



# Chapter 12

## Detection and Quantification of Yeast Species in Food Samples for Quality Control

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### Abstract

Yeasts grow in a high range of environmental conditions, which make them potent contaminants of foods and beverages. Thus, detect and quantify yeast species are essential for quality control of the foods. The isolation, enumeration, and identification of yeasts from foods and beverages follow the same principles and strategies that are used for yeasts in general. Different approaches have been used to enumerate yeast species, including traditional methods based on physiological characteristics, molecular characterization, and protein profile evaluation of microorganisms.

**Key words** Physiological identification, Specific pair primers, PCR, qPCR, MALDI-TOF, Yeast spoilage, Culture-independent method

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### 1 Introduction

Foods and beverages are spoiled when they are no longer pleasing the consumer's eyes and taste. Generally, they have an undesired odor, appearance, taste, or texture, or combinations of these defects. Microbial spoilage, especially by yeast, can occur at any stage throughout the production chain from the raw material, harvest, during handling and processing, to the final packaged product. The consequences of a spoilage outbreak can be impacting [1–3] and include:

- Economic loss due to wasted product or product with inferior quality and value.
- Losses with product disposal.
- Costly litigation and forensic investigation to determine cause, liability, and compensation, in the event of a massive outbreak.
- Adverse impact on company reputation and brand image.
- Effect on human health, causing pathologies.



**Fig. 1** Colony morphology of the main yeast genera associated with spoilage under different growth conditions

Although bacteria and molds (filamentous fungi) are the most frequently perceived organisms of spoilage, yeast damage is also significant. Generally, food containing high acid concentration (low pH), high sugar or salt contents, products preserved with weak acids, and long-term frozen products are prone to spoilage by yeasts. While a great diversity of yeast species can be isolated from foods and beverages, only a few yeast genera are frequently associated with spoilage outbreaks (Fig. 1). Food and beverage spoilage by yeasts is well documented in the scientific literature, and it is addressed from ancient times to recent times [1–8].

Spoilage is a result of yeast growth in the product, and a vast array of metabolites are formed. In this way, the characteristics of the food, such as chemical, physical, and sensory parameters, are changed. Carbon dioxide (gas) production by yeasts causes the

containers of packaged products to swell and eventually explode as a consequence of the internal pressures increase, which can be as high as 5 atm. As yeasts grow on the surfaces of solid products, they become visually evident as individual colonies, or as a film of dull, dry, or slimy biomass. In liquid products, yeast growth may occur as a film of biomass that floats on the surface of the product or as sediments of cells within the product. These visual signs of spoilage are usually accompanied by the development of off-odors and off-flavors in the product [2, 9]. These defects become noticeable when yeast growth reaches approximately  $10^5$  cells or colony-forming units (CFU) per gram or milliliter and are evident at  $10^7$  CFU/g or mL.

The ubiquitous occurrence of yeasts in the environment ensures that most foods become contaminated with yeasts during different stages in the production chain. The main classes of food that suffer spoilage by yeast are as follows [2]:

*Meat products.* Freshly slaughtered beef, lamb, pork, poultry, and seafood meats harbor low populations ( $10^1$  to  $10^3$  CFU/g) of yeasts that generally represent about 5–10% of the total microbiota. Yeasts grow during the storage of meats at 5 °C.

*Dairy products.* Yeasts are found in raw and pasteurized milk at  $10^1$  to  $10^3$  CFU/mL. However, their growth is limited under refrigeration, as psychotropic bacteria quickly overgrow them. Nevertheless, milk is an adequate substrate for their growth, and in the absence of bacterial competition, they reach populations of  $10^8$  to  $10^9$  CFU/mL. Fermented dairy products are prone to spoilage by yeasts due to their higher acidity, which restricts bacterial competition. Yogurts undergo gaseous, fermentative spoilage, usually from yeast contaminants that originate from raw materials, such as fruits, and from ineffective cleaning and sanitation of processing equipment.

*Vegetable products.* Vegetables are more prone to spoilage by bacteria than by yeasts. It is due to the proteinaceous nature and neutral pH of vegetable tissues. However, some reports about yeast species causing problems in tomatoes have been described. Generally, yeast spoilage is more frequently related to fermented vegetables, such as the pink discoloration of sauerkraut.

*Fruit products.* Yeasts are present as part of the surface microbiota of healthy, undamaged fruits at populations of  $10^1$  to  $10^3$  CFU/cm<sup>2</sup>. By damaging the structural integrity of fruits, their sugary and acidic tissues are exposed, which are excellent substrates for fermentative yeast growth, causing eventual product spoilage. Fermentative spoilage yeast may also spoil processed fruit products, such as fruit juices, juice, and pulp concentrates, canned fruits, dried fruits, glazed fruits, ready-to-

eat fruit slices, and fruit salads. Fruit and fruit products are ingredients used in the elaboration of other commodities such as yogurts, jams, syrups, pies, cakes, and several beverages, and they can be a primary source of yeast contamination and spoilage of these products.

*Nonalcoholic beverages.* Carbonated and noncarbonated soft drinks, energy beverages, sports drinks, and various health beverages are typical nonalcoholic beverages. These beverages are characterized by the presence of approximately 10% carbohydrates as well as having ingredients that include fruit juices, flavors, organic acids (e.g., citric, malic, lactic, and acetic acids), vegetable extracts, colorants, antioxidants, vitamins, and carbonation. Their sugary, acid (pH 3.0–3.5) and relatively oxygen-free conditions make them highly susceptible to fermentative yeast spoilage.

*Alcoholic beverages.* These products present another specialized habitat for yeast spoilage. Ethanol tolerance and the ability to grow at low pH values are the fundamental properties that enable yeasts to grow and spoil these products selectively. Bottled beers that have been appropriately processed by filtration and/or heat pasteurization rarely undergo spoilage. However, wines are more likely to spoilage.

*Bakery products.* Although yeasts are primarily responsible for the fermentative production of bakery products (e.g., the baker's yeast *Saccharomyces cerevisiae*), they can also cause spoilage of these products. During storage, bread can develop alcoholic, fruity, and acetone-like off-flavors, and some may show visible yeast growth as white, chalky, or other colored spots.

*Products with high contents of sugar or salt.* Foods containing high concentrations (40–70%) of sugar such as sugar cane, sugar syrups, molasses, honey, malt extract, fruit juice concentrates, jams, jellies, confectionery products, and dried fruits are prone to yeast spoilage.

Prevention of yeast spoilage requires good manufacturing practices, including the hygienic practices, the application of the principles of Hazard Analysis Critical Control Point (HACCP) analysis, or other quality management programs. Prevention and minimization of contamination are, therefore, essential requirements in the management of yeast spoilage. Raw materials and ingredients used in food and beverage processing should be free of yeast contamination through the adoption of appropriate specifications. Effective cleaning and sanitation of equipment and process lines to eliminate yeast contamination is another essential requirement. Routine monitoring of end products to ensure that they conform to appropriate specifications is usually part of an overall strategy to manage yeast spoilage [1, 2, 4–6].

Generally, the ecological principles associated with the occurrence and growth of microorganisms in foods, are also applied to yeast spoilage of foods. For a better understanding and management of food and beverage spoilage by yeasts, a systematic process of investigation and knowledge acquisition is required, and this information includes isolation, enumeration, and taxonomic identification of the species and strains responsible for the spoilage [1–8]. These involve the sequential operations, also called as culture methods. Start by rinsing or maceration of samples, followed by dilution and enumeration of yeast cells [4, 10]. Over recent decades, the classical methods for microbiology and physiology studies have been, in part, replaced by sophisticated methods such as polymerase chain reaction (PCR)–based DNA techniques, fluorescent microscopy, and cytometry flow. These methods are known as uncultured methods [5–7].

The most frequent procedure for the enumeration of yeast is viable plate counting. In this method, samples are serially diluted and plated onto a suitable growth medium. The diluted samples may be spread onto the surface of agar plates, or mixed with molten agar, poured into plates, and allowed to solidify. Then, the plates are incubated under proper conditions that permit yeast reproduction so that colonies develop and can be observed without the aid of a microscope. It is assumed that each yeast colony arises from an individual cell that has undergone cell division. By counting the number of colonies and considering the dilution factor, the population of yeasts in the original sample can be determined. Specific yeasts can be tolerant or intolerant of various conditions or chemical compounds. Also, they may be able to use specific carbon or nitrogen sources. These properties can be used to obtain information about the possible identity of an unknown yeast, by observing yeast growth on plates containing various additions and different conditions. The information gained from the differential plating can be associated with information on the yeast morphology observed through microscopy, to reach a tentative identification, or at least to eliminate some possibilities [4]. Although the use of a combination of physiological tests is laborious and time-consuming, it is traditionally employed for yeast identification in food samples. For this reason, a protocol is described in this chapter.

A simple method for yeast enumeration is the direct yeast cell counting. Direct microscopic counts are performed by adding a known volume of the sample over a specific area of a slide, counting representative microscopic fields, and relating the averages back to the appropriate volume-area factors. Counting chambers, such as the Petroff-Hauser and Levy counting chambers, are commonly used to perform the direct counting because they are produced with depressions in which a known volume overlies an area that is ruled into squares. The ability to count a defined area and convert the numbers observed directly to volume makes the direct

enumeration procedure relatively straightforward. Direct counting procedures are rapid and used to assess the sanitation level of a food product [4]. This method may be used to count total yeast in the samples; however, it is not possible to distinguish among different yeast species.

Another method used for the quantification of yeasts is carried out by cultivation in liquid medium and characterization of the produced cell biomass. Cell mass may be determined by weighing whole cells; biomass can be correlated with cell numbers by reference to a standard curve. Wet weight or dry weight of yeast may be used for the estimation of cell numbers. Microbial biomass is estimated by measuring constant biochemical components of microbial cells, such as protein, adenosine triphosphate (ATP), lipopolysaccharides, peptidoglycan, and chlorophyll. Biomass can also be estimated by measured turbidity that can then be correlated with cell numbers by reference to a standard curve [4]. These methodologies are employed as indirect ways of yeasts' quantification in food samples; however, as mentioned for direct microscopic counts, these tools do not allow to distinguish different yeast species in a sample.

The use of molecular DNA-based methods of yeast detection and enumeration has increased in recent years due to their specificity, rapid identification, and independence of the metabolic state of the cell. Individual yeast species may be quickly determined from comparing the nucleotide sequence of domains 1 and 2 (D1/D2) of the LSU rRNA gene or internal transcribed spacer (ITS) obtained by sequencing analysis with those deposited in databases of DNA sequences [11, 12]. Some laboratories perform DNA sequencing or have contracts with companies that perform it for them. However, this is not the case for many laboratories; thus, other molecular methods can be employed for yeast identification. Species-specific primer pairs, random amplified polymorphic DNA (RAPD), repetitive element palindromic (rep-PCR), amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs), pulsed-field gel electrophoresis (PFGE), and others are some examples of alternative methods. The use of species-specific primer pairs is a simple method and may be useful to identify a target and known species as the case of some spoilage yeast species [13–17]. The protocol of this technique is detailed in this chapter. In practice, it consists of a PCR, followed by separation of amplicons by gel electrophoresis and verification of the band presence, which characterizes the target species. However, the inclusion of several species-specific primer pairs in the same PCR may provide an unclear profile of bands.

A technique that has been successfully employed for microbial identification, including yeast species, is the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). This method is simple and rapid and consists of the extraction of abundant structural proteins such as ribosomal proteins

from microbial colonies. The ionizing laser vaporizes these extracted proteins with mass-to-charge ratio ( $m/z$ ) peaks with varying intensities, generating mass spectra. The obtained spectra for each isolate are compared with those available in a reference database for microbial identification. Unknown yeasts can be identified by matching their spectrum to the most similar spectrum in the database. The MALDI-TOF software provides scores that allow us to identify microbial genus and species with accuracy. This method has been used for yeast identification [18–20], and a protocol is proposed in this chapter.

Fast and efficient techniques can be used to detect spoilage yeasts in food by uncultured methods. Fluorescence microscopy procedure for quantitation of viable and nonviable yeasts in beverages and other liquid samples eliminates the need for incubation time, thus reducing the analytical time required. Total yeasts can be counted; however, different yeast species in the same sample are not detected. An advantage of this technique is that living and dead yeast cells can be differentiated [5].

Flow cytometry (FCM) is a technique used to detect and measure the physical and chemical characteristics of a population of microbial cells (bacteria and yeast). In this process, a suspension of cells (bacteria and yeast) is injected into the flow cytometer equipment. The sample is focused to ideally flow one cell at a time through a laser beam, where the light scattered is characteristic to the cells and their components. Microbial cells are often labeled with fluorescent markers, so light is absorbed and then emitted in a band of wavelengths. High number of microbial cells (bacteria and yeast) can be quickly examined, and the data gathered are processed by a computer. Specific fluorescent markers for yeast species may be used, allowing to evaluate specific species [6].

The real-time PCR is well established internationally as a method in brewing microbiology, primarily concerning the detection of beverages spoilage bacteria/yeast [21, 22]. Large wine and brewing companies, the central laboratories of beverages groups, and commercial service laboratories use real-time PCRs for the detection and identification of beverages spoilage bacteria/yeast and, to some extent, also for wild yeast as well as wine/brewing yeast. Real-time PCR provides a rapid and reliable means for identifying and differentiating *Saccharomyces* and non-*Saccharomyces* wine/brewing species. Real-time PCR can be used to identify single unknown yeast strains at the species level. It can also serve as a tool for finding trace contaminations in mixed populations at concentrations of one contaminating cell in 1000 culture yeast cells (e.g., one cell of *Saccharomyces cerevisiae* in 1000 cells of *Saccharomyces pastorianus* ssp. *carlsbergensis*). Identifying the correct species to which a wine/brewing yeast strain belongs can rapidly be carried out [7]. Due to its high applicability for yeast species detection from food samples, a protocol of this technique is described in detail below.

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## 2 Materials

### 2.1 Yeast Culture

For yeasts counting from food samples, different media may be used depending on the investigation interest. Chapter 11 shows some examples of selective and differential media frequently employed for quality analysis in food samples such as MYGP Copper Agar and Lysine medium, both used for wild yeast growth in the brewing industry; DG18 agar (dichloran 18% glycerol agar) used for osmophilic and xerophilic yeasts growth in food samples; WL nutrient agar used for wild yeasts from brewing and fermentation process (medium at pH 5.5 is employed for brewers' yeast while at pH 6.5 for bakers' and distillers' yeasts); and DBDM (*Dekkera-Brettanomyces* differential medium) described by [23] is specially used for counting of the contaminants yeast (*Dekkera* spp. and *Brettanomyces* spp.) in the fermentation process. Prepare and sterilize the culture medium according to the manufacturer's instructions (*see Note 1*).

### 2.2 Identification by Physiological Characterization (See Note 2)

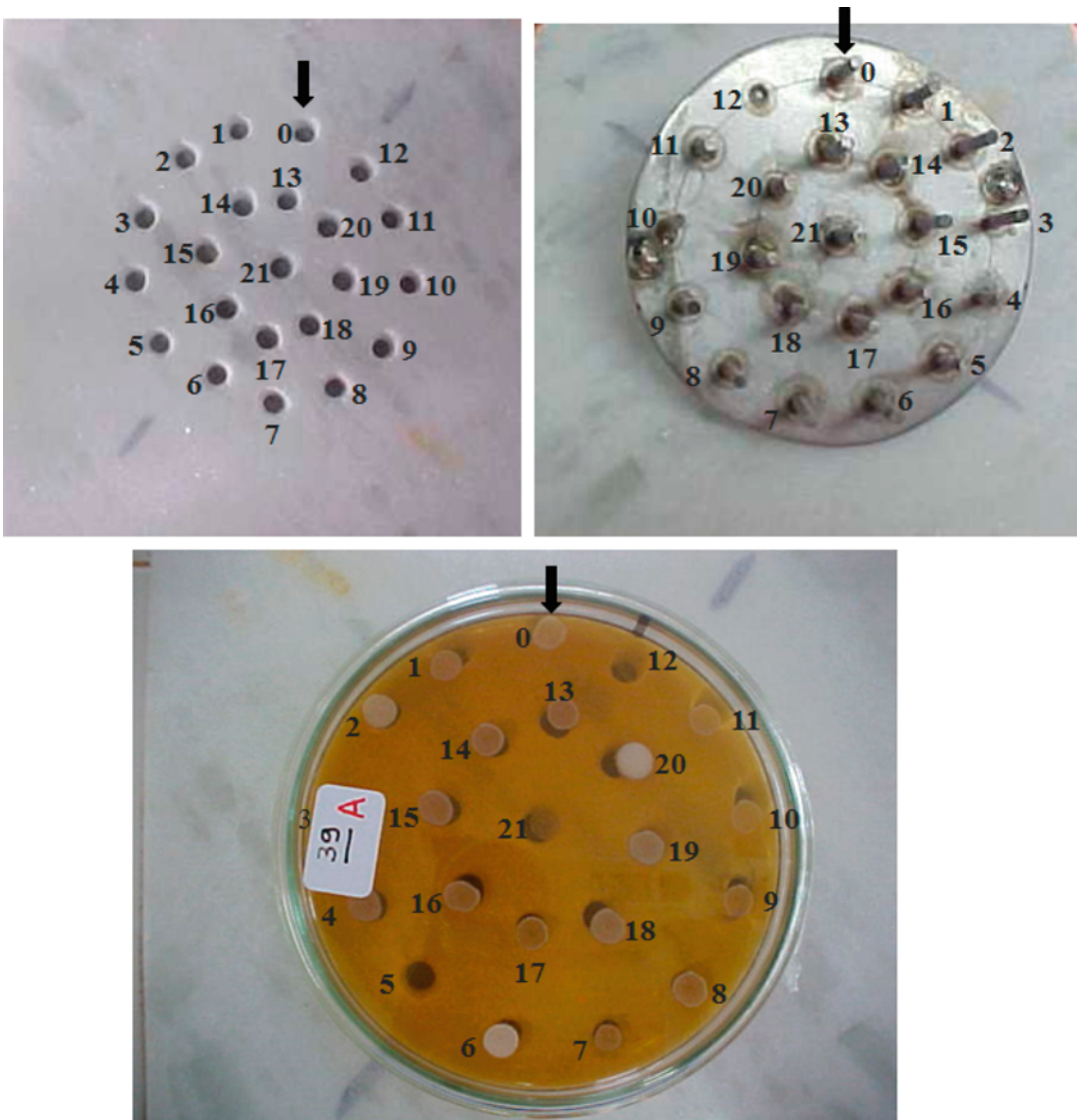
1. Sterilize distilled water for yeast suspension preparation.
2. Prepare a stock solution of each carbon and nitrogen source at 1 M (e.g., maltose, glucose, sucrose, inulin, raffinose, melibiose, galactose, lactose, trehalose, melezitose, methyl- $\alpha$ -D-glucoside, soluble starch, cellobiose, salicin, L-sorbose, L-rhamnose, D-xylose, L-arabinose, D-ribose, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, Myo-inositol, DL-lactate, succinate, citrate, D-gluconate, D-glucosamine, N-acetyl-D-glucosamine, hexadecane, 2-keto-D-gluconate, 5-keto-D-gluconate, saccharate, xylitol, L-arabinitol, arbutin, propane 1,2-diol, butane 2,3-diol, cadaverine, creatinine, L-lysine, ethylamine, nitrate, nitrite) and sterilize (*see Note 3*).
3. Prepare and sterilize plates containing base medium for carbon (YNB) and nitrogen (YCB) source assimilation tests according to the manufacturer's instruction (*see Note 4*). Add carbon and nitrogen sources to the plates at a final concentration of 0.025 M (*see Note 5*).
4. Prepare and sterilize plates containing a basal medium, such as YPD (yeast extract 1.0%, peptone 2.0%, dextrose 2.0%, agar 1.5%) or MEA (malt extract 2.0%; dextrose 2.0%, peptone 0.6%, agar 1.5%).
5. Prepare and sterilize Fermentation Medium (FM): peptone soya 0.75%, yeast extract 0.5%, bromothymol blue 0.04%. Add FM and the carbon sources at a final concentration of 0.025 M (e.g., maltose, glucose, sucrose, inulin, raffinose, melibiose, galactose, lactose, trehalose, melezitose, starch, D-xylose, methyl- $\alpha$ -D-glucoside) into the tubes containing Durham tubes (*see Note 3*).



6. Sterilize the “stamp” by autoclaving at 121 °C for 20 min. This “stamp” allows inoculating 21 yeast isolates at the same time onto the plate, as shown in Fig. 2.

**2.3 Identification by Specific Primer Pairs by PCR**

1. Commercial kits for DNA extraction (*see Note 6*).
2. Commercial kits for PCR.
3. Specific pair primers. Table 1 shows some specific pair primers described for the identification of different yeasts regarding quality interest in food samples (*see Note 7*).



**Fig. 2** “Stamp” used to inoculate multiple yeasts at the same time onto plates for yeast identification by physiological characterization method

4. Thermal cycler, gel electrophoresis equipment, loading buffer, agarose, and DNA marker (molecular weight standard with the bands ranging from 100 bp and longer).
5. Prepare stock buffer solution 50× TAE: 242 g/L Tris base, 57.1 mL glacial acetic acid, 20 mL of 0.5 M EDTA (pH 7.5). The buffer solution for use is 1×, dilute it before use.
6. Ethidium bromide (stock solution 1 mg/mL) or other DNA intercalating dye (SYBR Safe or SYBR Green).
7. Ultraviolet transilluminator system and Polaroid camera.

#### **2.4 Identification by MALDI-TOF**

1. Protein extraction: deionized water, absolute ethanol, formic acid 70%, acetonitrile, and matrix solution  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA, Fluka<sup>®</sup>).
2. Analysis: 96-well MALDI flex plates (Bruker<sup>®</sup> Daltonics), MALDI-TOF microflex LT spectrometer (Bruker Daltonics<sup>®</sup>), and the automatic MALDI Biotyper system.

#### **2.5 Independent Culture Method: Identification and Quantification by qPCR**

1. Commercial kits for extraction of total DNA from food samples.
2. Commercial kits for qPCRs.
3. Specific pair primers. Table 1 shows some specific pair primers described for identification and quantification of different yeasts regarding quality interest in food samples.
4. Real-time PCR thermal cyclers and software system.

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## **3 Methods**

### **3.1 Yeast Culture**

1. For yeast counting, inoculate the prepared samples (as described in Chapter 8) in the specific media (according to investigation interest) and incubate for 48–72 h at 30 °C (*see Note 8*). Representatives of each morphotype (the square root of the total is recommended) may be submitted to the evaluations described below (biochemical or specific primer pairs or MALDI-TOF) to confirm the species. For calculation of yeast population, *see* Chapter 10.

### **3.2 Identification by Physiological Characterization**

1. Transfer each colony to sterilized microtubes containing 1.0 mL of sterile distilled water. Then, incubate the tubes at 30 °C for 24 h for reserves exhaustion. After incubation, standardize yeast inoculum at 620 nm for OD of 1.0.
2. Fermentation assay: Inoculate 150  $\mu$ L of inoculum into the tubes containing MF added to the carbon source. Incubate until 21 days, however, evaluate at 7 days intervals. The media showing yellow color are positive.

**Table 1**  
**Primers used for yeast identification by PCR using specific primer pairs and qPCR**

Microorganism	Primers	Fragment size (pb)	Annealing temperature (°C)	References
Pair specific primer				
<i>Brettanomyces anomalus</i> /B. <i>Bruxellensis</i>	DB90F 5' GAYACTAGAGAGRRGGARGGC 3' DB394R 5' ACGAGGAACGGGCCGCT 3'	305	65	[13]
<i>Debaromyces hansenii</i>	DhPadF 5' GCGACTATGAACAAGGTTTCCAACGA 3' DhPadR 5' CCTTCAATGTAAACATCAGCGGCC 3'	400	67	[15]
<i>Saccharomyces bayanus</i>	LgHOF 5' TGGAAAGTCTACGAGAACAAGCC 3' LgHOR 5' CCTCTATGTAAAGTCCGTATACTG 3'	700	55	[17]
<i>S. cerevisiae</i>	ScHOF 5' GTTAGATCCCAGGCGTAGAACAG 3' ScHOR 5' GCGAGTACTGGACCAAACTCTTAG 3'	400	61	[17]
<i>S. pastorianus</i>	ScHOF 5' GTTAGATCCCAGGCGTAGAACAG 3' ScHOF 5' GCGAGTACTGGACCAAACTCTTAG 3'	300	61	[17]
<i>Trigonopsis cantarellii</i>	CAN-F 5' CCGTCCITGCAATATGTTCCCT 3' CAN-92 5' GGTAGCTGATCACCCGAAAAGC3'	290	67.5	[16]
qPCR				
<i>Brettanomyces bruxellensis</i>	BbF1 5' CACGAGGGTGTGTTTTCTTCAAAG 3' BbR1 5' AAATTACAACCTCGGTTGCCC 3'	243	66	[22]
<i>Candida californica</i>	CcF3 5' CATTAAACAAGCTCGACCTCAGATC 3' CcR3 5' CACTGTGCTCCGCAGAGACTC 3'	187	64.5	[22]
<i>Candida zeylanoides</i>	CZ-5f 5' CGATGAGATGCCCAATTCCA 3' CZ-3bw 5' GAAAGGAACGCAAAATACCAA 3'	191	58	[21]
<i>Curvibasidium pallidicorallinum</i>	CpR1f 5' GCCGAGTTTTGTAAAGTAGAAGCT 3' CpR3 5' TCCAAACAGACTTGTATGCGGTC 3'	243	67	[22]
<i>Debkeria bruxellensis</i>	DBRUXF 5' GGATGGGTGCACCTGGTTTACAC 3' DBRUXR 5' GAAGGGCCACAITCACGAACCCCG 3'	79	69	[24]

(continued)

**Table 1**  
(continued)

Microorganism	Primers	Fragment size (pb)	Annealing temperature (°C)	References
<i>Hanseniaspora uvarum</i>	HU-5fw 5' GGCGAGGATACCTTTCTCTG 3' HU-3bw 5' GAGGCGAGTGCATGCAA 3'	172	59	[21]
<i>Metschnikowia</i> species	Mr8F 5' TTCCTCACCCCTCGTAAGACTACC 3' Mr8R 5' CGGACCCAAATCTCTCAAAT 3'	155	66	[22]
<i>Metschnikowia pulcherrima</i>	MP-5fw 5' CAACGCCCTCATCCAG 3' MP-3bw 5' AGTGTCTGCTTGAAGCC 3'	253	60	[21]
<i>Meyerozyma guilliermondii/caribbica</i>	MeF3 5' GAGATCAGACTCGATATTTGTGAG 3' MeR5 5' GTCTAGGCAGGCAGCATCAAC 3'	156	64	[22]
<i>Pichia angusta</i>	PANG-5fw 5' GTGTCCATTTCCGTGTAAGA 3' PANG-3bw 5' AGCCCCACCCACAAG 3'	175	56	[21]
<i>Pichia anomala</i>	PA-5fw 5' ACGTCATAGAGGGTGAGAA 3' PA-3bw 5' AAACACCAAGTCTGATCTAATG 3'	197	57	[21]
<i>Pichia kluyveri</i>	PK-5fw 5' AGTCTCGGGTTAGACGT 3' PK-3bw 5' GCITTTTCATCTTTCCITTCACA 3'	169	55	[21]
<i>Rhodotorula mucilaginosa</i>	RM-5fw 5' GCGCTTTGTGATACATTTTC 3' RM-3bw 5' CCATTATCCATCCCGGAAAA 3'	169	54	[21]
<i>Saccharomyces cerevisiae</i>	SC-5fw 5' AGGAGTGGGTCTTTTCTAAAG 3' SC-3bw 5' TGAATGCGAGATTCCCCCA 3'	215	59	[21]
<i>S. cerevisiae/bayanus</i>	SeF1 5' GCGTCTAGGCGAACAAATGTTCTTA 3' SeR1 5' CCCCAAAAGTTGCCCTCTCCA 3'	199	67	[22]
<i>Torulaspota delbrueckii</i>	TD-5fw 5' GTGGCGAGGATCCAG 3' TD-3bw 5' CTATCGGTCTCTCGCAA 3'	186	58	[21]
<i>Williopsis saturnus</i>	WS-5fw 5' GGGTGTCCAGTGCITTTG 3' WS-3bw 5' CCCAAGAAGGGAAGATAATCAC 3'	199	56	[21]

3. Assimilation assay: Add 300  $\mu\text{L}$  of each yeast into the “stamp” wells (Fig. 2) and stamp onto the plates containing the different carbon and nitrogen sources. Incubate at 30 °C for 48–72 h. Apparent growth is considered positive (*see Note 9*).
4. Growth temperature assay: Inoculate the yeasts (using the “stamp” according to that described in **step 3 for Assimilation assay**) onto basal media (YPD or MEA) and incubate at different temperatures (e.g., 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C).
5. For yeast species identification, compare the obtained results with those described by [25, 26]. An online tool may be used by simply adding the data to the online form available in CBS Database (<http://www.wi.knaw.nl/Collections/BiolomicsID.aspx?IdentScenario=Yeast2011ID>).

### 3.3 Identification by Specific Primer Pairs by PCR (See Note 10)

1. Extract DNA from each yeast isolate by using a commercial kit according to manufactures’ instruction or using the heating method (*see Note 6*).
2. Prepare the PCRs using a PCR kit according to manufacturer protocol. Use specific pair primers (Table 1) depending on investigation interest.
3. Using a thermal cycler, perform the amplification according to conditions described in Table 1.
4. Using agarose gels 0.8–1.5% (*see Note 11*), the amplification products are separated by electrophoresis apparatus using buffer TAE 1 $\times$ . Then, the gel is stained using a DNA intercalating dye (e.g., Ethidium Bromide, SYBR Safe, SYBR Green). DNA fragments are visualized by UV transillumination system, and images are captured and stored using a polaroid camera and specific software. Use a molecular weight standard to compare the amplicons fragments (*see Note 12*).

### 3.4 Identification by MALDI-TOF (See Note 13)

1. Add portions of yeast colonies into microtubes containing 300  $\mu\text{L}$  of deionized water, vortex for 30 s and then add 900  $\mu\text{L}$  of absolute ethanol. Mix again for 30 s and centrifuge for 2 min at 10,000  $\times g$ .
2. Remove the supernatant and add 50  $\mu\text{L}$  of formic acid 70%, and 50  $\mu\text{L}$  of acetonitrile to the pellet. Vortex for 30 s and centrifuge for 2 min at 10,000  $\times g$ .
3. Remove the supernatant. The obtained precipitate contains yeast proteins to be evaluated by MALDI-TOF.
4. Add the obtained precipitate to the 96-well MALDI flex plates (Bruker Daltonics<sup>®</sup>) containing 1  $\mu\text{L}$  of matrix solution  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA), wait for complete

evaporation of the liquid, and then insert the plates into the equipment for analysis (*see Note 14*).

5. Before the analyses, perform an external calibration of MALDI-TOF MS using a standard bacterial protein test (provided by Bruker Daltonics®).
6. Perform the analysis in triplicate, using the MALDI-TOF microflex LT spectrometer (Bruker Daltonics®). Use the automatic MALDI Biotyper system for yeast identification (*see Note 15*).

### **3.5 Independent Culture Method: Identification and Quantification by qPCR**

1. Extract DNA from food samples by using a commercial kit according to manufactures' instruction (*see Note 16*).
2. Prepare the PCRs using a PCR kit according to manufacture protocol. Use specific pair primers (Table 1) depending on investigation interest.
3. Using a thermocycler, perform the amplification according to conditions described in Table 1 (*see Note 17*).
4. Standard curves: For identification and quantification of yeast specie by qPCR method, a standard curve from samples containing a known number of yeast cells needs be performed. Thus, identified yeast species (the subject of investigation) are cultivated in YPD or MEA at 30 °C for 24 h. Then, the cells are counted using a Neubauer chamber. The DNA from the known yeast populations is extracted using commercial kits and serially diluted (1:10), e.g., from 10<sup>8</sup> to 10<sup>7</sup> down to 10 cell/mL. Each point on the calibration curve is measured in triplicate (*see Note 18*).
5. For results analysis, some parameters need be considered: percentage of efficiency,  $R^2$  for standard curves, and slope (*see Note 19*). The data are analyzed by the software provided by the qPCR equipment by comparing the quantification obtained from food samples and standard curve samples.

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## **4 Notes**

1. Most of the media employed for yeast cultivation are available in commercial form.
2. There are commercial kits available in the market for yeast identification. Otherwise, it is possible to perform batch tests in the laboratory. In this chapter, several tests are described; however, there are additional tests that can also be performed for more reliable identification as described by [25, 26].

3. Use all sources of carbon and nitrogen that are available in the laboratory. A higher number of tests will provide more reliable results. The carbon and nitrogen solutions are filter-sterilized.
4. YNB (Yeast Nitrogen Base) is used for carbon source evaluation, and YCB (Yeast Carbon Base) is used for nitrogen source evaluation. Both are available in commercial form.
5. Carbon and nitrogen sources are added after medium sterilization when pouring into the plate at a temperature around 45 °C.
6. There are several commercial kits available for DNA extraction from yeast isolates. Perform the DNA extraction according to manufactures' instruction. Otherwise, the genomic DNA from several yeast species (e.g., *Saccharomyces* spp., *Pichia* spp., *Candida* spp., and others) may be extracted by heating at 95 °C in ultra-pure water solution. Check quantity and quality using, for instance, NanoDrop (Thermo Scientific<sup>®</sup>) and Bioanalyzer 2100 (Agilent<sup>®</sup>) or similar. The DNA may be stored at -20 °C for further use.
7. Table 1 shows the same examples of specific pair primers for conventional PCR and qPCR described in the literature. However, it is essential to evaluate and validate it before the test. Novel primers may be designed according to investigation interest.
8. The temperature of incubation may vary according to the yeast and food sample analyzed.
9. Mark the yeast "zero" on the plates, as shown in Fig. 2. Furthermore, note the yeast order. Use a basal medium plate, such as YPD or MEA, as a positive control.
10. Species-specific primer pairs tool for yeast species identification is valid when used for known species is the subject of the search. Otherwise, for unknown species, the sequence analysis of rDNA regions, such as the 18S, the D1/D2 domains of the 26S, and the internal transcribed spacer (ITS1-5.8S rRNA-ITS2 region) may be indicated.
11. The concentration of agarose in the gel can vary according to the expected size of the PCR product. For a lower molecular weight of PCR products, use a higher concentration of agarose.
12. It is crucial to perform the PCR using positive and negative controls, which means to use DNA from known yeasts.
13. The MALDI-TOF protocol is based on Bruker<sup>®</sup> equipment. For other equipment, it is essential to follow the manufacturer's instructions.

14. Other matrix solutions and extraction methods may be used for microbial identification by MALDI-TOF, as described by [27].
15. The organism databases are the key components for the microbial identification of commercial MALDI platforms. The manufacturers have continually updated them with the discovery of new microbial species and annotations. For microbial identification, follow the standard identification scores provided by the Bruker Biotyper<sup>®</sup> software: ID score < 1.7 indicates not reliable identification “not reliable ID”; ID score  $\geq 1.7$  and < 2.0 indicate a reliable identification and probable genus-level ID “Genus level ID”; and ID score  $\geq 2.0$  indicates a reliable identification and secure genus level “Genus level ID and probable Species-level.”
16. There are several commercial kits available for DNA extraction from different samples. Perform the DNA extraction according to manufactures’ instruction or with some adaptations depending on the food matrix. Check quantity and quality using, for instance, NanoDrop (Thermo Scientific<sup>®</sup>) and Bioanalyzer 2100 (Agilent<sup>®</sup>) or similar. The isolated DNA may be stored at  $-20\text{ }^{\circ}\text{C}$  for further use.
17. Following the cycling temperatures, it is increased by  $1\text{ }^{\circ}\text{C}$  every 5 s from  $50\text{ }^{\circ}\text{C}$  to  $99\text{ }^{\circ}\text{C}$  to obtain the melting curve. Perform all analyses in triplicate. For an excellent resolution, the DNA concentration in the samples must be limited to 50 ng per analysis, except for standard curves.
18. It is essential to repeat the standard curve every run with the food samples for accurate comparison. In the case of many food samples to be analyzed, the standard curve may be performed in a separate run. However, add at least samples from three points of the standard curve in the run with the DNA from food samples.
19. Consider acceptable values ranging from 85 to 110% (1.85–2.1) for percentage of efficiency; desired  $R^2 \geq 0.99$ , and slope ranging from  $-3.6$  to  $-3.3$ .

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