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Incidence of osmophilic yeasts and *Zygosaccharomyces rouxii* during the production of concentrate grape juices

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ABSTRACT

Zygosaccharomyces rouxii is the main spoilage yeast of grape juice concentrates. Detection and identification of *Z. rouxii* during the production of grape juice concentrate is critical to prevent spoilage in the final product. In this work, three grape juice concentrate processing plants were assessed by identifying osmophilic yeasts in juices and surfaces during different stages of a complete production line. Subsequently, molecular typing of *Z. rouxii* isolates was done to determine the strain distribution of this spoilage yeast. Osmotolerant yeast species, other than *Z. rouxii*, were mainly recovered from processing plant environments. *Z. rouxii* was only isolated from surface samples with grape juice remains. *Z. rouxii* was largely isolated from grape juice samples with some degree of concentration. Storage of grape juice pre-concentrate and concentrate allowed an increase in the *Z. rouxii* population. A widely distributed dominant molecular *Z. rouxii* pattern was found in samples from all three processing plants, suggesting resident microbes inside the plant.

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1. Introduction

Yeasts have been used during centuries in the production of diverse foods and alcoholic beverages. However, they have also shown to be involved in the spoilage of an extensive range of foods. Yeasts are able to grow at low pH values, high sugar content and refrigeration temperature, making them potential spoilers of refrigerated or concentrated fruit juices (Stratford, 2006).

Mendoza and San Juan are the most important producers of grape juice concentrates in Argentina, and they are responsible for more than 97% of the total production (Bruzone, 1998). In addition to a low pH (pH 2.5–3.2), grape juice concentrates also have a low water activity (a_w 0.70–0.85) because of the high sugar concentration. Consequently, juices are rarely spoiled by microorganisms that typically grow at a_w values of 0.89 or higher (Combina et al., 2008). However, microorganisms such as osmophilic yeasts are able to grow (Akdeniz et al., 2013; Combina et al., 2008; Guo et al., 2013; Rojo et al., 2014). The yeasts most frequently isolated from products with high sugar content are species of the genus *Zygosaccharomyces* (Stratford, 2006). Their high resistance to weak acid preservatives and their extreme osmotolerance and vigorous fermentation of hexose sugars make them potential spoilage vectors in the food and beverage industry (Casas et al., 2004; Martorell et al., 2007; Rojo et al., 2015; Stratford et al., 2013).

Little information is available on frequent spoilage yeasts of grape juice concentrates. A previous study carried out in Argentina shown that *Z. rouxii* was the only species isolated from spoiled grape juice concentrate and it was also detected at a higher frequency in unspoiled samples (Combina et al., 2008). Subsequently, the inhibitory effect of physical factors and chemical compounds in the grape juice concentrate was assessed, in an attempt to control growth of this yeast species. Results showed that the pH was the physical factor with the highest impact on delaying the spoilage of the product, independently of water activity and temperature

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assayed. A pH value below 2.0 was enough to increase the shelf life of the product for more than 60 days in both isothermal and non-isothermal conditions (Rojo et al., 2014). Additional studies were carried out in order to assess the individual effects of different chemical preservatives to control growth of *Z. rouxii*. Results showed that only four preservatives (potassium sorbate, sodium benzoate, dimethyl dicarbonate and vanillin) were able to reduce yeast growth approximately 40% (Rojo et al., 2015).

In the last years, many efforts have been made by juice companies to improve control and prevention of yeast spoilage. Frequently, techniques used are derived from traditional food microbiology without any special consideration with regard to substrate composition that could lead to false negative results, e.g. composition and temperature of diluents commonly used for sample dilution are not suitable to prevent osmotic shock and to allow recovery of sublethally injured yeasts. Consequently, the results obtained are frequently misleading and underestimate the actual sanitary conditions of the product.

Elimination and control of *Z. rouxii* in plants that produce grape juice concentrates are very difficult and little information is available on how certain manufacturing practices and processing treatments affect *Z. rouxii* during the production of grape juice concentrates (Ocón et al., 2010). Concentration of grape juice involves a number of steps including sulfiting, heating from 40 to 110 °C, pasteurization, clarification and filtration that could remove or inactivate *Z. rouxii* cells.

Very little has been written to where such as yeasts originate and the ecology of these yeasts in the natural environment, in the food production factory or in the home or domestic environment (Stratford, 2006). Detection and identification of *Z. rouxii* during the production of grape juice concentrate is critical to prevent spoilage in both the final product and derivatives that are sweetened with this product. This knowledge would help to accomplish corrective measures and develop an appropriate plan for prevention and control of spoilage.

In this context, three grape juice concentrate processing plants were assessed by identifying osmophilic yeasts in juices and surfaces during different stages of a complete production line. Subsequently, molecular typing of *Z. rouxii* isolates was done in order to know the strain distribution of this spoilage yeast.

2. Materials and methods

2.1. Sampling and growth conditions

Samples were collected from three processing plants in Mendoza and San Juan provinces, Argentina, during concentration of grape juice. The processing plants were chosen as a result of their different concentration processing and different production scale (processing plant A: 3500 tons/year, B: 25,000 tons/year and C: 10,000 tons/year). The simplified flowchart of the manufacturing of grape juice concentrate is shown in Fig. 1. Samples of grape juice and samples from different processing plant environments included a complete processing line from raw material to final product (Table 1). Two hundred and fifty milliliter of juice samples were aseptically collected in sterile flasks. Samples from surfaces and equipment were taken less than 15 min before juice processing, when the equipment had been cleaned and disinfected. Samples were taken by streaking 400 cm^2 with sterile cotton plugs, which were subsequently placed in 10 mL of sterile 30% (v/v) glucose-water and stored at 4 °C until further laboratory analysis.

2.2. Osmophilic yeast enumeration

Fifty grams of grape juice samples were aseptically placed in a

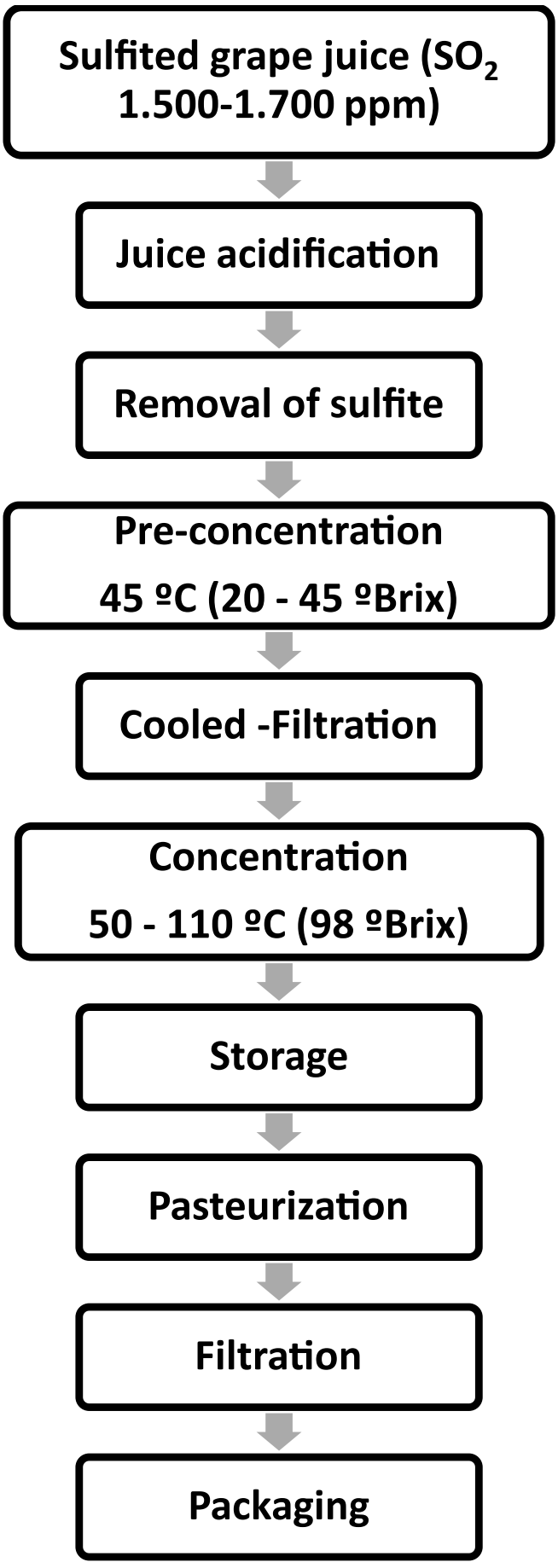

Fig. 1. Process flow diagram of grape juice concentration.

Table 1
Samples taken at three different processing plants during grape juice concentration.

Samples		Processing plant A	Processing plant B	Processing plant C
Grape juice	Sulfited grape juice	✓	✓	✓
	Acidified grape juice		✓	✓
	De-sulfited grape juice	✓		✓
	Grape juice pre-concentrate (GJPC)	✓	✓	
	Filtered GJPC	✓	✓	
	Grape juice concentrate (GJC)	✓	✓	✓
	Spoiled GJC	✓		
	Pasteurized GJC			✓
Surfaces and wash water	Sulfited grape juice tank		✓	
	Acidified grape juice tank		✓	
	GJPC tank	✓	✓	
	GJC tank	✓		✓
	Floors	✓	✓	✓
	Walls	✓	✓	✓
	Hoses			✓
	Wash water filter	✓		✓
	Plate Filtration	✓		
	Pump and connectors	✓		✓
	Operator gloves		✓	
	Filling pipe	✓		✓

Table 2
Osmophilic yeast count and isolation percentage of yeasts species identified from samples taken during grape juice concentration in processing plant A.

Samples		Osmophilic yeast[a]		Yeast species identification	Isolation (%)
		Processing line 1	Processing line 2		
Grape Juices	Sulfited grape juice	<1	NS		
	De-sulfited grape juice	<1	NS		
	Fresh grape juice pre-concentrate (GJPC)	1	NS	*C. matritensis*	100
	Stored and filtered GJPC	$>4.10^4$	NS	*C. matritensis*	50
				Z. rouxii	50
	Fresh grape juice concentrate (GJC)	<1	NS		
	Stored GJC	<1	NS		
	GJC during packaging	<1	NS		
	Spoiled GJC	NS	$>4.10^4$	*Z. rouxii*	100
Surfaces and wash water	GJPC Tank	<1	NS		
	GJPC filtration Tank	<1	NS		
	GJC Tank	$>4.10^4$	NS	*M. pulcherrima*	100
	Floor of GJPC area	<1	NS		
	Floor of GJC area	<1	NS		
	Floor of packaging area	<1	NS		
	Pump and connectors	$>4.10^4$	NS	*W. anomalus*	100
	Walls	<1	NS		
	Filling pipe	$>4.10^4$	NS	*W. anomalus*	100
	Wash water filter	$>4.10^4$	NS	*C. matritensis*	25
				T. delbrueckii	75
	GJPC plate filtration	$>4.10^4$	NS	*C. matritensis*	33
				L. thermotolerans	33
				W. anomalus	33

[a] Grape juice samples in CFU/50 g and surface samples in CFU/400 cm^2. NS: not sampled.

sterile flask containing 50 mL of sterile 30% (w/v) glucose-water diluents to prevent osmotic shock and allow sublethally injured cells to recover (Combina et al., 2008; Rojo et al., 2014). Grape juice and surface/equipment samples were filtered by cellulose nitrate filter (0.45 μm pore size) (Sartorius, Germany). Filters were aseptically placed onto osmophilic yeast count agar medium MY50G (Combina et al., 2008). Plates were incubated at 28 °C until visible colonies appeared. Representative isolates (4–5 colonies) of every colony type was streaked out to isolate single colonies in YPD medium (0.40 g/L glucose, 5 g/L bacteriological peptone, 5 g/L yeast extract, 20 g/L agar).

2.3. Molecular identification of osmophilic yeasts

Isolates were grown aerobically in 10 mL of YPD broth (0.40 g/L glucose, 5 g/L bacteriological peptone, 5 g/L yeast extract) at 28 °C for 48 h. The cultures were centrifuged for 5 min at 13,000 rpm and cell pellets were collected. DNA extraction was carried out following the protocol by Hoffman and Wiston (1987). The region between the 18S rRNA and 28S rRNA genes was amplified using two specific internal transcribed spacers: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White et al., 1990). Briefly, amplification was performed using an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with initial denaturation at 95 °C for 5 min, followed by 40 PCR cycles with denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min. An additional extension at 72 °C for 10 min was carried out at the end of the 40 cycles (Esteve-Zarzoso et al., 1999). The amplified fragments were purified with a Pure Link PCR purification kit (Invitrogen by Life Technology, Carlsbad CA, USA) according to the manufacturer's instructions. Both strands of the rDNA region were sequenced

Table 3
Osmophilic yeast count and isolation percentage of yeasts species identified from samples taken during grape juice concentration in processing plant B.

Samples		Osmophilic yeast [a]	Yeast species identification	Isolation (%)
Grape Juices	Sulfited grape juice	<1		
	Acidified sulfited grape juice	<1		
	Grape juice pre-concentrate (GJPC)	>4.10^4	*Z. rouxii*	100
	After gross filtration GJPC	>4.10^4	*Z. rouxii*	100
	Before fine filtration GJPC	>4.10^4	*Z. rouxii*	25
			C. magnoliae	75
	After fine filtration GJPC	>4.10^4	*Z. rouxii*	75
			C. magnoliae	25
	Before plate filtration GJPC	>4.10^4	*Z. rouxii*	100
	After plate filtration GJPC	<1		
	Filtered GJPC stored	>4.10^4	*W. anomalus*	100
	Fresh grape juice concentrate (GJC)	<1		
	GJC in holding tank (before clean-in-place circuit)	>4.10^4	*Z. rouxii*	100
Surfaces and wash water	Sulfited grape juice tank	<1		
	Acidified sulfited grape juice	<1		
	GJPC tank	3	*C. matritensis*	25
			T. delbrueckii	75
	Filtered GJPC tank	<1		
	Floor of GJPC area	>4.10^4	*Z. rouxii*	100
	Floor of packaging area	<1		
	Walls	<1		
	Operator gloves	<1		

[a] Grape juice samples in CFU/50 g and surface samples in CFU/400 cm^2. NS: not sampled.

with the Sanger capillary sequencing method, using a Premix BigDye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK). The BLAST search (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov) was used to compare the sequences obtained with databases of the National Center for Biotechnology Information (NCBI) (Altschul et al., 1990). Identification was considered correct when gene sequences showed an identity of 99% or higher.

2.4. Strain typing of Z. rouxii isolates

Isolates identified as *Z. rouxii* were differentiated at strain level by RAPD-PCR analysis. RAPD profiles were generated using primer OPA 3 (AGTCAGCCAC) (Martorell et al., 2005). Three *Z. rouxii* outgroup strains were included in this study in order to validate the discriminatory capacity of the technique. Moreover, primers OPA E-12 (CCGAGCATTC), OPA R-08 (GTGAATGCGG) and OPA S-05 (GTCACCTGCT) were included in order to confirm strain typing. PCR reactions were carried out in 25 μL reaction volumes containing 1–5 ng of DNA, 3.5 mM $MgCl_2$, 200 ng of primer OPA-3 (or the other OPA primers), 25 μM of each dNTP, and 1 U of Taq polymerase (Invitrogen Co.). The thermal cycler was programmed as follows: initial denaturation at 94 °C for 5 min, followed by 45 cycles of 92 °C for 1 min, annealing at 36 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The RAPD-PCR products were visualized on a 1.5% agarose gel after staining with ethidium bromide. The molecular sizes of DNA fragments were obtained after comparison with a 100-bp molecular marker.

Table 4
Osmophilic yeast count and isolation percentage of yeasts species identified from samples taken during grape juice concentration in processing plant C.

Samples		Osmophilic yeast[a]		Yeast species identification	Isolation (%)
		Processing line 1	Processing line 2		
Grape Juices	Sulfited grape juice	2.4.10^4	NS	*Z. rouxii*	50
				Sch. pombe	50
	Acidified sulfited grape juice	<1	NS		
	De-sulfited grape juice	<1	NS		
	Fresh grape juice concentrate (GJC)	<1	NS		
	GJC in holding tank	1.4.10^5	1.4.10^5	*Z. rouxii*	100
	GJC stored	>4.10^4	>4.10^4	*Z. rouxii*	100
	Re-processing GJC before pasteurization	1	1.44.10^2	*Z. rouxii*	100
	GJC after pasteurization	NS	15	*Z. rouxii*	100
	GJC pasteurized, filtered and stored	39	>4.10^4	*Z. rouxii*	100
Surfaces and wash water	GJC Tank	<1	NS		
	Floor of concentration area	15	NS	*W. anomalus*	100
	Floor GJC area	NS	>4.10^4	*W. anomalus*	50
				T. delbrueckii	50
	Floor of packaging area	<1	<1		
	Pump and connectors	<1	NS		
	Walls	NS	20	*C. orthopsilosis*	50
				W. anomalus	50
	Air hoses	NS	2.10^3	*Z. rouxii*	100
	Filling pipe	NS	<1		
	Wash water filter	NS	>4.10^4	*W. anomalus*	75
				C. apicola	25

[a] Grape juice samples in CFU/50 g and surface samples in CFU/400 cm^2. NS: not sampled.

Table 5
Molecular pattern of *Zygosaccharomyces rouxii* isolated from samples taken during grape juice concentration in three different processing plants.

Plant	Sample		RAPD pattern	Incidence (%)
A	Processing line 1	Stored grape juice pre-concentrate (GJPC)	I	67%
			II	33%
	Processing line 2	Spoiled grape juice concentrate (GJC)	I	75%
			III	25%
B	Grape juice pre-concentrate (GJPC)		IV	100%
	After gross filtration GJPC		IV	100%
	Before fine filtration GJPC		IV	100%
	After fine filtration GJPC		IV	100%
	Before plate filtration GJPC		IV	100%
	GJC in holding tank (before clean-in-place circuit)		V	25%
			IV	75%
	Floor of GJPC area		V	28%
			IV	58%
			VI	14%
C	Processing line 1	Sulfited grape juice	VII	100%
		GJC in holding tank	VII	100%
		GJC stored	VII	14%
			VIII	57%
			IX	29%
		Re-processing GJC before pasteurization	VIII	100%
		GJC pasteurized, filtered and stored	VIII	100%
	Processing line 2	GJC in holding tank	VII	100%
		GJC stored	VII	75%
			IX	25%
		Re-processing GJC before pasteurization	VII	66%
			VIII	34%
		GJC after pasteurization	VII	34%
			VIII	66%
		GJC pasteurized, filtered and stored	VII	100%
		Air hoses	VII	100%

3. Results

In order to assess the distribution of osmophilic yeasts during the processing of grape juice concentrate, a total of 63 samples were taken along the production line at the three processing plants chosen for this study. Twenty nine of the samples corresponded to grape juice and 34 to surface/equipment. The sampling points were determined "*in situ*" and depended on concentration processing carried out in each plant (Table 1).

3.1. Osmophilic yeasts in grape juice samples

Out of the total of grape juice samples taken during the different stages of juice concentration, 76% were positive for osmophilic yeasts (22/29) and 69% (20/29) were positive for *Z. rouxii* (Tables 2–4).

Because of the differences between the processing plants, each of them was individually analyzed. Plants A and B perform a two-stage concentration process, consisting of pre-concentration of grape juice followed by filtration and storage for 1–2 weeks at 5 ± 3 °C until the concentration process and subsequent packaging are conducted. Osmophilic yeast counts exceeded 4×10^4 CFU/50 g in 78% (7/9) of grape juice pre-concentrate samples. Identification of the yeast isolates revealed that *Z. rouxii* was the most prevalent species in samples of grape juice pre-concentrate, and it was the only yeast species isolated in grape juice concentrate (Tables 2 and 3). In processing plant A, fresh grape juice pre-concentrate showed a very low number of *Citeromyces matritensis* (anamorph *Candida globosa*), but after a week the juice showed increasing numbers of *Z. rouxii* accompanied by the species previously detected (50%–50%) (Table 2).

In processing plant B, three filtration stages (gross filtration, fine filtration and cellulose plate filtration) of grape juice pre-concentrate are carried out throughout the concentration process. In this plant, samples were obtained during each of the filtration stages of the juice pre-concentrate. Between each of the filtration stages, the grape juice pre-concentrate is stored at 5 °C in tanks. The residence time in each of these tanks ranged from one day to one week, according to the processing plant capacity and market demand. High numbers of osmophilic yeasts were observed in grape juice pre-concentrates during different filtration stages previous to plate filtration (Table 3). Plate filtration retained most of the yeast showing less than one cell in 50 g of grape juice pre-concentrate. However, after 12 days of storage, yeast counts increased again as previously observed in processing plant A, although in this case yeasts were identified as *Wickerhamomyces anomalus* (Table 3). In both processing plants (A and B) the concentration stage allowed to reduce the yeast population below the detection limit of the technique used (1 CFU/50 g), but in processing plant B, storage of the grape juice concentrate during a week enabled the *Z. rouxii* population to increase (Table 3).

Processing plant C followed a different concentration protocol. Sulfited grape juice is directly concentrated and stored in tanks at room temperature until packaging according to market demand. Before packaging, grape juice concentrate is re-processed (dilution, acidification, clarification and concentration) to condition it according to customer requirements. This grape juice re-concentrate is then mixed and homogenized with other production batches by injecting air in order to reach the total volume requested by the customer. This batch is then pasteurized and packaged. In this processing plant and prior to concentration, equal proportions of two species of osmophilic yeasts, *Z. rouxii* and *Schizosaccharomyces pombe*, were detected in sulfited grape juice (Table 4). *Z. rouxii* was the only species identified in all samples of the grape juice concentrate. As previously observed, the concentration process of grape juice reduced the population of osmophilic yeasts below one cell in 50 g, which then increased during 2 months of storage at room temperature (Table 4). Re-processing and pasteurization of

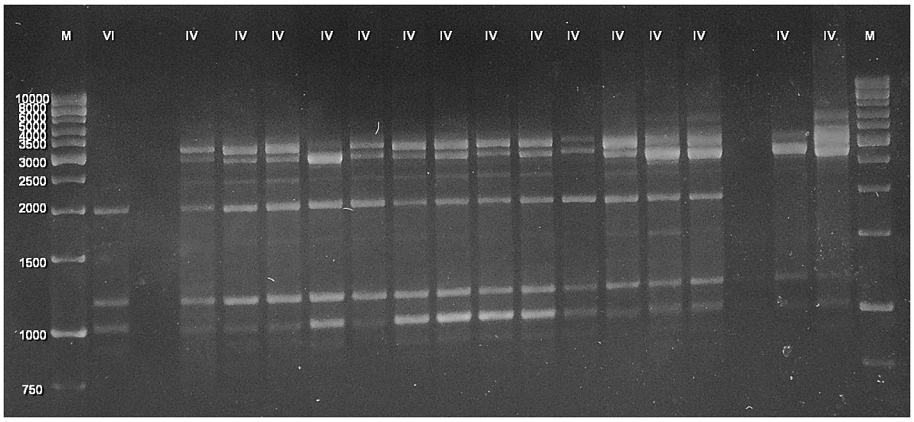

Fig. 2. Example of OPA 3 RAPD PCR dominant patterns (IV and VI) exhibited by *Z. rouxii* isolates from samples in processing plant B. Lane M corresponds to 1 kb DNA ladder (Thermo Fisher Scientific, USA).

the grape juice concentrate reduced the population of *Z. rouxii* again. The pasteurization program carried out (94 °C for 62 s) left a detectable residual population of *Z. rouxii*, which increased during the 4 days of storage of the juice before packaging (Table 4).

3.2. Osmophilic yeasts in processing plant environment samples

From the 34 samples obtained from processing plant environments, 38% were positive for osmophilic yeasts (13/34) showing different counts depending on the surface/equipment analyzed and only 6% of them were identified as *Z. rouxii* (2/34) (Tables 2–4). All positive samples belonged to surface areas or equipment related to grape juice pre-concentrate and concentrate, whereas in the previous processing stages samples were negative. Counts were different between processing plants. While processing plant A showed high osmophilic counts in the tank that received the grape juice concentrate and surface samples associated with filtration and filling of the final product, the other two processing plants (B and C) showed high counts in floor and wash water filter samples (Tables 2–4). Unexpectedly, most of the yeast species identified in the processing plant environments did not belong to *Z. rouxii*. Seven other yeast species were isolated: *W. anomalus*, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *C. matritensis*, *Candida orthopsilosis* and *Candida apicola* (Tables 2–4). *Z. rouxii* was present only in two surface samples, the floor of the grape juice pre-concentrate area in processing plant B, and the air hoses used to homogenize the grape juice concentrate in processing plant C (Tables 3 and 4).

3.3. Distribution of Z. rouxii strains during grape juice concentrate production lines

In order to know the strain distribution of *Z. rouxii* during the processing of grape juice concentrate RAPD-PCR analysis of all *Z. rouxii* isolates was carried out. RAPD with OPA 3 gave nine different patterns for all the *Z. rouxii* isolates. This technique allowed discrimination between the three *Z. rouxii* outgroups strains and the isolates from different processing plants. Findings were confirmed using three other OPA primers (OPA E-12, OPA R-08 and OPA S-05) in RAPD-PCR reactions carried out in a subset of *Z. rouxii* strains including three isolates from each processing plant and the three *Z. rouxii* outgroup strains.

Overall, limited polymorphism (three molecular patterns) was observed in *Z. rouxii* isolates from each processing plant. Each of the three processing plants exhibited a dominant molecular pattern for *Z. rouxii* isolates, and the main molecular pattern was different for each of the processing plants (Table 5). In processing plant A, pattern I was exhibited by 67% (processing line 1) and 75% (processing line 2) of the *Z. rouxii* isolates, accompanied by two molecular patterns present at smaller proportions. The same molecular pattern I was found in samples belonging to two different processing lines (Table 5).

In processing plant B, molecular pattern IV was detected in *Z. rouxii* isolates from grape juice pre-concentrate and concentrate samples at a percentage between 75% and 100% (Table 5 and Fig. 2). The same molecular pattern was also found in 58% of the *Z. rouxii* isolates present in samples of the floor of the grape juice pre-concentrate area, accompanied by two other molecular patterns at a smaller proportion (Table 5).

In processing plant C, two different production lines were sampled in order to compare the strains that were present in either. *Z. rouxii* isolates from all the grape juice concentrate samples taken from both processing lines shared two dominant molecular patterns (VII and VIII) (Table 5). Pattern VII was also detected in sulfited grape juice prior to concentration from processing line 1. Pattern IX was detected in *Z. rouxii* isolates from both processing lines when the grape juice concentrate was stored. The air hose used for homogenizing the grape juice concentrate showed the same molecular pattern as in the grape juice concentrate (Table 5).

4. Discussion

Due to the high economic losses caused by food spoilage, identification of spoilage microorganism and evaluation of their distribution in processing plants is a high priority. Only when the nature of the spoilage is completely understood a decision can be made on cleaning procedures and product recall (Harrison et al., 2011; James and Stratford, 2003; Rawsthorne and Phister, 2006).

To enable identification of the distribution of spoilage yeasts during grape juice concentration it is necessary to have proper techniques for accurate identification at different levels. For this reason, several molecular-based methodologies have been used to identify spoilage yeasts (Loureiro and Querol, 1999). Sequencing of the 5.8S-ITS rDNA region has proven to be a suitable methodology for a rapid and accurate identification of *Zygosaccharomyces* species (Scorzetti et al., 2002). Analysis of genetic polymorphisms within species and population variability have shown to be very helpful to determine the source contamination during food processing (Loureiro and Querol, 1999). In the present study, OPA 3 was able to discriminate between different *Z. rouxii* isolates.

Cell counts of samples from the processing plants environment showed the presence of osmophilic yeasts in areas associated with grape juice with some degree of concentration (grape juice pre-concentrate and concentrate). Osmophilic yeasts were also detected in all grape juice samples after pre-concentration or concentration. This was to be expected because of the correlation between the type of microorganism isolated and the substrate characteristics, which explains the fact that osmophilic yeasts only appear in foods with a high sugar concentration.

Unexpectedly, most yeast species isolated from surfaces were not identified as *Z. rouxii*. Three yeast species, *W. anomalus*, *T. delbrueckii* and *C. matritensis*, were more frequently isolated, followed by *L. thermotolerans*, *M. pulcherrima*, *Candida apis* and *C. orthopsilosis*. All these species displayed osmotolerant characteristics and were previously described in fresh concentrates and/or pasteurized fruit juices, condensed milk, refined sugar and raisins (Deák, 2008; Deák and Beuchat, 1993; Jay et al., 2005; Kurtzman and Fell, 1998; Kurtzman et al., 2011; Moreno Arribas and Polo, 2008). In line with our work, Stratford (2006) described yeast genera as *Candida* sp., *Torulaspora* sp. and *Wickeramomyces* sp. on surfaces of fruit juice factories.

Presence of osmotolerant yeast species in areas of grape juice concentrate processing plants suggests that these yeasts harbor characteristics that enable them to survive and persist in sanitized surfaces, as they were often found at high numbers. These species have previously been described as yeasts associated with processing plant environments of sugary products, even though they have not been classified as potential spoilers of the product *per se*. A study carried out by Stratford (2006) supports this fact, confirming that despite a far larger number of yeast species present in certain beverages only few of them could be referred to as "spoilage yeasts". Similar to our observations, Davenport (1996) found yeasts within the factory environment particularly concentrated in areas where sugary products are spilled or washed away and diluted into the soakaways and drains. Recently, Wang et al. (2015) reported that species such as *C. glabrata*, *C. orthopsilosis*, *Candida zemplinina* and *H. opuntiae* were recovered from apple juice plant environments. Consequently, and based on our findings, it may be concluded that the main yeast species present in processing plant environments do not represent any spoilage risk to grape juice concentrate.

Zygosaccharomyces bailii and *Z. rouxii* species have been described as not highly resistant to biocides such as peracetic acid or hypochlorite (Frisón et al., 2014; Martorell et al., 2007). Both compounds are used as a sanitizing agent in the processing plants sampled in the present study. In line with this, Stratford (2006) described *Z. rouxii* as a highly susceptible species to heat and acetic acid, unable to survive after cleaning procedures. These previous works could explain the absence of *Z. rouxii* in areas of the processing plants examined in our work.

Unlike the findings in the environments of the processing plants, grape juice concentrate obtained during different processing stages showed a clear predominance of *Z. rouxii* species. Presence of this species was associated with juices that had been concentrated to some degree. Osmophilic yeasts were under the detection limit of the technique used (<1 CFU/50 g) in freshly obtained grape juice concentrate, while positive counts were recorded when the grape juice concentrates were stored for some time (5–60 days). In agreement with our results, Wang et al. (2015) found that all in-line apple juice concentrates were negative for osmophilic isolates whereas juice concentrates that had been stored for a longer time did contain osmophilic isolates. A comparable result was reported by Combina et al. (2008), who found that grape juice concentrates prior to container filling showed a lower number of yeasts (0.12 Log10 CFU/g) than the concentrate stored in containers for an extended period of time (4.40–7.06 Log10 CFU/g). During the concentration process there are stages that include temperatures up to 110 °C. It is generally accepted that binomial thermal treatments such as 74 °C/16 s or 85 °C/1 s guarantees the juice stability (Graumlich et al., 1986). However, high sugar concentrations in processed fruit concentrates cause an increase in the heat resistance of the microbes, which is partly due to the decrease in the water activity (Hui et al., 2006; Steyn et al., 2011). In addition, previous studies with *S. cerevisiae* in fruit juices and beers demonstrated that ascospores were 25–350 times more resistant to heat than vegetative cells (Milani et al., 2015; Put et al., 1976; Put and Jong, 1982). Further research is needed to determine the heat resistance of *Z. rouxii* spores that allow knowing if they could survive the concentration and pasteurization processes.

The fact that *Z. rouxii* was isolated only from surfaces with juice remains, suggesting that these "difficult to clean" surfaces could be a reservoir of this spoilage yeast. Moreover, *Z. rouxii* was coincidentally found on these surfaces and in grape juice concentrates and repeatedly isolated from different production lines independently of the raw material used. *Z. rouxii* strains were different in each of the three processing plants, suggesting resident microbes inside the plant. This result is pertinent as previous studies reported that fruit juice concentrates may contain isolates previously detected in facilities with unsuitable hygiene (Wang et al., 2015). However, how concentrates are contaminated by these isolates is not clearly understood yet.

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