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Selection in yeast
I: Assessing genetic stability and relative fitness of commercial yeasts

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SUMMARY

The potential for changes in allele frequencies in yeast populations by selection was examined. Cells from the wine yeast *Saccharomyces cerevisiae* (strain Montrachet) were grown over a large number of generations using two different culturing techniques, each with two variations: serial transfers on WLN agar plates with and without UV irradiation, and continuous culture in autoclaved and in filter-sterilized grape must. A low frequency of variant isozyme patterns was found in samples taken at the end of the experiment. Growth rates in must and on agar plates were also examined, and it was found that all samples were faster-growing than the original strain, to varying degrees. Applications for the selection system developed are discussed.

INTRODUCTION

The initial studies on the microflora of grapes, musts and naturally fermenting wines were concerned primarily with the origin, systematics and group successions during the progress of fermentation [2,6,13,17]. Since the advent of pure culture starter yeasts [25] there has been a succession of attempts to isolate or construct improved wine yeast strains through clonal selection [7], mutagenesis [1], recombination by sexual breeding [8,9,21], cell fusion [4] or transformation [27]. A similar series of reports can be found for the construction of Brewer's and other industrial yeasts [22].

There have been few reports, however, on the efficacy of isolating selected pure culture starter yeasts with superior initial growth rates or on the genetic stability of selected strains. Contaminant or mutant yeasts with better initial growth rates or "fitness" are of particular concern in situations where the cell numbers are relatively small and the number of generations is large, as is the case with the expanding of starter cultures where such yeasts tend to dominate populations both numerically and metabolically [23]. In the past, many wineries and breweries kept their stock cultures on fresh, frozen or oil-covered agar slants and expanded them immediately before fermentation. For the last 20

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years, many wineries have used rehydrated "active dry wine yeast" that had been cultured aerobically on a molasses-based medium with scant regard for selection effects of the previous growth regime. A few industries use ultra-frozen selected strains [18,26] or chemostats and fermentometers [3] to store or evaluate commercially available yeast performance and genetic stability. The use of chemostats to culture microbes and select mutant strains has been known for some time [12] but to our knowledge has not been used for fitness or stability studies of wine yeasts.

This work assesses the improvement in fitness attainable with a common commercial wine yeast maintained on an agar slant in a stock culture by examining changes in growth rate and isozyme patterns when grown for a large number of generations on various media and when subjected to ultraviolet mutagenesis.

MATERIALS AND METHODS

Strains and culture

The wine yeast *Saccharomyces cerevisiae* strain 522 (Montrachet from R.E. Kunkee, University of California at Davis) was used throughout the study. For a control yeast the G74 strain (from H. Becker, Geisenheim Institute, F.R.G.) was used during electrophoresis. For long-term storage, the Montrachet strain was kept at −80°C in 2-ml glass vials containing 1:1:2 glycerol/water/YEPD (10.0 g yeast extract, 20.0 g D-glucose and 20.0 g peptone per litre). A working culture was maintained at 4°C on WLN (Difco) agar plates.

Four culturing techniques were used to grow the yeast over a large number of generations: serial transfer on WLN plates, serial transfer on WLN plates with periodic UV irradiation, continuous culture in autoclaved grape must, and continuous culture in filter-sterilized grape must. All cultures were begun from an inoculum taken from the original strain.

Serial transfer on WLN plates

The serial transfers were done by forming a two-character alphanumeric inoculum (maximum of four per plate) on WLN plates. Inocula were kept large (about 8×10^5 cells) to minimize the risk of random genetic drift of allele frequencies. Growth reached 3.72×10^8 cells on the average by the time the next transfer was due (48–72 h). A total of 165 transfers over a period of over 14 months were made, on each of six parallel lines of plates.

Serial transfer on WLN plates with UV irradiation

The method used was similar to that for unirradiated serial transfers. At each transfer, however, the inocula were irradiated for 60–120 s with a 254 nm Mineralight model UVG-54 (Ultra-Violet Products, San Gabriel, CA) UV lamp positioned 30 cm from the plates. Growth by the time of the next transfer was highly variable and, therefore, not measured. Four replicates were used, with a total of 16 transfers over 50 days.

Continuous culture in autoclaved grape must

The apparatus used was a modified version of the original chemostat described by Novick and Szilard [12] using a stir bar-agitated 500-ml fermentation flask and a 4-l substrate reservoir. A commercially blended white grape must was first centrifuged for 20 min at $3000 \times g$, autoclaved for 15 min at 121°C and 15 p.s.i., then poured into the input flask while hot. A small amount of fermentation had preceded preparation of the medium but was not considered significant for the purposes of these experiments. A total of 201 volume-doublings of the culture were obtained over a period of 3 months. Culture volume in the chemostat was maintained at 150–200 ml.

Continuous culture in filter-sterilized grape must

When filter-sterilized must was required, centrifuged must containing 0.1 g/l $K_2S_2O_5$ was first prefiltered through a 6.0-μm plate filter then thrice through 0.45-μm Gelman filters, and then finally was put into the substrate reservoir of the chemostat through a 0.45-μm Acrodisc filter. A total of 105 doublings (150–200 ml culture) over 2 months were obtained.

Electrophoresis

At the end of the growth periods, the four cultures, including all replicates plus the original strain and another wine yeast for a control (strain G74), were grown both on WLN plates (two plates/sample) and in filter-sterilized reconstituted concentrated white grape must. Isozyme patterns for the 28 samples are presented in Fig. 1. The 14 plate-grown samples were scraped off the plates into a Tris/ascorbate buffer [19] with an excess of glass beads and ground in a Mickle homogenizer for three 5-min intervals with 3-min ice cooling. Samples were then frozen at −80°C until used. The 14 liquid-grown samples were harvested by centrifugation, washed and recentrifuged before homogenization as with the plate-grown samples. Cell homogenates were loaded onto Whatman 3 MM paper wicks and inserted approximately a third of the distance from the cathodal end of a 12.5% horizontal starch (Electrostarch) gel. Starch gel recipes and stains were obtained from a number of sources [5,10,15,16,20]. All gels were run at 300 V and 60 mA for 6.5–8 h at 4°C.

Growth rates

The growth rates (biomass values) in grape must of the four cultures were compared to that of the starter strain. An inoculum of 8×10^3 cells was placed into each of 33–35 test tubes containing 8 ml of filter-sterilized juice for each sample. Cells used to make the inoculum were first grown to near stationary phase in grape juice in order to equally acclimate cells to that medium and to diminish problems in population variation due to cell mass–cell number effects on lag phase duration. Tubes were left to grow for 70 h at 25°C without agitation, after which relative cell concentration from 660 nm absorbance was determined.

The two serial transfer samples and the original strain were also compared with respect to growth on WLN plates. Inocula were scraped from the plates, washed in 0.8% saline, sonicated to break clusters and examined for bud frequency. Dilutions were calculated so as to apply about 5–10 cells per WLN plate with 50 plates per sample. Colonies were photographed onto slide film after 5 and 8.5

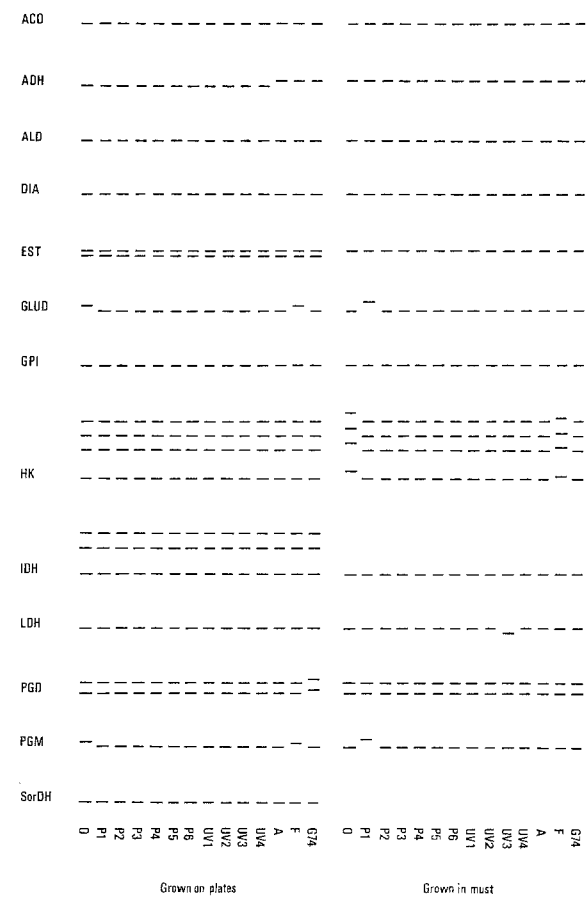

Fig. 1. Banding patterns observed for the thirteen clearly resolved enzymes. The symbols used to designate the samples are the following:
O: original strain, Montrachet; P1–P6: the six replicates of serial transfers on WLN agar plates; UV1–UV4: the four replicates of serial transfers on WLN agar plates with UV irradiation; A: continuous culture in autoclaved must; F: continuous culture in filter-sterilized must; G74: a second wine yeast strain (used as a control).

days of growth and their diameters measured. A correlation between colony diameter and cell number was derived and used as an estimate of growth (data not shown).

RESULTS AND DISCUSSION

The foregoing experiments gave some measure of the rate of genetic change and selection in yeasts as a function of the number of generations and se-

lection pressure exerted by the growth conditions. The serial transfer on WLN plates amounted to approximately 1461 generations. This value was calculated from the equation $G = N (\log_2 (C/I)$ where G = the number of generations, N = the number of transfers (165), C = the colony size at transfer (3.72×10^8 cells) and I = inoculum size (8.05×10^5 cellls). The number of generations for the UV-irradiated serial transfers on WLN plates was calculated to be a minimum of 140, though the value cannot be precise due to variation in colony size.

The maximum throughput for the 150–200-ml chemostat culture of Montrachet wine yeast was three volume-doublings per day before cell washout occurred. The steady-state cell population was calculated to be 3×10^8 to 2×10^9 cells, which provides sufficient generations for the selection process [23]. Chemostat throughput would have to be adjusted downwards considerably if environmental conditions or media composition was adjusted to select for tolerance to cold, SO_2 or high ethanol concentrations. It is interesting to note that if one were to grow cells through 200 generations (which we obtained in the 500-ml chemostat flask) in a batch lot culture, one would need a vessel considerably larger than the sun.

Of 31 isoenzymes assayed, only 13 gave acceptable resolution (Table 1, Fig. 1). Of these, most enzymes were found to be monomorphic throughout the range of samples but there were several samples that clearly showed electrophoretic variants (Fig. 2). The sample of 13 enzymes is a small fraction of the cell's total enzyme complement and only 22% [14] of new mutations to a gene will result in detectable enzyme variants, so the successful introduction and fixing of several new isozyme variants in a population is quite significant. Calculations of the rate of genetic change with respect to fitness require computer simulation and will be presented in later work.

The existence of isozyme diversity in commonly used commercial wine yeasts has been previously reported [24]. In the present study it was interesting to note that strain G74 isolated from German wines and run as an electrophoretic control had a considerable number of isozyme patterns in common with the Californian Montrachet. Isozyme analysis

Table 1

Yeast isozymes used

Enzyme	Abbrev.	Buffer*	Observed activity**
Aconitase	ACO	A	+/+
Alcohol dehydrogenase	ADH	B	+/+
Aldolase	ALD	C	+/+
NADH diaphorase	DIA	A	(+)/(+)
Esterase	EST	B	+/+
L-Glutamate dehydrogenase	GLUD	C	+/+
Glucose phosphate isomerase	GPI	B	+/+
Hexokinase	HK	C	+/+
Isocitrate dehydrogenase	IDH	A	+/(+)
Lactate dehydrogenase	LDH	C	(+)/(+)
Phosphogluconate dehydrogenase	PGD	A	+/+
Phosphoglucomutase	PGM	C	+/+
Sorbitol dehydrogenase	SorDH	B	+/−

* A = morpholine citrate [5]; B = lithium-borate [15]; C = Tris citrate [10].

** The slash (/) represents aerobic/anaerobic sample preparations. + = activity observed; (+) = faint activity; − = no activity discernible.

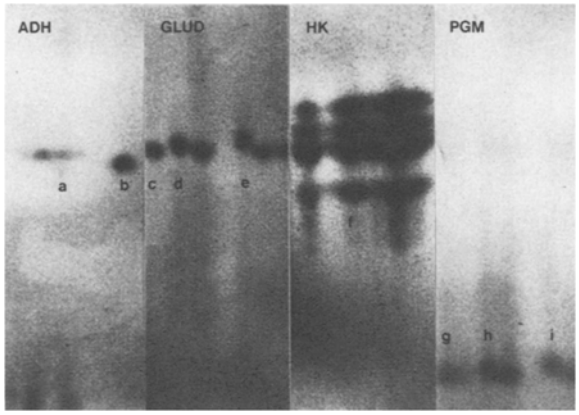

Fig. 2. Some phenotypes in the polymorphic enzyme systems. The sample designations are as in Fig. 1. a: ADH fast phenotype, observed in plate-grown A, F, G74, and all must-grown samples; b: ADH slow phenotype, observed in plate-grown O through UV4; c: GLUD slow (common) phenotype; d: GLUD medium phenotype, observed in plate-grown O, F; e: GLUD fast phenotype, observed in must-grown P1; f: HK common phenotype (medium and fast phenotypes do not appear on this gel); g: PGM slow (common) phenotype; h: PGM medium phenotype, observed in plate-grown O, F; i: PGM fast phenotype, observed in must-grown P1.

of the aerobic plate cultures produced several isozymes that were missing (IDH, EST, SorDH) or altered (ADH) in the anaerobic flask cultures reflecting physiological states associated with glucose catabolite repression rather than some genetic change [11]. The reason for the difference in the two hexokinase isozyme patterns of the original culture is not known.

The endpoint growth rates approximated through biomass values in the batch culture flask were established by absorbance readings of the 70-h cultures as presented in Table 2. All samples were found to be highly significantly faster-growing in the juice than the original strain from which they were derived. It should be noted that generation times were considerably longer than observed for axenic laboratory cultures. The two chemostat selections were especially fast-growing, with the sample with the most generations (205 in autoclaved must) being the fastest. The endpoint growth rates on WLN plates showed similar results (Table 3). The UV-irradiated serial transfer sample, however, appeared to significantly outgrow the original strain only after 8.5 days of incubation. Although the plate-selected samples also grew more quickly in fermenting must, they nevertheless grew significantly slower than the samples which had been selected in must (Table 2). The accelerated growth of the plate-selected samples in must may have been due to an increase in overall fitness, whereas the must-selected samples had specific adaptations to growth in fermenting must in addition to an increased general fitness. This observation would indicate that fermentation industries using chemostat selections should use substrates as similar as possible to the industrial substrate to obtain maximal

Table 2

Absorbance readings of the 70-h-old cultures in must (reconstituted concentrated white grape juice) for the four samples plus the original strain

Strain	No. of gens.	No. of readings	Mean optical* density	**
Original (Montrachet)	0	32	0.1940 ± 0.0937	a
Serial transfer on WLN plates	1461	35	0.3032 ± 0.1088	b
Serial transfer with UV irradiation	140	32	0.3386 ± 0.0933	b
Continuous culture in autoclaved must	201	35	0.6411 ± 0.1433	c
Continuous culture in filter-sterilized must	105	35	0.4653 ± 0.1080	d

* Absorbance at 660 nm is a measure of cell concentration.

** The symbols a–d represent the significance groups, at > 99.5% confidence. Readings with the same letter are not significantly different.

Table 3

Diameters of the colonies from the two plate-selected samples and from the original strain after 5 and after 8.5 days of growth on WLN plates

Cells were made single before spreading them, at a concentration of roughly six cells per plate. Only colonies not physically touching other colonies were measured. Colony diameter is a measurement of cell number.

Strain	No. of gens.	Days incubation	*N*	Mean diameter (mm)	*
Original (Montrachet)	0	5	308	5.569 ± 0.3883	a
Serial transfer on WLN plates	1461	5	272	5.711 ± 0.5575	b
Serial transfer with UV irradiation	140	5	215	5.580 ± 0.5304	a
Original (Montrachet)	0	8.5	261	8.770 ± 0.6672	c
Serial transfer on WLN plates	1461	8.5	221	8.980 ± 0.8026	d
Serial transfer with UV irradiation	140	8.5	201	8.983 ± 0.8762	d

* The symbols a–d represent the significance groups at > 95% confidence. Means with the same letter are not significantly different.

gains in fitness. The fact that the yeast in the autoclaved media grew faster than the filtered media yeast is probably due to the greater number of generations for selection.

It should also be noted that the system described addresses only selection for the initial growth phase in a specific medium and does not consider growth in later phases nor the organoleptic quality of the product. At the present time there is little information available on either the genetics or physiology of organoleptically significant metabolites of yeasts, and selection in this regard must await the relevant basic studies of fermentation yeast metabolism.

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