40 Debaryomyces

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40.1 INTRODUCTION

Species of the ascomycetous genus *Debaryomyces* are among the most common yeasts isolated from many natural habitats such as air, soil, pollen, tree exudates, plants, fruits, insects, feces, gut of vertebrates, and sea water.[1] Some of their species, especially *Debaryomyces hansenii*, have been found in a wide variety of foods, mainly those with low water activity (a_w) as well as in high-sugar products such as fruit juices, soft drinks, wine, beer, sugary products, bakery products, dairy products and meat or processed meats.[1–12] Also *Candida famata*, anamorph of *D. hansenii*, formerly known as *Torulopsis candida*, can be found in many foods, in particular cheese and other dairy products and sausages.[2,13,14]

The genus *Debaryomyces* is normally considered a nonpathogenic yeast[15] and has rarely been isolated from humans. Some species have been reported to exert positive effects in the ripening of fermented foods such as dairy and meat products. However, some diseases have been related to *D. hansenii* and its anamorph *C. famata*, mainly in immunocompromised patients, such as a case of bone infection,[16] an allergic alveolitis,[17] or septicemia.[18] The positive effects of *Debaryomyces* in foods and its pathogenic relevance will be discussed in this chapter.

For the diagnosis of *Debaryomyces*, different physiological and morphological methods have been traditionally used. However, these methods are laborious, lack discriminatory power and misidentification occurs frequently.

Progress in the molecular nucleic acid methods may allow the possibility to characterize yeasts at species and strain level. We here will analyze the main physiological and morphological methods, as well as the molecular techniques for the diagnosis of *Debaryomyces*.

40.1.1 Classification and Morphology of *Debaryomyces*

Genus *Debaryomyces* was established by Klocker[19] with the single species of *D. globosus*, and currently 18 species have been included: *D. carsonii*, *D. castellii*, *D. coudertii*, *D. etchelsii*, *D. hansenii*, *D. maramus*, *D. melissophilus*, *D. nepalensis*, *D. occidentalis*, *D. polymorphus*, *D. pseudopolymorphus*, *D. robertsiae*, *D. udenii*, *D. vanrijiae*, *D. yamadae*,[20] *D. prosopidis*,[21] *D. mycophilus*,[22] and *D. singareniensis*.[23]

The members of this genus show spherical cells, and pseudomycelium is absent, primitive or occasionally well developed. All species are perfect, haploid, and have a vegetative reproduction by multilateral budding.[24] The sexual reproduction proceeds via heterogamous conjugation of two cells of different form or size, generally mother and bud, although the isogamous conjugation also occurs.[24] The conjugation commonly leads to a diplophase followed by meiosis and ascospore formation.[25] One to two spherical, globular, ovoidal or lenticular smoothy or warty spores are usually formed per ascus, but in some species up to four spores could be present.[24] *Debaryomyces* species are distinguished from

other ascomycetous yeast genera by the special internal ultrastructure of their ascospores.[24]

The nuclear base composition of the *Debaryomyces* species is 37 mol% G + C or higher.[26] The kariotype analysis revealed a high degree of polymorphism.[27] Thus, in the most frequent species of the genus, *D. hansenii*, two varieties are differentiated, according to the current taxonomy: *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii*. They can be discriminated by their maximum growth temperatures, the sequence divergences of their 26S rRNA genes, and differences in the electrophoretic mobility of their glucose-6-phosphate dehydrogenase.[28,29]

The anamorph of *D. hansenii* is *C. famata*. Phylogenetically, *D. hansenii* is related to *Candida albicans*[30] and belongs to a monophyletic clade containing organisms that translate CTG as serine instead of leucine. For this, Fitzpatrick et al.[31] suggested that *D. hansenii* and *Candida guilliermondii* are sister taxa.

40.1.2 Biology, Pathogenesis, and Medical Importance

Debaryomyces species are osmotolerant and can grow in media containing up to 4 M NaCl.[32] Species of this genus are characterized physiologically by their inability to assimilate nitrate, as well as their weak or nonexistent fermentation capacities,[24] and chemotaxonomically by their expression of coenzyme Q-9.[33] These and others characteristics, which are used for testing species of genus *Debaryomyces* are summarized in Table 40.1.

The main species of this genus, *D. hansenii* is able to grow at 10% NaCl or 5% glucose, and these characteristics are used to discriminate *D. hansenii* from other ascomycetous yeasts. On the other hand, *D. hansenii* is one of the lipid-accumulating, "oleaginous" yeasts, and can accumulate lipids to concentrations up to 70% of its dry biomass[34] and its metabolism is clearly dominated by pathways that contribute to lipid metabolism. *D. hansenii* is a heterogeneous species, with remarkable phenotypic differences between strains, such as variations in their ability to metabolize various carbon sources, the expression of different lipase and protease activities, and their diverse optimal growth conditions.[4,35] The biological characteristics of *D. hansenii* have been reviewed recently by Breuer and Harms.[36] *D. hansenii* can be cultivated in media with up to 25% NaCl or 18% glycerol. It is isolated from environments with high salt concentrations, such as sea water or several types of food. In fact, moderate NaCl concentrations improve growth of *D. hansenii* cells. The positive effect of NaCl on *D. hansenii* growth is even more evident in the presence of several stress conditions, such as high temperature and low or high pH levels in the media. All these characteristics make that *D. hansenii* is regarded as a halophilic yeast.[37]

D. hansenii is reported to grow optimally at 20–25°C, which might be a consequence of its natural occurrence in habitats such as sea water. *D. hansenii* can grow at 5°C and even below 0°C. At 10°C this yeast is able to grow at pH 4.0–6.0 when a_w is up to 0.99. *D. hansenii* can grow at a_w values as low as 0.65.[36,38]

The growth of *Debaryomyces* in foods is not considered usually as harmful, and some species have positive effects in the ripening of fermented foods like dairy and meat products.[39–41] Due to their ability to grow at low pH, high salt concentration and low temperature, yeasts are the first microorganisms that develop on the cheese surface. They contribute in cheese ripening by assimilation of lactic acid causing an increase in pH, which will enhance the growth of other microorganisms. In addition, *D. hansenii* metabolizes lactose,

TABLE 40.1
Characteristics of Genus *Debaryomyces*

Characteristics	Possible Results for *Debaryomyces* spp.
Fermentation of: galactose, glucose, lactose, maltose, melibiose, raffinose, sucrose	+, –, s, v, w
Assimilation of carbon compounds: L-arabinose, cellobiose, citric acid, erythritol, galactose, inositol, lactose, maltose, D-mannitol, raffinose, L-rhamnose, ribitol, D-ribose, soluble starch, succinic acid, sucrose, trehalose, D-xylose	+, –, v
Splitting of arbutin	+, –, v
Assimilation of nitrite	+, –, v
Growth in vitamin-free medium	+, –
Growth on 50% (w/w) glucose-yeast extract agar	+, –, w
Growth at 37°C	+, –, v
G + C (mol%)	37–40%
Shape of the ascospores	spherical, oval
Wall of the spores	warty, smooth
Number of spores per ascus	1, 2, 3, 4
Formation of pseudomycelium	+, –

Source: Kreger-van Rij, N.J.W., *The Yeast, A taxonomic Study*, 3rd ed. Elsevier Science Publishers, Amsterdam, 1984.
Notes: +, positive reaction; w, weak reaction; s, slow reaction; –, negative reaction; v, variable reaction.

as well as multiple organic carbon and nitrogen sources, to generate volatile sulphur compounds, esters, alcohols, aldehydes, and ketones that contribute to alcoholic, acidic, and cheesy flavour.[42] In addition, *D. hansenii* isolated from meat products showed proteolytic and lipolytic activities,[43–49] and it has been suggested that *D. hansenii* enhances the sensory characteristics and may contribute to the flavour in dry-cured fermented sausages,[41,50] ripened loins[51] or dry-cured hams.[12,52] *Debaryomyces* species also contribute to the ripening of pickles, where they oxidize the acids produced by lactic acid bacteria during fermentation.[3,53]

It is also possible to find *Debaryomyces* spp. in other kinds of foods. They were found in ready-to-eat fufu and lafun-fermented cassava products.[54] *Debaryomyces polymorphus* was one of the most frequently reported yeasts in fruit salads.[55] However, *D. hansenii* has been the most reported species in fermented tea plant (*Camellia sinensis*) leaves, seasoned green table olives, and processed fresh edible sea urchins.[56–58]

An excessive growth of *Debaryomyces* may cause undesirable sensory changes in the formation of bad aromas and flavors, gas production, discoloration, and changes in texture.[3,59]

Proteolytic *D. hansenii* was isolated from decayed and damaged, uncooked, ripe tomatoes. Growth of a proteolytic, alkalinizing yeast such as *D. hansenii* in raw tomatoes enhances conditions for growth of *Salmonella,* because of increased pH. Thus, the risk of human diseases caused by pathogenic bacteria favoured by increased pH of decayed pulp tissue is enhanced by this yeast.[60]

Debaryomyces spp. have rarely been isolated from humans, but they are considered opportunistic pathogens. Wong et al.[16] reported some infections caused by *Debaryomyces* species. *Trichosporon candida* (*C. famata*) was isolated from a patient with chronic skin lesions on the hands and feet. *D. hansenii* was also found in one case of bone infection that a 23-year-old woman suffered over 4 years. Several clinical samples were identified as *D. hansenii* (and its anamorph *C. famata*) in superficial infections.[61] *D. hansenii* was also responsible for a persistent candidemia observed in a patient heavily treated with various fungicides.[18] A 65-year-old female was diagnosed of extrinsic allergic alveolitis resulting from exposure to inhaled organic dusts, being *D. hansenii* the dominant species in indoor air samplings.[17]

Different species of *Candida* are part of the natural microbiota and, thus, are regarded as commensal organisms in humans. *C. famata* was thought to be nonpathogenic for humans. However, this yeast was isolated in combination with other *Candida* spp. from a relevant number of clinical cases, including ocular endophthalmitis, retinopathy, and central nervous system infection. In addition, *C. famata* is rarely implicated in human fungemia.[62–64] New treatments of hospitalized patients seem to have favored the emergence of *C. famata* as a pathogen, but they may be under-reported.[65] In this sense, *C. famata* has been associated with catheter-related bloodstream infection[66] and rarely with other infections,[16,67–71] generally in immunocompromised patients. Thus, medical importance of *D. hansenii* and its anamorph *C. famata* may rely on susceptibility of immunocompromised patients and on its resistance to the treatments applied to different pathologies.

40.1.3 Diagnosis of *Debaryomyces*

Different physiological and morphological methods have been traditionally used in taxonomic differentiation of *Debaryomyces.*[1,33,72–74] However, several studies have shown that traditional identification methods, based on phenotypic properties of yeasts (morphological, biochemical, and physiological tests) are laborious, lack discriminatory power and misidentification occurs frequently.[75] In addition, identification on the basis of morphological properties generates the so-called double binomial nomenclature, with one name for the vegetative state (anamorph) and another for the sexual state (teleomorph). This is the case of *C. famata*/*D. hansenii*. Furthermore, these methods generally produce ambiguities and inaccuracies in the results, because the morphological and physiological characteristics are strongly influenced by growing conditions.

Progress in molecular biology in the last decade has opened up possibilities of characterizing yeasts at the genomic level. The sequencing of the genes coding for 18S and 26S ribosomal RNA (rRNA), as well as internal transcribed spacer (ITS), has brought about many changes in the identification and classification of yeasts.[76] In addition, techniques based on random amplified polymorphic DNA (RAPD-PCR) and restriction fragment length polymorphism (RFLP), have already been recognized as reliable tools for the rapid identification of yeasts.[9] We here review the main physiological and morphological methods, as well as the molecular techniques.

40.1.3.1 Physiological and Morphological Analysis

The physiological and morphological identification of *Debaryomyces* is done on the basis of several characteristics of this genus which are listed below:

Characteristics of vegetative reproduction. (i) Modes of vegetative reproduction: by budding, by fission or a combination of both processes; (ii) characteristic of vegetative cells: morphology grown in liquid and solid media; (iii) formation of pseudomycelium and true mycelium; (iv) formation of asexual endospores; (v) formation of chlamydospores; and (vi) formation of germ tubes.

Sexual characteristics. Characteristics of ascospore or basidiospore formation.

Physiological and biochemical characteristics. The physiological tests used for identifying purposes are those associated with the utilization of carbon and nitrogen sources, growth factor requirements, growth at elevated temperatures and on media of high sugar or sodium chloride content, formation of typical characteristic metabolites, and susceptibility to antibiotics. The utilization of these tests for *Debaryomyces* characterization requires considerable experience and skill for evaluating specified

tests. Furthermore, great difficulties for the differentiation at species level in this genus could be found, since many biochemical and physiological tests show the same result for different species. Among the physiological biochemical tests of characterization, in addition to those in Table 40.1, the following have been used:

- Formation of extracellular, amyloid compounds
- Production of ammonia from urea
- Splitting of fat
- Ester production
- Cycloheximide resistance
- Tolerance of 1% acetic acid
- Gelatin liquefaction
- Diazonium Blue B (DBB) color test
- Coenzyme Q structure
- Tetrazolium indicator medium (TTC medium)

The main biochemical tests are electrophoresis of proteins, coenzyme Q analysis, allozyme analysis, and ultrastructure and chemical composition (polysaccharides, fatty acids) of the cell wall.[24] Missoni et al.[77] found the evaluation of cell fatty acids by gas chromatography very useful in the routine diagnosis and epidemiological monitoring of the infection due to *Candida* spp. (including *C. famata*). These techniques are not always stable or reproducible because they depend on the physiological status of the strains. For example, fermentation of sugars is not very accurate because the slow release of CO_2 is not so immediate to be trapped in a tube Durham.

Many commercial methods based on the above morphological and physiological characteristics have been developed for the identification of yeasts, although most of them focus on clinical isolates related to different diseases that do not include species of genus *Debaryomyces.* Some examples of this are the Yeast Identification System API 20C (BioMérieux), the Uni-Yeast-Yek system (Remel), the Minitek system (BBL),[72,78–80] or enzyme-based system such as Yeast Identification Panel (Baxter-MicroScan) and MicroScan Rapid Yeast Identification (Innovative Diagnostic Systems).[73,81] The basic principle of these systems is carbohydrate assimilation, which requires a minimum incubation period of 24 h for growth. The enzymatic activity systems use chromogenic substrates and can identify yeasts within 4 h after inoculation.

Due to the increasing importance of yeasts in the production and spoilage of foods, the database of the above methods has been completed with foodborne yeasts. Thus, the API ID32C system allows the identification of three species of the genus *Debaryomyces*: *D. hansenii*, *D. marama*, and *D. polymorphus.*[82] Similarly, the Vitek Yeast Biochemical card (BioMérieux) allows identification of *D. hansenii.*[83,84]

Furthermore, several methods have been developed for automatic identification of yeasts on the basis of biochemical tests, such as the highlighting system Vitek 2® (BioMérieux) with colorimetric and fluorimetric VITEK 2 yeast cards[85,86] and Biolog YT Microplate® (AES Laboratories).

In addition, selective, and differential chromogenic solid media have been developed for the detection of *Debaryomyces* spp. For food samples, *Debaryomyces* differential medium (DDM) has been reported to be very satisfactory.[87,88]

40.1.3.2 Molecular Analysis

The molecular methods for identifying genus *Debaryomyces* and yeasts in general, are based on the study of DNA and RNA. The yeast nucleic acid sequences contain the primary information which determines all the physiological, biotechnological, and pathogenic characteristics and potential of a particular organism. The use of genotypic rather than phenotypic characteristics for identification is potentially more accurate, reproducible, and rapid.[83] Nucleic acid-based methods have the advantage over phenotypic identification methods by not being influenced by environmental conditions of the cells, because the nucleotide sequence of the DNA does not change during growth.

(i) Methods based on nucleic acid hybridization

Nucleic acid hybridization is typically between a DNA or RNA molecule present in the target organism and a DNA probe which has a sequence complementary to the target sequence.

18S rRNA-targeted oligonucleotide probes were designed for rapid and reliable identification of yeasts like the genus *Debaryomyces* and the species *D. hansenii.*[89–91]

(ii) Methods based on nucleic acid amplification

The most popular method of amplification is the polymerase chain reaction (PCR) technique. The PCR can detect one copy of the target sequence by using two oligonucleotide primers.

Conventional PCR has been used for identification of *D. hansenii* only in some cases.[92] However, different variations of this technique (PCR-RFLP, RAPD, Q-PCR, NASBA, etc.) are frequently used to differentiate the genus *Debaryomyces.*

One of the problems when using PCR with clinical samples or isolates related to pathogenesis is the possibility of detecting naked DNA derived from dead and degrading yeast cells instead of live yeasts, which results in false-positives. An alternative to PCR consists in using nucleic acid sequence-based amplification (NASBA) system that selectively amplifies RNA. This method has been used with yeasts of genus *Candida.*[93]

The RAPD-PCR is a variation of PCR used for the identification of yeasts. This method is based on PCR amplification of the genomic DNA in the presence of a single short primer. Due to low-temperature hybridization primer joins unspecific sites throughout the genome, allowing the amplification of DNA fragment of different length. The use of RAPD-PCR permits to obtain fingerprints which are specific for species and even strains. The RAPD-PCR technology with different primers, mainly from micro- and minisatellites, has been used for the correct identification of several species of *Debaryomyces.*[75,83,94–96]

One of the most promising PCR techniques in the detection of microorganisms is real-time PCR or quantitative PCR (Q-PCR). Q-PCR assays have been developed for detecting and enumerating yeasts, specially for yeasts in wine[97,98] and foods,[99] as well as in fungal infections by *C. famata*.[64]

(iii) Methods based on RFLP

The determination of the RFLP is the differentiation of organisms by analyzing patterns of rupture that are generated in a specific site of the genome when it is cut by restriction enzymes. Then, gel electrophoresis displays a pattern of polymorphic bands corresponding to the fragments of different sizes, which are generated on the cut of each endonuclease. This fragment length polymorphism appears because the organisms of different species and even strains differ in the distance of cleavage sites for each restriction enzyme. The similarity of the patterns generated allows for correlations between species and strains.

The PCR-RFLP is a useful method for identification of some yeast genus, using the restriction analysis of different regions of ribosomal RNA/DNA genes (ITS, 5.8S rDNA, 18S rDNA, etc.). This technique has two steps: first, the rDNA region is amplified and then the PCR product is digested. Therefore, this technique is slower than some current PCR methods, which achieve identification and typing with just the PCR products. The results of this technique depend on the region of genome amplified and the enzymes used. For example, PCR-RFLP of ITS1-5.8S rDNA-ITS2 and 18S rDNA regions has been reported as a good method for the differentiation of *Saccharomyces* species,[100,101] but it does not allow for separation of *Debaryomyces* spp.[94] However, the PCR-RFLP of the intragenic spacer (IGS) of rDNA is proposed as a clear technique for the practical discrimination species of the genus *Debaryomyces*.[102]

In the technique of pulsed-field gel electrophoresis (PFGE), restriction enzymes digest the complete genome and large DNA molecules are resolved by continuous reorientation of the electric field during gel electrophoresis, determining chromosome length polymorphism (CLP). This pattern is specific for yeast species, due to genetic and evolutionary phenomena that have taken place in the chromosomes (insertions, deletions, and translocations). This method has been reported as a useful tool in the differentiation of species and strains of *Debaryomyces*.[27,35]

Another technique based on restriction fragment length polymorphism is the mitochondrial DNA (mtDNA) restriction analysis. Among all the molecular techniques described in literature, mtDNA restriction analysis appears as one of the most suitable methods to differentiate between yeast strains. Querol et al.[103] developed a new mitochondrial restriction analysis method based on the extraction of total yeast DNA and the use of GC-rich restriction endonucleases that recognise a high number of sites in the yeast nuclear DNA, but few sites in the mtDNA. This technique has successfully been used to characterize strains of genus *Debaryomyces*.[94,104–106]

The most reliable, simple, and fast methods to differentiate *Debaryomyces* spp. at strain level rely first on RFLP analysis of mtDNA and then on RAPD-PCR using micro- or minisatellite primers.[94]

40.2 METHODS

In this chapter, a differentiation method for *Debaryomyces* spp. that includes first mtDNA restriction analysis and then RAPD-PCR using micro- and minisatellite primers is described. For this, total yeast DNA is first isolated and then either digested with *Hae*III for RFLP of mtDNA or amplified by RAPD-PCR using microsatellite primers $(GACA)_4$, $(GAC)_5$ and $(GTG)_5$ and the minisatellite primer M13.

40.2.1 Reagents and Equipment (Table 40.2)

40.2.1.1 Sample Preparation

Samples must be taken aseptically and homogenized in a Stomacher lab-blender with sterile peptone water (0.1%, w/v). Decimal dilutions are obtained with the same diluent and 0.1 ml is spread onto the surface of different selective media for yeasts, such as Dichloran Rose-Bengal Chloramphenicol Agar (DRBC, Oxoid, Cambridge, UK), Malt Extract Agar (2% w/v malt extract, 2% w/v glucose, 0.1% w/v peptone, 2% w/v agar) and Dichloran-Glycerol Agar (DG18, Oxoid). They are incubated at 25°C for 5 days.

The isolates obtained can be placed in cryotubes containing Malt Extract Broth (2% w/v malt extract, 2% w/v glucose, 0.1% w/v peptone) and stored at –80°C in 20% (v/v) glycerol.

Total DNA is isolated from broth cultures. Yeast cells are grown in 10 ml of Yeast Peptone Glucose broth (1% w/v yeast extract; 2% w/v peptone; 2% w/v glucose) in a 50 ml conical tube at 25°C in an orbital shaker at 200 rpm (Figure 40.1). In addition, for mitochondrial DNA isolation, conical tubes should be placed inclined in the orbital shaker. To reach an adequate cell density, incubation for 48 h is recommended. Cells are pelleted by centrifugation for 5 min at 4,000 rpm, resuspended in 500 µl of 1 M sorbitol, 0.1 M EDTA, pH 7.5, and transferred to a 2 ml sterile microtube. Subsequently, 40 µl of lyticase (20 mg/ml) are added to digest cell walls of yeasts and obtain spheroplasts. After 45 min of incubation at 37°C in a water bath with occasional shaking, the suspension is centrifuged at 13,000 rpm for 1 min and the supernatant is discarded. The pellet is resuspended in 500 µl of 50 mM Tris-HCl, 20 mM EDTA pH 7.4 to release cellular DNA from spheroplasts. After that, 50 µl of 10% (w/v) sodium dodecyl sulfate (SDS) are added and the mixture is heated at 65°C for 10 min in a water bath. To remove proteins, 200 µl of 5 M potassium acetate are added, the solution is shaken and stored on ice for 15 min. Next, it is centrifuged at 13,000 rpm for 5 min. A volume of 500 µl of the resulting supernatant is transferred to a new sterile microtube together with an equal volume of ice-cold isopropanol and it is left at room

TABLE 40.2
Reagents and Equipment Required for RFLP Analysis of Mitochondrial DNA and RAPD-PCR

Reagents and Culture Media	Equipment
Glycerol	Laminar flow cabinet
Lyticase	Stomacher lab-blender
Sodium dodecyl sulfate (SDS), molecular biology grade	Incubator
Ethyllendiaminetetraacetic acid (EDTA) disodium salt, dihydrate	Orbital shaker
Tris-(hydroxymethyl)-aminomethane	Vortex
D-Sorbitol extrapure	Freezer
Hydrochloric Acid (HCl) 35%	Water bath
Potassium acetate, extra pure	Centrifuge for 50 ml conical tubes
Isopropanol	Centrifuge for 2 ml and 0.5 ml microtubes
Ethanol absolute	1 ml, 200 µl, 50 µl, 5 µl, 1 µl and 0.5 µl pipettes
RNase	5 ml cryotubes
Ultrapure water	Spectrophotometer
*Hae*III and 10 × buffer	Microwave oven
Agarose D-1	UV transilluminator
DNA molecular markers	Thermal cycler and PCR tubes
Bromophenol blue, indicator	Horizontal electrophoresis unit with the appropriate gel casting tray and combs
Ethidium bromide	
PCR reagents (see Table 40.3).	
Dichloran 18% Glycerol (DG18) Agar	
Dichloran Rose Bengal Chloramphenicol (DRBC) Agar	
Bacteriological agar	
Malt extract	
Yeast extract	
D-Glucose anhydrous, extra pure	
Bacteriological peptone	

temperature for 5 min. DNA is pelleted by centrifugation at 13,000 rpm for 10 min, washed with 1 ml of ice-cold 70% (v/v) ethanol and centrifuged at 13,000 rpm for 1 min. Ethanol is aspirated with a pipette and the pellet is dried at 37°C for 45 min. Dried DNA is dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA pH 8.0). For RNA digestion, 1 µl of RNase (20 mg/ml) is added and the solution is incubated at 37°C for 30 min in a water bath and immediately placed at –20°C. The DNA obtained can be used for RFLP analysis and RAPD-PCR. To be used for PCR the DNA has to be spectrophotometrically quantified and brought to a final concentration of 100 ng/µl.

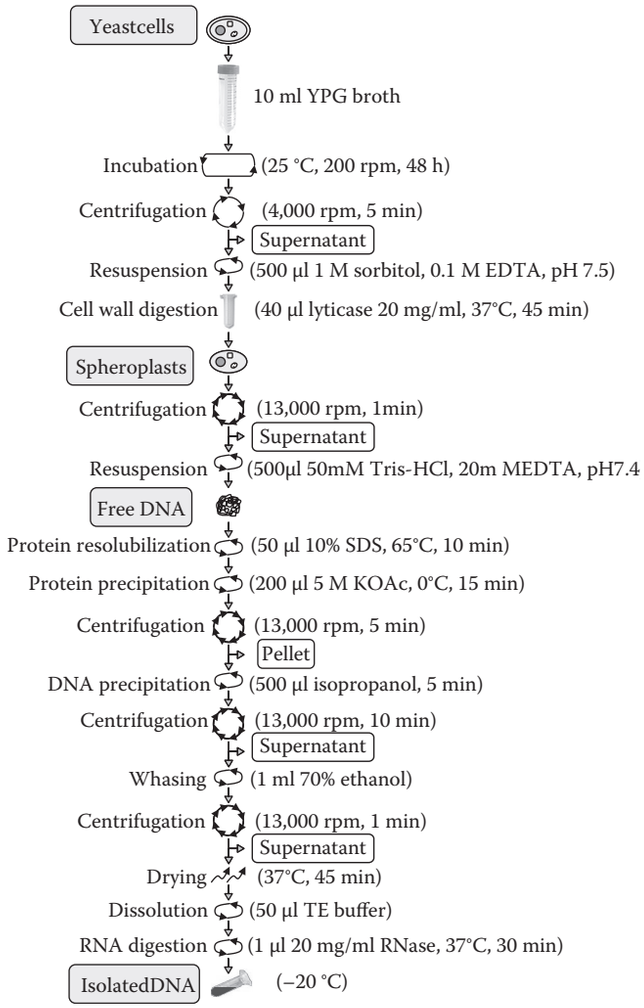

FIGURE 40.1 Diagram of DNA isolation from *Debaryomyces* yeasts.

40.2.2 Detection Procedures

40.2.2.1 RFLP Analysis of Mitochondrial DNA

Mitochondrial DNA restriction analysis consists in the digestion of total DNA with restriction endonucleases with recognition sites rich in GC, such as *Hae*III, that results in an overdigestion of the nuclear DNA to render specific bands from mtDNA.

The digestion mixture is prepared on ice for a final volume of 15 µl as follows: 5 µl of DNA isolated according to the above method, 20 U of the restriction enzyme *Hae*III and 1.5 µl of the appropriate 10 × buffer.[94] Then, the reaction is performed overnight at 37°C in a water bath (Figure 40.2).

An alternative method using a microwave oven for DNA digestion has been described.[107,108] The digestion mixture is placed inside a water bath and incubated in three heating times at maximum level of the microwave oven (1,250 W) for 20 s each, giving a spin between each time.

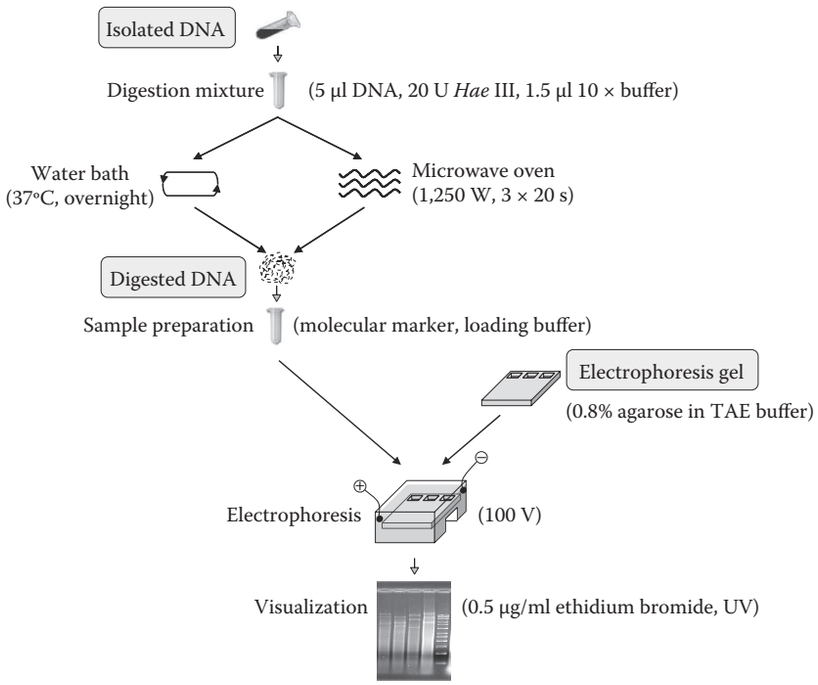

FIGURE 40.2 Diagram of RFLP analysis of mitochondrial DNA from *Debaryomyces* yeasts.

Restriction fragments are electrophoretically separated in a horizontal 0.8% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) at 100 V. Each digested DNA sample, as well as an appropriate DNA molecular marker, are mixed with loading buffer (50% glycerol, 0.25% bromophenol blue and 25 mM EDTA) and loaded into the gel wells. Restriction fragments are visualized in an UV transilluminator after ethidium bromide (0.5 µg/ml) staining. Sizes of restriction fragments can be estimated by comparison to the DNA molecular marker.

40.2.2.2 RAPD-PCR

For RAPD-PCR a single primer with an arbitrary sequence of oligonucleotides is used. Thus, knowledge of DNA template sequence from the tested yeasts is not required. Core sequence of phage M13 (5´-GAGGGTGGCGGTTCT-3´)[109] and the microsatellite primers $(GACA)_4$, $(GAC)_5$ and $(GTG)_5$ have proved useful to characterize *Debaryomyces* spp. using RAPD-PCR.[94]

The PCR mixture must be prepared on ice with the reagents and concentrations summarized in Table 40.3 to reach a final volume of 50 µl. The reaction is performed in a thermal cycler following the amplification programs shown in Table 40.3. After the program ends, RAPD-PCR products are kept in the thermal cycler at 4°C. The reaction mixture adding water instead of DNA sample can be used as negative control.

RAPD-PCR products are examined by electrophoresis in horizontal 1% (w/v) agarose gels at 100 V (Figure 40.3). A DNA molecular marker can be incorporated into the gel to estimate the size of the amplification products. Gels are stained with an ethidium bromide (0.5 µg/ml) solution and visualized under UV light.

40.3 CONCLUSIONS

Yeasts of genus *Debaryomyces* have been usually found as microbial population of food, specially ripened foods. These yeasts are considered normally as nonpathogenic. However, some species of this genus have been occasionally isolated from human diseases such as bone infection, allergic alveolitis or septicaemia in immunocompromised patients.

Eighteen different species have been included in the genus *Debaryomyces*. All of them are osmotolerant and can grow in media containing up to 4 M NaCl. Species of this genus are characterized physiologically by their inability to assimilate nitrate, as well as their weak or nonexistent fermentation capacities.

Among the physiological, morphological, and molecular methods used for characterizing *Debaryomyces* species, the most reliable, simple, and fast way to characterize *Debaryomyces* spp. relies on RFLP analysis of mtDNA and RAPD-PCR using micro- or minisatellite primers. In the present work, a combined procedure that includes characterization by both above methods to differentiate *Debaryomyces* spp. at strain level has been described.

TABLE 40.3
RAPD-PCR Reagents and Programs for Different Primers

		M13	$(GACA)_4$	$(GAC)_5$	$(GTG)_5$
Reagents	**Stock Concentration**	**Volume (μl)**			
Mg^{2+}-free reaction buffer	10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100	5	5	5	5
$MgCl_2$	50 mM	2	3	4	4
PCR nucleotide mix	10 mM	1	1	1	1
Primer	100 ng/μl	1	2	2	2
DNA	100 ng/μl	2	1	1	1
Taq DNA polymerase	2 U/μl	0.5	0.5	0.5	0.5
Sterile deionized water		38.5	37.5	36.5	36.5
PCR stages	**Number of Cycles**	**PCR Program**			
Initial denaturation	1	94°C, 3 min			94°C, 5 min
Denaturation		94°C, 45 s	94°C, 1 min		94°C, 30 s
Annealing	30	50°C, 1 min	36°C, 1 min		45°C, 1 min
Extension		60°C, 3 min	55°C, 5 min		60°C, 5 min
Final extension	1	60°C, 3 min	55°C, 5 min		60°C, 5 min

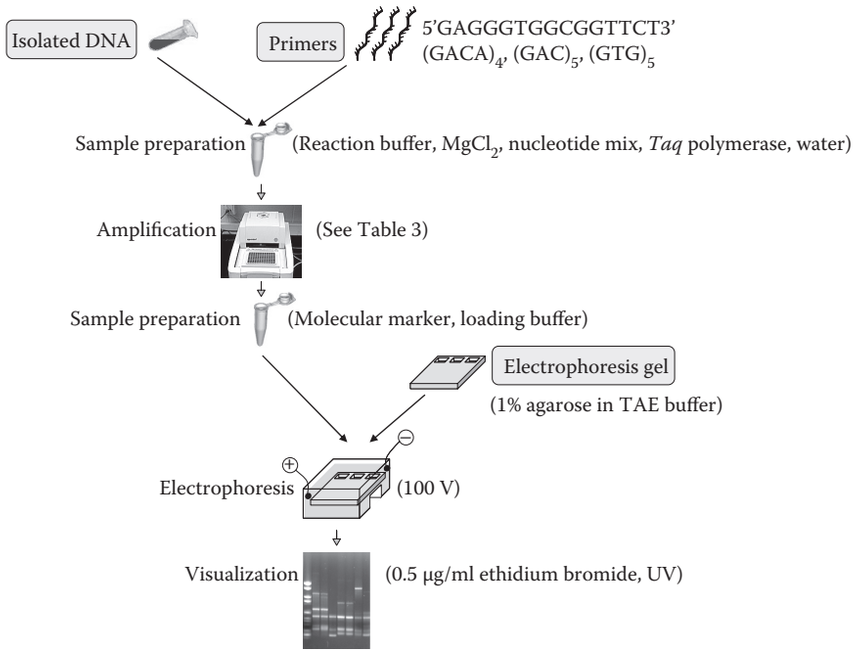

FIGURE 40.3 Diagram of RAPD-PCR from *Debaryomyces* yeasts.

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