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Yeast identification in grape juice concentrates from Argentina

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Abstract

Aims: The purpose of this study was to identify yeast species present in spoiled and unspoiled grape juice concentrates from Argentine industries.
Methods and Results: Osmophilic and osmotolerant yeasts were isolated from spoiled – visually effervescent – and unspoiled – without any visible damage – grape juice concentrates by the spread-plate technique in two culture media. Yeast identification was done by classical and molecular methods. *Zygosaccharomyces rouxii* was the only species isolated from spoiled grape juice concentrates. In unspoiled samples, five different species were identified: *Z. rouxii* was isolated at a higher frequency, followed in decreasing order by *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia anomala* and *Kluyveromyces delphensis*.
Conclusions: Yeasts isolated from grape juice concentrates were characterized by a limited taxonomic diversity, where *Z. rouxii* was the main species isolated.
Significance and Impact of the Study: Grape production in Argentina is mainly devoted to the industry where wine and grape juice concentrates represent major types of commercial products. Little information on common yeast contaminants is available for grape juice concentrates. This study constitutes the first report of osmophilic yeast species present in spoiled and unspoiled grape juice concentrates elaborated in Argentina.

Introduction

Grape juice and by-products represent an important percentage of the food industry in the world. Argentine grape production is mainly devoted to the industry, where wine and grape juice concentrate are the two major types of commercial products [Instituto Nacional de Estadísticas y Censos (INDEC) 2007]. Grape juice concentrate has a great importance as an additive to several massively consumed products. Owing to their natural qualities, it is employed to elaborate baby foods, pharmaceutical products, candies, jellies, jams and fruit syrups (Bruzone 1998).

Microbial food spoilage is commonly the result of the combined activities of yeasts, moulds and bacteria; however, depending upon the environmental conditions, one of them may prevail. Juice concentrates are more stable than other juice products; high sugar concentrations and low pH preserve these products. The combination of these factors supports the development of a reduced number of micro-organisms as xerophilic yeasts and moulds. However, considering that grape juice concentrates are stored in sealed drums with a minimal head space, they are less susceptible to spoilage by xerophilic moulds, as these organisms are obligate aerobes. Consequently, xerophilic yeasts represent the primary spoilage cause in this substrate [International Commission on Microbiological Specifications of Foods (ICMSF) 1980].

Yeasts capable of growing at low a_w values have been described as osmophilic, osmotolerant, osmoduric, xerophilic and xerotolerant. The lowest a_w at which nonosmophilic yeast can grow is in the range of 0·85–0·92 (ICMSF

1980). According to the definition, in this work, yeasts capable of growing in high-sugar foods with $a_w < 0{\cdot}85$ were named osmophilic yeasts. Moreover, there are yeasts which support high osmotic pressures and have some heat resistance, but they are incapable of growing at low a_w or they do it very slowly. For a practical differentiation, this group was named osmotolerant yeasts. When grape juice concentrate is used as a component or food additive, osmotolerant yeasts become important.

There is little information on the level of yeast contamination considered to be acceptable or unacceptable in some specific foods, such as grape juice concentrate. In Argentina, microbiological specifications for this substrate are often set by the purchaser, who is usually a drink or food processor. A survey of beverage manufacturers in Australia provide one of the few specifications available for grape juice concentrates (Andrews 1992). Unacceptable levels for concentrates varied from 1 cell per ml up to >1000 cells per ml depending on the storage conditions.

The must concentrate industries routinely apply techniques that come from traditional food microbiology to make microbiological analyses on grape juice concentrate without any special consideration on substrate composition. Consequently, the results obtained are frequently misleading and underestimated. Yeast a_w tolerance is related to the solute involved; hence, the media chosen for the detection and enumeration of xerotolerant yeasts should reflect the content of the food being analysed. Seeding techniques – spread *vs* pour plate – and diluent compositions must be carefully selected to allow a good recovery of stressed or sublethally injured cells (Andrews *et al.* 1997).

The rapid identification of spoilage yeasts is of great importance to the food industry. Based on the classical taxonomy criteria, a simplified identification key for yeast species associated with foods has been proposed by Deák (1986, 1992). On the other hand, several molecular-based methodologies have also been proposed to identify these yeasts (Loureiro and Querol 1999). The restriction analysis of the 5·8S-internal transcribed spacer (ITS) rDNA region has proven to be a suitable methodology for rapid and accurate yeast identification (Loureiro and Querol 1999). In this work, both methods to assess the yeast species populations in grape juice concentrates were applied.

It may be assumed that each type of food may be altered by a specific group of yeasts (Loureiro 2000). These selected species could thus be considered as potentially harmful in this context, and should be specifically controlled in the particular food industry. The purpose of this study was to identify the yeasts present in spoiled and unspoiled grape juice concentrates from Argentine industries.

Materials and methods

Samples

A total of 21 grape juice concentrate samples were taken from five grape concentrating industries located in Mendoza and San Juan states, Argentina. Grape juices were concentrated using falling film evaporator with multiple effects followed by pasteurization. Sixteen samples were obtained, few minutes before container filling to determine the quality of the samples to be sealed. These samples, without any visible damage, were considered as 'unspoiled'. Five spoiled samples, visually effervescent, were provided by industries to assess the yeast species directly associated with product spoilage. Duplicate 500-ml samples were collected in sterile flasks and stored at 4°C until laboratory arrival. The Brix degree of juice concentrates was measured using a digital refractometer (Atago PAL-3, Tokyo, Japan), and the pH was determined using a digital pHmeter (Orion Research 701A, Cambridge, MA).

Yeast enumeration and isolation

Five grams of juice concentrate were decimal diluted in 30% (w/v) glucose to prevent osmotic shock and allow sublethally injured cells to recover. Dilutions were spread in two culture media. Simultaneously, the smaller dilution was filtered through a membrane (0·45-μm pore) and placed in two culture media. Selective high sugar media – malt extract, yeast extract 50% glucose agar (MYG50) (Beuchat 1993) – was used to assess the presence of osmophilic yeasts. Tryptone glucose yeast (TGY) extract agar (Beuchat *et al.* 2001) was chosen to allow the growth of all (osmotolerant and osmophilic) yeasts present in the concentrate samples. Plates were incubated at 25°C for a week before yeast colonies were counted. Representative isolates of each colony type were purified by streak plating and subcultured onto yeast extract peptone dextrose (YEPD).

Traditional and molecular yeast identification

All yeast isolates were identified according to the simplified identification method (SIM) confined to the most frequent foodborne yeasts described by Deák (1992). Representative isolates from each species group according to SIM were confirmed by molecular identification. DNA extraction was carried out with the maxiDNA kit (Promega, Madison, WI, USA) according to manufacturer's instructions. The region between the 18S rRNA and 28S rRNA genes was amplified using specific ITS, namely ITS1 and ITS4 primers (White *et al.* 1990). To achieve greater polymorphism, the amplified genes were treated

with the restriction enzymes *Cfo*I, *Hinf*I and *Hae*III for an identification of yeasts at the species level (Esteve Zarzoso *et al.* 1999). The PCR amplified 5·8S rRNA ITS region was sequenced in those yeast species where identification was doubtful or unclear, using the ABI Prism 3100 genetic analyser. The sequence comparisons were performed using the basic local alignment search tool (BLAST) within the NCBI database (National Center for Biotechnology Information).

Results

The methodology employed allowed yeast recovery from 90% (19/21) of the analysed samples. Simultaneous spread in two culture media, one high-sugar selecting and another to recover the total yeast population, provided a rapid assessment of osmophilic or osmotolerant yeast populations present in grape juice concentrates prior to species identification. No statistical difference on colony counts were observed in the same sample spread in both culture media, indicating that only osmophilic yeast populations were present in all samples (Table 1).

Yeast populations in grape juice concentrates varied considerably (Table 1). Spoiled concentrates showed a high contamination level, which ranged between $\log_{10}$ 4·4 and 7·1. When unspoiled juice concentrates were analysed, only two of the 16 samples were negative – less than one viable yeast per gram – in the analysed conditions. Forty per cent of the samples (7/16) contained less than 10 CFU g^{-1} and 25% of samples (4/16) ranged from 10 to 30 CFU g^{-1}. Nineteen per cent of unspoiled samples (3/16) showed elevated yeast counts. Juice concentrates with these loads did not exhibit visible product spoilage at the moment of examination, although one of them had a similar contamination level to the spoiled samples (Table 1).

A total of 200 colonies isolated from spoiled and unspoiled grape juice concentrates were identified by traditional taxonomical criteria and confirmed by molecular taxonomy. SIM allowed accurate genera assignation in most of the isolates, but failed at the species-level designation in comparison with the molecular methods employed. In Table 2, correlation between species assignation with two identification methods used is shown. SIM produce

Table 1 Yeast populations, colour, pH and Brix degree of spoiled and unspoiled grape juice concentrate samples from Mendoza and San Juan, Argentina

Sample code	Origin	Colour	°Brix	pH	Yeast counts* (log CFU g^{-1} ± SD) TGY	MYG50
Spoiled						
A	San Juan	White	ND	3·9	7·06 ± 5·72	6·96 ± 5·51
B	San Juan	White	69	4·3	4·39 ± 2·81	4·64 ± 2·49
C	San Juan	White	66·5	2·9	4·40 ± 3·18	4·48 ± 3·30
D	San Juan	White	75·5	2·8	5·60 ± 4·00	5·54 ± 4·78
E	Mendoza	White	65·4	2·4	5·23 ± 3·56	5·14 ± 3·56
Unspoiled						
F	Mendoza	White	68	2·5	1·49 ± 0·30	1·32 ± 0·87
G	Mendoza	White	68	2·6	0·82 ± 0·18	0·37 ± 0·24
H	Mendoza	White	68·5	2·9	0·12 ± 0·24	0·12 ± 0·24
I	Mendoza	White	69·5	2·6	0·60 ± 0·30	0·52 ± 0·24
J	Mendoza	White	66·5	2·3	< 1	< 1
K	Mendoza	White	68·5	2·6	0·12 ± 0·24	0·12 ± 0·24
L	Mendoza	Rosé	69	1·7	1·05 ± 0·18	1·09 ± 0·4
M	Mendoza	Rosé	69	1·7	2·43 ± 1·18	2·28 ± 1·33
N	Mendoza	White	69·5	2·7	1·44 ± 0·40	1·23 ± 0·42
O	Mendoza	Red	66·5	2·7	0·12 ± 0·24	0·12 ± 0·24
P	Mendoza	Red	67	3·7	4·21 ± 2·81	4·31 ± 2·88
Q	Mendoza	Red	67·5	3·8	0·12 ± 0·24	0·22 ± 0·24
R	San Juan	White	68	2·5	1·49 ± 0·06	1·47 ± 0·49
S	San Juan	White	68	2·4	0·12 ± 0·24	0·12 ± 0·24
T	San Juan	White	67	2·4	< 1	< 1
U	Mendoza	White	65	2·4	3·84 ± 2·00	3·86 ± 2·48

TGY, tryptone glucose yeast; MYG50, malt extract, yeast extract 50% glucose agar; ND, not determined.

*Mean values obtained on three replicate counts ± standard deviations. No significant differences between yeast counts in both culture media were found within the same sample (Fisher's LSD test, $P \leq 0{\cdot}05$).

Table 2 Comparison between yeast species assignations applying two different identification methods in yeast isolates from grape juice concentrates samples from Mendoza and San Juan, Argentina

Identification method			
SIM		RFLP 5·8-ITS and sequencing	
Yeast species assignation	Isolation frequency (%)	Yeast species assignation	Isolation frequency (%)
Spoiled samples		Spoiled samples	
Zygosaccharomyces rouxii	39	*Zygosaccharomyces rouxii*	100
Zygosaccharomyces microellipsoides	28		
Zygosaccharomyces bisporus	16·5		
Zygosaccharomyces bailii	16·5		
Unspoiled samples		Unspoiled samples	
Z. rouxii	46	*Z. rouxii*	76
Z. microellipsoides	21		
Z. bisporus	9		
Saccharomyces cerevisiae	5	*S. cerevisiae*	14
Saccharomyces pastorianus	3		
Torulospora delbruekii	3		
Zygosaccharomyces microellipsoides	3		
Schizosaccharomyces pombe	6	*Sc. pombe*	6
Pichia subpelliculosa	2	*Pichia anomala*	2
Kluyveromyces marxianus	2	*Kluveromyces delphensis*	2

SIM, simplified identification method; ITS, internal transcribed spacer;

misidentification when variable, delayed or weak test results were found. Variations (±) in certain characters which are important in the master key or subgroup assignation by SIM have been detected. Some specific examples from our experience were *Zygosaccharomyces* species differentiation by raffinose and maltose assimilation, where isolates showed variable or delayed positive response. Variable response to erytritol and galactose assimilation by *Pichia anomala* makes difficult the distinction of this species from others. Elsewhere, *Kluyveromyces delphensis* is not included in SIM species key, conducting to erroneous assignation. *Saccharomyces* species showed misidentifications at genera level when SIM was applied. Molecular method employed allowed accurate yeast species assignations. Figure 1 showed PCR products from three representative isolates amplified with ITS1 and ITS4 primers followed by restriction pattern. *Saccharomyces cerevisiae* assignation was confirmed by sequencing, because restriction enzymes used in restriction fragment length polymorphism (RFLP) 5·8 ITS do not provide enough information to distinguish between species included in *Sacharomyces sensu stricto* group. Therefore, yeast species assignation obtained by RFLP 5·8-ITS, followed by sequencing, was considered more appropriate to show the real incidence isolated from grape juice concentrates (Table 2).

Yeasts isolated from grape juice concentrates were characterized by a limited taxonomic diversity. *Zygosaccharomyces rouxii* was the only species isolated from spoiled grape juice concentrates (Table 2). In samples without visible spoilage, five different species were identified: *Z. rouxii* was isolated at a higher frequency, followed by *S. cerevisiae* and *Schizosaccharomyces pombe*; both *Pichia anomala* and *Kluyveromyces delphensis* appeared in only one sample, always accompanying a greater population of *Z. rouxii* or *S. cerevisiae*.

Discussion

The analytical methods used by the industry to evaluate yeasts present in foods and drinks are still yeast and mould count plates, making use of a rich culture medium. Under this condition, it is not possible to distinguish between dangerous and innocuous yeasts in product stability, making it difficult to determine the preventive measures that must be taken in cases of high levels of contamination (Loureiro 2000). In this study, diluents and culture media were selected from the recommendations of several collaborative studies on media for the enumeration of yeasts in foods (Deák 1992; Beuchat *et al.* 2001). Rehydration in sugar-supplemented diluents has been used before homogenizing to prevent osmotic shock and help recovery of sublethally injured yeasts. Isolation and enumeration have been done by spread plating of samples on the surfaces of an appropriate medium. Different researchers have demonstrated the high performance of TGY medium for enumerating yeasts compared with other media (Beuchat *et al.* 1998). The media chosen for the detection and enumeration of xerotolerant yeasts should reflect the content of the food being analysed. In our work, MYG50 has proven to be a good media for the recovery of osmo-

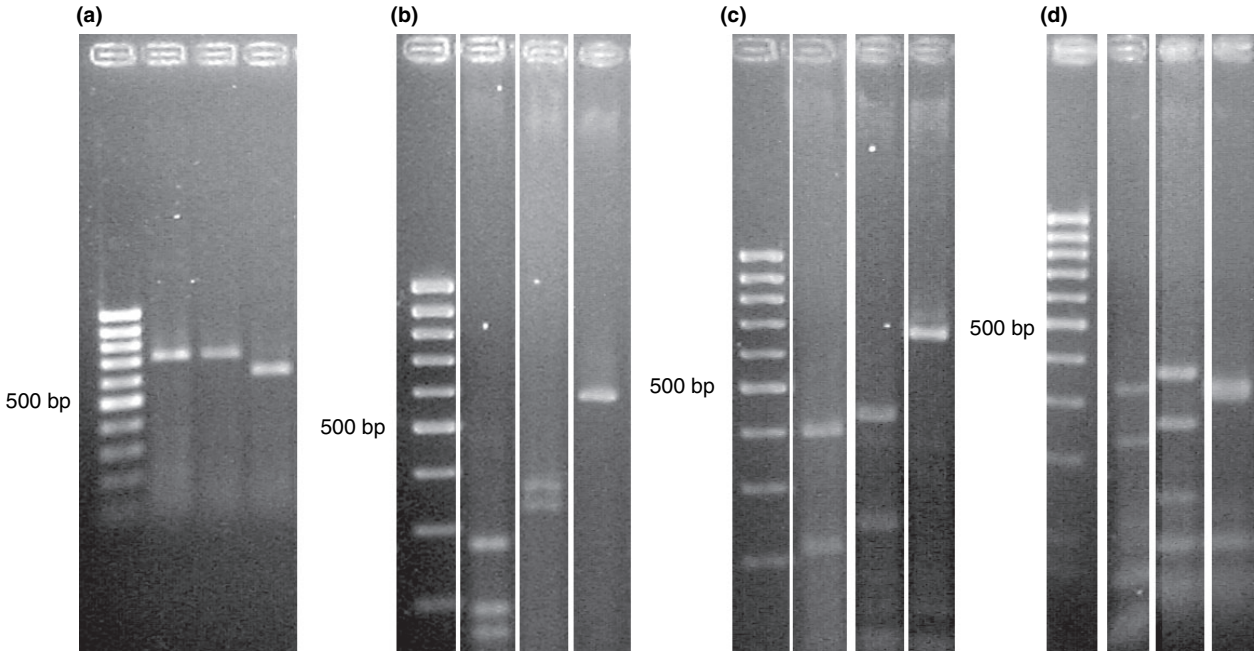

Figure 1 5·8 internal transcribed spacer restriction analysis. PCR products (a) from three representative isolates digested with *Cfo*I (b), *Hae*III (c) and *Hin*fI (d). From left to right, 100-bp DNA leader, *Zygosaccharomyces rouxii*, *Kluyveromyces delphensis* and *Pichia anomala*.

philic yeasts from grape juice concentrates. Simultaneous spread into two culture media, one high-sugar selecting and another to recover the total yeast population, allow the osmophilic and osmotolerant yeast populations to be rapidly recognized. This methodology provides a clear approach to the real risk of concentrate juice spoilage.

A total yeast population of less than 10 cells per ml is generally considered as an appropriate limit to evaluate the quality of unfrozen grape juice concentrates (Andrews 1992). In our work, 56% of unspoiled samples were below this level. Failure to reach these levels may be attributed to poor factory sanitation, especially after pasteurization, and less commonly to poor pasteurization or the poor quality of raw materials (Andrews 1992).

In order to design adequate strategies to prevent spoilage, it is advantageous to know the identity of the spoilage micro-organisms present in the product and to get an insight into the source of contamination (Loureiro 2000). Yeast classical taxonomy is primarily based on physiological properties. There is no single standardized method for many of these tests, and their results are often dependent on the technique employed. In addition, many results of the tests are variable for different strains of the same species, giving rise to frequent misidentification (Loureiro and Querol 1999). Obviously, this identification scheme cannot be routinely utilized by the food industry, and therefore, various simplified methods have been developed (Loureiro and Querol 1999). In this work, a SIM was applied. SIM species assignations were confirmed applying PCR-RFLP of 5·8-ITS fragments, followed by sequencing. In opposition to King and Török (1992), who proved that SIM produced a correct identification in over 80% of the cases in yeast isolates from fruit and vegetable samples; in our experience, SIM was not enough to allow an accurate species identification. A molecular methodology employed proved to be adequate for species assignment. However, this analysis must be complemented with nucleotide sequencing of 5·8-ITS gene to resolve mis- or nonidentification.

Yeast populations in different fruit juice concentrates have been investigated in different countries, but only a few taxonomic studies have been made in grape juice concentrates (Suarez *et al.* 1981; Deák and Beuchat 1993; Fugelsang 1998). Works have shown that a limited number of yeast species can survive the fruit concentrate process and growth in this kind of substrate with low a_w and pH values. *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii* emerged as the main yeasts that cause spoilage in these foods (Deák and Beuchat 1993; Arias *et al.* 2002; Martorell *et al.* 2005). *Zygosaccharomyces* species have been primarily associated with must and wine spoilage. Moreover, the practice of concentrating must has increased the importance of this genus. The ability of *Z. rouxii* and *Z. bailii* to grow in the presence of high sugar concentrations and their resistance to preservatives (sorbic acid, benzoic acid and sulfur dioxide) makes them one of the main causes of fermentative food spoilage (Loureiro and Malfeito-Ferreira 2003).

Other yeast species involved in the spoilage of preserved liquid acid foods are *Sc. pombe*, *P. membranaefaciens*, *S. cerevisiae, Torulaspora delbruekii* and *Candida* species (Suarez *et al.* 1981; Deak and Beuchat 1993). *Kluyveromyces delphensis* has only been described in dried figs (Barnett *et al.* 2000). Strains of *S. cerevisiae* have frequently been associated with re-fermentation of red wines and concentrated juices. *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii*, although dangerous spoilers, are not regarded as common contaminants because of their low incidence (Loureiro and Malfeito-Ferreira 2003). Species found in Argentine grape juice concentrates match well with previously described species in substrates with high sugar concentrations in different countries (Suarez *et al.* 1981; Deák and Beuchat 1993; Fugelsang 1998).

This study represents the first report of osmophilic yeast species contamination present in spoiled and unspoiled grape juice concentrates elaborated in Argentina.

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