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# Reduction of volatile acidity of wines by selected yeast strains

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**Abstract** Herein, we isolate and characterize wine yeasts with the ability to reduce volatile acidity of wines using a refermentation process, which consists in mixing the acidic wine with freshly crushed grapes or musts or, alternatively, in the incubation with the residual marc. From a set of 135 yeast isolates, four strains revealed the ability to use glucose and acetic acid simultaneously. Three of them were identified as *Saccharomyces cerevisiae* and one as *Lachancea thermotolerans*. Among nine commercial *S. cerevisiae* strains, strains S26, S29, and S30 display similar glucose and acetic acid initial simultaneous consumption pattern and were assessed in refermentation assays. In a medium containing an acidic wine with high glucose–low ethanol concentrations, under low oxygen availability, strain S29 is the most efficient one, whereas *L. thermotolerans* 44C is able to decrease significantly acetic acid similar to the control strain *Zygosaccharomyces bailii* ISA 1307 but only under aerobic conditions. Conversely, for low glucose–high ethanol concentrations, under aerobic conditions, S26 is the most efficient acid-degrading strain, while under limited-aerobic conditions, all the *S. cerevisiae* strains studied display acetic acid degradation efficiencies identical to *Z. bailii*. Moreover, S26 strain also reveals capacity to decrease volatile acidity of wines. Together, the *S. cerevisiae* strains characterized herein appear promising for the oenological removal of volatile acidity of acidic wines.

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## Introduction

Acetic acid is the main component of volatile acidity and is critical for wine quality. Its concentration in wines is approximately 0.5 g $l^{-1}$ and must remain below 1.1 g $l^{-1}$ according to current legislation. This acid is mainly produced by bacterial spoilage in *Botrytis-cinerea*-infected grapes. The concentration of acetic acid bacteria can increase drastically on *Botrytis*-infected grapes (up to $10^7$ cells per milliliter, whereas the concentration on healthy grapes ranges usually between $10^2$ and $10^4$ cells per milliliter). The ruptured grape berry skin, caused by the infection, allows bacteria to access the berry's interior. Usually, *Gluconobacter* species occur on grapes, but *Acetobacter* species can dominate on the surface of rotten grapes, using ethanol as preferential carbon source that is produced in small amounts by yeast (Du Toit 2002). Acetic acid can also be formed by yeasts during alcoholic fermentation. Enzymatic reactions that can lead to acetic acid formation in yeast include (1) reversible formation from acetyl coenzyme A (CoA) and acetyl adenylate through acetyl CoA synthetase, (2) cleavage of citrate by citrate lyase, (3) production from pyruvate by pyruvate dehydrogenase, (4) reversible formation from acetyl phosphate by acetyl kinase, and (5) oxidation of acetaldehyde by aldehyde dehydrogenase (Jost and Piendl 1975). Yeast cultures exposed to oxygen, actively synthesizing fatty acids for growth, may produce acetic acid upon entry into anaerobic conditions as a mechanism for the regeneration of free CoA needed for other biosynthetic activities (Boulton et al. 1998). Acetate formation may also play a

physiological role in the regeneration of reducing equivalents (NADH and NADPH) that are essential for the maintenance of the redox balance (Remize et al. 2000; Saint-Prix et al. 2004). Several other studies have linked the production of acetic acid to increased glycerol production (Englinton et al. 2002; Remize et al. 1999).

Other explanations for the excessive formation of acetic acid during grape must fermentation are, among others, effects derived from nutrient imbalance and competition between coexisting yeasts and bacterial populations during concurrent malolactic fermentations (Boulton et al. 1998).

Sugars are the preferential carbon and energy source of the yeast *Saccharomyces cerevisiae*, but nonfermentable substrates such as ethanol, glycerol, lactate, or acetate can also be used for the generation of energy and cellular biomass (Schüller 2003). *S. cerevisiae* is one of the yeast species that can use acetic acid as a sole carbon and energy source. During growth in acetate-containing media, this substrate is metabolized via acetyl CoA formed by one of the two acetyl CoA synthetases: the Acs1p (peroxisomal) and Acs2p (cytosolic). Acetyl CoA is then consumed in the glyoxylate shunt by isocitrate lyase and malate synthase outside the mitochondria or enters the mitochondria to be oxidized in the tricarboxylic acid cycle. Gluconeogenesis also needs to be active (Dos Santos et al. 2003).

Acetic acid present at the beginning of fermentation is partially metabolized by wine yeasts at the middle–end of this process in the presence of residual sugars (Ribéreau-Gayon et al. 2000). In *S. cerevisiae*, acetate transport and metabolism are subjected to glucose repression, similar to almost all other nonsugar carbon sources. Hence, growth of *S. cerevisiae* in a medium containing glucose and acetic acid displays a diauxic growth with consumption of acetic acid only after glucose exhaustion (Rodrigues 1998; Casal et al. 1998). This behavior is also described for other yeasts species like *Candida utilis* (Leão and Van Uden 1986), *Torulaspora delbrueckii* (Casal and Leão 1995), and *Dekkera anomala* (Gerós et al. 2000). In contrast, *Zygosaccharomyces bailii* ISA 1307 displays a biphasic growth in a medium containing a mixture of glucose and acetic acid; the first phase is associated with a simultaneous consumption of glucose and acetic acid and the second with the utilization of the remaining acid (Sousa et al. 1998). These authors propose that both membrane transport and acetyl CoA synthetase and their regulation are important for the ability of *Z. bailii* to metabolize acetic acid in the presence of glucose. These physiological traits are responsible for the species' high resistance in environments containing mixtures of sugars and acetic acid such as those often present during wine fermentation (Sousa et al. 1998). Perfusion experiments also showed that *Z. bailii* is more resistant than *S. cerevisiae* to short-term intracellular pH changes caused by acetic acid (Arneborg et al. 2000).

Anaerobic growth of *S. cerevisiae* in mixed culture media containing glucose and acetic acid is determined by the extracellular pH and acetic acid concentration. Without acetic acid, growth is possible at a pH as low as 2.5, whereas in the presence of 10 g $l^{-1}$ of acetic acid the minimum growth-permissive pH value increased up to 4.5, and the concentration of the undissociated form should not exceed 5.0 g $l^{-1}$ (Mohammad et al. 1997).

Winemakers have been using an empirical biological deacidification procedure to lower acetic acid contents of wines with high volatile acidity (above 0.8 g $l^{-1}$) and which consists in a refermentation associated to acetic acid consumption by yeasts. According to Ribéreau-Gayon et al. (2000), this enological practice is performed by mixing the acidic wine with freshly crushed grapes or musts in a proportion of no more than 20–30% (*v*/*v*). The initial volatile acidity of this mixture should not exceed 0.6 g $l^{-1}$ and the final volatile acidity of the newly made wine rarely exceeds 0.3 g $l^{-1}$. The added wine should be microbiologically stable before incorporation to avoid bacterial growth. Alternatively, the acidic wine can be incubated with the residual marc from a finished wine fermentation. Though this practice has been based on the assumption that fermentative yeasts are able to metabolize a large portion of the accumulated acetic acid during an alcoholic fermentation of the first 50–100 g of sugar (Ribéreau-Gayon et al. 2000), such physiological traits of wine yeasts are generally less well known among enologists. The aim of the present study was to isolate and characterize yeasts species with ability to reduce the acetic acid content of grape musts or wines with high volatile acidity. It is anticipated that the identified yeasts can be used as starters in an efficient and controlled biological procedure to decrease volatile acidity of acidic must or wines.

## Materials and methods

### Microorganisms

From a refermentation process of a wine with excessive acetic acid concentration (1.36 g $l^{-1}$), 135 yeast isolates were collected. The strains were tested regarding their growth patterns in a differential medium containing glucose (0.2% *w*/*v*) and acetic acid (5.0 g $l^{-1}$), at pH 4.0 or 6.0 (Schuller et al. 2000; data not shown). The strains 43C, 44C, 45C, and 30C showed both growth and color change of the medium (due to pH changes), indicating simultaneous glucose and acetic acid consumption. Strain 30C was collected at the beginning of a refermentation process with the residual marc from a finished wine fermentation. Strains 43C, 44C, and 45C were collected at the end of the same refermentation process.

Several other *S. cerevisiae* commercial strains (S19, S23, S24, S25, S26, S28, S29, S30, and S36) were also tested

regarding acetic acid and glucose consumption. The mentioned designations (S19–S36) are our internal references in order to avoid revealing commercial names. The strain *Z. bailii* ISA 1307 was used as a control strain.

## Culture media and growth conditions

Strains were kept at −80°C in microtubes containing yeast peptone dextrose (YPD) broth (glucose 2% *w*/*v*, peptone 1% *w*/*v*, yeast extract 0.5% *w*/*v*) supplemented with glycerol (30% *v*/*v*). Prior to each experiment, strains were streaked onto YPD plates and incubated during 48 h at 25°C. Acetic acid utilization was assessed in a minimal mineral medium (Van Uden 1967) containing acetic acid 5.0 g $l^{-1}$ and glucose (0.5% *w*/*v* to 5% *w*/*v*) at 25°C and pH 3.0. The medium was then filtered through a membrane (Milipore 0.22-μm pore size). A preculture was grown overnight in 10 ml of culture medium, transferred to Erlenmeyer flasks (initial OD of about 0.03), and incubated at 25°C. Acetic acid and glucose consumption was evaluated under aerobic (250-ml Erlenmeyer flask containing 100 ml of minimal medium, 120 rpm) and limited-aerobic conditions (250-ml Erlenmeyer flask containing 230 ml of minimal medium, 100 rpm). In all experiments in liquid media, the flaks were sterilized before use and were only opened to collect the samples in a controlled sterile environment. Commercial *S. cerevisiae* strains were assessed only under limited-aerobic conditions and adjusting acetic acid and glucose concentration to 2.5 g $l^{-1}$ and 0.75% (*w*/*v*), respectively. In these assays, the initial concentration of acetic acid was lowered to 2.5 g $l^{-1}$ to evaluate the capability of yeasts to decrease maximum volatile acidity levels usually present in acidic spoiled wines and which are about 2.0 g $l^{-1}$.

## Refermentation assays

In order to simulate the refermentation of a wine with excessive amounts of acetic acid, mixtures were prepared containing two thirds of a mineral medium (Van Uden 1967) and one third of acidic white wine. The chemical characteristics of the wines used are summarized in Table 1. Volatile acidity of the mixture was adjusted to 1.13 g $l^{-1}$ using glacial acetic acid (Merck) and the pH of the medium to 3.5 using NaOH. The medium containing wine 1 was further supplemented with glucose (13% *w*/*v*) and ethanol (4% *v*/*v*) in order to simulate the refermentation of a wine with must from the beginning of fermentation. The mineral medium containing wine 2 was supplemented with glucose (3.3% *w*/*v*) and ethanol (10% *v*/*v*) in order to simulate the refermentation of a wine with the residual marc from a finished wine fermentation. The mixtures, mineral medium and acidic white wine, were filtered through a membrane (Milipore 0.22-μm pore size). Ten milliliter of the inoculated media were incubated overnight and used as a preculture for growth experiments that occurred at 25°C under both aerobic and limited-aerobic conditions, as described before. All experiments were performed in triplicate.

## Removal of acetic acid from an acidic wine under different oxygenation conditions

Strain S26 was used to assess the influence of aeration on acetic acid removal from an acidic wine (wine 3, Table 1). Volatile acidity was adjusted to 1.44 g $l^{-1}$ using glacial acetic acid (Merck) and the pH set to 3.55 using NaOH. The acidic wine was filtered through a membrane (Milipore 0.22-μm pore size) prior to use. Ten milliliter of the inoculated wine were incubated overnight and used as a preculture for growth experiments that occurred at 25°C under the previously described aerobic and limited-aerobic conditions and also under anaerobic conditions (230-ml acidic wine in a 250-ml Erlenmeyer flask, without mechanical shaking).

## Analytical determinations

Cellular growth in liquid media was followed by optical density at 640 nm in a UV-VIS Scanning Spectrophotometer

**Table 1** Chemical characteristics of the wines used in refermentation simulation assays

| Chemical characteristics | Wine 1 | Wine 2 | Wine 3 | Analytical methods (CEE N.° 2676/90)[a] |
|---|---|---|---|---|
| Density at 20°C | 0.9940 | 0.9925 | 0.9905 | Densitometry |
| Free $SO_2$ (mg $l^{-1}$) | 1.92 | 7.04 | 11.9 | Ripper method |
| Total $SO_2$ (mg $l^{-1}$) | 76.5 | 78.1 | 28.0 | Ripper method |
| Volatile acidity (g $l^{-1}$ acetic acid) | 0.51 | 0.54 | 0.36 | Distillation using a Cazenave-Ferré followed by titration with phenolphthalein |
| Residual sugar (g $l^{-1}$) | n.d. | n.d. | 1.10 | Lane-Eynon method |
| Titratable acidity (g $l^{-1}$ tartaric acid) | 5.13 | 5.17 | 8.89 | Titration with bromothymol blue |
| pH | 3.17 | 3.22 | 3.01 | Potentiometer |
| Alcoholic degree (*v*/*v*) ethanol (%) | 11.8 | 11.3 | 10.4 | Distillation |

[a] CEE N.° 2676/90—Official Journal of the European Communities, 33, 3.10.1990. (ISSN 0257-7771)
*n.d.* Not determined

(Shimadzu UV-2101PC). Glucose, acetic acid, and ethanol concentrations were determined by high-performance liquid chromatography with a Perkin-Elmer series 10 Liquid Chromatographic System equipped with an ion exclusion, cation exchange column (BIORAD–HPX–87H), and an RI detector. The column was eluted with sulfuric acid (0.013 N) at 62°C, at a flow rate of 0.6 ml $min^{-1}$. Samples were filtered through a membrane (Milipore 0.22-μm pore size) prior injection of a volume of 6 μl. The components were identified by their relative retention times in comparison to the values obtained for the respective standards, using the program JCL 6000 Software (Jones Chromatography). Acetic acid concentration was also determined enzymatically with an Enzytec Laboratories kit.

Analysis of the density, pH, alcohol concentration, volatile acidity, $SO_2$, and titratable acidity was performed according to published methods, outlined in Table 1.

### DNA isolation

Yeast cells were cultivated in 1-ml YPD medium (36 h, 28°C, 160 rpm). DNA isolation was performed as described (Lopez et al. 2001) with a modified cell lysis procedure, using 25 U of Zymolase (SIGMA). Cell lysis was dependent on the strain and lasted between 20 min and 1 h (37°C). DNA was used for sequencing and microsatellite analysis.

### D1–D2 region amplification and sequencing

The D1–D2 variable domain at the 5' end of the 26S rDNA (nucleotides 63–642 for *S. cerevisiae*) was amplified with primers NL-1 (GCATATCAATAAGCGGAGGAAAAG) and NL-4 (GGTCCGTGTTTCAAGACGG; O'Donnell 1993), obtained from MWG Biotech, Germany. Polymerase chain reactions (PCR) were performed in a reaction mixture (30 μl) containing 60-ng genomic DNA, 12 pmol of each primer, 0.2 mM of deoxynucleotide triphosphate (MBI Fermentas), 1.5 mM $MgCl_2$, 3 μl of 10× reaction buffer (MBI Fermentas), and 0.6 U of Taq polymerase (MBI Fermentas). Amplification reactions were performed on a BioRad iCycler thermal cycler with the following cycling parameters: initial denaturation at 94°C for 4 min, followed by 36 cycles at 94°C for 20 s, 53°C for 20 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplified fragment was purified (Nucleospin Extract II PCR cleanup kit, Machery-Nagel) and sequenced by using the ABI Big Dye Terminator kit (version 3.1). Processing of the samples for loading onto the sequencer was performed as per manufacturer's instructions. Sequence similarity search was done using GenBank BLASTN search (Altschul et al. 1990).

### Microsatellite amplification

Six trinucleotide microsatellite loci (ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5, and ScAAT6), originally described by Pérez et al. (2001), were used as molecular markers for *S. cerevisiae* strain delimitation. Their amplification and analysis was performed as previously described (Schuller et al. 2004). PCR products were resolved in an ABI Prism 310 DNA sequencer (Applied Biosystems) and analyzed using the corresponding Genescan software.

### Statistical analysis

A one-way analysis of variance (ANOVA) or single-factor ANOVA (Excel, Microsoft) and the program STATISTICA (factorial ANOVA) were used to evaluate the differences between the yeasts strains concerning glucose and acetic acid consumption in the different assays.

## Results

### Molecular identification and characterization of the isolated wine strains

Strains 30C, 43C, 44C, and 45C were identified by the amplification of the D1–D2 variable domain at the 5' end of the 26S rDNA (nucleotides 63–642 for *S. cerevisiae*) with primers NL-1 and NL-4 (O'Donnell 1993) and the amplified fragments were subsequently sequenced. Sequence similarity search was done using GenBank BLASTN search (Altschul et al. 1990). D1–D2 sequence of strain 30C, 43C, and 45C showed 99–100% identity with deposited *S. cerevisiae* sequences (accession numbers U53879, AY130346, and U44806, respectively). D1–D2 sequence of strain 44C shows 99% identity with *Lachancea thermotolerans* NRRL Y-8284 (accession number U69581; Kurtzman 2003; Kurtzman and Robnett 2003). Consulting the Centraalbureau voor Schimmelcultures, this strain is similar to the type strain of *Zygosaccharomyces thermotolerans,* isolated from mirabelle plum and identified by Lachance (1998). Until October 2004, this species was considered as *Kluyveromyces thermotolerans.*

Microsatellite analysis (Table 2) shows that *S. cerevisiae* strains 30C and 45C are genetically very similar, showing identical alleles for five loci. These two strains could be merely distinguished by locus ScAAT5, being strain 30C homozygous (216 bp) and strain 45C heterozygous (216 or 219 bp).

**Table 2** Allelic diversity of *S. cerevisiae* strains 30C, 45C, and 43C

| | Microsatellite (bp) | | | | | |
|---|---|---|---|---|---|---|
| Strain number | ScAAT1 | ScAAT2 | ScAAT3 | ScAAT4 | ScAAT5 | ScAAT6 |
| 30C | 171 | 381 | 271 | 329 | 216 | 259 |
| 45C | 171 | 381 | 271 | 329 | 216 or 219 | 259 |
| 43C | 158 | 378 | 247 | 308 | 219 | 259 |

Numbers indicate the length (bp) of alleles for the six microsatellite loci ScAAT1 to ScAAT6

## Characterization of the isolated strains regarding their ability to degrade acetic acid

Subsequently, we studied the effects of glucose and acetic acid concentrations and aeration conditions on the consumption of acetic acid by the previously mentioned isolates. The strain *Z. bailii* ISA1307, previously shown to use acetic acid simultaneously with glucose (Sousa et al. 1998), was used as reference. As shown in Table 3, strains *Z. bailii* ISA1307 and *L. thermotolerans* 44C revealed as most efficient in consuming acetic acid, being able to degrade 61.6% and 28.2% of the initial acid, respectively, when grown under limited-aerobic conditions in a mixed substrate medium containing glucose (5.0% *w*/*v*) and acetic acid (5.0 g $l^{-1}$). Under limited-aerobic conditions, and for an initial glucose concentration of 0.75% (*w*/*v*), strains 43C and S26 displayed an identical ability ($P>0.05$, statistical class "b") to degrade acetic acid although not so efficient as strain *Z. bailii* ISA 1307 (statistical class "a") because they consumed just about 50% of the acetic acid after 312 h. Strain 44C has an identical ability to degrade acetic acid in the media with 5.0% or 0.75% *w*/*v* of glucose under limited-aerobic conditions (statistical class "c"). The higher initial concentration of glucose does not alter the rate of acetic acid consumption by strain 44C; however, this strain decreased the rate of glucose consumption (statistical classes "a" and "b"). Only under aerobic conditions and for 0.5% (*w*/*v*) of glucose, all the four isolated strains completely consumed the acetic acid. Even though, regarding the relatedness of the strains in terms of acetic acid consumption, they behaved significantly different ($P\leq0.05$) in respect to the time necessary to complete the acid consumption, varying between 72 and 216 h (*Z. bailii* ISA 1307 and 44C, respectively).

## The ability of commercial wine strains to degrade acetic acid

With the purpose to further evaluate whether the ability to degrade acetic acid in the presence of glucose was a characteristic of the isolated strains, nine commercial *S. cerevisiae* strains were chosen for further analysis. These assays were carried out in minimal medium containing acetic acid 2.5 g $l^{-1}$ and glucose 0.75% (*w*/*v*), under limited-aerobic conditions, at 25°C and pH 3.0. Strain S26 was the most efficient acetic-acid-consuming strain, leading to values below 0.7 g $l^{-1}$ at the end of 168 h (Fig. 1). Besides, this strain exhausted glucose very quickly. Table 4 shows that the strains display significant differences ($P\leq0.001$) regarding acetic acid consumption ability. However, strains S29 and

**Table 3** Concentration of acetic acid and glucose (g $l^{-1}$) present in the minimal media with different initial concentrations of glucose (0.5% to 5% *w*/*v*) and acetic acid (5.0 g $l^{-1}$), after fermentation by the four yeast isolates in comparison with *S. cerevisiae* strains S26 and *Z. bailii* ISA 1307, under aerobic (216 h) or limited-aerobic conditions (312 h), at 25°C and pH 3.0

| Yeasts strains | Aerobic conditions | | Limited-aerobic conditions | | | |
|---|---|---|---|---|---|---|
| | Glucose (0.5% *w*/*v*), acetic acid (5.0 g $l^{-1}$) | | Glucose (0.75% *w*/*v*), acetic acid (5.0 g $l^{-1}$) | | Glucose (5% *w*/*v*), acetic acid (5.0 g $l^{-1}$) | |
| | Glucose (g $l^{-1}$) | Acetic acid (g $l^{-1}$) | Glucose (g $l^{-1}$) | Acetic acid (g $l^{-1}$) | Glucose (g $l^{-1}$) | Acetic acid (g $l^{-1}$) |
| ISA 1307 | 0 a | 0 (72 h)[a] a | 0 a | 0.02±0.03 a | 0 a | 1.92±0.03 b |
| S26 | 0 a | 0 (144)[a] a | 0 a | 2.09±0.09 b | 0 a | 4.41±0.03 d, e |
| 30C | 0 a | 0 (192 h)[a] a | 0 a | 4.40±0.04 b, e | 0 a | 4.90±0.04 e |
| 43C | 0 a | 0 (168 h)[a] a | 0 a | 2.02±0.09 b | 0 a | 4.77±0.02 e |
| 44C | 0 a | 0 (216 h)[a] a | 0 a | 3.99±0.13 c, d | 15.11±0.06 b | 3.59±0.06 c |
| 45C | 0 a | 0 (168 h)[a] a | 0 a | 4.01±0.08 c, d | 0 a | 4.71±0.01 d, e |

Results obtained for strains and culture conditions with the same letter are not significantly different ($P<0.001$).
[a] Time needed to exhaust acetic acid from the medium.

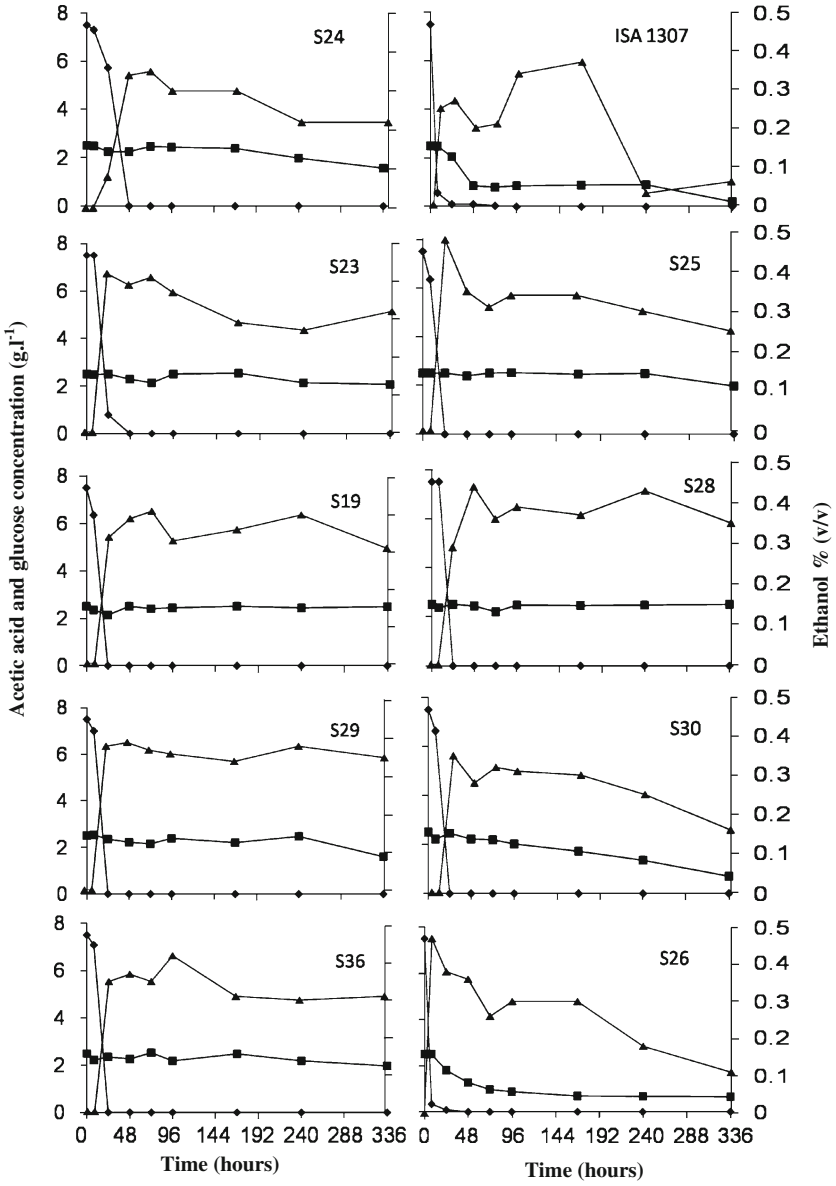


**Fig. 1** Acetic acid (*squares*), glucose (*diamonds*), and ethanol (*triangles*) consumption by nine commercial strains and *Z. bailii* ISA 1307 (control strain), in minimal medium containing acetic acid 2.5 g $l^{-1}$ and glucose 0.75% (*w*/*v*), under limited-aerobic conditions, at 25°C and pH 3.0

S30 showed the most similar behavior to S26 and therefore were included in the refermentation simulation assays with acidic wines.

## Refermentation simulation assays with acidic wines

As previously shown, both the isolated strains (43C, 45C, and 44C) and the commercial *S. cerevisiae* strains S26, S29, and S30 have demonstrated capacity to remove acetic acid. To verify their potential application in a refermentation process under wine cellar conditions, they were inoculated in a mixed medium containing two thirds of minimal medium (Van Uden 1967) supplemented with one third of an acidic white wine. The volatile acidity of the mixture was 1.13 g $l^{-1}$, corresponding to the values usually found in acidic wines. Two wine-supplemented mineral media were tested, according to Table 1; the first medium simulated the refermentation of a wine with fresh crushed grapes or with grape must; the second wine-supplemented mineral medium simulated the refermentation of a wine with the residual marc from a finished wine fermentation. Acetic acid consumption was again evaluated under aerobic or limited-aerobic conditions to assess whether aeration was a limiting factor of the process. We evaluated the rate of acetic acid and glucose consumption at the end of 48 and 72 h for the first and second medium, respectively (Table 5). Statistical analysis (factorial ANOVA) showed that each strain has a characteristic pattern of glucose and acetic acid consumption ($P \leq 0.001$) for the four growth conditions tested. Besides, the strains displayed significant differences regarding acetic acid consumption and glucose consumption for each condition tested. Among *S. cerevisiae* strains,

**Table 4** Consumption of acetic acid (g $l^{-1}$), after 336 and 504 h, by nine commercial strains and *Z. bailii* ISA 1307 (control strain), in minimal medium containing acetic acid 0.25% (*v*/*v*) and glucose 0.75% (*w*/*v*), under limited-aerobic conditions, at 25°C and pH 3.0

| Yeast strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Time (h) | ISA 1307 | S26 | S24 | S23 | S25 | S19 | S28 | S29 | S30 | S36 |
| 336 | 0±0 b | 0.02±0 b | 1.56±0.23 a, c | 2.13±0.28 a | 1.96±0.07 a | 2.53±0.07 a | 2.12±0.21 a | 1.59±0 a, c | 0.70±0.23 b, c | 2.48±0 a |
| 504 | 0±0 a | 0±0 a | 0.31±0.02 a, b | 0.46±0.07 a, b, c | 0.79±0.10 b, c | 1.49±0.39 d | 0.76±0.23 b, c | 0.12±0.04 a | 0±0 a | 0.92±0.11 c, d |

Results obtained for strains and culture conditions with the same letter are not significantly different ($P<0.001$).

except strain S29, the glucose and acetic acid consumption patterns were most similar for limited-aerobic growth conditions (both in high- and low-glucose conditions).

The most efficient strains–conditions regarding acetic acid consumption (letters g and h, Table 5) were the two non-*Saccharomyces* strains *Z. bailii* ISA 1307and *L. thermotolerans* 44C in the high-glucose medium (aerobic condition), followed by strain S29 in the same medium (limited-aerobic condition), and by strains S26 and ISA1307 in the medium containing glucose (3.3% *w*/*v*) and ethanol (10% *v*/*v*) under aerobic conditions (Table 5). Some of the strains showed intermediate acetic acid removal efficiency (between 8.6% and 46.8%, statistical classes "b–f") under certain conditions. The lowest acetic acid removal efficiency (statistical class "a") was observed for strains 43C, 45C, and S30 (high-glucose-concentration medium, aerobiosis) and for *L. thermotolerans* 44C (high-glucose-concentration medium, limited aerobiosis).

Strains 43C, 45C, S26, and S30 consumed glucose efficiently (removal of 87–100%, statistical class "g") from both culture media under limited-aerobic conditions and from the high-glucose culture medium under aerobic conditions. In this respect, strain S26 was one exception displaying high glucose consumption for both media, and under aerobic and anaerobic conditions. Strains ISA 1307 and 44C were intermediate to poor glucose consumers in high-glucose medium (aerobic conditions) and the remaining three culture conditions, respectively. A similar pattern, although not so pronounced, was found for strain S29.

Strains 43C, 45C, S26, and S30 showed identical glucose and acetic acid consumption patterns for both media under limited-aerobic conditions. Strains 43C and 45C that are genetically highly related (Table 2) showed distinct glucose consumption efficiency in the low-glucose medium under aerobic conditions. Taken all together, strain S26 revealed the most desirable combination of glucose and acetic consumption (100% and 86.7%, respectively) when grown in a medium containing low glucose and high ethanol concentrations, under aerobic conditions. Moreover, under limited-aerobic conditions and for both media, strain S26 displayed acid removal efficiencies identical to strain ISA 1307 but associated to higher glucose removal efficiency comparatively with strain ISA 1307.

## Removal of acetic acid from an acidic wine under different oxygenation conditions

As previously shown, *S. cerevisiae* S26 has demonstrated capacity to remove acetic acid under aerobic and limited-aerobic conditions. We further evaluated the capacity of this strain to remove acetic acid from an acidic wine that was not supplemented with glucose (Table 1, wine 3, residual sugar of 1.10 g $l^{-1}$) under different oxygen conditions. Acetic acid

**Table 5** Percentage of acetic acid and glucose consumption after refermentation of wine-supplemented culture medium containing glucose 13% (*w*/*v*) and ethanol 4% (*v*/*v*) or glucose 3.3% (*w*/*v*) and ethanol 10% (*v*/*v*), after 48 and 72 h of incubation, respectively

| Yeast strains | | Glucose (13% *w*/*v*) and ethanol (4% *v*/*v*) | | Glucose (3.3% *w*/*v*) and ethanol (10% *v*/*v*) | |
|---|---|---|---|---|---|
| | | Aerobic conditions | Limited-aerobic conditions | Aerobic conditions | Limited-aerobic conditions |
| ISA 1307 | Acetic acid | 94.8±3.30 h | 40.9±9.80 e, f | 71.2±3.02 g | 41.6±2.64 e, f |
| | Glucose | 52.4±2.62 e, f | 38.8±6.36 d, e | 23.1±5.60 a, b, c | 39.4±2.10 d, e |
| 44C | Acetic acid | 94.6±4.79 h | 15.25±3.30 a, b, c | 28.1±1.70 c, d, e | 17.4±7.16 b, c, d |
| | Glucose | 58.5±8.60 f | 31.0±5.69 c, d | 16,4±1.76 a, b | 30.4±5.79 c |
| 43C | Acetic acid | 0±0 a | 31.2±9.70 c, d, e, f | 36.4±9.88 e, f | 37.5±3.17 e, f |
| | Glucose | 100±0 g | 96.94±3.17 g | 40.7±7.42 d, e | 100±0 g |
| 45C | Acetic acid | 16.0±4.06 a, b, c | 40.3±6.60 e, f | 33.4±6.88 d, e, f | 40.1±6.58 e, f |
| | Glucose | 100±0 g | 97.4±2.28 g | 23.8±6.61 a, b, c | 100±0 g |
| S26 | Acetic acid | 46.8±4.99 f | 45.9±5.60 f | 86.7±2.63 g, h | 44.6±3.58 e, f |
| | Glucose | 100±0 g | 87.7±10.72 g | 100±0 g | 100±0 g |
| S30 | Acetic acid | 8.6±4.44 a, b | 39.9±5.70 e, f | 36.3±4.91 e, f | 35.1±6.37 e, f |
| | Glucose | 100±0 g | 98.2±3.15 g | 31.7±5.40 c, d | 100±0 g |
| S29 | Acetic acid | 31.4±2.47 c, d, e, f | 82.5±3.03 g, h | 9.6±3.03 a, b | 43.3±4.75 e, f |
| | Glucose | 92.7±1.15 g | 56.8±4.65 f | 17.3±2.86 a, b | 14.85±4.98 a |

Results obtained for strains and culture conditions with the same letter are not significantly different ($P<0.001$).

consumption and growth require the presence of oxygen as provided under our limited-aerobic or aerobic experimental setup. The differences between the three culture conditions were statistically significant ($P<0.001$). As shown in Fig. 2, maximum growth occurred under aerobic conditions and the consumption of acetic acid was close to 90% (after 432 h of incubation), accompanied by a considerable consumption of ethanol. Under limited-aerobic condition, the percentage of acetic acid reduction (61.5%) and the decrease in the alcoholic degree (1.4) achieved were lower (Table 6).

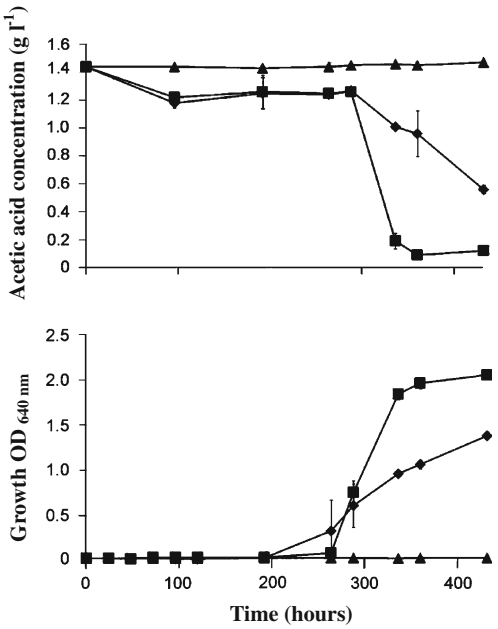


**Fig. 2** Growth (OD 640 nm) of the *S. cerevisiae* strain S26 and acetic acid consumption (g $l^{-1}$) under aerobic (*squares*), limited-aerobic (*diamonds*), and anaerobic conditions (*triangles*) when cultivated in wine 3 (see Table 1) with ethanol 10.4% (*v*/*v*), a volatile acidity of 1.44 g $l^{-1}$ (see wine 3 in Table 1), at 25°C and pH 3.55

## Discussion

From a set of 135 yeast isolates, four strains revealed ability to use glucose and acetic acid simultaneously. Three of the four isolated strains were identified as *S. cerevisiae* species and one as *L. thermotolerans*. The commercial wine strains *S. cerevisiae* S26, S29, and S30 also revealed ability to degrade glucose and acetic acid simultaneously. These three strains together with the isolated *S. cerevisiae* strains 43C and 45C and *L. thermotolerans* strain 44C were further assessed regarding their ability to use acetic acid in refermentation assays with media containing acidic wines.

The isolated strain *L. thermotolerans* 44C displays a behavior similar ($P>0.05$) to the reference strain *Z. bailii* ISA 1307 both regarding acetic acid and glucose degradation in the presence of high glucose and low ethanol concentrations, under aerobic conditions. *L. thermotolerans*

**Table 6** Consumption of acetic acid in wine by *S. cerevisiae* strain S26, under aerobic, limited-aerobic, and anaerobic conditions, after 432 h

| Aeration conditions | Final ethanol degree % (v/v) | Final volatile acidity g $l^{-1}$ |
|---|---|---|
| Aerobic | 6.5±0.21 | 0.12±0.04 |
| Limited-aerobic | 9.0±0.28 | 0.56±0.06 |
| Anaerobic | 8.6±0.14 | 1.47±0.00 |

The initial alcoholic degree of the wine was 10.4% (*v*/*v*); the initial volatile acidity was 1.44 g $l^{-1}$ (see wine 3 in Table 1)

has been used for wine production as active dry yeast in combination with *T. delbrueckii* and *S. cerevisiae* for enhancing the aroma and flavor of wine (Hansen et al. 2001). Under such conditions, *S. cerevisiae* strains display highly significant differences ($P<0.001$) when compared to the same reference strain. Better performance of *L. thermotolerans* regarding acetic acid degradation, comparatively with the *S. cerevisiae* strains, is lost under limited-aerobic conditions. This fact is consistent with the lesser tolerance of *L. thermotolerans* to low oxygen availability than *S. cerevisiae* strains as described by Hansen et al. (2001). On the other hand, in a medium containing low glucose (3.3% *w*/*v*) and high ethanol (10% *v*/*v*) under aerobic conditions, S26 is the most efficient acid-degrading strain ($P<0.001$). These results suggest that under aerobic conditions *S. cerevisiae* strains are less affected by the higher ethanol concentration. However, in a refermentation process with low oxygen availability, and in a medium containing high glucose (13% *w*/*v*) and low ethanol (4% *v*/*v*) initial concentrations, the *S. cerevisiae* strain S29 is the most efficient strain. Under these same limited-aerobic conditions but in a medium containing initial low glucose (3.3% *w*/*v*) and high ethanol (10% *v*/*v*), all *S. cerevisiae* strains studied display acetic acid degradation efficiencies identical to *Z. bailii* ISA 1307. Therefore, our data show that *S. cerevisiae* strains, under limited-aerobic conditions, can be more or equally efficient as *Z. bailii* ISA 1307 regarding acetic acid consumption.

Taking into account that the limited-aerobic conditions are more realistic from the perspective of future enological implementation and considering that *Z. bailii* is undesirable for enological applications, our data show that *S. cerevisiae* strains tested can be used to decrease in about 80% and 40% of the volatile acidity of an acidic wine containing culture medium under limited-aerobic conditions both for high glucose–low ethanol and low glucose–high ethanol concentrations, after 72 h, respectively. Therefore, the *S. cerevisiae* strains characterized are capable of removing acetic acid independently of the relative amounts of glucose and ethanol. These findings are important from an applied point of view because the strains appear versatile and can be applied in less restrictive conditions regarding glucose–ethanol concentrations. At the same time, we find out that *S. cerevisiae* can decrease volatile acidity of wines with an elevated content of acetic acid (1.44 g $l^{-1}$) and low residual sugar (1.1 g $l^{-1}$, see Table 1), even without further sugar addition, in conditions where oxygen is limited (strain S26).

In this line, the selected *S. cerevisiae* strains will efficiently conduct a refermentation process for volatile acidity reduction. Although it was generally accepted that *S. cerevisiae* strains were unable to consume acetic acid in the presence of glucose, the results obtained show physiological diversity among strains of this species, validating our initial purpose to search for interesting strains among indigenous and commercial yeasts. It is noteworthy that the acetic acid removal efficiencies were obtained for initial concentrations about twofold higher (1.1 g $l^{-1}$) than the values proposed for a typical refermentation assay (0.6 g $l^{-1}$). It can be therefore anticipated that, in a refermentation process in industrial settings, the desired acetic acid reduction occurs in less than 72 h and the respective experiments are now underway.

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