Identification of different species of *Rhodotorula* using Internal Transcribed Spacers

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Abstract

The identification and classification of yeasts have traditionally been based on morphological, physiological and molecular identification. In the present study, six yeast isolates were isolated from potatoes and maize all the isolates able to produce carotenoid. *Rhodotorula spp.* was identified by physiological and molecular genetic tools. Physiological identification were carried out by assimilation of carbon sources, assimilation of nitrogen sources and antibiotic resistance pattern using disk diffusion method. The fragment size polymorphism (FSP) were used for molecular genetic identification. The Internal Transcribed Spacer (ITS1) and (ITS2) regions of the isolates were amplified separately. The isolates were grouped based on fragment size polymorphism (FSP) of the amplicon. For precise and final identification, the ITS-PCR products were subjected to sequencing followed by Blast analysis. As a result, three isolates were identified as *Rhodotorula mucilaginosa*, and the other three were identified as *R. glutinis*.

Introduction

Rhodotorula and *Rhodosporidium* produce the major carotenoid pigments, carotene, torulene and torularhodin. However, the type, concentration and yield of various carotenoids are depending on the genetic makeup *i.e.* species interacted with environment *i.e.* culture conditions. A natural system of yeast classification should be based on the coordinate use of morphological, physiological, biochemical and molecular information (**Viljoen, 1989).**

Carotenogenic yeasts are a diverse group of unrelated organisms (mostly *Basidiomycota*) and the majority of the known species are distributed in four taxonomic groups subdivided in two classes the first class *Urediniomycetes* (*Sporidiobolales* and *Erythrobasidium*), and the second class *Hymenomycetes* (*Cystofilobasidiales* and *Tremellales*). The genus *Rhodotorula* includes three active species; *Rhodotorula glutinis*, *Rhodotorula minuta* and *Rhodotorula mucilaginosa* (formerly known as *Rhodotorula rubra*).

Up to the present time the carbon sources used in assimilation tests in the major attempts at yeast classification have been limited to glucose, fructose, mannose, galactose, maltose, sucrose,

lactose, and ethyl alcohol. A number of workers have attempted to use pentoses, polysaccharides, organic acids, and polyhydric alcohols, but the results have been quite variable among strains of the same species. There has as yet been no comprehensive testing of large numbers of strains of many species, followed by an analysis of the data to determine which compounds give consistent, taxonomically valuable results in each group of related species **(Lynfer, 1948).**

(Zahra *et al,* 2013) were examined for susceptibility tests against three groups of antifungals, polygenes (Amphotericin B, nystatin), azoles (clotrimazole, miconazole, fluconazole) and allylamine (terbinafine). Resistance to Amphotericin B was found in 5.8% of isolates whereas 52.2% and 42.0% of isolates were dose dependent and sensitive to drug, respectively .Most isolates were sensitive to nystatin (71.0%) and only 11 isolates (16.0%) showed resistance.

Conventional yeast identification based on phenotypic characteristics is often misleading and inconclusive, and usually needs to be emphasized by molecular methods. Currently, one of the methods widely used in molecular taxonomy is the characterization of rDNA regions that have been preserved during evolution. Fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. In each unit there are 3 rRNA genes: a small rRNA gene (18S), the 5.8 S rRNA genes, and a large rRNA gene (28S). Conserved sequences exist in large subunit (LSU) and small subunit (SSU) genes, and these genes have been used in many taxonomic studies. The spacer regions between the subunits are called internal transcribed spacers (ITS), and those between the gene clusters are called intergenic spacers (IGS). These spacer regions are considerably more variable than the subunit sequences and have been widely used in studies on the relationships among species within a single genus or among intraspecific populations **(Melike, 2008**).

Molecular phylogenetic analysis based on internal transcribed spacer region (including 5.8S rDNA) and large-subunit rDNA D1/D2 domain sequences indicated that they represent a novel basidiomycetous yeast species. Multiple sequence alignment is discussed in light of homology assessments in phylogenetic research. Pairwise and multiple alignment methods are reviewed as exact and heuristic procedures. Since the object of alignment is to create the most efficient statement of initial homology, methods that minimize no homology are to be favored. Therefore, among all possible alignments, the one that satisfies the phylogenetic optimality criterion the best should be considered the best alignment. Since all homology statements are subject to testing and explanation. This way, consistency of optimality criteria is desirable. This consistency is based on the treatment of alignment gaps as character information and the consistent use of a cost function (e.g., insertion-deletion, transversion, and transition) through analysis from alignment to phylogeny reconstruction **(Phillips *et al.,* 2000).**

Material and Methods

Samples collection

Ten samples from different plant leaves tomato and maize were collected from various localities of Egypt and used for the isolation of red pigment yeast strains by a inoculating the potato Dextrose Agar (PDA) plate in the laboratory by plating method.

Phenotypic tests

Morphological properties were determined for each group according to **(Kreger, 1984).** The production of pigments and shape of colonies of yeast was examined based on Direct Mounts.

Physiological characterization

Assimilation of carbon sources to characterize physiologically, the yeast isolates was inoculated in medium containing different carbon and/or nitrogen compound as sole carbon or nitrogen source in order to determine the capability to assimilate them depends on the rate of growth.

Assimilation of Carbon sources

Glucose, fructose, lactose, sucrose, starch, maltose, mannitol, xylose, (D) Mannitol, Inositol and glycerol were tested as different carbon sources for growth as well as pigment formation for each of different isolates according to **(Frengova, 2009)** yeast extract used as nitrogen sources.

Assimilation of nitrogen sources

The culture medium having glucose (30 g/L) as carbon source and different nitrogen compounds as nitrogen sources Yeast extract (2.8 g/L), peptone (2.4 g/L), casein acid (4.4 g/L), Urea (0.64 g/L) Sodium nitrate (2 g/L), ammonium nitrate(0.8 g/L) were the different nitrogen sources evaluated (**Iathal**, **2005**)

Antibiotic sensitivity

The different isolates were subjected to antibiotic resistance screening by disc diffusion method. For this purpose, commercially available antibiotic discs (Five antibiotics having different mode of actions such as cell wall synthesis inhibitors, membrane permeability alternatives, protein synthesis inhibitors and DNA synthesis inhibitors) were inoculated on previously prepared lawn of culture on PDA agar plates. The plates were incubated at 37°C for 24 h. The presence or absence of inhibition zone around the antibiotic discs after 48h were recorded (**Mona, 2007**).

Identification by Molecular tests PCR-FSP

The rDNA regions are unique and can be used to identify common yeasts at the species level **(Iwen *et al.,* 2002; Mirhendi *et al*., 2008)**. A preliminary grouping of the isolates was performed on the basis of fragment size polymorphism (FSP) of both the internal transcribed spacer (ITS1) and (ITS2) regions in rDNA using PCR **(Mirhendi *et al*., 2008**).

The forward ITS1 primers (ITS1f) (5'-TCCGTAGGTGAAC CTGCGG-3') and reverse ITS1 primers (ITS1r) (5'-GCTGCGTTCTTCATCGATGC-3') were used to amplify the ITS1 region and the forward ITS2 primers (ITS2f) (5'-GCATCGATGAAGAACGCAGC-3') and the reverse ITS2 primers (ITS2r) (5'-TCCTCCGCTTATTGATATGC-3') primers were used for amplification of ITS2 region, respectively. The amplicon of each region for each individual yeast isolate were mixed and subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. The molecular sizes of the DNA fragments were judged in comparison with molecular standards 1Kb DNA ladder. The identification of the yeasts was based on the electrophoretic pattern for each species.

PCR Conditions

ITS1 region included (ITS1f and ITS1r). The conditions of PCR amplification were: 7 min at 94 ºC, followed by 35 cycles to denaturing,45 s at 94 ºC, 1 min at 56 ºC to annealing, and 1min at 72 ºC with a final extension of about 7 min at 72 ºC **(Mokatar *et al*, 2011).**

ITS2 region included (ITS2f and ITS2r) and total region from ITS1f primer forward and ITS2r primer reverse used conditions of PCR amplification were 95ºC for 120 sec followed by 35 cycles to denaturation at 95ºC for 30 sec, annealing at 55ºC for 30 sec and 72ºC for 60 sec with a final extension of about 10 min at 72ºC.

Purification of PCR Products

Amplified PCR product for 9ITS1-5.8rDNA-ITS2 six *Rhodotorula* isolates were purified using EZ-10 spin column PCR products purification.

ITS-sequencing and phylogenetic analysis

A representative four isolate of red yeasts was selected for sequencing and phylogenetic analysis. A part of the rDNA region ITS1–5.8S rDNA–ITS2 was amplified using the forward (ITS1) and reverse (ITS2) primer pairs.

The sequencing of the product PCR was carried through in an automatic sequencer ABI PRISM 3730XL Analyzer using BigDyeTM Terminator Cycle Sequencing Kits following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using ITS1f-ITS2r primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Computational analysis (BLAST) and construction of a phylogenetic tree.

The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequences were aligned using Align Sequences Nucleotide BLAST; a neighbor-joining phylogenetic tree was constructed using MegAlign software.

RESULTS AND DISCUSSION

Identification of yeast isolates

The classification of yeast isolates (Rh.1*, Rh.2, Rh.3, Rh.4, Rh.5, Rh.6*) was based on morphological, physiological, biochemical test **(Ahmed, 2009)** and molecular genetic (**Mokhtari, 2011).**

1. Morphological Identification

Morphological and cultural characteristics of the yeast isolates were identified by direct mounts which are made in order to study yeast morphology microscopically and to determine the purity of the isolates **(Bowman and Ahearn., 1975; Haley., 1971; Hupert and Harper 1975; Land *et al.,* 1979; Salkin *et al.,* 1987; Kreger., 1984; Larone., 1987; Mcginnis., 1980; Pincus *et al.,* 1988; El zoutari *et al.,* 1990).**

All of isolates are sediment in broth culture, oval in cell shape, smooth in colony texture, orange colony color, no diffusion pigment and Bipolar budding in asexual reproduction. Similar results were also obtained **(Ahmed, 2009)**.

2. Physiological and biochemical

Normally the growth medium for yeast *Rhodotorula* is mainly contains glucose as carbon source and yeast extract as nitrogen sources.

In the present study, in order to test the assimilation of different carbon sources and/or different nitrogen sources, one of the eleven different sources of carbon (Table 1) was used instead of glucose and seven nitrogen type sources (Table 2) were examined. For the carbon source tests yeast extract used as nitrogen source. For the nitrogen source test, glucose was used as carbon sources **(Dufoss, 2006).**

2.1. Assimilation of different carbon sources

Carbon assimilation is an important criterion in the taxonomy and identification of yeast, which depends on organic carbon sources for the energy supply and growth. The carbohydrates are being the sources of greater important. Sugars such as glucose, fructose, lactose, sucrose, starch, maltose, mannitol, xylose, Inositol and glycerol were examined (table 1).

Table (1): Assimilation of different carbon sources by *Rhodotorula ssp.*

Sugar	*Rh.1*	*Rh.2*	*Rh.3*	*Rh.4*	*Rh.5*	*Rh.6*
Glucose	+++	+++	+++	+++	+++	+++
Fructose	++	++	++	++	++	++
Lactose	---	---	---	---	---	---
Sucrose	+++	+++	+++	+++	+++	+++
Starch	++	++	++	++	++	++
Maltose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
xylose	+	+	+	+	+	+
Glycerol	+	+	++	++	++	+
Inositol	---	---	---	---	---	---

+++: High growth ++: moderate growth +: Low growth ---: No growth

In Table (1) growth of all isolates are compered and divided into high growth (glucose and sucrose), moderated growth (Fructose and starch), low growth (maltose and xylose) and no growth (lactose and inositol) this result is according to (**Ahmed, 2009; Letha and Manja, 2004).**

As shown in table (1) the three local isolated yeast *Rh.3, Rh.4* and *Rh.5* were found to be very similar. The yeast growth and pigment formation were high on (Glucose and Sucrose), moderate growth (Fructose, Starch and Glycerol), low growth (Maltose, Mannitol and xylose) and no growth (lactose and inositol).

Rh.1,Rh.2 and *Rh.6* were found to be very similar table (1). The yeast growth and pigment formation were high on (Glucose and Sucrose), moderate growth (Fructose, Mannitol and Starch), low growth (glycerol, Maltose and xylose) and no growth (lactose and inositol).

Based on yeast growth and pigment formation, different responses were obtained. When disaccharides are used as carbon sources, a maximum growth and pigmentation were observed on sucrose while very little of both growth and pigment were noticed when maltose is used . On the other hand, lactose resulted in growth and hence no pigment formation. Xylose supported a high rate of pigmentation and growth. This is due to the ability of yeast under study to metabolize Glucose, Sucrose and Mannitol are considered to get positive assimilation and disables to hydrolyze lactose so

lactose is get negative assimilation. The result obtained are accordance with **Frengova *et al*, 2003.** The result of Inositol is according to (**Golubev, 2010)**

2.2 Assimilation of different nitrogen sources

One of the objectives of these experiments was to study the effect of different nitrogen sources on yeast growth as well as on the carotenoid pigments biosynthesis of yeasts under study. Thus Sodium nitrate, Yeast extract, Peptone, Sodium peroxynitrite, Urea, Ammonium nitrate and casine as nitrogen sources were used as marker identification table, (2).

Table (2): Assimilation of different nitrogen sources by *Rhodotorula ssp.*

Nitrogen Sources	*Rh.1*	*Rh.2*	*Rh.3*	*Rh.4*	*Rh.5*	*Rh.6*
Sodium nitrate	--	--	+	+	+	--
Yeast extract	+++	--	+++	+++	+++	+++
Peptone	+++	++	+++	+++	+++	+++
Sodium peroxynitrite	----	---	++	++	++	----
Urea	++	++	++	++	++	++
Ammonium nitrate	++	---	---	++	++	++
Casine	+	--	+	+	+	+

+++: High growth ++: moderate growth +: Low growth ---: No growth

Table (2) observed that all isolates have moderate growths on urea were observed and no or low growth on sodium nitrate and casine.

Based on assimilation of different nitrogen sources, *Rh.3, Rh.4* and *Rh.5* were found to be similar. High growth and Pigmentation formation were observed on (yeast extract and peptone), moderated growth (Sodium peroxynitrite, ammonium nitrate and Urea), low growth (casine and sodium nitrate

Based on assimilation of different nitrogen sources, *Rh.1*and *Rh.6* were found to be similar. High growth and Pigmentation formation were observed on (yeast extract and peptone), moderated growth (urea), low growth (casine), and *Rh.3* is low growth (sodium nitrate), moderated growth (Sodium peroxynitrite) and no growth (ammonium nitrate) but *Rh.1* and *Rh.6* were observed no growth (sodium nitrate and sodium peroxynitrite) and moderate growth (ammonium nitrate).

Yeast extract and peptone gave the maximum growth of the most isolated yeast under study and poor growth was observed of the yeasts with Sodium peroxynitrite, sodium nitrate and casine. The results obtained are in accordance with (**Ferrao and Garg, 2011)** and in disaccordance (**Latha *et al,* 2005)** who found that sodium nitrate was the best nitrogen source for their *Rhodotorula* strain, while the ammonium nitrate was the poorest nitrogen source.

2.3. Antibiotic resistance patterns

The isolated of *Rhodotorula* spp. are tested for resistant of several types of antibiotics that may could be used as marker identification. Table (3) shows different response of *Rhodotorula* isolates by different five antifungal discs.

Table (3): Antibiotic resistant pattern of yeast isolates (*Rh.1-Rh.6*) measured as inhibition zone diameters (mm) by disc assay.

Antibiotic	**Symbol**	**ug**	***Rh.1***	***Rh.2***	***Rh.3***	***Rh.4***	***Rh.5***	***Rh.6***
Amphotericin B	AMB	100	R	LR	R	R	R	10
Nystatin	NY	100	10	20	18	15	15	20
Kanamycin	K	30	LR	10	R	10	LR	10
Trimethoprim	SxT	25	R	R	R	R	R	R
Gentamicin	CN	10	LR	10	LR	LR	LR	10

R: Resistant (no inhibition zone)

LR: low resistant (< 10 mm)

Table (3) showed the antibiotics resistance patterns. Discs of Amphotericin B (100ug), Nystatin(100ug), Kanamycin(30ug),Trimethoprim/sulphmethoxazo(25 ug), Gentamicin(10ug) were used for antibiotic resistance test. All isolates were found to be resistant to one or more of the antibiotics in each of yeast strain. All this results indicated that, in each of yeast strain there are different gene(s) controlled the resistance of their group of antibiotics.

The result obtained showed that all isolates have resistance and low resistance to Amphotericin B, Trimethorim/sulphmethoxazo, Kanamycin and Gentamicin but isolates *Rh.2, Rh.3, Rh.4, Rh.5* and *Rh.6* are sensitive to nystatin compared *Rh.1* is resistance.

The resistance and low resistance to Amphotericin B studied is disaccorded the result obtained by **(Zaas *et al*, 2003)** They showed that all isolates were most sensitive to amphotericin B.

Rh.1 is a Lowest sensitive to nystatin. This result obtained is in accordance to **Zahra *et al*., 2013** who showed that the most of isolates were sensitive and only isolate *R*. *muciaginosa* was resistant to nystatin

3. Molecular identification (5.8 rDNA gene sequences test)

In order to obtain a precise identification, it is necessary to use molecular techniques based on DNA sequence analysis. The use of ribosomal DNA (rDNA) genes for identification of fungal species based on the detection of conserved sequences in 5.8S rDNA and 28S rDNA enable the amplification of the ITS2 region between these two genes. Based on the similarity in size of the banding patterns of the PCR-FSP products (ITS1 and ITS2) were used to identify the *Rhodotorula* isolates.

Genomic DNA of six isolates of *Rhodotorula Rh.1, Rh.2,Rh.3,Rh.4 Rh.5* and *Rh.6* were analyzed by PCR amplification of rDNA gene including 5.8S gene and the flanking intragenic transcribed spacer ITS region of rDNA.

ITS1 and ITS2 which are separated by the 5.8S gene are widely used for species identification of *Rhodotorula*. On the basis of the similarity in size of the bands of the PCR-FSP products (ITS1 and ITS2), the six isolates were compered.

3.1 PCR amplification of ITS1 region of rDNA of *Rhodotorula* isolates

Using of PCR technique to detect of ITS1 primer (ITS1f-ITS1r) revealed species specific band of amplification product for various *Rhodotorula* isolates (figure 1).

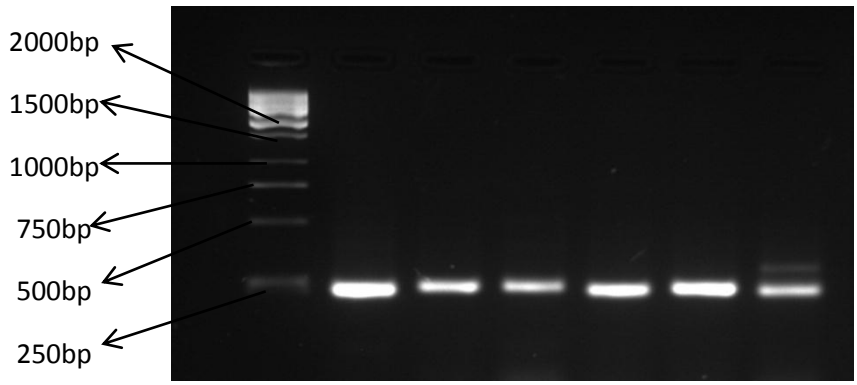
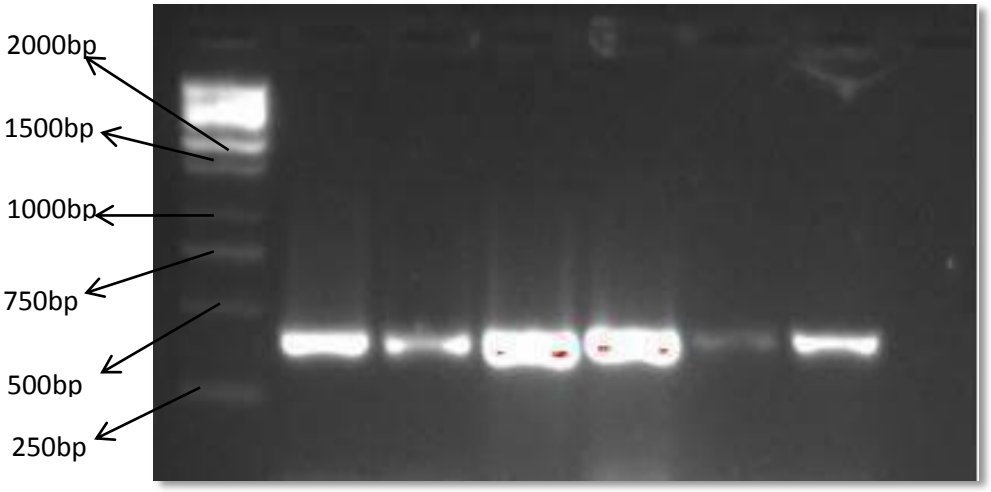


Figure (1): PCR products from amplification of ITS1 primer (ITS1f-ITS1r) region of rDNA of *Rhodotorula* isolates.

The result obtained by ITS 1 were similarity of all isolates *Rhodotorula* spp. They have one band was about 221 bp. The result is in accordance to **(Mokhtari, 2011).**

3.2. PCR amplification of ITS2 region of rDNA of *Rhodotorula* isolate.

ITS2 primer (ITS2f-ITS2r) was detected in all isolates *Rhodotorula spp*. by one band shown figure (2).

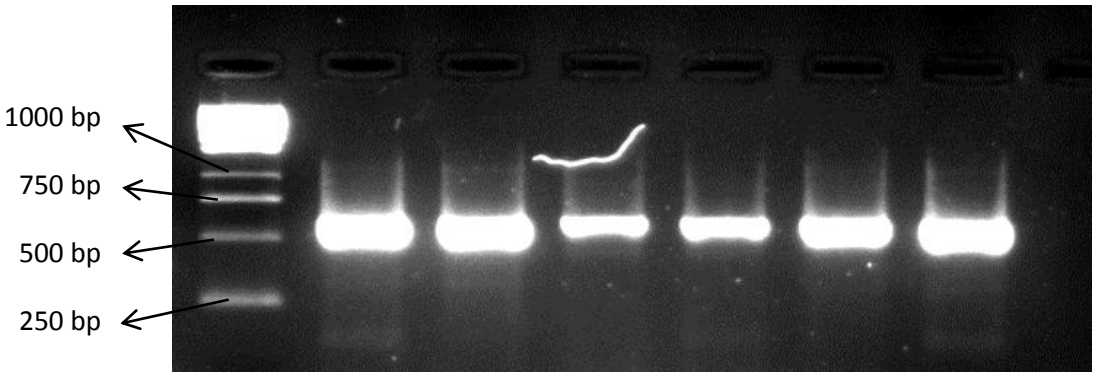


Figure(2): PCR products from amplification of ITS2 (ITS2f–ITS2r) region of rDNA of *Rhodotorula* isolates

Figure(2) showed one band in all *Rhodotorula* isolates (about 353 bp) the result is in accordance with **(Mokhtari, 2011).**

3.3. PCR amplification of ITS1-ITS2 region of rDNA of *Rhodotorula* isolates.

Amplification of the ITS1-5.8-ITS2 with primers ITS1f and ITS2r yielded a single product estimated by gel electrophoresis of approximately 530 to 588 bp was obtained from PCR amplification for all six isolates of *Rhodotorula spp*. (figure. 3).



Figure(3): PCR products from amplification of ITS1 – ITS4 region of rDNA of *Rhodotorula* isolates**.**

The ITS PCR has helped to detect polymorphism at ITS1-ITS2 region of rDNA among the *Rhodotorula* isolates. The lengths of amplified fragments using the primers ITS1f and ITS2r about 554 bp for six isolates of *Rhodotorula ssp.*

Its work to note that ITS-1 and ITS-2 region of the rDNA gene complex are known to be highly variable and suitable for phylogenetic studies of fungi at the inter and intraspecific level (**Mokhtari, 2011 and Nada, 2013**).

Identification by sequencing analysis

Based on morphological, physiological identification and ITS pattern, three isolates *Rh.3*, *Rh.4* and *Rh.5* were similar so, one of them (*Rh.5*) was taken for ITS sequence analysis.

The representative isolates were then subjected to sequence and phylogenetic analysis. BLAST analysis of the nucleotide sequence of the ITS region of *Rh.1* and *Rh.6* isolates showed 99% similarity with *Rhodotorula mucilaginosa*. On the basis of the Blast *Rh.2* and *Rh.5* appeared high similarity with *Rhodotorula glutinis*. The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/ BLAST/).

A neighbor joining phylogenetic tree was constructed using MegAlign software were retrieved from the Gene Bank database and included in the analysis (figures 4 and 5).

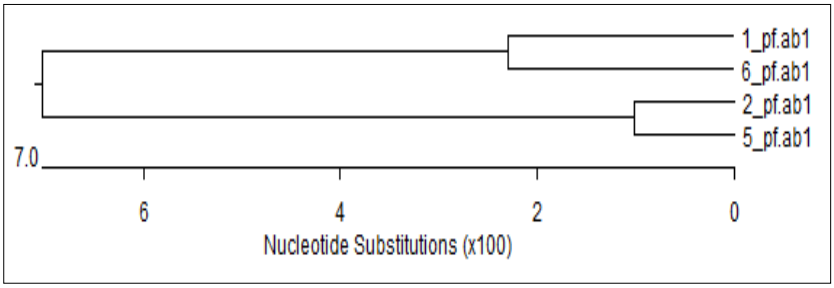


Figure (4): The phylogenetic tree showing the relationship between the *Rhodotorula ssp.* By forward primer.

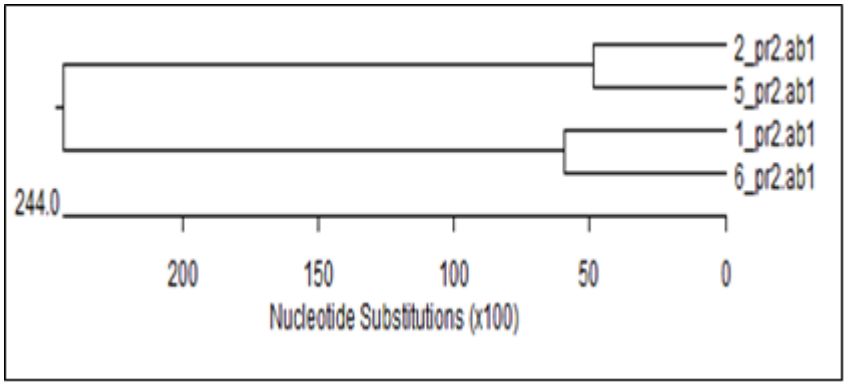


Figure (5): The phylogenetic tree of the relationship between the *Rhodotorula ssp.* by reverse primer.

Based on the results obtained all the six isolates can be grouped into two main clusters. one clusters represents *Rhodotorula mucilaginosa* (*Rh.1* and *Rh.6*) and other cluster contains *Rhodotorula glutinis* (*Rh.2, Rh.3, Rh.4* and *Rh.5*) as shown in figures (4 and 5). The results obtained are in accordance to **(Mokhtari, 2011)** who said that there are no different genetic at a high level but differ in their phylogenetic location and evolutionary divergence.

Conclusion

By morphological identification, no difference between all of *Rhodotorula* isolates. On the other hand, different gets in physiological and biochemical characterization, based on assimilation of different carbon sources and different nitrogen sources obtained *Rh.3*, Rh.4 and *Rh.5* were similarity between them and *Rh.1* , Rh.6 were found similarity between them also. In antibiotic resistance patterns, all isolates were sensitive to nystatine and low resistance to resistance for Amphotericin B, Trimethoprim, Kanamycin and Gentamicin were studied to response *Rhodotorula* on antifungal agent.

By molecular identification, all result concert all species in genera *Rhodotorula*, under *Rhodotorula mucilaginosa* (*Rh.1* and *Rh.6*) and under *Rhodotorula glutinis* (*Rh.2, Rh.3, Rh.4* and *Rh.5*) by Identical 99% from BLAST.

Reference

Ahamed.H.S.S. (2009). Evaluation and application of carotenoids producer from food wastes by some microorganisms. PHD. Agric. Sc (food technology Ain Shams University

Bowman, Pl, Ahearn, DG (1975). Evaluation of the Uni-Yeast-Tek Kit for the identification of medically important yeasts. J. Clin. Microbiol. 2:354-357.

Dufossé (2006). Microbial Production of Food Grade Pigments *Food Technol.* Biotechnol. 44 (3) 313–321

El-Zaatari, M, Pasarell, L, McGinnis, MR, Buckner, J, Land, GA, Salkin, IF (1990). Evaluation of the updated Vitek yeast identification data base. J. Clin. Microbiol. 28:1938-1941.

Frengova and Dora (**2009**). Carotenoids from *Rhodotorula* and *Phaffa*: yeasts of biotechnological importance Ind Microbiol Biotechnol 36:163–18

Frengova G., E. Simova, K. Pavlova, D. Beshkova, and D. Grigorova (1994). Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafiltrate. Biotechnol. Bioeng. 44, 888D894.

Golubev.W.I (2010). *Myo*_Inositol Assimilating New Species of *Rhodotorula* Harrison Microbiology, Vol. 79, No. 6, pp. 862–864.

Haley, LD (1971). Identification of yeasts in clinical microbiology laboratories. Am J. Med. Technol.37:125-131.

Iwen, P.C., S.H.Hinrichs, and M.E. Rupp (2002). Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Med Mycol* 40, 87-109.

Kreger-v. R. (1984). The Yeasts. A Taxonomic Study, NJW (ed), 3rd Edition, Amsterdam, Elsevier Publishers.

Land, GA, Harrison, BA, Hulme, KL, Cooper, BH, Byrd, JC (1979). Evaluation of the New API 20C strip for yeast identification against a conventional method. J. Clin. Microbiol. 10:357-364.

Larone, DH: Medically Important Fungi (1987). A Guide to Identification. 2nd Edition, New York, Elsevier Publishers.

Latha.B.V. K. jeevaratnam, H.S.Murali and K.S. Manja (2005). Influence of growth factors on carotenoid pigmentation of *Rhodotorula glutinis* DFR-PDY from natural Source, journal of biotechnology vol.4, July 2005, 353-357.

McGinnis, MR (1980). Laboratory Handbook of Medical Mycology, New York, Academic Press.

Melike and İbrahim (2008). Molecular characterization of *Rhizoctonia solani* AG4 using PCR-RFLP of the rDNA-ITS region

Mirhendi S.H., H.Adin, M.R Shidfar, P. Kordbacheh, S.J. Hashemi, M.Moazeni, L. Hosseinpur, and A.M. Rezaie (2008). Identification of Pathogenic Candida Species: PCR-Fragment Size Polymorphism (PCR-FSP) Method. Tehran University Medical Journal, **66**, 639-645.

Mokhtari M., H.R. Etebarian, S.H. Mirhendi and M. Razavi (2011). Identification and phylogeny of some species of the genera *Sporidiobolus* and *Rhodotorula* using analysis of the 5.8S rDNA gene and two ribosomal internal transcribed spacers Arch. Biol. Sci., Belgrade, 63 (1), 79-88

Mona (2007). Genetic Improvement of some bacterial strains used in biological control M.Sc. Dep. of Genetics, Fac. of Agriculture, Ain Shams University

Nada.F (2013). Identification and characterization of biocontrol genes in Trichoderma isolates and their functions against soil borne fungal plant pathogens. PHD in department of genetics, faculty of agriculture, Fayoum University.

Phillips A1, D. Janie, W. Wheeler (2000). Multiple sequence alignment in phylogenetic analysis. Mol Phylogenet Evol. Sep; 16 (3):317-30.

Pincus, DH, Salkin, IF, McGinnis (1988). MR: Rapid methods in medical mycology. Lab. Med.19:315-320.

Salkin, IF, Land, GA, Hurd, NJ, Goldson, PR, McGinnis, MR (1987). Evaluation of Yeast dent and Uni-Yeast-Tek yeast identification systems. J. Clin. Microbiol. 25:624-627.

Viljoen, B. C. and J. L. F. Kock (1989). A taxonomic study of the yeast genus *Candida Berkhout* Syst.App!. Microbio!., 12, 91-102

Zaas AK, M. Boyce, W. Schell, B.A. Lodge, J.L. Miller and J.R.Perfect (2003). Risk of fungemia due to *Rhodotorula* and antifungal susceptibility testing of *Rhodotorula* isolates. J Clin Microbiol.; 41(11):5233-5.

Zahra S. 1, A. Z. Mahmoudabadi and S. Hydrinia (2013). Isolation, Identification and Susceptibility Profile of *Rhodotorula* Species Isolated from Two Educational Hospitals in Ahvaz Jundishapur Journal of Microbiology; Aug 6(6): e8935.