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short communication

Advances in the Development of a Methodology to Identify Common Yeast Contaminants of High Sugar Food Products

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Summary

Sixty-eight strains belonging to seven yeast species isolated in our laboratory from high sugar foods (Debaryomyces hansenii, Issatchenkia orientalis, Saccharomyces cerevisiae, Torulaspora delbrueckii, Zygosaccharomyces bailii, Zygosaccharomyces rouxii), were studied in order to provide small industries with simple and rapid methods for detecting contaminations. Different strategies were used based on (i) the design of selective/differential culture media by adding to a basal medium for yeasts inhibitory agents, dyes or chromogenic substrates, and (ii) the detection of specific enzymatic activities. The mentioned yeast species could be differentiated on the basis of their growth on three isolation media (eosin/methylen blue, acetic acid, and acetic acid/potassium tellurite) and the use of an additional test for discrimination (growth on 2-keto-D-gluconate, and β-glucosidase or alkaline phosphatase activities).

Key words: differential-detection, high sugar foods, yeasts

Introduction

Yeasts from the environment may contaminate raw and processed foods, but only part of this primary microflora will survive under selective pressures exerted by physical, chemical and biological factors, and other environmental conditions such as temperature, humidity, or gaseous atmosphere. It can be assumed that each type of food is typically attacked only by specific yeasts (*1*). These selected species could thus be considered as potentially harmful in this context, and should be specifically controlled in the particular food industry. We studied several problems of contamination by osmotolerant yeasts in three Spanish food industries (sugar syrups, fruit concentrates, and marzipan factories). The isolated yeasts were identified by morphological and physiological methods as *Debaryomyces hansenii, Issatchenkia orientalis, Saccharomyces cerevisiae, Torulaspora delbrueckii, Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii*. Nevertheless, traditional identification methods are laborious and time consuming and are not used in routine practice in industries. In the present work we carried out a search for distinctive features of the mentioned yeasts common in high sugar foods, with the objective of providing the small food industries with simple and rapid methods for detecting the contamination and preventing the spoilage of their products.

Material and Methods

Yeast strains

Thirty-eight yeast strains isolated in our laboratory were selected. These strains came from different food products containing high proportions of sugar (marzipan, fruit concentrates and syrups). In addition, thirty strains were obtained from Culture Collections (CECT: Coleccion Española de Cultivos Tipo, PYCC: Portuguese Yeast Culture Collection), most of them being food isolates.

Culture conditions

Strains were maintained on Yeast Morphology Agar slants (YMA, Difco Laboratories, Detroit, MI) 10 g/L glu-

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Table 1. Growth or colour of the studied yeast strains on YMA basal medium with the addition of selective or differential compounds (No. of strain assayed betwen brackets)

Species	A	B	C
Debaryomyces hansenii	Vi (5)	– (10)	– (10)
Issatchenkia orientalis	Vi (5)	+ (5)	+ (5)
Saccharomyces cerevisiae	MGr (8)	– (8)	– (8)
Torulaspora delbrueckii	Vi (3)	V (7)	V (7)
Zygosaccharomyces bailii	Bl (2)	+ (8)	– (8)
Zygosaccharomyces rouxii	Bl/Vi (22)	– (30)	– (30)

A, eosin/methylene blue; B, acetic acid (10 g/L); C, acetic acid (5 g/L)+ potassium tellurite (0.2 g/L); Vi, violet; Bl, black; MGr, metallic green; + = 100 % growth; – = 100 % absence of growth; V= variable results.

cose at 4 °C. For inocula, a suspension of an actively growing culture of the strains on YMA was prepared in distilled water (6 Mc Farland, c. a. 10^6 cells/mL). Growth was checked daily.

Study of different culture media

Several culture media were designed for this study using YMA as a basal medium added of chloramphenicol (0.5 g/L) to inhibit bacterial growth. In order to observe changes in the colour of the colonies, two dyes, eosin (0.4 g/L) and methylene blue (0.065 g/L), and a chromogenic substrate, triphenyltetrazolium (0.1 g/L) (all from Merck, Darmstadt, Germany), were added to YMA basal medium. When selective growth was investigated, acetic acid (5 and 10 g/L) (Panreac, Barcelona, Spain) and potassium tellurite (0.15, 0.20, 0.25 and 0.30 g/L) (Merck, Darmstadt, Germany) were used. Mixtures of both inhibitors were also used to improve the selectivity (5 g/L of acetic acid + 0.2 g/L of potassium tellurite; 10 g/L of acetic acid + 0.2 g/L of potassium tellurite). Drops of fifty microliters of inocula prepared as described were used to inoculate the plates, which were incubated at 30 °C for 15 days. The results were recorded according to growth and colour, the latter standardized using a colour pattern for computer graphic design (Hewlett-Packard, Co. Minneapolis, USA).

Detection of enzymatic activities

The enzymatic profile of several selected strains of each yeast species was studied using the ApiZym kit of BioMérieux (BioMérieux S.A., Marcy-L'Etoile, France) which includes 19 different enzymes listed in Table 2.

The specific detection of β-glucosidase was investigated by adding 0.1 g/L of a fluorogenic substrate MUC (4-methylumbelliferyl β-D-glucopyranosyde, Sigma-Aldrich, Madrid, Spain) to YMA basal medium. The assays were performed on microtiter plates. Wells were inoculated with 5 μL of a suspension of the yeast strains as described, and incubated at 30 °C for 7 days. Growth and fluorescence on a UV lamp (320 nm) were recorded daily.

Results and Discussion

In a previous work (2) we studied a number of physiological traits and commercial or modified solid culture media to search for distinctive features useful for the rapid detection of the mentioned osmotolerant yeasts isolated from Spanish industries. It included 75 physiological charactheristics commonly used in yeast identification. Solid culture media contained different dyes or chromogenic substrates (basic fuchsin/sodium bisulphite, brilliant green/phenol red, esculin/ammonium citrate, methylene blue), pH indicators (acid fuchsin, bromocresol green, bromocresol purple, bromothymol blue, neutral red, phenol red) or inhibitory compounds (acetic acid, brilliant green, crystal violet, malachite green, potassium tellurite). An overall examination of the results showed that: *(i)* there was an elevated heterogeneity when fermentation or assimilation of different compounds were considered, or when certain dyes were added to the basal medium, *(ii)* changes in pH, revealed by using pH indicators, were generally homogeneous among the yeasts studied. These results prompted us to investigate modified or new strategies in the present work.

The incorporation of different inhibitory and chromogenic substrates into a basal medium for yeasts was evaluated in order to establish a pattern for species discrimination based on growth, colour and morphology of

Table 2. Enzymatic activities detected by using the ApiZym kit of BioMérieux.
+ = presence of the enzyme in all of the strains; – = absence of the enzyme in all of the strains; V = variable results.

Species (No. of strains)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Debaryomyces hansenii (5)	+	+	+	–	+	–	V	–	–	+	V	–	–	–	V	V	–	V	–
Issatchenkia orientalis (5)	V	+	+	–	+	V	V	–	–	+	+	–	–	–	–	–	–	–	–
Saccharomyces cerevisiae (9)	V	+	+	V	+	V	V	–	–	V	V	–	–	–	+	–	–	–	–
Torulaspora delbrueckii (3)	+	+	+	–	+	+	V	–	–	+	+	–	–	–	V	–	–	–	–
Zygosaccharomyces bailii (2)	–	+	V	–	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–
Zygosaccharomyces rouxii (23)	V	+	V	–	+	V	–	–	–	V	V	–	–	–	V	–	–	–	–

1. Alkaline phosphatase
2. Esterase (C4)
3. Esterase-lipase (C8)
4. Lipase (C4)
5. Leucine arylamidase
6. Valine arylamidase
7. Cystine arylamidase
8. Trypsin
9. Chymotrypsin
10. Acid phosphatase
11. Naphtol-AS-BI-phosphohydrolase
12. α-galactosidase
13. β-galactosidase
14. β-glucoronidase
15. α-glucosidase
16. β-glucosidase
17. N-acetyl-β-glucosaminidase
18. α-mannosidase
19. α-fucosidase

Table 3. Simplified scheme for differentiation of osmotolerant yeasts isolated in this study

Growth on isolation media[1]			Yeasts	Additional tests
A	B	C		
MGr	–	–	*Sacharomyces cerevisiae*	
				β-Glucosidase activity[2]
Bl or Vi	–	–	*Debaryomyces hansenii*	+
			Zygosaccharomyces rouxii	–
				Alkaline phosphatase activity[2]
Bl or Vi	+	–	*Zygosaccharomyces bailii*	+
			Torulaspora delbrueckii	–
				Growth on 2-keto-D-gluconate[1]
Bl or Vi	+	+	*Issatchenkia orientalis*	–
			Torulaspora delbrueckii	+

(1) A, eosin/methylene blue; B, acetic acid (10 g/L); C, acetic acid (5 g/L) + potassium tellurite (0.2 g/L); MGr, metallic green; Bl, black; Vi, violet; +, growth; –, absence of growth.
(2) +, presence of enzyme; –, absence of enzyme.

the colonies. Quindos *et al.* (3) described the utility of triphenyltetrazolium for typing *Candida* subspecies of clinical origin. However, in a second study, the same authors reported a poor correlation of this typing with the groups obtained when using molecular methods (*i.e.* karyotyping by PFGE, $RAPD_s$) (4). The colour pattern obtained using triphenyltetrazolium for food spoilage yeasts in this work did not permit a clear discrimination among the studied yeasts, as intraspecific colour variations were observed (data not shown). A better colony colour typing was obtained by adding eosin/methylene blue to the yeast basal medium YMA. As shown in Table 1, only the colonies of *Saccharomyces cerevisiae* strains exhibited a green metallic appearance, thus permiting its differential isolation, and the other species produced black or violet colonies. Selective isolation was studied using acetic acid and potassium tellurite at different concentrations, as well as combinations of both compounds. The most significant results are shown in Table 1. *Z. bailii* grew on 10 g/L of acetic acid, while *Issatchenkia orientalis* and some strains of *Torulaspora delbruekii* were able to grow also in the presence of acetic acid + potassium tellurite (0.5 g/L + 0.2 g/L).

The enzymatic characterization of species using ApiZym strips was one of strategies used in this work. As shown in Table 2, some of the tested enzymes were present in all (+), none (–), or in only some (V) of the strains tested for each species. One of the enzymes, β-glucosidase, was detected only in some of the strains of *Debaryomyces hansenii*, but not in the rest of the yeast species studied. On the basis of this preliminary result, several strains of each species were tested for the detection of β-glucosidase on a solid basal medium with the fluorogenic substrate MUC. This compound, when cleaved by the enzyme β-glucosidase, produced methylumbelliferone which fluoresces under UV light (5). Only the strains of *D. hansenii* produced fluorescence in the conditions of the assay, thus permitting the differential detection of this yeast species.

As a summary, Table 3 provides a simplified scheme for differentiation of osmotolerant yeasts most commonly isolated from high sugar products studied in this work (sugar syrups, fruit concentrates and marzipan). It is based on growth on three isolation media designed by us (eosin/methylene blue (A), acetic acid (10 g/L) (B) and acetic acid/potassium tellurite (10 g/L + 0.2 g/L) (C), and the use of an additional test when needed for discrimination between yeast species with a similar growth pattern on the isolation media. For example, the detection of β-glucosidase using the fluorescent substrate MUC for differentiation of *D. hansenii* and *Z. rouxii*; or alkaline phosphatase activity (Table 2) for *Z. bailii* and *T. delbruekii*; or the ability of growing on 2-keto-D-gluconate (6) to distinguish between *I. orientalis* and *T. delbrueckii*.

We think that this experimental approach has produced promising results for the improvement of rapid detection of some of the spoilage yeasts that should be specifically controlled in high sugar food industries. More expanded studies are currently being carried out in our laboratory in order to design new differential culture media based on the detection of enzymatic activities that could be introduced in the routine quality control in food factories.

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Napredak u razvoju metodologije identifikacije općih kontaminanata kvasaca u proizvodima s velikim udjelom šećera

Sažetak

Ispitano je šezdesetosam sojeva koji pripadaju u sedam vrsta kvasaca izoliranih u našem laboratoriju iz proizvoda s velikim udjelom šećera (Debaryomyces hansenii, Issatchenkia orientalis, Saccharomyces cerevisiae, Torulaspora delbrueckii, Zygosaccharomyces bailii, Zygosaccharomyces rouxii), kako bi omogućili primjenu jednostavnih i brzih postupaka za otkrivanje kontaminacija. Različiti su bili pristupi zasnovani na: (i) konstrukciji selektivne/diferencijalne kulture, dodavajući inhibitorske reagense, boje ili kromogene supstrate u osnovni medij za kvasce te (ii) detekciji specifičnih enzimskih aktivnosti. Navedene su se vrste kvasaca mogle međusobno razlikovati prema njihovu rastu na trima izolacijskim medijima (eozin/metilensko plavilo, octena kiselina i octena kiselina/kalijev telurit) i primjenom dodatnog testa za diskriminaciju (rast na 2-keto-D-glukonatu i aktivnosti β-glukozidaze ili alkalne fosfataze).