ORIGINAL ARTICLE

Zymological indicators: a new concept applied to the detection of potential spoilage yeast species associated with fruit pulps and concentrates

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In a survey of the microbial quality of raw materials used in fruit juice processing, yeast counts in fruit concentrates and pulps were found to range from <1 to $2 \cdot 9 \times 10^3$ cfu g^{-1}. Ascomycetous yeasts were represented by 76% of the isolates while 24% were basidiomycetes. The identification of strains isolated by the simplified identification system (SIM) revealed 19 yeast species representing 12 genera. The most frequently isolated yeasts belonged to the genera Saccharomyces, Pichia, Cryptococcus, Kluyveromyces and Candida.

Fatty acid yeast composition allowed the separation of contaminating yeasts into one of three major groups. Group I included yeasts without linoleic (C 18:2) and linolenic (C 18:3) fatty acids such as Saccharomyces cerevisiae. Group II comprised yeasts without C 18:3 fatty acid like Zygosaccharomyces rouxii and Torulaspora delbrueckii, and group III included yeasts with C 18:2 and C 18:3 acids that belong, among others, to one of the following yeast genera: Pichia, Candida, Kluyveromyces or Cryptococcus.

Species-specific PCR primers were used for the rapid detection and identification of the most dangerous species affecting fruit concentrate stability. The simplified protocol used consisted of PCR-amplification of conserved tracts in the ITS region of the rDNA unit, thus enabling the detection of potentially dangerous flora such as Zygosaccharomyces species and T. delbrueckii in contaminated fruit concentrates. Results from PCR-typing were in full agreement with the fatty acid compositions of these species.

The grouping of contaminant yeasts into three main groups showed that fatty acid composition may be used to differentiate yeasts according to their technological significance. Yeasts isolated in this work as being most dangerous to product stability belong to either group II (Z. rouxii and T. delbrueckii) or group I (Saccharomyces spp.). Group III was comprised of several species regarded as indicators of deficiencies in 'good manufacturing practices'. Thus, each of the groups delineated may be considered to be a zymological indicator of technological significance. The conjugation of fatty acid profiles with PCR-typing methods may be used as a rapid detection system for contaminant yeasts. The fatty acid profiles provide a preliminary identification of yeasts potentially dangerous to product stability present

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within 48 h of isolation. Whereas the PCR-typing method is mainly used to confirm isolate identity, when required, after the initial diagnosis has been performed, over a period of 4 h.

Introduction

Yeasts are common contaminants of fruit concentrates and represent a major problem to industries that process fruits or fruit products (Davenport 1996). The low pH and high sugar content of these products favour yeast growth and consequently product deterioration is predominantly due to yeast activity (Deak and Beuchat 1996). Numerous studies have been published on this subject, which are covered by extensive and excellent reviews (Thomas 1993, Tudor and Board 1993, Deak and Beuchat 1996). These works report that the most common yeast contaminants isolated from fruit concentrates are *Candida* spp., *Debaryomyces hansenii*, *Hansenula* spp., *Rhodotorula* spp., *Pichia* spp., *Dekkera* spp., *Lodderomyces elongisporus*, *Hanseniaspora* spp., *Issatchenkia orientalis*, *Kloeckera* spp., *Kluyveromyces marxianus*, *P. anomala*, *Saccharomyces* spp., *Torulaspora delbrueckii* and *Zygosaccharomyces* spp. Among these, the species *Z. rouxii*, *Z. bisporus* and *Z. bailii* are particularly dangerous to product stability due to their high resistance to low pH and low A_w (Maimer and Busse 1992, Thomas 1993). *Saccharomyces cerevisiae* is also considered to be a predominant spoilage species in concentrates, juices and fruit beverages (Gardini and Guerzoni 1986, Deak and Beuchat 1993b), and in that respect is considered to be the source of most problems associated with processed fruits (Maimer and Busse 1992).

Several methodologies may be used to differentiate the yeast flora present in foods. Traditional plating techniques may be adapted using selective and/or differential media but there is no satisfactory medium for detecting, isolating and enumerating all yeasts in all foods (Beuchat 1993). Selective-differential media have been developed to identify broad microbial groups in certain types of foods such as preservative-resistant yeasts or osmotolerant yeasts (Beuchat 1995). However, yeast differentiation by differential media is poorly developed when compared with similar works

applied to bacteria (see book edited by Corry et al. 1995). Other phenotypic methods include conventional yeast identification by means of assimilation or fermentation tests and the use of morphological characteristics (Kurtzman and Fell 1998). Conventional methodologies are not suited to industrial laboratories even when these procedures are automated and computerised (Deak and Beuchat 1996). Simplified identification schemes (SIM) have been used successfully and appear to be more efficient than miniaturized systems (e.g. API systems) in the identification of foodborne species (Torok and King 1991, Deak and Beuchat 1993a), even though their reliability has been questioned (Rohm and Lechner 1990). Another approach concerns the use of biomarkers, such as the composition of cellular molecules like fatty acids, nucleic acids (DNA or RNA), among others (Deak and Beuchat 1996). Fatty acid composition has been used successfully in differentiating the most important foodborne yeasts (Augustyn et al. 1992, Malfeito-Ferreira et al. 1997) and it is complementary to yeast identification (Noronha-da-Costa et al. 1996, Welthagen and Viljoen 1997). Molecular methods of identification are being utilised with some success in the identification of commercial *S. cerevisiae* strains (Johnson and Mortimer 1986, Querol et al. 1992). Several methods exist for verifying strain identification by amplification of random (Baleiras-Couto et al. 1994, Molnár et al. 1995) or specific target sequences (Ibeas et al. 1996, Lavallée et al. 1994). The simplicity of PCR-based detection methods and the highly specific nature of the results has an advantage over most conventional differential methods known today (van der Vossen et al. 1996).

Davenport (1996) proposed dividing the contaminating yeasts of fruit concentrates and juices according to their spoilage potential. The most dangerous species belong to the taxa *Z. bailii* and *S. cerevisiae* while, for instance, *P. anomala* and *R. glutinis* are merely indicators of poor hygiene. Therefore, microbiological

control in the industry should not only give information on overall yeast contamination but should also provide information on the species that are dangerous to product stability. With this purpose in mind, the concept of zymological (zymo = yeast) indicators would be of great interest to industries that process foods susceptible to yeast spoilage.

The aim of this work was the establishment of zymological indicators in the fruit processing industry. For this purpose, three different yeast differentiation techniques were compared (SIM, fatty acid profiles and PCR-based detection), bearing in mind the importance of early detection of dangerous spoilage yeasts.

Material and Methods

Product samples

The fruit pulps and concentrates analysed in this study were used as raw materials for the production of fruit juices and beverages in a Portuguese processing plant.

Yeast strains

The yeast strains analysed were obtained from culture collections or isolated from product samples. Samples were taken from the surface of 200 kg containers using a sterile syringe. Serial dilutions with Ringer solution (Oxoid, Unipath Ltd., Basingstoke, UK) were prepared when necessary and 0·5 ml were spread on agar plates. Duplicate plates of Malt Extract Agar (MEA, Oxoid) were incubated at 25°C and counts were made after a maximum of 10 days.

Yeast isolates were selected from counting plates according to different colony morphology and the proportion of each species on the total flora was based on distinct morphologies. Strains were purified by subsequent re-streaking on MEA. Slants of pure cultures in MEA were kept at 4°C until used.

Utilization of the Simplified Identification Method (SIM)

The identification followed the procedures described by Deak and Beuchat (1987) and Deak

(1992). The following biochemical tests were performed as described by these authors: urea splitting, glucose fermentation, nitrate assimilation, carbon source assimilation (maltose, raffinose, galactose, cellobiose, trehalose, erythritol, mannitol, melibiose, xylose and inositol). Whenever necessary complementary tests were used as described by Kreger-van Rij (1984): acetic acid production, growth in 10 ppm cycloheximide, growth at 37°C, pseudomycelium formation, bipolar budding and other morphological observations.

Determination of cellular fatty acid methyl ester compositions (FAME)

Pure cultures were grown on GYP (20 g l^{-1} glucose (Merck, Darmstadt, Germany), 5 g l^{-1} yeast extract (Difco Laboratories, Detroit, Michigan, USA), 10 g l^{-1} peptone (Difco Laboratories) and 20 g l^{-1} agar, pH 6·0) slants for 24 h at 25°C. Biomass was suspended in 1 ml Ringer solution (Oxoid) and inoculated in triplicate slants of 24-mm diameter tubes with cotton plugs. Incubation was carried out at 25°C for 48 h.

Fatty acid extraction and derivatization followed the procedures described by Noronha-da-Costa et al. (1994). Fatty acids were quantified as a percentage of the total amount of fatty acids from myristic (C 14:0) to linolenic acids (C 18:3). Identification of fatty acids was made by relative retention times of pure compounds (Sigma Chemical Co., St. Louis, USA) using palmitic acid (C 16:0) as the reference. Calculations were performed by the Chrom-Card for Windows program (Fisons Instruments, model 8130, Rodano, Italy). Data statistical treatment was performed by principal component and cluster analysis (PCA) using the software SPAD.N version 2·5 PC (Centre International de Statistique et d'Informatique Appliquées, Saint-Mandé, France). In the PCA the fatty acid compositions of identified strains obtained from culture collections were used as active variables while the profiles of yeast isolates were taken as illustrative variables which do not affect the statistical discrimination of the active variables (Anonymous, 1993).

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PCR-mediated DNA analysis

Potential spoilage yeasts were identified by the use of a species-specific PCR based detection method. Species-specific primers for *Zygosaccharomyces* and *Torulaspora* were used in PCR experiments to amplify fragments of known size. Certified culture collection strains of *Z. bailii*, *Z. bisporus*, *Z. rouxii* and *T. delbrueckii* were tested initially to assess the specificity and fragment sizes produced by each primer set. Universal primers (ITS1 and ITS4), based on published sequences of conserved regions of the 18S and 26S rRNA genes were used to amplify the ITS region (White et al. 1990), and served as a positive control. A negative control using PCR components and primers, but no added DNA, was included to ensure that the reagents used were not contaminated with extraneous template DNA. Additional type strains belonging to other yeast genera (*Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Pichia* and *Saccharomyces*) were tested to settle the question of false positive signals. DNA amplifications of all strains examined were performed in duplicate, using the universal primer set and at least one species-specific oligonucleotide primer pair.

PCR amplification. The yeast strains were selected from culture collections or isolated during the course of this work. Pure cultures were grown on YM plates ($3 \, g \, l^{-1}$ malt extract (Difco), $3 \, g \, l^{-1}$ yeast extract (Difco), $5 \, g \, l^{-1}$ peptone (Difco), $10 \, g \, l^{-1}$ glucose (Merck) and $20 \, g \, l^{-1}$ agar) at $25°C$ for $48 \, h$. DNA amplifications were performed using a Robocycler 96 thermal cycler (Stratagene, California, USA).

Oligonucleotide primers were designed by James (1999) from conserved tracts of the ITS1 and ITS2 transcribed spacers. The primer sequences used ranged in size from 19 to 21 bases in length (Table 1) and were synthesized by Medprobe A.S. (Norway).

DNA amplifications were performed directly on yeast cells. A single yeast colony from a plate was resuspended in $50 \, \mu l$ of sterile demineralized water. The cells in suspension were diluted in water (1:5) and boiled for $5 \, min$ and placed on ice for an equal period of time. A cell suspension of $25 \, \mu l$ was used for each PCR amplification reaction.

Amplifications were performed in a $50 \, \mu l$ reaction volume containing $3·0 \, mM \, MgCl_2$, $0·1\%$ gelatine (w/v), $0·1\%$ Triton X-100, $200 \, \mu mol \, l^{-1}$ each of dATP, dCTP, dGTP and dTTP, $0·2 \, \mu mol \, l^{-1}$ of each of the two amplification primers and one unit of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut, USA). Twenty-five microliters of a cell suspension was added to $25 \, \mu l$ of the PCR mix. The reaction mix was overlaid with sterile mineral oil ($50 \, \mu l$) to prevent evaporation during PCR cycling. The cycling parameters were as follows: an initial denaturation step of $94°C$ for $2 \, min$ followed by two cycles of $90 \, s$ at $94°C$, $90 \, s$ at $54°C$ and $90 \, s$ at $72°C$. Thirty-five additional cycles were performed as follows: $90 \, s$ at $92°C$, $90 \, s$ at $54°C$ and $90 \, s$ at $72°C$. The annealing temperature varied from $52–54°C$, depending on the strains tested. For *Z. rouxii* an annealing temperature of $52°C$ was used instead of $54°C$. After the cycling steps were completed, the mixture was kept at $72°C$ for $5 \, min$ to allow complete extension of amplified products. PCR-amplified DNA fragments were

Table 1.   Primers sequences used in the PCR-based detection method

Primer	Primer sequence (5'–3')	Target/Species
PZba1	AATTTCTGATTGACGAGTTCT	*Z. bailii*
PZba2	ACTCACCCAATCTCCTAGT	*Z. bailii*
PZbi1	TCTGATTGGCAAGTCAATGG	*Z. bisporus*
PZbi2	CAATTTCAAGCTAACCCAGT	*Z. bisporus*
PZrx1	ACGGAGTTCTCTCAAAGTG	*Z. rouxii*
PZrx2	ACCTAATACGACTATCGTG	*Z. rouxii*
PTd1	GATACTGTAAGAGAGGATCAG	*T. delbrueckii*
PITS1	TCCGTAGGTGAACCTGCGG	Universal
PITS4	TCCTCCGCTTATTGATATG	Universal

analysed by conventional gel electrophoresis in a 3% (w/v) agarose gel (Nusieve® 3:1) at $5\,Vcm^{-1}$. Tris-Acetate (TAE, 1X) buffer (0·04 M Tris-acetate and 0.001 M EDTA, pH 8·0) was used as electrolyte. A 1 kb DNA ladder (Boehringer-Mannheim, Germany) was used as the molecular weight reference. PCR fragments were visualised under UV illumination and photographed with a Polaroid® camera.

Results

Contamination of fruit pulps and concentrates

The results of yeast enumeration in the products analysed are shown in Table 2. The highest counts were observed in orange concentrate and peach pulp. The pear pulp and apple concentrate showed lower counts while microbial contamination was not detected in the pineapple and passion-fruit concentrates. All containers of orange concentrate and peach pulp were contaminated by yeasts while lower percentages of contaminated samples were obtained in apple concentrate and pear pulp (Table 2). However, the observed microbial loads did not induce visible product spoilage at the moment of examination.

Yeast characterisation by analysis of FAME

The FAME composition of food relevant yeast species from culture collections facilitated their division into three major groups based on the presence or absence of linoleic (C 18:2) and linolenic (C 18:3) fatty acids (Table 3):

group I, without C 18:2 and C 18:3 fatty acids; group II, with C 18:2 and without C 18:3 fatty acid and group III, with C 18:2 and C 18:3 fatty acids. Group I was comprised of species of the genus *Saccharomyces*; group II comprised the species *Z. bailii*, *Z. rouxii*, *T. delbrueckii* and *Dekkera* sp.; and group III comprised the species *P. anomala*, *P. membranifaciens*, *I. orientalis*, *D. hansenii* and *K. marxianus*. All strains of each of the three major groups were subjected to principal component and cluster analysis (the resulting projection plans are shown in Fig. 1 only for group II). The distribution of the yeast species in the clusters of the major groups is shown in Table 3. In group I it was not possible to discriminate the different species of *Saccharomyces* spp.. In group II, the genera *Dekkera*, *Torulaspora* and *Zygosaccharomyces* were clearly separated into distinct clusters. The separation of Z. rouxii and *Z. bailii* was not achieved for all strains because some *Z. rouxii* strains were clustered together with Z. bailii. Group III was characterized by the heterogeneity of the clusters. Most of them involved more than one species while only two clusters were constituted by one species.

The fatty acid profiles of the strains isolated in this study were introduced into the corresponding database of each major group and subjected to principal component and cluster analysis. The isolates were allocated to the major groups and clusters shown in Table 3. In group I all isolates were necessarily clustered with species of the genus *Saccharomyces* spp.. In group II the isolates were placed in one cluster containing only strains of *Z. rouxii* and in another containing only *T. delbrueckii* strains.

Table 2.   Enumeration of yeast flora on the surface of fruit concentrate and pulp containers

Product and Brix (%)	Average $(cfu\,g^{-1})$	Maximum $(cfu\,g^{-1})$	Minimum $(cfu\,g^{-1})$	Number of containers	Contaminated containers (%)
Pineapple concentrate (59·5–60·5)	<1	<1	<1	5	0
Orange concentrate (63·0–65·0)	503	2895	2	63	100
Apple concentrate (70)	3	25	<1	13	30
Passionfruit concentrate (49·5–50·5)	<1	<1	<1	5	0
Pear pulp (11·0–12·0)	14	126	<1	8	25
Peach pulp (10·0–12·0)	283	1360	4	22	100

Table 3.   Allocation of yeast isolates to clusters obtained by principal component and cluster analysis of their long-chain fatty acid compositions

Major group	Clusters	Database species[a]	Strain number[b]
I	A	*S. cerevisiae* (2), *S. pastorianus* (2)	52
	B	*S. cerevisiae* (5)	
	C	*S. paradoxus* (4), *S. pastorianus* (1), *S. cerevisiae* (1)	
	D	*S. bayanus* (5), *S. pastorianus* (2), *S. cerevisiae* (1)	15, 35, 39, 68, 94, 113
II	A	*D. bruxellensis* (8)	
	B	*T. delbrueckii* (9)	75
	C	*D. anomala* (4), *D. naardenensis* (1)	
	D	*D. naardenensis* (2)	
	E	*Z. rouxii* (6)	61, 70, 79, 82, 89, 110, 180
	F	*Z. bailii* (18), *Z. rouxii* (5)	
III	A	*D. hansenii* (8), *I. orientalis* (6), *P. membranifaciens* (2)	3, 4, 7, 14, 19, 22, 40, 45, 49, 53, 55, 60, 65, 72, 93, 104, 109, 117, 1121
	B	*P. anomala* (9)	8, 18, 23, 29, 30, 33, 34, 83, 84, 105, 111, 132
	C	*P. anomala* (5), *P. membranifaciens* (1)	13, 41, 57, 77, 96, 101, 1120
	D	*I. orientalis* (2), *P. membranifaciens* (1)	
	E	*P. membranifaciens* (15), *I. orientalis* (1)	21, 28, 112
	F	*P. membranifaciens* (15), *K. marxianus* (2)	16, 48
	G	*K. marxianus* (6)	

[a]Number of strains of each species, originating from culture collections, is shown in parentheses.
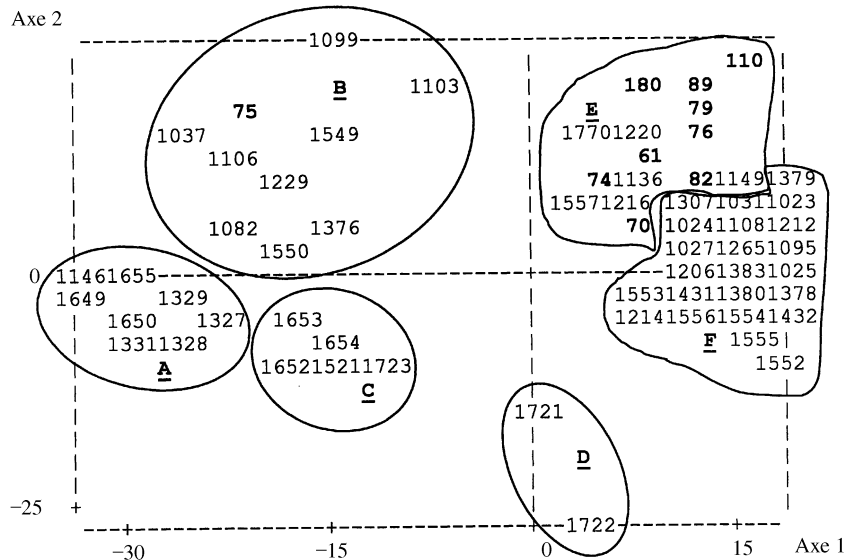[b]Strains isolated from samples analysed in this work.

Figure 1.   Projection plan of the principal component and cluster analysis of the long-chain fatty acid compositions of yeasts belonging to group II. The letters correspond to the clusters described in Table 3. The strains written in bold were isolated during the course of this work and were considered illustrative variables. The strains from culture collections are identified by the respective ISA number.

In group III the strains isolated were distributed over various clusters composed by single or several species (Table 3). Overall, according to the FAME compositions, 12% of the strains belong to group I, 14% to group II and 74% to group III, with variable proportions in each product (Table 4). The isolates of group III were present in all types of products, whereas the representatives of group II were recovered from orange and apple concentrates and from pear pulp. Strains with profiles included in group I were only detected in orange concentrates. The highest counts ($\sim 10^3$ cfu g^{-1}) were obtained with yeasts belonging to group II or III, while yeasts from group I were not recovered in levels higher than 30 cfu g^{-1}. In particular, the isolates with profiles similar to *Z. rouxii* were detected as the sole kind in four container samples, and as 66% of the isolates in one sample (see Table 4). The isolates with profiles similar to *Saccharomyces* sp. were in the following proportions: 100% (three samples from one concentrate container), 75% (one sample), 65 % (one sample) and 18% (one sample). The isolates of group III represented the total yeast flora of 19 samples, from which 12 showed profiles belonging to two different clusters whereas seven samples presented profiles from only one cluster (see Table 4).

Comparison of SIM and FAME results

The identification resulting from the utilization of the dichotomic keys of the SIM is shown in Table 4. The results of the urea splitting showed that most strains belong to the Ascomycota (76%) and the remaining 24% belong to the Basidiomycota. The isolates were distributed over 19 yeast species representing 12 genera. The comparison of SIM results with the clustering of yeasts according to their FAME composition is shown in Table 4. The SIM assigned nine strains to *S. cerevisiae*, of which six showed compatible fatty acid profiles. The remaining strains were placed in groups II and III. The three isolates assigned to *Z. rouxii* by the SIM were consistent with the fatty acid profiles. The species assigned by the SIM to the genus *Candida, Pichia, Kluyveromyces, Rhodotorula, Cryptococcus, Yarrowia, Rhodosporidium* and *Sporobolomyces* were placed in group III,

with the exception of one strain of *K. marxianus* which was placed in group II.

The incompatibilities detected in species belonging to FAME groups I and II led to the execution of further biochemical tests and the utilization of PCR-typing methods as described below. In relation to group III the possible incompatibilities were not investigated because rapid diagnostic tools were not available for the species given by the SIM and because the differentiation would not be relevant to the fruit processing industry. In fact, the species of group III are not regarded as dangerous spoilers in the industries concerned with this study (Deak and Beuchat 1996).

Strains 65 and 132 were identified by the SIM as *S. cerevisiae*, however their FAME composition indicated their inclusion in group III which is not characteristic of this species. In fact, the positive result in the additional test of growth in the presence of 0·01% cycloheximide showed that these strains could not be *S. cerevisiae*.

The phenotypic identification of strain 75 as *S. cerevisiae* resulted from the negative assimilation of mannitol. The positive mannitol reaction would have lead to the identification as *T. delbrueckii*. The result provided by the FAME was *T. delbrueckii* which was confirmed by PCR (see Table 5).

The identification of strain 70 as *K. thermotolerans* was determined by the weak glucose fermentation and positive maltose assimilation. A strong glucose fermentation would have lead to the identification as *Z. rouxii*. The FAME profile was similar to *Z. rouxii*. This identity was confirmed by PCR (see Table 5).

Rapid identification of spoilage yeasts by PCR typing

Direct PCR amplification was performed on crude cell lysates, using three primer sets and ITS universal primers. The specificity of the primers used in DNA amplification reactions (Table 5) was determined by using type strains of representative species as well as strains from distinct culture collections. A total of 65 strains from culture collections and the isolates belonging to FAME groups I and II were examined to evaluate the feasibility of using

Table 4.   Enumeration, identification (SIM) and fatty acid clustering of the isolated yeast strains

Origin (container number)	Sample	Strain nr.	cfu g^{-1}	%	FAME group/cluster[a]	SIM identification
Orange 1	1	7	1053	70	III/A	*C. diddiense*
		8	451	30	III/B	*P. ohmeri*
	2	111	22	44	III/B	*K. thermotolerans*
		55	20	40	III/A	*K. thermotolerans*
		23	5	10	III/B	*P. anomala*
		72	3	6	III/A	*R. mucilaginosa*
Orange 2	1	53	1362	90	III/A	*C. humicolus*
		96	137	10	III/C	*P. anomala*
	2	61	564	50	II/E	*Z. rouxii*
		70	564	50	II/E	*K. thermotolerans*
Orange 3	1	4	427	58	III/A	*C. humicolus*
		112	264	35	III/E	*C. stellata*
		65	56	7	III/A	*S. cerevisiae*
Orange 4	1	15	2	50	I/D	*S. cerevisiae*
		35	2	50	I/D	*S. cerevisiae*
	2	39	4	100	I/D	*S. cerevisiae*
	3	68	1	100	I/D	*S. cerevisiae*
Orange 5	1	28	4	30	III/E	*Y. lipolytica*
		105	2	14	III/B	*C. diddiense*
		132	2	14	III/B	*S. cerevisiae*
Orange 6	1	40	7	43	III/A	*K. thermotolerans*
		49	3	19	III/A	*K. thermotolerans*
		45	1	7	III/A	*Y. lipolytica*
Orange 7	1	48	124	82	III/F	*P. farinosa*
		94	28	18	I/D	*S. cerevisiae*
Orange 8	1	52	24	75	I/A	*S. cerevisiae*
		75	8	25	II/B	*S. cerevisiae*
Orange 9	1	83	17	36	III/B	*P. subpelliculosa*
Orange 10	1	16	1	100	III/F	*K. thermotolerans*
Orange 11	1	82	79	33	II/E	*Z. rouxii*
		89	79	33	II/E	*Z. rouxii*
		93	79	33	III/A	*C. laurentii*
Orange 12	1	113	30	65	I/A	*S. cerevisiae*
		84	16	35	III/B	*P. subpelliculosa*
Apple 1	1	110	1	100	II/E	-b
Apple 2	1	180	1	100	II/E	-
Apple 3	1	19	1	50	III/A	*C. diddiense*
		13	1	50	III/C	*C. albidus*
Peach pulp 1	1	104	171	50	III/A	-
		34	171	50	III/B	*P. anomala*
Peach pulp 2	1	77	1360	100	III/C	*P. anomala*
Peach pulp 3	1	21	208	73	III/E	*D. hansenii*
		18	76	27	III/B	*R. informiniatum*
Peach pulp 4	1	41	4	100	III/C	-
Peach pulp 5	1	60	37	50	III/A	*D. hansenii*
		1120	19	25	III/C	*C. laurentii*
		1121	12	16	III/A	*C. albidus*
		101	7	10	III/C	-
Pear pulp 1	1	3	1	100	III/C	*R. mucilaginosa*
Pear pulp 2	1	30	3	50	III/B	*C. sake*
		14	2	25	III/A	*S. roseus*
Pear pulp 3	1	22	3	25	III/A	*R. mucilaginosa*
		57	1	8	III/C	-
		109	1	8	III/A	-
Pear pulp 4	1	29	1	100	III/B	-
Pear pulp 5	1	33	1	33	III/B	-
		117	1	33	III/A	*D. hansenii*
Pear pulp 6	1	79	1	100	II/E	-

[a]The composition of the clusters is shown in Table 3.
[b]Not determined.

Table 5.   Amplification results with primers

Species/Strains[a]	PCR response with primer pair				
	pZba1/ pZba2	pZbi1/ pZbi2	PZrx1/ pZrx2	pTd1/ pITS4	pITS1/ pITS4
Z. bailii					
IGC 4227, IGC 4531, IGC 4805, IGC 4806, IGC 5188, IGC 5190, IGC 5200, IGC 5202, IGC 5203, IGC 5204, IGC 5208, IGC 5221, IGC 5222, IGC 5224, IGC 5225, ISA1022, ISA 1023, ISA1024, ISA 1025, ISA 1027, ISA 1031, ISA 1095, ISA 1148, ISA 1206, ISA 1214, ISA 1378, ISA 1381, ISA 1383, ISA 1430, ISA 1433	+	−	−	nd[b]	+
IGC 5167[T]	+	−	−	−	+
IGC 5207, IGC 5316	−	−	−	nd	+
Z. rouxii					
IGC 4802, IGC 4879, IGC 5226, ISA 1552, ISA 1553, ISA 1554, ISA 1555, ISA 1557, ISA 1558	−	−	+	nd	+
ISA 1770[T], IGC 3701, ISA 1136, ISA 1188	−	−	+	−	+
Z. bisporus					
IGC 5335[T], TNO R-7	−	+	−	−	+
IGC 5336, IGC 5337, TNO R-66, TNO R-68, TNO R-216, TNO R-211	−	+	−	nd	+
T. delbrueckii ISA 1082[T], IGC 2613, ISA 1099	−	−	−	+	+
D. hansenii IGC 2693[T]	−	−	−	−	+
I. orientalis ISA 1528[T]	−	−	−	−	+
K. marxianus ISA 1538[T]	−	−	−	−	+
P. anomala ISA 1478[T]	−	−	−	−	+
P. membranifaciens ISA 1005[T]	−	−	−	−	+
S. bayanus ISA 1634[T]	−	−	−	−	+
S. cerevisiae ISA 1630[T]	−	−	−	−	+
S. pastorianus IGC 4570[T]	−	−	−	−	+
Isolated strains[c]					
Saccharomyces sp. 15, 35, 39, 94, 68	−	−	−	−	+
T. delbrueckii 75	−	−	−	+	+
Z. rouxii 61, 70, 74, 76	−	−	+	−	+
Z. rouxii 79, 82, 89, 110, 180	nd	nd	+	nd	+

[a]IGC, Instituto Gulbenkian de Ciência (Portuguese Yeast Culture Collection) Oeiras, Portugal; ISA, Instituto Superior de Agronomia, Lisboa, Portugal; TNO, TNO Nutrition and Food Research Institute, Zeist, The Netherlands. T-type strain.
[b]Not determined.
[c]Probable identification given by fatty acid compositions.

various primer pairs to differentiate important spoilage yeasts. In each case, amplification products were verified by using appropriate positive and negative controls. At an annealing temperature of 52–54°C discrete amplification products were observed with the ITS1-ITS4 primer pair in all the yeast strains examined. A discrete fragment of DNA of known size was amplified when the strain tested corresponded to a specific species and the corresponding primer pair was utilised in the PCR reaction. Species could be differentiated by the presence of amplification product and the size of the amplified fragment. DNA amplification products varied in size, from 450 bp (for *Z. bisporus*) to 600 bp (*Z. bailii*). Most *Zygosaccharomyces* strains examined showed the expected primer specificity. All species-specific primers produced distinct DNA fragments only when chromosomal DNA from the target species was

present, and did not yield amplification products with DNA from other species. In each case, the specific primer sets produced one discrete band for the species in question (results not shown). Strain identifications were confirmed for all *Z. bisporus*, *Z. rouxii* and *T. delbrueckii* examined in the present work. PCR amplification with primers pZba1/pZba2 produced a band of approximately 600 bp in length with strains belonging to the species *Z. bailii*. However, two strains identified as *Z. bailii* (IGC 5207 and IGC 5316) failed to produce the expected amplification product. Negative PCR responses with all other available primer pairs led to the conclusion that the strains in question do not belong to any of the spoilage yeasts targeted and so were incorrectly identified by the conventional methodologies.

Discussion

Yeast enumeration and characterisation

The microbial loads of the fruit pulps and concentrates tested are in accordance with other values from literature (Deak and Beuchat 1993a, b, Cava and Hernández 1994). The levels of contamination were variable not only in the different raw materials but also in the same lot of products as reported elsewhere (Deak and Beuchat 1993a, Tudor and Board 1993).

The comparison of results given by SIM and FAME in relation to the most dangerous species isolated (*Z. rouxii* and *T. delbrueckii*) showed that FAME results completely agreed with the results of PCR tests. This suggests that FAME is more adequate for typing these species than the SIM. The reasons for most of the observed discrepancies were related with weak or variable responses of one or two tests used in the dichotomic keys of the SIM, as already pointed out by Rohm and Lechner (1990). Recently, Deak and Beuchat (1996) presented a new dichotomic key in which other tests are proposed and variable responses for the same species are included. For instance, the incorrect identification of some strains of *S. cerevisiae* would not have occurred because the growth in cycloheximide is included in the current key. However, the novel key increased the number of tests from 19 to 33 which makes the technique more difficult and time consuming for use in food industry laboratories. Thus, for the purpose of this work, besides being more accurate for typing *Z. rouxii, T. delbrueckii* and *S. cerevisiae*, the FAME profiling is a faster technique than the SIM.

In relation to the species of group III, the FAME database is, at the moment, constituted by few ascomycetous species and has not been enlarged to basidiomicetous yeasts. The heterogeneity of the profiles for the same species or their similarity for different species demonstrate that this technique cannot be used as the sole means of yeast identification, as stated by Augustyn et al. (1992). However, this lack of precision is not relevant in technological terms where the main concern is to assess the presence of yeasts dangerous to product stability.

Definition of zymological indicators

The division of contaminant yeasts into three main groups showed that FAME composition may be used to differentiate yeasts according to their technological significance. The species considered to be the most dangerous yeasts to product stability isolated in this work were placed in groups II (*Z. rouxii* and *T. delbrueckii*) and I (*Saccharomyces* spp.). The former was detected in pear pulp, orange and apple concentrates. The latter was only recovered from orange concentrates. Strains belonging to group III were isolated from all products and involved a series of species considered to be an indicator of 'poor manufacturing practices' such as poor process hygiene or environmental contamination. The variability observed in yeast counts for the same product was also present as regards the types of FAME profiles detected, particularly in orange concentrate where containers were contaminated either by mixed populations or by isolates showing only one of the three FAME major groups. However it should be noted that *Z. rouxii*, when present, dominated the sample flora.

The major FAME groups are comparable to those presented by Davenport (1996) in a forensic approach to the problem of yeast contamination in the fruit industry. From a technological point of view, Davenport (1996)

stated that 'micro-organism behaviour patterns and traits are paramount over correct names'. Therefore, FAME profiles may be used as a zymological indicator that enables a rapid characterization of the contaminant yeast flora. The products analysed were characterized by the predominance of yeasts associated with poor manufacturing practices (FAME group III). The presence of dangerous yeasts of FAME groups I and II, was observed, generally, in lower numbers but represents a warning as to occasional spoilage of processed products based on raw materials bearing these yeasts.

The utilization of highly specific molecular probes for yeast typing enabled the most dangerous species to fruit concentrate stability to be detected. The simplified protocol adapted from James (1999) showed specific amplification for the species *Z. bailii, Z. bisporus, Z. rouxii* and *T. delbrueckii*. In this case study only the two last species were detected confirming that orange and apple concentrates and pear pulp harbour a dangerous contaminant flora. This method has several advantages over other genotypic identification typing methods. First, it can be performed on whole yeast cells, greatly reducing the amount of time necessary to prepare samples, secondly the PCR primers are species-specific. The PCR-based identification method on the other hand is easy to perform, fast (taking less than 4 h after strain purification), economical and capable of widespread application in the food industry. However, this technique is very practical only when a probable species is to be identified. In this case the choice of the primer may be limited to few species. Complications arise when the species is not known and so a wide range of primers may be necessary, being tedious and/or expensive to spot the unidentified species, because the negative results do not give information on the contaminant flora.

To overcome this problem we propose the conjugation of FAME profiling with molecular probes in order to type contaminant yeasts rapidly. FAME provides a broad primary picture of the isolated flora giving a correct identification in the case of the species most dangerous to product stability, in an overall procedure that takes 48 h after yeast isolation. Then the range of molecular probes to be tested is restricted to the cases where confirmation of identity may be necessary. The advantage of use of FAME profiling is not based on the availability of a highly specific differentiation technique but on the availability of a tool for a broad separation of yeast isolates which was shown to have sound technological significance. In addition, this approach may be a useful tool for the definition of specifications for raw-materials and products where yeasts play the main role as spoiling agents, like in the fruit processing industry.

Acknowledgements

References

Anonymous (1993) *Système portable d'analyse des données numériques* (SPAD.N, version 2.5 P.C.). Centre International de Statistique et d'Informatique Appliquées. Saint-Mandé, France.

Augustyn, O., Kock, J. and Ferreira, D. (1992) Differentiation between yeast species, and strains within a species, by cellular fatty acid analysis 5. A feasible technique? *System. Appl. Microbiol.* **15,** 105–115.

Baleiras-Couto, M., van der Vossen, J. M., Hofstra, H. and Huis in't Veld, J. (1994) RAPD analysis: a rapid technique for differentiation of spoilage yeasts. *Int. J. Food Microbiol.* **24,** 249–260.

Beuchat, L. (1993) Selective media for detecting and enumerating foodborne yeasts. *Int. J. Food Microbiol.* **19,** 1–14.

Beuchat, L. (1995) Media for detecting and enumerating yeasts and moulds. In *Culture media for food microbiology* (Eds J. Corry, G. Curtis, and R. Baird) pp. 229–242. Amsterdam, Elsevier Science B.V.

Cava, R. and Hernández, P. (1994) Comparison of media for enumerating osmotolerant yeasts in orange juice concentrates. *Int. J. Food Microbiol.* **22,** 291–295.

Corry, J., Curtis, G. and Baird, R. (1995) *Culture media for food microbiology.* Amsterdam, Elsevier Science B.V.

Davenport, R. (1996) Forensic microbiology for soft drinks business. *Soft Drinks Managem. Int.*, **4,** 31–32.

Deak, T. (1992) Experiences with, and further improvement to, the Déak and Beauchat simplified identification scheme for foodborne yeasts. In *Moderns Methods in Food Mycology* (Eds R. Samson, A. Hocking, J. Pitt and A. King) pp. 47–54. Amsterdam, Elsevier Science B.V.

Deak, T. and Beuchat, L. (1987) Identification of foodborne yeasts. *J. Food Prot.* **50,** 243–264.

Deak, T. and Beuchat, L. (1993a) Comparison of the SIM, API 20C, and ID 32C systems for identification of yeasts from fruit juice concentrates and beverages. *J. Food Prot.* **56,** 585–592.

Deak, T. and Beuchat, L. (1993b) Yeasts associated with fruit juices concentrates. *J. Food Prot.* **56,** 777–782.

Deak, T. and Beuchat, L. R. (1996) *Handbook of food spoilage yeasts*. New York, CRC Press.

Gardini, F. and Guerzoni, M. (1986) Evaluation of the spoilage potential of the yeasts colonizing a soft-drink factory (in italian). *Industrie delle Bevande* **10,** 367–373.

Ibeas, J. I., Lozano, I., Perdigones, F. and Jiménez, J. (1996) Detection of *Dekkera-Brettanomyces* strains in sherry by a nested PCR method. *Appl. Environ. Microbiol.* **62,** 998–1003.

James, S. (1999) *Molecular systematics of ascomycetous yeasts*. Ph.D. Thesis, University of East Anglia, Norwich, U.K.

Johnson, J. and Mortimer, R. (1986) Electrophorectic karyotyping of laboratory commercial strains of *Saccharomyces* and other yeasts. *Int. J. Syst. Bacteriol.* **35,** 569–572.

Kreger-van Rij, N. (1984). *The Yeasts, a taxonomic study* 3rd edn. Amsterdam, Elsevier Science Publisher, B.V.

Kurtzman, C. and Fell, J. (1998) *The Yeasts, a taxonomic study* 4th edn. Amsterdam, Elsevier Science Publisher B.V.

Lavallée, F., Salvas, Y., Lamy, S., Thomas, D.Y., Degré, R. and Dulau, L. (1994). PCR and DNA fingerprinting used in quality control in the production of wine yeast strains. *Am. J. Enol. Vitic.* **45,** 86–91.

Maimer, E. and Busse, M. (1992) Growth properties and gas formation by yeasts isolated from processed fruits in media with various Brix values and sorbic acid contents. *J. Food Prot.* **55,** 192–197.

Malfeito-Ferreira, M., Tareco, M. and Loureiro, V. (1997) Fatty acid profiling: a feasible typing system to trace yeast contamination in wine bottling plants. *Int. J. Food Microbiol.* **38,** 143–155.

Molnár, O., Messner, R., Prillinger, H., Stahl, U. and Slavikova, E. (1995) Genotypic identification of *Saccharomyces* species using random amplified polymorphic DNA analysis. *System. Appl. Microbiol.* **18,** 136–145.

Noronha-da-Costa, P., Rodrigues, C., Spencer-Martins, I. and Loureiro, V. (1996) Fatty acid patterns of film forming yeasts and new evidence for the heterogeneity of *Pichia membranaefaciens*. *Lett. Appl. Microbiol.* **23,** 79–84.

Querol, A., Barrio, E., Huerta, T. and Ramon, D. (1992) A comparative study of different methods of yeast strain characterisation. *System. Appl. Microbiol.* **15,** 439–446.

Rohm, H. and Lechner, F. (1990) Evaluation and reliability of a simplified method for identification of food-borne yeasts. *Appl. Environm. Microbiol.* **56,** 1290–1295.

Thomas, D. (1993) Yeasts as spoilage organisms in beverages. In *The Yeasts* 2nd edn. *Yeast Technology* (vol. 5) (Eds A. Rose and J. Harrison) pp. 517–561. London, Academic Press.

Tudor, E. and Board, R. (1993) Food-spoilage yeasts. In *The Yeasts* 2nd edn. *Yeast Technology* (vol. 5) (Eds A. Rose and J. Harrison) pp. 435–516. London, Academic Press.

Torok, T. and King, A. (1991) Comparative study on the identification of food-borne yeasts. *Appl. Environ. Microbiol.* **57,** 1207–1212.

Van der Vossen, J. M. and Hofstra, H. (1996) DNA based typing, identification and detection systems for food spoilage microorganisms: development and implementation. *Int. J. Food Microbiol.* **33,** 35–49.

Welthagen, J. and Viljoen, B. (1997) The value of certain chemotaxonomic methods in the identification of food related yeasts. *Food Microbiol.* **14,** 231–245.

White, T., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: a guide to methods and applications* (Eds M. Innis, D. Gelfand, J. Sininsky and T. White) pp. 315–322. San Diego, Academic Press.

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