)
MMY2[a]	24.16 ± 0.45		
145[b]	64.22 ± 1.10		
145111	61.33 ± 0.28	145111A	56.43 ± 0.20
145112	64.35 ± 0.18	145112A	56.75 ± 0.20
145113	59.69 ± 0.75	145113A	52.46 ± 0.45
145114	52.01 ± 0.15	145114A	64.04 ± 0.47
145211	61.82 ± 0.11	145211A	62.87 ± 0.09
145212	77.11 ± 0.26	145212A	58.37 ± 0.37
145213	67.46 ± 0.37	145213A	56.88 ± 0.06
145214	67.81 ± 0.28	145214A	61.15 ± 0.04

Data are means of three independent experiments ± standard deviation.
[a]Haploid strain.
[b]Wild-type.

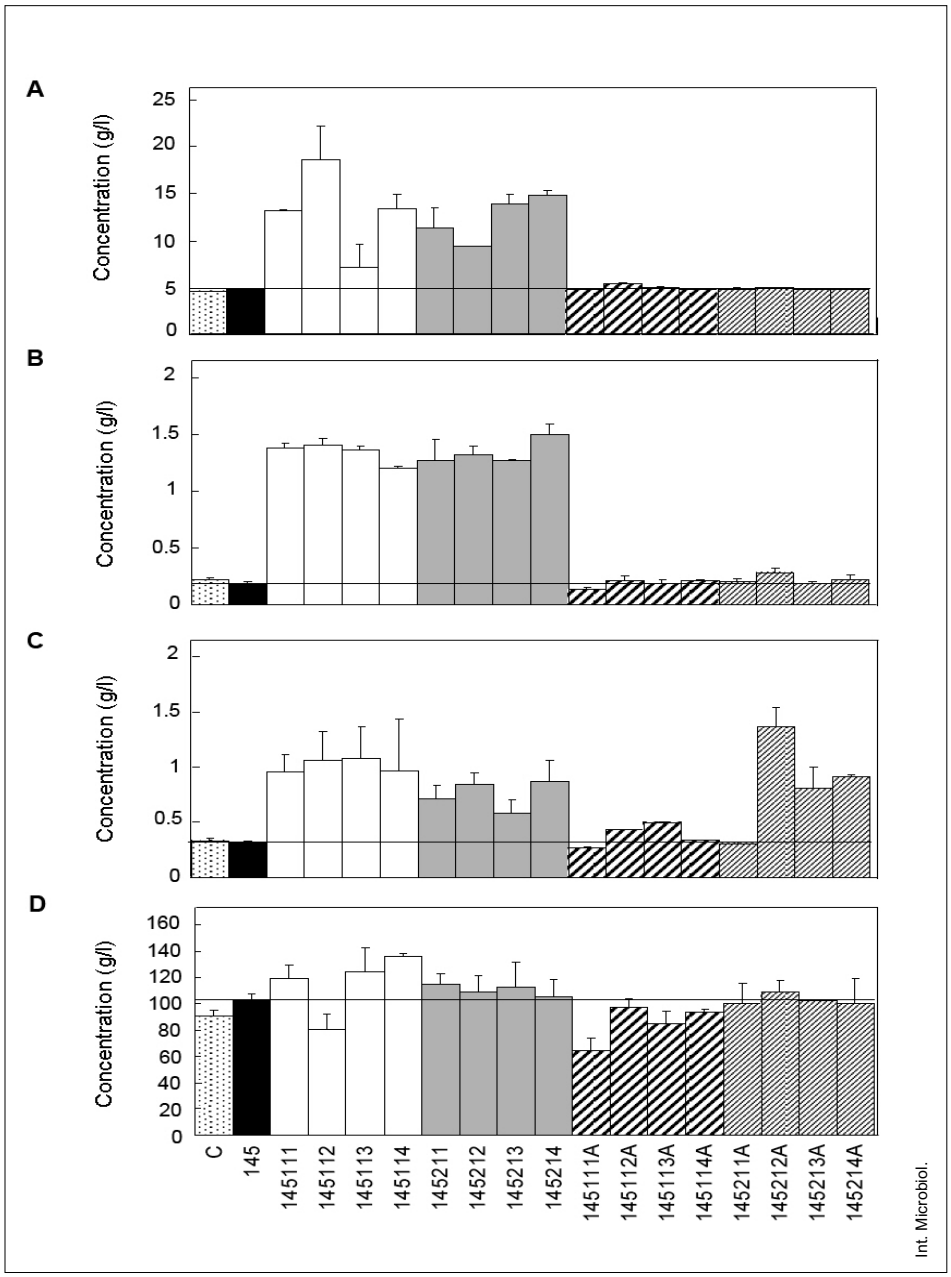

Fig. 1. Concentration of oenological compounds after wine fermentation with meiotic products of two asci treated with benomyl and two without benomyl. (**A**) Glycerol, (**B**) lactic acid, (**C**) acetic acid, (**D**) ethanol. Data are means of three independent experiments analyzed statistically (α = 0.05). Error bars show the standard deviation. Horizontal lines mark the result obtained for wild-type 145.

properties. The treatment of yeast strains with benomyl, followed by sporulation and single spore cultures, could be used to reduce the ploidy level and hence the genetic instability of polyploid or aneuploid strains. The improvement of sporulation capacity, tetrad production, spore viability, and spore clone selection is the first step in optimizing yeast genetics to obtain more tractable strains from a wild-type isolate [7,8,12].

In the present work, sporulation ability improved under all conditions. After each cycle of meiosis, only colonies able to sporulate with complete tetrads were selected. This would explain why, in the case of treatment A, sporulation rates increased after each cycle, in that cells which had lost those alleles limiting sporulation were selected. The same effect was observed for cells subjected to treatment B and cells that were not treated; in these cases, however, sporulation ability decreased after the third meiosis. The presence of sporulation-hampering alleles in heterozygosis in the parental homothallic strain implies that in both treatment B and untreated cells these alleles were in homozygosis after meiosis, thus causing the observed decrease in sporulating cells. Although aneuploid cells may lose chromosomes during vegetative growth, this is uncommon [5] but occurred more fre-

quently in the presence of benomyl. Moreover, as seen for treatment A, the effect was cumulative.

The combination of benomyl treatment with spore selection provided the highest levels of tetrad formation and tetrad spore viability. In fact, due to the loss of detrimental and deleterious alleles before the cells had become homozygotic, such strains became more stable. Moreover, the homozygosis caused by homothallism maintains the genetic properties of the strain, thus eliminating any potential lethal allele in a single meiosis [10,19].

Chromosome loss and a decrease in ploidy due to benomyl treatment were evidenced by the increased auxotrophies after each meiosis and by the reduced DNA content of cells subjected to treatment A. The latter result suggested that parental strain 145 was aneuploid, with a ploidy level close to triploidy. Oenological trait analysis of treated and untreated cells confirmed that strains treated with benomyl according to protocol A (strains 145111A to 145214A) underwent a chromosome loss that involved a loss of heterozygosity. The differences concerning acetic acid content in spore clones from ascus 2 were probably due to the high heterozygosity of the parental strain for the genes involved in the production of this metabolite, as these genes are located on different chromosomes.

Heterozygosity and high numbers of chromosomes were maintained in strains derived from the parental strain, which were not subjected to any treatment (strains 145111 to 145214), so that their oenological traits differed from those of the parental strain. Consistent with our findings, other authors have previously reported that oenological traits are inheritable. Hence, strains derived from spore clones of euploid, homozygous parentals have the technological profile of the parental strain; but high heterozygosity, together with a variable number of alleles, will cause differences in the final product of the fermentation [15,22]. Benomyl treatment therefore achieved stable, highly sporulating oenological *S. cerevisiae* strains of low ploidy, while preserving those properties from the parental strain that are desirable in winemaking.

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