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COMPLEX IDENTIFICATION OF RED YEAST ISOLATE FROM GASTROINTESTINAL TRACT OF HUCUL LONG-LIVER (CARPATHIANS, UKRAINE)

The red yeasts are currently widely discussed and controversial group of yeasts because of the growing number of reports of their ability to become opportunistic pathogens of plants, animals and humans. The aim of this work was complex identification of the red yeast culture isolated from gastrointestinal tract of healthy Hucul long-liver from the Carpathians highland region of Ukraine. Torularhodin was found to be a major component within yeast culture carotenoids complex. According to conventional biochemical and morphological approaches as well as to molecular biological investigation of internal transcribed spacer region (ITS) of ribosomal operon it was concluded that isolate belonged to species Rhodotorula mucilaginosa.

K e y w o r d s: red yeasts, identification, carotenoids, ITS, phylogram.

Synthesis of carotenoids is present in many microorganisms: bacteria, actinobacteria, algae and fungi. Fungal ability for the carotenogenesis and the formation of coloured colonies is found only among families of basidiomycetous yeasts, belonging to the genera *Rhodosporidium*, *Cystofilobasidium*, *Sporidiobolus* and anamorphic genera *Rhodotorula*, *Cryptococcus*, *Sporobolomyces*. Considering their colonies pigmentation, these yeasts were incorporated in the group of red (pink) yeasts [1, 9]. The common red yeasts representatives are yeasts species belonging to genus *Rhodotorula*, which are ubiquitous saprotrophic organisms that can be isolated from various environmental sources: soil, fresh and sea water, air, foods, and household items like shower curtains and toothbrushes.

Rhodotorula species were traditionally considered to be non-virulent saprotrophs and common contaminant microorganisms. However, over last two decades, these yeasts have emerged as the potential opportunistic pathogens [22]. It is known that yeasts are common normal microbiota of the skin, respiratory and digestive tracts of the humans. The total number of fungi in the content of human large intestine usually does not exceed 10^3–10^4 CFU/g of faeces [2, 13]. In the studies of yeast diversity of different age groups of Abkhaz highlanders, the red yeasts were reported to be isolated from human gastrointestinal tract (GIT) mainly starting from the age of 35 years old and considerably increasing with ageing [2]. It is believed that carotenoids synthesizing yeasts get into a body of humans and animals with food [13]. For example, ubiquitous

Rhodotorula mucilaginosa can be often isolated from dairy products, beverages, fruits and vegetables [22].

It has been also reported that isolated from different sources yeast strains demonstrated growth stimulating effect on important agricultural crops [19]. Boby et al. found the positive effect of a combination of soil yeast *Rhodotorula mucilaginosa* with arbuscular mycorrhizal fungus *Glomus mosseae* on the cowpeas growth [5].

Traditionally, yeasts have been identified and classified by morphological and biochemical-physiological methods [17]. Such classic methods include descriptional approaches as well as the studies of the yeasts ability for fermentation and assimilation of 30–80 different chemical substances [17]. Also, various molecular biology-based methods have been increasingly applied to the yeasts identification [4]. Some of them use, for example, the differences in the structure of the rRNA internal transcribed spacer (ITS) in taxonomic studies of fungi including yeasts [11, 12, 14].

The aim of this work was a taxonomic study of the red yeast strain isolated from Hucul long-liver (Carpathians highlands, Ukraine) using morphological, physiological and molecular biological methods.

Materials and methods. *Microorganisms.* The strain S1 of red yeast was isolated from GIT of healthy Hucul long-liver from the highland region (village Biloberezka) of the Eastern Carpathians, Ukraine, in May 2013. The isolation was performed by serial dilution method (with strain accounting $1 \cdot 10^3$ CFU/g of faeces) and the culture was maintained on malt agar medium at 32 ^{0}C.

Phenotypic identification. Yeast identification was based on the determination of morphological and physiological characteristics and was carried out according to Kurtzman and Fell [17]. Yeast morphology was described after 72 h cultivation at 32 ^{0}C on malt agar using light microscopy. The physiological characteristics were determined by the ability of the yeast isolate to assimilate a number of carbon sources: glucose, sucrose, raffinose, melibiose, galactose, lactose, trehalose, maltose, melizitose, soluble starch, cellobiose, salicin, L-sorbose, L-rhamnose, D-xylose, L-arabinose, D-arabinose, D-ribose, ethanol, glycerol, erythritol, D-mannitol, ribitol, DL-lactate, succinate, citrate, D-gluconate, D-glucosamine, N-acetyl-D-glucosamine, inulin, methyl-α-D-glucoside and to ferment carbohydrates: glucose, galactose, sucrose, maltose, lactose, raffinose, trehalose [17].

Characterization of red pigment produced by yeast. For biomass yield yeast was cultivated on malt agar for 4 days at 28 ^{0}C [10]. Yeast biomass was removed from the medium surface by a sterile scalpel, mechanically homogenized with quartz sand and then dried in the open air at room temperature. Stepwise pigment extraction from both dry and wet biomass was carried out by using ethanol, hexane, petroleum ether, chloroform and acetone. Quartz sand was removed from the obtained extracts by centrifugation at 8050 g for 5 min [10].

Preliminary pigment identification was performed by thin layer chromatography (TLC) [6]. Separation of the extract was carried out using plates Cromatofolgas AL HPTLC Silicagel 60 F254 by "Merck". Fractionation of carotenoid extracts was performed in two different solvent systems: petroleum ether – diethyl ether (99:1) and petroleum ether – acetone (1:4) [10]. The TLC chromatogram for the red yeast strain S1 was compared to the

acetone extracts of biomass of established carotenoids producing yeast strains *Rhodotorula glutinis* UCM Y-1330 and *Sporobolomyces roseus* UCM Y-1938 from Ukrainian Collection of Microorganisms (UCM). The letter extracts were provided by Dr. S. Golembiovska (Zabolotny Institute of Microbiology and Virology NASU, Kyiv, Ukraine).

The separated by TLC components of strain S1 then were scraped off from the plates and pigment was extracted by using ethanol or acetone [6]. The spectrophotometric analysis of the separated pigments was performed using spectrophotometer Beckman DU-8B at 400–600 nm (visible light).

Yeast identification based on ITS-fragment of rDNA. The genomic DNA was extracted using mechanical disruption with glass beads combined with the SDS-based enzymatic lysis method adapted from Wach et al. [21]. Polymerase Chain Reaction (PCR) and sequencing were performed with the universal primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and amplicons were sequenced using the Sanger di-deoxynucleotide method in an ABI Prism 3100 automated sequencer (Applied Biosystems, CA) [8, 12]. Species identification was performed using the Basic Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov); an E value of $< 10^5$ was used as cutoff for species identification [8]. The program MEGA 6 with Neigbour-Joining algorithm was used to construct a phylogram of phylogenetic relationships between the representative genera of basidiomycetous yeasts belonging to the red yeasts group.

Results and discussion. The species incorporated into the so-called "red yeast" group are very common and can be isolated from various environments [2]. For example, being ubiquitous contaminant microorganisms, *Rhodotorula* spp. was reported to be the most common microorganism isolated from the hands of hospital employees and patients [22].

Carotenoids forming yeasts, for example *Rhodotorula* species, for decades were regarded as a perspective group in terms of the development of microbes-based biotechnologies and bioremediation techniques. However, previously considered nonpathogenic, *Rhodotorula* species nowadays demonstrated the ability for the opportunistic pathogenic behaviour towards the susceptible, especially immunocompromised patients [22]. At the same time it was well documented that red yeasts, including *Rhodotorula* species, were common and numerous representatives of the normal yeasts microbiota of the GIT of healthy adult Abkhaz highlanders increasing their occurrence with ageing and reaching the maximum number for the long-livers age group [2].

Here we performed the complex study of taxonomic characteristics of the red-pigmented yeast strain S1 isolated from the gastrointestinal tract of the healthy and fit Carpathian long-liver.

The first step of taxonomic identification of yeast strain S1was the study of its macro- and micromorphology (Fig. 1).

Macromorphology. After 72 h cultivation at 32 ^{0}C on malt agar, the colonies of yeast strain S1 had butyrous texture, glistening surface and round shape (Fig. 1 left). Yeast synthesized pigments of salmon colour.

Micromorphology. Cells shape varied from spherical to ovoid with size being (2–8)´(2–12)μm (Fig. 1 right). The reproduction of cells by polar budding was observed.

The red pigmentation of yeasts is known to indicate their belonging

exclusively to the division *Basidiomycota* [17]. One of the main criteria in taxonomic studies of yeasts is their ability for sexual reproduction and sporulation. We did not observe the sexual reproduction process or spore formation for yeast isolate S1. It is known that genus *Rhodotorula* is anamorphic [15, 17] which supported our assumption about the strain S1 belonging to this genus.

Assimilation of carbon sources. In the course of phenotypic identification, the biochemical profile of the red yeast isolate S1 was studied showing its

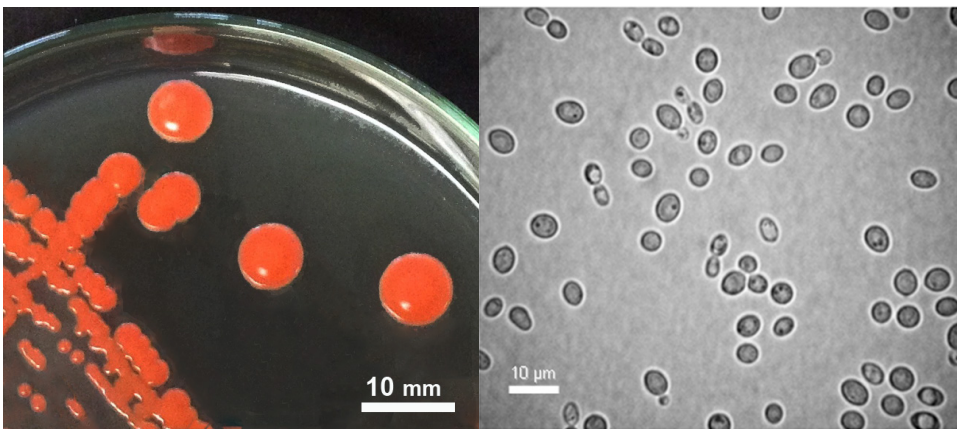

Fig. 1. Morphology of colonies (left) and cells (right) of yeast strain S1

ability to assimilate various carbon sources. To refine our data on strain S1 identification we compared the obtained results to the available database information using on-line program CBS – KNAW Fungal Biodiversity (http://www.cbs.knaw.nl/Collections/). The highest similarity of our data was found towards the reference standard strain *Rhodotorula mucilaginosa* CBS 316^T (99.83%), followed by *R. dairenensis* (98.49 %), *R. pacifica* (97.10 %) and *Rhodosporidium sphaerocarpum* (94.32 %). Table 1 shows the comparison of carbon sources assimilation ability between the red yeast strain S1 and the standard strain *Rhodotorula mucilaginosa* CBS 316^T.

As a result, the isolated yeast strain was preliminarily identified as *Rhodotorula mucilaginosa.* The assimilation ability for both strains was characterized with certain variability which is typical for this species (Tab. 1). Generally, due to this variability among strains in the utilization of the majority of the test carbon compounds, *R. mucilaginosa* has a long list of synonyms [17]. Some variability of these strains could signify separate species requiring the further examination by nucleotide sequence analysis using molecular biological and bioinformatic methods.

Carotenoids analysis. Because the strain S1 was characterized by red colouration, the nature of the pigment(s) was studied by using conventional methods: TLC, spectrophotometry, etc. [6]. All results obtained testified to the carotenoids nature of the S1 pigments. For example, Figures 2 A and B show the typical absorption spectra for the major pigment of the tested strain S1 with the retardation factor (R_f), representing the fraction of an analyte in the mobile phase of a TCL system, being equal 0.2. This substance was characterized by the maximum values of the absorbance at the wavelength

Table 1

Assimilation of carbon sources by red yeast isolate S1 and the reference strain *Rhodotorula mucilaginosa* CBS 316^T

No	Carbon source	Strain S1	Strain CBS 316^T	No	Carbon source	Strain S1	Strain CBS 316^T
1	Glucose	+	+	**17**	L-Arabinose	+	v
2	Sucrose	+	+	**18**	D-Arabinose	+	v
3	Raffinose	+	+	19	D-Ribose	+	v
4	Melibiose	-	-	**20**	Ethanol	+	v
5	Galactose	w	v	**21**	Glycerol	+	v
6	Lactose	-	-	22	Erythritol	-	-
7	Trehalose	+	+	**23**	D-Mannitol	-	v
8	Maltose	+	v	**24**	Ribitol	-	v
9	Melezitose	+	v	**25**	DL-Lactate	+	v
10	Soluble starch	-	-	**26**	Succinate	+	+
11	Cellobiose	+	v	**27**	Citrate	+	v
12	Salicin	+	v	**28**	D-Gluconate	w	+
13	L-Sorbose	+	v	**29**	D-Glucosamine	-	v
14	Methyl-α-D-glucoside	-	v	**30**	N-Acetyl-D-glucosamine	-	-
15	D-Xylose	+	+	**31**	Inulin	-	-
16	L-Rhamnose	-	v				

"+" – positive; "–" – negative; "v" – variable; "w" – weak [17].

485 nm and 491 nm when dissolved in ethanol and hexane, respectively (Figs. 2A and B). It is known that such absorption spectra are characteristic for carotenoid acid torularhodin which is accumulated by basidiomycetous yeasts [6, 7]. To compare our findings with other red yeasts, the reference torularhodin absorption spectra for *Rhodotorula glutinis* UCM-1330 and environmental red yeast isolate from the cave ("Mushkarova Hole", Western Ukraine) are presented in Figure 2C [3].

ITS-based identification of yeast strain. The modern approaches to taxonomic studies include also the analysis of the homology of nucleotides sequences of the ribosomal operon [11]. For the tested red yeast strain S1, we identified the nucleotide sequence of ITS-fragment of the ribosomal operon which was 654 bp long. This fragment consisted of partial sequence of 18S ribosomal RNA gene, complete sequences of internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, and partial sequence of 28S ribosomal RNA gene. The universal fungal primers ITS1 and ITS4 we used for amplification of this fragment [13]. The obtained DNA sequence for the studied yeast strain *Rhodotorula* sp. S1was submitted to the NCBI da-

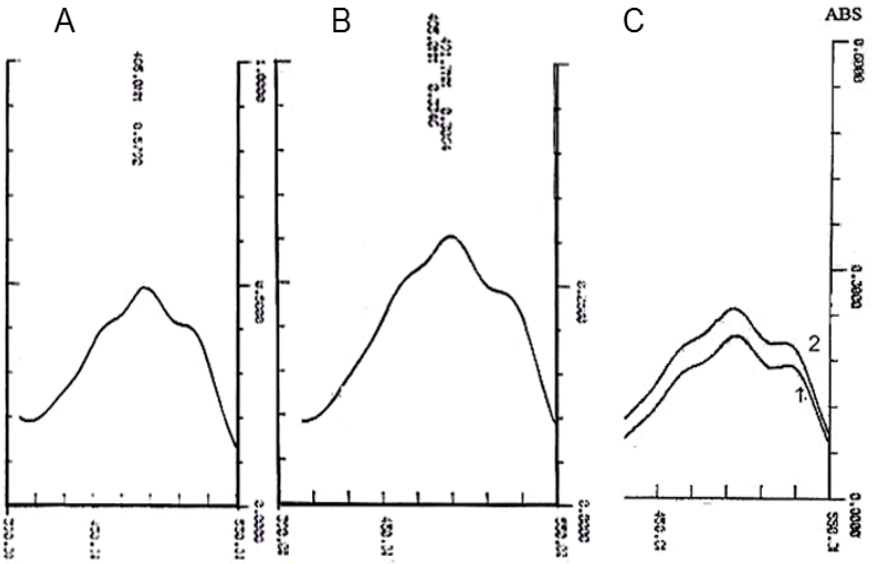

Fig. 2. Typical absorption spectra of the pigments of red yeasts: (A, B) major pigment of the tested strain S1 with R_f 0.2, when dissolved in ethanol (A) and hexane (B); (C) dissolved in ethanol reference torularhodin pigments (R_f 0.21) of (1) "Mushkarova Hole" red yeast isolate and (2) reference strain *Rhodotorula glutinis* UCM-1330 [3]

tabase with accession number KR779874 (http://www.ncbi.nlm.nih.gov/nuccore/939608912).

In silico analysis of the nucleotides sequence of ITS-fragment KR779874 was performed using NCBI database "Nucleotide collection" focusing on its part containing information on the primary structure of DNA for basidiomycetous fungi (taxid: 5204). The BLASTN analysis revealed 100 % similarity of the strain S1 ITS-fragment to the ITS sequences of some *Rhodotorula mucilaginosa* strains. Generally, over 150 GenBank sequences of *R. mucilaginosa* strains demonstrated 94–100 % homology to ITS-fragment of studied strain S1.

The phylogram of genetic relationships (or dendrogram) between different species of the red yeasts group belonging to genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces*, *Sporidiobolus* genera, including the studied isolate S1, was constructed on the basis of ITS-sequences using Neigbour-Joining algorithm (Fig. 3). The majority of strains used were standard reference strains from the reputable international collections.

The dendrogram showed that all analysed species formed two large clades. The first clade incorporated the representatives of three species of *Rhodosporidium* genus (Fig. 3). The second clade was a polyphyletic group including the representatives of different species of the genera *Rhodotorula*, *Sporobolomyces*, *Sporidiobolus* as well as *Rhodosporidium*.

It is worth notice that anamorph-teleomorph relationships of the considerable number of strains of the genera *Rhodotorula* and *Rhodosporidium* have been described in the scientific literature from the viewpoint of chemosystematics. For example, the red yeast species *Rhodotorula rubescensis* was reported as

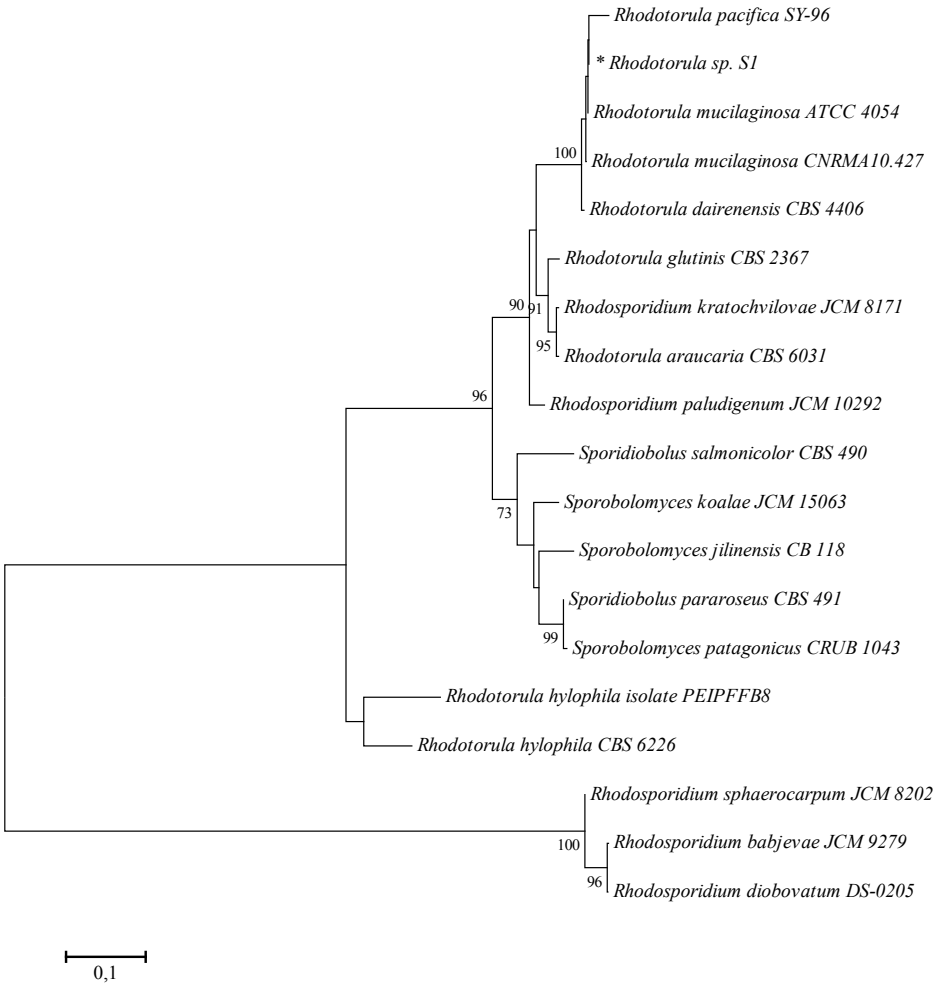

Fig. 3. ITS-sequences based dendrogram of the phylogenetic relationships between the strains of genera *Rhodotorula, Rhodosporidium, Sporobolomyces* and *Sporidiobolus* and the studied *Rhodotorula sp.* S1 (marked with an asterisk)

anamorph of the species *Rhodosporidium toruloides*, which is the synonym of *Rhodotorula gracilis* [15].

The location of the studied strain S1 in the dendrogram revealed its grouping with other strains belonging to the entirely *Rhodotorula mucilaginosa* species. According the bootstrap analysis this group was significantly different from the species of genera *Rhodosporidium, Sporobolomyces, Sporidiobolus* and other *Rhodotorula* species.

One of the most closely phylogenetically related to the studied *Rhodotorula* sp. S1 within the corresponding clade was found to be a standard reference strain *Rhodotorula mucilaginosa* ATCC 4054^{T} from the American collection (Fig. 3). So, the phylogram data confirmed the *Rhodotorula mucilaginosa* identity of the studied red yeast strain S1.

Despite the controversy caused by *Rhodotorula* sp. ability for opportunistic pathogenesis, it should kept in mind that the incidences of fungemia caused by *Rhodotorula* species were comparatively rare, for example in the USA

not exceeding 2 %, and mostly associated with central catheters in patients with haematologic malignancies [22]. The biological effects, both positive and negative for human health and longevity, of the red yeasts survival and growth in the gastrointestinal system still remain unclear and need further interdisciplinary studies.

Similarly to the previous data on the increasing numerous occurrence of red yeasts in the gastrointestinal tract of the Abkhaz healthy long-livers, our research demonstrated the presence of *Rhodotorula* species in GIT of healthy long-liver in the highland region of Ukrainian Carpathians. The question about the possible interactions between the long-liver macroorganism and the widespread red yeasts as a component of its normal microbiota might be also relevant from the gerontological point of view.

Thus, in the course of a complex study using morphological, physiological, biochemical and molecular biological methods, the taxonomic identity of the red yeast GIT isolate S1 was established to belong to the species *Rhodotorula mucilaginosa*.

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КОМПЛЕКСНА ІДЕНТИФІКАЦІЯ ШТАМУ ЧЕРВОНО-ПІГМЕНТОВАНИХ ДРІЖДЖІВ, ВИДІЛЕНОГО З ШЛУНКОВО-КИШКОВОГО ТРАКТУ ГУЦУЛЬСЬКОГО ДОВГОЖИТЕЛЯ (КАРПАТИ, УКРАЇНА)

Резюме

Червоні дріжджі в даний час є широко обговорюваною та суперечливою групою дріжджів через зростаюче число повідомлень про їхню здатність викликати опортуністичні захворювання рослин, тварин і людини. Метою даної роботи була комплексна ідентифікація культури червоних дріжджів, що була ізольована із шлунково-кишкового тракту (ШКТ) здорового гуцульського довгожителя з високогір'я Карпат України. У даного штаму дріжджів мажорним компонентом у комплексі накопичених каротиноїдів був торулородин. Відповідно до результатів загальноприйнятих морфологічних та фізіологічних, а також молекулярно-біологічних досліджень внутрішньої спейсерної, що транскрибується, області рибосомного оперона (ITS) був зроблений висновок, що даний ізолят належить до виду *Rhodotorula mucilaginosa*.

Ключові слова: Rhodotorula mucilaginosa, ідентифікація, каротиноїди, ITS, філограма.

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КОМПЛЕКСНАЯ ИДЕНТИФИКАЦИЯ ШТАММА КРАСНО-ПИГМЕНТИРОВАННЫХ ДРОЖЖЕЙ, ВЫДЕЛЕННОГО ИЗ ЖЕЛУДОЧНО-КИШЕЧНОГО ТРАКТА ГУЦУЛЬСКОГО ДОЛГОЖИТЕЛЯ (КАРПАТЫ, УКРАИНА)

Резюме

Красные дрожжи в настоящее время являются широко обсуждаемой и спорной группой дрожжей через возрастающее количество сообщений об их способности вызывать оппортунистические заболевания растений, животных и человека. Целью данной работы была комплексная идентификация культуры красных дрожжей, выделенной из желудочно-кишечного тракта (ЖКТ) здорового гуцульского долгожителя из высокогорья Карпат Украины. В соответствии с результатами как общепринятых биохимических и морфологических, так и молекулярно-биологических исследований внутренней транскрибируемой спейсерной (ITS) области рибосомного оперона был сделан вывод, что данный изолят принадлежит к виду *Rhodotorula mucilaginosa*.

Ключевые слова: Rhodotorula mucilaginosa, идентификация, ITS, филограмма.

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