INDO AMERICAN JOURNAL OF PHARMACEUTICAL SCIENCES CODEN [USA]: IAJPBB

ISSN: 2349-7750

INDO AMERICAN JOURNAL OF PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.3366248

Available online at: <u>http://www.iajps.com</u>

Research Article

PHYLOGENETIC BIODIVERSITY OF YEASTS IN THE REPUBLIC OF NORTH OSSETIA-ALANIA (second report)

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| Article Received: June 2019 | Accepted: July 2019 | Published: August 2019 |
|---------------------------------------------|---------------------------------|---------------------------------------------------------------------------------------------------------------------------|
| Abstract: | | |
| The authors isolated yeast strains from the | surface of grape cultivars Burg | gund (strain I7), hops cones grown in the |
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The authors isolated yeast strains from the surface of grape cultivars Burgund (strain **I7**), hops cones grown in the botanical garden of Gorsky state agrarian university (Vladikavkaz, Russia) (strain **DEN4**), as well as from soil samples of the molasses distillery stillage drainfields (strain **B1**). The primary identification of the selected strains was performed by studying cultural, tinctorial, morphological, physiological-biochemical, and technological properties in the Research Institute of Biotechnology of Gorsky SAU, using traditional research methods. For the final identification based on the analysis of ribosomal genes sequence of the studied strains, colonies on the slant wort agar were sent to the Bioresource Centre – All-Russian Collection of Industrial Microorganisms (BRC VKPM) National Research Centre "Kurchatov Institute" – State Research Institute of Genetics analysis of the nucleotide sequence, coding part of rRNA genes it was found that I7 strain is the closest to the species **Torulaspora delbrueckii**.

Key Words: yeasts, strain, gene encoding 18S rRNA, DNA, PCR mode.

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Please cite this article in press Boris Georgievich Tsugkiev et al., Phylogenetic Biodiversity Of Yeasts In The Republic Of North Ossetia-Alania (Second Report)., Indo Am. J. P. Sci, 2019; 06[08]. IAJPS 2019, 06 [08], 14615-14623

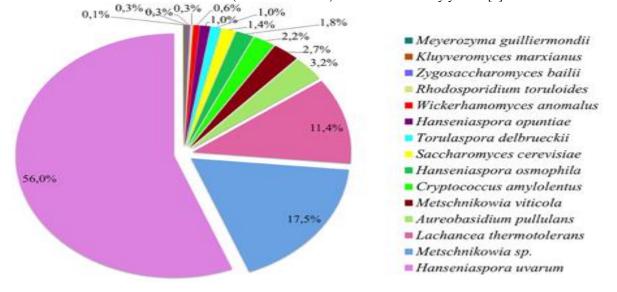
RELEVANCE:

Currently, one of the most dynamically developing areas of industrial production is biotechnology. The progress in the industry makes new demands on the properties of strains-producers, used in various fields of human activity. The list of technological demands on yeast in various sectors of the food industry is very large. They are due to physiological and morphological features that make up the generic, species, racial characteristics. The combination of these features is responsible for the effective use of the production culture, which is the main part of the technological chain.

Researchers report that yeasts *Hanseniaspora uvarum* can be both beneficial, in particular as the main source of protein when cultivating fodder biomass, and harmful. In particular, the work of Kethireddy, V. (2016) provides information on one of the innovative methods to control the growth of wild yeasts during wine maceration, as well as in the early fermentation stages, which can change the wine taste profile. This study examines the effect of irradiation electric fields on *Saccharomyces cerevisiae, Picchia kluyveri* and *Hanseniaspora uvarum*. [6].

Nowadays, Saccharomyces cerevisiae is the most widely used yeast species in oenology. However, over the past decade, several other yeast types have become actively used in winemaking, as they can positively affect the wine quality. Some yeast types are still the subject of various studies (Hanseniaspora uvarum, Starmerella bacillaris, etc.). Together with their biotechnological benefit, the knowledge of their unconventional properties has significantly extended the application of these yeasts during last 10 years. Thus, Masneuf-Pomarede, I., (2016) describes how genomics and genetics tools provide new data on the structure and biodiversity population of unconventional yeasts in the wine industry. [7].

Studies on isolating new wild yeast strains from grapes and wine were made by Slovak researchers. They managed to identify seven different *Saccharomyces cerevisiae* strains, two *Kloeckera apiculata* [teleomorph *Hanseniaspora uvarum*] strains and one strain *Pichia kluyveri* (1; 3%), *Pichia occidentalis* [anamorph *Candida sorbosa*] (1; 3%) and *Metschnikowia pulcherrima* (1; 3%). To isolate new strains 15 samples of wine "Federweisser" were used. Ethanol/formic acid extraction method was used in these studies. Mass spectrometer



Researchers studying wild yeast species found that fifteen different species of wild yeast are most commonly found in grapes. *Hanseniaspora uvarum* was the most common species, accounting for more than half of the total number of isolates, then follows *Metschnikowia* sp. (composed of *M. pulcherrima* and *M. fructicola*) and *Lachancea thermotolerans* (Fleet, 2008 [3]; Jolly et al., 2014 [4]. As for the largest species population in this study, the analysis of *H. uvarum* enzymatic profile gave rise to the most complex clustering, although in some cases it was possible to determine the enzymatic profile depending on *H. uvarum* origin, which was isolated from all the studied vineyards and received in total 431 isolates [2].

MALDI-TOF (Microflex LT/SH) was used to identify yeasts. [5].

The development of new fermented foods and beverages requires a growing use of new yeast types. In his study Antonio de Anchieta Câmara Jr. et al. focused on the resistance to dehydration-oxidation of three wine yeast types *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans*, cultivated and dehydrated under different conditions, compared to *Saccharomyces cerevisiae*, as a reference source. [1].

When fermenting wine, yeasts *Saccharomyces cerevisiae* are usually used. However, many other yeast types are also involved in the fermentation process, some with interesting oenological features. *Torulaspora delbrueckii* is sometimes used in mixed or sequential fermentation with *Saccharomyces cerevisiae* to improve the sensory wine profile. Main differences in the ways of fermenting glucose and the formation of aroma and flavour compounds such as glycerin, esters and acetic acid are of special interest. Paralogous genes are lacking in *Torulaspora delbrueckii* glycerol glycolysis and biosynthesis. Results of Tondini, F. (2019) research indicate a tendency for *Torulaspora delbrueckii* to produce less acetic acid. [9].

In view of the foregoing, it seems advisable to study yeast species in North Ossetia (Russia), as well as the possibility of using pure cultures of the identified yeast strains in biotechnological industry.

The **aim** of the present study was to isolate new yeast strains from various environmental sources.

MATERIAL AND RESEARCH METHODS:

The present study used grape berries of Burgund cultivar, hops cones, as well as soil samples to isolate yeasts strain – decomposer of the drainfields, which received xenobiotic – molasses distillery stillage for three years.

Malt wort and agar wort were used as a culture medium.

The primary identification of the selected strains was performed by studying cultural, tinctorial, morphological, physiological-biochemical, and technological properties in the Research Institute of Biotechnology of Gorsky SAU, using traditional research methods [11] and [12].

For the final identification based on the analysis of ribosomal genes sequence of the studied strains, yeast cultures on the slant wort agar were sent to the Bioresource Centre – All-Russian Collection of

Industrial Microorganisms (BRC VKPM) National Research Centre "Kurchatov Institute" – State Research Institute of Genetics.

At the next stage, genetic research of the studied yeast strains was performed. Sequencing was performed using an automatic sequencer AE3000.

Methods of genetic research: Researches in Bioresource Centre – All-Russian Collection of Industrial Microorganisms (BRC VKPM) NRC "Kurchatov Institute" – State Research Institute of Genetics included:

I. Culture screening to individual colonies and biomass obtaining for RNA 18S analysis.

II. DNA extraction. (Genomic DNA Purification Kit) III. Strain identification based on 18S rDNA sequence

III.1 Selection of primers and PCR modes.

Conservative primers to develop gene sequence coding 18S rRNA [8]:

NS1 – gtagtcatatgcttgtctc NS4 - cttccgtcaattcctttaag

N54 - cuccgicaatteettaag

PCR mode:

1. 95°C – 3 min. 2. 35 cycles 95°C - 30 sec. 57°C - 30 sec. 72°C - 30 sec. 3. 72°C – 5 min.

Conservative primers to develop gene sequence coding 5,8S rRNA and internal transcribed spacers ITS1 and ITS2 [10]: ITS1 – TCCGTAGGTGAACCTGCG

ITS4 – TCCTCCGCTTATTGATATGC

PCR mode:

1. $95^{\circ}C - 3 \text{ min.}$ 2. 35 cycles $95^{\circ}C - 30 \text{ sec.}$ $57^{\circ}C - 30 \text{ sec.}$ $72^{\circ}C - 30 \text{ sec.}$ 3. $72^{\circ}C - 5 \text{ min.}$

Consecutive primers to amplify D1/D2 domain of 26S rRNA gene: NL-1 GCATATCAATAAGCGGAGGAAAG NL-4 GGTCCGTGTTTCAAGACGG PCR mode:

95°C - 3 min.
35 cycles
95°C - 30 sec.

52°C – 30 sec. 72°C – 30 sec. 3. 72°C – 5 min. III.2 18S rRNA and 5,8 rRNA genes sequencing, comparison of sequences and building phylogenetic trees.

Sequencing was performed using automatic sequencer AE3000.

To analyze sequences the specialized computer program BLAST was used [13].

Validity of the results: Sequences sufficient to classify strains into a certain taxonomic group of microorganisms were determined.

IV. Identification using species-specific primers Primers specific to *Fusarium equiseti* [4]: FEF1 5' - CATACCTATACGTTGCCTCG-3'

FER1 5' - TTACCAGTAACGAGGTGTATG-3' Primers specific to *Fusarium oxysporum* [4]: FOF1 5' - ACATACCACTTGTTGCCTCG-3' FOR1 5' - CGCCAATCAATTTGAGGAACG-3'

PCR mode:

1. 95°C – 3 min. 2. 35 cycles 95°C – 30 sec. 52°C – 30 sec. 72°C – 30 sec. 72°C – 5 min.

V. Conditions for PCR electrophoresis of the samples under study:1,0% agarose gel, electrophoresis at an electric field strength of 5 V/cm.

Results and their discussion: Macro - and micromorphological features were determined in isolated yeast strains. The results are given in table 1.

| | Table 1 - | Morphological | properties | of isolated | veast strains |
|--|-----------|---------------|------------|-------------|---------------|
|--|-----------|---------------|------------|-------------|---------------|

| Table 1 - Morphological properties of isolated yeast strains | | | | | | | | |
|--------------------------------------------------------------|--------|----------|----------|----------|----------|-------------|--|--|
| Sources of isolation | Cells | Cells | Colonies | Colonies | Colonies | Colonies | | |
| | shape | size, µm | shape | size | colour | consistency | | |
| | 1 | • | 1 | | | • | | |
| Grape berries of | lemon- | 3-4x6-7 | rounded | medium | whitish- | viscous | | |
| Burgund cultivar | shaped | | | | matte | | | |
| 5 | 1 | | | | | | | |
| Hops cones | round | 7-8 | rounded | large | cream | viscous | | |
| 1 | | | | U | | | | |
| Soil samples of | round | 5-6 | rounded | medium | white | viscous | | |
| drainfields | | | | | | | | |
| | | | | | | | | |

The analysis of the data given in table 1 found that the isolated cultures of microorganisms by macro-and micromorphological features can be presumably considered as unicellular yeast fungi.

The results of the identification research.

I7 strain identification based on ribosomal genes sequence analysis.

Analysis stages:

a) Sequencing of sections of the sequence coding 18S rRNA gene: When sequencing the DNA section coding 18S rDNA gene of the studied strain, the following sequence was obtained:

AGTATAGCAATTTATACAGTGAAACTGCGAA TGGCTCATTAAATCAGTTATCGTTTATTTGAT AGTTTCTTTACTACATGGAATACCTGTGGTAA TTCTAGAGCTAATACATGCTTAAAATCCCAA CTTACGAAGGGATGTATTTATTAGATAAAAA

ATCAATGTCTTCGGACTTCTGATGATTCATAA TAACTTTTCGAATCGCATGGCTTTATGCTGGC GATGGTTCATTCAAATTTCTGCCCTATCAACT TTTGATGGTAGGATAGTGGCCTACCATGGTTT CAACGGGTAACGGGGGAATAAGGGTTCGATTC CGGAGAGGGAGCCTGAGAAACGGCTACCAC ATCCAAGGAAGGCAGCAGGCGCGCAAATTA CCCAATCCTAATTCAGGGAGGTAGTGACAAT AAATAACGATACAGGGCTTTATGTCTTGTAA TTGGAATGAGTACAATTTAAATACCTTAACG AGGAACAATTGGAGGGCAAGTCTGGTGCCAG CAGCCGCGGTAATTCCAGCTCCAATAGCGTA TATTAAAGTTGTTGCAGTTAAAAAGCTCGTA GTTGAACTTTGGGCTTGGAGAATCGGTCCAA CTTTGTTGTGTACTGTCTTCTCCAGGTCTTTCC TTCTGGTTCTCATTTGGGGGTTTACTCCATTTG TTGATCCAGGATTTTTACTTTGAAAAAATTAG AGTGTTCAAAGCAGGCGTTTTGCTCGAATAT ATTAGCATGGAATAATAGAATAGGACGATTG GTTCTATTTTGTTGGTTTCTAGGACCATCGTA ATGATTAATAGGGACGGTCGGGGGGCATCAGT

ATTCAGTTGTCAGAGGTGAAATTCTTGGATTT ACTGAAGACTAACTACTGCGAAAGCATTTGT CAAGGGACGTTTTCATAATCAGACGAAGTAG GGGATCGTAGATGATCAGAWTACCGT

b) Analysis of the gene sequences coding 18S **rRNA:** The similarity of the nucleotide sequence of the gene coding 18S rDNA of the studied strain was analyzed using BLAST server [13].

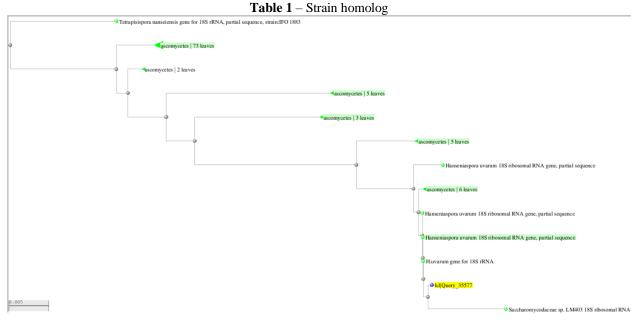
RESULTS:

Primary screening on the GenBank database showed that the studied strain belongs to the following systematic group: *Eukaryota; Fungi; Dikarya;*

Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycodaceae; Hanseniaspora.

The strains involved in the analysis and the similarity level of 18S rDNA sequence of the studied strain are shown in figure 1.

Homology of at least 97% is considered to be the criterion for classifying a microorganism to a particular species [10]. As shown by fig. 1, the analyzed strain can be classified into several species.



The method to compare nucleotide sequences coding D1/D2 domain of 26S rRNA gene was also used to establish phylogenetic affinity of similar species.

When sequencing the DNA section coding D1/D2 domain of the 26S rRNA gene, the following sequence was obtained:

TGTTTTTTGCATGCACTCGCCTCTCGTGGGCT TGGGCCTCTCAAAAATTTCACTGGGCCAACA TCAATTCTGGCAGCAGGATAAATCATTAAGA ATGTAGCTACTTCGGTAGTGTTATAGCTTTTT GGAATACTGTTAGCCGGGATTGAGGACTGCG CTTCGGCAAGGATGTTGGCATAATGGTTAAA TGCCGCCCGTCTTGAAACACGGGACACAAAG A

Phylogenetic analysis, made by using strains of closely related microorganisms, showed that species *Hanseniaspora uvarum* (99 %) is the closest to the studied strain.

The analysis of the nucleotide sequence coding a part of rRNA genes showed that the studied **I7** strain is the closest to the species *Hanseniaspora uvarum*.

DEN4 strain identification based on ribosomal genes sequence analysis.

Analysis stages:

a) Sequencing of sections of the sequence coding 18S rRNA gene: When sequencing the DNA section coding the 18S rDNA gene of the studied strain the following sequence was obtained:

AACKGCGATTGGCTCAAAAAGTGGGTTATCG TTTATTTGATAGTTCCTTTACTACATGGTATA ACTGTGGTAATTCTAGAGCTAATACATGCTT AAAATCTCGACCCTTTGGAAGAGATGTATTT ATTAGATAAAAAATCAATGTCTTCGGACTCT TTGATGATTCATAATAACTTTTCGAATCGCAT GGCCTTGTGCTGGCGATGGTTCATTCAAATTT CTGCCCTATCAACTTTCGATGGTAGGATAGT GGCCTACCATGGTTTCAACGGGTAACGGGGA ATAAGGGTTCGATTCCGGAGAGGGGAGCCTGA GAAACGGCTACCACATCCAAGGAAGGCAGC AGGCGCGCAAATTACCCAATCCTAATTCAGG GAGGTAGTGACAATAAATAACGATACAGGG CCCATTCGGGTCTTGTAATTGGAATGAGTAC AATGTAAATACCTTAACGAGGAACAATTGGA GGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT TCCAGCTCCAATAGCGTATATTAAAGTTGTTG CAGTTAAAAAGCTCGTAGTTGAACTTTGGGC CCGGTTGGCCGGTCCGATTTTTTCGTGTACTG GATTTCCAACGGGGCCTTTCCTTCTGGCTAAC CTTGAGTCCTTGTGGCTCTTGGCGAACCRGG ACTTTTACTTTGAAAAAATTAGAGTGTTCAA AGCAGGCGTATTGCTCGAATATATTAGCATG

GAATAATAGAATAGGACGTTTGGTTCTATTT GTTGGTTTCTAGGACCATCGTAATGATTAATA GGGACGGTCGGGGGGCATCAGTATTCAATGTC AGAGTGAAATTCTTGGATTTATTGAAGACTA ACTACTGCGAAAGCATTTGCYAGAACGTTCA TAATCA

b) Analysis of the gene sequences coding 18S **rRNA:** The similarity of the nucleotide sequence of the gene coding 18S rDNA of the studied strain was analyzed using BLAST server [13].

RESULTS:

Primary screening on GenBank database showed that the studied strain belongs to the following systematic group: Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetas; Saccharomycetales; Saccharomycetaceae; Saccharomyces.

The strains involved in the analysis and the sequence similarity level of 18S rDNA of the studied strain are shown in fig. 2.

Homology of not less than 97% is considered to be a criterion for identifying microorganisms to a particular species [10].

As shown by fig. 2, the analyzed strain can be classified into several species.

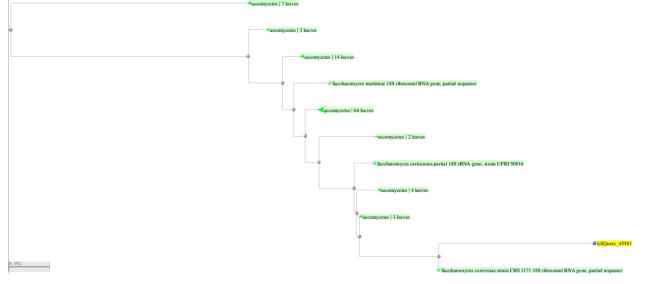


Figure 2 – Strain homology

The method to compare nucleotide sequences coding D1/D2 domain of 26S rRNA gene was also used to establish phylogenetic affinity of similar species.

When sequencing the DNA section coding D1/D2 domain of the 26S rRNA gene, the following sequence was obtained:

ARSRGGGGGGAATGGCCTTAGTAACGGCGAGT GAAGCGGCAAAAGCTCAAATTTGAAATCTGG TACCTTCGGTGCCCGAGTTGTAATTTGGAGA GGGCAACTTTGGGGGCCGTTCCTTGTCTATGTT CCTTGGAACAGGACGTCATAGAGGGTGAGAA TCCCGTGTGGCGAGGAGTGCGGTTCTTTGTA AAGTGCCTTCGAAGAGTCGAGTTGTTTGGGA ATGCAGCTCTAAGTGGGTGGTAAATTCCATC TAAAGCTAAATATTGGCGAGAGACCGATAGC GAACAAGTACAGTGATGGAAAGATGAAAAG AACTTTGAAAAGAGAGAGTGAAAAAGTACGTG AAATTGTTGAAAGGGAAGGGCATTTGATCAG ACATGGTGTTTTGTGCCCTCTGCTCCTTGTGG GTAGGGGAATCTCGCATTTCACTGGGCCAGC ATCAGTTTTGGTGGCAGGATAAATCCATAGG AATGTAGCTTGCCTCGGTAAGTATTATAGCCT GTGGGAATACTGCCAGCTGGGACTGAGGACT GCGACGTAAGTCAAGGATGCTGGCATAATGG TTATATGCCGCCCGTCTTGAAACACGGACCC AAAAATG

Phylogenetic analysis, made by using strains of closely related microorganisms, showed that species *Saccharomyces cerevisiae* (99 %) is the closest to the studied strain.

The analysis of the nucleotide sequence coding a part of rRNA genes showed that the studied **DEN4** strain is the closest to the species *Saccharomyces cerevisiae*.

B1 strain identification based on ribosomal genes sequence analysis.

Analysis stages:

a) Sequencing of sections of the sequence coding 18S rRNA gene: When sequencing the DNA section coding the 18S rDNA gene of the studied strain the following sequence was obtained:

AACTGCGAATGGCTCAAAAAGTCAGTTATCG TTTATTTGATAGTTCCTTTACTACATGGTATA ACTGTGGTAATTCTAGAGCTAATACATGCTT AAAATCTCGACCTTTGGAAGAGATGTATTTA TTAGATAAAAAATCAATGTCTTCGGACTCTTT GATGATTCATAATAACTTTTCGAATCGCATG GCCTTGTGCTGGCGATGGTTCATTCAAATTTC TGCCCTATCAACTTTCGATGGTAGGATAGTG

GCCTACCATGGTTTCAACGGGTAACGGGGAA TAAGGGTTCGATTCCGGAGAGGGGAGCCTGAG AAACGGCTACCACATCCAAGGAAGGCAGCA GGCGCGCAAATTACCCAATCCTAATACAGGG AGGTAGTGACAATAAATAACGATACAGGGCC CATTCGGGTCTTGTAATTGGAATGAGTACAA TGTAAATACCTTAACGAGGAACAATTGGAGG GCAAGTCTGGTGCCAGCAGCCGCGGTAATTC CAGCTCCAATAGCGTATATTAAAGTTGTTGC AGTTAAAAAGCTCGTAGTTGAACTTTGGGCC TGGTTGGCCGGTCCGATTTTTTCGTGTACTGG TTTCCAACCGGGCCTTTCCTTCTGGCTAACCT TGGGTCCTTGTGGCCCTTGGCGAACCAGGAC TTTTACTTTGAAAAAATTAGAGTGTTCAAAG CAGGCGTATTGCTCGAATATATTAGCATGGA ATAATAGAATAGGACGTTTGGTTCTATTTGT TGGTTTCTAGGACCATCGTAATGATTAATAG GGACGGTCGGGGGGGGCATCAGTATTCAATTGT CAGAGGTGAAATTCTTGGATTTATTGAGACT AACTACTGCGAAGCATTTGCCAAGACGTTTT CATATCAGACGAAGTTAGGGGGATCGAGATGA ATCA

b) Analysis of the gene sequences coding 18S **rRNA:** The similarity of the nucleotide sequence of the gene coding 18S rDNA of the studied strain was analyzed using BLAST server [11].

RESULTS:

Primary screening on GenBank database showed that the studied B1 strain belongs to the following systematic group: Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetaceae; Torulaspora.

The strains involved in the analysis and the sequence similarity level of 18S rDNA of the studied strain are shown in fig. 3.

Homology of not less than 97% is considered to be a criterion for identifying microorganisms to a particular species [10]. As shown by fig. 3, the analyzed strain can be classified into several species.

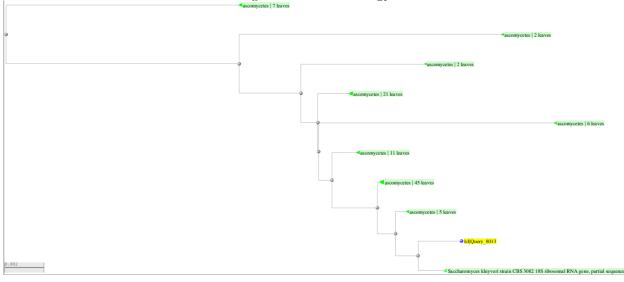


Figure 3 – Strain homology

The method to compare nucleotide sequences coding D1/D2 domain of 26S rRNA gene was also used to establish phylogenetic affinity of similar species.

When sequencing the DNA section coding D1/D2 domain of the 26S rRNA gene, the following sequence was obtained:

AGTGATTCCTTAGTACGGCGAGTGAAGCGGC AAAAGCTCAAATTTGAAATCTGGTACCTTCG GTGCCCGAGTTGTAATTTGTAGAAGGTAACT TTGGGGCTGGTCCTTGTCTATGTTCCTTGGAA CAGGACGTCATAGAGGGTGAGAATCCCGTGT GGCGAGGATCCCAGTTCTTTGTAAAGTGCTTT CGAAGAGTCGAGTTGTTTGGGAATGCAGCTC TAAGTGGGTGGTAAATTCCATCTAAAGCTAA ATATTGGCGAGAGACCGATAGCGAACAAGTA CAGTGATGGAAAGATGAAAAGAACTTTGAA AAGAGAGTGAAAAAGTACGTGAAATTGTTGA AAGGGAAGGGCATTTGATCAGACATGGTGTT TTGCGCCCTCTGCTCCTTGTGGGTGGGGGGAAT CTCGCAGCTCACTGGGCCAGCATCAGTTTTG GCGGCAGGATAAATCTGCAGGAATGTAGCTT GCCTCGGTAAGTGTTATATCCTGTAGAAATA CTGCCAGCTGGGACTGAGGACTGCGACTTTA CGTCAAGGATGCKGGCATAATGGTTATRTGC CGCCCGYCTTYAAMMMRRRRMMMMACAAA AAAAA

Phylogenetic analysis, made by using strains of closely related microorganisms, showed that species Torulaspora delbrueckii (99 %) is the closest to the studied strain.

The analysis of the nucleotide sequence coding a part of rRNA genes showed that the studied **B1** strain is the closest to the species *Torulaspora delbrueckii*.

The analysis of the nucleotide sequence coding a part of rRNA genes showed that the studied strain isolated from the surface of Burgund grape berries is the closest to the species *Hanseniaspora uvarum*. The yeast strain *Saccharomyces cerevisiae* was isolated from the surface of hops cones growing in the botanical garden of Gorsky state agrarian university. Yeasts isolated from the soil samples of drainfields belong to the species *Torulaspora delbrueckii*.

CONCLUSIONS:

Identified yeast strains can be of interest for different biotechnological production. Thus, yeasts Saccharomyces cerevisiae and Hanseniaspora uvarum can be used in winemaking. Isolated yeast strain Torulaspora delbrueckii can be recommended as a decomposer of the molasses distillery stillage because it was isolated from places of lasting xenobiotic emission.

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