Morphological and phylogenetic identification of *Pichia* species associated with foods in Basrah, Iraq

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Abstract

Eight yeasts isolates belong to six species of *Pichia* were isolated from different food sources of fresh fruits, fruit juices, honey and molasses. Six species of isolates were first obtained in Iraq: *Pichia kluyveri*, *Pichia fermentans*, *Pichia kudriavzevii*, *Pichia manshurica*, *Pichia membranaefaciens* and *Pichia sp*. Which were identified by morphological and molecular methods. The later was done depending on DNA sequencing of ITS1 and ITS2 regions of yeast rDNA.

KEYWORDS: *Pichia*, Strains, Food, Molecular, New record, Iraq.

1. Introduction

Yeasts are characterized as single celled that reproduce asexually by budding and less common fission. This helps yeasts to increase their numbers rapidly in liquid environments which are suitable for the reproduction of single celled microorganisms. Many yeasts grow easily under anaerobic conditions, which are also suitable for growth in the liquid media. On the other hand, the proliferation of individual cells determines their diffusion or penetration of solid surfaces, unlike the fungus [1].

There has been a close association between yeasts and foods since the beginnings of human civilization, the first humans discovered fermentation of food and beverages added nutritional value to those substances and perhaps made them better preserved, consequently, fermented food has contributed to human life during the historical ages [2].

Pichia yeast is associated with food and feed production and has the potential to grow in preserved food due to their low pH, high osmotic pressure and low oxygen content. Some species such as *P. anomala* has the ability to produce lethal toxins that act as antimicrobial agents. In addition, it can utilizes a wide range of sources of nitrogen and phosphorus, making it a potential factor to reduce environmental pollution caused by organic wastes from agriculture. On the other hand, this genus can cause certain diseases, especially for people with immune deficiency [3].

Many of yeasts found in juices were isolated by Boboye *et al*., [4]. *Pichia* species were isolated from fresh fruits such as grapes [5, 6] and citrus [7]. Corbaci *et al*., [8] conducted a study to isolate yeasts from dairy products: *Pichia anomala* and *Pichia guilliermondii*, was isolated from honey [9].

The aim of this study was to isolate, characterize and identify *Pichia* species, which have not been recorded before from food sources in Basrah/ Iraq.

2. Materials and methods

2-1: Sample collection

Fifty samples were collected from different food sources of honey, molasses and fresh fruits from local markets in Basrah city, Iraq. Samples were transferred aseptically to the laboratory.

2-1-1: Fresh Fruits:

The isolation method from fresh fruits was described by Kurtzman *et al*., [10], that was achieved by weight 10 g of each type of fruit and placed in blender jar. Then 90 ml of 0.1% peptone water was added and mixed for 45 seconds to homogenize. A series dilutions were prepared for each suspension, then the YPGA plates were inoculated from each sample and incubated at 25°C for 2-7 days. After the emergence of growth on media, the various colonies were selected for secondary subculture to obtain pure yeast isolates on MEA and stored at 4°C for subsequent transactions.

2-1-2: Fruits juices:

To isolate yeasts from fruit juices, we applied the procedure described by Kurtzman *et al*., [10] by spreading 0.1 ml of each sample of juices directly on to YPGA medium, and then streaked by the Loop. The plates were incubated at 25^{o}C for 2-7 days.

2-1-3: Honey and molasses:

Ten grams of each sample of honey and molasses were weighed separately under aseptic conditions and added to 90 ml of 0.1% Peptone water. This represents the primary dilution 10^{-1}. From this dilution, series of dilutions were done up to 10^{-3}.0.1 ml, of each dilution of honey or molasses samples was transferred to YPGA plates and cultured by spreading method, then were incubated at 25^{o}c for 2-7 days [11].

2-2 Yeast strains identification

Isolated yeast strains were identified depending on their morphological characteristics according to [2, 1, and 10], thereafter, they were diagnosed by molecular techniques as mentioned by Nisiotou *et al*., [12].

2-2-1: Morphological characteristics

Morphological features of selected yeast strains were examined based on growth manner on solid media. Moreover, specific texture features were recorded; color elevation; colony surface, in addition to margins.

2-2-2: Microscopically identification

Yeast strains which were grown for 2-3 days at 25^{o}C, were further examined microscopically to determine yeast cell shape, fission or budding (Mono, Bi, or multipolar) and examined for conidial formation by staining with lactophenol cotton blue dye.

2-2-3: Molecular diagnosis

Pure yeast isolates were diagnosed by molecular techniques depending on large subunit (LSU) of rRNA gene sequences [13]. First, genomic DNA of each isolate was extracted according to [14] as following steps:

Single colony of freshly grown yeast strain was dropped in an eppendorf tube contains 200µl of DNA extraction buffer (Triton X- 100, SDS, NaCl , Tris – HCl and EDTA) and 200 µl of Phenol: Chloroform: Isoamyl alcohol mixture (25: 24: 1, v: v), cells were lysed by adding glass beads and vigorously vortexed for 5 min. Thereafter, cells lysate was removed and centrifuged for 5 min, 4^{o}C at 10000 rpm. The upper layer (representing DNA) was re-mixed by vortex with pre-cooled 1 ml of absolute ethanol and precipitated by centrifugation as mentioned previously. Nucleic acids pellet were re-suspended in a mixture of 400 µl (1x) TE buffer (Tris – HCl, EDTA) and 5 µl of RNase and incubated for 30 min at 37^{o}C for RNA removal, then 10µl of (4M) NH4OAc and 1 ml of pre-cooled ethanol were added and the mixture was vortexed for few seconds. Finally, pure DNA pellets were obtained after centrifugation as mentioned previously, and re-suspended by 50µl of (1x) TE buffer. Samples were stored at - 20 ^{o}C until use.

Genomic DNA samples were examined by gel electrophoresis for 30 min using 0.8% agarose gel and 60 voltage. For yeast strains identification, large subunit (LSU) of rDNA (ITS1-ITS2 regions) was amplified according to [13] by polymerase chain reaction test (PCR), using the primers set mentioned in table (1).

Table (1) Sequences of nitrogen bases of primers used in the ITS1 and ITS2 amplification process [13]

Primer	Primer Sequences (5`-3`)	length	Tm	Ta
ITS1	F-5'-TCC GTA GGT GAA CCT GCG G-3''	19 base	62 ^{o}C	56 ^{o}C
ITS4	R-5'-TCC TCC GCT TAT TGA TAT GC-3'	20 base	58 ^{o}C	53 ^{o}C

Tm: Melting temperature

Ta: Annealing temperature

PCR was done by using Sprint Thermal cycler and the Master Mix kit (Bioneer, Korea) following the manufacturer instructions. Thereafter, PCR products were investigated by gel electrophoresis on 0.8% agarose gel, at 60 v. for 1 hr.

Table (2) Polymerase Chain Reaction Program

Stage	Steps	Temperature	Time	No. of cycles
I	Denaturation 1	95 °C	1 min	1
II	Denaturation 2	95 °C	1 min	35
	Annealing	55 °C	0.45 min	
	Extension	72 °C	1 min	
III	Final extension	72 °C	10 min	1

PCR products of amplified ITS1-ITS2 regions were sent to Macrogen Company (Korea) for DNA sequencing.

3. Results and discussion:

A one week old yeast colonies, that were expected to be *Pichia* species isolates, were selected for subsequent morphological and molecular identification.

3-1 Morphological characteristics:

Morphological features of the selected yeast colonies, which were recorded in the primary isolation are summarized in Table (3).

Table (3) Morphological features of the selected yeast isolates.

Isolate no.	Species	Colony on solid media	Cell shape
R1, R32	*Pichia sp.*	White-colored with a rough surface	Spherical
R6, R20	*Pichia kluyveri*	White to dark yellow, dull, with entire edges.	Ovoid to elongate
R12	*Pichia fermentans*	White to Cream-colored curly colony and the lobed edges.	Ovoid to ellipsoidal
R19	*Pichia kudriavzevii*	Creamy colored, butyrous	Ovoid to elongate
R34	*Pichia manshurica*	White to brown	Ovoid to cylindrical
R45	*Pichia membranaefaciens*	White, convex, irregular edges.	Ovoid to elongate

3-2 Molecular diagnosis:

Eight yeast isolates belong to six species were diagnosed by DNA sequencing of ITS1-ITS2 regions related to rDNA gene. Phylogenetic tree was drawn for the mentioned isolates as shown in figure 1 using MEGA 6 program. Six different types of *Pichia* species were isolated for the first time in Iraq.

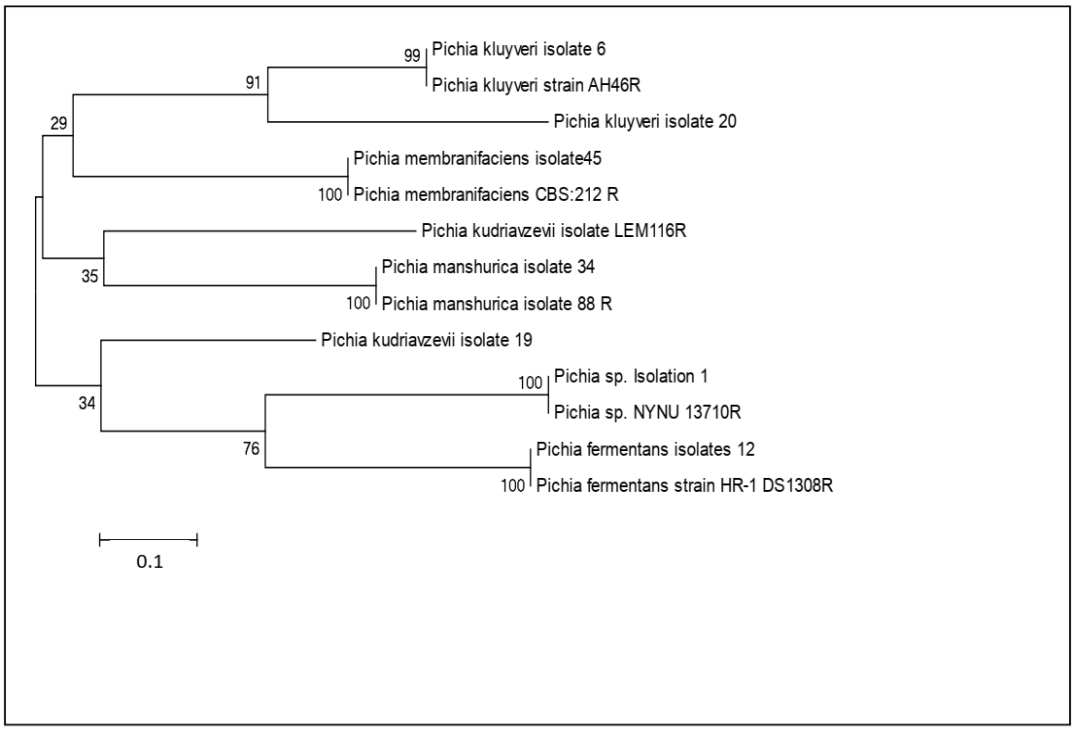

Figure (1) Rooted Neighbor Joining phylogenetic tree of the isolated *Pichia* sp. It was constructed from concatenated sequences of 316 bp for each strain derived from an alignment of 18S ITS gene sequences, then produced from analysis conducted by MEGA 6 program. This N-J tree showing the distribution and phylogenetic relationship between 7 different strains, which were isolated from different food sources and 6 Reference strains (R). All vertical branches lengths were drawn to scale Boot strap values after 1000 repetitions are indicated.

3-3 Isolates description:

1. ***Pichia fermentans*** **Lodder Figure (2)**

Anamorph: *Candida lambica* (Linder & Genoud) van Uden&H.R.Buckley

Colonies on MEA were 1-3 mm diameter in 3 days at 25°C, White to cream-colored, dull, surface smooth to wrinkled, margins irregular lobed. Yeast cells ovoidal to ellipsoidal, 5-7 X2 - 4 µm, occur singly, in pairs or in short chains. Asci 2-4 spared, ascospores hat-shaped, the ledges are not easily detected under the light microscope.

Material examined: R12 (living culture), isolated from peaches, Basrah, Iraq, 31/11/2017. A pure culture was deposited at Mycology Laboratory, Department of Biology, Collage of Science, University of Basrah.

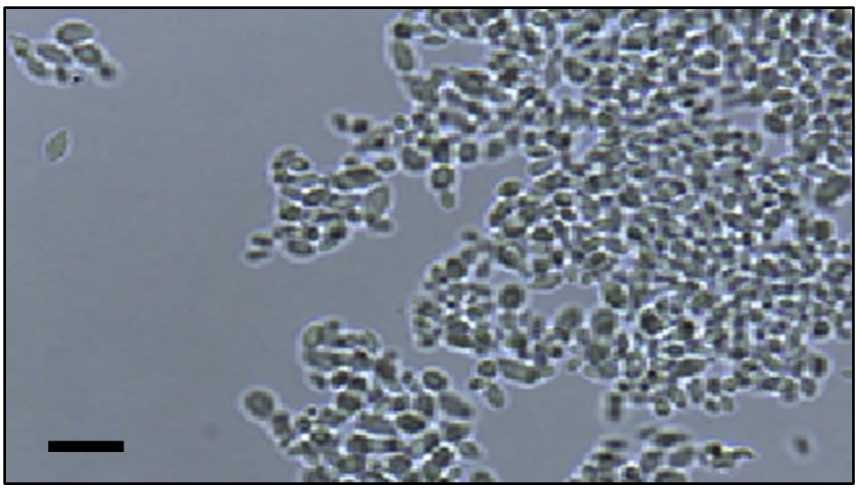

Figure (2) Vegetative cells of *Pichia fermentans*, scale bar=10µm

This species is first time recorded in Iraq.

Matching ratio was 100%

This yeast was isolated from cheese, butter, orange juice, as well as isolated from some clinical specimens such as sputum [10].

2. *Pichia kluyveri* Bedford ex Kudryavtsev Figure (3)

Anamorph: *Candida eremophila* Paff, Starmer&Tredick- Kline

Colonies on MEA were 48 mm. diameter in 3 days at 25°C, white to dark yellow, dull, with entire to finely margins. Yeast cells are ovoid to elongate, 3-7X2-4 µm, occur in pairs or in short chains. Asci are evanescent, 2-4spored, hat- shaped.

Material examined: Two strains were obtained, R6 (living culture) isolated from melon, Basrah, Iraq, 20/11/2017; R 20 (living culture) isolated from apples, 5/1/2017. Pure cultures were deposited at Mycology laboratory, Biology Department, College of Science, Basrah University.

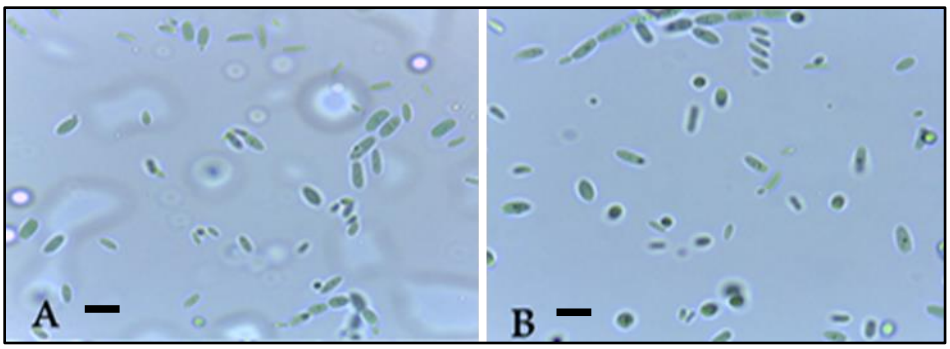

Figure (3) A. Vegetative cells. B. Asci and Ascospores, bar = 5µm.

This species is common to cause fruits rots [15]. Also, it has been isolated from olive, fermented cacao, potato, and spinal figs [10]

> R20_ITS1

***Pichia kluyveri* partial 18S rRNA gene, strain AH46 99% 380 bp**

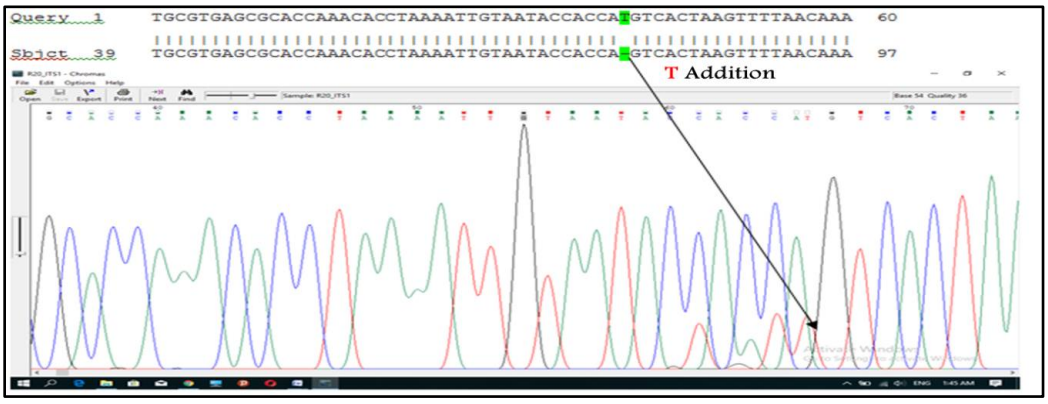

Figure (4): Location and type of mutation in *Pichia kluyveri*

3. ***Pichia kudriavzevii* Boidin, Pignal&Besson Figure (5)**

Anamorph: *Candida krusei* (Castellani) Berkhout

Colonies on MEA were 1-3 mm diameter in 3 days at 25°C, cream-colored, butyrous. Yeast cells 4-7 x 2-4 µm, ovoid to elongate, occur singly or in pairs. Ascospores were not observed for this strain.

Material examined: R19 (living culture), isolated from pears, 19/11/2017, Basrah, Iraq. Pure culture was deposited at Mycology Laboratory, Biology Department, College of Science, University of Basrah.

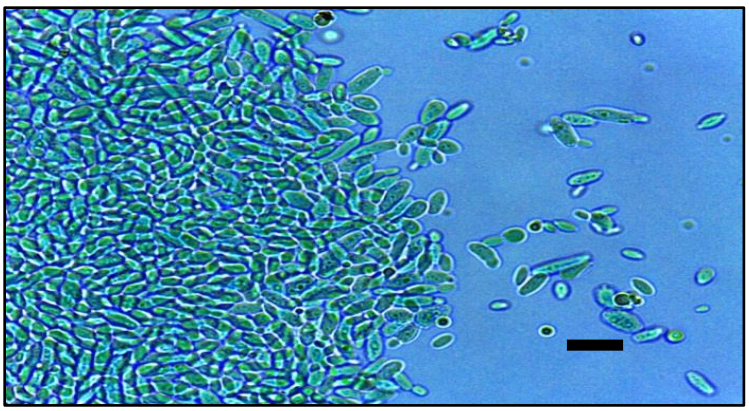

Figure (5) Vegetative cells of *Pichia kudriavzevii,* bar=10µm

This species is first time recorded in Iraq.

This species was transferred to *Pichia* sp. and nominated as *P. orientalis* [16], but others [17], proposed the new name *Pichia kudriavzevii*. Recently, it was emerged through the analysis of multiple genes (Multi-gene analysis), that Issatchenkia represents the members of the *P. membranaefaciens* clade, which resulted in species being returned to the *Pichia* species [18].

The description of this type is similar to that of [10], as well as the absence of ascospores on MEA 2% and 5% MEA.

This type isolated from berries and fruit juice in Russia and from Sake syrup in Japan and chicken eggs in the USA [18].

Pichia kudriavzevii strain E20662_ITS small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed 99% 445 bp

Figure (6): Location and type of mutation in *Pichia kudriavzevii*

4. ***Pichia manshurica*** **Saito Figure (7)**

Colonies on MEA were 1-3 mm diameter in 3 days at 25°C, white to brown colored, margins irregular. Yeast cells 3-6 X2-3 µm, ovoid to cylindrical, Occur singly, in pairs or in small clusters. Asci are early deliquescing at maturity, contain 1-4 hat-shaped ascospores.

Material examined: R34 (living culture), isolated from molasses 22/2/2018, Basrah, Iraq. Pure culture was deposited at Mycology Laboratory, Department of Biology, Collage of Science, University of Basrah.

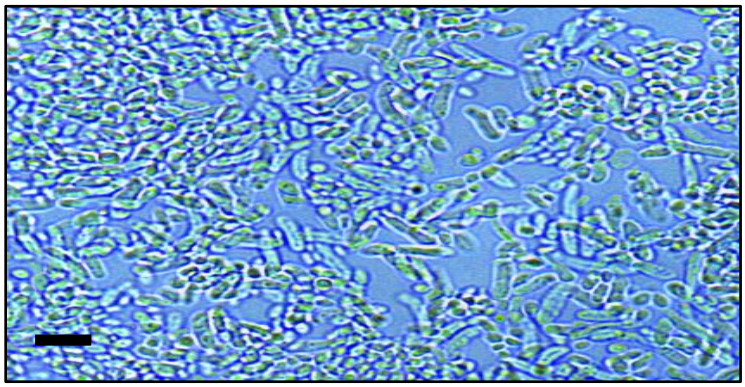

Figure (7) Vegetative cells of *Pichia manshurica*, bar=10µm

This species is first recorded in Iraq.

Pichia manshurica yeast is common in natural fermentations, including rotting plant materials, but its role is uncertain in food fermentation, food and beverage spoilage. This yeast was isolated from feces and its ability to grow at 37 ^{o}C was examined, so it is likely to cause human infection [10].

This isolate is considered as a new strain with 98% identical percentage to that one found in NCBI gene bank, since there are many mutations occurred: the substitution of cytosine with thymine (C-T), two thymine replacements of adenine (T-A) and a deletion of a cytosine nitrogen base (C).

>R34_ITS1

Pichia manshurica strain 88 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

98% 318

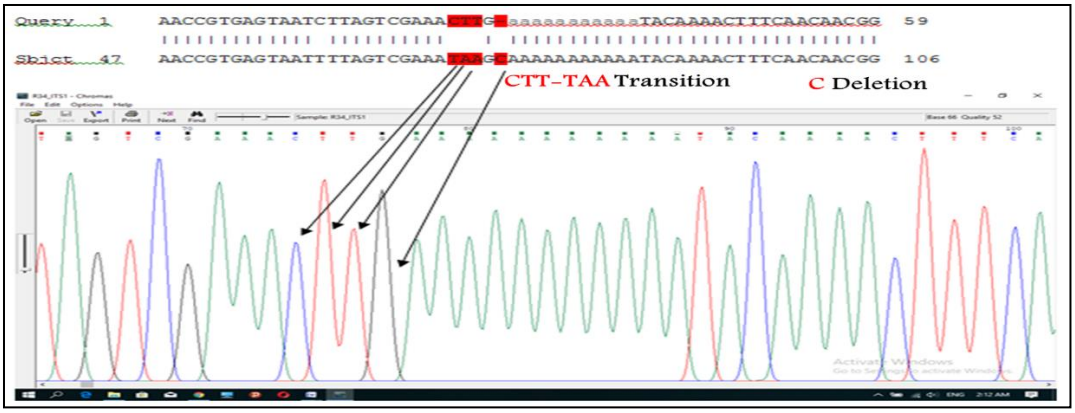

Figure (8): Location and type of mutations in *Pichia manshurica*

5. *Pichia membranaefaciens* E.C.Hanse Figure (9)

Anamorph: *Candida valida* (Leberle)Uden& H.R. Bukley

Colonies on MEA were 2-3 mm diameter in 3 days at 25 °C, off- white, convex, irregular margins. Yeast cells are ovoid to elongate, 4 -7 X 2-4 µm, occur singly, in pairs, in chains or in clusters. Asci formed from single cells, 4-spored tiny, hat –shaped ascospores, formed on Malt Acetic Acid (MAA) after one week at 25 °C.

Malarial examined: R45 (1iving culture), isolated from fresh fruit (Kiwi) 6/11/2017, Basrah, Iraq. Pure culture was deposited at Mycology Laboratory, Biology Department, College of Science, University of Basrah.

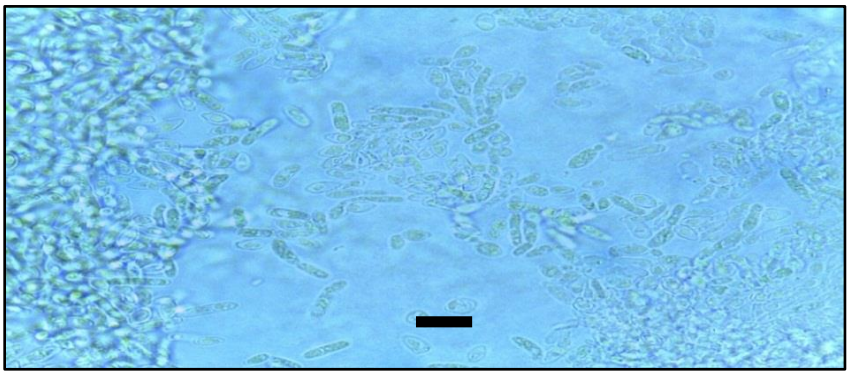

Figure (9) Vegetative cells of *Pichia membranaefaciens*, bar=10µm

This species is recorded for the first time in Iraq.

This species is often associated with olive brines [19], but it is also found in brine shrimp [20]. This species has also been isolated from various foods, fruit juices and beverages [1].

Pichia membranaefaciens is a promising member in biocontrol against *Monilia fructicola* on cherries [21], *Botrytis cinerea* on grapes [22], and against post-harvest pathogens of apples [23]. This effect appears to be due to the ability of this yeast to produce killer toxin [22] and extracellular enzymes such as B-1,3-glucanase and chitinase [24].

6. ***Pichia sp.*** **Figure** (10)

Colonies on MEA were 3mm-diameter in 3 days at 25 °C, white-colored, surface appears rough. Yeast cells are spherical, 1.92 to 3.84 µm.

Material examined: Two strains were reported. R1 (living culture), isolated from orange juice, 1/12/2017, Basrah, Iraq; R32 (living culture), isolated from carrot jam, 27/12/2017 Basrah, Iraq. Pure cultures were deposited at Mycology Laboratory, Biology Department, Collage of Science, University of Basrah.

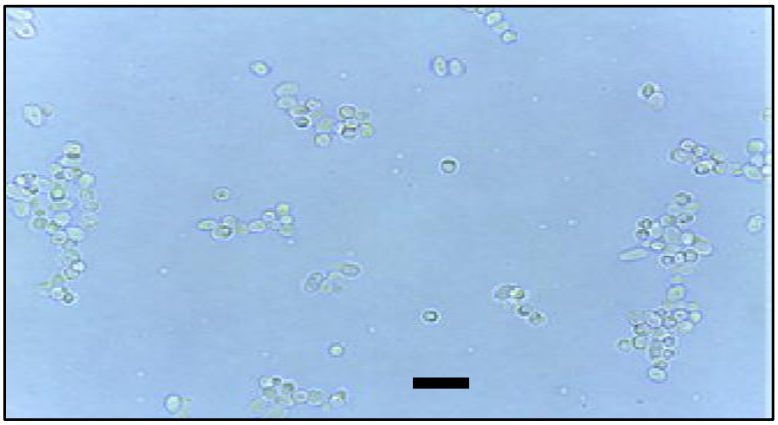

Figure (10) Vegetative cells of *Pichia sp.*, bar=10µm

The species was not diagnosed, although it coincided with the NYNU13710 strain 100%.
Diagnosis of this species may require the use of special primers as D1 / D2 to determine the nucleotides sequences for genus diagnosis, therefore need a broader study.

Conclusion:

Based on the molecular characterization, an interesting species of the yeast *Pichia* were added to Iraqi mycobiota, as well as strains are considered to be new in GenBank.

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التشخيص المظهري والجيني لانواع الجنس Pichia المصاحبة للاغذية في البصرة/العراق

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المستخلص

من مختلف المصادر الغذائية،والتي شملت الثمار Pichia تم في هذا البحث عزل ثمانية سلالات تعود لستة انواع تابعة للجنس الطازجة وعصائر الثمار والعسل والدبس.تم الحصول على ستة انواع تسجل لاول مرة في العراق،شخصت

Pichia kluyveri, Pichia fermentans, Pichia kudriavzevii, Pichia manshurica, Pichia membranaefaciens and *Pichia sp*.

بالطرق المظهرية والجزيئية.