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PRODUCERS, BIOLOGY, SELECTION, AND GENE ENGINEERING

Selection of *Saccharomyces bayanus* Strains with High Pectinolytic Activity and Phylogenetic Analysis of *PGU* Genes

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Abstract—For the first time, a large-scale screening has been carried out of pectinolytic activity in *Saccharomyces bayanus* strains isolated in various regions of the world from fermentation processes and natural sources. The ability to secrete active endo-polygalacturonase was shown to be a feature of this species. Regardless of the source and place of isolation, *S. bayanus* var. *uvarum*, *S. bayanus* var. *bayanus* and *S. eubayanus* have the *PGU1b PGU2b PGU3b* genotype. According to phylogenetic analysis, the pectinase genes (*PGU*) of the hybrid brewer's yeast *S. pastorianus* are originated from the cryophilic *S. bayanus* yeast and not from *S. cerevisiae*. Five selected *S. bayanus* strains with the highest pectinolytic activity are promising for further molecular genetic studies and breeding of wine yeasts.

Keywords: wine yeasts, *Saccharomyces bayanus* var. *uvarum*, *Saccharomyces bayanus* var. *bayanus*, *Saccharomyces eubayanus*, endo-polygalacturonase, pectinase, *PGU* genes, phylogenetic analysis

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INTRODUCTION

Pectin is a plant polysaccharide consisting of galacturonic acid residues partially esterifed with methanol and linked by the α(1–4)-glycosidic bonds. Pectin compounds, as structural elements of plant tissues, are contained in all higher plants and contribute to their tolerance to both biotic and abiotic environmental factors. The greatest amount of pectin is characteristic of berries, fruits and vegetables. As an example, in grapes, this is 0.5–5.0 g/L depending on the cultivar. Mechanical grinding of fruits rich in pectin results in obtaining a highly viscous juice, which remains bound to the pulp and forms a jelly-like mass. Even a small content of pectins in wine may cause colloidal turbidities and filter clogging [1].

Hydrolysis of high-molecular plant compounds is a complex process involving several enzymes; the main one is pectinase (endo-polygalacturonase, EC 3.2.1.15). Pectinolytic enzymes are widely used in biotechnology for the clarification of fruit juices and wines, fermentation of tea and coffee, purification of plant fibers and waste water, bleaching paper, etc. [2–7]. Pectinases account for approximately 25% of the global food enzyme purchases and their production is constantly growing [8]. The pectinolytic enzymes are used most actively in winemaking and fruit juice manufacture. The treatment of pulp with pectinases improves the separation and clarification of the wort, reduces excessive foaming during fermentation and increases the organoleptic characteristics of the final product [9].

Almost all commercial enzyme preparations are obtained from the *Aspergillus* and *Trichoderma* filamentous fungi. Apart from endo-polygalacturonases, these fungal preparations also contain admixtures and enzymes with undesirable side activities, for instance, pectinesterase, which leads to an enhanced content of toxic methanol in wine [10]. Yeast usually do not secrete pectinesterase; therefore, their pectinases are safe for wine and juice clarification [11, 12]. It has been shown that the use of *Saccharomyces cerevisiae* strains with endo-polygalacturonase activity in winemaking results in an efficient wine clarification and a two-fold decrease in its filtration time [9].

The *Saccharomyces* genus includes eight species: *S. cerevisiae*, *S. arboricola, S. cariocanus*, *S. bayanus*, *S. kudriavzevii*, *S. jurei*, *S. mikatae* and *S. paradoxus* [13–17]. *S. arboricola*, *S. kudriavzevii* and *S. mikatae* have low pectinolytic activity, while some strains of *S. cariocanus*, *S. paradoxus* and *S. jurei* are able to readily degrade pectin compounds [18]. The above

Abbreviations: dNTP, deoxyribonuceloside triphosphate; Pgu1, endo-polygalacturonase; UV, ultraviolet.

yeasts are not associated with human economic activities; usually, they are isolated from the exudate and bark of broad-leaved trees, various insects, soils, etc. Two *Saccharomyces* species, *S. cerevisiae* and *S. bayanus*, are involved in winemaking together with their interspecific hybrids: *S. cerevisiae* × *S. bayanus*, *S. cerevisiae* × *S. kudriavzevii* and *S. cerevisiae* × *S. bayanus* × *S. kudriavzevii* [19–24].

As a rule, the *S. cerevisiae* yeast is characterized by extremely low pectinolytic activity or is even unable to degrade pectin compounds [11, 25, 26]. At the same time, some strains of the related species *S. bayanus* actively secrete endo-polygalacturonase [18, 27, 28]. The *S. bayanus* species has a complex composition, including two varieties, *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum* [21, 29]. The latter occupies the specific ecological niche in viticulture and winemaking at low temperatures [30]. These yeasts are associated with the production of white, sweet, and sparkling wines, as well as cider [19, 31–36]. *S. bayanus* var. *bayanus* is mainly represented by brewery contaminating strains [21]. In 2011, the related yeast *S. eubayanus* was isolated from the bark of beeches in Argentina [37]. Later, this species was found in China [38], the United States, and Canada [38, 40]. The yeast has been partially genetically isolated; its hybrids are characterized by low survival of ascospores [41]. The European bottom fermenting brewer's yeast *S. pastorianus* (syn. *S. carlsbergensis*) is an interspecific hybrid of *S. cerevisiae* and *S. bayanus*, and its cold resistance is inherited from *S. bayanus*. Whole genome sequencing of several *S. eubayanus* strains revealed their high similarity with the cold-resistant parent of hybrid yeast *S. pastorianus* [40, 42, 43].

The goal of this work was to study the pectinolytic activity of the *S. bayanus* var. *bayanus*, *S. bayanus* var. *uvarum* and *S. eubayanus* yeasts using strains of different ecological and geographical origin, as well as to select strains with high pectinolytic activity that are of interest for the wine yeast selection.

MATERIALS AND METHODS

The origin of the studied *S. bayanus* strains is indicated in Table 1. The yeast was cultivated at 28°C on a complete YPD nutrient medium containing, g/L: yeast extract, 10; peptone, 20; glucose, 20; and agar, 20. To prepare native chromosomal DNA, the yeast was grown in 15 mL of liquid YPD at 28°C for 12–16 h.

Yeast Pectinolytic Activity

Screening for pectinolytic activity was carried out by the Louw et al. method [26] with some modifications. Yeasts were grown on YPD solid medium. Overnight yeast cultures were inoculated with a loop onto a minimal medium with polygalacturonic acid, containing, g/L: yeast nitrogen base with amino acids (Difco, United States), 6.7; polygalacturonic acid (Sigma, United States), 12.5; glucose, 10; agar (Difco), 20; and Na_2HPO_4, 6.8 (pH 4.0). Cells were grown for 3 days at 28°C; the formed yeast colonies were washed off with distilled water, after which Petri dishes were filled with 6 M HCl for 5–10 min. Enzymatic activity was evaluated by clear hydrolysis zones (halos) of polygalacturonic acid around the yeast colonies. Petri dishes were photographed and the size of the halos was determined using the IC Measure_2.0.0.272 software (www.helicon.ru). Two independent experiments were carried out for each strain. The patented *S. cerevisiae* VKPM Y-718 strain (a polyploid of the Kokur-3 wine strain) with high pectinolytic activity [44] was used as a control.

Pulsed-Field Gel Electrophoresis of Native Chromosomal DNA (Molecular Karyotyping) and Southern Hybridization

Chromosomal DNA was isolated as described in [31] and separated on a CHEF-DR III device (Bio-Rad, United States). Samples were placed in the wells of 1% agarose gel. Pulsed-field gel electrophoresis was performed at 200 V for 15 h at a field switching time of 60 s and for 9 h at a field switching time of 90 s. 0.5 × TBE (45 mM Tris, 10 mM EDTA, and 45 mM boric acid, pH 8.0) cooled to 14°C was used as electrophoresis buffer. The *S. cerevisiae* YNN 295 strain (Bio-Rad) with a known order and size of chromosomes served as a reference karyotypic. After electrophoresis, the gel was stained with ethidium bromide for 2–3 h, then washed in distilled water for 2 h and photographed in UV light.

Chromosomal DNA was transferred onto a nitrocellulose membrane using a Vacuum blotter (Bio-Rad). DNA was fixed on the membrane by annealing at 80°C for 2 h. A PCR-amplified fragment covering the main portion of the gene *PGU1b* coding region of *S. bayanus* var. *uvarum* CBS 7001 served as a probe. The label was introduced by a nonradioactive method using digoxigenin-labeled dUTP (dig-II-dUTP) from the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Switzerland), according to the producer's instructions. Hybridization and development of hybridization bands were also carried out according to these instructions.

Sequencing and Phylogenetic Analysis

PCR was performed using a DNA amplifier (Bio-Rad). Yeast DNA was isolated by the Lõoke et al. method [45]. *S. bayanus* pectinase genes *PGU1b*, *PGU2b* and *PGU3b* were amplified with the following primer pairs: PGU13/PGU14 (5'-CCACCAAACGCAATGATTT-3'/5'-ATGATGCACCTGAGCCAGAT-3'); PGE11/PGE12 (5'-GCTTTATGCGCTTTTGCTGT-3'/5'-AACCAGATGGGATTCCAGAA-3') and PGB51/-PGB52 (5'-TTTTGCTGTCTCAGCAGCTC-3'/5'-TTCCAGAACAGCCAGAAAAGG-3'). Primers

Table 1. The origin and pectinolytic activity of *Saccharomyces* strains

Number	Strain[a]	Source/Location	Diameter[b], mm	Group number
		S. bayanus var. *bayanus*		
1	CBS 425	Apple juice/Switzerland	11.5	2
2	CBS 424	Pear juice	13.6	2
3	CBS 380^T	Cloudy beer/Europe	14.1	2
4	NBRC 1948	Spoiled beer/Europe	18.7	3
5	CBS 378	Beer/Europe	23.7	4
		S. bayanus var. *uvarum*		
6	PJS1.94	Fermenting pulp/Bordeaux, Sauternes, France	0	1
7	SRC410	Apple juice/Normandy, France	8.6	1
8	LC1.95	Fermenting pulp/Sancerre, France	9.1	1
9	PJP1.95	Fermenting pulp/Pouilly-Fume, France	10.4	2
10	PJP11.94	Fermenting pulp/Pouilly-Fume, France	10.5	2
11	UWO(PS) 99-808.3	Beech *Nothofagus* sp. sap/Patagonia, Argentina	10.7	2
12	VKM Y-362	Tokay wine/Slovakia	10.9	2
13	PJS2.95	Fermenting pulp/Sancerre, France	11.0	2
14	SCU 74	Muscadet wine/ Loire Valley, France	11.1	2
15	M300	Red wine/Rostov, Russia	11.2	2
16	CECT 1369	Wine/Spain	11.4	2
17	SCU 299	Muscadet wine/ Loire Valley, France	11.4	2
18	CCY21-31-12	*Amanita citrine* mushroom/Slovakia	11.5	2
19	PYCC 6330	*Cyttaria hariotii* fruit body/Patagonia, Argentina	11.7	2
20	T4/1	Tokay wine/Hungary	11.8	2
21	VS2.94	Fermenting pulp/Bordeaux, Sauternes, France	11.8	2
22	DBVPG 1690	Grapes/Italy	12.2	2
23	SRC306	Apple juice/Normandy, France	12.4	2
24	17e1	Grape juice, Bordeaux, France	12.4	2
25	SRC55	Apple juice/Bretagne, France	12.7	2
26	SC4	Champagne/Champagne, France	12.7	2
27	Sμ1-5A	Grape berries/Sancerre, Loir Valley, France	12.7	2
28	IFI 371	Wine/Spain	12.9	2
29	PYCC 6864	*Cyttaria gunni* on *Nothofagus menziesii*/New Zealand	13.0	2
30	VKM Y-509	Tokay wine/Slovakia	13.1	2
31	CECT 1884	Wine/Spain	13.3	2
32	YIIC2.93	Fermenting pulp/Bordeaux, Sauternes, France	13.3	2
33	SRC274	Apple juice/Normandy, France	13.3	2
34	TBIIbI3.92	Fermenting pulp/Bordeaux, Sauternes, France	13.6	2
35	IFI 373	Wine/Spain	13.7	2
36	IFI 369-4B	Wine/Spain	13.7	2
37	M471	Grapes/Moldova	13.9	2
38	VKM Y-364	Tokay wine/Slovakia	13.9	2
39	148.01	Elm exudate *Ulmus pumila*/Blagoveshchensk, Russia	13.9	2
40	PYCC 6868	*Nothofagus solandri* var. *solandri* bark/Lewis Pass, New Zealand	14.0	2
41	SCU 397	Muscadet wine/ Loire Valley, France	14.2	2
42	PYCC 7082	*Cyttaria* sp. on *Nothofagus dombeyi*/Patagonia, Argentine	14.5	2

Table 1. (Contd.)

Number	Strain[a]	Source/Location	Diameter[b], mm	Group number
43	SCU 197	Muscadet wine/ Loire Valley, France	14.5	2
44	136.01	Elm exudate *Ulmus pumila*/Blagoveshchensk, Russia	14.7	2
45	SCU 374	Muscadet wine/ Loire Valley, France	15.1	3
46	CBS 377	Pear wine/Germany	15.2	3
47	VKM Y-508	Tokay wine/Slovakia	15.2	3
48	TBVIc2.95	Fermenting pulp/Bordeaux, Sauternes, France	15.3	3
49	DBVPG 1693	Grapes/Italy	15.5	3
50	CBS 7001	Caddisfly *Mesophylax adopersus*/Spain	15.5	3
51	D13	White wine/Alsace, France	15.6	3
52	VKM Y-363	Tokay wine/Slovakia	15.6	3
53	PYCC 7083	*Nothofagus pumillio* bark/Argentina	15.6	3
54	M369	Wine/ Slovakia	16.0	3
55	PYCC 6867	*Nothofagus solandri* var. *solandri* bark/New Zealand	16.3	3
56	M488	Grapes/Moldova	16.4	3
57	M478	Grapes/Moldova	16.6	3
58	SCU 11	Muscadet wine/ Loire Valley, France	16.6	3
59	T5/6	Tokay wine/Hungary	17.0	3
60	VKM Y-1146	Grapes/Michurinsk, Russia	17.2	3
61	DDI4.95	Fermenting pulp/Bordeaux, Barsac, France	17.5	3
62	PYCC 6865	*Nothofagus cunninghamii* bark/Tasmania, Australia	17.7	3
63	PYCC 6869	*Nothofagus solandri* var. *solandri* bark, Lewis Pass, New Zealand	17.7	3
64	VKM Y-361	Tokay wine/Slovakia	17.7	3
65	SCU 13	Muscadet wine/ Loire Valley, France	18.0	3
66	SRC258	Apple juice/Normandy, France	18.0	3
67	CBS 395^T	Black current *Ribes nigrum* juice/Netherlands	18.0	3
68	M477	Grapes/Moldova	18.5	3
69	M489	Grapes/Moldova	18.6	3
70	T13/30	Tokay wine/Hungary	18.8	3
71	NCAIM Y-00677	Alcoholic drink/Hungary	18.9	3
72	CECT 10560	Wine/Spain	19.2	3
73	CBS 8711	Wine/Loir Valley, Tours, France	20.1	4
74	M472	Grapes/Moldova	20.7	4
75	CBS 431	Fermented Marx pear juice	23.7	4
76	NCAIM Y-00676	Alcoholic drink/Hungary	23.8	4
		S. eubayanus		
77	PYCC 7088	Soil under *Nothofagus pumilio* tree/Argentina	13.6	2
78	PYCC 7089	Soil under *Nothofagus oblique*/Argentine	13.6	2
79	PYCC 7085	*Nothofagus antarctica* bark/Argentina	14.2	2
80	yHKS212	*Acer saccharum* bark/Wisconsin, United States	14.3	2
81	PYCC 7084	*Cyttaria harioti* on *Nothofagus dombeyi*/Argentine	14.7	2
82	PYCC 7087	Soil under *Nothofagus pumilio* tree/Argentina	16.2	3
83	CBS 12357^T	*Cyttaria hariotti*/Argentina	16.6	3
84	yHKS210	*Fagus grandifolia* bark/Wisconsin, United States	17.1	3

Table 1. (Contd.)

Number	Strain[a]	Source/Location	Diameter[b], mm	Group number
85	yHKS211	*Fagus grandifolia* bark/Wisconsin, United States	17.1	3
86	PYCC 7086	Soil under *Nothofagus antarctica*/Argentina	18.6	3
		S. pastorianus		
87	CBS 1538	Beer/Europe	13.2	2
		S. arboricola		
88	CBS 10644^T	Oak *Quercus fabric* bark/China	3.5	1
		S. cariocanus		
89	UFRJ 50816^T	*Drosophila* sp./Brazil	11.8	2
		S. cerevisiae		
90	CBS 1171^T	Oranjeboom brewery/Netherlands	0	1
91	VKPM Y-718	Kokur-3 polyploid mutant, dry white wine/Crimea	27.0	4
		S. jurei		
92	NCYC 3947^T	Oak *Quercus robur* bark/France	17.2	3
		S. kudriavzevii		
93	NBRC 1802^T	Rotting leaves/Japan	5.0	1
		S. mikatae		
94	NBRC 1815^T	Soil/Japan	5.0	1
		S. paradoxus		
95	CBS 432^T	Oak *Quercus* sp. bark/Russia	16.2	3

[a] Acronyms for culture collections are as follows: VKM, All-Russian Collection of Microorganisms, Moscow, Russia; VKPM, All-Russian Collection of Industrial Microorganisms, Moscow, Russia; M, Magarach Collection of Winemaking Microorganisms, Magarach All-Russian National Institute for Vine and Winemaking, Russian Academy of Sciences, Yalta, Russia; CBS, The Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CECT, Spanish Type Culture Collection, University of Valencia, Valencia, Spain; CCY – Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia; DBVPG, Dipartimento di Biologia Vegetale Universita di Perugia, Italy; NBRC/IFO, National Institute of Technology and Evaluation, Tokyo, Japan; NCAIM, National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary; NCYC, National Collection of Yeast Cultures, Norwich, United Kingdom; PYCC, Portuguese Yeast Culture Collection, Lissabon, Portugal; SRC, The Yeast Collection of Station de Recherche Cidricoles, Le Rheu, France; UCDFST, Herman J. Phaff Yeast Culture Collection of the Department of Food Science and Technology, University of California, Davis, California, USA; UFRJ, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Brazil; UWO (PS), Culture collection of the Departament of Biology, University of Western Ontario, Ontario, Canada; D13, PJP1.95, TBIIb13.92, TBVIc2.95, YIIC2.93, VS2.94, PJS1.94, LC1.95, PJS2.95, DDI4.95, strains from the collection of Institut des Sciences de la Vigne et du Vin (ISVV), Villenave-d'Ornon, France; IFI, Instituto de Fermentaciones Industriales, Madrid, Spain; SCU, Institut Technique de la Vigne et du Vin, Centre d'Expérimentation de Nantes, Nante, France; yHKS, strains from the collection of Dr. Hittinger C.T., Madison, Wisconsin, USA. The remaining strains are from the collection of the Laboratory of Molecular Genetics of Yeasts, National Research Center "Kurchatov Institute," Kurchatov Complex for Genetic Research (GosNIIgenetika), Moscow, Russia. Correspondence of strains from different collections: CBS 8711 = L19, CBS 7001 = MCYC 623, NBRC 1815 = CBS 8839, NBRC 1802 = CBS 8840, NCYC 3947 = CBS 14759, UFRJ 50816 = CBS 8841. T, type culture.
[b] Diameter of the polygalacturonic acid hydrolysis zone around yeast colonies.

PGU11 (5'-CACATTGATGGACAAACGCA-3')/PGU12 (5'-AGGATTAACAGCTTGCACCA-3') were used to amplify the *S. cerevisiae PGU1* gene.

PCR was carried out in a buffer (30 μL) containing 2.5 mM $MgCl_2$, 0.1 mmol of each dNTP, 50 pmol of each primer, 2.5 U of *Taq* polymerase (Syntol, Russia) and 20–200 ng of DNA. The reaction was performed according to this format: initial denaturation, 94°C for 4 min; 35 cycles [denaturation at 94°C for 60 s, primer annealing at 55°C for 60 s, DNA synthesis at 72°C for 120 s], and final elongation at 72°C for 10 min. The amplification products were separated by electrophoresis in 1% agarose gel at 60–65 V in 0.5× TBE buffer for 2–3 h. The gel was stained with ethidium bromide, washed in distilled water and photographed in UV light on a Vilber Lourmat transilluminator (Vilber, France). The 1 kb DNA Ladder kit (Fermentas, Thermo Fisher Scientific, United States) was used as a MW marker.

The amplified fragments were eluted from the gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific) according to the producer's protocol. The nucleotide sequences of the *PGU* genes were identified by two strands by direct Sanger sequencing on an

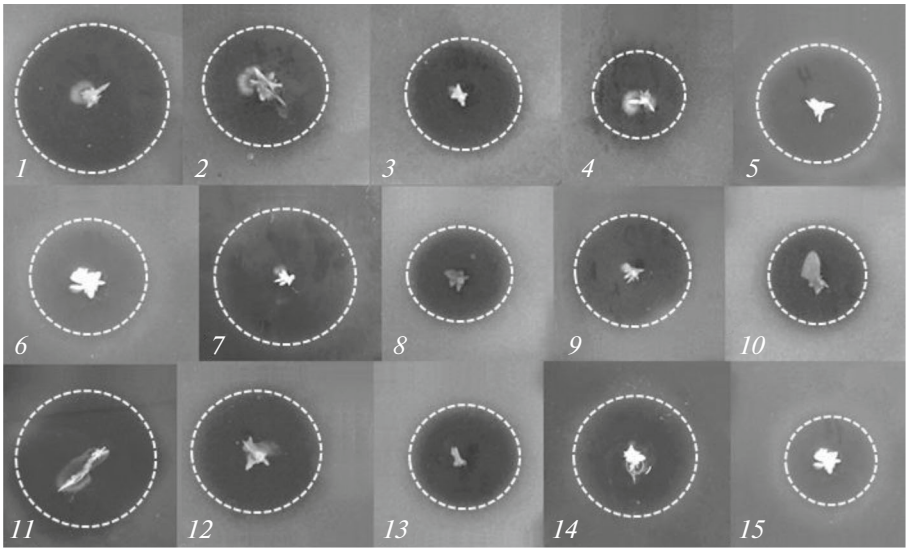

Fig. 1. Screening of *Saccharomyces bayanus* strains grown on a medium containing polygalacturonic acid for the presence of pectinolytic activity: reference strain *S. cerevisiae* VKPM Y-718 (*1*); *S. bayanus* var. *uvarum* M489 (*2*); VKM Y-361 (*3*); SCU 197 (*4*); M472 (*5*); CBS 395 (*6*); CBS 431 (*7*); PYCC 6867 (*8*); PYCC 6869 (*9*); PYCC 7083 (*10*); *S. bayanus* var. *bayanus* CBS 378 (*11*); NBRC 1948 (*12*); *S. eubayanus* CBS 12357 (*13*); PYCC 7086 (*14*); and yHKS 212 (*15*). Dashed circles outline polygalacturonic acid hydrolysis zones around yeast colonies.

Applied Biosystems 3730 automatic sequencer (Applied Biosystems, United States). Similarity of the obtained and the known nucleotide sequences was determined using the BLAST program in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and SGD (http://www.yeastgenome.org/). Multiple alignments of the known and hypothetical amino-acid sequences were carried out using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic trees were built by the neighbor-joining method in the MEGA 7 program [46]. *Kluyveromyces marxianus* CBS 6566 yeast endo-polygalacturonase (Epg1) was used as an outgroup.

RESULTS AND DISCUSSION

Pectinolytic Activity

Pectinolytic activity was studied in the type *S. pastorianus* CBS 1538 culture and in 86 *S. bayanus* strains isolated from various wines, grapes, fruit and berry juices, and natural sources (exudate and bark of broad-leaved trees, insects, soil, and others) (Table 1). The *S. cerevisiae* VKPM Y-718 polyploid strain with high endo-polygalacturonase activity was used as a control. The type cultures of *S. cerevisiae* CBS 1171, *S. arboricola* CBS 10644, *S. cariocanus* UFRJ 50816, *S. kudriavzevii* NBRC 1802, *S. jurei* NCYC 3947, *S. mikatae* NBRC 1815 and *S. paradoxus* CBS 432 were also used for comparison.

The strains under study were divided into four groups depending on the diameter of the polygalacturonic acid hydrolysis area; (1), less than 10 mm; (2), 10–15 mm; (3), 15–20 mm; and (4), more than 20 mm (Table 1). Group 1 was represented by the *S. bayanus* var. *uvarum* LC1.95 (halo diameter 9.1 mm) and SRC410 (8.6 mm) strains isolated in France from fermenting pulp and apple juice, respectively (Table 1). Pectinolytic activity was completely absent only in one wine strain, PJS1.94, isolated from fermenting pulp in France. Most of the studied strains exhibited a fairly high pectinolytic activity: 44 strains were assigned to group 2 and 34 strains to group 3. The latter included 23 *S. bayanus* var. *uvarum* strains from grapes and various wines from France, Spain, Hungary and Slovakia, plus 5 natural isolates (Table 1 and Fig. 1, lanes *2*, *3*, *6*, *8–10*). Halo diameter sizes from 15 to 20 mm were observed in five out of ten studied *S. eubayanus* strains and in the *S. bayanus* var. *bayanus* NBRC 1948 strain (18.7 mm) (Table 1 and Fig. 1, lane *12*).

The highest pectinolytic activity was found in the following five strains: M472 (20.7 mm), CBS 8711 (20.1 mm), CBS 431 (23.7 mm), NCAIM Y-00676 (23.8 mm) and CBS 378 (23.7 mm) (Fig. 1). The first four strains belong to *S. bayanus* var. *uvarum*, and the fifth one, to *S. bayanus* var. *bayanus* (Table 1). The strains CBS 431 (fermenting pear juice), NCAIM Y-00676 (alcoholic drink) and CBS 378 (beer) were comparable in efficiency of polygalacturonic acid hydrolysis with the control strain *S. cerevisiae* VKPM Y-718 (27.0 mm). The latter is a tetraploid strain obtained by treating the wine diploid strain Kokur-3 with a colchicine solution [44]. It should be noted that all the studied strains of *S. bayanus* var. *bayanus*, *S. bayanus* var. *uvarum* and *S. eubayanus* are diploid and are represented by fertile monospore cultures.

Previously, we found that the type culture of the *S. pastorianus* CBS 1538 hybrid yeast is also diploid [21].

Chromosomal Mapping of PGU Genes in the S. bayanus Genome

The *S. cerevisiae* yeast has only one pectinase gene, *PGU1*, located on the X chromosome [11, 25, 26]. We have previously shown that *S. bayanus* var. *uvarum* yeasts have three *PGU* genes with different chromosomal location: *PGU1b*, chromosome X; *PGU2b*, chromosome I and *PGU3b*, chromosome XIV [18, 47, 48]. It was established that the *PGU1b* gene nucleotide sequence has 86–87% similarity to the *PGU2b* and *PGU3b* genes, while the last two genes are 96% identical to each other.

In this work, we determined the number of the *PGU* genes in the strains of *S. bayanus* var. *bayanus* (CBS 380, CBS 378, CBS 425, CBS 424, and NBRC 1948) and *S. eubayanus* (CBS 12357, PYCC 7085, PYCC 7084, PYCC 7086, PYCC 7087, PYCC 7088, PYCC 7089, yHKS210, yHKS211, and yHKS212). Using the three primer pairs described in the MATERIALS AND METHODS section, PCR fragments corresponding to the *PGU1b*, *PGU2b* and *PGU3b* genes of the studied strains were amplified. Southern hybridization confirmed the presence of these *PGU* genes in the genomes of the *S. bayanus* var. *bayanus* and *S. eubayanus* yeasts. According to PCR analysis and Southern hybridization, the type culture of the hybrid *S. pastorianus* CBS 1538 yeast also has the three pectinase genes. On the other hand, the CBS 1538 genome was shown not to contain the *PGU1* gene characteristic of *S. cerevisiae* yeasts.

Thus, *S. bayanus* var. *bayanus*, *S. bayanus* var. *uvarum* and *S. eubayanus* have the *PGU1b PGU2b PGU3b* genotype regardless of the source and region of their isolation. The only exception is the French wine strain *S. bayanus* var. *uvarum* PJS1.94, which possesses only two pectinase genes, *PGU1b* (chromosome X) and *PGU3b* (chromosome XIV) [48]. Interestingly, this is the only one of the 87 studied strains that is unable to hydrolyze polygalacturonic acid (Table 1).

Phylogenetic Analysis of S. bayanus PGU1b Genes and their Encoded Amino-Acid Sequences

We have sequenced the *PGU1b* genes from 18 *S. bayanus* strains of different origins with different pectinolytic activity: *S. bayanus* var. *uvarum* (SC4, SRC258, CBS 8711, TBVIC2.95, PJS2.95, CECT 10560, PYCC 6330, PYCC 7082 and PYCC 7083), *S. bayanus* var. *bayanus* (CBS 380, CBS 378 and NBRC 1948) and *S. eubayanus* (PYCC 7084, PYCC 7085, PYCC 7086, PYCC 7087, PYCC 7088 and yHKS 210). The *PGU1b* gene of the type *S. pastorianus* CBS 1538 strain was also sequenced. The nucleotide sequences of 14 *S. bayanus* var. *uvarum* strains (CBS 7001, CBS 395, CBS 377, M300, VKM Y-361, VKM Y-1140, NCAIM Y.00677, T5/6, T13/30, PJS1.94, UWO (PS) 99-808.3, CCY21-31-12, 136.01 and 148.01) and of the type *S. eubayanus* CBS 12357 were taken from [18, 48] and the GenBank database. The resulting sequences contained 1077 nucleotides, which covers the main part of the *PGU1b* gene-encoding region.

Comparative analysis of the obtained nucleotide sequences revealed two groups of *S. bayanus* var. *uvarum* strains, which differ by 18–20 nucleotide substitutions. The first group includes 16 strains differing in the diameter of the polygalacturonic acid lysis zone, i.e., in endo-polygalacturonase activity. The nucleotide sequences in the following strains were shown to be identical and differ by one substitution from the *PGU1b* sequence of strains CBS 395 (18.0 mm) and SC4 (12.7 mm): PJS1.94 (0 mm), UWO(PS) 99–808.3 (10.7 mm), PJS2.95 (11.0 mm), M300 (11.2 mm), CCY21-31-12 (11.5 mm), PYCC 6330 (11.7 mm), PYCC 7082 (14.5 mm). CBS 377 (15.2 mm), TBVIc2.95 (15.3 mm), CBS 7001 (15.5 mm), VKM Y-1146 (17.2 mm), T5/6 (17.0 mm), VKM Y-361 (17.7 mm) and T13/30 (18.8 mm). The second group contains nine strains: 148.01 (13.9 mm), 136.0 (14.7 mm), VKM Y-508 (15.2 mm), PYCC 7083 (15.6 mm), M488 (16.4 mm), CECT 10560 (19.2 mm), SRC258 (18.0 mm), NCAIM Y.00677 (18.9 mm) and CBS 8711 (20.1 mm). The *PGU1b* gene of the latter strain contained two substitutions compared to the rest of the members of this group. It should be noted that most of the nucleotide substitutions were synonymous and did not affect the protein structure. Pectinases of the *S. bayanus* var. *uvarum* strains from both groups only differed in two amino-acid substitutions.

The *S. bayanus* var. *bayanus* CBS 380 (14.1 mm) and CBS 378 (23.7 мм) strains have identical nucleotide sequences of the *PGU1b* gene, which differs by one position (C–T) from that of the NBRC 1948 strain (18.7 mm), and by three positions (A–G and C–T transitions, and T–A transversion) from the gene sequence of the hybrid *S. pastorianus* CBS 1538 yeast (13.2 mm). The amino-acid sequences of pectinases from *S. bayanus* var. *bayanus* and the *S. pastorianus* CBS 1538 hybrid yeast were identical. Interestingly, the nucleotide sequence of the *PGU1b* gene of the CBS 1538 strain is similar to that of other yeasts deposited in GenBank, including both old collection strains CBS 1486, CBS 1503, CBS 1513 and the modern bottom fermenting brewer's yeast strains W34/70 and Weihenstephan 34/70. It is important to note that the *S. cerevisiae*-type pectinase genes were not observed in the genome of all the listed *S. pastorianus* strains. The differences between the pectinase genes of the *S. eubayanus* strains range from zero to five nucleotides; in *S. eubayanus,* four to seven substitutions were observed compared to *S. pastorianus* and seven to ten substitutions compared to *S. bayanus* var. *bayanus*. The *PGU1b* nucleotide sequences of *S. bayanus* var.

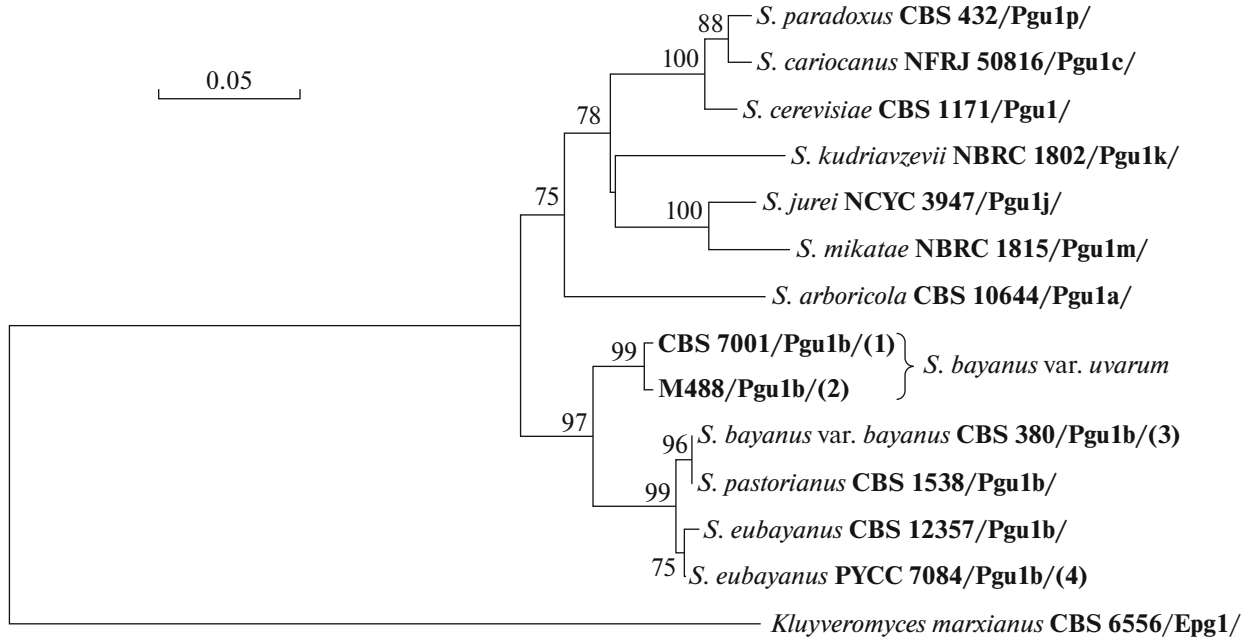

Fig. 2. Phylogenetic analysis of endo-polygalacturonase amino-acid sequences of *Saccharomyces bayanus* and other species of the *Saccharomyces* genus. Endo-polygalacturonase of *Kluyveromyces marxianus* (Epg1) was used as an outgroup. Bootstrap support values higher than 70% are represented. The scale bar corresponds to 50 amino-acid substitutions per 1000 amino-acid positions. The numbers in brackets indicate groups of strains with identical amino-acid sequences: (1) M300, VKM Y-1146, T5/6, T13/30, UWO (PS)99-808, PYCC 6330, PYCC 7082, CCY21-31-12, CBS 377, PJS1.94, PJS2.95, TBVIc 2.95, VKM Y-361, SC4; (2) 136.01, 148.01.3, NCAIM Y.00677, VKM Y-508, CBS 8711, CECT 10560, SRC258, PYCC7083; (3) NBRC 1948; (4) yHKS210, PYCC 7085, PYCC 7086, PYCC 7087, PYCC 7088.

uvarum and *S. bayanus* var. *bayanus/S. eubayanus* differ by more than 80 positions, while the pectinase gene sequences from *S. bayanus* and other seven *Saccharomyces* species (*S. arboricola* CBS 10644, *S. cariocanus* UFRJ50816, *S. jurei* NCYC 3947, *S. cerevisiae* CBS 1171, *S. kudriavzevii* NBRC 1802, *S. mikatae* NBRC 1815 and *S. paradoxus* CBS 432) differ by more than 190 nucleotides.

The resulting *PGU* gene nucleotide sequences served as the basis for determining the corresponding protein sequences, which in turn, were used to construct a phylogenetic tree (Fig. 2).

Kluyveromyces marxianus Epg1 was used as an outgroup. There are two main clusters on the phylogenetic tree. The first includes with a 97% bootstrap support pectinases from *S. bayanus* var. *uvarum, S. bayanus* var. *bayanus, S. eubayanus* and the hybrid *S. pastorianus* brewer's yeast, which are 94–99% identical. The highest similarity (99–100%) is characteristic of Pgu1 from the last three taxa.

The second cluster contains the remaining seven *Saccharomyces* species. Two subclusters within it differ from the rest. The first subcluster is composed by the *S. cerevisiae*, *S. paradoxus* and *S. cariocanus* strains, the Pgu1 of which are 97–98% similar. The second subcluster includes *S. mikatae* and *S. jurei*, which have endo-polygalacturonases identical by 96%. *S. kudriavzevii* pectinase Pgu1k adjoins this subcluster; it has 88–92% similarity with the others. *S. arboricola* Pgu1a protein also adjoints the second cluster; its identity with the corresponding *S. cerevisiae*, *S. cariocanus*, *S. jurei*, *S. kudriavzevii*, *S. mikatae* and *S. paradoxus* proteins is 87–88%. The similarity between Pgu1 of *S. bayanus* and the remaining *Saccharomyces* species is 86–88%.

CONCLUSIONS

Thus, based on a phylogenetic analysis, it can be concluded that the *Saccharomyces* yeast *PGU* genes are species-specific. As an example, the *PGU* genes of the hybrid brewer's yeast *S. pastorianus* are likely to originate from the cold-tolerant *S. bayanus* and not from *S. cerevisiae*. This is in good agreement with the fact that *S. bayanus* is common in breweries, while the *S. cerevisiae* beer yeast does not have pectinolytic activity. The type culture *S. cerevisiae* CBS 1171 isolated from the brewing process is unable to degrade pectin (Table 1). All *S. bayanus* strains studied in this work have rather high pectinolytic activity, which is likely to be a feature of this species. Regardless of where and from what source they were isolated, the studied strains of *S. bayanus* var. *uvarum, S. bayanus* var. *bayanus* and *S. eubayanus* have the *PGU1b PGU2b PGU3b* genotype. The only exception is the French wine strain *S. bayanus* var. *uvarum* PJS1.94 with its

PGU1b PGU3b genotype, which is unable to decompose polygalacturonic acid.

Strains M472, CBS 8711, CBS 431, NCAIM Y-00676, and CBS 378, which secrete active endo-polygalacturonase, are of interest for further molecular genetic research and selection, including those aimed at creating new wine strains.

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