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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SOME YEAST ISOLATES

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Abulhamd[1], A.; M.M. Mokhtar[2] and Farrag, Rasha, Mohamed[2]

1. Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Cairo, Egypt
2. The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt

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ABSTRACT

Sixteen yeast isolates from various sources including molasses, foodstuffs, concentrated juices and soils were identified using simplified API 20 C AUX yeast identification system and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). The isolates were found to belong to 11 species; *Candida famata, C. glabrata, C. krusei / inconspicua, C. norvegensis, C. colliculosa, Cryptococcus humicolus, Rhodotorula mucilaginosa, Kloeckera* spp., *Geotrichum capitatum and Saccharomyces cerevisiae*. API and RAPD-PCR proved to be useful and convenient taxonomic tools for rapid identification at the species level, proving that at least two techniques are needed for precise identification. RAPD-PCR procedure is faster than traditional characterization techniques; the identification of a single colony was typically achieved in less than 10 hr.

INTRODUCTION

Various molecular techniques have been developed which permit species identification and typing of food-borne microorganisms, including yeast. Pulsed-field gel electrophoresis (PFGE; karyotyping), restriction enzyme analysis, PCR-based techniques, sequencing **(Deák, 1995**; **Van der Vossen & Hofstra 1996; Smole and Raspor, 1997)** and Southern hybridization with moderately repetitive DNA probes **(Odds *et al* 1992 and Espinel-Ingroff *et al* 1999)** are among the technique used. Some of these techniques have been applied successfully to characterize yeast isolated from various food products; however, most are too sophisticated or cumbersome for use in routine industrial practice.

In recent years, PCR-based techniques targeting ribosomal RNA genes that can be performed with relative ease have emerged. Of these, restriction analysis of variable internal transcribed spacer (ITS) sequences framing the more conservative 5.8S rRNA gene (rDNA) has proven most useful, allowing both species identification and typing of isolates (**Valente *et al* 1997; Guillamón *et al* 1998 and Fernández *et al* 1999**). Based on an extensive database, this technique has been proposed for rapid and routine identification of yeast (**Esteve-Zarzoso *et al* 1999**).

The most frequently used PCR-based technique currently in use is random amplified polymorphic DNA-PCR (RAPD-PCR) which has been described by **Williams *et al* (1990)**. In this technique, single or a pair wise combination of primers, typically 9 to 10 nucleotides in length, are used to amplify target genomic DNA by polymerase chain reaction (PCR). Fragments of DNA are generated by PCR amplification if the primer target sites for the primer happen to occur within approximately 5 kb of each other on opposite DNA strands.

A similar procedure was simultaneously discovered by **Welsh and McClelland (1990)**, who called it arbitrary primed-PCR. This procedure results in the amplification of small segments of the bacterial genome, the size and pattern of the fragments are characteristic of the bacterial species. The amplified products, which form strain-specific fingerprints, are then analyzed by separation through an agarose gel and ethidium bromide stained.

Specific PCR amplification of the 5S and 16S rRNA genes has been used to detect lactic acid bacteria in beer **(DiMichele & Lewis, 1993 and Tompkins *et al* 1996**). **Tsuchiya *et al* (1994)** have

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combined the specific PCR with temperature gradient gel electrophoresis to identify lactic acid bacteria. RAPD-PCR technology can also be used for the identification of non-brewing yeast without changes to the procedure (**Lieckfeldt *et al* 1993**).

Randomly amplified polymorphic DNA (RAPD) analysis seemed to be efficient in distinguishing different isolates; it has a high discriminatory power, it is easy to perform, does not require radiolabelled probes, and it is applicable to several microorganisms (**Robert *et al* 1995**). It can be used to reliably type yeast strains (**Baleiras *et al* 1995 & 1996 and Molnár *et al* 1995**).

The major advantage of using RAPD-PCR technology is that a pure single colony can be identified by its fingerprint pattern in less than one day. In the fermentation industry, this procedure has been useful for the characterization of different isolates of yeast (**Lieckfeldt *et al* 1993 and Grando *et al* 1994**), the identification of *Obesumbacterium proteus* (**Savard *et al* 1994**), the investigation of barley variants (**Ko and Henry, 1994**), and the identification of hopleaf tissue (**Abbott and Fedele, 1994**). *Obesumbacterium proteus* has also been differentiated using a PCR technique called enteric repetitive intergenic consensus-PCR (ERIC-PCR) (**Prest *et al* 1994**).

Another advantage of the RAPD-PCR for the identification of microorganisms is that the identification is based on the genotype rather than the phenotype. Phenotypic identification is the basis of identification by other "rapid" methods, such as the API system or the Biolog GN microplate system, which rely on the metabolic activity of a microbe.

In this study, RAPD-PCR was applied to confirm the identification of several yeast isolates from different sources by API system. The ability to differentiate rapidly between a pure colony isolate of yeast among contaminants within one working day represents a significant improvement in reducing hold times while traditional phenotypic characterization is undertaken. In addition, this technology has application at any location where pure colony isolates are routinely obtained by traditional plating methods.

MATERIALS AND METHODS

Isolation and culture media

Isolation and subculturing of the yeast isolates were performed on different media including yeast extract peptone dextrose (YPD) broth medium (glucose, 10; yeast extract, 3; peptone, 3 g/L), yeast extract peptone dextrose (YPD) agar medium (YPD broth + 15 g agar), yeast extract Malt extract (YM) broth medium (glucose, 10; yeast extract, 3; peptone, 3; malt extract, 3 g/L) and Yeast extract Malt extract (YM) agar medium (YM broth + 15 g agar). pH of the media was adjusted at 4.5. The investigated yeast isolates were isolated from molasses, foodstuffs, concentrated juices and soils. Samples were introduced into YPD and YM broth media and incubated at 28°C for 24 hr. The samples were diluted 10^4, 10^6 and 10^8 fold with distilled water and then 0.1ml of dilutes were plated onto YPD and YM agar media. After incubation at 28°C, the appearing yeast colonies were purified. *Saccharomyces cerevisiae* TCY1 (*MAT* $\propto$ *lys2 ura3*), was the used type strain (**Abul-Hamd, 1999**).

Biochemical identification of yeast

The API 20 C AUX yeast identification system (BioMerieux Vitek, Inc., Hazelwood, Mo) was used for biochemical identification of the yeast isolates. The system consist of a disposable plastic strip with twenty cupules containing dehydrated reagents sufficient for 18 biochemical tests, a negative control and a couple containing glucose as positive control along the reaction. Suspensions were made by picking up a separate colony for each isolate from 48-72 hr agar plates and were added to the provided medium. Suspensions were used to fill the cupules of the test strips as directed by the provider. All test strips were incubated ed at 30°C and the growth was recorded after 48 and 72 hr. Based on the observed reactions, a profile number was generated for each isolate. Identification was made by reference to the API analytical profile index.

DNA extraction

The investigated yeast isolates were grown on YPD agar plates at 28°C for 24 to 48 hr. A single colony was then subcultured overnight on YPD broth medium and incubated at 28°C with shaking at 200 rpm. DNA was extracted from this culture by an existing protocol (**Lehmann *et al* 1992**). Yeast DNA extract could be stabilized by precipitating the nucleic acids with two volumes of ethanol for 3 hr at -20°C. The yeast nucleic acid precipitate was collected by pelleting in a microfuge, and the supernatant was decanted and discarded. The nucleic acid pellet was dried briefly under

vacuum then resuspended in 1 ml of sterile water.

DNA concentrations were determined spectrophotometrically with a spectrophotometer (Lambda 1A; Perkin-Elmer). DNA purity was determined by A_{260}/A_{280} ratio and 1.8 to 2.1 was considered acceptable.

RAPD-PCR

The two primers used in this study, primer 1 (5'-GGTGCGGGAA) and primer 6 (5'-CCCGTCAGCA) were purchased from Amershambioscience. Amplification reaction solutions were prepared in a final volume of 50 µl containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 100 M each of dATP, dGTP, dCTP, and dTTP (Boehringer Mannheim), 2.5 M primer, 1.25 units of *Taq* DNA Polymerase (Boehringer Mannheim) and approximately 50 ng of DNA. The amplification was performed in an MJ Research programmable thermal cycler (PTC-100/60) where the program was as follows: universal denaturation cycle (5 min at 94°C), 45 cycles of annealing/extension reactions (1 min at 94°C, 1 min at an optimum annealing temperature 36°C for each used universal primer and 2 min at 72°C) and cycle of final extension step (5 min at 72°C) was followed by soaking at 4°C.

Horizontal Gel Electrophoresis

RAPD-PCR-amplified products were separated by agarose gel electrophoresis using a horizontal submarine gel system (E-C Apparatus Corp., Holbrook, N.Y.). Agarose (Gibco BRL Life Technologies) at a concentration of 2% (wt/vol) was used to separate RAPD products. Electrophoresis was conducted in 0.5× TBE buffer (5.4 g of Tris base, 2.75 g of boric acid, and 2 ml of 0.5 M EDTA [pH 8.0] in 1 liter of distilled water) at 10 V/cm for various times, depending on the size of the gel unit; DNA size markers (Boehringer XII and XIV) were used as standards. DNA bands were stained with ethidium bromide (10mg/ml) and then visualized and photographed under UV light using a Gel Doc.2000 Transilluminator (Bio-Rad).

Cluster analysis

Genetic relationships and divergence between RAPD patterns of the yeast isolates were calculated from the Pearson coefficient using Bio-Rad Molecular Analyst software (**Anonymous, 1999**) and are illustrated in a dendrogram constructed using the unweighted pair-group method with arithmetic averaging (UPGMA) and single linkage.

RESULTS

Isolation and identification of yeast isolates

Sixteen yeast isolates (symbolized 2-17) were subcultured and purified on Yeast extract peptone dextrose (YPD) agar medium and Yeast-Malt extract media. The Yeast isolates were isolated form molasses, foodstuffs, concentrated juices and soils. The API 20 C AUX yeast identification system was used for identification of the sixteen yeast isolates. The yeast identification system (API 20 C AUX) confirmed that the sixteen yeast isolates belong to *Candida famata* (isolate 16), *C. glabrata* (3 isolates; 3, 6, 15), *C. krusei / inconspicua* (2 isolates; 13, 17), *C. norvegensis* (isolate 5), *C. colliculosa* (2 isolates; 9 & 10), and *Cryptococcus humicolus* (isolate 14), *Rhodotorula mucilaginosa* (isolate 12), *Kloeckera* spp. (2 isolates; 4 & 7) and *Geotrichum capitatum* (2 isolate; 8 & 11) and *Saccharomyces cerevisia*, TCY1 (lane 2 in **Fig. 1 and 3**)

RAPD reaction

Two oligonucleotide primers were tested for their ability to generate RAPD markers from genomic DNAs of sixteen yeast strains.

Figures (1 and 3) illustrate random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) fingerprint patterns of the yeast isolates with both primers. Lane 2, *Saccharomyces cerevisiae*, TCY1; Lanes 3, 6 &15, *Candida glabrata*; Lanes 4 & 7, *Kloeckera* spp.; Lane 5, *C. norvegensis*; Lanes 8 & 11, *Geotrichum capitatum*; Lanes 9 & 10, *C. colliculosa*; Lane 12, *Rhodotorula mucilaginosa*; Lanes 13, 17, *C. krusei / inconspicua*; Lane 14, *Cryptococcus humicolus*; Lane 16, *Candida famata* **(Figs. 1 and 2).** Lane 1 represents 250 bp ladder **(Figs. 1 and 3)** as DNA size standard.

The developed bands with various molecular weights were subjected to statistical analysis where the phylogenetic relationship among the biochemically-identified yeast isolates was investigated using a tree diagram with Euclidean distances and dice coefficient being the joining metric and the calculation method respectively. **Figure (2)** represents the tree diagram for DNA bands amplified by primer-1.

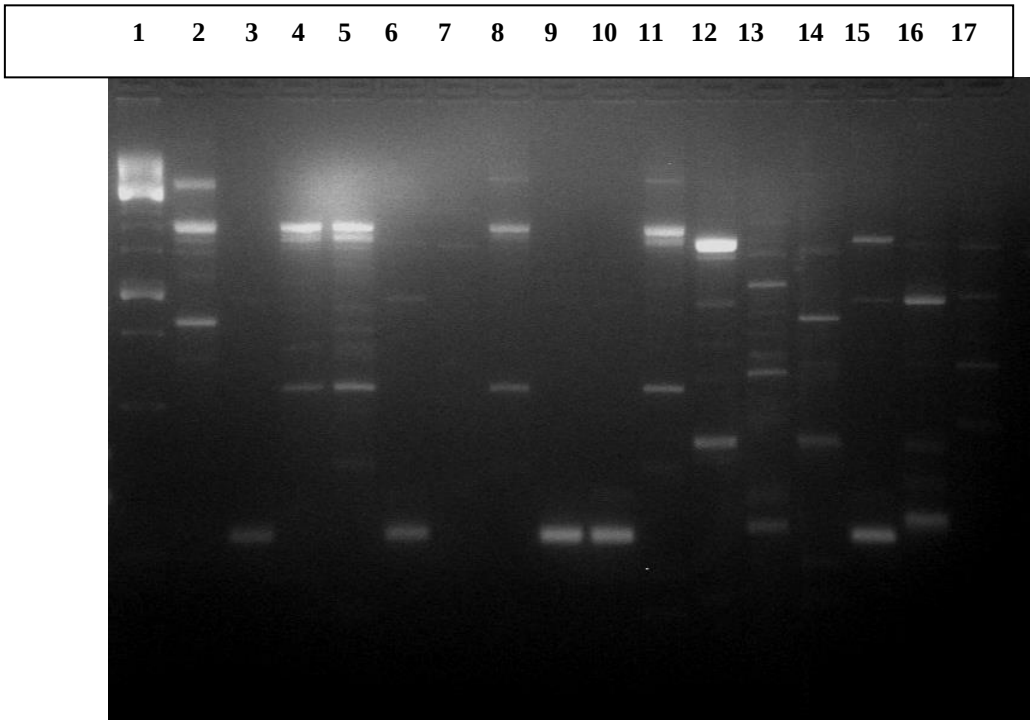

Figure 1. Banding pattern of RAPD- PCR of yeast isolates using primer-1.
Lane 1, 250bp ladder; Lane 2, *Saccharomyces cerevisia*, TCY1; Lanes 3-17 yeast isolates.

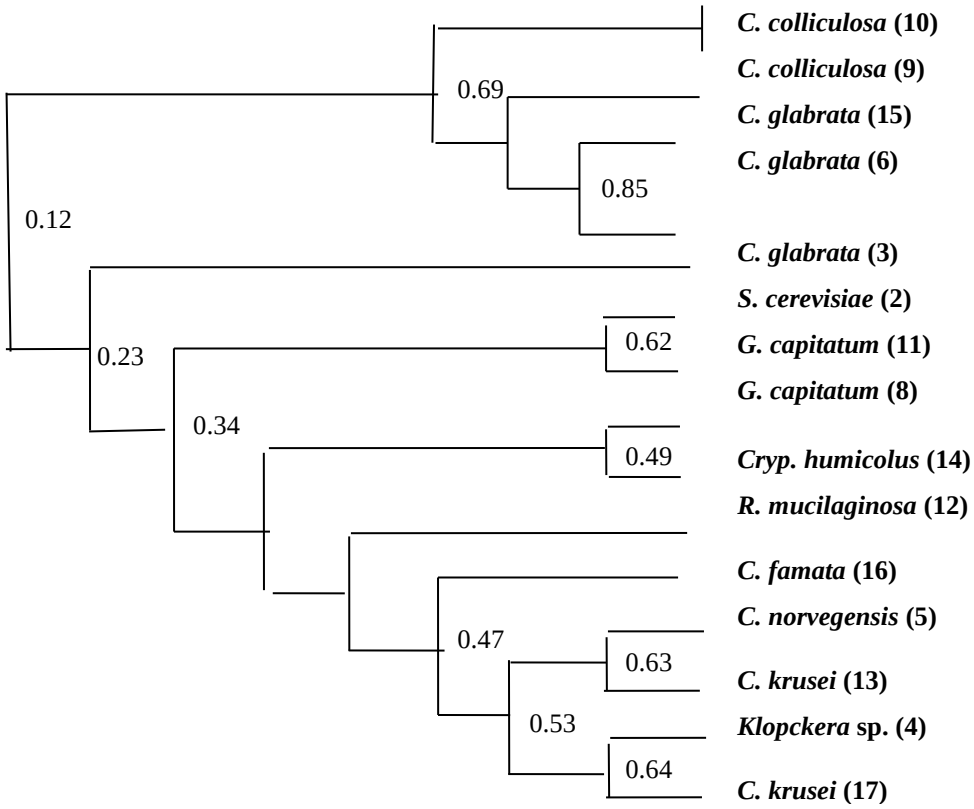

Fig. 2. Dendrogram of the yeast isolates based on the similarity matrix of RAPD analysis using primer-1.

Figure 3. Banding pattern of RAPD- PCR of yeast isolates using primer-6
Lane 1, 250bp ladder; Lane 2, *Saccharomyces cerevisiae*, TCY1; Lanes 3-17 yeast isolates.

The tree diagram classifies the investigated yeasts into four clades (groups). Clade one includes five yeasts of *Candida glabrata* (3 isolates; 3, 6, 15 and *C. colliculosa* 2 isolates; 9 & 10) with two of which (isolates; 9 and 10) being identical; their similarity matrix is 100 (a linkage distance of one). The two strains of *C. glabrata*, 3 and 6, follows *C. colliculosa*, 9 and 10 in linkage where they are joined together by a distance of 0.85. *C. glabrata*, 15 is linked to the other two strains belonging to the same organism *C. glabrata* (3 and 6) by a distance of 0.84 and 0.80 respectively, while joined to *C. colliculosa*, 9 and 10 at a distance of 0.69. Isolates of this clade are linked to the rest of the yeasts investigated by a distance of 0.12.

The two isolates 8 and 11 of *Geotrichum capitatum* represent clade two, they are linked together at a distance of 0.62. Clade two is the nearest clade to isolate 2 (*Saccharomyces cerevisiae* TCY1) being linked at a distance of 0.23. Clade three includes isolates *Cryptococcus humicolus* (isolate 14), *Rhodotorula mucilaginosa* (isolate 12), linked together at a distance of 0.49. Clade three is linked to clade two at a distance of 0.34, while linked to the last clade (clade four) at an average distance of 0.40.

Clade four is represented by six isolates of *Kloeckera* spp. (isolates 4 & 7); *Candida krusei / inconspicua* (isolates 13, 17); *C. norvegensis* (isolate 5), and *Candida famata* (isolate 16). In clade four, 7 (*Kloeckera* sp.) and 17 (*Candida krusei / inconspicua*) are the most related to each other (joined by a distance of 0.64) followed by 13 (*Candida krusei / inconspicua*) and (*Kloeckera* sp.) 4 (linked by a distance of 0.63), the last two isolates are joined to 7 (*Kloeckera* sp.) and 17 (Candida *krusei / inconspicua*) at a distance of 0.53, with all these four isolates being joined to *C. norvegensis* (isolate 5), at a distance of 0.47. *Candida famata*, isolate 16 is the least related isolate to isolates of clade four being joined to all of them at an average distance of 0.37.

Figure (4) illustrates the tree diagram developed from the amplified DNA fragments by primer 6. The relationship among the isolates developed by primer six are more or less close to that developed by primer one. From **Figure (4)**, three clades could be observed, clade one includes five isolates of *Candida glabrata* (isolates 3, 6, 15) and *C. colliculosa* (isolates 9 & 10; the same isolates forming clade one in case of primer one with differences in the values of the joining distances). The two strains belonging to *Candida glabrata* (3 and 6) are the most closely related

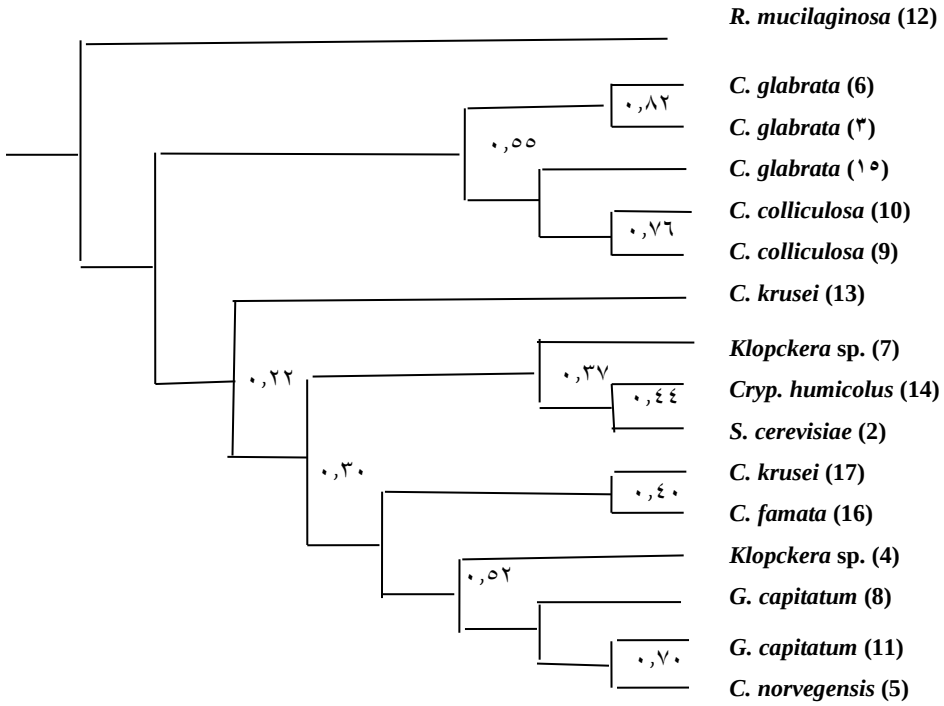

Figure. 4. Dendrogram of the yeast isolates based on the similarity matrix of RAPD analysis using primer- 6.

isolates in case of primer 6 where they are joined together by a distance of 0.82.), *C. colliculosa* (isolates 9 & 10) follows *Candida glabrata* (3 and 6) for being joined at a distance of 0.76. *Candida glabrata*, 15 is linked to *C. colliculosa* (isolates 9 & 10) by an average distance of 0.66, while joined to *Candida glabrata* (3 and 6) at an average distance of 0.55. Isolates of this clade are linked to the rest of the isolates investigated at an average distance of 0.04 representing the uniqueness of the isolates of this clade.

Clade two includes three isolates *Saccharomyces cerevisiae*, TCY1 (isolate 2), *Cryptococcus humicolus* (isolate 14), *Kloeckera* sp. (isolate 7). *Saccharomyces cerevisiae*, 2 and *Cryptococcus humicolus*, 14 are linked at a distance of 0.44 while they are joined to *Kloeckera* sp., 7 at a distance of 0.37.

Primer six reorganized the isolates of clade four of primer one where isolate *Kloeckera* sp. (isolate 7). and *C. krusei / inconspicua* (isolate 13) were replaced by *Geotrichum capitatum* (isolates 8 & 11) incase of clade three of primer six, so clade three is represented by six isolates of *C. norvegensis* (isolate 5), *Geotrichum capitatum* (isolates 8 & 11), *Kloeckera* sp. (isolate 4), *Candida famata* (isolate 16), and *C. krusei / inconspicua* (isolate 17). In clade three of primer six, *C. norvegensis* (isolate 5) and *Geotrichum capitatum* (isolate 11) are the most related to each other (joined by a distance of 0.70) followed by *Geotrichum capitatum* (isolate 8) which is linked to *C. norvegensis* (isolate 5) and *Geotrichum capitatum* (isolate11) by a distance of 0.65 and 0.67 respectively (in case of primer one *Geotrichum capitatum* (isolates 8 & 11) were closely related at a distance of 0.62 representing clade two). The former three isolates are linked to isolate *Kloeckera* sp. (isolate 4), at a distance of 0.52, while all of the mentioned isolates are linked to *Candida famata* (isolate 16), and *C. krusei / inconspicua* (isolate 17) at an average distance of 0.3. *Candida famata* and *C. krusei / inconspicua*, (16 and 17) are the least linked of clade three (0.4). Isolate 13 representing another strain of *C. krusei / inconspicua* is linked to clade one at a distance of 0.04 while to clade two at 0.22. Clade two and clade three are joined together at a distance of 0.30.

DISCUSSION

Identification of microorganisms has changed dramatically over the past decade through direct examination of the tremendous variation present in

DNA (**McEwen *et al* 2000**). Typing methods based on phenotypic characteristics are usually cheap and easy to perform but they have shown a lack in their reproducibility. On the contrary, genotypic methods are expensive and require sophisticated technology but they usually have good reproducibility (**Del Castillo *et al* 1997**).

In the current study RAPD-PCR procedure was used successfully along with the API biochemical kit as a research tool to identify and characterize yeast isolates from various locations. **Corte *et al* (2005)** stated that the combination of different typing techniques was useful when discriminating similar organisms. So, the introduction of a second typing technique can be more advantageous than increasing the number of characters obtained with a single method. **Pina *et al* (2005)** used the combination of PCR-fingerprinting and RAPD assays to discriminate fifty-eight yeast isolates from carbonated orange juice factory that showed to be very useful in tracking the route of contamination in a carbonated juice production chain.

This study demonstrates that the identification of yeast isolates by their distinctive fingerprint pattern can be achieved in less than 10 hr using RAPD-PCR technology. The procedure requires only a small amount of crudely extracted DNA from a single colony isolate. The method is not useful for the characterization of mixtures of unknowns, but single-colony isolates from a nutrient plate containing a mixed culture are sufficient for a clean fingerprint pattern. In food and beverage industry laboratories, of great significance is the time saved by analytical methods. The time required for identification of an isolate can be as short as 10 hr, which includes DNA extraction, RAPD amplification, and electrophoretic analysis. So, the technology is multifaceted in that it can be applied at any point along the brewing process.

RAPD-PCR produced a profile of bands that allowed the identification of intra- and interspecific polymorphisms among isolates. The technique was performed with each of the two primers as outlined in experimental. Both primers yielded strong distinctive patterns for the 16 yeast isolates while the number and sizes of the generated fragments being entirely differnt from one another. **Rieseberg (1996)** stated that primers with arbitrary sequences give different banding patterns with the same DNA even by applying on the same individual genotypes of species which may be obtained due to the recombination generating species.

Our study and those of others reported previously (**King *et al* 1995; Thanos *et al* 1996; Lockhart *et al* 1997; Steffan *et al* 1997; Melo *et al* 1998; Cadez *et al* 2002; Vasdinyei and Deak, 2003; Fadda *et al* 2004; Foschino *et al* 2004 and Pinto *et al* 2004**) have shown that RAPD methods performed with different oligonucleotides basically generated consistent patterns, with several shared fragments unique to each species. The use of different primers for the identification of the microorganisms increased the level of confidence of the identification.

According to the results obtained, RAPD fingerprinting, combined with API yeast identification system was useful when discriminating similar organisms. Additionally, it should be noted that the results of biochemical identification are very close to those of molecular characterization of primer 1 which grouped the isolated yeasts into clades very similar to those of biochemical identification. However, primer 6 was different from the API identification in a number of isolates where it grouped differently identified API isolates together confirming the need for more than one technique for identification and that biochemical identification is not enough. This is ensured by the percentage of error admitted worldwide for biochemical identification.

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