Isolation, Characterization, and Identification of Yeasts Associated with Foods from Assiut City, Egypt

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**Authors' contributions**

Author SMRK designed the study, wrote the protocol, molecular identify yeasts isolates, revised the manuscript and submit it. Authors AMAH and NFAD wrote the protocol, supervised and revised the manuscript. Author OMA carried out the experiments and write the first draft of the manuscript. All authors read and approved the final manuscript.

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**ABSTRACT**

The objective of this work was to isolate, characterize physiologically, and identify yeasts associated with foods from Assiut city, Egypt. Fifty-two colonies of yeasts associated with orange, mandarin, tomato, squash, sobia drink, mango juice, sugarcane juice, yogurt and buttermilk samples, collected from Assiut City, Egypt, were isolated. Out of which, Eleven isolates were selected randomly and subjected to morphological, biochemical studies and molecular identification techniques employing sequence of internal transcribed spacer (ITS) regions and partial D1/D2 large-subunit domains of the 26S ribosomal RNA. Identified yeasts were belonged to six genera and species; four species belonged to ascomycetes: Debaryomyces hansenii (five isolates), Saccharomyces cerevisiae (one isolate), Candida tropicalis (one isolate), and Pichia kudriavzevii
Candida environments for the growth of yeasts, where apiculate yeasts: (dairy products are especially favorable growing, fermentative, low assimilative profiles of vitamins promote occurrence of yeasts [8,9]. The for several yeast species [2]. Colonization of fast highest amount of nitrogenous compounds and [1]. Decaying fruits are an important microhabitat supporting the growth of yeasts, where the addition to, an association with soil and insects [4,5]. Basidiomycetous genera such as Cryptococcus and Sporobolomyces yeasts were dominated on leaves of mango trees [6]. In addition, ascomycetous yeasts associated the wounded parts of the phylloplane [7].

Fruit juices and soft drinks constitute suitable environment for growth of most microorganisms. Actually, beverages are excellent substrates for supporting the growth of yeasts, where the highest amount of nitrogenous compounds and vitamins promote occurrence of yeasts [8,9]. The dairy products are especially favorable environments for the growth of yeasts, where Candida (C. sphaerica) Debaryomyces, Mycoderma, Rhodotorula, and Saccharomyces (S. dairenis and S. unisporus) are the most common isolated genera in these environments [10]. Other yeasts species like Issatchenkia orientalis, C. albicans, Clavispora lusitaniae (Candida lusitaniae), Kodamaea ohmeri (Pichia ohmeri), Kluyveromyces marxianus, and C. catenulate were associated with traditional Egyptian dairy products and karish cheese were found to be the most diverse in its yeast florase [11]. Similarly, the most prevalent isolates in Egyptian karish cheese were belonged to Trichosporon cutaneum (25%), C. catenulata (23%), Yarrowia lipolytica (13%), Debaryomyces hansenii (13%), Kluyveromyces lactis (6%), Geotrichum candidum (7%), C. zeylanoides (5%), C. lambica (3%), C. albicans (2%), Cryptococcus formans (1%), Rhodotorula glabrata (1%) and S. cerevisiae (1%) [12]. Generally, yeasts are important microflora of many food products due to their ability to grow on a substrate rich by proteins, lipids, sugars, and organic acids [13].

Molecular identification techniques employing sequencing the internal transcribed spacer (ITS) regions and partial D1/D2 large-subunit domains of the 26S ribosomal RNA (rRNA) have become the most frequently and convenient method for identification of yeasts isolates. This study aimed to isolate yeasts associated with milk products, fruits, and juices from Assiut city, Egypt. The yeasts were identified morphologically, physiologically as well as by molecular techniques.

2. MATERIALS AND METHODS

2.1 Samples and Isolation

Three samples from each food source; orange, mandarin, tomato, squash, sobia drink, mango juice, sugarcane juice, yogurt, and buttermilk were collected from the local markets in Assiut city, Egypt. The isolation was achieved according to Lodder and Kreger [14], and Kurtzman et al. [15], where the samples were settled at room temperature (25~30°C) for 48 hours (h), then used for isolation. Liquid samples were direct spread and streaked on yeast peptone dextrose agar (YPD), as well of a 1/10 w/v of solid samples/ sterile water were homogenized and used for direct plating techniques during this study. A medium composed of 10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of dextrose, 20 g/L of agar and added ampicillin (100 mg/L), and chloramphenicol (100 mg/L) to prevent bacterial contamination, and then the plates were incubated at 28±2°C for 10 days. Purification of yeast colonies were achieved by streaked methods. The cell/colony morphologies of the purified yeasts isolates were investigated and monitored under microscope. Yeast cultures were maintained on 2% YPD slants at 4°C for short period storage. In addition, equal volumes of propagated yeast cultures and 80% glycerol were mixed well and stored at 80°C for long time preservation.
2.2 Identification of Yeast Strains

Selected yeast isolates were identified based on their morphological, biochemical properties according to Kurtzman et al. [15]; Walt and Yarrow [16] While, molecular techniques according to Nisiotou et al. [17].

2.2.1 Morphological characteristics

The strains were first checked for their morphological aspects and growth patterns on solid and liquid media. Characteristic features of textures (mucoid, fluid or viscous, butyrous); elevation (flat or raised); color (yellow, orange and red); surface (glistening or dull, smooth, rough, and sectored) and margin (entire, undulating, lobed, and filaments) were investigated. The cells of a young actively growing culture from 2–3 days at 25°C were stained by lacto phenol-cotton blue and investigated microscopically to determine the shape of cells, budding (Mono, Bi, and multipolar) or fission and conidial formation. Moreover, the formation of compact, coherent, flocculent, or mucoid sediment, a ring, floating islets or a pellicle of yeasts was examined. Additionally, formation of germ tubes was checked using 24 h cultivated yeasts and incubated at 37°C for up to 28 days. The fermentation abilities were inspected at frequent intervals for accumulated gas in the Durham tubes, as well as changes in indicator (phenol red) color were investigated according to Kurtzman et al. [15].

Moreover, other physiological characteristics were performed to examine the osmotic pressure of yeast isolates such as growth abilities on 50, 60% w/v glucose, and 10% NaCl plus 5% glucose. In addition to, the ability of growth at 37°C, urea hydrolysis, starch formation, tolerance to 1% acetic acid. Furthermore, acid production from glucose of Custer’s chalk medium at 25°C after incubation of two weeks was carried out according to the methods of Kurtzman and Fell [18].

2.2.2 Physiological and biochemical characteristics

The ability of eleven yeasts isolates to utilize and grow aerobically on carbon and nitrogen as a sole source of energy was studied. Several carbon sources (D-glucose, D-galactose, lactose, maltose, sucrose, raffinose, soluble starch, D-ribose, L-rhamnose, D-xyllose, glycerol, D-mannitol, ethanol, and methanol) used in this study. While the nitrogen sources used were nitrate, nitrite, and L-lysine. Assimilation tests were achieved by replica plate method, where a set of plates contained different carbon or nitrogen source in a carbon or nitrogen basal agar medium were inoculated by yeasts isolates. The growth of colonies of negative control plates (without carbon or nitrogen sources) was compared with plates supplemented with carbon or nitrogen sources after 24–48 h of incubation [15].

On the other hand, fermentation abilities of yeast isolate to ferment 2% sugar solutions of (glucose, lactose, maltose, raffinose, galactose, sucrose, and xylose) and 4% of raffinose was tested. Pure yeast isolates were cultivated on YPD liquid medium for 48 h and then the cell pellets were collected by centrifugation. Fermentation was started by addition of sugar solutions to cell pellets and resuspended prior to insert Durham tubes (inverted without any air in aseptic condition) to collect any gas produced during fermentation. Thereafter, the tubes were incubated at 25°C for up to 28 days. The fermentation abilities were inspected at frequent intervals for accumulated gas in the Durham tubes, as well as changes in indicator (phenol red) color were investigated according to Kurtzman et al. [15].

Moreover, other physiological characteristics were performed to examine the osmotic pressure of yeast isolates such as growth abilities on 50, 60% w/v glucose, and 10% NaCl plus 5% glucose. In addition to, the ability of growth at 37°C, urea hydrolysis, starch formation, tolerance to 1% acetic acid. Furthermore, acid production from glucose of Custer’s chalk medium at 25°C after incubation of two weeks was carried out according to the methods of Kurtzman and Fell [18].

2.2.3 Molecular identification

Purified yeast isolates were identified based on sequences of rRNA gene of large subunit (LSU) according to Nisiotou et al. [17] as the following: Genomic DNA was extracted from overnight grown yeast cells in broth YPD medium at 28°C and 150 rpm. Yeasts cells pellets were collected by centrifugation and resuspended in a lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA) with glass beads and mixture of phenol, chloroform, isoamyl alcohol (25:24:1). Thereafter, vigorously vortex the mixture to lysis yeast cell walls and DNA precipitated by chilled absolute alcohol as described previously [19].

PCR thermal cycler started amplification of rDNA in 50 µL reaction mixture, containing 5 µL 10X KOD buffer, 5 µL dNTP mixtures, 3 µL MgSO₄, 15 pmol of each primer, 1 µL KOD polymerase and 100 ng of genomic DNA. PCR conditioned to denaturation at 94°C for 10 s, annealing at 50°C for 30 s, and extension at 68°C for 1.5 min /kb. Amplification of the ITS1, 5.8S-ITS2, and D1/D2 domains rDNA region was achieved by forward primer ITS1 (5'-TCCGTAGGTGAAAGCTGCGG-3') and reverse primer NL-4 (5'-
GGTCCGTGTTCGAAGACGG-3'). PCR product was purified, dyed and sequenced as described previously [20]. Sequencing data were submitted to NCBI/BLAST (blast.ncbi.nlm.nih.gov/BLAST.cgi) for sequence alignment, to construct a phylogenetic tree, and to assign an accession numbers for the selected yeasts isolates.

3. RESULTS

3.1 Isolation Incidences and Sources of Isolates

Fifty-two yeast colonies were isolated from twenty-seven samples (nine sources) on YPD agar medium. A total of 52 isolates were obtained: yogurt (N=6), orange (N=3), tomato (N=7), squash (N=2), mandarin (N=2), mango juice (N=11), buttermilk (N=10), and Egyptian sobia drink (N=4). The isolates subjected to further streaking until completely purified then eleven isolates were selected for further studies of characterizations and identification.

3.2 Morphological Characterization

The morphological characters of selected yeast isolates were summarized in Table 1.

3.3 Biochemical Characterization

3.3.1 Assimilation tests

All selected yeast isolates assimilated glucose and maltose and were unable to assimilate methanol as a sole carbon source. Only isolate Y.5 well-assimilated citrate and xylose as reflected in the results shown in Table 2. Isolates Y.5 and Y.16 negatively and poorly assimilated sucrose, respectively, while the other isolates showed a well assimilation. Lactose efficiently assimilated by Y.7-10, 13 and 16 whereas the rest was not. For starch, only Y.5 strongly assimilates it from among the eleven-tested yeasts isolates while, Y.7-10 and 13 were weakly dissociation abilities (Table 2).

3.3.2 Fermentation tests

Seven sugars (glucose, lactose, maltose, raffinose, galactose, sucrose, D-xylose) were used during study fermentation abilities of yeasts isolates. Isolate Y.2 was able to ferment all sugars except xylose and lactose, while Y. 5 and 6 fermented only glucose. On the other hand, Y.14, 15 and 16 were unable to ferment any sugar. Yeasts isolates Y.7-10 and 13 were efficient to ferment glucose and less for galactose, while unable to ferment the rest of sugars (Table 3). The results of additional physiological characteristics were summarized in Table 4.

3.4 Molecular Identification

Eleven yeasts isolates were identified, based on molecular identification techniques using amplified fragments of ITS and D1/D2 domains of large subunit, which belonged to six genus: D. hansenii (five isolates), S. cerevisiae (one isolate), C. tropicalis (one isolate), Pichia kudriavzevii (one isolate), R. mucilaginosa (two isolates), and Trichosporon dulcitum (one isolate). In the phylogenetic tree, all strains

| Isolate no. | Colony on solid medium | Cells shape | Growth in broth media |
|-------------|------------------------|-------------|-----------------------|
| Y.2         | Smooth surface, raised, cream-color | Globose, ovoidal, multilateral budding | White precipitate |
| Y.5         | White, smooth, butyrous, and soft | Subglobose to ovoidal- single cells, and in pairs. | Rings |
| Y.6         | Butyrous and light cream colored with small lobes | Ovoidal to elongate | Pellicles |
| Y.7-10 and 13 | Smooth surface, raised, entire margin, white color | Spheroidal, short chains, multilateral budding | Pellicles |
| Y.14 and 15 | Smooth surface, flat, entire margin, pink- to deep red color | Ovoidal, multilateral budding | Pink ring precipitate |
| Y.16        | Folded, rough surface, flat, with volcano-shaped, fringed with filaments, yellowish, pale cream-colored | Hyphae disarticulating into arthroconidia | Pellicles and rings |
clustered with their nearest phylogenetic neighbors strains (Fig. 1). Their accession numbers were coordinated with identified names and listed in Table 5 and Fig. 1.

4. DISCUSSION

Isolation of yeasts from natural resources is the most successful technique to obtain yeast isolates that have abilities to utilize and ferment various exogenous compounds [21]. Isolation of yeast species associated with different food samples collected from Assiut city, Egypt, was investigated. The species *S. cerevisiae*, *C. tropicalis*, *P. kudriavzevii*, *D. hansenii*, *R. mucilaginosa*, *T. dulcitum* were isolated. The results showed that all these species were found in lower frequency. This could be due to that normal yeast flora on fresh, undamaged fruits are generally low, presence of preservatives for juices, and presence of lactic acid bacteria for milk products. *D. hansenii* was associated only with fruit samples and mango juice, whereas *R. mucilaginosa* was isolated only from mango juice.

Table 2. Assimilation of carbon sources using selected yeasts isolates

| Isolate no. | Glucose | Galactose | L-rhamnose | D-ribose | D-xylene | Maltose | Lactose | Sucrose | Raffinose | Starch | Citrate | D-mannitol | Ethanol | Glycerol | Methanol |
|-------------|---------|-----------|------------|----------|----------|---------|---------|---------|-----------|--------|---------|------------|---------|----------|----------|
| Y.2         | +       | s         | -          | -        | -        | +       | -       | +       | w         | -      | -       | w          | -       | -        | -        |
| Y.5         | +       | +         | -          | -        | +        | -       | -       | +       | +         | +      | w       | w          | -       | -        | -        |
| Y.6         | +       | w         | -          | -        | -        | -       | -       | -       | +         | -      | -       | +          | -       | -        | -        |
| Y.7         | +       | s         | w          | w        | +        | +       | +       | +       | w         | w      | s       | w          | +       | -        | -        |
| Y.8         | +       | s         | w          | w        | s        | +       | +       | w       | w         | w      | w       | w          | w       | -        | -        |
| Y.9         | +       | +         | s          | w        | w        | s       | +       | w       | w         | w      | w       | w          | w       | -        | -        |
| Y.10        | +       | s         | w          | w        | s        | +       | w       | w       | w         | w      | w       | w          | w       | -        | -        |
| Y.13        | +       | s         | w          | w        | s        | +       | w       | w       | w         | w      | w       | w          | w       | -        | -        |
| Y.14        | +       | w         | -          | +        | w        | w       | -       | +       | w         | w      | -       | w          | w       | -        | -        |
| Y.15        | +       | w         | -          | +        | w        | +       | -       | s       | -         | -      | w       | w          | w       | -        | -        |
| Y.16        | +       | -         | -          | s        | w        | +       | +       | w       | +         | +      | +       | +          | +       | +        | +        |

Symbols: (+) positive; (-) Negative; (s) Strong positive; (w) Weak positive. Data obtained from three independent experiments

Table 3. Fermentation abilities of selected yeasts isolates

| Isolate no. | Glucose | Galactose | D-xylene | Maltose | Lactose | Sucrose | Raffinose |
|-------------|---------|-----------|----------|---------|---------|---------|-----------|
| Y.2         | +       | +         | -        | w       | -       | +       | +         |
| Y.5         | +       | -         | -        | -       | -       | -       | -         |
| Y.6         | +       | -         | -        | -       | -       | -       | -         |
| Y.7         | W       | w         | -        | -       | -       | -       | -         |
| Y.8         | +       | w         | -        | -       | -       | -       | -         |
| Y.9         | +       | w         | -        | -       | -       | -       | -         |
| Y.10        | +       | w         | -        | -       | -       | -       | -         |
| Y.13        | +       | w         | -        | -       | -       | -       | -         |
| Y.14        | -       | -         | -        | -       | -       | -       | -         |
| Y.15        | -       | -         | -        | -       | -       | -       | -         |
| Y.16        | -       | -         | -        | -       | -       | -       | -         |

Symbols: (+) positive; (-) Negative; (s) Strong positive; (w) Weak positive. Data obtained from three independent experiments
## Table 4. Additional biochemical tests using isolated yeasts

| Strain no. | L-lysine | Nitrite | Nitrate | Growth at 30°C | Growth at 37°C | Starch formation | Glucose 50% | Glucose 60% | NaCl 10%+ glucose 5% | Acid production | Hydrolysis of urea | Acetic acid 1% | Starch hydrolyses |
|------------|----------|---------|---------|---------------|---------------|-----------------|-------------|-------------|------------------------|----------------|-------------------|---------------|------------------|
| Y.2        | -        | -       | -       | +             | +             | -               | w           | +           | w                      | -              | -                 | -             | -                |
| Y.5        | -        | +       | +       | +             | -             | -               | -           | +           | s                      | -              | W                 | -             | -                |
| Y.6        | s        | -       | -       | +             | s             | -               | +           | w           | s                      | w              | -                 | -             | -                |
| Y.7        | +        | -       | -       | +             | -             | s               | s           | w           | w                      | -              | -                 | -             | -                |
| Y.8        | +        | -       | -       | +             | s             | s               | s           | w           | w                      | +              | -                 | -             | -                |
| Y.9        | +        | -       | -       | +             | s             | s               | w           | w           | w                      | -              | -                 | -             | -                |
| Y.10       | w        | -       | -       | +             | -             | s               | s           | w           | w                      | s              | -                 | -             | -                |
| Y.13       | +        | -       | -       | +             | -             | s               | s           | w           | w                      | +              | -                 | -             | -                |
| Y.14       | -        | -       | -       | +             | +             | w               | -           | -           | s                      | -              | -                 | -             | -                |
| Y.15       | w        | -       | -       | +             | +             | w               | -           | -           | s                      | -              | -                 | -             | -                |
| Y.16       | s        | -       | -       | -             | -             | +               | +           | +           | +                      | s              | -                 | -             | -                |

Symbols: (+) positive; (-) Negative; (s) Strong positive; (w) Weak positive. Data obtained from three independent experiments.

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**Fig. 1.** Phylogenetic tree relationships of rRNA sequences genes of identified yeasts.
Table 5. Identification of yeast strains based on molecular bases and their accession numbers

| Strain no. | Source            | Accession number | Strain name                      |
|-----------|-------------------|------------------|----------------------------------|
| Y.2       | Sugarcane juice   | KM504287         | Saccharomyces cerevisiae         |
| Y.5       | Egyptian Sobia drink | KR264907          | Candida tropicalis               |
| Y.6       | Yogurt            | KR264908         | Pichia kudriavzevi               |
| Y.7       | Orange            | KM504284         | Debaryomyces hansenii            |
| Y.8       | Mandarin          | KR264906         | Debaryomyces hansenii            |
| Y.9       | Tomato            | KR264905         | Debaryomyces hansenii            |
| Y.10      | Squash            | KR264904         | Debaryomyces hansenii            |
| Y.13      | Mango juice       | KR264903         | Debaryomyces hansenii            |
| Y.14      | Mango juice       | KR264902         | Rhodotorula mucilaginosa         |
| Y.15      | Mango juice       | KR264901         | Rhodotorula mucilaginosa         |
| Y.16      | Buttermilk        | KM504286         | Trichosporon dulcitum            |

The complex identification yeasts by a conventional methodology of biochemical that requires 60~90 tests for correct species identification [22]. Nonetheless, these methodology are necessary to detect any special physiological character. Furthermore, due to high accuracy of identification by molecular techniques, it was used in parallel with biochemical tests for the identification of yeast species. The obtained results from morphological characteristics of the isolated colonies coupled with the biochemical tests were highly significant in yeast identification and correlated with molecular technique identification.

_D. hansenii_ showed the most frequented strain where five isolates of _D. hansenii_ isolated from different sources (fruit samples and mango juice). It has an ability to assimilate all tested sources of carbon except methanol, in addition to, ferment glucose and galactose. Galactose is a ‘non-conventional’ nutrient for yeasts, which however can be used as a sole carbon source when glucose is absent from the medium. Thus, the ability of the yeast cells to assimilate galactose indicated the expression of the GAL genes [23]. As a consequence of that, _D. hansenii_ may has GAL genes. It has caused spoilage of fruit, juice, marzipan, and canned figs [24,25]. It has been isolated from a variety of fruit [24], soft drinks [26], raw tomatoes [27], sugarcane [28] and been reported in high numbers in yoghurt [29]. It proved to be genetically and biochemically interesting yeasts with considerable biotechnological promise [30].

_P. kudriavzevi_ was associated only with yogurt during our study. It initially classified as a strain of _Issatchenka orientalis_, but Kreger-van Rij [35] noted that this strain (CBS 5459) differed from the others in being sorbose-positive and citrate-negative. An isolated strain has an ability to assimilate glucose, galactose, maltose, sucrose, ethanol and glycerol, in addition to, ferment only glucose. _P. kudriavzevi_ gained much attention recently in the biotechnology. Recombinant _P. kudriavzevi_ VTT C-79090T had been able to produce up to 171 g L\(^{-1}\) of D-xylonate from 171 g L\(^{-1}\) D-xylose at low pH [36]. Yuangsaard et al. [37] reported that newly isolated thermotolerant _Pichia kudriavzevi_ had been produced 7.86% (w/v) ethanol from cassava starch hydrolysate, which adjusted to 18% glucose at 40°C within 24 h. Moreover, the ability of _P. kudriavzevi_ to utilize glycerol makes it an excellent source for biodiesel production. Recently, Thiru et al. [38] have carried out a detailed investigation on the production of biodiesel from crude glycerol using _Cryptococcus curvatus_. _S. cerevisiae_ was isolated from sugarcane juice, _C. tropicalis_ from Egyptian sobia drink, and _T. dulcitum_ from buttermilk. Mendoza et al. [39] stated that _Rhodotorula, Pichia, Candida, and Saccharomyces_ were
frequently isolated from pasteurized fruit juices while; *Candida* and *Saccharomyces* spp. have often been reported as spoilage-causing organisms in citrus juices [40, 41].

5. CONCLUSION

This study reported the occurrence of yeast species associated with dairy products, fruits, and juices collected from Assiut City, Egypt. Eleven yeast isolates belonged to six genera were successfully identified. Four species belonged to ascomycetes: *D. hansenii* (five isolates), *S. cerevisiae* (one isolate), *C. tropicalis* (one isolate), and *P. kudriavzevii* (one isolate). In addition, two yeasts species belonged to basidiomycetes: *R. mucilaginosa* (two isolates), and *T. dulcitum* (one isolate). The biochemical identification was highly consistent with molecular identification techniques using amplified fragments of ITS and D1/D2 domains of large subunit. Dairy products, fruits, juices were commonly associated by different yeasts and it can be used for isolation of different genera and species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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