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Fermentative stress adaptation of hybrids within the *Saccharomyces* sensu stricto complex

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Abstract

Along the fermentation process yeasts are affected by a succession of stress conditions that affect their viability and fermentation efficiency. Among the stress conditions the most relevant are high sugar concentration and low pH in musts, temperature and, as fermentation progresses, ethanol accumulation.

Nowadays, due to the demanding nature of modern winemaking practices and sophisticated wine markets, there is an ever-growing search for particular wine yeast strains possessing a wide range of optimized, improved or novel enological characteristics. Traditionally, the species *S. cerevisiae* and *S. bayanus* within the *Saccharomyces* sensu stricto species are considered some of the most important yeast species involved in fermentation processes. However, in the last years, hybrid strains between the species *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii* have been described as yeasts conducting the alcoholic fermentations and some of them are commercially available.

Our results indicate that yeasts in the *Saccharomyces* sensu stricto complex were not affected by low pH or high glucose content in the media; however temperature and ethanol concentration variables appreciably affected their growth. The strains pertaining to *S. cerevisiae* were able to tolerate high temperature stress, whereas strains within *S. bayanus* and *S. kudriavzevii* were better adapted to growth at lower temperatures. Regarding to alcohol tolerance, *S. cerevisiae* is tolerating alcohol better than *S. bayanus* or *S. kudriavzevii*. Surprisingly, the natural hybrids between these species have adapted to growth under ethanol and temperature stress by inheriting competitive traits from one or another parental species.

These results open new perspectives in the construction of new hybrid strains with biotechnological interest, as the characteristics of the parents may result in interesting combinations in the hybrids.

Keywords: *Saccharomyces* sensu stricto; *Saccharomyces* hybrids; Fermentation stress; Domestication

1. Introduction

Fermentation has been used for several thousands of years as an effective and low-cost resource to preserve the quality and safety of foods. Among the fermenting microorganisms, yeasts are the most important group of microorganisms that have been used in production of alcoholic beverages such as wine and beer (Romano et al., 2006).

The *Saccharomyces* sensu stricto species complex contains some of the most important species involved in the fermentation processes. The species *S. cerevisiae* is considered the agent of wine, bread, ale beer, and sake fermentations. The species *S. bayanus* or *S. bayanus* var. *bayanus* (Naumov, 2000) is involved in lager beer fermentation, whereas *S. uvarum* (Nguyen and Gaillardin, 2005; Pulvirenti et al., 2000) or *S. bayanus* var. *uvarum* (Naumov, 2000) has been isolated from wine and cider fermentations (Naumov et al., 2000a, 2001; 2002). Other species in the sensu stricto group, namely *S. paradoxus*, has been described as the main yeast in Croatian wines (Redzepovic et al., 2002). The other three species in the sensu stricto complex, *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* have been found in natural environments (Naumov et al., 2000b) and are never present in fermentative environments.

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Hybrid strains between the species in the *Saccharomyces* sensu stricto complex have also been described as yeasts involved in alcoholic fermentations. In 1998, Masneuf et al., described a hybrid yeast strain possessing nuclear genomes from both a *S. cerevisiae* and a *S. bayanus*-like yeast and, since then, several wine and beer yeasts have been described as hybrids of these two species (Naumov et al., 2000a; Casagerola et al., 2001; de Barros Lopez et al., 2002). Recently, Rainieri et al. (2006) have identified up to three different hybrid types in lager brewing yeasts, namely, *S. cerevisiae*×*S. bayanus* (*S. pastorianus*), *S. bayanus*×*S. uvarum* and a triple hybrid by *S. cerevisiae*×*S. bayanus*×*S. uvarum*. Moreover, Gonzalez et al. (2006) have described wine yeasts as hybrids between *S. cerevisiae* and *S. kudriavzevii*, the latter being a yeast species which isolates have been found growing on decayed leaves in Japan.

The *Saccharomyces* yeasts that are isolated for industrial purposes are selected for their special characteristics. Selective pressures always favour the yeasts with the most efficient fermentative catabolism, particularly strains of *S. cerevisiae* and strains of closely related species such as *S. bayanus* (Pretorius, 2000). Similarly, several hybrids in the *Saccharomyces* sensu stricto complex have been selected and commercialised like the yeasts conducting the alcoholic fermentation; therefore, the hybrid strains must be well adapted to the stress conditions occurring during the alcoholic fermentation.

At the beginning of fermentation, yeast cells are affected by osmotic stress due to the high sugar concentration, as well as low pH; and as fermentation progresses other stress conditions as ethanol accumulation become relevant. Moreover, depending on the fermentation process, other factors such as temperature can be considered as stress factors (Cardona et al., 2007).

Several authors have reported on variation of yeast growth with sugar concentration (Carrasco et al., 2001; Zuzuarregui and del Olmo, 2004). The concentration of fermentable sugars (glucose and fructose) in grape musts varies between 125 and 250 g/L (Fleet and Heard, 1993) thus, it is likely that the initial concentration of sugar in grape must will selectively influence the species and strains of yeast responsible for the fermentation. Different studies (Lafon-Lafourcade, 1983; Monk and Cowley, 1984) indicated that growth rate and completeness of fermentation by *S. cerevisiae* were decreased as the initial concentration of sugar in grape must increases above 200 g/L.

Grape must acidity is also considered as important for the survival and growth of yeasts. Fleet and Heard (1993) observed that growth rate and must fermentation by *S. cerevisiae* were decreased as the pH was decreased from 3.5 to 3.0. On the other hand, a recent study of pH influence on growth of *S. bayanus* var. *uvarum* showed that pH does not have a significant influence on growth (Serra et al., 2005).

The temperature at which the alcoholic fermentation is conducted affects the yeast growth and duration of fermentation (Fleet and Heard, 1993). The rate of yeast growth and alcoholic fermentation increases as the temperature increases, with maximum rates occurring at temperatures between 20 and 25 °C (Amerine et al., 1980). On the other hand, Serra et al. (2005) reported that below the optimal growth temperature yeast growth rate increases as the temperature increases, but after the optimal temperature yeast growth rate decreases very fast due to damage of cellular membranes and enzymes denaturation. In recent years, there has been a preference by winemakers to ferment white wines at temperatures in than range of 10 to 15 °C to minimize the loss of aromatic volatiles, and red wine fermentations performed at higher temperatures (18–30 °C) to enhance extraction of anthocyanin pigments. Similarly, beer fermentation occurs at different temperatures depending on the type of beer; ale beer fermentation occurs between 16 and 23 °C and lager beer fermentation occurs between 8 and 15 °C (Hammond, 2000).

Grape must is typically prepared from fully matured grapes, making for high flavour intensity, but also a considerable concentration of sugar. This much sugar invariably leads to the production of wines with high levels of ethanol, sometimes reaching concentrations above 15% (v/v) (de Barros Lopes et al., 2003). In spontaneous fermentations there is a progressive growth pattern of indigenous yeasts in the middle stages when the ethanol rises to 3–4% (Fleet and Heard 1993). The latter stages of natural wine fermentations are invariably dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae*. Similarly, in beer fermentation ethanol content ranges between 4 and 9% although high gravity beer contains at least 8% of ethanol (Campbell, 2003). The analysis of resistance to ethanol of 14 wine strains by Zuzuarregui and del Olmo (2004) showed that ethanol affected growth of some strains severely.

The aim of this study was to investigate the tolerance to stress conditions occurring during wine fermentation of different wine and beer yeasts pertaining to the *Saccharomyces* sensu stricto complex and the hybrids between them. Besides, we tried to resolve the question of which parental species bequeath the hybrids with the abilities to deal with the different stress tolerances.

2. Materials and methods

2.1. Strains

Table 1 lists the strains used in this study. Species name, strain number in different culture collections and isolation source are indicated.

2.2. Medium and inoculum's preparation

Yeast cells were grown in GPY (peptone 0.5%, yeast extract 0.5%, glucose 2%). Medium was solidified by adding 1.5% agar. 48 h fresh yeast cultures on GPY agar were used to inoculate 5 mL of fresh GPY and shaken overnight at 30 °C. The next day the cultures were adjusted to an absorbance at 655 nm of 0.3 by dilution with fresh GPY. After shaking at 30 °C for another 4 h the cells were diluted in sterile water to an A655 of 0.3 and 5 μL of a 5-fold dilution series in water were spotted onto all stress media. All plates were incubated aerobically and checked 1 to 5 consecutive days for colony development.

Table 1
List of strains used in this study

Species	Strains	Origin	pH			Glucose (g l^{-1})			Temperature (°C)				Ethanol (%)			
			2,8	3,0	3,2	200	250	300	10	16	30	37	5	10	12	15
S. cerevisiae	CECT 1942^T = CBS 1171^T	Ale beer, The Netherlands	6	6	6	6	6	6	3	5	6	0	4	0	0	0
	CECT 1384=CBS 1636	Spoiled beer, Ireland	6	6	6	6	6	6	3	6	6	6	6	6	6	5
	CECT 1387 = CBS 7372	Spoiled draught beer, UK	6	6	6	6	6	6	4	6	6	6	6	6	6	6
	CECT 11001 = NCYC 2340	Lager beer, Belgium	6	6	6	6	6	6	3	6	3	0	3	0	0	0
	CECT 11036 = CBS 381	Spoiled beer, Japan	6	6	6	6	6	6	4	6	6	6	6	6	6	5
	Lalvin T73, Lallemand	wine, Alicante, Spain	6	6	6	6	6	6	3	6	6	6	6	6	6	6
	Uvaferm CEG, Danstar	Wine, Eppernay, France	6	6	6	6	6	6	3	6	6	6	6	6	6	5
	Fermiblanc Arom DSM-Gist Broc.	Wine, Cognac, France	6	6	6	6	6	6	3	6	6	6	6	6	5	2
	Fermicru Primeur DSM-Gist Broc.	Wine, Beaujolais, France	6	6	6	6	6	6	3	6	6	6	6	6	6	5
	UCLM S-377, Springer Oenologie	wine, Spain	6	6	6	6	6	6	3	6	6	6	6	6	6	2
S. bayanus	CECT 11035^T = CBS 380^T	Turbid beer	6	6	6	6	6	6	5	6	6	0	6	6	2	0
	CECT 1991 = DSM 70411	Turbid beer	6	6	6	6	6	6	6	6	6	0	6	6	5	3
	CECT 11185 = NCYC 114	Beer contaminant, UK	5	6	6	5	4	4	6	6	6	0	6	4	0	0
	CECT 12627	Wine, Valladolid, Spain	6	6	6	6	6	6	6	6	6	0	6	6	2	0
	CECT 12629	Must, Zaragoza, Spain	6	6	6	6	6	6	6	6	6	0	6	6	2	0
	CECT 12638	Must, Cadiz, Spain	6	6	6	6	6	6	6	6	6	0	6	6	4	0
	CECT 12669	Grapes, La Rioja, Spain	6	6	6	6	6	6	6	6	6	0	6	6	3	0
	CECT 12930	Wine, Spain	6	6	6	6	6	6	6	6	6	0	6	6	5	5
S. paradoxus	54	Grapes, Croatia	6	6	6	6	6	6	4	6	6	6	6	6	6	0
	11157 = CBS 2908	Soil, South Africa	6	6	6	6	6	6	4	6	6	6	6	6	6	0
	120 M	Pulque, Mexico	6	6	6	6	6	6	4	5	6	6	6	6	6	5
	CECT 1939^T = CBS 432 ^{T}	Tree sap, Russia	6	6	6	6	6	6	4	6	6	6	6	6	6	0
S. kudriavzevii	IFO 1802^T	Decayed leaf, Japan	5	6	6	6	6	5	6	6	6	0	4	0	0	0
S. cariocanus	CBS 8841^T	*Drosophila* sp., Brazil	6	6	6	6	6	6	3	6	6	6	6	6	2	0
S. mikatae	CBS 8839^T	Soil, Japan	6	6	6	6	6	6	6	6	6	6	6	6	0	0
Hybrid strains																
S.c.×S.b.	Lalvin S6U, Lallemand	Wine, Italy	6	6	6	6	6	6	3	6	6	6	6	6	6	4
S.c.×S.b.	CECT 11000 = BRAS 12	Ale beer	6	6	6	6	6	6	3	6	6	6	6	6	6	6
S.c.×S.b.	CECT 11037 = CBS 1513	Lager beer	6	6	6	6	6	6	5	6	6	0	6	5	2	2
S.c.×S.k.	Lalvin W 27, Lallemand	Wine, Wädenswill, Switzerland	6	6	6	6	6	5	5	6	6	6	6	6	6	0
S.c.×S.k.	Lalvin W 46, Lallemand	Wine, Wädenswill, Switzerland	6	6	6	6	6	6	5	6	6	6	6	6	6	1
S.c.×S.k.	SPG 16-91	Wine, Wädenswill, Switzerland	6	6	6	6	6	6	5	6	6	6	6	6	6	0
S.c.×S.k.	SPG 441	Wine, Wädenswill, Switzerland	6	6	6	6	6	6	4	6	6	6	6	6	6	0
S.c.×S.k.	CECT 1388=NCYC 447	Draught beer	5	6	6	6	6	6	3	6	6	6	6	6	6	5
S.c.×S.k.	CECT 1990=DSM 1848	Draught beer, UK	6	6	6	6	6	6	3	6	6	6	6	6	6	4
S.c.×S.b.×S.k.	CBS 2834	Wine, Wädenswill, Switzerland	6	6	6	6	6	6	5	6	6	6	6	6	6	1
S.c.×S.b.×S.k.	CID 1	Cider, France	6	6	6	6	6	6	6	6	6	6	6	6	6	2

Tolerance to the different fermentation stress factors is indicated by numbers from 0 (absence of growth) to 6 (colony development at the sixth dilution).
Growth at low pH was observed at 24 h of incubation at 30 °C.
Growth at high glucose concentration was observed at 24 h of incubation at 30 °C.
Growth at 10 °C was recorded after 6 days of incubation; growth at 16 °C was recorded after 3 days of incubation; growth at 30 °C and 37 °C was recorded at 24 h of incubation.
Growth at 5%, 10%, 12% and 15% of ethanol was observed at 48 h of incubation at 30 °C.

2.3. Analysis of yeast tolerance to several stress conditions

Four stress factors considered as key features in wine fermentation were studied: pH, glucose, temperature and ethanol. For each stress factor, several conditions were tested: pH (2.8, 3.0 and 3.2), glucose (200 g/L, 250 g/L and 300 g/L glucose), temperature (10 °C, 16 °C, 30 °C and 37 °C), ethanol (5%, 10%, 12% and 15% ethanol), and combined stress (250 g/L glucose and pH 2.8). Stress media were prepared using GPYA (peptone 0.5%, yeast extract 0.5%, glucose 2%, agar 1.5%) supplemented with the adequate amounts of ethanol and glucose. pH stress was checked on GPYA adjusted to the desired pH, and temperature stress was performed on GPYA medium incubated at the above mentioned temperatures. Except for temperature stress, all plates were incubated at 30 °C. In all cases, a positive growth control was carried out with yeast cells that were not exposed to any stress condition and incubated at 30 °C until colonies appeared in all dilutions (24 h). To detect variations in our results due to experimental errors, the tests were repeated at least three times. However, the experimental design and the method studying growth do not permit proper statistical evaluation or error calculation.

3. Results and discussion

The primary role of fermenting yeast in the production of alcoholic beverages is to catalyze the rapid, complete and efficient conversion of grape sugar to ethanol, carbon dioxide and other minor metabolites without the development of off-flavours (Pretorius, 2000). As in other industrial processes,

yeast cells never find a physiologically optimal environment during their use in wine production. In fact, they are exposed to a continuous mix of several stress conditions (Attfield, 1997; Bauer and Pretorius, 2000; Cardona et al., 2007).

In the present study we have tested the tolerance to four stress conditions, namely, pH, high glucose concentration, temperature and ethanol, of strains in the species within the *Saccharomyces* sensu stricto complex and hybrids between them. Table 1 shows the results strain growth of the strains at different stress conditions considered. Colony development at the sixth dilution is indicated with 6, colony development at the fifth dilution is indicated with 5 and so on for the rest of dilutions. Zero means that colony growth was not observed.

3.1. Growth under pH stress conditions

The majority of yeasts can grow at pH as low as 3.5 (Yarrow, 1998), however, pH in grape musts ranges between 2.75 and 4.2 (Fleet and Heard, 1993), whereas beer fermentation starts at pH 5.0–5.2 and falls to a final pH of 3.8–4.0 (Campbell, 2003).

Tolerance to low pH was observed by colony growth on plates at pH 2.8, 3.0 and 3.2 after 24 h of incubation at 30 °C.

In general, the majority of strains were able to develop colonies in all dilutions at the three pH conditions. Our results showed similar pH tolerance for strains within all species, with independence of their isolation source, wine or beer, or natural environments. Only the type strain of *S. kudriavzevii* and the strain CECT 11185 within *S. bayanus* were slightly affected by pH 2.8 and were able to growth up to the fifth dilution only. Our results indicate that ability to growth at low pH could be considered common to all species and hybrids within the group of *Saccharomyces* sensu stricto. Consequently, grape must or beer with low pH should not be considered a stress factor for yeasts in alcoholic fermentations. Other authors have obtained similar conclusions; Charoenchai et al. (1998) observed that variation of pH did not affect the growth rate of natural grape species or *S. cerevisiae*, and Serra et al. (2005) also reported that pH does not have a significant influence on *Saccharomyces* growth.

3.2. Growth under osmophilic stress conditions

The majority of yeasts culture media contain 20 g/L of sugar, although many yeast species are able to growth in glucose concentrations up to 40% (Yarrow, 1998). Sugar content in grape musts for table wine varies from 140 to 260 g/l (Amerine et al., 1980). In the case of beer, sugar content in an all-malt infusion mash produces wort with approximate glucose and fructose content of 9 g/L and maltose content about 40 g/L (Nevoigt et al., 2002; Campbell, 2003).

Colony growth on plates at 200 g/L, 250 g/L and 300 g/L was observed after 24 h of incubation at 30 °C.

In general, the majority of strains were able to develop colonies in all dilutions at the three glucose concentrations after 24 h of incubation at 30 °C. Even the type strain of *S. paradoxus*, isolated from tree sap in Russia, was able of growing at 300 g/L of glucose, although tree saps have very low glucose content (around 1 g/L) (Escher et al., 2004). Also, the type strain of *S. cariocanus*, isolated from soil, showed growth at all sugar concentrations. The type strain of *S. kudriavzevii*, isolated from decayed leaves, was slightly affected by 300 g/L of glucose in the culture media. The strain CECT 11185 within *S. bayanus*, isolated from beer, was clearly affected by high glucose content in the culture media. Our results indicate that, in general, species within the group of *Saccharomyces* sensu stricto are able to growth at sugar concentrations in grape must. Additional reports on winemaking of must from dried grapes showed that yeast growth is though severely affected at 40° Brix (approximately 400 g/l glucose) (Caridi et al., 1999).

3.3. Growth under pH and glucose stress conditions

Yeast growth was not affected by high glucose concentration and lower pH separately. However, these stress conditions occur simultaneously in grape must at the beginning of the fermentation; consequently, yeast tolerance to both stress conditions was simultaneously tested on agar plates at pH 2.8 and 250 g/L of glucose, which are normal conditions in wine musts. In general, the majority of yeast strains were able to develop colonies at all dilutions after 24 h of incubation at 30 °C (data now shown) and no differences were observed between these results and the ones obtained at pH 2.8. Consequently, the ability to growth at both low pH and high sugar concentration could be considered common for the species within the group of *Saccharomyces* sensu stricto, and therefore these parameters do not have a significant influence on yeast growth, even when both are simultaneously considered.

Other combined stress conditions happening in alcoholic fermentations such as temperature and ethanol were not studied because these parameters produced the highest variations in growth and, therefore, the evaluation of the accurate influence of each parameter in the combined results might be very difficult without complementary studies involving transcription analysis of genes involved in tolerance to one and both conditions simultaneously.

3.4. Growth under temperature stress conditions

Colony growth on GPY plates incubated at 10 °C, 16 °C, 30 °C and 37 °C was used to test yeast tolerance to low and high temperatures. Growth at 30 °C and 37 °C was recorded after 24 h of incubation. Growth at 16 °C was registered after 3 days of incubation. At 10 °C growth was observed at 6 days of incubation.

The majority of yeasts are incubated between 20 °C and 25 °C because most of them are mesophilic; however, temperatures between 4 °C and 15 °C are essential for psychrophilic taxa. Higher temperatures 30 °C and 37 °C are often required for yeasts that are strictly associated with warm-blooded sources (Yarrow, 1998). Most of the yeast strains of our study were able to develop colonies at 16 °C and 30 °C in all tested dilutions.

At 37 °C, the majority of strains within *S. cerevisiae* were able to grow except for the type strain of *S. cerevisiae*,

isolated from ale beer and the strain CECT 11001, isolated from lager beer, which was growing poorly at 30 °C and was not able to grow at 37 °C. On the contrary, two *S. cerevisiae* strains isolated from spoiled beer were able to grow at 37 °C. The remaining *S. cerevisiae* strains isolated from wine were able to grow at 37 °C. All strains within *S. paradoxus* were able to grow at 37 °C independently of their isolation source or geographical origin.

None of the strains within the species *S. bayanus* was able to grow at 37 °C. The species *S. bayanus* is currently divided into two varieties: var. *bayanus* and var. *uvarum*. Serra et al. (2005) reported that temperature was the factor which had the main influence on the yeast growth, and found that a strain of *S. bayanus* var. *uvarum* (*S. uvarum*) was less sensitive to temperature falls than a strain of *S. cerevisiae*, which is in agreement with the classification of *S. uvarum* as cryotolerant yeast in winemaking (Kishimoto and Goto, 1995; Naumov, 1996). On the other hand, *S. bayanus* var. *bayanus* (*S. bayanus*) is a hybrid from *S. uvarum* and from the same non-*S. cerevisiae* parental strain as that of lager brewing strains (Casagerola et al., 2001; Tamai et al., 1998). Modern beer production was initiated in temperate countries of Central Europe (Germany, Czech Republic and Denmark) where rather seldom fermentation temperatures raise up to 37 °C. Our results suggest that low fermentation temperatures for beer production might have favoured the cryotolerant trait in beer strains, and as a consequence *S. bayanus* beer strains and also *S. cerevisiae* ale and *S. pastorianus* lager beer strains are not able to grow at 37 °C. On the other hand, beer contaminants are able to grow at 37 °C, therefore they can contaminate not only at low fermenting temperatures but also at higher temperatures during transport or storing of beer.

Finally, the type strains of *S. cariocanus* and *S. mikatae* were both able to grow at 37 °C, however the type strain of *S. kudriavzevii* was not able to grow at 37 °C.

All hybrids between *S. cerevisiae*×*S. bayanus* (*S. pastorianus*) and *S. cerevisiae*×*S. kudriavzevii* were able to grow at 37 °C with the exception of CECT 11037 isolated from lager beer. Even CID1, the triple hybrid between *S. cerevisiae*×*S. bayanus*×*S. kudriavzevii*, showed colony growth in the first dilution at 37 °C.

These results indicate that wine yeast hybrids between *S. cerevisiae* and *S. bayanus* or *S. kudriavzevii* might have inherited the capability to growth at 37 °C from a wine strain of the *S. cerevisiae* parental because *S. bayanus* and *S. kudriavzevii* are not able to grow at 37 °C.

Nowadays, wine fermentation is done at controlled temperatures; however, grape must fermentation was traditionally done at the end of summer, during the warm days of September in the Mediterranean countries at average temperatures between 25 to 30 °C and, in central Europe countries between 20 to 25 °C. Additionally, anaerobic fermentation generates waste energy in the form of heat and yeasts must be able to maintain viability and ferment at temperatures approaching 38 °C (Boulton et al., 1996). Probably, after natural hybridization occurred, natural selection and winemakers have favoured keeping the ability to grow at 37 °C in wine yeasts.

Similarly, the beer hybrids are also able to grow at 37 °C. However, the *S. cerevisiae* strains conducting ale and *S. pastorianus* conducting lager beer fermentations are not able to grow at 37 °C, for that reason, our results suggest that a *S. cerevisiae* strain of possible wine origin might have been the parental strain of the beer hybrids.

Most of the yeast strains were able to develop colonies in all dilutions at 10 °C and 16 °C. Colony growth at 16 °C was detected after 3 incubation days. Colony development at 10 °C was recorded after 6 incubation days (Fig. 1). We observed that strains within *S. bayanus* and *S. kudriavzevii* developed visible colonies in the sixth dilution after 6 incubation days. However, *S. cerevisiae* and *S. paradoxus* developed visible colonies in the sixth dilution after 10 incubation days (data not shown). Cryotolerance of *S. bayanus* strains was already observed by several authors (Kishimoto and Goto, 1995; Naumov, 1996; Giudici et al., 1998), and our results show that *S. kudriavzevii* is better adapted to grow at lower temperatures than *S. cerevisiae* and therefore could also be considered a cryotolerant strain.

All hybrids between *S. cerevisiae*×*S. bayanus* (*S. pastorianus*) and *S. cerevisiae*×*S. kudriavzevii* were able to grow at 10 °C better than any *S. cerevisiae* strain but not so well like the strains of *S. bayanus* or *S. kudriavzevii* (Fig. 1). These results indicate that wine yeast hybrids between *S. cerevisiae* and *S. bayanus* or *S. kudriavzevii* might have inherited the capability to growth at lower temperatures from the non-*S. cerevisiae* parental. Accordingly, wine hybrids might be also better adapted to low-temperature fermentations as they have inherited the cryotolerance from *S. bayanus* or *S. kudriavzevii*. Nowadays, technological advances in winemaking favour fermentations at controlled low temperatures to increase the fruity aromas from the grapes. Therefore wine yeast hybrids able to ferment in the lower range of temperatures could be of great biotechnological interest.

Research in this line has already been done for improving the fermentative capabilities of beer-fermenting yeasts (Sato et al., 2002). Ale beer-fermenting yeasts (top-yeasts) generally exhibit good fermentability at a temperature of about 20 °C, but do not function well at low-temperature as bottom-fermenting yeasts. Therefore, hybridization with *S. bayanus* is useful for improving the low-temperature fermentability of the top-fermenting yeast *S. cerevisiae* (ale beer). Additionally, the cryophilic

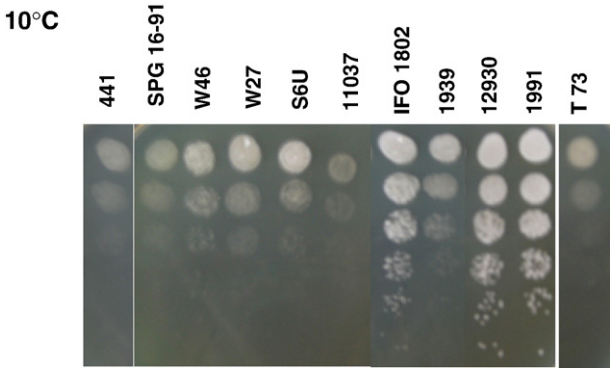

Fig. 1. Colony development at 10 °C after 6 incubation days.

performance of bottom-fermenting yeasts (lager beer) may mainly be due to *S. bayanus* (Sato et al., 2002).

3.5. Growth under ethanol stress conditions

Colony growth on GPY plates containing 5%, 10%, 12% and 15% ethanol was used to test tolerance to ethanol on agar plates. Colony growth was verified after 48 h of incubation at 30 °C.

The majority of the yeasts produce ethanol in low percentages around 3 to 4% (Yarrow, 1998) and, consequently, they are able to growth at low ethanol percentages. Yeasts found on the surface of grape skins and the indigenous microbiota associated with winery surfaces such as *Kloeckera*, *Hanseniaspora*, *Candida*, *Metschnikowia* and *Pichia* predominate in natural fermentations when the ethanol rises to 3–4%. The latter stages of wine fermentation, when ethanol level is around 15%, are dominated by alcohol-tolerant strains of *S. cerevisiae* (Pretorius, 2000). On the other hand, ethanol content in beer fermentation ranges between 4 and 9% although high gravity beer contains at least 8% of ethanol (Campbell, 2003).

The yeast strains investigated in our study were able to develop colonies in all dilutions on plates containing GPY media supplemented with low percentages of ethanol (up to 5%). All strains within the species *S. cerevisiae* were able of colony development on plates containing 10% of ethanol at the majority of dilutions, with the exception of the type strain of *S. cerevisiae* isolated from ale beer and CECT 11001 isolated from lager beer. All the strains within *S. bayanus* and *S. paradoxus* were able to grow on media containing 10% of ethanol. The type strain of *S. kudriavzevii* was not capable of growing at 10% of ethanol; however, the type strains of *S. cariocanus* and *S. mikatae* displayed growth in all tested dilutions. Finally, all hybrids between *S. cerevisiae*×*S. bayanus* and *S. cerevisiae*×*S. kudriavzevii*, and even the triple hybrids *S. cerevisiae*×*S. bayanus*×*S. kudriavzevii* were able of growing at 10% of ethanol.

At 12% of ethanol (Fig. 2), the strains within the species *S. cerevisiae* were able of colony development except for the strains that could not grow at 10%. Most of the strains within *S. bayanus* showed difficulties to grow at 12% of ethanol and, the strain CECT 11185, isolated from contaminated beer could not grow at any dilution. All *S. paradoxus* were able of colony development in all tested dilutions. The type strain of *S. mikatae* could not grow at 12% of ethanol; however, the type strain of *S. cariocanus* was able to grow at the second dilution. The majority of hybrids between *S. cerevisiae*×*S. bayanus* (*S. pastorianus*) and *S. cerevisiae*×*S. kudriavzevii* were able to grow at all tested dilutions in media containing 12% of ethanol. The only exception was the strain CECT 11037, isolated from lager beer, which presented difficulties to growth at the second dilution. These results indicate that wine yeast hybrids between *S. cerevisiae* and *S. bayanus* or *S. kudriavzevii* might have inherited the capability to growth at high ethanol percentages from a wine strain of the *S. cerevisiae* parental because *S. kudriavzevii* is not able to grow in media containing 10% of ethanol and the majority of strains in *S. bayanus* have difficulties to grow at 12% of ethanol. In the case of the beer

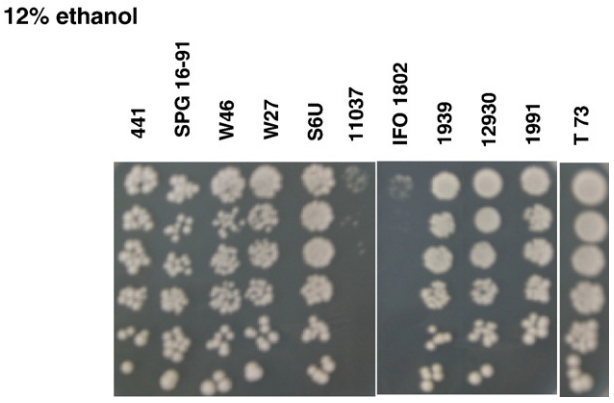

Fig. 2. Colony development at 12% of ethanol after 2 incubation days.

yeast hybrids, they were able to grow at 12% of ethanol better than the *S. cerevisiae* strains conducting ale and lager beer fermentations, for that reason, our results suggest that a wine strain or a beer contaminant strain of *S. cerevisiae* able to grow at high ethanol concentrations might have been the parental strain of the beer hybrids.

When the percentage of ethanol in the culture media increased up to 15% we observed strain specific behavior. Among the alcohol-tolerant species *S. cerevisiae*, most of them could grow up to the fifth or sixth dilution except for Fermiblanc Arom and UCLM S-377, which were able to grow up to the second dilution only. The majority of strains within *S. bayanus* were not able to grow but for the strains CECT 1991, isolated from turbid beer, and CECT 12930, isolated from wine. Among *S. paradoxus*, only the strain 120 M isolated from pulque was able to grow at 15% of ethanol. The type strain of *S. cariocanus* could not grow at 15% of ethanol.

Most of the hybrids showed reduced tolerance to grow at 15% of ethanol. The beer hybrids between *S. cerevisiae* and *S. bayanus* or *S. kudriavzevii* were able to grow in most of the dilutions. These results indicate that beer yeast hybrids might have inherited the capability to growth at high alcohol percentages the *S. cerevisiae* parental because the majority of strains pertaining to *S. bayanus* or *S. kudriavzevii* are not able to grow at high alcohol percentages. Among the wine strains only the hybrid of *S. cerevisiae* and *S. bayanus* isolated from Italian wine was able to grow up to the fourth dilution at 15%, whereas the hybrids isolated from Switzerland wines were not able to growth or were able to growth in the first dilution only.

As a general conclusion, wine hybrids showed better adaptation to growth at high percentages of ethanol (12% and 15%) than the *S. bayanus* or *S. kudriavzevii* parental species, therefore they must have inherited this ability from the *S. cerevisiae* parental.

Recent studies on enological characterization of hybrids demonstrated that wine yeasts natural hybrids are not only well adapted to fermentation conditions but, they produce higher amounts of aromatic compounds than the parental species (Gonzalez et al., 2007). Consequently, the construction of laboratory hybrids imitating the natural evolution process represents a promising method for genetic improvement of wine yeast. Moreover, selection of laboratory hybrid strains

with the abilities important for wine and beer making should take into account the different stress conditions occurring during alcoholic fermentation.

In this study we have used a simple platting method to test the ability of parental species of *Saccharomyces* and their hybrids to growth under different stress circumstances during wine fermentation. A similar platting approach has been used by several authors to test wine yeast viability following several stress treatments (Carrasco et al., 2001; Garay-Arroyo et al., 2004; Ogawa et al., 2000; Zuzuarregui and del Olmo, 2004) and measuring growth at different temperature conditions of *Saccharomyces cerevisiae* clinical strains (de Llanos et al., 2006).

The results obtained in our study reveal that hybrid strains are able to resist high glucose concentration and low pH in grape musts; they are well adapted to grow at low temperatures like the parental species *S. bayanus* and *S. kudriavzevii* and at high temperatures like *S. cerevisiae*, and they tolerate alcohol percentages as well as the alcohol-tolerant strains of *S. cerevisiae*. The stress conditions tested using this simple plating method must be considered an approximation to the true stress conditions during fermentation. However, wine fermentations conducted at different temperatures using *S. cerevisiae* and *S. kudriavzevii* parental and hybrid strains (Gonzalez et al., 2007) show similar conclusions than in our study, therefore we believe that this platting method is suitable for testing the yeasts ability to grow at independent stress conditions.

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