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# Selection of Wine Yeasts for Growth and Fermentation in the Presence of Ethanol and Sucrose

TAHÍA BENÍTEZ,* LUCAS DEL CASTILLO, ANDRÉS AGUILERA, JAIME CONDE, AND E. CERDÁ-OLMEDO

*Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain*

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To optimize the conversion of carbohydrates to ethanol, strains of several *Saccharomyces* species were examined for the ability to grow and ferment in a range of sucrose and ethanol concentrations. A total of 632 wine yeasts, most of them isolated from wineries in Andalusia and Extremadura, southwestern Spain, were subjected to screening and selection. Growth and fermentative capacity in different ethanol and sucrose concentrations varied from one strain to another. There was no correlation between growth and fermentative capacity. The best 35 strains grew in 15% ethanol and fermented in 18% ethanol. Ethanol accumulated, although at a reduced rate, after the cells stopped growing. Most yeast strains were highly fermentative in 50% sucrose. Some of them effectively utilized the carbohydrates of the culture, yielding final ethanol concentrations of >14%. Of the 35 selected strains, 16 were promising for genetic analysis and breeding because of their capacity to sporulate. These strains were homothallic, and their spores were viable. The meiotic products analyzed so far were also homothallic.

Ethanol is an ideal fuel and starting point for countless chemical transformations. Optimal conversion of carbohydrates to ethanol requires cells that are tolerant of high concentrations of both and able to efficiently produce ethanol at relatively high temperatures. Although yeasts have been the basis of several traditional industrial conversions of carbohydrates to ethanol, little is known about the genetic or molecular basis of their ethanol tolerance.

Ethanol is clearly inhibitory for yeasts: cell growth stops at relatively low ethanol concentrations, and fermentation stops at relatively higher ones (17). Decreases in the rate of ethanol production are related to decreases in viable cell count (12). Cell growth inhibition by ethanol is noncompetitive and has been described as either a linear (7, 8) or an exponential function of ethanol concentration (1).

The physiological basis of ethanol inhibition is obscure. Highly tolerant strains have been reported to store fewer lipids, compared with other strains (5). The plasma membrane is the first sensitive organelle to make contact with ethanol. Since ethanol is an amphipathic compound, the lipid composition of the plasma membrane may be important for ethanol tolerance (11, 19). Spheroplasts made from ethanol-tolerant cells seem to be stable in 20% ethanol, and cell membranes have been reported to become ethanol tolerant in proteolipid-supplemented medium (6).

Ethanol tolerance of yeasts may be related to ethanol concentration inside the cell. The intracellular ethanol concentration of Brewers' yeast appears to be lower than those of less-tolerant yeasts. Intracellular ethanol concentrations above a certain threshold inactivate alcohol dehydrogenase and kill cells (13).

Highly tolerant strains accumulate less storage carbohydrate, compared with less-tolerant strains (5). Some of the sugar-tolerant yeasts described in this paper were also alcohol tolerant, but these two features are not necessarily related.

Ethanol tolerance has seldom been studied from the genetic point of view. Strain improvement has been accomplished by creating hybrids between highly tolerant *Saccharomyces cerevisiae* and *S. diastaticus* strains (3), although data on the tolerance of the meiotic products have not been reported. Ismail et al. (9) have studied the meiotic segregation of the tolerance factor in many diploids and have carried out crosses between haploid products with different levels of tolerance. These workers have shown that *Saccharomyces* hybrids tolerate higher ethanol concentrations, compared with the parents, and have demonstrated the polygenic character of ethanol tolerance in the strains studied.

TABLE 1. Systematic classification of ethanol-tolerant yeast strains

| Species | No. of strains | | |
|---|---|---|---|
| | Pre-selected | Selected | Sporulating |
| *S. cerevisiae* | 69 | 28 | 12 |
| *S. fermentati* | 9 | 2 | 2 |
| *S. chevalieri* | 8 | 1 | 0 |
| *S. rosei* | 5 | 1 | 0 |
| *S. capensis* | 1 | 0 | 0 |
| *S. bayanus* | 1 | 0 | 0 |
| *S. pretoriensis* | 1 | 0 | 0 |
| *Saccharomyces* sp. | 12 | 3 | 2 |

As part of a project to investigate the physiology and genetics of ethanol tolerance and to obtain more-tolerant strains, we studied the main features of natural strains obtained from Spanish wine musts.

## MATERIALS AND METHODS

**Organisms.** Yeast strains were obtained from the following sources: 615 *Saccharomyces* strains were isolated by J. A. Casas (Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla) from musts taken from different Andalusian and Extremaduran wineries (strains ACA1 to ACA615); 11 *S. cerevisiae* strains, generously provided by Victor Ar-

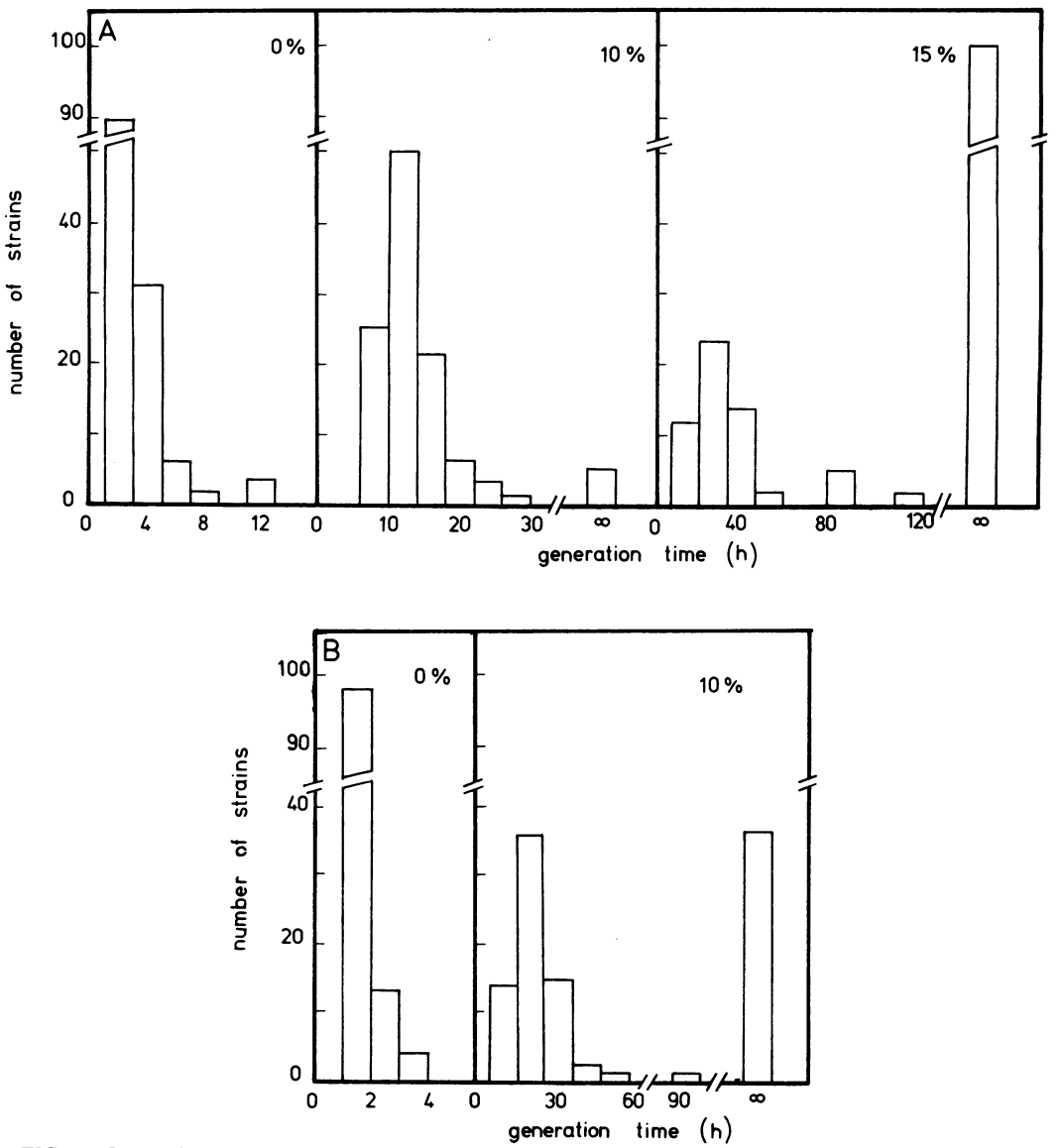


FIG. 1. Generation times of 106 preselected wine yeast strains in YPD containing the indicated concentrations of ethanol. (A) Incubation at 22°C; (B) incubation at 37°C.

TABLE 2. Growth rates of laboratory strain S288C (haploid) and DA1 (diploid) at various temperatures and ethanol and carbohydrate concentrations

| Temp (°C) | Strain(s) | Growth rate (generation time [h]) | | | |
|---|---|---|---|---|---|
| | | YPD + indicated ethanol concn (% [vol/vol])[a] | | | YPD + 50% (wt/vol) sucrose |
| | | 0 | 10 | 15 | |
| 22 | S288C | 0.214 | 0.040 | 0 | 0.026 |
| | Wine yeasts[b] | 0.196 ± 0.045 | 0.064 ± 0.013 | 0.016 ± 0.024 | 0.026 ± 0.015 |
| 26 | S288C | 0.285 | 0.085 | 0 | 0.040 |
| | DA1 | 0.375 | 0.142 | 0 | ND[c] |
| 37 | S288C | 0.396 | 0 | 0 | 0.051 |
| | DA1 | 0.475 | 0 | 0 | ND |
| | Wine yeasts[b] | 0.348 ± 0.097 | 0.016 ± 0.022 | 0 | 0.031 ± 0.027 |

[a] No strain grew in 18% ethanol at any temperature.
[b] Averages (± standard deviations) for 16 selected wine yeast strains (Table 5).
[c] ND, Not determined.

royo (Instituto de Fermentaciones Industriales, Madrid), were isolated from wines made in different areas of Spain (strains IFI3 to IFI277), except for one strain, which came from bread (IFI251); and 5 *S. cerevisiae* strains, a gift from J. Conde, were from Jerez de la Frontera and were isolated from sherry wines (strains FJF135 to FJF414; FJF305 was a "flor" yeast, rather than an *S. cerevisiae* strain). As reference strains, we used the laboratory haploid *S. cerevisiae* S288C (ATCC 26108) and the diploid *S. cerevisiae* DA1. The latter was obtained by crossing strains D585-11C (**a** *lys1*) and X30-3C (α *ade2.1 his4-Δ15*) from Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

**Media and culture conditions.** Yeasts were inoculated into 10-ml tubes containing 4-ml portions of liquid YPD (1% yeast extract, 2% peptone, 2% dextrose [16]) and incubated for 2 days at 22 or 37°C; 10-μl portions were then inoculated into 10-ml tubes containing 4-ml portions of YPD supplemented with 10, 15, or 18% ethanol or 50% sucrose and incubated at 22 or 37°C. Growth, expressed as generation time, was determined by measuring the optical density of the cultures at 595 nm.

Preliminary assays of fermentative capacity were

TABLE 3. Ethanol production by selected *Saccharomyces* strains grown for 7 days in YPD with 50% sucrose

| Ethanol produced at 22°C (% [vol/vol]) | Strain(s) | Ethanol produced at 37°C (% [vol/vol]) | Ethanol produced at 22°C (% [vol/vol]) | Strain(s) | Ethanol produced at 37°C (% [vol/vol]) |
|---|---|---|---|---|---|
| 14.6–15.0 | FJF305 | 13.0 | 12.1–12.5 | ACA346 | 10.0 |
| | | | | FJF212 | 11.2 |
| 14.1–14.5 | FJF206 | 11.4 | | IFI85 | 12.0 |
| | | | | IFI275 | 8.7 |
| 13.6–14.0 | ACA4 | 12.5 | | | |
| | ACA7 | 11.2 | 11.6–12.0 | ACA345 | 13.7 |
| | ACA180 | 11.2 | | ACA500 | 11.2 |
| | FJF135 | 13.4 | | FJF338 | 13.0 |
| | IFI256 | 11.8 | | IFI251 | 12.0 |
| 13.1–13.5 | ACA174 | 13.7 | 11.1–11.5 | IFI87 | 12.5 |
| | ACA490 | 12.7 | | | |
| | IFI3 | 11.2 | 8.5–9.0 | IFI6 | 13.7 |
| | | | | IFI82 | 12.8 |
| 12.6–13.0 | ACA167 | 12.5 | | | |
| | FJF414 | 9.6 | ND | ACA318 | 13.2 |
| | IFI274 | ND[a] | | ACA347 | 11.2 |
| | | | | ACA390 | 13.0 |
| 12.1–12.5 | ACA21 | 12.0 | | ACA405 | 13.0 |
| | ACA161 | 12.5 | | ACA407 | 13.7 |
| | ACA166 | 12.5 | | ACA450 | 12.0 |
| | ACA178 | 11.9 | | ACA501 | 12.0 |

[a] ND, Not determined.

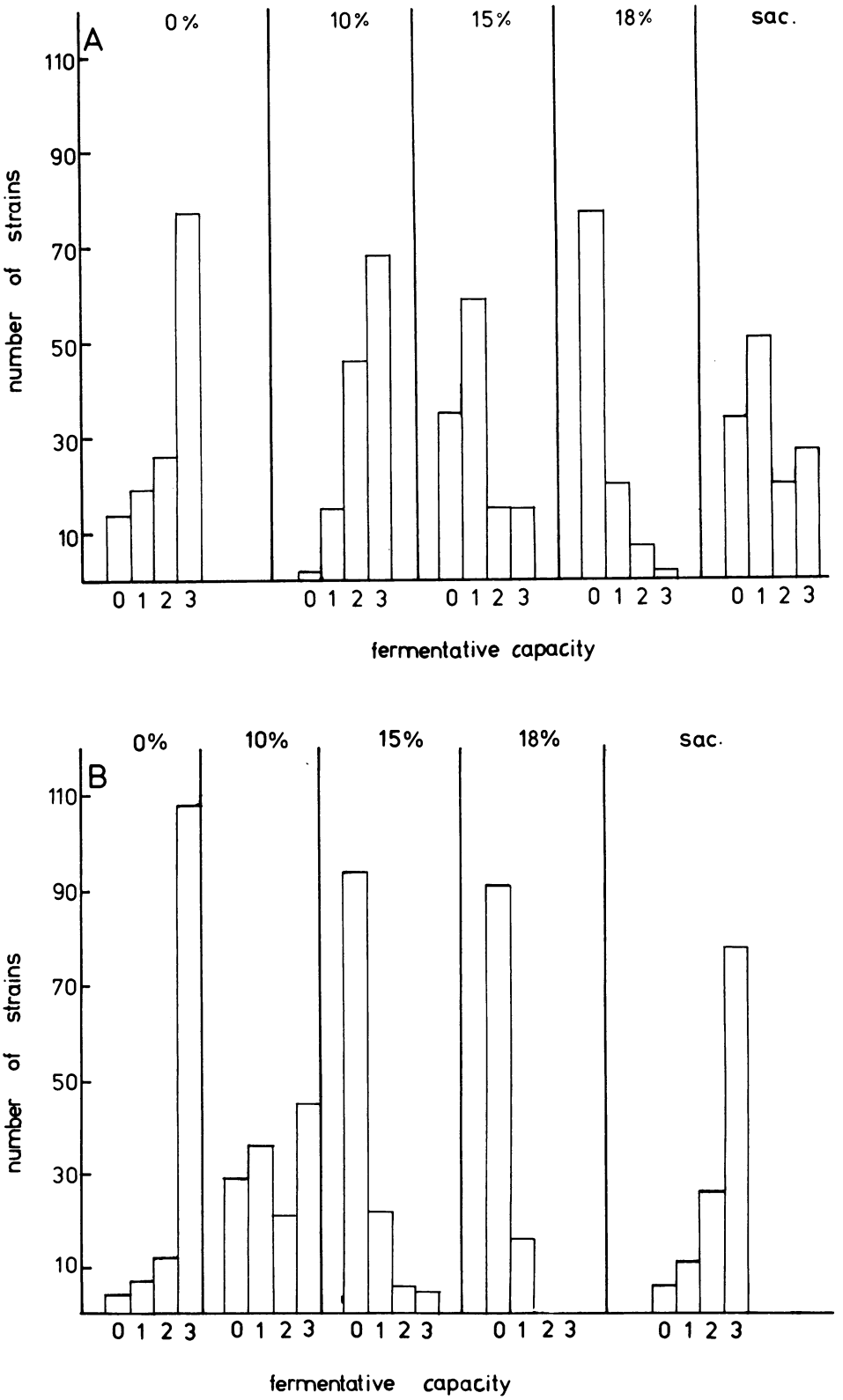


FIG. 2. Fermentative capacities (0, none; 1, minimal; 2, moderate; 3, maximal) of 106 preselected wine yeast strains in YPD containing the indicated concentrations of ethanol. (A) Incubation at 22°C; (B) incubation at 37°C.

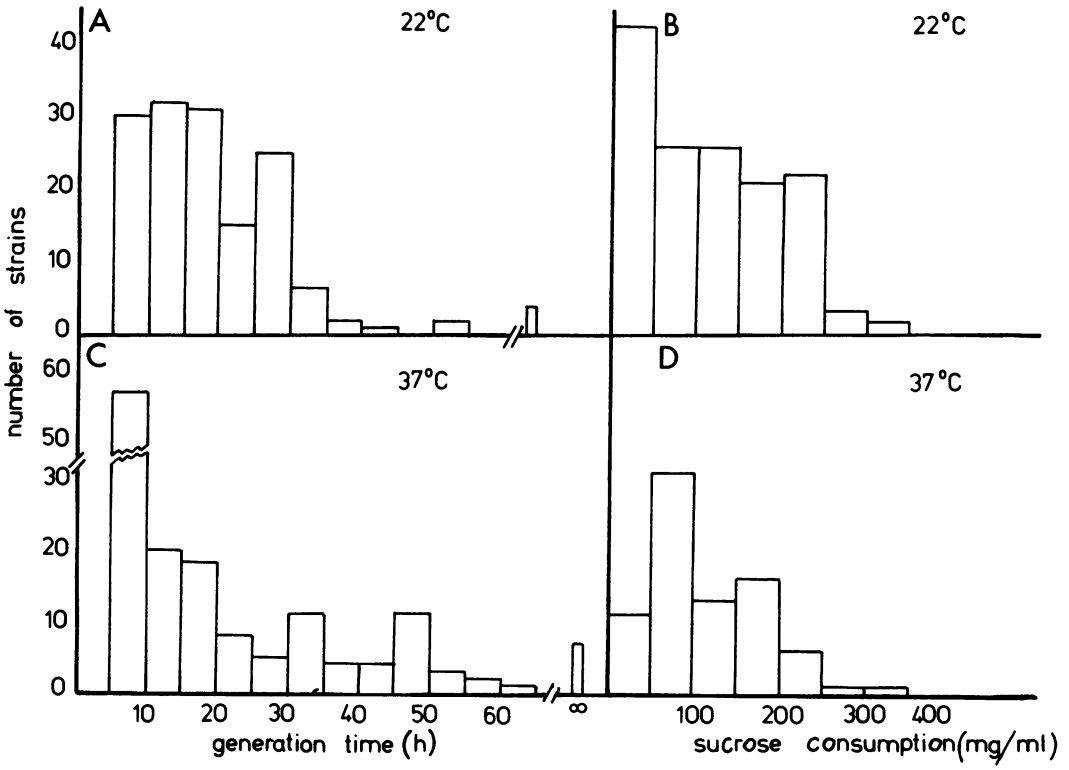


FIG. 3. (A and C) Generation times of selected *Saccharomyces* strains at 22 and 37°C in 50% sucrose; (B and D) consumption of sucrose after 7 days of incubation.

made by inoculating 250-μl portions of stationary-phase cultures into 10-ml tubes containing 4-ml portions of YPD supplemented as described above. An inverted tube (Durham) was used to collect the $CO_2$ liberated through fermentation. A semiquantitative estimate of fermentative capacity, based on the amount of $CO_2$ liberated, was used (see legend to Fig. 2).

**Sucrose consumption and ethanol production.** Sucrose was determined by the anthrone (2) and phenol (4) methods. Ethanol concentrations were calculated from the remaining sucrose concentration or were measured directly with alcohol dehydrogenase (10).

**Genetic characterization.** Selected yeast strains were allowed to sporulate, and tetrads were analyzed by usual procedures at an incubation temperature of 22°C (16). Cell suspensions containing abundant tetrads were incubated with helicase (Suc d'Helix Pomatia; L'Industrie Biologique Française, Clichy, France) at different concentrations and for different periods of time, depending on the strain. Tetrads were dissected with a micromanipulator.

## RESULTS

**Broad characterization and preselection of wine yeasts.** The criterion followed for a first round of selection was growth in YPD with 10% ethanol at 22°C without shaking; 106 of 632 strains grew exponentially under these conditions, with generation times of about 8 h. The final optical density was similar to that reached in the absence of ethanol. The taxonomic classification of the strains, carried out by J. A. Casas Asín, is shown in Table 1.

The ethanol-tolerant strains were inoculated into 5-ml portions of YPD supplemented with 0, 10, 15, or 18% ethanol and incubated at 22 or 37°C without shaking (Fig. 1). No strain grew in 18% ethanol at 22°C or 15% ethanol at 37°C. Even in cultures of highly tolerant strains, the generation time increased with ethanol concentration. Some of the strains showed nonexponential growth.

In comparison, the maximal ethanol concentration allowing growth of reference strain S288C was about 10% up to 30°C but only 8% at 37°C. At variance with many wine yeasts, the laboratory strain was incapable of growing in 15% ethanol at any temperature (Table 2).

Fermentative capacity was as varied as growth parameters (Fig. 2). Fermentation was less sensitive than growth to high temperatures and ethanol concentrations but was still drastically reduced. Fermentative capacity and growth were not correlated. Some strains had a high fermentative capacity but were unable to

TABLE 4. Sporulation of selected wine strains

| Strain | Species | Conjugation | | Sporulation[a] | % Spores germinated |
|---|---|---|---|---|---|
| | | With **a** | With α | | |
| ACA4[b] | *S. fermentati* | | | 3, NA | 100 |
| ACA7[b] | *S. fermentati* | | | 3, NA | 90 |
| ACA21[b] | *Saccharomyces* sp. | | | 3, NA | 80 |
| ACA161 | *S. cerevisiae* | | | 1, NA | |
| ACA166[b] | *S. cerevisiae* | | | 2, NA | 64 |
| ACA167[b] | *S. cerevisiae* | | | 2, NA | 60 |
| ACA174[b] | *S. cerevisiae* | | | 3, NA | 100 |
| ACA178 | *S. rosei* | No | No | 0, — | |
| ACA180 | *S. cerevisiae* | No | No | 0, — | |
| ACA318 | *S. cerevisiae* | No | No | 0, — | |
| ACA345 | *S. chevalieri* | | | 3, AA | |
| ACA346 | *S. cerevisiae* | No | No | 0, — | |
| ACA347 | *Saccharomyces* sp. | No | No | 0, — | |
| ACA390 | *S. cerevisiae* | | | 3, NA | 1 |
| ACA405 | *S. cerevisiae* | | | 1, NA | |
| ACA407[b] | *S. cerevisiae* | | | 2, NA | 48 |
| ACA450[b] | *S. cerevisiae* | | | 2, NA | 50 |
| ACA490 | *S. cerevisiae* | | | 2, NA | 1 |
| ACA500[b] | *S. cerevisiae* | | | 3, NA | 10 |
| ACA501 | *S. cerevisiae* | | | 3, NA | 5 |
| FJF135 | *S. cerevisiae* | No | No | 0, — | |
| FJF305[b] | *Saccharomyces* sp. | | | 1, NA | |
| FJF414[b] | *S. cerevisiae* | | | 2, NA | 20 |
| FJF338 | *S. cerevisiae* | No | No | 0, — | |
| FJF212 | *S. cerevisiae* | No | No | 0, — | |
| FJF206[b] | *S. cerevisiae* | | | 2, NA | 73 |
| IFI3 | *S. cerevisiae* | No | No | 0, — | |
| IFI6 | *S. cerevisiae* | No | No | 0, — | |
| IFI82[b] | *S. cerevisiae* | | | 2, NA | 70 |
| IFI85 | *S. cerevisiae* | No | No | 0, — | |
| IFI187[b] | *S. cerevisiae* | | | 2, AA | |
| IFI251 | *S. cerevisiae* | No | No | 0, — | |
| IFI256[b] | *S. cerevisiae* | | | 3, AA | 95 |
| IFI274[b] | *S. cerevisiae* | | | 1, AA | |
| IFI275 | *S. cerevisiae* | | | 1, AA | |
| S288C | *S. cerevisiae* | | | 3, NA | 100 |

[a] 0, No sporulation; 1, <1% sporulation; 2, moderate sporulation; 3, maximal sporulation (~80%); NA, normal asci (four spores); AA, abnormal asci (<4 spores); —, no asci.

[b] One of the strains most amenable to genetic analysis.

grow under restrictive temperatures or ethanol concentrations. These are probably the most interesting findings as far as industrial applications are concerned.

Similar experiments were made with all preselected strains in YPD with 50% sucrose. Many of the strains grew rather well and had a high fermentative capacity at this high sugar concentration (Fig. 3). In some of the cultures, fermentation was better at 37°C. Production of ethanol, as estimated from final sucrose concentrations, was quite variable.

**Selected strains.** From the 106 preselected strains, 35 strains were chosen because of their achievement in one or more of the tests. Ethanol production by these strains in YPD with 50% sucrose is shown in Table 3. Some strains gave a final ethanol concentration of >14% (vol/vol), as measured with alcohol dehydrogenase. The flor yeast (FJF305) grew more slowly than did other strains, even in YPD, but fermented well and was highly tolerant of ethanol and sucrose; this strain produced the highest ethanol yields from carbohydrates.

We subjected the 35 selected strains to several variations of the usual laboratory protocol for obtaining sporulation (Table 4); optimum sporulation was obtained by incubation at 22°C. Nonsporulating strains can be haploid; if so, they should be capable of crossing with haploid strains of the opposite sex. The conjugation with known α or **a** haploid strains gave negative results (Table 4). Nonsporulating strains can also be highly polyploid or aneuploid or can have genetic defects in their conjugation or sporulation mechanisms.

TABLE 5. Physiological characteristics of 16 selected wine yeast strains[a]

| Strain | Growth (generation time [h]) | | | | | | | (Em) F | |
|---|---|---|---|---|---|---|---|---|---|
| | 22°C | | | | 37°C | | | 22°C | 37°C |
| | 0% E | 10% E | 15% E | 50% S | 0% E | 10% E | 50% S | | |
| ACA407 | 3.0 | 13 | 10.0–15.0 | 18 | 1.7 | 23 | 8 | 18 | 15 |
| ACA450 | 2.5 | 10 | | 14 | 1.5 | 26 | 8 | 18 | 15 |
| ACA174 | 3.2 | 10 | 15.1–20.0 | 24 | 1.8 | 12 | 45 | 15 | 15 |
| ACA161 | 3.0 | 12 | 20.1–25.0 | 40 | 1.5 | 18 | 54 | 18 | 18 |
| ACA166 | 3.5 | 10 | 25.1–30.0 | 38 | 1.7 | – | 48 | 18 | 18 |
| ACA167 | 3.0 | 10 | | 28 | 2.0 | 13 | 23 | 15 | 15 |
| ACA4 | 4.0 | 12 | ∞ | 30 | 1.7 | – | 42 | 15 | 15 |
| ACA7 | 3.2 | 9 | ∞ | 43 | 1.5 | – | 54 | 18 | 18 |
| ACA21 | 4.0 | 13 | ∞ | 26 | 2.0 | – | 80 | 18 | 18 |
| FJF206 | 4.5 | 11 | ∞ | 60 | 3.5 | – | 24 | 18 | 15 |
| FJF305 | 6.0 | 12 | ∞ | 34 | 4.0 | – | 18 | 15 | 10 |
| FJF414 | 4.5 | 11 | ∞ | 54 | 3.5 | – | 60 | 15 | 15 |
| IFI82 | 3.5 | 15 | ∞ | – | 2.5 | – | – | 18 | 18 |
| IFI87 | 4.0 | 9 | ∞ | 15 | 2.5 | – | 30 | 18 | 18 |
| IFI256 | 4.5 | 7 | ∞ | 30 | 2.0 | – | 15 | 15 | 15 |
| S288C | 4.7 | 20 | ∞ | 40 | 2.6 | – | 25 | 10 | 10 |
| ACA500 | 2.5 | 11 | ND | 12 | 1.7 | 16 | 10 | 15 | 15 |

[a] (Em) F, Maximal ethanol (E) concentration at which fermentation was detectable after 120 h in YPD with ethanol or sucrose (S) at the indicated concentrations; ND, not determined; –, no growth. No strains grew in 18% ethanol at 22 or 37°C or in 15% ethanol at 37°C. All strains but IFI82 fermented in 50% sucrose at 22 and 37°C.

From the 35 selected strains, 16 were chosen because of their physiological characteristics and potential for genetic manipulation (Table 4). Of the 16 yeasts, 12 were *S. cerevisiae* strains, 2 were *S. fermentati* strains (ACA4 and ACA7), and 2 were *Saccharomyces* strains (ACA21 and FJF305). They were homothallic, and their capacity for sporulation and spore germination varied, depending on the strain. Their meiotic products were also homothallic. Table 5 shows the physiological growth and fermentative capacities of these strains at different concentrations of ethanol and sucrose and temperatures of 22 and 37°C, respectively.

## DISCUSSON

We found that yeast growth inhibition increased with ethanol concentration and that fermentative capacity was only inhibited at higher ethanol concentrations (Table 5). Growth inhibition of *S. carlsbergensis* by ethanol is related to the retention of ethanol inside the cells; i.e., yeast cells stop dividing when the intracellular ethanol concentration reaches a critical value. There was a linear relationship between external and internal ethanol concentrations, as determined with washed, centrifuged cells. Higher intracellular ethanol concentrations and stronger inhibition were found at higher temperatures (14, 15).

Laboratory strain S288C did not grow at sucrose concentrations of 60%, and growth was nonexponential at sucrose concentrations of >40%. Similar results have been reported for glucose when a different strain of *S. cerevisiae* was used (18).

Wine yeasts vary enormously in the ability to grow and ferment at high ethanol or sugar concentrations. Growth and fermentative capacities were not correlated. High fermentation and lack of growth are desirable industrial features. Our best strains grew at 15% ethanol and fermented at 18% ethanol. When inoculated into YPD supplemented with 50% sucrose, they fermented the carbohydrate in the culture and gave final ethanol concentrations of >14%. In growth media, cells tolerate ethanol levels that are higher than those normally produced in fermentation. Similar results have been reported by Day et al. (3) for brewing yeasts. They have found that although the ability of different yeasts to tolerate high levels of ethanol varies widely, *Saccharomyces* strains brewing are fairly uniform in their response to ethanol, tolerating ethanol concentrations of 7 to 13%; osmophilic *Saccharomyces* strains are not very ethanol tolerant. We found that some carbohydrate-tolerant yeasts are also alcohol tolerant, but this does not imply a general correlation between the two phenotypes. The taxonomic classification of our strains leads us to conclude that ethanol tolerance is not a reproducible feature of yeast species.

Many wine yeast strains sporulated very poorly, giving abnormal asci with one, two, or three spores, or were unable to sporulate at all. We

suspect that they are polyploid or aneuploid and therefore of uncertain promise for genetic studies.

Our results are encouraging and provide added evidence that concerted breeding of yeast strains can improve ethanol production.

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