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MOLECULAR-GENETIC AND BIOCHEMICAL CHARACTERIZATION OF *SACCHAROMYCES CEREVISIAE* STRAIN 25-G, ISOLATED FROM FERMENTED CEREAL BEVERAGE

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Abstract: *Yeast strain 25-G was isolated from naturally fermented cereal beverage (boza). By biochemical (API 20 C Aux) and molecular-genetic (partial sequencing of the 26S rRNA gene) methods, a representative of the species Saccharomyces cerevisiae var. diastaticus was identified. The enzymatic profile of the strain was determined by applying a kit system API ZYM (BioMerieux, France). Its proteolytic and amylase activities were examined as well. Saccharomyces cerevisiae var. diastaticus strain 25-G exhibits amylase activity, which makes it suitable for being included in the composition of starter cultures used at the production of fermented cereal foods and beverages.*

Key words: *boza, identification, sequencing, enzyme profile, amylolytic activity*

1. Introduction

Cereal foods and beverages are a major source of nutrients. Boza is a traditional low-alcohol fermented cereal beverage. It is made of millet, corn, wheat, rice and others [1]. It is defined as a beverage thick in texture, light or dark beige in colour, slightly sharp or slightly sour in taste, with specific odor, which is naturally fermented by lactic acid bacteria and yeasts such as: *Lactobacillus fermentum*, *Lactobacillus sanfranciscensis*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Leuconostoc paramesenteroides*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc raffinolactis*, *Lactococcus lactis* subsp. *lactis*, *Oenococcus oeni*, *Weissella paramesenteroides*, and *Weissella confusa* and yeasts: *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces carlsbergensis*, *Candida glabrata*, *Candida tropicalis*, *Geotrichum candidum*, and *Geotrichum penicilatum* [1, 2, 3, 4].

The purpose of the present study was the biochemical and molecular-genetic identification of yeast strain 25-G isolated from naturally fermented cereal beverage – boza, and determination of its enzyme profile.

2. Materials and methods

2.1. Microorganisms

Yeast strain 25-G was isolated from naturally fermented cereal beverage (boza).

2.2. Nutrient media

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

2.2.2. ***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 [5].

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.

2.2.4. ***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.

2.3. Culturing and storage of the test microorganism

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

2.4. Physiological Methods

2.4.1. Agar-diffusion method for determining the amylase activity

This method comprised in determining the ability of the tested strain to hydrolyze starch. The solid medium for the determination of amylase activity was 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 were made. The cellular suspension of the tested strain was pipetted into the wells. This test was performed in quadruplicates. After inoculation, the plates were cultured at 30°C for 48 hours. The results were reported as positive if there was a more turbid halo around the wells in the Petri dishes. The lack of a halo was a sign of the inability of the strain to hydrolyze starch.

2.4.2. Agar-diffusion method for determining the proteolytic activity

This method comprised in determining the ability of the tested strain to digest milk proteins. The solid medium for the determination of proteolytic activity was 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 were made. The cellular suspension of the tested strain was pipetted into the wells. This test was performed in quadruplicates. After inoculation, the plates were incubated at 30ºC for 48 hours. The results were reported as positive if there was a bright halo around the wells of the Petri dishes. The absence of a halo was a sign of the inability of the strain to hydrolyze milk proteins.

2.5. Biochemical methods

2.5.1. Determination of the biochemical profile

The system API 20 C Aux (BioMerieux SA, France) for identification of yeast species based on the consumption of 19 carbon sources was used for the determination of the biochemical profile of the tested strain. Fresh 24-hour culture of the tested strain, developed on malt agar, was resuspended according the instructions of the manufacturer in API C resuspension medium. The honeycomb wells on the bottom of the incubation boxes were filled with sterile physiological solution. The API 20 C strips were placed in the

incubation boxes and the microtubules were inoculated with the prepared cell suspension. The sample was incubated for 48h to 72h at the optimum temperature for the studied strain. Results were recorded according the change in turbidity in comparison to the control (microtubule 0). The results were processed with apiweb® identification software.

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

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

2.6. Genetic methods

2.6. 1. Isolation of total DNA

The isolation of DNA was performed by the method of Delley et al. [6].

2.6.2. 26S rDNA amplification and visualization

All PCR reactions were 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 were 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') [7]. The amplification program included: 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 was visualized on a 2% agarose gel stained with ethidium bromide solution (0.5 µg/ml), using an UVP Documentation System (UK).

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

The purification of fragment of the 26S rDNA was conducted using DNA-purification kit (GFX MicrospinTM) according to the manufacturer's instructions.

2.6.4. Partial sequencing of the 26S rRNA gene.

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

3. Results and discussion

The yeast strain 25-G was isolated from naturally fermented cereal beverage.

By determining the ability of the strain to utilize the 19 carbon sources included in the kit system for rapid identification of yeasts API 20 C Aux the yeast strain 25-G was identified with poor reliability:

Cryptococcus laurentii - 46,1%, *Cryptococcus huminicula* - 29,5%, *Trichosporon mucoides* - 23,4%.

Therefore a molecular-genetic method for identification was applied - sequencing of the gene for the 26S rRNA. The yeast strain 25-G belongs to the species *Saccharomyces cerevisiae* with a percentage of similarity between the partial sequence of the 26S rDNA of yeast strain 25-G and the partial sequence of the 26S rDNA of *Saccharomyces cerevisiae* strain LQC 10089 - 99% (Fig. 1).

***Saccharomyces cerevisiae* strain LQC 10089 26S ribosomal RNA gene, partial sequence**

Score	Expect	Identities	Gaps	Strand
877 bits(972)	0.0	492/496 (99%)	0/496(0%)	Plus/Plus

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Query  44   GCTCAAATTTGAGTCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTG  103
            ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  40   GCTCAAATTTGAATCTGGTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTG  99

Query  104  GGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGT  163
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100  GGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGT  159

Query  164  GGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAG  223
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160  GGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAG  219

Query  224  ATCTAAGTGGGTGGTAAATTCCATGTAAAGCTAAATATTGGCGAGAGACCGATAGCGAAC  283
             ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  220  CTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAAC  279

Query  284  AAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAAT  343
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280  AAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAAT  339

Query  344  TGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGG  403
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340  TGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGG  399

Query  404  TAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTTGGTGGCAGGATAAATCCATAG  463
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400  TAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTTGGTGGCAGGATAAATCCATAG  459

Query  464  GAATGTAGCTTGCCTCGGTAAGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAG  523
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  460  GAATGTAGCTTGCCTCGGTAAGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAG  519

Query  524  GACTGCGATGTAAGTC  539
            |||||||| |||||||
Sbjct  520  GACTGCGACGTAAGTC  535
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Fig.1 Comparison of the partial sequence of the 26S rDNA of yeast strain 25- G and the partial sequence of the 26S rDNA of *Saccharomyces cerevisiae* strain LQC 10089.

The enzyme profile of the studied strain was determined using the kit system API ZYM. It demonstrated leucine - aminopeptidase, acid phosphatase, phosphohydrolase, α-glucosidase activity. The strain does not possess the ability to synthesize β-galactosidase, which is why it oes not develop in nutrient media containing lactose as substrate (Table 1).

Experimental data demonstrate that the studied strain can be included in the composition of starter cultures for fermented cereal foods and beverages.

The amylolytic and proteolytic activity of *Saccharomyces cerevisiae* strain 25-G was determined by the agar-diffusion method with wells.

Table 1
Enzyme profile of *Saccharomyces cerevisiae 25-G*

	Enzyme	Activity* *Saccharomyces cerevisiae* 25-G
1	Control	-
2	Alcaline phosphatase	1
3	Esterase	2
4	Esterase-lipase	1,5
5	Lipase	-
6	Leucine-aminopeptidase	5
7	Valine-aminopeptidase	1
8	Cysteine-aminopeptidase	-
9	Trypsin	-
10	Chimotrypsin	-
11	Acid phosphatase	5
12	Phospohydrolase	2
13	α-galactosidase	-
14	β-galactosidase	-
15	β-glyucoronidase	-
16	α-glyucosidase	4
17	β- glyucosidase	-
18	α-glyucosaminidase	-
19	α-manosidase	-
20	α-fucosidase	-

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

The strain demonstrated significant amylolytic activity but lacks proteolytic activity (Table 2).

Table 2.
Proteolytic and amylolytic activity of *Saccharomyces cerevisiae* strain 25-G.
d_{well} = 6mm

d_{zone}, mm / Strain	Amylolytic activity	Proteolytic activity
Saccharomyces cerevisiae 25-G	14	-

4. Conclusion

By modern physiology, biochemistry and molecular-genetic methods the newly isolated yeast strain 25-G, isolated from naturally fermented cereal beverage, was identified. It was shown that the system for rapid identification API 20 C Aux does not usually have enough discriminative ability. *Saccharomyces cerevisiae* 25-G exhibited leucine - aminopeptidase, acid phosphatase, phosphohydrolase, α-glucosidase and amylolytic activity.

5. References

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