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Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations

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Abstract

Multistarter fermentations of *Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* together with *Saccharomyces cerevisiae* were studied. In grape musts with a high sugar content, mixed trials showed a fermentation behaviour and analytical profiles of wines comparable to or better than those exhibited by a pure culture of *S. cerevisiae*. Sequential trials of *T. delbrueckii* and *K. thermotolerans* revealed a sluggish fermentation, while those of *H. uvarum* exhibited an unacceptable increase in ethyl acetate content (175 ml l^{-1}). A low fermentation temperature (15 °C) of multistarter trials of *H. uvarum* resulted in a stuck fermentation that was not due to a deficiency of assimilable nitrogenous compounds since lower amounts of these compounds were used. Sequential fermentation carried out by *H. uvarum* at 15 °C confirmed the high production of ethyl acetate. The persistence and level of non-*Saccharomyces* yeasts during multistarter fermentations under stress conditions (high ethanol content and/or low temperature) can cause stuck fermentations.

Keywords: Wine; Fermentation; Analytical profile; *Hanseniaspora uvarum*; *Torulaspora delbrueckii*; *Kluyveromyces thermotolerans*

1. Introduction

The natural fermentation of grape must is usually started by low-alcohol-tolerant apiculate yeasts (*Kloeckera*/*Hanseniaspora*) that predominate the first stages of fermentation. After 3–4 days, they are replaced by elliptical yeasts (*Saccharomyces cerevisiae*) that continue and finish the fermentation process (Amerine et al., 1980; Martini, 1993). In addition, during the various stages of fermentation, it is possible to isolate other cultures belonging to other yeast genera, such as *Candida*, *Torulaspora*, *Kluyveromyces* and *Metschnikowia* (Fleet et al., 1984; Heard and Fleet, 1986; Pardo et al., 1989). Recently, there has been a re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking (Fleet and Heard, 1993; Ciani, 1997; Heard, 1999). Their presence and permanence throughout inoculated and non-inoculated fermentations are well documented (Heard and Fleet, 1985; Martinez et al., 1989), as well as their contributions to the analytical composition and the sensorial characteristics of wine (Romano et al., 1992; Schütz and Gafner; Lema et al., 1996; Egli et al., 1998). Several studies have indicated that the contribution of non-*Saccharomyces* yeasts leads to a more complex aroma and an improved wine quality (Romano et al., 1997; Egli et al., 1998; Ciani and Maccarelli, 1998; Henick-Kling et al., 1998). However, natural multistarter cultures remain uncontrolled processes and multistarter cultures need to be used under better-defined conditions.

For these reasons, several studies have evaluated the possibility of using controlled multistarter cultures to improve the quality of wines (Mora et al., 1990; Herraiz et al., 1990; Zironi et al., 1993; Ciani and Ferraro, 1996, 1998; Erten, 2002; Toro and Vazquez, 2002). In this context, our knowledge of the metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* wine yeasts under winemaking conditions needs to be improved. Recently, the effect of oxygen on the survival of non-*Saccharomyces* yeasts (Hansen et al., 2001) and esters formation (Rojas et al., 2003) during mixed cultures has been investigated.

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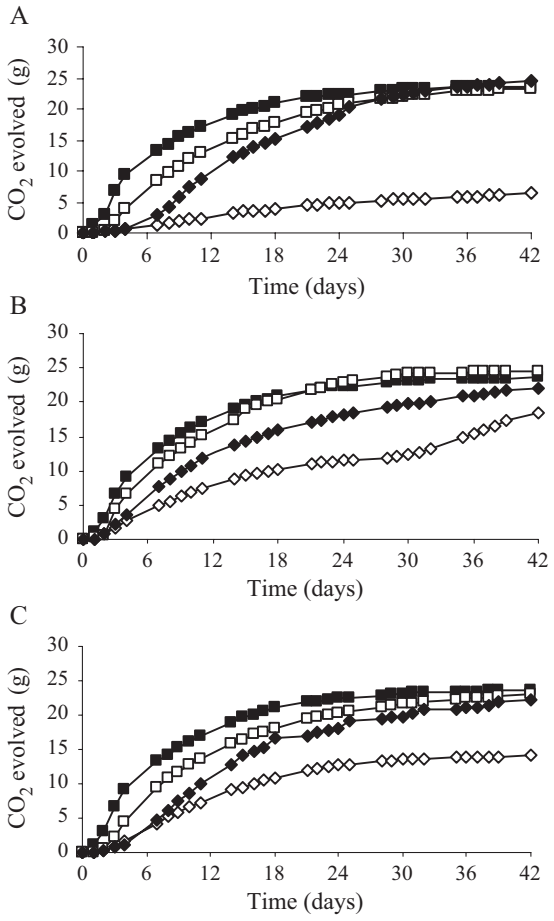

Fig. 1. Fermentations at 20 °C. ■, pure *S. cerevisiae* culture. (A) □, mixed *H. uvarum*/*S. cerevisiae* culture. ◆, sequential *H. uvarum*/*S. cerevisiae* culture. ◇, pure *H. uvarum* culture. (B) □, mixed *K. thermotolerans*/*S. cerevisiae* culture. ◆, sequential *K. Thermotolerans*/*S. cerevisiae* culture. ◇, pure *K. thermotolerans* culture. (C) □, mixed *T. delbrueckii*/*S. cerevisiae* culture. ◆, sequential *T. delbrueckii*/*S. cerevisiae* culture. ◇, pure *T. delbrueckii* culture.

In the present study, we report on the fermentation behaviour and the metabolic interactions of mixed and sequential cultures of *S. cerevisiae* and three potential non-*Saccharomyces* wine yeast starter species, *Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans*.

2. Materials and methods

2.1. Microorganisms and media

S. cerevisiae DBVPG 1014, *H. uvarum* DBVPG 3037 and *T. delbrueckii* DBVPG 6168 were from the Industrial Yeast Collection of the University of Perugia (DBVPG), and *K. thermotolerans* (known as M8 (Ciani and Pepe, 2001) was isolated from the winery environment. All of the strains were sub-cultured at 6-month intervals on malt agar medium and maintained at 6 °C.

The grape must used in the fermentation tests (cultivar pinot grigio, 18.5% (w/v) sugar, pH 3.10, 149.1 mg l^{-1} ammonia, 139.4 mg l^{-1} primary amino acids) was brought to a sugar content of 27% (w/v) by the addition of sucrose, and pasteurized at 90 °C for 15 min.

The total yeast populations were prepared in YPD medium (10 g l^{-1} Bacto yeast extract, 10 g l^{-1} Bacto peptone, 20 g l^{-1} glucose). Lysine agar (Unipath, Basingstoke Hampshire, England), which is unable to support the growth of *S. cerevisiae* (Lin, 1975), was used to assess the non-*Saccharomyces* yeasts. In order to check the *S. cerevisiae* populations, the following selective medium containing SO_2 was used: YPD medium buffered at pH 3.0 (citrate–phosphate buffer) was used after sterilization and following the addition of a concentrated solution of SO_2 (as $K_2O_5S_2$) to achieve a final concentration in the medium of 50 mg l^{-1} SO_2. Plates with YPD medium were incubated at 25 °C for 2 days before counting, whereas plates with lysine agar were incubated at 25 °C for 4 days before counting.

2.2. Fermentation conditions

Duplicate experiments were carried out in 300 ml Erlenmeyer flasks containing 200 ml of grape must at 20 or 15 °C by inoculation with 48-h pre-cultures (grown in the same medium at 20 °C) to obtain an initial inoculation level of 10^6 cells ml^{-1}. Mixed fermentation trials were simultaneously inoculated with 10^6 cells ml^{-1} of the non-*Saccharomyces* cultures and 10^6 cells ml^{-1} of the *S. cerevisiae* strain. Sequential fermentations trials were inoculated with 10^6 cells ml^{-1} of the non-*Saccharomyces*

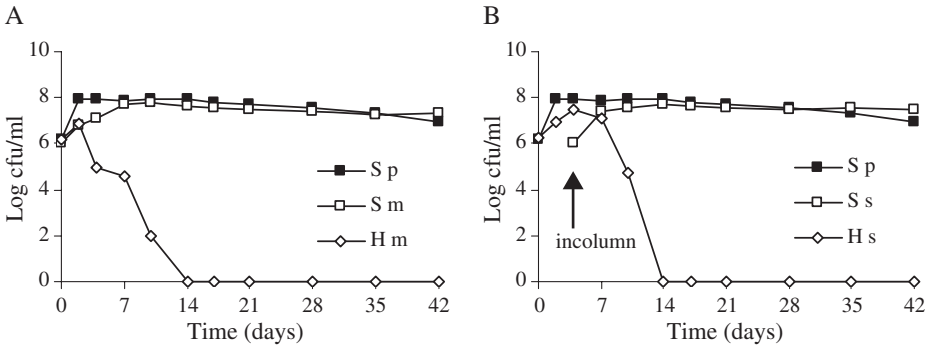

Fig. 2. Biomass evolution in *S. cerevisiae*/*H. uvarum* multistarter fermentations at 20 °C. ■, pure *S. cerevisiae* culture (S p). (A) □, mixed *S. cerevisiae* culture (S m). ◇, mixed *H. uvarum* culture (H m). (B) □, sequential *S. cerevisiae* culture (S s). ◇, sequential *H. uvarum* culture (H s).

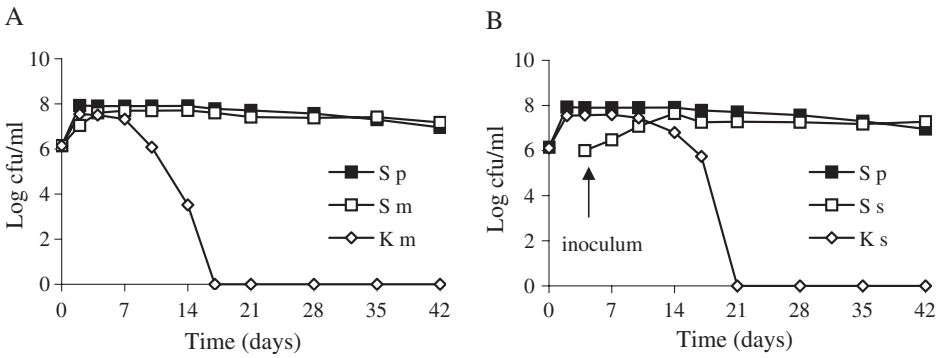

Fig. 3. Biomass evolution in *S. cerevisiae*/*K. thermotolerans* multistarter fermentations at 20 °C. ■, pure *S. cerevisiae* culture (S p). (A) □, mixed *S. cerevisiae* culture (S m). ◇, mixed *K. thermotolerans* culture (K m). (B) □, sequential *S. cerevisiae* culture (S s). ◇, sequential *K. thermotolerans* culture (K s).

cultures with the addition of the *S. cerevisiae* after 4 days (fermentations at 20 °C) or 7 days (fermentations at 15 °C).

After the aseptic stoppering of the flasks with a special valve containing sulphuric acid to allow only CO_2 to escape from the system (Ciani and Rosini, 1987), the weight loss was followed for several days until the end of fermentation (constant weight for two consecutive days). Fermentations were carried out in static conditions.

2.3. Analytical determinations

Viable cell counts were evaluated by a traditional plate counting technique using three different substrates, YPD medium, lysine agar, and YPD+SO_2, to estimate the total yeast populations, the non-*Saccharomyces* yeasts, and the *S. cerevisiae* cultures, respectively.

Ethanol was measured by gas-liquid chromatography (GLC) analysis (AOAC, 1990). Acetaldehyde, ethyl acetate and acetoin were determined by direct injection into the GLC system. Samples were injected into a 2 m by 2 mm (inner diameter) glass column packed with 80/100 Carbopack C/0.2% Carbowax 1500 (Supelco, Sigma-Aldrich, Milan, Italy), with an internal standard of 3-methyl-2-butanol. Nitrogen (20 ml min^{-1}) was used as the carrier gas. A Mega gas chromatograph (Carlo Erba, Milan, Italy), equipped with a flame ionization detector and linked to a DP 700 integrator (Carlo Erba, Milan, Italy), was used. The oven temperature ranged from 45 to 160 °C (4 °C min^{-1}). The temperature of the injector and the detector was 220 °C. Glucose and fructose (kit no. 139106) were determined by using specific enzymatic kits (Boehringer, Mannheim, Germany). Volatile acidity was quantified by steam distillation according to official analytical methods (EEC, 1990). Ammonia was assayed by diagnostic kits (no. 171-UV, Sigma Aldrich, S. Louis MI), while primary amino acids (assimilable nitrogen) were evaluated following the procedures described by Dukes and Butzke (1998).

3. Results and discussion

3.1. Course of fermentation and biomass evolution

It is well known that non-*Saccharomyces* wine yeasts can influence the fermentation kinetics of fermentations inoculated with starter cultures of *S. cerevisiae* (Heard and Fleet, 1998; Erten, 2002). Our results for the fermentation kinetics of multistarter fermentations initially confirmed this behaviour (Fig. 1). All of the multistarter cultures showed a reduced fermentation rate when compared with the pure cultures of *S. cerevisiae*. Moreover, sequential trials showed a further decrease in comparison with mixed trials. However, the final CO_2 evolution was quite similar between the pure and the multistarter fermentations at 20 °C.

The evolution of viable cells of multistarter fermentations of *S. cerevisiae*/*H. uvarum* is shown in Fig. 2, where apiculate

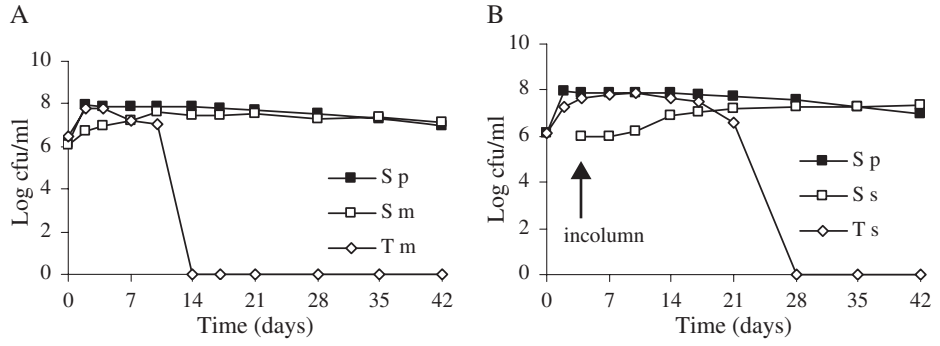

Fig. 4. Biomass evolution in *S. cerevisiae*/*T. delbrueckii* multistarter fermentations at 20 °C. ■, pure *S. cerevisiae* culture (S p). (A) □, mixed *S. cerevisiae* culture (S m). ◇, mixed *T. delbrueckii* culture (K m). (B) □, sequential *S. cerevisiae* culture (S s). ◇, sequential *T. delbrueckii* culture (K s).

Table 1
Principal enological characters of multistarter fermentations at 20 °C

Culture trial	Ethanol (vol.%)	Volatile acidity (g l^{-1})	Glucose residue (g l^{-1})	Fructose residue (g l^{-1})	Assimilable nitrogenous compounds used (mg l^{-1})
Pure *S. cerevisiae*	15.9±0.1[a]	0.71±0.01[a]	0.0	0.0	236.7±6.4[a]
Pure *H. uvarum*	4.5±0.1[b]	1.24±0.12[b]	53.8±2.0	51.8±2.3	235.0±1.0[a]
Mixed *S. cerevisiae*/*H. uvarum*	15.8±0.1[a]	0.71±0.05[a]	0.0	0.0	193.9±4.2[b]
Sequential *S. cerevisiae*/*H. uvarum*	16.0±0.1[a]	0.66±0.01[a]	0.0	0.0	194.9±5.3[b]
Pure *K. thermotolerans*	13.6±0.8[c]	0.45±0.05[a c]	0.0	30.2±0.5	236.0±4.9[a]
Mixed *S. cerevisiae*/*K. thermotolerans*	15.9±0.1[a]	0.60±0.14[a]	0.0	0.0	262.2±0.9[c]
Sequential *S. cerevisiae*/*K. thermotolerans*	15.6±0.2[a]	0.51±0.04[a]	0.0	2.9±2.0	259.9±1.0[c]
Pure *T. delbrueckii*	10.7±0.8[c]	0.22±0.04[c]	31.3±8.4	70.5±14.5	148.8±6.0[d]
Mixed *S. cerevisiae*/*T. delbrueckii*	15.7±0.1[a]	0.40±0.05[c]	0.0	1.9±0.5	145.4±0.3[d]
Sequential *S. cerevisiae*/*T. delbrueckii*	15.6±0.1[a]	0.32±0.04[c]	0.0	5.5±3.7	208.8±0.6[b]

The amount of sugar at the start was 270 g l^{-1}.
The initial yeast assimilable nitrogenous compounds were 288.5 mg l^{-1} (149.1 mg l^{-1} ammonia and 139.4 mg l^{-1} primary amino acids).
Values are means±standard deviations.
Values displaying different superscript letters ([a, b, c, d]) within each column are different according to the Duncan test (0.01%).

yeasts in sequential cultures exhibited a persistence and amounts of cells higher than those seen for mixed trials. As expected, in multistarter cultures, *S. cerevisiae* did not reach the cell concentration of a pure culture (approximately 10^8 CFU ml^{-1}).

The biomass evolutions of multistarter trials of *K. thermotolerans*/*S. cerevisiae* cultures and *T. delbrueckii*/*S. cerevisiae* cultures are shown in Figs. 3 and 4, respectively. These show that in mixed trials, non-*Saccharomyces* yeasts persist during the first stages of fermentation (10–14 days), while in the sequential cultures they persist for longer period (21–28 days). Moreover, in sequential cultures, both non-*Saccharomyces* yeasts were the dominant species also after the inoculation of the *S. cerevisiae* strain (for 7–10 days).

3.2. Analytical profile of wines

The results of the principal enological characters of microfermentations of our *H. uvarum*/*S. cerevisiae* trials (Table 1) show that despite its consistent production of volatile compounds in pure culture, *H. uvarum* did not cause an increase in volatile acidity in mixed cultures (Erten, 2002). This behaviour was also confirmed in sequential cultures, where a prolonged cell survival of *H. uvarum* was seen (Fig. 2). Both mixed and sequential cultures did not show sugar residues, highlighting the correct evolution of the fermentations. Furthermore, in all multistarter trials, less nitrogen (present in non-deficient concentrations: 288.5 mg l^{-1}) was used than in pure cultures, indicating the absence of competition for assimilable nitrogenous compounds between the apiculate and the *S. cerevisiae* yeasts.

In both the *K. thermotolerans*/*S. cerevisiae* and the *T. delbrueckii*/*S. cerevisiae* multistarter trials, acetic acid production was lower than that seen for pure *S. cerevisiae* cultures, confirming the peculiar characteristic under these conditions, of the low acetic acid production of these yeasts (Castelli, 1969; Mora et al., 1990). In contrast to the mixed fermentations that did not exhibit sugar residues (<2 g l^{-1}), sequential trials exhibited fructose residues, confirmed by a feeble reduction of final ethanol concentration compared with the pure cultures of *S. cerevisiae* showing a sluggish fermentation. Moreover, in both mixed and sequential cultures of *T. delbrueckii*/*S. cerevisiae*, lower assimilable nitrogenous compound consumption was seen than that exhibited by pure *S. cerevisiae* cultures, showing a similar behaviour to the

Table 2
Secondary products of multistarter fermentations at 20 °C

Culture trial	Acetaldehyde (mg l^{-1})	Ethyl acetate (mg l^{-1})	Acetoin (mg l^{-1})
Pure *S. cerevisiae*	99.7±15.3[a]	59.6±5.3[a]	11.9±3.7[a]
Pure *H. uvarum*	72.5±2.1[b]	1606.0±22.6[b]	87.7±3.4[b]
Mixed *S. cerevisiae*/*H. uvarum*	69.0±2.0[b]	111.5±18.0[c]	8.5±6.8[a]
Sequential *S. cerevisiae*/*H. uvarum*	59.6±2.2[b]	180.9±18.1[d]	6.9±1.3[a]
Pure *K. thermotolerans*	18.7±1.4[c]	77.3±0.3[a]	3.4±1.4[a]
Mixed *S. cerevisiae*/*K. thermotolerans*	63.7±6.6[b]	69.4±1.3[a]	7.5±2.4[a]
Sequential *S. cerevisiae*/*K. thermotolerans*	36.2±10.1[c]	75.0±7.6[a]	2.2±0.6[a]
Pure *T. delbrueckii*	122.3±2.1[d]	61.7±13.9[a]	7.3±0.8[a]
Mixed *S. cerevisiae*/*T. delbrueckii*	66.9±8.6[b]	59.7±5.6[a]	5.4±0.8[a]
Sequential *S. cerevisiae*/*T. delbrueckii*	32.5±7.2[c]	60.5±5.8[a]	2.7±1.0[a]

Values are means±standard deviations.
Values displaying different superscript letters ([a, b, c, d]) within each column are different according to the Duncan test (0.01%).

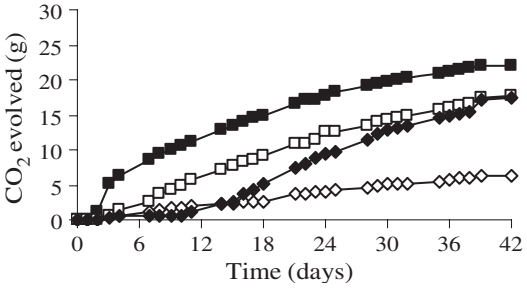

Fig. 5. Fermentations at 15 °C. ■, pure *S. cerevisiae* culture. □, mixed *H. uvarum*/*S. cerevisiae* culture. ◆, sequential *H. uvarum*/*S. cerevisiae* culture. ◇, pure *H. uvarum* culture.

Table 3
Enological profiles of multistarter fermentations of *S. cerevisiae* and *H. uvarum* at 15 °C

Culture trial	Ethanol (vol.%)	Volatile acidity (g l^{-1})	Glucose residue (g l^{-1})	Fructose residue (g l^{-1})	Assimilable nitrogenous compounds used (mg l^{-1})	Acetaldehyde (mg l^{-1})	Ethyl acetate (mg l^{-1})	Acetoin (mg l^{-1})
Pure *S. cerevisiae*	15.4±0.4[a]	0.78±0.08[a]	0.0	7.9±1.6	234.0±5.0[a]	50.3±4.4[a]	64.9±6.1[a]	3.3±0.7[a]
Pure *H. uvarum*	4.3±0.2[b]	0.99±0.20[a]	67.3±3.0	52.7±1.1	127.5±1.0[b]	24.2±2.8[b]	648.0±11.1[b]	340.1±4.4[b]
Mixed *S. cerevisiae*/ *H. uvarum*	14.1±0.6[c]	0.78±0.11[a]	0.0	25.0±14.2	214.3±14.6[ac]	72.1±2.1[a]	117.0±19.2[c]	12.1±4.8[a]
Sequential *S. cerevisiae*/ *H. uvarum*	14.2±0.1[c]	0.71±0.01[a]	3.3±1.3	30.6±0.5	196.4±8.4[c]	100.6±7.6[c]	263.4±13.6[d]	15.6±1.5[a]

The amount of sugar at the start was 270 g l^{-1}.
The initial yeast assimilable nitrogenous compounds were 288.5 mg l^{-1} (149.1 mg l^{-1} ammonia, 139.4 mg l^{-1} primary amino acids).
Values are means±standard deviations.
Values displaying different superscript letters ([a, b, c, d]) within each column are different according to the Duncan test (0.01%).

H. uvarum strain. In contrast, all of the *K. thermotolerans*/*S. cerevisiae* trials exhibited an increase in nitrogen consumption, suggesting possible competition at the critical nitrogen concentrations during mixed and sequential fermentations.

Evaluations of some of the volatile compounds confirmed that apiculate yeasts consistently formed higher amounts of ethyl acetate in pure cultures (Table 2) (Ciani and Picciotti, 1995; Ciani and Maccarelli, 1998; Romano, 2002). In mixed and sequential cultures, apiculate yeasts influenced the final amounts of ethyl acetate in wines. However, in mixed cultures, the levels of ethyl acetate concentrations achieved could contribute to the fruity notes and add to the general complexity (Gil et al., 1996; Ciani, 1997), while the sequential cultures surpassing the threshold taste level (150 mg l^{-1}) (Jackson, 1994) produced a sour-vinegar off odour.

The amounts of ethyl acetate produced by apiculate yeasts appear to be linked to their level of contribution to the fermentation (more in sequential than in mixed cultures). These results are of interest considering the possible use of apiculate yeasts in winemaking. A different behaviour was shown by *H. uvarum* regarding acetoin production in multistarter trials. Pure cultures of *H. uvarum* were high producers of acetoin (Table 2), confirming the results of previous studies (Ciani and Picciotti, 1995; Ciani and Maccarelli, 1998; Romano, 2002). However, in contrast to this ethyl acetate production, in multistarter fermentations the presence of apiculate yeasts did not cause an increase in acetoin, which was probably metabolised by the actively fermenting *S. cerevisiae* yeast strain (Romano, 2002).

The amounts of acetaldehyde, an important by-product of fermentation (Ciani, 1997), did not appear to be negatively influenced by mixed or sequential cultures of *H. uvarum*, since lower amounts were exhibited in the presence of apiculate yeasts.

As expected, *K. thermotolerans* and *T. delbrueckii* multistarter trials did not cause substantial modifications in the secondary products evaluated, as compared with the pure *S. cerevisiae* cultures. Moreover, the constant reduction of acetaldehyde content in multistarter fermentations of these two species compared with pure culture of *S. cerevisiae* is also worth highlighting.

3.3. Influence of low temperature on apiculate multistarter cultures

Since several studies have indicated a higher persistence of apiculate yeasts and different fermentation behaviours at low temperatures (Gao and Fleet, 1988; Heard and Fleet, 1988; Erten, 2002), we investigated the influence of low temperature on the fermentation behaviour of *H. uvarum* in multistarter cultures. Fermentations carried out at 15 °C showed that the pure *S. cerevisiae* cultures had a limited sugar residue (8 g l^{-1} fructose). Mixed and sequential trials of *H. uvarum*/*S. cerevisiae* carried out at this temperature showed a reduction in CO_2 formation and lower amounts of ethanol production, as

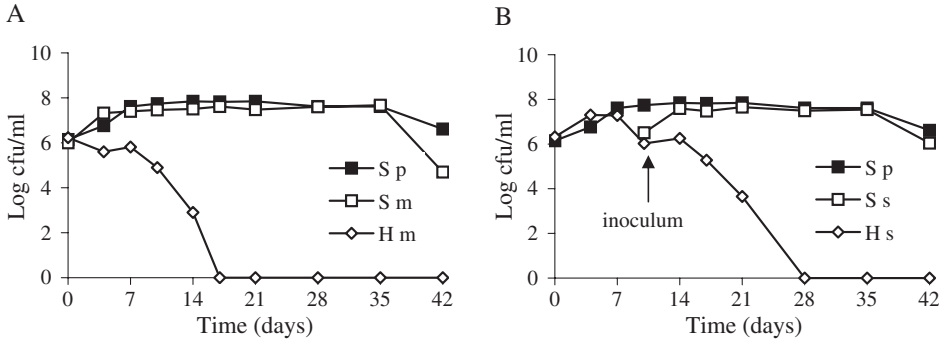

Fig. 6. Biomass evolution in *S. cerevisiae*/*H. uvarum* multistarter fermentations at 15 °C. ■, pure *S. cerevisiae* culture (S p). (A) □, mixed *S. cerevisiae* culture (S m). ◇, mixed *H. uvarum* culture (H m). (B) □, sequential *S. cerevisiae* culture (S s). ◇, sequential *H. uvarum* culture (H s).

compared to the pure cultures of *S. cerevisiae* (Fig. 5, Table 3). This lower fermentation activity was confirmed by a consistent presence of final sugar residues, indicating a stuck fermentation. However, this reduced fermentation activity was not due to a deficiency of assimilable nitrogenous compounds, since lower amounts of these compounds were used (Table 3). At the low fermentation temperature, all multistarter trials showed higher cell numbers and increases in persistence of viable biomass of *H. uvarum* than was seen at 20 °C (Fig. 6), confirming the results of previous studies (Heard and Fleet, 1988; Erten, 2002). With respect to the analytical compounds, the fermentations of apiculate yeasts at 15 °C confirmed the behaviours showed at 20 °C.

4. Conclusions

To enhance the complexity of wine flavours in winemaking, multistarter fermentations could be an interesting alternative to a guided fermentation using a starter culture of *S. cerevisiae* (Grossman et al., 1996; Ciani, 2001). In this context, interactive behaviours between the different species need to be considered (Fleet, 2003). The results of the present study show that mixed fermentations of all of the species evaluated do not negatively influence the evolution of fermentation and wine flavour, producing dry wines with similar or better analytical profiles to those produced by pure *S. cerevisiae* cultures. Due to the higher persistence of non-*Saccharomyces* yeasts during fermentation, the sequential trials show some possible limitations. Sequential cultures of *T. delbrueckii* and *K. thermotolerans* cause a limited sugar residue, indicating the endurance of fermentation, while sequential trials with *H. uvarum* reveal an excessive increase in ethyl acetate, which surpasses the threshold taste level (150 mg l^{-1}) (Jackson, 1994). Low temperatures (15 °C) negatively influence the evolution of fermentations, causing stuck multistarter fermentations of *H. uvarum*. This behaviour does not appear to be due to the competition for nitrogen, but rather to the competition for other nutrients (e.g. thiamine) (Bisson, 1999).

In conclusion, multistarter fermentations of *H. uvarum*, *T. delbrueckii* and *K. thermotolerans* are influenced by the level and persistence of these yeasts during fermentation. Prolonged persistence and high levels of these yeast species during multistarter fermentations could result in stress conditions (high ethanol contents and/or low temperatures), stuck or sluggish fermentations.

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