Ciencia y Tecnología Alimentaria

Ciencia y Tecnología Alimentaria
Sociedad Mexicana de Nutrición y Tecnología de Alimentos
somenta@gmail.com
ISSN (Versión impresa): 1135-8122
ISSN (Versión en línea): 1696-2443
MÉXICO

2005
C. M. Carvalho / A. Rocha / M. L. F. Estevinho / A. Choupina
IDENTIFICATION OF HONEY YEAST SPECIES BASED ON RFLP ANALYSIS OF THE ITS REGION
Ciencia y Tecnología Alimentaria, diciembre, año/vol. 5, número 001
Sociedad Mexicana de Nutrición y Tecnología de Alimentos
Reynosa, México
pp. 11-17

Red de Revistas Científicas de América Latina y el Caribe, España y Portugal
Universidad Autónoma del Estado de México
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ISSN 1135-8122

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IDENTIFICACIÓN DE ESPECIES DE LEVADURAS DE MIEL BASADA EN ANÁLISIS RFLP DE LA REGION ITS

IDENTIFICACIÓN DE ESPECIES DE LEVADURAS DE MEL BASADA EN ANÁLISES RFLP DA REXIÓN ITS

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Recibido: 15 de Septiembre de 2005; recibida versión revisada: 9 de Noviembre de 2005; aceptado: 9 de Noviembre de 2005
Received: 15 September 2005; revised version received: 9 November 2005; accepted: 9 November 2005

Abstract

In the present study, the restriction patterns generated from the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene were used to identify a total of seven honey yeast species belonging to six different genera. Polymerase chain reaction (PCR) products of this rDNA region showed a high length variation for the different species. The size of the PCR products and the restriction patterns obtained with endonucleases HhaI, HaeIII and HinfI yielded a unique profile for each species, except for *Zygoscharomyces mellis*. The use of this molecular approach is proposed as a new rapid and easy method of routine honey yeast identification.

Keywords: Honey yeast identification, RFLP, 5.8S-ITS region

Resumen

En el presente estudio, el perfil de restricción generado por la región que abarca los separadores transcritos internos (ITS1 y ITS2) y el gen 5,8S rRNA se usó para identificar un total de 7 levaduras de mieles pertenecientes a 6 géneros diferentes. Los productos de la reacción en cadena de la polimerasa (PCR) en esta región rDNA mostraron una alta variabilidad en la longitud para las diferentes especies. El tamaño de los productos de PCR y el perfil de restricción obtenido con endonucleasas *Hha*I, *Hae*III y *Hinf*I rindieron un perfil único para cada especie, excepto para *Zygoscharomyces mellis*. El uso de esta aproximación molecular se propone como un nuevo método rápido y fácil de usar para la identificación rutinaria de levaduras de mieles.

Palabras clave: Identificación, levaduras de la miel, RFLP, región 5,8S-ITS

Resumo

No presente estudo, o perfil de restricción xerado pola rexión que abarca os separadores transcritos internos (ITS1 e ITS2) e o xen 5,8S rRNA empregouse para identificar un total de 7 levaduras de meles pertencentes a 6 xéneros diferentes. Os productos da reacción en cadea da polimerasa (PCR) nesta rexión rDNA amosaron unha alta variabilidade na lonxitude para as diferentes especies. O tamaño dos productos de PCR e o perfil de restricción obtido con endonucleasas HhaI, HaeIII e HinfI rindiron un perfil único para cada especie, excepto para *Zygoscharomyces mellis*. O emprego desta aproximación molecular proponse como un novo método rápido e fácil de usar para a identificación rutinaria de levaduras de meles.

Palabras chave: Identificación, levaduras da mel, RFLP, region 5.8S-ITS

INTRODUCTION

Honey has been highly appreciated as an alimentary product, and has been largely used, since ancient times, either preventing or curing infirmity and illness or in cosmetic manufacturing (Gonnet and Vache, 1985). Honey is a sugary substance obtained from the nectar of the flowers or from the secretions which come from or lie on the living parts of the plant and which honey bees' crop, transform and combine with their own specific substances, and store in the honeycomb of the beehive. Honey is a product extremely rich in sugars of which glucose and fructose are outstanding It also possesses vitamins, mineral salts and enzymes. Honey, however and despite having a high tenor in sugars and consequently a reduced water activity, an acid pH, and bactericidal substances (H_2O_2 and inhibins), it frequently suffers fermentations driven by yeasts, which make this product inappropriate for consumption. Fermentation is an irreversible phenomenon that can run in honey mainly during storage, causing significant economical losses. In such a case, honey presents a characteristic odour, increasing acid flavour and gas bubbles. The honey of Trás-os-Montes is characterized by its dark colour, accentuated floral aroma and an intense, persistent odour.

Traditionally, identification and characterization of yeast species have been based on morphological traits and especially on their physiological abilities (Barnett *et al.*, 1990; Kreger-Van Rij, 1984). This conventional methodology requires evaluation of some 60-90 tests, and the process is complex, laborious and time consuming (Deák, 1995; Deák and Beuchat, 1996). In recent years, to improve the conventional methods, rapid kits for yeast identification have been developed. However, they were initially designed for clinical diagnosis and their application is restricted to 40-60 yeast species of medical interest (Deák, 1993).

Recent progress in molecular biology has lead to development of new techniques for yeast identification based on similarity or dissimilarity of DNA, RNA or proteins. These include allozyme patterns (Naumov *et al.*, 1997) DNA-DNA hybridization (Torok *et al.*, 1993; Vaughan Martini and Martini, 1985, 1987), electrophoretic karyotyping (Guillamón *et al.*, 1996; Nadal *et al.*, 1996; Perez *et al.*, 1995; Querol *et al.*, 1992b; Schuctz & Gafner, 1993; Torok *et al.*, 1993), microsatellite analysis (Baleiras Couto *et al.*, 1996), nested-PCR (Ibeas *et al.*, 1996), random amplified polymorphic DNA (RAPD) analysis (Baleiras Couto *et al.*, 1994; Lopandic *et al.*, 1996; Quesada and Cenis, 1995), RFLP of chromosomal DNA (Versavaud and Hallet, 1995) or RFLP of mitochondrial DNA (Belloch *et al.*, 1997; Guillamón *et al.*, 1994, 1997; Ibeas *et al.*, 1997; Nadal *et al.*, 1996; Pérez *et al.*, 1995; Querol *et al.*, 1992a; Romano *et al.*, 1996). However, these techniques are impractical for the routine identification of a large number of species since they were developed for species characterization and there is no available database which would permit the analysis of new results (Esteve-Zarzoso *et al.*, 1999).

Restriction fragment length polymorphisms (RFLP) have been widely used at different taxonomic levels in fungal systematics. A large selection of both mithocondrial and chromosomally derived probes have been used, including those derived from the ribosomal RNA gene complex, and these have yielded important information on relationships both within, and between fungal species (Paterson *et al.*, 1994).

Molecular biology techniques provide alternative and additional methods and are becoming an important tool in solving industrial problems. The application of these techniques has generated a larger number of studies on the classification, identification, and ecology of the yeast species (Guillamón *et al.*, 1994, 1996; Querol *et al.* ,1992b; Schutz and Gafner, 1993, 1994).

PCR amplification of specific sequences for the identification of organisms has become common because of the relative ease of manipulation and the high reproducibility. Previous results have demonstrated that the complex ITS (internal transcribed spacer) regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful in measuring close fungus genealogical relationships since they exhibit far greater interspecific differences than 18S and 25S rRNA genes (Cai *et al.*, 1996; James *et al.*, 1996; Kurtzman, 1992, 1993). Because ribosomal regions evolve in a concerted fashion, they show a low intraspecific polymorphism, and a high interspecific variability (Li, 1997) has been proved very useful for the classification of *Saccharomyces* species (Huffman *et al.*, 1992; Molina *et al.*, 1992; Valente *et al.*, 1996; Wyder and Puhan, 1997), *Kluyveromyces* species (Belloch *et al.*, 1998) and, for the identification of a collection of wine yeast species (Guillamón *et al.*, 1998) and yeast species associated with orange juice (Arias *et al.*, 2002).

In the present study, we identified a total of seven yeast species described as been present in Trás-os-Montes honey by using the restriction patterns generated from the region spanning the internal transcribed spacers (ITS1 and IRS2) and the 5.8S rRNA gene. For this purpose, we have obtained restriction patterns of PCR products.

METHODS

Yeast strains

We analysed twenty different strains belonging to seven different yeast species of six genera, obtained from Trás-os-Montes honey. To keep this study to a manageable size, we have mainly relied on type strains representing the yeast species most frequently isolated from honey. For some species, when substantial heterogeneity was suspected, or due to their biotechnological importance, additional strains were also characterized. The designations of strains are listed in Table 1.

PCR reaction and DNA digestions

DNA was isolated according to J. M. Beckerich (Institute National de la Recherche Agronomique, France) and diluted to 1-50 ng/µg.

The rRNA gene region was amplified in a UNO II Biometra® Thermocycler. Primer pairs used to amplified

Table 1.- Size in bp of the PCR products and the restriction fragments of the 5.8S-ITS analysis. [a]PCR amplified product.

References	Species	AP[a] (bp)	Restriction fragments		
			***Hha*I**	***Hae*III**	***Hin*fI**
TAB M109 M125 5B1	*Rhodotorula mucilaginosa*	640	320+240+80	425+215	340+225+75
ESA1 7ER	*Saccharomyces cerevisiae*	880	385+365+130	320+230+180+150	365+180+155
M41	*Zygosaccharomyces rouxii*	750	290+200+170+90	400+210+90	350+260+140
5B3 M70	*Candida parapsilosis*	550	300+240	400+115	290+260
M1 M26 M27 ESA17 M16	*Candida magnoliae*	425	200+190	285+140	225+200
ESA11	*Pichia membranifaciens*	500	175+110+90+75	330+90+50	275+200
1Lisa 2Lisa 1694-P011	*Zygoscharomyces mellis*	850	350+250+210	560+200+90	400+270+180

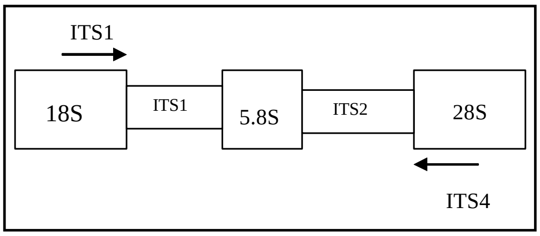

Figure 1. Nuclear rRNA gene region amplified by PCR using primers ITS1 and ITS4 (ITS internal transcribed spacer).

the ITS region (see Figure 1), ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') is to a conserved 3' domain in the 18S nuclear subunit and ITS4 (5´-TCCTCCGCTTATTGAT ATGC-3') is a reverse primer to a conserved region of the nuclear large rDNA (Lott *et al.*, 1993).

PCR conditions were as follows: initial denaturing at 95 ºC; 35 cycles of denaturating at 94 ºC for 1 min, annealing at 55.5 ºC for 2 min and extension at 72 ºC for 2 min; and final extension at 72 ºC for 10 min, using 100 ng genomic DNA, 0.5 µM of each primer, 10 µM deoxynucleotides, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, and 10X Buffer (Promega, Madison, Wis.). PCR products (approximately 0.5-1.0 µg) were digested without further purification with the restriction endonucleases *Hha*I, *Hae*III and *Hinf*I (Promega, Madison, Wis.) according to the supplier's instructions (Arias *et al.*, 2002). The PCR products and their restriction fragments were separated on 1.5 % and 3 % agarose gels, respectively, with 1 X TAE (Tris-acetic acid-EDTA) buffer. Gels were stained with ethidium bromide, visualized, and photographed under UV light. Fragment sizes were estimated by comparison against a DNA standard (100-bp ladder; Promega).

RESULTS AND DISCUSSION

Yeast species present in Trás-os-Montes honey

The ITS1 and ITS4 primers were used to amplify a region of the rRNA gene repeat unit that includes the 5.8S rRNA gene and the two non-coding regions designated the internal transcribed spacers (ITS1 and ITS2) of the twenty strains belonging to seven species. Figure 2 show the sizes of the PCR products and the restrictions fragments obtained using the restriction endonucleases *Hha*I, *Hae*III and *Hinf*I.

PCR products showed a high lenght variation in this region for the different species: 880 bp for the type strains *Saccharomyces cerevisiae*, 640 bp for the strain *R. mucilaginosa* and 425 for the type strains *Candida magnoliae* (Table 1). PCR products from strains of the same species and from species of the some genus had identical or very similar molecular sizes.

When the rRNA gene region was digested with *Hinf*I each species exhibited a specific restriction pattern. Species of the some genus showed a very similar restriction patterns with this restriction endonuclease. Similar results were also obtained with *Hha*I. Each species yielded an exclusive pattern.

Table 2 shows the two yeast species that have not been previously described. Using the restriction endonucleases *Hha*I, *Hae*III and *Hinf*I, we found new

Table 2.- Nucleotide fragment length of new 5.8-ITS profiles described in this study. [a]PCR amplified product.

ESAB Reference	Species	AP[a] (bp)	Fragment length(s) (bp) after restriction endonuclease analysis with:		
			***Hha*I**	***Hae*III**	***Hin*fI**
ESA 51	*Trichosporon mucoides*	500	300+100	500	300+150
1694-P015	*Candida sorbosivorans*	400-500	200+100+70+50	210+190+50	230+200

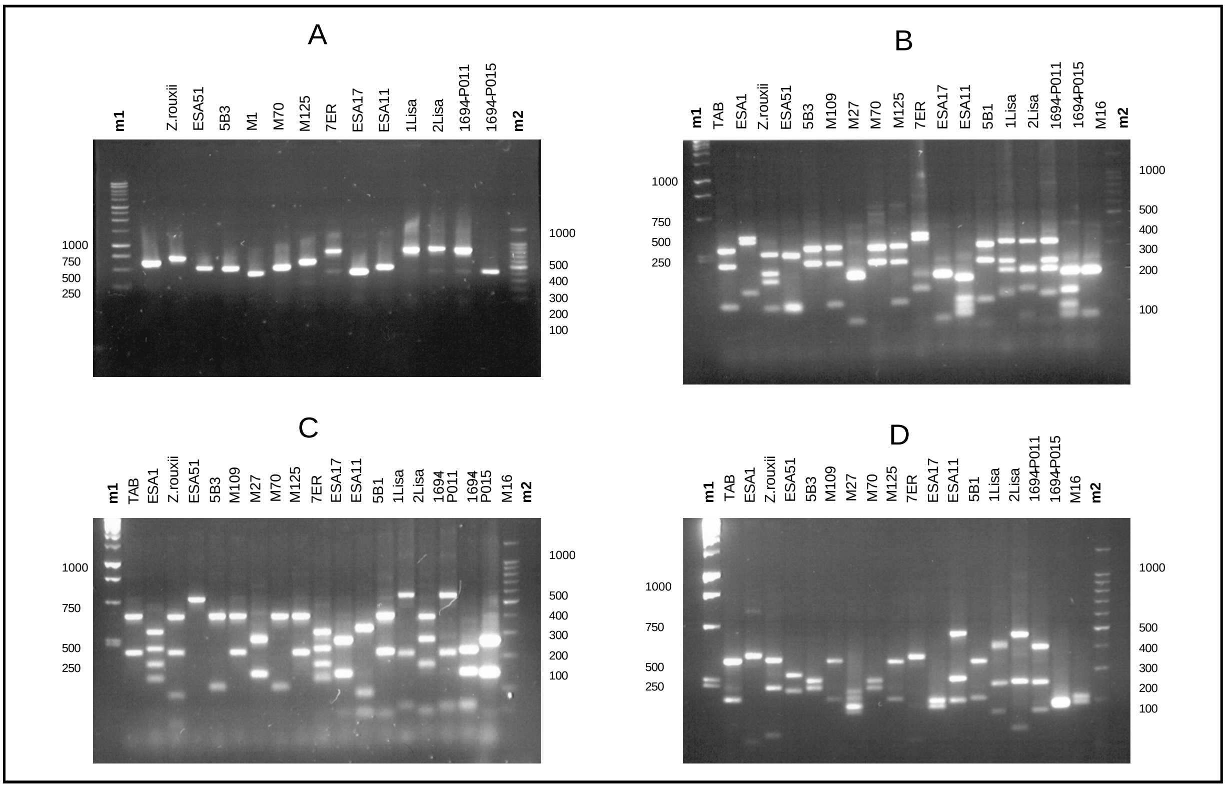

Figure 2. Size of the PCR-amplified rDNA region of some yeast strains (A) and restriction analysis with the endonucleases *Hha*I (B), *Hae*III (C), and *Hinf*I (D). Lanes m1 and m2 correspond to molecular size standards (1kb DNA Ladder and 100bp DNA Ladder from Promega).

profiles corresponding to *Trichosporon mucoides* and *Candida sorbosivorans.*

The genus Candida includes all yeast species that cannot be classified in other asexual ascomycetous yeast genera. This genus is very heterogeneous and the perfect state of most Candida species is still unknown (Krejer-vanRij, 1984).Different molecular techniques have been used to identify and characterize *Candida* species. However, except in the case of clinical species (Botelho and Planta, 1994; Iwaguchi *et al.*, 1990; Jordan, 1994), a standard method of classification has not been developed. In the present study, the honey strains showed a unique restriction pattern for each species with the three endonucleases used. However, the size of the fragments was not very variable.

The genus *Pichia*, in the description given by Kurtzman (1984) is, with 57 species, the largest ascomycetous yeast genus. A wide variation exists in their morphological and physiological responses, and some have been transferred to the genera *Issatchenkia* and *Debaryomyces* (Krejer-van Rij, 1987). In this work we only analyzed one specie recovered from honey, corresponding to *Pichia membranifaciens.*

The species of the genus *Zygosaccharomyces* are important osmotolerant food spoilage ascomycetous yeasts (Esteve-Zarzoso *et al.*, 1999), and one of the most common in honey.

In this study we identified two species corresponding to *Zygosaccharomyces rouxii and Zygosaccharomyces mellis*. One strain of *Zygosaccharomyces mellis* showed different pattern indicating that this taxon includes two different types of strains.

The genera *Rhodotorula* include basidiomycetous yeasts producing red-or orange-pigmented colonies (Esteve-Zarzoso *et al.*, 1999). An important characteristic of *Rhodotorula* species is the inability to assimilate inositol, which distinguishes *Rhodotorula* from *Cryptococcus* (Krejer-vanRij, 1987).

Yeast identification based on this analysis has proven to be a rapid, reliable, and accurate tool for yeast identification. In our study, this technique provided good results in terms of time and accuracy, but the existent database should be continuously updated. After we updated the previous database with the new profiles founded in this study, all of isolates would be correctly identified. However, as more profiles are added to the database, identification will become increasingly difficult due to no or slight differences between the 5.8S-ITS profiles (Arias *et al*., 2002).

Unfortunately, similar or identical 5.8S-ITS patterns do not necessarily belong to related species (Esteve-Zarzoso *et al*., 1999). Furthermore, it has to be considered that one single mutation in the 5.8S-ITS region could lead to the loss or gain of a restriction site, resulting in a completely different pattern (Arias *et al*., 2002). One alternative to overcome such an occurrence would be to sequence the 5.8S-ITS region and contrast them with the presently available databases (Arias *et al*., 2002). This region provides enough variability to distinguish between most yeast species due to their high taxonomic value (Kurtzman *et al*., 1998). However, the sequencing time requirement and cost are still too high to facilitate use in common quality control labs but may be affordable in the future.

CONCLUSIONS

In our study, restriction patterns generated from the DNA region comprising the internal transcribed spacers (ITS) and the 5.8S rRNA gene made it possible to differentiate among the studied species using a pattern of bands characteristic for each species observed. The ITS regions, which are much less evolutionarily conserved than the rRNA coding genes (Burns *et al*., 1991), appear to be useful in detecting genetic variability among species, which is valuable for taxonomic purposes and also for species identification.

With this method we differentiate seven yeast species belonging to six different yeast genera isolated from honey. Analysis of the Table 1 and 2 confirmed that this method can be used to differentiate all the species with the exception of *Trichosporon mucoides* and *Candida sorbosivorans*, and one strain in the specie *Zygosaccharomyces mellis*. In all most cases, the method proved to be reproducible and very useful to easily and rapidly identify and classify all the species included in the present work. This special feature has been used for identification of honey yeast species.

The difficulty in RFLP analysis is that too many small fragments are produced, it is worth nothing that fragments smaller than 50 bp could not be clearly visualized and were not consider in this analysis.

Finally, the results presented in this work constitute a supplement to the initial database. To prevent errors we recommend the use of same conditions that we have applied in this study, namely to separated the PCR products on the 1.5 % and the restriction fragments on 3 % agarose gels.

ACKNOWLEDGEMENTS

This work was supported by Agro 242 Project.

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