Two Verdant Types of *Saccharomyces uvarum*[1]

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ABSTRACT

Wallerstein Laboratories Nutrient (WLN) agar has been used for differentiating normal colonies of *Saccharomyces uvarum* from mutant colonies and for subdividing the latter into petites and verdants. In our hands, petites all formed small colonies with dark green centers and white edges. Verdants, however, formed colonies with very different shades of green. This study was undertaken to explore the differences among verdants. Cytochrome absorption spectra of verdant isolates grown aerobically in a low-glucose broth at 25°C revealed two cytochrome patterns. The moderately green verdant, L3G, possessed a normal cytochrome absorption spectrum, while the dark green verdant, 16-7, lacked cytochromes aa_3, b, and c_1. In addition, the dark green verdant accumulated a substance absorbing at 575 nm, possibly the cytochrome precursor, protoporphyrin IX. Oxygen uptake studies indicated that the moderate green verdant was respiratory-sufficient, while the dark green one was respiratory-deficient. Although the moderate green verdant did not differ from the normal control in respiration, a metabolic difference was found. The moderate green verdant used maltotriose more rapidly than the normal control. These results demonstrate at least two types of verdants which may be isolated on WLN agar. Both verdants produced inferior beer and are undesirable in pitching yeast.

Key words: *Cytochromes, Mutants, Protoporphyrin IX, Respiration.*

There are numerous microbiological causes for undesirable aromas and flavors in beer. Therefore, among other measures, the routine examination of pitching yeast for contamination by bacteria, wild yeasts, and culture-yeast variants has become a necessity which no brewer can afford to neglect. While there are many methods for detecting bacteria and wild yeasts, there are few for detecting culture-yeast variants.

One method for detecting culture-yeast variants (5) describes a particularly interesting class of spontaneous variants of bottom-fermenting yeasts which were termed "verdant" because of the color of their colonies on Wallerstein Laboratories Nutrient (WLN) agar (Difco No. 0424-01). On this medium, colonies of normal cells were uniformly white to light green in color, while colonies of petites had white edges with dark green centers. Colonies of verdants were described as uniformly colored, moderate to dark green. Curiously, verdants were considered respiratory-deficient, in spite of their ability to use ethanol and lactate.

In our hands, a normal, bottom-fermenting yeast also formed white to light green colonies on WLN (Fig. 1-A) and petites formed small colonies with white edges and dark green centers (Fig. 1-B). Verdants, however, were not uniform in size and exhibited various patterns of color and various shades of green. Because of this diversity and because the perception of shades of green is so subjective, we undertook to determine what other differences, if any, existed among verdants.

EXPERIMENTAL

Cultures

A bottom-fermenting strain of *Saccharomyces uvarum*, formerly *S. carlsbergensis* (6), designated MC-2, was used as the normal strain in this study because of its color reaction on WLN agar. Spontaneous variant colonies of MC-2 growing on WLN were isolated and subsequently designated L3G, 16-7, and petite. These cultures were maintained on malt-extract, yeast-extract, glucose, and peptone (MYGP) agar slants or broth.

Growth of Cultures on WLN Agar

Cultures to be plated on WLN agar were first grown in 50 ml of MYGP broth and incubated at 25°C for 48 hr with a constant agitation (rotary shaker at 300 rpm). Each culture was then diluted and plated to yield 50–100 colonies per petri dish after incubation at 28°C for 14 days. The colonial characteristics of each culture were recorded.

Absorption Spectra

Cells for cytochrome absorption were grown in 1 liter of MYGP broth in a 6-liter Erlenmeyer flask and incubated at 25°C for 72 hr with constant agitation (300 rpm). The cells were harvested by centrifugation, washed with deionized water, and adjusted to approximately a 25% cell suspension in deionized water. Cytochromes were scanned at 120 nm/min using a Perkin Elmer Spectrophotometer (Model 356) and recorded at 60 nm/min.

Cytochrome absorption spectra were measured at room temperature using a quartz cuvet with a 10-mm light path. At low temperature (liquid N_2, 77°K), spectra were obtained using a cryogenic accessory equipped with a cuvet with a 3-mm light path. Light scatter was balanced with strips of filter paper (Whatman No. 1).

The absorption spectrum of protoporphyrin IX (Sigma Chemical Co.) was made in pyridine at room temperature.

Oxygen Assimilation

Cells for determination of oxygen uptake were grown in 50 ml of MYGP broth in a 250-ml Erlenmeyer flask and incubated at 25°C for 48 hr with constant agitation (300 rpm). They were then harvested by centrigugation, washed three times with 0.05*M* sodium phosphate buffer at pH 7.4, and adjusted to a 25% cell suspension. Oxygen uptake was monitored with an oxygen electrode in a closed reaction vessel containing 3.0 ml of the above buffer and 50 μl of yeast suspension. After establishing the endogenous rate of oxygen uptake, 50 μl of a 20% solution of glucose was added to the reaction vessel and oxygen measurements were resumed. Dry weight of yeast was estimated by evaporating 2.0 ml of the yeast suspension to dryness. The rate of oxygen consumption was reported as μl O_2/hr/mg dry yeast (QO_2).

Laboratory Fermentations

Pitching yeast for laboratory fermentations was propagated in 100 ml of commercial wort at 15°C for 5 days with gentle agitation twice daily. One liter of the same commercial wort was pitched at 10^7 cells/ml. The fermentations were carried out at 15°C for 7 days with gentle agitation twice daily. They were held at this temperature for two additional days without agitation, then cooled to approximately 0°C and held for 2 more days. The degrees Balling and the pH of the decanted beers were measured.

Pilot-Scale Fermentation

Pilot-scale fermentations were carried out as described for the laboratory fermentations, except that the volume of wort fermented was approximately 36 liters. The resulting beers were finished in the usual manner and evaluated organoleptically.

Liquid Chromatography

Residual carbohydrates were determined by the liquid chromatography method of Brobst *et al.* (3), with some modifications. Operating parameters were as follows:

Instrument—Waters 201 with differential refractometer;
Temperature—Water jacket at 85°C;
Sample size—15 μl Valco Sample Valve;
Pressure—250–500 psi;
Flow rate—0.4 ml/min;
Column—24 in. × 3/8-in. o.d. SS column, Aminex 50W × 4 cation exchange resin, 2–30 μ, Ca^{++} form; and

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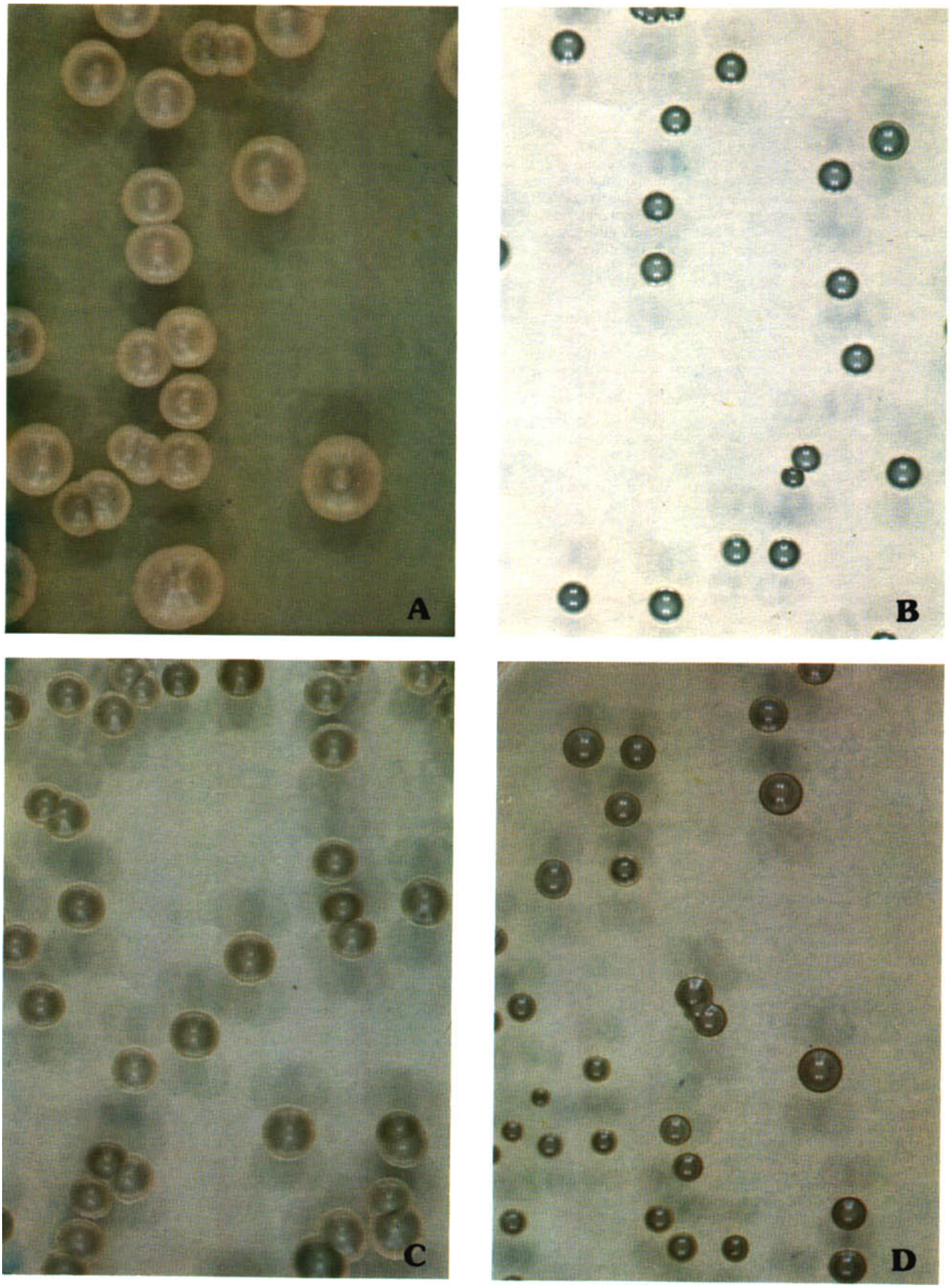

Fig. 1. Representative colonies of *Saccharomyces uvarum* after 14 days' growth on WLN agar. A, Normal strain; B, petite; C, verdant L3G; and D, verdant 16-7.

Standard—external, 2.0, 1.0, 0.5 g/100 ml glucose solutions.

RESULTS AND DISCUSSION

Growth of Variant Strains on Agar

Verdants, which occurred at a frequency of less than 1% in MC-2, were picked and transferred to MYGP broth. After three days' growth, verdant cultures were diluted and plated on WLN. Well-isolated colonies were picked and the above growth and plating procedures were repeated. In this manner, a number of verdants were obtained. Two of these were selected for further study because of their morphological differences and because of their genetic stability on this medium.

One verdant (designated L3G and shown in Fig. 1-C) formed moderate green colonies which were uniform in size and color, but formed considerably larger colonies than the second verdant. The latter (designated 16-7 and shown in Fig. 1-D) formed dark green colonies, about the same size as those formed by the petite. However, these 16-7 verdants had three concentric colored bands: a dark green outer band; a light green middle band; and a moderately green center.

Cytochrome Absorption Spectra

Since cytochrome-deficient mutants tend to form smaller colonies on solid media than do cytochrome-complete cells, cytochromes were determined. Each culture was grown in MYGP broth under highly aerobic conditions. The normal culture, MC-2, contained the α cytochrome absorption bands for cytochromes aa_3 at approximately 600 nm, cytochrome b at 557 nm, cytochrome c_1 at 553 nm, and cytochrome c at 546 nm (Fig. 2A). The petite contained only cytochrome c (Fig. 2B). The verdant L3G (Fig. 2C) had a spectrum nearly identical to that of the normal culture (cf. Figs. 2C and 2A). Verdant 16-7 (Fig. 2D), on the other hand, was devoid of cytochromes aa_3, b, and c_1; but contained cytochrome c. In addition to the cytochrome c band, this variant had a pronounced absorption band at 575 nm (which was only a slight shoulder in the spectrum of the petite) and a somewhat less pronounced band at about 540 nm.

Material Absorbing at 575 nm

Figure 3 depicts the absorption spectrum at room temperature of verdant 16-7 after growth in a low glucose medium under highly aerobic conditions together with that of protoporphyrin IX. Three prominent absorption bands at 506, 538, and 575 nm were present in the spectrum of this verdant in addition to the absorption band for cytochrome c. The first three bands were in good agreement with three of the four absorption bands of protoporphyrin IX. The fourth band at 629 nm in free solutions of protoporphyrin IX was absent in verdant 16-7. However, since the absorption spectrum of this verdant was made on whole cells, the absence of this band could be due to the bound state of protoporphyrin IX

Bassel *et al.* (2) recently described a mutant of *Saccharomycopsis lipolytica* which not only accumulated protoporphyrin IX but excreted it. The *S. lipolytica* mutant, which requires a functional respiratory system for growth, appears to accumulate protoporphyrin IX due to a mutation affecting regulation of porphyrin biosynthesis. Other possible explanations for the accumulation of protoporphyrin IX have been discussed by Cox and Charles (4). However, our verdant differs from the mutants described by Bassel *et al.* (2) and Cox and Charles (4) in that the accumulation of protoporphyrin IX is coupled with the complete loss of cytochromes aa_3 and b, but not c. Thus, our mutant appears similar to the true petite with the exception of protoporphyrin IX accumulation. Mutants similar to these and to the porphyrin mutants described by Bard *et al.* (1) appear to be impaired prior to protoporphyrin IX and will be useful for elucidating the porphyrin and cytochrome biosynthetic pathways.

Respiratory Characteristics

The respiratory quotients (QO_2) of the four cultures used in this study are shown in Table I. The normal culture and the L3G verdant culture were found to have relatively high endogenous respiratory rates. Verdant 16-7 and the petite cultures did not respire appreciably. The addition of glucose to the normal and

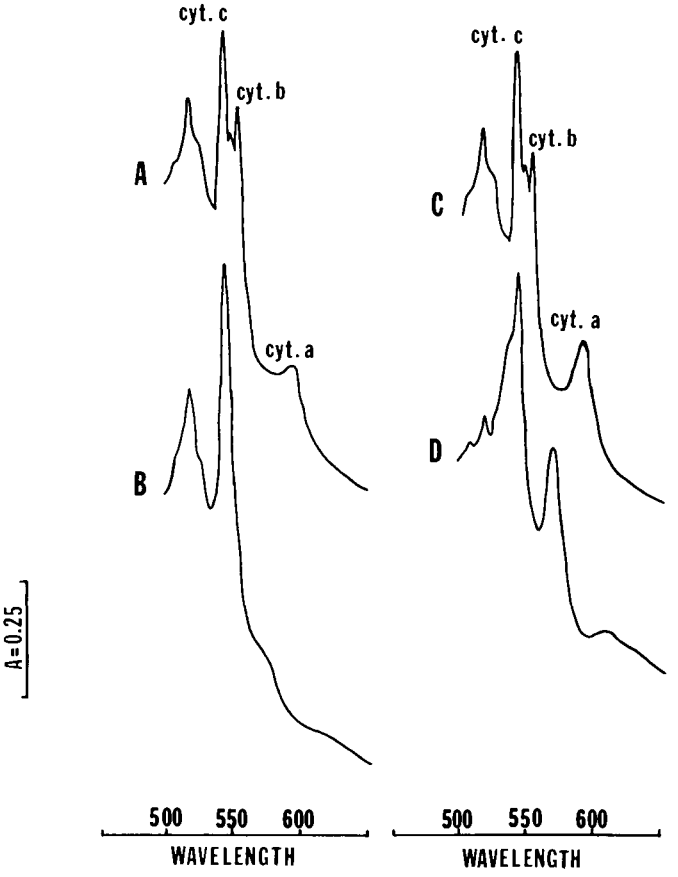

Fig. 2. Cytochrome absorption spectra of: A, normal MC-2; B, petite; C, verdant L3G; and D, verdant 16-7.

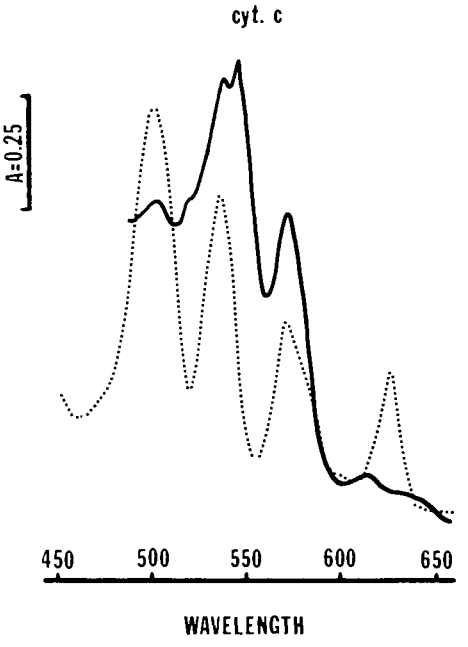

Fig. 3. Absorption spectra of verdant 16-7 (solid line) and protoporphyrin IX (dotted line).

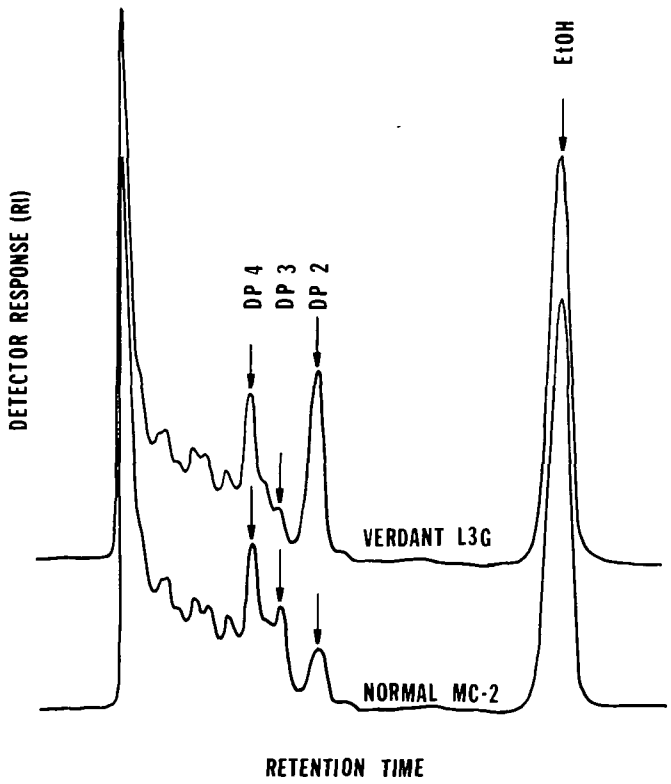

Fig. 4. Chromatograms (HPLC) of residual carbohydrates in partially fermented worts. Maltose, maltotriose, and maltotetraose are designated DP-2, DP-3 and DP-4, respectively.

verdant L3G cultures abruptly increased their respiratory rates. That of the normal culture increased 42%, while that of verdant L3G increased 32%. In contrast, the addition of glucose either to the verdant 16-7 or to the petite did not alter the rate of endogenous respiration.

When ethanol was substituted for glucose, the same results were obtained in all cases. The cultures MC-2 and L3G were stimulated, but culture 16-7 and the petite were not.

Laboratory Fermentations

The possibility of fermentative differences between normal and verdant L3G cultures was investigated in laboratory fermentations. After approximately three days of fermentation, the verdant culture was observed to sediment much faster than the normal culture. Microscopic examination of the fermenting verdant yeast revealed that many of the daughter cells had failed to separate from the mother cells, while the fermenting normal cells had separated completely. This fact may explain the differences in sedimentation.

Carbohydrate analyses of partially fermented worts revealed another major difference between these cultures. The chromatograms of these partially fermented worts are shown in Fig. 4. Glucose and fructose have been completely fermented by both cultures. However, the normal yeast metabolized maltose faster than the verdant L3G. Integration of the chromatograms indicated 0.27 g residual maltose/100 ml beer for the normal culture but 0.78 g/100 ml for the verdant—about a threefold difference. However, the verdant L3G metabolized maltotriose faster than the normal, but at the expense of maltose. From the chromatogram in Fig. 4, it was estimated that 0.12 g maltotriose remained per 100 ml beer for the verdant culture, but 0.32 g/100 ml for the normal yeast culture—again approximately a threefold difference.

TABLE I
Respiration of Glucose by the Normal and Three Variant Strains of *S. uvarum*

Culture	Respiration Rates[a]		% Stimulation by Glucose
	Endogenous	Glucose added	
MC-2 (normal)	26	37	42
L3G (verdant)	28	37	32
16-7 (verdant)	0.6	0.6	0
Petite	0.6	0.6	0

[a]Expressed as QO_2, μl O_2/hr/mg.

Pilot-Scale Fermentations

The fermentation of commercial wort by verdant L3G proceeded very slowly (probably due to the rapid sedimentation of this yeast), requiring twice as much time to end-ferment as the normal MC-2. Triangular taste tests of these beers by a qualified panel were highly significant. Nine out of ten panel members preferred the normal control beer. Comments by panel members indicated that the verdant beer had a fruity aroma and contained diacetyl.

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Literature Cited

1. BARD, M., WOODS, R. A., and HASLUM, J. M. *Biochem. Biophys. Res. Commun. 56*: 324 (1974).
2. BASSEL, J., HAMBRIGHT, P., MORTIMER, R., and BEARDEN, A. M. *J. Bacteriol. 123*: 118 (1975).
3. BROBST, K. M., SCOBELL, H. D., and STEELE, E. M. *Amer. Soc. Brew. Chem., Proc. 1973,* p. 43.
4. COX, R., and CHARLES, H. P. *J. Bacteriol. 113*: 122 (1973).
5. CZARNECKI, H. T., and VAN ENGEL, E. L. *Brew. Dig. 34*(3): 52 (1959).
6. LODDER, J. (ed.) The yeasts (2nd ed.). North-Holland: Amsterdam (1970).

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