Molecular-genetic and biochemical characterization of *Saccharomyces cerevisiae* strain 36-6G

Remzi Cholakov, Rositsa Denkova, Velichka Yanakieva, Zapryana Denkova, Zoltan Urshev, Safie Syuleyman

Molecular-genetic and biochemical characterization of Saccharomyces cerevisiae strain 36-6G. *Yeast strain 36-6G isolated from naturally fermented cereal beverage (boza) is selected. It is identified as a representative of the species Saccharomyces cerevisiae applying molecular-genetic method – partial sequencing of the 26S rDNA. The enzymatic profile of the strain is determined applying the kit system API ZYM (BioMerieux, France). Its proteolytic and amylase activities are examined as well. It is shown that the Saccharomyces cerevisiae strain 36-6G exhibits amylase activity. The strain is suitable for inclusion in the composition of starter cultures for the production of fermented cereal foods and beverages.*

Key words: *Saccharomyces, boza, cereal beverage, identification, sequencing, enzymatic profile, amylolytic activity*

INTRODUCTION

Boza is a traditional low-alcoholic fermented cereal beverage. Millet, maize, wheat, rice and many other cereal crops are used for the production of boza [1]. The origin of that beverage dates back to ancient Mesopotamian and Anatolian civilizations [8]. Nowadays it is a widespread drink in Turkey and Bulgaria [9], popular among all age groups. Because of its physical properties it is defined as a drink with thick texture, light or dark beige in colour, with slightly sharp or slightly sour taste, with a specific scent. Boza is obtained by natural fermentation of cereal substrates by lactic acid bacteria: *Lactobacillus fermentum, Lb. sanfrancisco, Lb. rhamnosus, Lb. plantarum, Lb. pentosus, Leuconostoc paramesenteroides, Leu.mesenteroides ssp. mesenteroides, Leu. raffinolactis, Lactococcus lactis* subsp. *lactis, Oenococcus oeni, Weissella paramesenteroides* and *W. confusa* and yeasts: *Saccharomyces cerevisiae, S. uvarum, S. carlsbergensis, Candida glabrata, C. tropicalis, Geotrichum candidum* and *G. penicilatum* [1, 5, 7, 10].

The purpose of the present study is the biochemical and molecular-genetic identification of Yeast strain 36-6G isolated from naturally fermented cereal beverage – boza, and determination of its enzymatic profile.

MATERIALS AND METHODS

1. Microorganisms

Yeast strain 36-6G is isolated from naturally fermented cereal beverage (boza).

2. Nutrient media

2.1. Saline solution. Composition (g/dm^3): NaCl - 5. Sterilization - 20 minutes at 121ºC.

2.2. Malt-agar. Composition: malt extract (Kamenica, Bulgaria), dilluted in a ratio of 1:1 with tap water + 2% agar, pH is adjusted to 6.5 – 7.0. Sterilization - 25 minutes at 121ºC [2].

2.3. Solid medium for the determination of amylase activity. Composition (g/dm^3): meat extract - 3, peptone - 5, soluble starch - 2, agar - 15. pH is adjusted to 7.2 ± 0.1. Sterilization - 25 minutes at 121ºC.

2.4. Solid medium for the determination of proteolytic activity. Composition (g/dm^3): skimmed milk - 28, casein hydrolyzate - 5, yeast extract – 2.5, glucose - 1, agar - 15. pH is adjusted to 7 ± 0.2. Sterilization - 25 minutes at 121ºC.

3. Culturing and storage of the test microorganism

The studied yeast strain is grown on malt-agar at 28ºC for 48 hours and is stored at 4 ± 2ºC for 2 months.

4. Physiological Methods

4.1. Agar-diffusion method for determining the amylase activity

This method comprises in determining the ability of the tested strain to hydrolyze starch. The solid medium for the determination of amylase activity is melted and poured in Petri dishes (15 cm^3 of the medium per Petri dish). After the hardening of the medium wells with a diameter of 6 mm are made. The cellular suspension of the tested strain is pipetted into the wells. This test is performed in quadruplicates. After inoculation, the plates are cultured at 30°C for 48 hours. The results are reported as positive if there is a more turbid halo around the wells in the Petri dishes. The lack of a halo is a sign of the inability of the strain to hydrolyze starch.

4.2. Agar-diffusion method for determining the proteolytic activity

This method comprises in determining the ability of the tested strain to digest milk proteins. The solid medium for the determination of proteolytic activity is melted and poured in Petri dishes (15 cm^3 of the medium per Petri dish). After the hardening of the medium wells with a diameter of 6 mm are made. The cellular suspension of the tested strain is pipetted into the wells. This test is performed in quadruplicates. After inoculation, the plates are incubated at 30ºC for 48 hours. The results are reported as positive if there is a bright halo around the wells of the Petri dishes. The absence of a halo is a sign of the inability of the strain to hydrolyze milk proteins.

5. Biochemical methods

5.1. Determination of the profile of the enzyme activity of the test cultures.

The determination of the profile of enzyme activity is performed, using the test kit API ZYM (BioMerioux, France) for semi-quantitative determination of the enzyme profile of the studied strain. Fresh 24-hour culture of the tested strain is centrifuged for 15 minutes at 5000 x g, the obtained biomass precipitate is washed twice and resuspended in API suspension medium. The API ZYM strips are placed in the incubation boxes and the microtubules are inoculated with the prepared cell suspension. The sample is incubated for 4 to 4,5 hours at 37°C. After the incubation one drop of reagent A and one drop of reagent B are pipetted into each microtubule. After 5 min staining is reported according to the color scheme described in the manufacturer's instructions. The enzyme activity is determined according to a color scale from 0 (no enzyme activity) to 5 (maximum enzyme activity).

6. Genetic methods

6. 1. Isolation of total DNA

The isolation of DNA is performed by the method of Delley et al. [4].

6.2. 26S rDNA amplification and visualization.

All PCR reactions are performed using the PCR kit – PCR VWR in a volume of 25 µl in a Progene cycler (Techne, UK) according to the instructions of the manufacturer. In each PCR reaction 50 ng total DNA of the tested strain and 10 pmol praimers are used. DNA of the studied strain is amplified using universal primers for the 26S rDNA gene - NL1 (5'-GCATATCAATAAGCGGAGGA AAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') [6]. The amplification program includes: denaturation - 95°C for 3 minutes; 40 cycles - 93°C for 30 s, 55°C for 60 s, 72°C for 2 minutes; final elongation - 72°C for 5 minutes. The resulting product is visualized on a 2% agarose gel stained with ethidium bromide solution (0.5 µg/ml), using an UVP Documentation System (UK).

6.3. Purification of the product of the PCR-reaction – end fragment of the 26S rDNA – from TAE agarose Gel

The purification of 16S rDNA is conducted using DNA-purification kit (GFX MicrospinTM) according to the manufacturer's instructions.

6.4. Partial sequencing of the 26S rRNA gene.

The partial sequencing of the 26S rRNA gene is conducted by "Macrogen Europe Laboratory", Netherlands, based on the method of Sanger.

RESULTS AND DISCUSSION

Yeast strain 36-6G is isolated from naturally fermented cereal beverage - boza.

By examining the ability of Yeast strain 36-6G to utilize 19 carbon sources included in the kit system API 20C Aux for quick identification and testing its ability to form pseudo mycelium the studied strain is identified to be a representative of the species *Cryptococcus laurentii* with percentage of reliability 82.2% [3].

In order to confirm the species identification of the studied strain obtained using biochemical methods for identification, molecular-genetic method for genotyping – partial sequencing of the gene for the 26S rRNA is applied. After careful comparison of the obtained sequence with the public online nucleotide BLAST database, Yeast strain 36-6G is determined to be a *Saccharomyces cerevisiae* strain with 100% complementation between the partial sequence of the studied strain and the partial sequence of the 26S rDNA gene of *Saccharomyces cerevisiae* strain UL139 (Fig. 1).

***Saccharomyces cerevisiae* strain UL139 26S ribosomal RNA gene, partial sequence**

Score	Expect	Identities	Gaps	Strand
1035 bits(538)	0.0	538/538(100%)	0/538(0%)	Plus/Minus

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Query  17   TGCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCAGCTGGCAGTATTCCCACAGGCT  76
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  TGCAGCATCCTTGACTTACGTCGCAGTCCTCAGTCCCAGCTGGCAGTATTCCCACAGGCT  488

Query  77   ATAATACTTACCGAGGCAAGCTACATTCCTATGGATTTATCCTGCCACCAAAACTGATGC  136
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  ATAATACTTACCGAGGCAAGCTACATTCCTATGGATTTATCCTGCCACCAAAACTGATGC  428

Query  137  TGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGGAGCAGAGGGCACAAAACACCATG  196
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  TGGCCCAGTGAAATGCGAGATTCCCCTACCCACAAGGAGCAGAGGGCACAAAACACCATG  368

Query  197  TCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAAG  256
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  TCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAAG  308

Query  257  TTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCT  316
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  TTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCT  248

Query  317  TTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCTTCGAAGG  376
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  TTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCTTCGAAGG  188

Query  377  CACTTTACAAAGAACCGCACTCCTCGCCACACGGGATTCTCACCCTCTATGACGTCCTGT  436
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  CACTTTACAAAGAACCGCACTCCTCGCCACACGGGATTCTCACCCTCTATGACGTCCTGT  128

Query  437  TCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTGCCCTCTCCAAATTACAACTCGGGC  496
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  TCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTGCCCTCTCCAAATTACAACTCGGGC  68

Query  497  ACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCGCTTCACTCGCCGTACTAAGCA  554
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   ACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCGCTTCACTCGCCGTACTAAGCA  10
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Fig. 1. Comparison between the partial nucleotide sequence of the 26S rDNA of Yeast strain 36-6G and the partial sequence of the 26S rDNA of *Saccharomyces cerevisiae* UL139

For a more accurate analysis of the possibilities of the studied strain for use in the production of fermented foods and beverages its enzymatic profile using the system API ZYM is examined. The studies reveal the presence of alkaline phosphatase, C_4 lipase, C_8 lipase, leucine-aminopeptidase, valine-aminopeptidase, cystine-aminopeptidase, acid phosphatase, naphthol-AS-BL-phosphohydrolase, α-glucosidase. The strain lacks β-galactosidase and therefore does not develop in media containing lactose as only carbon source (Table 1).

The amylolytic and the proteolytic activity of *Saccharomyces cerevisiae* strain 36-6G are examined by agar-diffusion methods. Results are recorded as positive (presence of enzymatic activity) if the diameter of the zones around the wells is bigger than 6 mm. Experimental data show that the strain possesses amylolytic activity but lacks proteolytic activity. The results of these experimental tests are shown in Table 2.

Table 1. Enzymatic profile of *Saccharomyces cerevisiae* strain 36-6G

	Enzyme	Activity* *Saccharomyces cerevisiae* 36-6G	
1	Control	-	
2	Alkaline phosphatase	1	
3	Esterase	2	
4	Esterase-lipase	1,5	
5	Lipase	-	
6	Leucine-aminopeptidase	4	
7	Valine-aminopeptidase	1	
8	Cysteine-aminopeptidase	1	
9	Trypsin	-	
10	Chymotrypsin	-	
11	Acid phosphatase	3,5	
12	Phosphohydrolase	1,5	
13	α-galactosidase	-	
14	β -galactosidase	-	
15	β -glucoronidase	-	
16	α -glucosidase	3	
17	β-glucosidase	-	
18	α -glucoseaminidase	-	
19	α –manosidase	-	
20	α –fucosidase	-	

* The enzymatic activity is determined according to a color scale from 0 (no enzymatic activity) to 5 (maximum enzymatic activity)

Table 2. Proteolytic and amylolytic activity of *Saccharomyces cerevisiae* strain 36-6 G

Activity, mm / Strain	Amylolytic activity	Proteolytic activity
Saccharomyces cerevisiae 36-6G	12	-

CONCLUSION

1. The species identification of Yeast strain 36-6G, isolated from naturally fermented cereal beverage, is achieved by applying modern physiological, biochemical and molecular-genetic methods for identification.

2. It is shown that the biochemical system for rapid identification of yeasts API 20C Aux does not give reliable identification results.

3. The enzymatic profile of *Saccharomyces cerevisiae* strain 36-6G is determined. The strain demonstrates alkaline phosphatase, C_4 lipase, C_8 lipase, leucine-aminopeptidase, valine-aminopeptidase, cystine-aminopeptidase, acid phosphatase, naphthol-AS-BL-phosphohydrolase, α-glucosidase and amylolytic activity.

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About the authors:

PhD Student Remzi Gyultekinov Cholakov, Department of “Microbiology”, University of Food Technologies, Plovdiv, 26 Maritza Blvd., Telephone: 0889-761 720, e-mail: inj.cholakov@gmail.com

This paper has been reviewed