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Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*

Ma. Jesús Torija, Nicolas Rozès, Montse Poblet, José Manuel Guillamón, Albert Mas*

Unitat d'Enologia del Centre de Referència de Tecnologia d'Aliments, Dept Bioquímica i Biotecnologia, Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, Ramón y Cajal, 70, 43005 Tarragona, Spain

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Abstract

The influence of fermentation temperature (from 15 to 35 °C) on a mixed strain population was studied. Mitochondrial DNA analysis was used to differentiate *Saccharomyces cerevisiae* strains and the frequency of each strain during the alcoholic fermentation was determined. The chemical analyses of resulting wines were carried out. The temperature determined how *Saccharomyces* strains developed and how effectively they fermented. Some strains performed better at high temperatures and others at low temperatures. The maximal population size was similar at all temperatures. At low temperatures, however, it was reached later though it remained constant throughout the alcoholic fermentation. On the other hand, viable cells decreased at high temperatures, especially at 35 °C. Obviously, the composition of the wines changed as the temperature of fermentation changed. At low temperatures, alcohol yield was higher. Secondary metabolites to alcoholic fermentation increased as the temperature increased. Glycerol levels were directly affected by temperature.

Keywords: Glycerol; Ethanol; Yeast; Population dynamics; Wine

1. Introduction

Alcoholic fermentation is a combination of complex interactions involving must variety, microbiota and winemaking technology. Obviously, some factors strongly affect alcoholic fermentation, and as a consequence, the quality of the wine. The most important ones are the clarification of grape juice, the levels of the sulphur dioxide, the temperature of fermentation, the composition of the grape juice, inoculation with selected yeasts and the interaction with other microorganisms (Ribéreau-Gayon et al., 2000). One of these factors, the temperature of fermentation, directly affects the microbial ecology of the grape must and the biochemical reactions of the yeasts (Fleet and Heard, 1993).

Several authors have suggested that some species of non-*Saccharomyces* have a better chance of growing at low temperatures than *Saccharomyces* (Sharf and Margalith, 1983; Heard and Fleet, 1988) because they can increase their tolerance to ethanol (Gao and

* Corresponding author. Tel.: +34-977-250000; fax: +34-977-250347.

E-mail address: amb@fe.urv.es (A. Mas).

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Fleet, 1988). Also the number of different species, as well as their endurance during alcoholic fermentation, is conditioned by both the temperature of the must and the temperature during fermentation. These changes determine the chemical and organoleptic qualities of the wine (Fleet and Heard, 1993). Temperature is also known to affect yeast metabolism, and as a result, the formation of secondary metabolites such as glycerol, acetic acid, succinic acid, etc. (Lafon-Lafourcade, 1983).

In a previous ecological study (Torija et al., 2001), we isolated several strains from a *Saccharomyces cerevisiae* "natural" population, which were used in the present study. This population had a high diversity of strains, probably related to the absence of killer phenotype, control of temperature and lack of previous inoculation with commercial strains. Very little is known about how fermentation temperature affects the dynamics of the *Saccharomyces* strain population. Competition during alcoholic fermentation carried out at different temperatures could therefore be a way of testing the natural endurance of indigenous strains. This could be used as a criterion for rapidly selecting one of several strains and at the same time, studying resistance to temperature in a controlled situation, i.e. under laboratory conditions. Inoculating selected yeasts to ensure that alcoholic fermentation begins properly has become common practice in enology, and new molecular biology techniques for identifying strains have made it easier to confirm the imposition of inoculated yeasts (Querol et al., 1992a).

The aim of this paper is therefore to evaluate how temperature affects the dynamics of a known population of *Saccharomyces* during alcoholic fermentation. We used the 20 most consistent strains present in natural vinifications. We report how *Saccharomyces* strains isolated from the same wine-producing area interact during alcoholic fermentations at different temperatures and how this affects the composition and characteristics of the wine.

2. Materials and methods

2.1. Yeast strains

The strains had previously been isolated and identified in an ecological study at wineries in the Priorat region (Spain) (Torija et al., 2001). Twenty strains of *S. cerevisiae* with killer sensitive phenotype were used. These strains were cultured for 48 h at room temperature on fresh YPD medium [Glucose 20 g l^{-1}; Peptone Bacteriological (Cultimed, Panreac, Barcelona, Spain) 20 g l^{-1}; Yeast extract (Cultimed) 10 g l^{-1}] before they were used in the fermentations.

2.2. Fermentation experiments

The medium was made from concentrated white must (Concentrados Palleja, Riudoms, Spain) that was diluted to obtain a final sugar concentration of 200 g l^{-1}. Total acidity was adjusted to 6 g l^{-1} with tartaric acid and the total sulphur dioxide was adjusted to 60 mg l^{-1} with potassium metabisulphite. The must pH was 3.3.

Fermentations were performed in 500 ml bottles filled with 450 ml of media and covered with a cotton cap. Twenty strains were inoculated in the same flask at the initial population of 1×10^5 cfu ml^{-1} each, which made the total population 2×10^6 cfu ml^{-1}.

The fermentations were incubated in duplicate at five different temperatures (15, 20, 25, 30 and 35 °C) without shaking. Everyday, the CO_2 released was assessed by measuring weight loss and the growth of yeast was measured by counting the viable yeast. Final fermentation was considered when no residual sugars were left (<2 g l^{-1}). Maximal fermentation rate was the maximal slope obtained from the representation of sugar consumption towards fermentation day and was expressed as concentration of consumed sugars (in g l^{-1}) per day.

Viable yeasts were determined by plating them on YPD agar at an adequate dilution. For the molecular study, 40–50 colonies were taken randomly and isolated from each plate. Sampling was done halfway through fermentation (MF; density ≅ 1,040 g l^{-1}) and at final fermentation (FF; density ≅ 990 g l^{-1}). It has to be considered that only 40–50 randomly selected colonies were analysed at each point, so the results should to be taken with the appropriate restrain of the random selection.

2.3. Mitochondrial DNA restriction analysis

DNA was extracted and mitochondrial DNA restriction patterns of the strains were determined as

previously was described by Querol et al. (1992b) with the restriction endonuclease *Hin*f I (Boehringer Mannheim, Germany). Restriction fragments were separated on a horizontal 0.8% agarose (Ecogen, Barcelona, Spain) gel in TBE buffer (Tris 89 mM; boric acid 89 mM; EDTA 2 mM; pH 8).

2.4. Chemical analysis

Fermentable sugar concentration and ethanol were analysed using a modified Rebelein method (Rebelein, 1973) and ebulliometry, respectively (García Barceló, 1990). Titratable acidity was analysed by titration with 0.1 M sodium hydroxide solution and phenolphthalein as the indicator (Ough and Amerine, 1987). Residual sugars, glycerol, acetaldehyde and acetic and succinic acids were assayed using specific Boehringer Mannheim enzymatic kits.

3. Results

3.1. Effect of temperature on yeast growth

Temperatures grouped in three different profiles of fermentation kinetics (see Fig. 1 and Table 1). Fermentations at 15 and 20 °C began more slowly, as we can see by their longer lag phase and slower rate of maximal fermentation, especially at 15 °C. This caused a delay in reaching the maximal population (10^8 cfu ml^{-1}), but once it was reached it remained at these high values for the whole process, i.e. there was a long stationary phase and no declining phase.

Fermentations at 25 and 30 °C reached similar maximal populations, although the initial fermentation rates were faster than at low temperatures. After the eighth day of fermentation, the population decreased down to values of 10^6 cfu ml^{-1}. Finally, fermentations at 35 °C had no lag phase, but they had a quick

Table 1
Effect of fermentation temperatures on yeast population, length and rate of fermentation

Temperature (°C)	Day of maximal population	Maximal population reached (cfu ml^{-1})	Length of fermentation (days)	Maximal fermentation rate (g l^{-1} day^{-1})
15	6	1.18×10^8	15	9.41
20	3	1.46×10^8	15	20.87
25	3	1.73×10^8	15	52.87
30	3	1.95×10^8	20	63.23
35	2	0.97×10^8	20	69.69

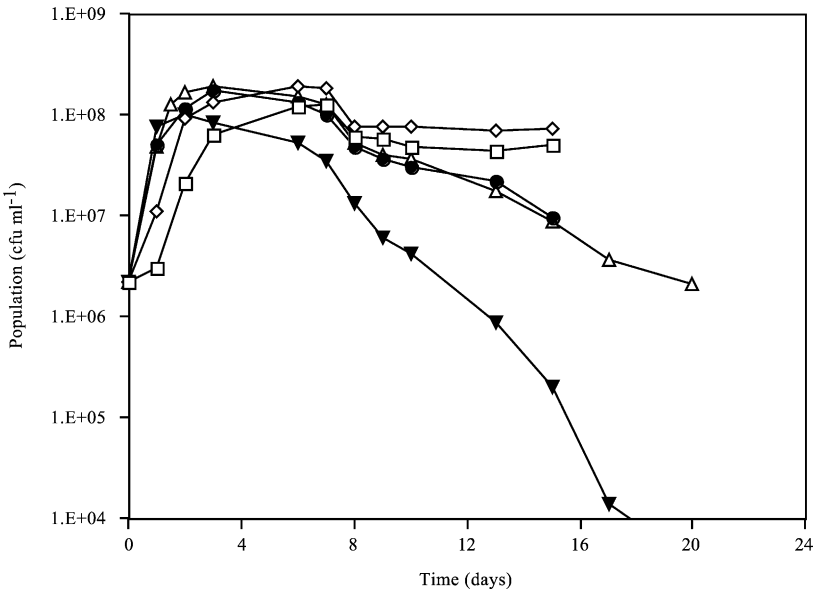

Fig. 1. Variation in population size during alcoholic fermentation at different temperatures. □ 15 °C, ◇ 20 °C, ● 25 °C, △ 30 °C, ▼ 35 °C.

exponential phase, and reached the maximal population earlier. However, the fermentation proceeded with a very short stationary phase. The decline phase began on the sixth day of fermentation and reached values as low as 10^2 cfu ml^{-1} at the end of the process. Cell viability therefore fell as temperature increased.

Interestingly, the length of fermentation, just like the maximal population, was similar for all the temperatures studied, although high temperature fermentations needed more time to consume all the available sugars. So, although these fermentations started faster, the higher cell death may have caused a final decrease in the use of available sugars. In contrast, low temperature fermentations, which started more slowly, consumed faster all the sugars because the high biomass was maintained throughout the process.

3.2. Effect of temperature on strain variability

We analysed the relative performances of the strains at different temperatures in competition (Fig. 2A and B) at the same starting population sizes (5%). In mid-fermentation, only more extreme and the optimal temperatures were studied. At 35 °C, at the end of fermentation, only one strain survived both the high temperature and the sugar content, and therefore, the ethanol content. However, more strains were present in the middle of fermentation when the alcohol concentration was lower, and therefore, the synergic effect of ethanol and temperature was less restrictive. At 25 and 30 °C, one strain (P29), led the fermentations, with a presence that ranged from 25% to 42%. This strain showed a better performance of high temperatures because it always increased by the end of fermentation at these temperatures.

At low temperatures, no strain led the fermentation; up to four strains had a relative presence of 12.5–17.5% at the end of the fermentation but their percentages in the middle of the process were almost undetectable. Interestingly, five strains did not appear at any temperature, while four strains were identified at the end of fermentation irrespective of the temperature (except at 35 °C when only one was isolated), although their percentages were different according to the temperatures. Also, the percentages of the strains changed during fermentation; some strains that were

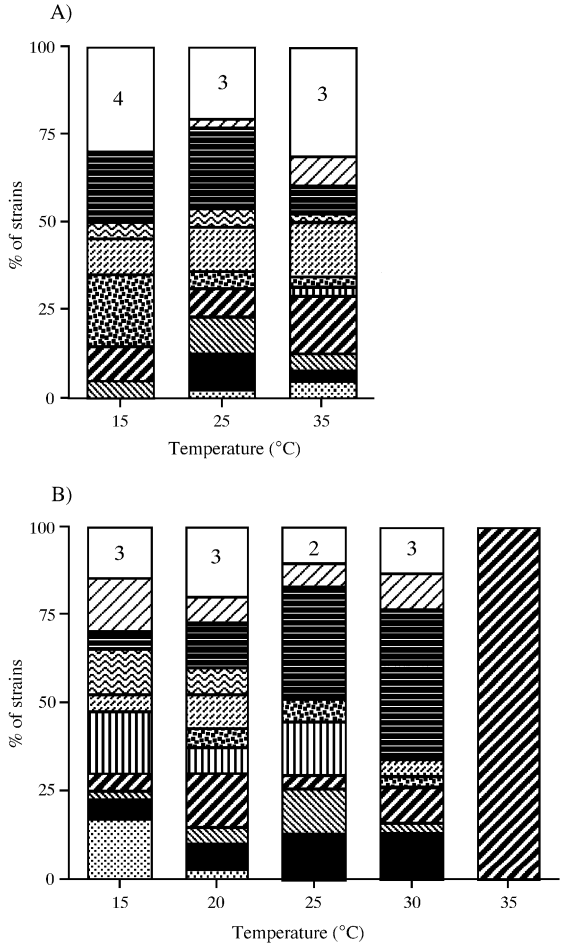

Fig. 2. Percentages of *S. cerevisiae* strains present at: (A) mid fermentation and (B) the end of fermentation. ⊡ P01, ■ P04, ▨ P06, ◥ P07, ◫ P08, ⊡ P12, ▨ P21, ▨ P25, ▬ P29, ▨ P33, □ others (the numbers on top of the bars represent the numbers of different minoritary strains, < 10%).

especially abundant in the middle phases clearly disappeared at the end of fermentation.

For the most usual vinification temperatures (Fig. 3), the results were grouped as high temperatures (25–35 °C) and low temperatures (15–20 °C). High temperatures are common for the production of red wines, which are generally fermented without cooling, while white wines are generally fermented at lower, controlled temperatures. From this, the strains could be chosen to make red or white wines according to their best performing temperatures. In our study, there were two main strains for the red wine process (P07 and

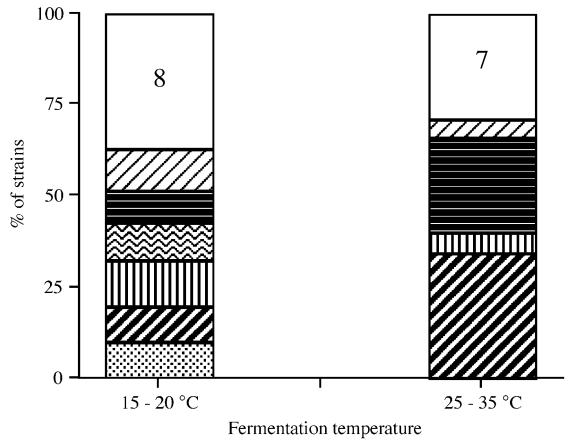

Fig. 3. Percentages of *S. cerevisiae* strains grouped for "vinification" temperatures. ⊡ P01, ◥ P07, ◫ P08, ⊠ P25, ▬ P29, ▨ P33, □ others (the numbers on top of the bars represent the numbers of different minoritary strains, < 10%).

P29). Although P07 is the only survivor strain at 35 °C, P29 is probably the most suitable strain for the temperatures that are normally used to produce red wines (25–30 °C), since 35 °C may be too high for winemaking. For white wines, there was no dominant strain; six strains had similar percentages at the end of fermentation.

3.3. Effect of temperature on analytical parameters

Chemical analyses were performed on the resulting wines (Table 2), all of which had residual sugars below 2 g l^{-1}. As expected, ethanol yield decreased as the temperatures increased. Acetic acid and, in particular, glycerol increased as the temperature increased. Acetaldehyde had maximal concentration when the fermentation was performed at 20 °C, and minimal concentration at 35 °C. The sum of all the secondary products (glycerol, acetic and succinic acids and acetaldehyde) increased as fermentation temperatures increased. In fact, these results may indicate two shifts in the yield of fermentation products. Although the differences between the yield at 15–20 °C and the yield at 25–30 °C may be considered unimportant (3 g l^{-1}), if we also take into account the loss of CO_2, there is a considerable reduction. On the other hand, the changes between 30 and 35 °C are dramatic.

4. Discussion

Few studies have analysed how temperature affects the population dynamics of *Saccharomyces* strains during alcoholic fermentation (Epifanio et al., 1999). Nearly all focused on different species of yeasts (Sharf and Margalith, 1983; Heard and Fleet, 1988; Mateo et al., 1991).

As expected, the growth in yeast varied according to temperature. The usual growth curve, with a series of short-lag, exponential, stationary and decline phases, was observed at 25 and 30 °C, whereas at 35 °C a high amount of yeast died. This high yeast mortality may have induced a slower final of fermentation and can produce stuck fermentations with higher sugar contents. In fact, these results agree with

Table 2
Chemical analyses of wines (end products)

Concentration (g l^{-1})	15 °C	20 °C	25 °C	30 °C	35 °C
Ethanol	93.60 ± 0.56[a]	93.04 ± 0.88	90.00 ± 0.56	89.60 ± 0.00	79.52 ± 1.84
Glycerol	6.05 ± 0.11	6.59 ± 0.07	6.91 ± 0.11	7.18 ± 0.02	7.38 ± 0.08
Acetaldehyde	0.05 ± 0.00	0.09 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.02 ± 0.00
Succinic acid	0.74 ± 0.06	0.89 ± 0.04	0.77 ± 0.06	0.92 ± 0.08	0.70 ± 0.03
Acetic acid	0.08 ± 0.01	0.13 ± 0.01	0.14 ± 0.00	0.13 ± 0.01	0.22 ± 0.04
Sum of products	100.52	100.74	97.86	97.87	87.84
CO_2	89.53	88.99	86.08	85.70	76.06
Products + CO_2	190.05	189.73	183.94	183.57	163.90
Ethanol yield[b]	47.51	47.23	45.68	45.48	40.36

[a] Mean ± SD.
[b] Ethanol produced (g l^{-1}) × 100/initial sugars (g l^{-1}).

previous reports that yeast viability decreases as the temperature increases (Ough, 1966; Nagodawithana et al., 1974; Casey et al., 1984). This decrease was thought to be due to a greater accumulation of intracellular ethanol at higher temperatures, which would produce cell toxicity (Nagodawithana et al., 1974) and would alter the structure of the membrane, decreasing its functionality (Lucero et al., 2000). However, at low temperatures there was no decline phase, so the stationary phase lasted until the end of fermentation.

Changing the temperatures clearly improved the development of some *Saccharomyces* strains. Some strains were predominant at low temperatures but others predominated at high ones. Our results agree with those of Fleet and Heard (1993) who reported that temperature affected the development of the indigenous yeast strains, and suggested that different strains were better or less suited for different temperatures (ranging from 10 to 30 °C). For example, when *Saccharomyces* and non-*Saccharomyces* were growing in the same medium, the latter could even dominate the fermentation at low temperatures (Sharf and Margalith, 1983; Heard and Fleet, 1988). However, as we took a semiquantitative approach in all these ecological studies (i.e. we analysed only a small percentage of all the population), these results do not mean that only those strains that are found in the population analysis can ferment at a given temperature. In fact, some of these strains were tested in pure culture at different temperatures and they fermented correctly. Another interesting result was that some strains were ubiquitous at all temperatures, which means that they were competitive in a broader temperature range than the others. However, the most important data for real winemaking is probably the ability of some strains to finish the fermentation, since any delay would allow undesirable compounds to appear.

It is well known that fermentation at 35 °C is very restrictive and that this fermentation probably ended because only one strain survived this high temperature. In our laboratory and experimental cellar, we had stuck fermentations at 35 °C that still had a high concentration of sugar, and this also happened in commercial cellars. The effect of higher temperatures is a premature end of fermentation, which means that fermentation is incomplete and the ethanol concentration is low (Larue et al., 1980). We might therefore assume that, at these restrictive temperatures, almost all the fermentations would proceed with difficulty and that there would be a higher probability of stuck fermentations, unless a resistant strain appeared, as in our case.

The percentage of the different *Saccharomyces* strains changed considerably during fermentation. This may be related to their sensitivity to ethanol toxicity; some strains always disappeared when the concentration of ethanol in the medium was high. Also, the ethanol tolerance of some yeast species depends on the temperature (Casey and Ingledew, 1986; D'Amore and Stewart, 1987; Gao and Fleet, 1988), and this could be the case of some *Saccharomyces* strains. This may explain why the presence of some strains decreased at some temperatures, but were able to finish the fermentation at other temperatures.

The resistance of a strain to specific temperature and ethanol concentration may be related to the natural conditions of their isolation area. In the AOC Priorat, red wines with a high alcohol content predominate, and fermentations are normally done at 25–30 °C. So, at lower temperatures, which are more usual for white wines, there were no "preferred" strains. Mixed innocula could be used to ensure fermentation at high sugar content and low temperatures.

However, temperature affected not only the fermentation kinetics (rate and length of fermentation) but also the yeast metabolism, which determined the chemical composition of the wine. As expected, the final concentration of alcohol decreased as the temperature increased, which has been related to a drop in the ethanol yield and a reduced use of substrate (Casey and Ingledew, 1986). In our experiments, the substrate was completely used, and although losses due to evaporation at high temperatures are expected, the main cause of this reduction is probably the increase of products of other metabolic pathways such as glycerol, acetic acid, etc. However, these secondary products could not completely explain why ethanol yield dropped so much as the temperature increased. We have not studied all the possible secondary products, yet the concentrations of those we have not studied are negligible though some of them could be relevant for tasting (i.e. esters, higher alcohols, etc.). Glycerol and succinic acid are the main secondary products in alcoholic fermentations led by *S. cerevi-*

siae. Although the total concentration of secondary products increased, the concentration of acetaldehyde decreased as the temperature increased, contrary to what had previously been reported (Ribéreau-Gayon et al., 1975). The difference in ethanol yield at different temperatures may also be related to biomass production, but at 35 °C the difference is too great for it to be explained in this way. So, adjustment of yeast metabolism to extreme conditions should account for this difference.

The temperature of fermentation can clearly affect the development of the different *Saccharomyces* strains. As a consequence, wine quality and characteristics are strongly affected by the *Saccharomyces* strain population dynamics and their endurance to different temperatures can be a criterion for indigenous microbiota selection for further purposes such as fermentation starters. The yield of ethanol and other fermentation by-products are also related to temperature during fermentation. So temperature of fermentation could also be used in winemaking to control some of the chemical characteristics of the wines such as glycerol or ethanol concentrations.

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