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Schizosaccharomyces selective differential media

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This study discusses the optimisation of a selective and differential medium which would facilitate the isolation of *Schizosaccharomyces* (a genus with a low incidence compared to other microorganisms) to select individuals from this genus for industrial purposes, especially in light of the recent approval of the use of yeasts from this genus in the wine industry by the International Organisation of Vine and Wine, or to detect the presence of such yeasts, for those many authors who consider them food spoilers. To this end, we studied various selective-differential agents based on the main the physiological characteristics of this species, such as its high resistance to high concentrations of sugar, sulfur dioxide, sorbic acid, benzoic acid, acetic acid or malo-ethanolic fermentation. This selective medium is based on the resistance of the genus to the antibiotic actidione and its high resistance to inhibitory agents such as benzoic acid compared to possible microorganisms which can give rise to false-positive results. Malic acid was used as a differential factor due to the ability of this genus to metabolise it to ethanol, which allows detecting of the degradation of this compound. Lastly, the medium was successfully used to isolate strains of *Schizosaccharomyces pombe* from honey.

Key words: *Schizosaccharomyces pombe*, benzoic acid, actidione, differential selective medium, malic acid.

INTRODUCTION

Yeasts of the genus *Schizosaccharomyces* have occasionally been described as spoilage yeasts, usually due to the production of negative sensory characteristics (Gallander, 1977; Snow and Gallander, 1979; Yokotsuka et al., 1993; Unterholzner et al., 1988; Pitt and Hocking, 1985; Suárez-Lépe et al., 2012). However, these are also used for industrial purposes, particularly in the wine industry, due to their deacidifying properties which they owe to their ability to metabolise L-malic acid to ethanol (Gallander, 1977; Snow and Gallander, 1979; Dharmadhikari and Wilker, 1998; Sousa et al., 1993; Gao and Fleet, 1995; Sousa et al., 1995; Benito et al., 2012b; Benito et al., 2013) or recently, in ageing over lees, due to their superiority to *Saccharomyces cerevisiae* in terms of polysaccharide release (Palomero et al., 2009). Immobilised cells of this genus are also used for wine deacidification in order to avoid its possible side effects (Yokotsuka et al., 1993; Yajima and Yokotsuka, 2001). This species has further applications in fields such as sugar cane fermentation for the production of rum (Pech et al., 1984; Fahrasmane et al., 1988), palm wine production (Christopher and Theivendrarajah, 1988; Sanni and Lonner, 1993) and cocoa fermentation (Ravelomanana et al., 1984; Mazigh 1994). The literature also describes the use of certain mutants of the genus *Schizosaccharomyces* to reduce the initial content of gluconic acid in spoiled grape musts (Peinado et al., 2007; Peinado et al., 2009) or to deacidify wines (Thornton and Rodriguez, 1996). Fermentation with *S. pombe* also provides an interesting way of increasing the overall pyranoanthocyanin content of red wines, and of stabilising their colour during ageing (Morata et al., 2012). The [Office International de la Vigne et du Vin (International Organisation of Vine and Wine)]

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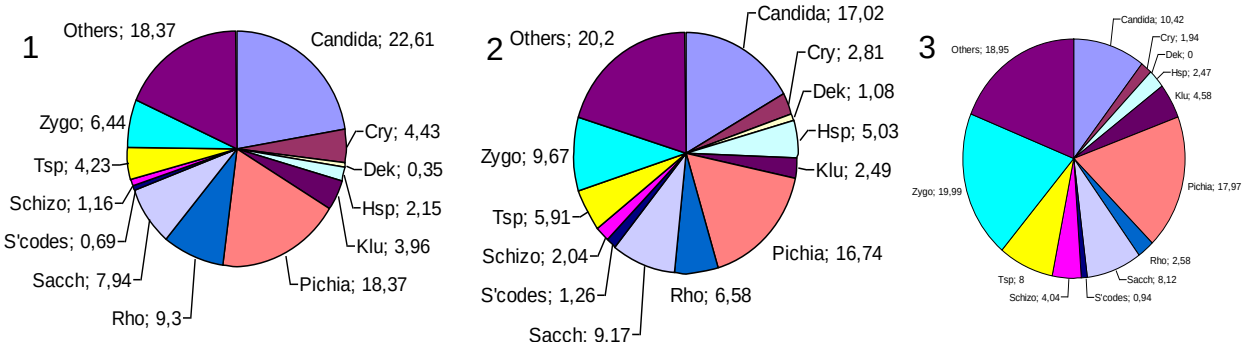

Figure 1. Simplified model of the estimated frequencies (%) of yeast species in food by genus; Deák (2008).

OENO/MICRO/97/75/Stage 7) 'Deacidification by *Schizosaccharomyces*' is an authorised practice, and yet, the number of commercial strains is very limited, probably due to the low incidence of this genus compared to other microorganisms (Pathania et al., 2010). Efforts should therefore focus on the isolation and selection of specimens from this species for industrial applications.

Although there are only few references in the literature which discuss the isolation of yeasts of the *Schizosaccharomyces* genus, the most relevant stem from grapes (Delfini, 1985; Messini et al., 1985), beer (Grieff, 1966), grape must, wine and palm wine (Kunkee and Goswell, 1977; Atputharajah et al., 1986; Delfini, 1985; Rojas, 2001). Most of these references however refer to products with a high sugar content, such as honey, sweets, molasses or dried fruit (Poncini and Wimmer, 1986; Tokouka et al., 1985; Tokouka and Ishinati, 1991; Vaughan Martini, 1991; Deák, 1988; Parfait and Sabin, 1975; Walker and Ayres, 1970); it would therefore seems logical to try and find these microorganisms in those niches. On the other hand, not one yeast species of the genus *Schizosaccharomyces* is found in the list of the 20 most frequent Food-Borne Yeasts compiled by Deák (2008). According to this author, the Calculated Frequencies of Yeast in Foods are as follows: *Schizo. Octosporus* (all foods: 0.18% /Fruit, beverages, wine and beer: 0.35%/ Low a_w products: 1.06%) and *Schizo. pombe* (All foods: 0.98% / Fruit, beverages, wine and beer: 1.69%/ Low a_w products: 2.98%). As a result, in the best case scenario and if looking at Low a_w products in which the incidence of this genus is higher, statistically speaking, we would obtain four strains from the genus *Schizosaccharomyces* (1.06 % + 2.98%) for every 100 strains isolated. Obtaining a selection of suitable yeasts on an industrial scale would therefore require painstaking efforts. Figure 1 proposes a simplified model of the estimated frequencies of the different species of yeast in foods by genus.

Low a_w Products. It is possible to appreciate the reduced incidence of *Schizosaccharomyces* compared to the rest. Zygo: *Zygosaccharomyces*, Tsp: *Torulospora*, Schizo: *Schizosaccharomyces*, S'codes: *Saccharomycodes*, Sacch: *Saccharomyces*, Rho: *Rhodotorula*, Klu: *Kluyveromyces*, Hsp: *Hanseniospora*, Dek: *Dekkera.*

Table 1 summarises the basis of the differential-selective media described in the literature for the isolation/ detection of yeasts. However, not a single selective medium specific for the isolation of yeasts of the genus *Schizosaccharomyces* has been described to date.

Selective media are based on the main metabolic and physiological characteristics of the microorganisms to be isolated and their main competitors. The main characteristics of *Schizosaccharomyces* are described below. Yeasts of this genus have been described as resistant to the antibiotic actidione, which only a small number of yeast species (Benito et al., 2012a) such as *Dekkera bruxellensis, Dekkera anomala, Hanseniaspora uvarum and Candida parapsilosis* are resistant. Figure 2 proposes an amended version of the previous frequency estimates of actidione resistant species, which shows that in the most favourable case (Low a_w Products) the frequency at which yeasts of the genus *Schizosaccharomyces* are isolated would increase to a value higher than 50% (*S. pombe* 41.62% + *S. octosporus* 14.8%).

These yeasts are also capable of growth on media containing high concentrations of sugar (Corry 1976) such as Malt Yeast 50% Glucose Agar and at temperatures of 37°C (Pitt and Hocking, 1999). Other authors have described them as highly resistant to antimicrobial agents such as benzoic acid or sorbic acid, at levels up to 600 mg/L (Warth, 1985, 1988), whereas most yeasts are inhibited at concentrations of 250 to 350 mg/L, although some species of *Zygosaccharomyces* can tolerate up to 800 to 1500 mg/L (James and Stratford, 2003). Some actidione resistant yeasts have however been described as benzoic acid-sensitive to 200 mg/L at pH 3.5 (Benito, 2009b) or *Kloeckera/Hanseniaspora* as sensitive to benzoate at concentrations below 188 mg/L at pH 3.5 (Warth, 1989). However, *D. bruxellensis* is described as particularly resistant to sorbate at levels near 1000 mg/L at pH 3.6 (Benito 2009b), and *Candida parapsilosis* as likewise resistant to sorbate concentrations of up to 10

Table 1. Summary of the differential-selective media described in the literature for the isolation/detection of yeasts. A number of these are patented and currently marketed.

Medium Selectivity	References	Basis
Non-*Saccharomyces* spp.	Morris and Eddy, 1957	Lysine as sole nitrogen source.
	Jerpensen and Jakobsen, 1996	$CuSO_4 5H_2O$ as an inhibitory agent.
Zygosaccharomyces bailii	Makdesi and Beuchat, 1996 Zoeklin, 1995	Addition of 10 ml glacial acetic acid as an inhibitory agent.
Brettanomyces /Dekkera ssp.	Benito et al., 2012a	Actidione, sorbic acid or ethanol assimilation as selective inhibitory factors. Bromocresol green as a differential agent for acetic acid-producing colonies.
Candida spp.	Tornai-Lehoczki et al., 2003	CHROMagar, chromogenic mixture
Yarrowia lipolytica	Carreira and Loureiro, 1998	L-tyrosine as a differential agent through the formation of brown pigment.
Kluyveromyces	Valderrama et al., 1999	Differential agents X-Gal and IPTG which produce blue colonies as a result of ß-galactosidase and ß-glucosidase activity.
Debaromyces hanseii	Siloniz et al., 2000	Salmon-Gluc and its inducer OMe-Gluc, as differential agents which give rise to salmon-coloured colonies.
Various *Zygo. bailii - Zygo. rouxii* *Tsp. delbrueckii* *Db. hansenii* *Iss. orientalis* *S. cerevisiae*	Siloniz et al., 2000	Methylene blue as a differential agent which produces different coloured colonies: Black to violet (*Zygo. Baily- Zygo. rouxii*), violet (*Tsp. Delbrueckii*, *Db. Hansenii and Iss. orientalis*), metallic green (*S. cerevisiae*).
Tsp. delbrueckii *Iss. orientalis*	Siloniz et al., 2000	Differential agent methylene blue, with K-tellurite and acetic acid as inhibitory agents.

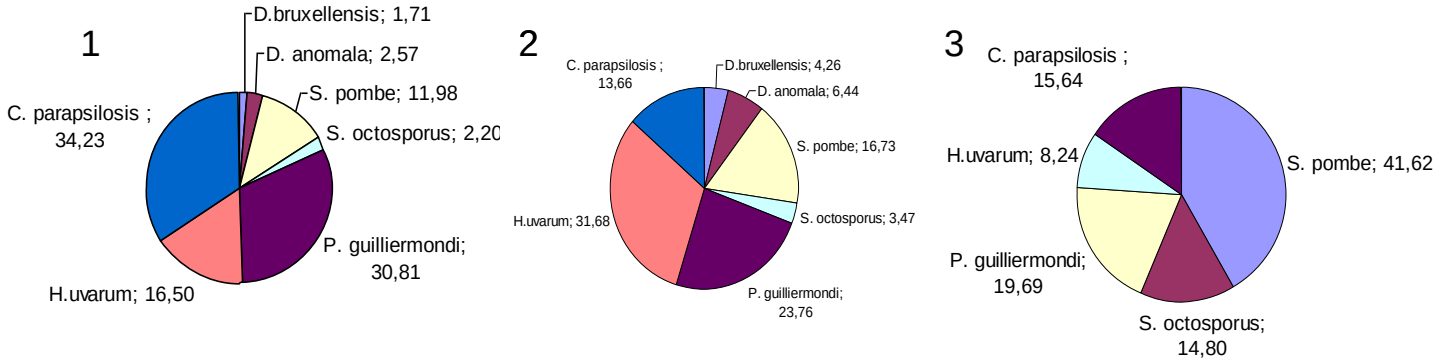

Figure 2. Model depicting the estimated incidence (%) of different yeast species in foodstuffs, by Deák 2008 amended to actidione resistant species. 1, All Foods; 2, Fruit, Beverages, Wine, Beer; 3, Low a_w Products. This shows a significant increase in the likelihood of finding yeast of the genus *Schizosaccharomyces* by using actidione as selective agent. C, *Candida*; H, *Hanseniaspora*; D, *Dekkera*; S, *Schizosaccharomyces*; P, *Pichia*.

to 20 g/L at pH 4 (Deák et al., 1992). *Schizosaccharomyces* have also been described as resistant to sulfur dioxide to levels of 120 mg/kg at pH 3.5 (Warth, 1985) or isolated in 45º Brix raspberry juice concentrate with a SO_2 content of 250 mg/kg at pH=3 (Pitt and Hocking, 1999), whereas other actidione resistant yeasts such as *Dekkera* or *Kloeckera* are described as more sensitive to concentrations of free sulphur dioxide of about 20 mg/L at pH 3.5 (Romano and Suzzi, 1993; Henick-Kling et al., 1998; Benito et al., 2009). In addition, *Schizosaccharomyces* can assimilate the sugars glucose, sucrose, maltose and raffinose, as well as D-gluconate as carbon source (Fugelsang and Edwards, 2007). They are also described as resistant to high concentrations of ethanol (Suárez and Iñigo, 2004) or able to thrive in media with a high content of acetic acid such as malt ace-

tic agar, which is not the case of actidione resistant yeasts such as *Dekkera*, *Kloeckera* (Deák, 2008) or *Candida parapsilosis*, which exhibit occasional growth (Pitt and Hocking, 1999). Lastly, it should be mentioned that one of the main characteristics of this species is its ability to carry out maloalcoholic fermentation and achieving malic deacidification of about 75 to 100%, depending on the strain and culture medium (Thornton and Rodríguez, 1996; Silva et al., 2003; De Fátima, Centeno and Palacios, 2007). Such degradation in a culture medium would be indicative of the presence of yeasts with this ability.

This study applies the principles outlined above to optimise a selective medium which would facilitate the isolation of *Schizosaccharomyces* yeasts compared to existing, traditional media, thus counterbalancing the drawbacks of their reduced presence. These principles are summarised in Table 2, which includes the main formulation concepts for possible differential-selective media applicable to the isolation of yeasts of this genus. This could be used to facilitate the detection of this microorganism when considered a food spoiler, or facilitate strain isolation/selection processes in order to cope with possible imminent requests for commercial strains suitable for industrial applications.

MATERIALS AND METHODS

Microbiological culture conditions

Several solid culture media were used in this study. 20 ml doses of each medium were added to individual sterile Petri dishes. These were then autoclaved for 15 min at 121°C, or for 5 minat 105°C in the case of media whose pH had been adjusted to 3.5 to avoid agar solidification problems. The following selective agents were then added: the antibiotic actidione, sorbic acid and benzoic acid in ethanol solution. Each medium was then checked by inoculating each Petri dish with type cultures belonging to different yeast strains using the streak method. The assays were performed in triplicate. Once inoculated, the plates were incubated isothermally at 25°C.

Yeasts indicative of the effectiveness of the growth media

The yeast strains used as indicator microorganisms were taken from type culture collections belonging to different Spanish associations (Table 3). These were selected so as to include genera and species which could produce false positives in the isolation/detection of *Schizosaccharomyces*.

Media used in the assessment of selective agents

In this study, we used growth media enriched with differential-selective agents the composition of which is provided in **Table 4.** Media which contained sodium benzoate or potassium sorbate were supplemented with varying concentrations of up to 600 mg/L in 50 mg/L increments. The pH values of the media were adjusted with phosphoric acid (Panreac, Barcelona, Spain). In addition to the differential - selective agents developed, chloramphenicol was included in some formulations (Mislivec et al. 1992) in order to inhibit bacterial growth, especially in honey and honeycomb isolates.

Glucose (J. T. Baker Chemicals B.V., Denventer, Holland), bacteriological peptone, yeast extract (all by Pronadisa, Madrid, Spain), *p*-Coumaric acid and actidione (Fluka Steinheim, Switzer land), ethanol, potassium sorbate, sodium benzoate, orthophos-phoric acid and malic acid (all supplied by Panreac, Barcelona, Spain) Chloramphenicol (Sigma-Aldrich, St. Louis, USA) and 100% glacial acetic acid (Merck, Darmstadt, Germany) were obtained.

Determination of malic acid

The determination was performed with a Y5 automatic enzymatic multianaliser and an enzymatic kit (Biosystems, Barcelona, Spain).

Isolation from honey and honeycomb

400 ml of YEPDActBzClMa liquid medium (Table 4) were combined with 100 mL of various affected supermarket honeys (10 honey samples) and eco-friendly bee farms (12). The cultures were incubated at 25°C in the original 500 ml glass honey containers, in order to prevent fungal growth and avoid air pockets. 18 Honeycomb samples were also immersed (Figure 3) in YEPDActBzClMa medium in sterile 50 ml containers (Deltalab, Barcelona, Spain). Cultures were then performed with dip-inoculated serial dilutions (10^{-1} to 10^{-6}) of each liquid medium considered positive (detection of significant malic acid degradation) in Petri dishes containing solid YEPDActCl agar.

Classifications of microorganisms

We used classical sugar assimilation and fermentation tests (Kreger-van Rij, 1984; Barnett et al., 2000; Kurtzman and Fell, 1998). Two additional tests were also introduced: significant malic acid degradation in liquid YEPDMa medium detected after 10 days by means of enzymatic analysis, and yeast fission observed with a microscope (Figure 4).

RESULTS AND DISCUSSION

Growth results obtained in culture media enriched with differential-selective agents

After culturing all of the strains under study (Table 3) in YEPDAct10 medium for five days, the only strains which exhibited growth were *S. pombe*, *D. bruxellensis*, *Dekkera anomala*, *Kloeckera apiculata*, *Hanseniaspora uvarum*, *Pichia guilliermondii*, and two of the six strains of *Candida parapsilosis* studied (1336 and 1355). *C. parapsilosis* resistance depended on the strain, and these displayed the slowest growth of all the yeasts studied, especially in YEPDA100 medium despite its rapid growth in the YEPD control medium without actidione. No growth whatsoever was detected for any of the other strains tested under these conditions, which validates the use of actidione as the main effective selective agent to isolate yeasts belonging to the genus *Schizosaccharomyces.*

Table 2. Table summarising the principles which could inspire the formulation of differential selective media based on the metabolic characteristics of *Schizosaccharomyces* yeasts and their main potential actidione-resistant false positives, or yeasts with high rates of incidence in low a_w products.

Yeast specie	Disadvantage	Advantage	Medium formulation
Schizosaccharomyces pombe	% Reduced incidence	**R** Actidione (100 mg/L) (Benito, 2012a)	Actidione up to 100 mg/L
		R $a_w\downarrow$ conditions (Corry, 1976)	High concentration of solutes
		R Benzoate/Sorbate (> 600 mg/L) (Warth, 1988)	Benzoate
		R SO_2 (120 mg/kg) pH 3.5 (Warth, 1985)	SO_2 up to 120 mg/kg) at pH 3.5
		R Acetic acid (Pitt and Hocking, 1999)	Acetic acid 1% v/v
		G Malt Acetic Agar	
		G 37ºC + (Pitt and Hocking, 1999)	Incubation Tº > 30ºC
		Malic acid degradation	Malic acid as a differential agent for malate reductase activity
Dekkera bruxellensis	**R** Actidione	**S** Benzoic acid (< 200 mg/L) (Benito, 2009)	Benzoic acid (> 200 mg/L)
	R Sorbate (> 1000 mg/L) (Benito, 2007)	**NG** Malt Acetic Agar (Pitt and Hocking, 1999)	Acetic acid 1% v/v
	G 37ºC	**NSP** $a_w\uparrow$	
		OG Malt yeast extract 50 % glucose agar (Pitt and Hocking, 1999).	
Dekkera_anomala	**R** Actidione	**S** Benzoic acid (< 200 mg/L) (Benito, 2009 b)	Benzoic acid (> 200 mg/L)
		S Ethanol (< 8%) (Benito, 2009 b)	Ethanol 8%
		NSP $a_w\uparrow$ (Déak, 2008)	
		NG Malt Acetic Agar (Pitt and Hocking, 1999).	
Hanseniaspora uvarum	**R** Actidione	**S** Benzoic acid (< 188 mg/L pH 3.5) (Warth, 1989 c.)	Benzoic acid (> 188 mg/L)
Kloeckera apiculata	High **SP** in grapes	**NG** Malt Acetic Agar (Pitt and Hocking, 1999).	SO_2 up to 120 mg/kg) at pH 3.5
		NG Malt yeast extract 50% glucose agar (Pitt and Hocking, 1999).	Acetic acid 1% v/v
		NG MEA 37º	
		S SO_2 (Romano and Suzzi, 1993; Henick-Kling *et al*., 1998)	
		S Ethanol (< 4%)(Suárez-Lepe, 2004)	
Candida parapsilosis	**R** Actidione up to 10 mg/L	Does not produce ascospores (Pitt and Hocking, 1999)	Acetic acid 1% v/v
	Optimum growth temp 35°C		
	OG Malt Acetic Agar (Pitt and Hocking, 1999)		
	G Malt yeast extract 50 % glucose agar (Pitt and Hocking, 1999)		
	R Sorbate (pH 4; 10-20 g/L) (Deák *et al*., 1992)		

Table 2 Contd.

Zygosaccharomyces bailii	**R** Benzoate (>1000 mg/L)	**S** Actidione (< 10 mg/L) (Benito, 2012a)	Actidione mg/L	10
	R Sorbate (> 1000 mg/L)	**NG** 37ºC (Pitt and Hocking, 1999)		
	G Malt Acetic Agar			
	G Malt yeast extract 50 % glucose agar (Pitt and Hocking, 1999)			
	VG 37 ºC			
	R Sorbate (> 1000 mg/L) (Warth, 1986)			
	R $a_w\downarrow$ conditions			
Saccharomycodes spp.	**R** Benzoate (> 600 mg/L)	**S** Actidione (< 10 mg/L) (Benito, 2012a)	Actidione mg/L	10
	G Malt Acetic Agar			
	G Malt yeast extract 50 % glucose agar (Pitt and Hocking, 1999)			

R, resistant; **S**, sensitive; **NSP,** no statistically significant presence in $a_w\uparrow$ foods; **G**, growth; **NG**, no growth; **OG**, occasional growth; **SP,** statistically significant presence; **VG,** variable growth.

All of the strains studied which tested resistant to the antibiotic actidione were cultured in the rest of the media enriched with additional differential-selective agents and yielded the results shown in Table 5. The study of these strains was supplemented with that of the species *Saccharomycodes ludwigii* and *Zygosaccharomyces bailii*, due to their high incidence in niches similar to those ascribed to *Schizosaccharomyces* and their high level of resistance to the above-named additives. Both species proved resistant to secondary selective agents at the highest concentrations used (Table 5), but were inhibited in the media containing actidione. The YEPDSb medium enriched with potassium sorbate at different concentrations inhibited the growth of the strains of *S. pombe* studied at the concentration of 400 mg/L at pH 3.5, although other authors have reported resistance at up 600 mg/L (Warth, 1989). According to this study, it does not seem worth including this selective agent in the formulation of differential-selective media used for *Schizosaccharomyces*, as other actidione resistant yeasts such as the strains of *D. bruxellensis* and *D. anomala* studied grew even at the maximum concentration used, that is 600 mg/L; in addition, according to some authors, these exhibit resistance at concentrations of up to 1000 mg/L (Benito et al., 2009b) at pH 3.5. Further, the actidione-resistant strains of *C. parapsilosis* studied (1336 and 1355) also resisted the maximum concentration used, although it should be noted that other authors have reported sorbate resistance at up to 10 to 20 g/L (Deak et al., 1992) at pH 4. The sodium benzoate enriched YEPDBz medium inhibited all actidione resistant yeast strains studied to concentrations of 300 mg/L, except for *S. pombe* which resisted the maximum concentration tested that is 600 mg/L. The YEPDActBzCl medium inhibited all actidione resistant strains tested, as well as *Saccharomycodes ludwigii* and *Zygosaccharomyces bailii*, although seven days were required for growth. The YEPDAcetAgar medium inhibited all actidione resistant strains except *S. pombe, Pichia guilliermondii* and *C. parapsilosis* although the growth of the latter in this medium has been described as occasional by other authors (Pitt and Hocking, 1999), meaning that whether or not growth occurs may depend on the strain. The YEP60%D medium inhibited all actidione resistant strains except for *S. pombe* and *C. parapsilosis*, which required seven days to grow. After inoculating liquid YEPDMA medium enriched with 2 g/L of malic acid with some representative strain selected among those resistant to actidione using an inoculation loop, significant degradation of this acid was only observed with the strains of *S. pombe* studied, although difference were observed between strains (Table 6). After observing these results, it appears that for the strain used in this study, the most accurate selective agent after actidione is sodium benzoate, although some other agents such as acetic acid or high concentrations of glucose could probably be added to prevent the emergence of resistant strains.

Isolation from honey and honeycomb: Classification of yeasts

It is worth pointing out that the incidence of microorganisms was insignificant in honeys bought from supermarkets and low in organic honeys, with only two of these testing positive for microbes able to degrade malic acid. Incidence in honeycombs was similarly low, with microorganisms capable of degrading malic acid only detected in three samples. Significant fungal growth was observed in some samples, especially those where the container

Table 3. Indicator microorganisms and their strains.

Yeast specie	Strain	Origin
Candida krusei	1245, 1250, 1251, 1253, 1255	**IFI**
Candida parapsilosis	1341, 1342, 1338, 1355, 1339, 1337, 1336, 1341	**IFI**
Candida pulcherrima	1200, 1198, 1199, 1204, 1205, 1206, 1207, 1209, 1210.	**IFI**
Dekkera anomala	CB52, CB60, CB61	**IFI**
Dekkera bruxellensis	D35, D36, D37, 2400, CB63, 6802, R3, 7801	**IFI**
	6802, R3, 7801	**ETSIA**
Hanseniaspora uvarum	899, 898, 910	**IFI**
Hansenula anomala	925, 926, 927, 929, 932, 933, 934, 1114, 1115, 1117, 1118, 1119, 1120	**IFI**
Hansenula holstii	943, 944, 945.	**IFI**
Hansenula polymorpha	1128	**IFI**
Hansenula saturnus	931	**IFI**
Hansenula subpelliculosa	1123, 1124, 1125.	**IFI**
Kloeckera apiculata	1010, 1015, 1059, 1045, 1065	**IFI**
Pichia guilliermondii	962	**IFI**
	513	**ETSIA**
Pichia membranifaciens	946, 947, 948, 949, 950, 951, 952, 954, 956	**IFI**
Saccharomyces bayanus	697	**IFI**
Saccharomyces cerevisiae	87, 88, 89, 90, 211, 212, 213, 2202, 2203, 2205	**IFI**
	7V, 9CV, S6U	**ETSIA**
Saccharomyces pastorianus	556	**IFI**
Saccharomyces veronae	1145, 602, 615, 617, 1135	**IFI**
Saccharomycodes ludwigii	974, 975, 976, 979, 980, 981	**IFI**
Schizosaccharomyces pombe	935, 936, 938, 939, 2139	**IFI**
Torulopsis stellata	1303	**IFI**
Torulospora rosei	717, 718, 719, 720, 722, 723, 724, 725, 726, 727, 728, 730, 731, 732, 733, 742.	**IFI**
Zygosaccharomyces bailii	708, 2789	**IFI**
Zygosaccharomyces veronae	1148, 615	**IFI**

IFI, Industrial Fermentation Institute. CSIC; **ETSIA**, 'Escuela Técnica Superior de Ingenieros Agrónomos' (Higher Technical School of Agricultural Engineering).

Table 4. Composition of media used to evaluate different selective factors.

Culture medium	YE (g/L)	Glc (g/L)	Pept (g/L)	Agar (g/L)	Act (mg/L)	Bz (mg/L)	SB (mg/L)	Cl (mg/L)	AceticA (g/L)	MA (g/L)	pH
YEPD	10	25	10	24							
YEPDBz	10	25	10	30		50-600					3.5
YEPDSb	10	25	10	30			50-600				3.5
YEPDAceticAgar	10	25	10	24					5		
YEPDActBzCl	10	25	10	30	10	350		200			
YEPDActCl	10	25	10	30	10			200			
YEP60%D	10	600	10	24							
YEPDAcBzClMa*	10	25	10	-	20	350				2	
YEPDAc10	10	25	10	24	10						
YEPDAct100	10	25	10	24	100						
YEPDMa*	10	200	10	-						2	

YE, yeast extract; Glc, glucose; Pept, peptone; Et, ethanol; Act, Actidione; Bz, Benzoate; Cl, chloramphenicol; SA, sorbic acid; MA, malic acid; AcetA, acetic acid. *Liquid media.

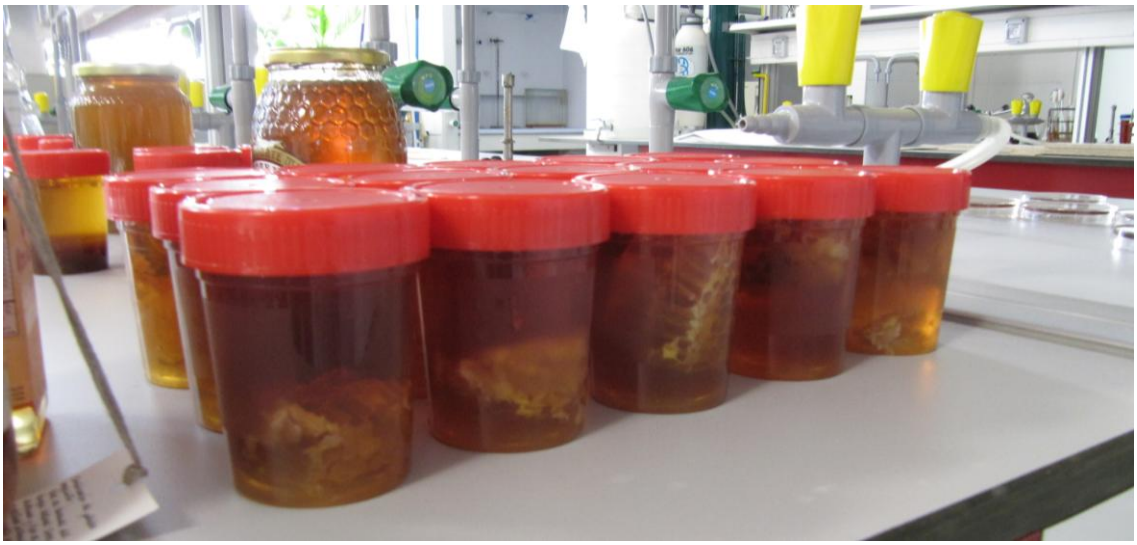

Figure 3. Honeycombs samples immersed in YEPDAcBzClMa medium in sterile 50 ml containers (Deltalab, Barcelona, Spain).

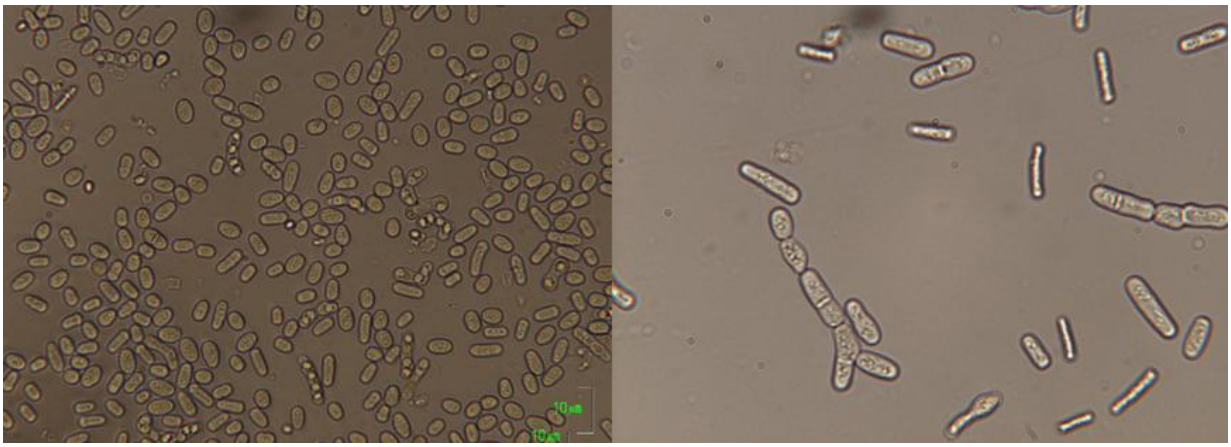

Figure 4. Detail of fission reproduction and sporulation of strains from the cultures studied.

was not completely full. Some authors report the presence of populations of yeasts and spore-forming bacteria of 10^2CFU/ml in honey (Snowdon and Cliver, 1996). Malic acid degradation was less than that observed in type cultures performed in YEPDMa medium, perhaps due to the presence of differential-selective additives or different sugar concentrations (Table 7), although honey may also contain malic acid.

Serial dilutions of 1 ml (10^{-1} to 10^{-6}) (obtained from cultures in liquid media in which malic acid degradation was detected) were performed and subsequently cultured by immersion in solid YEPD medium in Petri dishes. Ten strains were isolated randomly. The results of the classification of these strains are shown in Table 8. Based on the results obtained, it can be concluded that all of these strains belong to the species *S. pombe*, which has been described as the most common of this genus to be found in food according to the frequency estimates of Deák, 2008. Figure 4 shows photographed observations of fission reproduction sporulation and four-spore ascus formation. According to the above statistical ratio, we could expect 1-2 strains of *S. octosporus*. This discrepancy could be justified due to higher malic acid degradation of *S. Pombe* (it was known in the past as *Schizosaccharomyces malidevorans*) detected in malic acid degradation test during our isolation proposed methodology. In this case species with low malic acid degradation would be not detected.

Conclusions

There is a need to detect, isolate and select strains

Table 5. Growth results in Petri dishes after 10 days in culture.

Specie	YEPD	YEPDBz (mg/l)	YEPDSb (mg/l)	YEPDAcetAgar	YEP60%D	YEPDActBzCl	YEPDA10	YEPDA100
D. bruxellensis	+	- (250)	+ (600)	-	-	-	+	+
D. anomala	+	- (250)	+ (600)	-	-	-	+	+
Schizosaccharomyces pombe	+	+ (600)	- (400)	+	+	+	+	+
Pichia guilliermondii	+	- (300)	- (400)	+	-	-	+	+
Hanseniaspora uvarum /*Kloeckera apiculata*	+	- (250)	- (250)	-	-	-	+	+
Candida parapsilosis (Strains 1336 and 1355)	+	- (300)	- (600)	+	+	-	+	+/-
Saccharomycodes ludwigii	+	+ (600)	- (600)	+	+	-	-	-
Zygosaccharomyces bailii	+	+ (600)	+ (600)	+	+	-	-	-

+, positive growth; **-**, absence of yeast growth; +/-, peak growth; in media including varying concentrations of a selective agent, the inhibitory dose is indicated.

Table 6. Malic acid test 10 days after inoculation in YEPDMa medium.

Yeast strain	Malic acid (g/L)
	2.162 (initial concentration YEPDMa)
Dekkera bruxellensis D37	1.983 ± 0.104
Dekkera anomala D35	1.994 ± 0.092
Kloeckera apiculata 1010	1.974 ± 0.060
Kloeckera apiculata 1015	1.986 ± 0.083
Hanseniaspora uvarum 899	1.998 ± 0.098
Hanseniaspora uvarum 898	1.965 ± 0.115
Zygosaccharomyces bailii 708	1.956 ± 0.096
Saccharomycodes ludwigii 974	1.964 ± 0.090
Candida parapsilosis 1336	1.921 ± 0.106
Candida parapsilosis 1355	1.962 ± 0.035
Schizosaccharomyces pombe 935	0.097 ± 0.072
Schizosaccharomyces pombe 936	0.042 ± 0.096
Schizosaccharomyces pombe 938	0.067 ± 0.077
Schizosaccharomyces pombe 2139	0.089 ± 0.032

Table 7. Malic acid test after 15 days of honey and honeycomb culture in YEPDAcBzClMa medium (2 g/L malic acid).

Sample	Malic acid (g/L)
Honey 1	0.55
Honey 2	0.62
Honeycomb 1	0.42
Honeycomb 2	0.24
Honeycomb 3	0.37

belonging to the genus *Schizosaccharomyces* due both to their alterative properties and their interest in relation to modern industrial applications. However, there is a current shortage of specific techniques for their adequate isolation, coupled with the low incidence of these micro-organisms, so that it would be particularly worth devising and optimising a selective medium which would facilitate these tasks, especially in light of the fact that their use for industrial purposes was recently approved by the OIV and that the number of selected strains of this

Table 8. Classification of the ten yeast randomly isolated from honey and honeycombs.

Strain	Assimilation						Fermentation						Fission Reproduction	Sporulation	Malic acid degradation > 50 %
	Gal	Gl	L	M	R	S	Gal	Gl	L	M	R	S			
	-	+	-	+	-	+	-	+	-	+	+	+	+	4 spores	+
2	-	+	-	+	-	+	-	+	-	+	-	+	+	4 spores	+
2	-	+	-	+	-	+	-	+	-	+	+	+	+	4 spores	+
4	-	+	-	+	-	+	-	+	-	+	-	+	+	4 spores	+
5	-	+	-	+	-	+	-	+	-	+	-	+	+	4 spores	+
5	-	+	-	+	-	+	-	+	-	+	-	+	+	4 spores	+
6	-	+	-	+	-	+	-	+	-	+	+	+	+	4 spores	+
7	-	+	-	+	-	+	-	+	-	+	-	+	+	4 spores	+
8	-	+	-	+	-	+	-	+	-	+	+	+	+	4 spores	+
9	-	+	-	+	-	+	-	+	-	+	+	+	+	4 spores	+
10	-	+	-	+	-	+	-	+	-	+	+	+	+	4 spores	+

Gal, galactose; Gl, glucose; L, lactose; M, maltose; R, raffinose; S, saccharose.

genus is currently very limited. This differential-selective medium could therefore prove very helpful for any organisms which constitute likely candidates for such selection processes.

The selective agent actidione proved effective against most possible competitor yeast species. Of the species of yeast studied, only *S. pombe*, *D. bruxellensis*, *D. anomala*, *K. apiculata*, *H. uvarum*, *P. guilliermondii,* and some strains of *C. parapsilosis* were able to grow in the presence of actidione (10 mg/L), which could give rise to potential false positives. Benzoic acid stood out from the other selective agents studied and could be successfully used to eliminate the potential false positives observed in this study, due to the particular resistance of the genus *Schizosaccharomyces* to this compound. High sugar concentrations and the use of acetic acid could constitute further selective factors. The use of malic acid degradation in liquid media stands out as a differential factor which allows detecting the presence of yeasts able to degrade it.

Lastly, we managed to successfully isolate strains of *S. pombe* from theoretically unpasteurised honey and honeycomb from organic bee farms.

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