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Removing gluconic acid by using different treatments with a *Schizosaccharomyces pombe* mutant: Effect on fermentation byproducts

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Abstract

Three different treatments involving inoculation with *Schizosaccharomyces pombe* YGS-5 and *Saccharomyces cerevisiae* G1 strains were tested with a view to reducing the amount of gluconic acid in synthetic medium. The treatments involved (a) simultaneous inoculation with *S. cerevisiae* and *S. pombe* (**SpSc**); (b) depletion of gluconic acid with *S. pombe* and subsequent inoculation with *S. cerevisiae* following removal of *S. pombe* from the medium (**Sp** − **Sp** + **Sc**); and (c) as (b) but without removing *S. pombe* from the medium (**Sp** + **Sc**). The results thus obtained were compared with those for a control treatment involving fermentation with *S. cerevisiae* alone (**Sc**). The amounts of volatile compounds quantified in the fermented media were similar with the treatments where gluconic acid was previously depleted (*viz.* **Sp** − **Sp** + **Sc** and **Sp** + **Sc**). Amino acids were used in large amounts by *S. pombe* during removal of gluconic acid; this affected subsequent fermentation by *S. cerevisiae* and the formation of byproducts. Based on the gluconic acid uptake, fermentation kinetics, volatile composition and absence of off-flavours in the fermented media, both treatments (**Sp** − **Sp** + **Sc** and **Sp** + **Sc**) can be effectively used in winemaking processes to remove gluconic acid from must prior to fermentation.

Keywords: Gluconic acid; *Schizosaccharomyces pombe*; *Saccharomyces cerevisiae*; Volatile compounds; Amino acids; Fermentation

1. Introduction

Wine from rotten grapes contains high concentrations of gluconic acid formed in the metabolism of fungi (e.g., *Botrytis cinerea*) and acetic acid bacteria (e.g., *Acetobacter*, *Gluconacetobacter*) in rotten grapes. Gluconic acid affects wine stability during aging and storage, and poses a high risk of alteration, mainly through metabolization of the acid by various microorganisms such as lactic acid bacteria (Pérez, Valcárcel, González, & Domecq, 1991). Also, gluconic acid binds SO_2 very easily (Barbe, de Revel, & Bertrand, 2002), which requires entails using increased amounts of the oxide to ensure appropriate development of must fermentation and the absence of unwanted effects during wine storage.

Biological aging is the most crucial step in the production of sherry wines, which are aged in contact with microorganisms [mainly flor yeasts, which form a biofilm (velum) on the wine surface] for at least 4 years. During the process, a number of wine components are consumed and others formed that endow sherry wine with unique analytical and sensory features (Berlanga, Peinado, Millán, Mauricio, & Ortega, 2004).

High concentrations of gluconic acid can alter the biological aging of sherry wines through heterolactic fermentation; this is a result of the metabolism of lactic acid bacteria, which produce large amounts of lactic acid and increase volatile acidity – all of which affect wine quality (Pérez et al., 1991). Previous studies have shown that the flor yeast strain *Saccharomyces cerevisiae* var. *capensis* G1 (ATCC No. MYA-2451) metabolizes gluconic acid during the biological aging of sherry wines (Peinado, Moreno, Ortega, & Mauricio, 2003).

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Schizosaccharomyces pombe has been widely used for the biological deacidification of musts containing high concentrations of malic acid (Dharmadhikari & Wilker, 1998; Silva et al., 2003; Snow & Gallander, 1979; Sousa, Mota, & Leao, 1995). However, the resulting wines exhibit off-flavors (Gallander, 1977; Rankine, 1966; Snow & Gallander, 1979). Recently, we used a wild *S. pombe* yeast strain to reduce the gluconic acid content in wine (Peinado et al., 2004; Peinado, Mauricio, Medina, & Moreno, 2004).

Although both *S. cerevisiae* and *S. pombe* provide good results (they remove 30–50% of all gluconic acid present), neither yeast can fully deplete gluconic acid from treated wines. Therefore, a special treatment capable of efficiently removing gluconic acid from grape musts containing high levels of this compound prior to their alcoholic fermentation is required so that, after alcoholic fermentation, the resulting wines can be aged by using substantially reduced amounts of SO_2 without the above-described problems. The potential of a glucose-transport-deficient mutant of *S. pombe* for removing gluconic acid from grape must was recently studied in some respects (Peinado, Moreno, Medina, & Mauricio, 2005).

The principal aim of this work was to establish the influence of various *S. pombe* based treatments for the removal of gluconic acid from a synthetic medium mimicking grape must on its fermentation behavior.

2. Material and methods

2.1. Yeast strains and inocula

Two different yeast species were used. One was the yeast strain YGS-5, a glucose-transport-deficient, leucine-auxotrophic mutant of *S. pombe* that was obtained and supplied by Milbradt and Höfer (1994), and used here to deplete gluconic acid in synthetic media. The other was the flor yeast strain *S. cerevisiae* var. *capensis* (G1; ATCC No. MYA-2451), which was used to ferment the synthetic media.

The *S. pombe* YGS-5 yeast strain was grown on YNB medium containing amino acids (Difco) and supplemented with a 100 mg L^{-1} concentration of L-leucine (Sigma) and 3% D-gluconate potassium salt (Merck). The *S. cerevisiae* yeast strain was grown on YM medium (0.3% w/v yeast extract, 0.3% w/v malt extract and 0.5% w/v peptone, pH 6.5) containing 5% w/v glucose as carbon source (Merck). Both yeast strains were incubated at 28 ± 1 °C with shaking for 72 h. Cells were collected by centrifugation at 3500*g*. Media were inoculated with a population of 10^7 living cells mL^{-1} of *S. pombe* YGS-5 strain and/or 2×10^6 living cells mL^{-1} of *S. cerevisiae* G1 strain. The number of total and living cells was determined by counting under a light microscope in a Thoma chamber following staining with Methylene Blue (EBC, 1977).

2.2. Fermentation medium

The synthetic medium was prepared by diluting rectified concentrated grape must (840 g sugar L^{-1}) to a 200 g L^{-1} concentration of glucose + fructose with water and supplementing it with 0.3% w/v yeast extract, 2.5 g L^{-1} gluconic acid, 1 g L^{-1} malic acid, 3 g L^{-1} tartaric acid (Merck) and 100 mg L^{-1} L-leucine (Sigma). The medium was adjusted to pH 3.5 and sterilized by passage through Supra EK filters (Seitz, Germany).

2.3. Culture conditions

Simultaneous and sequential inoculations with both yeasts, and a control treatment involving inoculation with the fermentative yeast alone, were done. For sequential inoculation, 2 L of synthetic medium was inoculated with 10^7 living cells mL^{-1} of *S. pombe*, redistributed in three 1 L flasks that were continuously shaken on an Infors ag ch-4103 shaker at 150 rpm at 28 ± 1 °C until gluconic acid was depleted. Then, shaking was stopped and the resulting media were redistributed in six 500 mL flasks that were filled to 300 mL. The media contained in three of the flasks were centrifuged at 3500*g* to remove *S. pombe* prior to inoculation with 2×10^6 living cells mL^{-1} of *S. cerevisiae*; this treatment was designated **Sp − Sp + Sc**. The other three flasks were inoculated with 2×10^6 living cells mL^{-1} of *S. cerevisiae* without removing *S. pombe*, the treatment being designated **Sp + Sc**.

Three other flasks containing 300 mL of initial medium were simultaneously inoculated with 2×10^6 living cells mL^{-1} of *S. cerevisiae* G1 strain and 10^7 living cells mL^{-1} of *S. pombe*. This treatment was designed **SpSc**.

Finally, three other flasks containing 300 mL of initial medium were inoculated with *S. cerevisiae* G1 strain (2×10^6 living cells mL^{-1}) and used as control media (**Sc**).

Development of the fermentation process was monitored via the amount of CO_2 released as measured from the weight loss (Bely, Sablayrolles, & Barre, 1990; Sablayrolles, Barre, & Grenier, 1987).

2.4. Analytical methods

Ethanol, titratable acidity, pH and volatile acidity were determined in accordance with the Official Report of the European Community (EEC, 1990). Gluconic, malic and acetic acids were determined by using their respective enzymatic tests from Boehringer–Mannheim, Germany.

Major volatile compounds and polyols were quantified on a model 6890 gas chromatograph from Agilent Technologies (Palo Alto, CA), using the method of Peinado, Moreno, Muñoz, Medina, and Moreno (2004). A CP-WAX 57 CB capillary column (60 m long × 0.25 mm i.d., 0.4 μm film thickness) was used. Aliquots of 0.5 μL from 10 mL samples previously supplied with 1 mL of 1 g L^{-1} 4-methyl-2-pentanol as internal standard were used for injection into the instrument. Tartaric acid in the wine was previously removed by precipitation with 0.2 g of calcium carbonate and centrifugation at 300*g*.

Quantitation was based on the response factors obtained for standard solutions of each compound. A split

ratio of 30:1, an FID, and a temperature program involving an initial temperature of 50 °C (15 min), a 4 °C min^{-1} ramp and a final temperature of 190 °C (35 min) were used. The injector and detector temperatures were 270 and 300 °C, respectively. The flow rate of carrier gas (helium) was initially set at 0.7 mL min^{-1} (16 min) and followed by a 0.2 mL min^{-1} ramp to the final value (1.1 mL min^{-1}), which was held for 52 min.

Minor volatile compounds were determined by capillary column gas chromatography–mass spectrometry following continuous extraction of 100 mL of medium with 100 mL of Freon-11 for 24 h. Samples were adjusted to pH 3.5 and 5 mL of a 30 mg L^{-1} solution of 2-octanol was added as internal standard. The Freon extracts containing the volatile compounds were concentrated to 0.2 mL in a Kuderna–Danish microconcentrator and 1.5 μL aliquots injected into an HP-6890 gas chromatograph equipped with an HP MS 5972A mass detector (Agilent Technologies, Palo Alto, CA). An HP-Innowax fused silica capillary column (60 m long × 0.32 mm i.d., 0.25 μm film thickness) was used. The temperature program was as follows: initial temperature 40 °C, held for 10 min, and 1 °C min^{-1} ramp to 180 °C, held for 35 min. Helium at a constant flow rate of 0.9 mL min^{-1} was used as carrier gas, and a 30:1 split ratio employed at the injection port. The mass detector was used at a voltage of 1612 V in the scan mode to cover a mass range from 39 to 300 amu.

Retention times, spectral libraries supplied by Wiley, and pure chemical compounds obtained from Merck, Sigma–Aldrich, Riedel de Haën and Fluka, were used for identification, confirmation and preparation of standard solutions of the volatiles studied. Each compound was quantified from its response factor, which was obtained from standard solutions of known concentration subjected to the same treatment as the samples, using the target and qualifier ions for each compound selected by a Hewlett–Packard Chemstation (Palo Alto, CA).

Free amino acids were determined according to Botella, Pérez-Rodríguez, Domecq, and Valpuesta (1990). Amino acids were quantified from the absorbances at 254 nm of their dansyl derivatives, which were previously isolated by high-performance liquid chromatography on a Spectra-Physics P200 HPLC instrument equipped with an SP 8450 UV–vis detector and a 15 × 0.4 cm reversed-phase column packed with Spherisorb ODS2 resin of 5 μm particle size obtained from Tracer Analitica (Barcelona, Spain) and thermostated at 25 °C. A volume of 20 μl of 5 mmol L^{-1} L-norleucine was used as internal standard.

2.5. Odor perception threshold determination

The odor perception threshold is defined as the lowest concentration capable of producing a sensation. Such a sensation must be detected by at least 50% of the judges in a taste panel (Cutzach, Chatonnet, & Dubourdieu, 2000; Kotseridis & Baumes, 2000). Different solutions containing increasing concentrations of each compound were used for this purpose. Starting from the least concentrated solution, the judges were asked to identify the first solution whose stimulus departed from that perceived in the control. The control consisted of a 1:10 ethanol/water mixture containing none of the studied compounds. The judges, who were trained as recommended by AENOR (1997), were not selected by their ability to identify a particular aroma descriptor or the intensity of such an aroma; the panel consisted of 30 individuals of both sexes aged 20–55 years. All provided an odor descriptor for the studied compounds in the solution where the odor descriptor of concerned compound was clearly perceived.

2.6. Statistical treatment

The compounds exhibiting significant differences between fermentations were identified by one-way analysis of variance (ANOVA). Differences between treatments were established from a principal component analysis. The statistical software package Statgraphics Plus v. 2, from STSC, Inc. (Rockville, MD), was used in both cases. The results given are the averages of three independent tests each.

3. Results and discussion

3.1. Winemaking variables and fermentation kinetics

The gluconic acid concentration was decreased by 91% (2.3 g) of its initial level within 30 h after inoculation of the synthetic media with *S. pombe*. By contrast, the decrease was only 0.3 g after 5 days in the tests involving simultaneous inoculation with*S. pombe* and *S. cerevisiae* (**SpSc**), the gluconic acid concentration levelling off beyond that point; this treatment was thus discarded for subsequent tests. Similar results were previously obtained by Corte-Real and Leao (1992) with *Hansenula anomala* and *S. cerevisiae* yeasts inoculated at once: no malic acid uptake was detected, probably as a consequence of ethanol inhibiting malic acid transport through cell membranes.

Fig. 1 illustrates the kinetics of the three fermentation tests. The highest ethanol production rate was similar in all cases; however, the media that were previously inoculated with *S. pombe* (treatments **Sp + Sc** and **Sp − Sp + Sc**) exhibited a delayed kinetics in relation to the control treatment, which involved inoculation with *S. cerevisiae* alone (**Sc**).

Table 1 lists the winemaking variables in the initial media and at the end of the three fermentation tests. Titratable acidity and pH values exhibited significant differences as a result of gluconic acid uptake by *S. pombe*. Also, volatile acidity was significantly different in the three tests; its low values in all cases suggest appropriate fermentation development. As expected, *S. pombe* also used malic acid (Gallander, 1977; Rankine, 1966; Snow & Gallander, 1979).

Sugars were depleted in all tests; however, a slightly lower ethanol concentration was obtained in that where the mutant was removed by centrifugation after gluconic

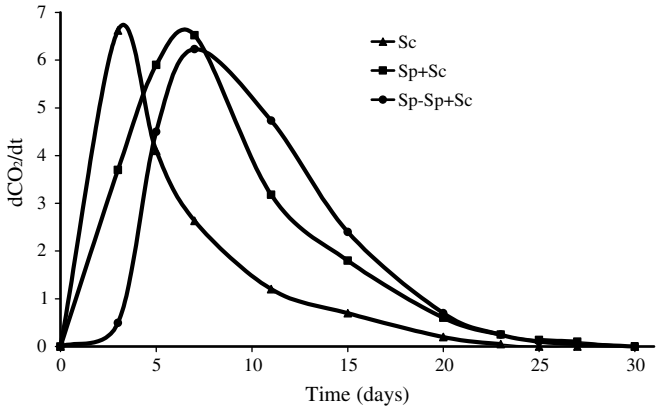

Fig. 1. Fermentation kinetics in media containing high gluconic acid concentrations. **Sc**, control fermentation with *Saccharomyces cerevisiae* G1; **Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5 and with *S. cerevisiae* to ferment the medium after gluconic acid was depleted; **Sp − Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5, removal of this yeast by centrifugation after gluconic acid was depleted and inoculation with *S. cerevisiae* to ferment the medium.

acid was depleted (**Sp − Sp + Sc**). This may have resulted from an increased activity of glyceropyruvic fermentation as suggested by the increased production of glycerol, acetaldehyde and its derivatives butanedione, acetoin and 2,3-butanediol (Tables 1 and 2). In the beginning of alcoholic fermentation, yeast grows in the presence of oxygen. Their pyruvate decarboxylase and alcohol dehydrogenase are weakly expressed. As a result, glycerol and some secondary products are formed. These secondary products are pyruvate derivatives (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2000).

Glycerol production by yeasts helps balance the intracellular redox potential by facilitating reoxidation of NADH previously formed during glycolysis. In this way, yeasts use accumulated pyruvic acid to produce butanedione, acetoin and 2,3-butanediol (Romano & Suzzi, 1996).

The yeasts produce 2,3-pentanedione via a mechanism similar to that for butanedione; removing *S. pombe* YGS-5 after gluconic acid was depleted resulted in the formation of increased amounts of the former (see Table 2). Both processes begin with activation of acetaldehyde as pyrophosphate thiamine, which results from the enzymatic decarboxylation of pyruvic acid. The main biosynthetic pathway from this intermediate to C4 derivates (butanedione) involves L-valine and L-leucine, whereas that leading to C5 derivatives (pentanedione) involves L-isoleucine (Bayonove, Baumes, Crouzet, & Günata, 2000).

Removing *S. pombe* affected the concentrations of glycerol, acetoin, 2,3-butanediol and 2,3-butanedione. Differences between the **Sp − Sp + Sc** and **Sp + Sc** treatments resulted from presence of *S. pombe* mutant and/or the yeast removal treatment.

3.2. Volatile compounds

The concentrations of major higher alcohols (*viz.* isobutanol, isoamyl alcohols and 2-phenylethyl alcohol) were minimal in the control fermentation and exhibited no significant differences between sequentially inoculated media (Table 2). Higher alcohols are synthesized via the pool of α-ketoacids, which are produced in amino acid catabolism and sugar anabolism in a respective proportion of 25% and 75%, respectively. Regulating these two pathways is very complicated and subject to a strong influence from the particular nitrogen source. The presence of substantial amounts of amino acids inhibits the anabolic pathway in favor of the catabolic production of higher alcohols, the absence of a nitrogen source having the opposite effect (Bayonove et al., 2000). The marked decrease in amino acid levels observed following the removal of gluconic acid by *S. pombe* (Table 3) suggests that the anabolic pathway may be favored during the subsequent fermentation with *S. cerevisiase*, and that the control fermentation may have been affected in the opposite way, thereby accounting for the differences observed.

The synthesis of propanol is a more complex process by effect of its being connected to the metabolism of both sugars and sulphur. The amount of propanol produced is inversely proportional to that of hydrogen sulfide and depends on the particular yeast strain when the amount of nitrogen available is not a limiting factor (Guidici, Zam-

Table 1
Winemaking variables for initial and fermented media[a] and the homogeneous group[b] for fermented media

Compound	IM	Sc	Sp + Sc	Sp − Sp + Sc
Reducing sugars (g L^{-1})	205 ± 5	3.1 ± 0.5a	2.7 ± 0.6a	2.5 ± 0.4a
Ethanol (% v/v)	0 ± 0	11.6 ± 0.2a	11.5 ± 0.2a	10.9 ± 0.2b
pH	3.4 ± 0.1	3.5 ± 0.1a	3.7 ± 0.1b	3.7 ± 0.1b
Titratable acidity (meq L^{-1})	73 ± 1	71 ± 1a	64 ± 1b	64 ± 1b
Volatile acidity (acetic acid g L^{-1})	0 ± 0	0.60 ± 0.06a	0.30 ± 0.2b	0.45 ± 0.04c
Glycerol (g L^{-1})	0 ± 0	4.7 ± 0.4a	3.8 ± 0.5b	7.5 ± 0.2c
Malic acid (g L^{-1})	1.0 ± 0.1	0.70 ± 0.03a	0.08 ± 0.01b	0.08 ± 0.01b
Gluconic acid (g L^{-1})	2.6 ± 0.1	2.4 ± 0.1a	0.22 ± 0.04b	0.22 ± 0.04b

[a] **IM**, initial medium; **Sc**, control fermentation with *Saccharomyces cerevisiae* G1; **Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5 and with *S. cerevisiae* to ferment the medium after gluconic acid was depleted; **Sp − Sp + Sc**, inoculation with *Schizosaccharomyces pombe* YGS-5, removal of this yeast by centrifugation after gluconic acid was depleted and inoculation with *S. cerevisiae* to ferment the medium.

[b] Different letters denote significant differences at $p \leqslant 0.05$.

Table 2
Concentrations of volatile compounds quantified in fermented media[a] and the homogeneous group[b]

Compound	Sc	Sp + Sc	Sp − Sp + Sc
Acetoin (mg L^{-1})	19 ± 1a	21 ± 1b	396 ± 38b
2,3-Butanediol, *levo* (g L^{-1})	1.3 ± 0.1a	0.30 ± 0.07a	1.9 ± 0.2c
2,3-Butanediol, *meso* (mg L^{-1})	335 ± 23a	226 ± 36b	475 ± 36c
1-Propanol (mg L^{-1})	13 ± 1a	8 ± 1b	10 ± 1b
1-Isobutanol (mg L^{-1})	10 ± 1a	40 ± 5b	38 ± 2b
Isoamyl alcohol (mg L^{-1})	75 ± 2a	115 ± 6b	109 ± 3b
2-Phenylethanol (mg L^{-1})	12 ± 1a	19 ± 2b	19 ± 1b
3-Ethoxy-1-propanol (mg L^{-1})	2.0± 0.4a	nd b	nd b
Benzyl alcohol (μg L^{-1})	125 ± 20a	351 ± 38b	274 ± 22c
Methionol (mg L^{-1})	1.6 ± 0.4a	10 ± 2b	10 ± 1b
Ethyl acetate (mg L^{-1})	13 ± 2a	14 ± 1a	7 ± 1b
Ethyl propanoate (μg L^{-1})	204 ± 32a	390 ± 20b	398 ± 37b
Ethyl lactate (mg L^{-1})	20 ± 1a	21 ± 1a	21 ± 1a
Ethyl butanoate (μg L^{-1})	71 ± 8a	66 ± 13a	82 ± 13a
Ethyl 3-hydroxybutanoate (μg L^{-1})	131 ± 20a	77 ± 20b	287 ± 16c
Ethyl hexanoate (μg L^{-1})	160 ± 16a	50 ± 4b	16 ± 3c
Ethyl octanoate (μg L^{-1})	119 ± 10a	nd b	nd b
Ethyl succinate (mg L^{-1})	3.1 ± 0.5a	23 ± 4b	16 ± 1c
Diethyl succinate (mg L^{-1})	3 ± 1a	3 ± 1a	3 ± 1a
Isobutyl acetate (μg L^{-1})	21 ± 3a	47 ± 4b	48 ± 2b
Isoamyl acetate (μg L^{-1})	76 ± 4a	85 ± 13a	19 ± 2b
2-Phenylethyl acetate (μg L^{-1})	99 ± 6a	53 ± 3b	50 ± 3b
Isobutanoic acid (mg L^{-1})	1.9 ± 0.3a	9.0 ± 0.9b	9.1 ± 0.4b
Butanoic acid (mg L^{-1})	1.8 ± 0.1a	1.5 ± 0.1b	1.8 ± 0.3ab
2 & 3-methylbutanoic acid (mg L^{-1})	1.5 ± 0.2a	3.4 ± 0.3b	5.2 ± 0.4c
Hexanoic acid (μg L^{-1})	1524 ± 85a	601 ± 40b	559 ± 84b
Octanoic acid (μg L^{-1})	3091 ± 290a	849 ± 102b	224 ± 34c
Decanoic acid (μg L^{-1})	357 ± 63a	110 ± 16b	72 ± 13b
2,3-Butanedione (μg L^{-1})	64 ± 16a	76 ± 8a	175 ± 20b
2,3-Pentanedione (μg L^{-1})	85 ± 6a	55 ± 5a	303 ± 28b
γ-Butyrolactone (mg L^{-1})	1.1 ± 0.1a	1.5 ± 0.2b	0.5 ± 0.1c
Pantolactone (μg L^{-1})	199 ± 28a	475 ± 60b	457 ± 30b
Acetaldehyde (mg L^{-1})	31 ± 5a	53 ± 5b	88 ± 5c
Benzaldehyde (μg L^{-1})	22 ± 3a	57 ± 12b	33 ± 4c

[a] **Sc**, control fermentation with *Saccharomyces cerevisiae* G1; **Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5 and with *S. cerevisiae* to ferment the medium after gluconic acid was depleted; **Sp − Sp + Sc**, inoculation with *Schizosaccharomyces pombe* YGS-5, removal of this yeast by centrifugation after gluconic acid was depleted and inoculation with *S. cerevisiae* to ferment the medium.
[b] Different letters denote significant differences at $p \leqslant 0.05$.

Table 3
Amino acid concentrations (mM) in the initial and fermented media[a]

Amino acids	IM	Sc	MAGAD	Sp + Sc	Sp − Sp + Sc
Aspartic acid	0.84 ± 0.03	0.61 ± 0.01	nd	nd	nd
Glycine	0.31 ± 0.02	0.44 ± 0.08	nd	nd	nd
Threonine	1.21 ± 0.14	nd	1.65 ± 0.09	nd	0.21 ± 0.03
Alanine	0.80 ± 0.03	0.74 ± 0.02	nd	0.01 ± 0.00	nd
α-Aminobutyric acid	0.22 ± 0.01	0.10 ± 0.01	nd	0.12 ± 0.02	0.09 ± 0.02
Proline	0.45 ± 0.02	0.56 ± 0.03	nd	nd	nd
Methionine	0.24 ± 0.03	0.23 ± 0.08	nd	0.13 ± 0.02	nd
Valine	0.54 ± 0.02	0.35 ± 0.04	0.26 ± 0.03	0.12 ± 0.02	nd
Isoleucine + phenylalanine	0.35 ± 0.02	0.29 ± 0.01	nd	nd	nd
Leucine	1.49 ± 0.09	1.38 ± 0.08	0.19 ± 0.03	1.22 ± 0.04	0.95 ± 0.05
Lysine	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.00	0.07 ± 0.01	0.06 ± 0.01
Tyrosine	0.04 ± 0.01	nd	nd	nd	nd
Glutamine	nd	0.48 ± 0.02	nd	nd	nd
γ-Aminobutyric acid	nd	0.21 ± 0.01	nd	0.14 ± 0.02	0.29 ± 0.02
Ornithine	nd	0.04 ± 0.01	nd	0.11 ± 0.02	0.06 ± 0.01
∑Amino acids	6.53	5.49	2.15	1.92	1.44

[a] **IM**, initial medium; **Sc**, control fermentation with *Saccharomyces cerevisiae* G1; **Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5 and with *S. cerevisiae* to ferment the medium after gluconic acid was depleted; **Sp − Sp + Sc**, inoculation with *Schizosaccharomyces pombe* YGS-5, removal of this yeast by centrifugation after gluconic acid was depleted and inoculation with *S. cerevisiae* to ferment the medium. **MAGAD**: medium after gluconic acid was depleted by *S. pombe* YGS-5.

bonelli, & Kunkee, 1993). In this work, the propanol concentration peaked in the control fermentation test (**Sc**).

Methionol (3-methyl-thio-propanol) exhibited a high concentration but no 3-ethoxy-1-propanol was detected in the media treated with *S. pombe* after gluconic acid was depleted (Table 2). A high production of methionol in wines containing high gluconic acid levels that were biologically aged with submerged cultures of *S. cerevisiae* (G1) was also previously observed by Peinado et al. (2003) and Peinado et al. (2004). These two compounds are mutually related; thus, 3-ethoxy-1-propanol is synthesized from homoserine via *O*-acetylhomoserine, which is a key compound in the biosynthetic pathway leading to methionine (the precursor of methionol) (Irwin, 1992).

Fatty acids with 2–10 carbon atoms, their ethyl esters and the acetates of higher alcohols are all derivatives of acyl-*S*-coenzyme, the biogenesis of which depends on the lipid and nitrogen metabolisms of the yeasts. Isobutanoic, 2-methylbutanoic and 3-methylbutanoic acids exhibited higher concentrations in the sequentially inoculated media (**Sp + Sc** and **Sp − Sp + Sc**) after fermentation. Similar results were previously obtained in wines containing high levels of gluconic acid that were treated with *S. cerevisiae* (Peinado et al., 2003) and *S. pombe* (Peinado, Moreno, Medina, et al., 2004) for its removal. Therefore, these acids must be related with the metabolism of gluconic acid. C_6–C_{10} acids and their ethyl esters are inhibitors of yeast growth, their formation being strongly influenced by the particular fermentation conditions (Geneix, Lafon-Lafourcade, & Ribéreau-Gayon, 1983); also, they are adsorbed by cell walls (Alexandre, Lubbers, & Charpentier, 1997; Ribéreau-Gayon, 1985). In this work, the control medium exhibited the highest concentrations of hexanoic and octanoic acids, which are clearly related with those of their ethyl esters; by contrast, butanoic acid and ethyl butanoate exhibited no significant differences in concentration between the three treatments (see Table 2).

Other ethyl esters such as ethyl propanoate and ethyl succinate exhibited significantly high levels in two fermenting media inoculated with *S. cerevisiae* after gluconic acid was depleted. The treatment not including removal by centrifugation of *S. pombe* (**Sp + Sc**) provided the highest concentrations of these esters. As regards the esters of acetic acid, only isobutyl acetate exhibited a significantly higher concentration in the two treatments with *S. pombe*; by contrast, 2-phenylethyl acetate exhibited the lowest concentrations in such treatments. Also, the isoamyl acetate uptake was affected by the removal of *S. pombe*: the compound exhibited a significantly decreased concentration in the medium subjected to the **Sp − Sp + Sc** treatment.

A relationship between benzyl alcohol and benzaldehyde was also observed. Thus, the control exhibited low concentrations of both compounds, whereas the treatment involving *S. cerevisiae* in the presence of *S. pombe* (**Sp + Sc**) provided the highest levels. According to Delfini et al. (1991), *Schizosaccharomyces* and *Zygosaccharomyces* yeasts are the most efficient producers of benzaldehyde and benzyl alcohol.

γ-Butyrolactone and pantolactone (α-hydroxy-β,β-dimethyl-γ-butyrolactone) are intramolecular esters of γ-hydroxybutyric acid and pantotenic acid, respectively. Pantolactone exhibited decreased concentrations in the control test, and so did γ-butyrolactone in the **Sp − Sp + Sc** test. Neither lactone appears to be related to gluconic acid metabolism (Peinado et al., 2003; Peinado, Mauricio, Medina, et al., 2004; Peinado, Moreno, Medina, et al., 2004), even though both have been reported as typical byproducts of alcoholic fermentation by yeasts (Bayonove et al., 2000).

The differences in volatile compounds between the two treatments and the control can be ascribed to the metabolic activity of *S. pombe* during its consumption of gluconic acid, and also to the disparate behaviour of *S. cerevisiae* in fermentation media with decreased amino acid levels relative to the control – as was the case with the two treatments involving sequential inoculation. As is recently reported by Peinado et al. (2005), *S. pombe* YGS-5 yeast strain produced no volatile compounds other than those obtained in fermentations conducted with *S. cerevisiae*. Nevertheless, significant differences in the concentration of some volatiles were obtained, after gluconic acid was removed by this fission yeast.

Gluconic acid is metabolized via the Entner–Doudoroff pathway (EDP) and pentose phosphate pathway (PPP), producing NADPH and glyceraldehyde-3-phosphate – which flows to acetylCoA. These pathways, together with glycolysis (combined with the tricarboxylic acid cycle), also have anabolic functions as they provide the precursors for amino acids, fatty acids, nucleotides, sugar phosphates and other compounds required for yeast growth and byproduct formation. Gluconate is directly phosphorylated to 6-phosphogluconate (6pg) by the enzyme gluconokinase, thereby directly entering PPP after the glucose-6-phosphate (g6p) branch point. By feeding yeast cells with gluconate, irreversible oxidation of g6p to 6-phosphoglucono-γ-lactone, catalyzed by g6p dehydrogenase, can be decreased. The oxidative branch of PPP is known to be significant; thus, it produces 2 mol of NADPH per mol of glucose catabolized – by contrast, only 1 mol of NADPH is produced per mol of gluconate catabolized. The equilibrium between the most oxidized forms (e.g., acids and aldehydes) and most reduced forms (alcohols) of some volatiles can be affected by the $NADP^+$/NADPH ratio. It is demonstrated that oxidative and no oxidative enzymes of the PPP in *S. pombe* are induced by gluconate, and that glucose tend to attenuate the synthesis of these enzymes (Mehta, Velnurugan, & Lobo, 1998). In contrast, *S. pombe* YGS-5, used in this study is not repressed by glucose (Milbradt & Höfer, 1994).

3.3. Free amino acid composition

The total amino acid concentration in the initial medium, 6.53 mM, decreased to 2.51 mM after gluconic acid

was depleted by *S. pombe*, the sole amino acids remaining in the medium being threonine, valine, leucine and lysine (see Table 3). This is consistent with the slower fermentation kinetics of the **Sp + Sc** and **Sp − Sp + Sc** treatments in relation to the control fermentation under *S. cerevisiae* alone (**Sc**). Several amino acids were released to the medium during alcoholic fermentation in all tests; such was especially the case with glutamine and γ-aminobutyric acid (GABA) in the control fermentation (relative to the initial medium), and leucine in the **Sp + Sc** and **Sp − Sp + Sc** treatments (relative to the media after gluconic acid was removed). The highest amino acid concentrations were found at the end of alcoholic fermentation in the control medium. The **Sp + Sc** treatment provided higher amino acid concentrations than did the **Sp − Sp + Sc** treatment. This was the likely result of the lysis of *S. pombe* yeast cells, which were not removed from the media, or of centrifugation, which may have caused aeration of the medium and boosted growth of *S. cerevisiae*, thereby increasing amino acid uptake (**Sp − Sp + Sc**).

3.4. Distinguishing gluconic acid removal treatments via odorant series

Volatiles are major contributors to the sensory quality of foods (particularly fermented beverages). The odor of a volatile compound can be described in terms of one or several descriptors agreed upon by experts (Etievant, 1991; Guth, 1997; López et al., 1999). Some authors have used odorant series to describe the aroma of wine and alcoholic beverages (Brugirard, Fanet, Seguin, & Torres, 1991; Torres, 1987). By grouping volatile compounds with a similar descriptor into odorant series, one can establish an odorant profile and determine the contribution of each individual compound to a particular series. Although this procedure cannot be interpreted as an arithmetic addition of odor sensations, it has the advantage over existing alternatives that it reduces the number of variables to be analysed. In addition, it is simpler, uses more objective criteria and facilitates comparison of the results as odorant series always comprise the same compounds (Moyano, Zea, Moreno, & Medina, 2002; Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004; Peinado, Mauricio, & Moreno, 2006).

The contribution of each compound to an odorant series is calculated from its odor activity value (OAV), which is defined as the concentration–odor perception threshold ratio (Table 4). In this way, a relation between the quantitative information provided by chemical analyses and sensory perceptions can be obtained. By combining the OAVs for the quantified volatile compounds with similar aroma descriptors, we established seven odorant series, namely: fatty, floral, fruity, herbaceous, roasty, solvent and sweet.

The fatty, fruity, solvent and sweet series exhibited significant differences between the three treatments (Table 5); on the other hand, the herbaceous and roasty series only differed significantly between the control and the two treatments involving *S. pombe* (**Sp + Sc** and **Sp − Sp + Sc**).

Table 4
Volatile compounds quantified in the wines, odor perception thresholds (OPT, in mg L^{-1}) odor descriptors and assignment to odorant series

Compound	OPT	Odor descriptor	Odorant series[a]
Acetoin	150	Buttery, cream	7
2,3-Butanediol, *levo*	150	Fruity	1
2,3-Butanediol, *meso*	150	Fruity	1
1-Propanol	306	Ripe fruit, alcohol	4, 1
Isobutanol	75	Alcohol, nail polish, wine-like	4
Isoamyl alcohols	60	Alcohol, nail polish	4
2-Phenyl ethanol	200	Rose, honey	2, 3
3-Ethoxy-1-propanol	0.1	Fruity	1
Benzyl alcohol	900	Roasted	5
Methionol	1.5	Cooked potato, cut hay	6
Ethyl acetate	12	Pineapple, varnish, balsamic	1, 4
Ethyl propanoate	1.8	Banana, apple	1
Ethyl lactate	150	Buttery, butterscotch, fruity	1, 7
Ethyl butanoate	0.4	Banana, pineapple, strawberry	1
Ethyl 3-hydroxybutanoate	1	Fruity, grape	1
Ethyl hexanoate	0.08	Banana, green apple	1
Ethyl octanoate	0.58	Banana, pineapple, pear, floral	1, 2, 3
Ethyl succinate	1200	Herbaceous	6
Diethyl succinate	1250	Fruity, floral	1
Isobutyl acetate	1.6	Sweet, fruity, apple, banana	3, 1
Isoamyl acetate	0.16	Banana, fruity	3, 1
2-Phenylethyl acetate	0.25	Fruity	2
Isobutanoic acid	30	Rancid butter	7
Butanoic acid	2.2	Cheesy, rancid, putrid	7
2 & 3-Methylbutanoic acids	1.5	Rancid	7
Hexanoic acid	3	Rancid, cheese	7
Octanoic acid	10	Rancid	7
Decanoic acid	6	Rancid	7
2,3-Butanodione	0.1	Yogurt, cake	3
2,3-Pentanodione	0.9	Buttery, sweet	3
γ-Butyrolactone	20	Caramel, coconut	3
Pantolactone	2.2	Balsamic, smoky, toasted bread	5
Acetaldehyde	110	Pungent, ripe apple	1
Benzaldehyde	2	Pungent, medicinal	2

[a] 1 – fruity, 2 – floral, 3 – sweet, 4 – solvent, 5 – roasted, 6 – herbaceous, 7 – fatty.

A principal component analysis (PCA) involving the odorant series as variables revealed that the first PC accounted for 82% of the variability in the data and the second PC for 15%. The contribution of each odorant series to PC1 and PC2 was calculated from their respective statistical values, which are shown Table 5. Only the floral and fruity series exhibited negative, similar values for PC1, all others except the solvent series having positive, also similar values. PC2 was primarily influenced by the solvent and sweet series owing to their high statistical weights. Plotting the values of the samples for PC1 against those for PC2 allowed the two treatments and the control to be clearly differentiated (see Fig. 2). Thus, the control samples (**Sc**)

Table 5
Values in odorant series for fermented media[a] and the homogeneous group,[b] and statistical values for PC1 and PC2[c]

Odorant series	**Sc**	**Sp + Sc**	**Sp − Sp + Sc**	Statistical value	
				PC1	PC2
Herbaceous	1.0 ± 0.3a	6 ± 1b	6.6 ± 0.7b	0.41	0.11
Fatty	2.8 ± 0.2a	4.1 ± 0.3b	5.3 ± 0.4c	0.39	−0.34
Floral	0.26 ± 0.02a	0.10 ± 0.01b	0.10 ± 0.01b	−0.41	−0.13
Fruity	4.7 ± 0.2a	3.5 ± 0.2b	2.6 ± 0.1c	−0.39	0.29
Roasty	0.11 ± 0.02a	0.27 ± 0.03b	0.25 ± 0.01b	0.39	0.26
Solvent	2.5 ± 0.2a	3.6 ± 0.2b	2.9 ± 0.1c	0.27	0.73
Sweet	2.2 ± 0.2a	3.0 ± 0.1b	4.2 ± 0.2c	0.37	−0.41

[a] **Sc**, control fermentation with *Saccharomyces cerevisiae* G1; **Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5 and with *S. cerevisiae* to ferment the medium after gluconic acid was depleted; **Sp − Sp + Sc**, inoculation with *Schizosaccharomyces pombe* YGS-5, removal of this yeast by centrifugation after gluconic acid was depleted and inoculation with *S. cerevisiae* to ferment the medium.
[b] Different letters denote significant differences at $p \leqslant 0.05$.
[c] From the principal component analysis: PC1 accounted for 82% and PC2 for 15% of variability in the data.

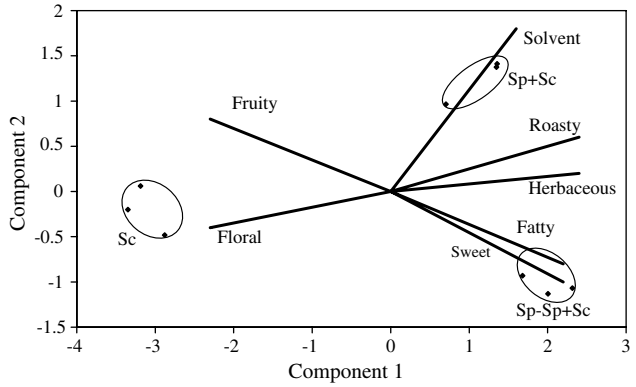

Fig. 2. Principal components analysis using odorant series as predictor variables. **Sc**, control fermentation with *Saccharomyces cerevisiae* G1; **Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5 and with *S. cerevisiae* to ferment the medium after gluconic acid was depleted; **Sp − Sp + Sc**, sequential inoculation with *Schizosaccharomyces pombe* YGS-5, removal of this yeast by centrifugation after gluconic acid was depleted and inoculation with *S. cerevisiae* to ferment the medium.

had negative values for PC1, while those of treatments **Sp + Sc** and **Sp − Sp + Sc** had similar, positive values, PC2 allowing the latter two treatments to be distinguished. Differences between treatments can be ascribed to the contribution of the floral and fruity series in the control treatment; the sweet and fatty series in **Sp − Sp + Sc**; and the solvent in **Sp + Sc**. These results do not allow one to identify the *S. pombe* treatment providing the better sensory results; in fact, this entails using standard tests, which, however, are only useful with samples for human consumption.

As reported by Gallander (1977), Rankine (1966) and Snow and Gallander (1979), wild strains of *S. pombe* produce off-flavors during alcoholic fermentation, probably as a result of their sugar metabolism. Although, the *S. pombe* YGS-5 mutant cannot metabolize sugars easily (Milbradt & Höfer, 1994; Peinado et al., 2005), our sensory analyses revealed the presence of no off-flavors with any treatment.

In conclusion, based on the fermentation kinetics, amino acid concentrations, volatile composition and odorant series, the two treatments with *S. pombe* YGS-5 may be effective with a view to removing gluconic acid from industrial grape must. Future tests on a pilot plant scale would be required in order to optimize the conditions of use of *S. pombe* in order to raise the amino acid concentrations in the must following depletion of gluconic acid and hence facilitate appropriate development of the fermentation process and the obtainment of a product with acceptable sensory quality.

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