BIOTECHNOLOGY

SCREENING AND IDENTIFICATION OF PIGMENTAL YEAST PRODUCING L-PHENYLALANINE AMMONIA-LYASE AND THEIR PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

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Abstract: The results of the analysis of DNA sequences encoding L-phenylalanine ammonia-lyase (PAL) synthesis, performed to obtain universal primers complementary to conserved regions of the *pal* gene, are presented in the article. The fragment of *pal* gene was amplified in organisms under study. Nucleotide sequence of the *pal* gene in microorganisms exhibiting L-phenylalanine ammonia-lyase activity was determined by DNA sequencing. The results of its comparison with the corresponding sequences of known species are presented. Phenotypic characteristics and biochemical properties of selected cultures were studied. An investigation aimed to choose a superproducer strain of L-phenylalanine ammonia-lyase was conducted. It was found that L-phenylalanine ammonia-lyase synthesis was the most active in the following strains: *Aureobasidium pullulans* Y863, *Rhodosporidium infirmominiatum* Y1569, *Candida glabrata* Y2813, *Candida maltose* Y242, *Debaryomyces robertsiae* Y3392, *Rhodosporidium diobovatum* Y1565, *Rhodotorula lactose* Y2770, *Saccharomyces cerevisiae* Y1127, *Tilletiopsis washingtonensis* Y1650, *Torulopsis apicola* Y566, *Tremella foliacea* Y1624, *Rhodotorula rubra* Y1193, and *Debaryomyces castellii* Y968. This allows recommending them for further research aimed to obtain the enzyme preparation of L-phenylalanine ammonia-lyase.

Keywords: L-phenylalanine ammonia-lyase, enzyme, *pal* gene, pigmental yeast, nucleotide sequence, amino acid sequence, phylogenetic tree

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INTRODUCTION

L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyzes the reaction of reverse deamination of L-phenylalanine to *trans*-cinnamic acid and ammonia [1]. It is the key enzyme of phenylpropanoid metabolism in plant and fungi, where it is involved in biosynthesis of secondary metabolites (flavonoids, furanocoumarines, and cell wall components), existing in multiple isoforms [2, 3].

PAL plays an important role in catabolic processes of microorganisms, providing for utilization of L-phenylalanine as a sole source of carbon and nitrogen [4]. Among the microorganisms, the highest PAL activity is exhibited by yeasts, especially the red basidiomycetes of the Rhodotorula family [5]. *Sporobolomyces roseus* and *Sporidiobolus pararoseus* are also PAL-producing yeasts [6].

Therapeutic potential of PAL with respect to neoplasms was evaluated due to its selective activity to phenylalanine and amino acids that are consumed by mammalian cells from external sources. PAL was shown to inhibit neoplasm growth in vitro [7].

The enzyme is also of interest as a therapeutic agent for phenylketonuria treatment and may be used for both direct therapy of phenylketonuria and production of food products free of phenylalanine [8]. Besides the medical applications, the enzyme may be used in biotechnology for L-phenylalanine production from *trans*-cinnamic acid [9].

Considerable contribution to the development and assimilation of the technology of specialized food products was made by G.B. Gavrilov, N.B. Gavrilova, V.I. Ganina, N.I. Dunchenko, I.A. Evdokimov, V.I. Kruglik, K.S. Ladodo, L.A. Ostroumov, A.N. Petrov, V.O. Popov, G.Yu. Sazhinov, V.A. Tutel'yan, V.D. Kharitonov, I.S. Khamagaeva, and A.G. Khramtsov, and to the technology of the enzyme preparation of PAL, by V.I. Mushtaev, M. Jason Mac Donald, H. Orum, and O.F. Rasmussen.

The development of new and improvement of existing technologies of the PAL preparation production requires new, more intensive sources of its superexpression, which is impossible without studies on the specific features of its genetics in known producers. Only 26 sequences of genomes of microorganisms exhibiting PAL activity were found in the databases of genetic sequences (EMBL and GenBank). Therefore, the search for microorganisms exhibiting L-phenylalanine ammonia-lyase activity based on sequence analysis of their genomes is urgent.

The aim of the work was to screen and identify pigmented yeasts producing L-phenylalanine ammonia-lyase and describe their physiological and biochemical characteristics.

OBJECTS AND METHODS OF RESEARCH

Agarose (Chemapol, Czech Republic); low gelling-temperature agarose (Ultra Pure, BRL, United States); β-mercaptoethanol, PEG-1500, PEG-6000 (Loba Feichemie, Austria); imidazole (Diaem, Russia); dATP, dCTP, dGTP, dTTP (Bioline, Germany); bactoagar, yeast extract (Difco, United States); bactotriptone (Ferak, Germany); D-glucose, urea, bromine (99.8%, for synthesis) (Merck, Germany); bromphenol blue, ammonium persulfate, *N,N'*-methylene-bis-acrylamide (Reanal, Hungary); acetyl phosphate, ethidium bromide, DTT, IPTG, Triton X-100, EDTA, $MgCl_2$, BSA, DMSO, Tris, $(NH_4)_2SO_4$, $CaCl_2$ (Sigma, United States); L-phenylalanine ammonia-lyase, acrylamide, *N,N'*-methylene-bis-acrylamide, ammonium persulfate (Sigma, Germany); RNase A, CH_3COOK, C_6H_5OH, $CHCl_3$, NaCl (Reakhim, Russia); MilliQ deionized water (Millipore, France); orthophosphoric acid (85.1%, imported) (Lenreaktiv, Russia); tris(hydroxymethyl)aminomethane, PIPES, SDS, X-Gal, tetracycline hydrochloride, chloramphenicol (Serva, Germany); L-phenylalanine (Acros Organics, Belgium); *trans*-cinnamic acid (Brituer Co. Ltd., China); boric acid (99.8%, extra pure), sodium tungstate dihydrate (99.1%, extra pure) (AppliChem, United States); (3-aminopropyl)triethoxysilane (98.0%, for synthesis), acrylic acid (99.8%, for synthesis), sodium caseinate (92.0%, extra pure), potassium monophosphate dihydrate (98.5%, extra pure), sodium carbonate (99.9%, for synthesis), sodium hydroxide (9.1%, pure for analysis), sodium chloride (99.8%, extra pure), hydrochloric acid (36.0%, extra pure) (Khimlaborpribor, Russia); sodium phosphate dibasic dodecahydrate (98.2%, extra pure), L-tyrosine (99.9%, extra pure), trichloroacetic acid (99.0%, for synthesis), acetic acid (98.5%, extra pure), phenolphthalein (98.5%, extra pure).

Sequences of the *pal* gene of ascomycetes and basidiomycetes presented in the NCBI international database are reported in Table 1.

Table 1. Sequences of microorganism strains possessing L-phenylalanine ammonia-lyase activity

Strain	Strain number	Species name
1	83976309	*Rhodotorula_graminis*
2	3293	*Rhodosporidium_toruloides*
3	3284	*Rhodotorula_mucilaginosa*
4	317034460	*Aspergillus_niger*
5	115437191	*Aspergillus_terreus*
6	497418	*Arabidopsis_thaliana*
7	242780352	*Talaromyces_stipitatus*
8	212533750	*Penicillium_marneffei*
9	169610841	*Phaeosphaeria_nodorum*
10	317157281	*Aspergillus_oryzae*
11	238493630	*Aspergillus_flavus*
12	121698870	*Aspergillus_clavatus*
13	119480760	*Neosartorya_fischeri*
14	71001127	*Aspergillus_fumigatus*
15	389639669	*Magnaporthe_oryzae*
16	116206211	*Chaetomium_globosum*
17	164422921	*Neurospora_crassa*
18	15824530	*Ustilago_maydis*

Table 1. Ending. Sequences of microorganism strains possessing L-phenylalanine ammonia-lyase activity

Strain	Strain number	Species name
19	331236172	*Puccinia_graminis*
20	507833891	*Letharia_vulpina*
21	4127288	*Amanita_muscaria*
22	299751359	*Coprinopsis_cinerea_okayama*
23	170097945	*Laccaria_bicolor*
24	409924409	*Tricholoma_matsutake*
25	482667462	*Pleurotus_eryngii*
26	1666264	*Agaricus_bisporus*

Strains of the microorganisms for study of their L-phenylalanine ammonia-lyase activity obtained form the All-Russian Collection of Industrial Microorganisms, GosNIIGenetika, are presented in Table 2.

Table 2. Strains of the microorganisms for investigation of the PAL activity

No.	Name	Microorganism number in the Collection of Microorganisms of GosNIIGenetika
1	*Aureobasidium pullulans*	Y863
2	*Bullera alba*	Y1581
3	*Bullera piricola*	Y1577
4	*Candida glabrata*	Y2813
5	*Candida maltosa*	Y242
6	*Cryptococcus laurentii*	Y227
7	*Cryptococcus macerans*	Y2763
8	*Cystofilobasidium capitatum*	Y1573
9	*Cystofilobasidium capitatum*	Y1852
10	*Debaryomyces castellii*	Y968
11	*Debaryomyces robertsiae*	Y3392
12	*Dioszegia hungarica*	Y3208
13	*Dioszegia sp.*	Y3320
14	*Geotrichum klebahnii*	Y3053
15	*Phaffia rhodozyma*	Y1666
16	*Phaffia rhodozyma*	Y1668
17	*Rhodosporidium capitatum*	Y1567
18	*Rhodosporidium diobovatum*	Y1565
19	*Rhodosporidium infirmo-miniatum*	Y1569
20	*Rhodotorula aurantiaca*	Y985
21	*Rhodotorula glutinis*	Y77
22	*Rhodotorula lactosa*	Y2770
23	*Rhodotorula minuta*	Y2777
24	*Rhodotorula rubra*	Y1193
25	*Saccharomyces cerevisiae*	Y1127
26	*Saccharomyces kluyveri*	Y2559
27	*Sporobolomyces holsaticus*	Y991
28	*Sporobolomyces roseus*	Y987
29	*Tilletiopsis washingtonensis*	Y1650
30	*Torulopsis apicola*	Y566
31	*Tremella foliacea*	Y1624
32	*Tremella mesenterica*	Y1625

BLAST2 software was used to search for homologous sequences; Generunner and Chromas, for analysis of nucleotide and amino acid sequences; Clustal Omega, for multiple nucleotide sequence alignment; and BioEdit 7.0.0, for editing and alignment, as well as translation into amino acid sequence. Analysis of an optimal model for amino acid substitutions was performed on a ProtTest 3 on-line server according to the Akaike information criterion (AIC) (the sequence of the *pal* gene of *Arabidopsis thaliana* was used as an out-group). The phylogenetic tree was built using a distance method in a Phylip 3.69 software package, and using the Bayesian method, in a MrBayes 3.2 software; the trees were visualized in a TreeGraph 2, and the logo diagram was built in a WebLogo software.

Sanger sequencing was performed on an ABI3730xl (Applied Biosystems, United States) automated sequencer according to the manufacturer's protocol using the BigDye® Terminator v3.1 Cycle Sequencing kit.

Oligonucleotides were obtained on an ABI3900 (Applied Biosystems) synthesizer. The results of the experiments were processed using the methods of mathematical statistics.

The primers RalF and RalR were synthesized by JSC Sintol. Primer operational parameters are presented in Table 3.

Table 3. Operational parameters of RalF–RalR primers

Primers	
Forward primer RalF	CTCACCAACTTCCTCAA CCACGGCA
Parameters	
Length	25 bp
Molecular weight	7475.9
CG content	56%
Melting temperature	69°C
Annealing temperature	64°C
Reverse primer RalR	ATGCCCTCGTCGTCCTT GACCTTGA
Parameters	
Length	25 bp
Molecular weight	7559.9
CG content	56%
Melting temperature	69°C
Annealing temperature	64°C

PCR amplification was performed on a SMART CYCLER (Cepheid, United States) thermocycler in 20–50 µL solution prepared on the basis of a 10-fold buffer for Taq polymerase, which contained 200 µmol of each of deoxyribonucleotides, 0.5 µmol primers, 2 µmol $MgSO_4$, 10 ng template, 2 units of Taq DNA polymerase, and 0.1 unit of Pfu DNA polymerase. Oligonucleotide annealing temperature was calculated using an empirical formula: $T_m = 67.5 + 34[\%GC] – 395/n$, where %GC = (G + C)/n, and n is the number of nucleotides. PCR products were analyzed by electrophoresis in 1-% agarose gel.

Table 4 presents the amplification parameters for RalF–RalR primers.

Table 4. Amplification parameters for RalF–RalR primers

Step	Temperature, °C	Time	
Initial heating	90–95	1 min	
Denaturation	95	30 s	30 cycles
Primer annealing	64	30 s	
Elongation	72	1 min	
Elongation	72	1 min	

DNA electrophoresis in agarose gel. Samples of DNA were separated by electrophoresis in Tris–acetate buffer (0.04 M Tris–acetate; 0.002 M EDTA) in a 0.7–0.8% agarose gel (Bio-Rad, United States) containing 0.5 µg/mL ethidium bromide under the voltage of 2–5 V/cm. GeneRuler™ 1 kb DNA Ladder (Fermentas, Lithuania) were used as standard molecular weight markers. DNA bands were detected upon gel irradiation with UV light using the Gel Doc XR Plus (Bio-Rad) system.

Isolation of DNA fragments from agarose gel. Samples of DNA were separated by electrophoresis in Tris–acetate buffer in a 0.7–0.8% agarose gel (Bio-Rad) containing 0.3 µg/mL ethidium bromide and analyzed by fluorescence under ultraviolet light at 254 nm. Gel pieces containing fragments of interest were cut out and transferred into microcentrifuge tubes, then DNA fragments were eluted from the gel using the "Isolation of DNA from agarose gels" kit (Boeringer Mannheim, Germany). Sodium perchlorate was added to the tubes in the amount of 400 µL per 100 mg weight of the cut out gel. The mixture was heated to 65°C, then agarose was dissolved in salt buffer. Glass milk microbeads were introduced into the suspension at the amount of 20 µL per 100 mg of gel weight. In the salt solution, DNA contained in the gel adsorbed on the surface of the microbeads. They were washed (consecutive precipitation–resuspension) with the same salt solution once and with 70% ethanol, two times. DNA was desorbed from the beads by resuspension in TE buffer (10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA) in the amount of 50 µL per 100 mg gel weight.

L-phenylalanine ammonia-lyase activity was determined according to a protocol by Sigma with little modifications in the preparation of the reaction mixture. All solutions were prepared in deionized MilliQ water.

Composition of standard incubation mixture (1 cm^3): 0.2 mol Tris-HCl, pH 8.5, 0.5 cm^3; 0.05 mol L-phenyl-alanine, 0.04 cm^3; deionized water, 0.42 cm^3.

After mixing and pre-incubation (at least 5 min at 30 ± 0.1°C), the reaction was initiated by the addition of 0.04 cm^3 diluted enzyme (0.025–0.125 U/cm^3 PAL).

In the control sample, 0.04 cm^3 water was added instead of the enzyme.

The reaction course was registered continuously at 270 nm in a Shimadzu UV-1800 (Shimadzu, Japan) spectrophotometer equipped with a thermocontrolled chamber in quartz cuvettes with a 1-cm optical path. Data collection and analysis was performed by a UV-probe v (Shimadzu) software. PAL activity was calculated according to the formula using the value of millimolar extinction coefficient of *trans*-cinnamic acid (Sigma's protocol):

$$Activity\ \left(U/_{cm^3}\right) = \frac{\left(\Delta OD_{270}/min_{exp} - \Delta OD_{270}/min_{control}\right) \times V_{reac.mix} \times f}{19.73 \times V_{sample}},$$

where $V_{reac.mix.}$ is the reaction mixture volume, mL; f, coefficient of dilution of the initial PAL preparation; 19.73, millimolar coefficient of extinction of *trans*-cinnamic acid at 270 nm; V_{sample}, sample volume, cm^3; min_{exp}, $min_{control}$, duration of enzymatic activity measurement in experiment and control samples, respectively.

The amount of PAL that catalyzed the transformation of 1 μmol L-phenylalanine into *trans*-cinnamic acid and NH_3 within 1 min at pH 8.5 at 30 ± 1°C was considered an activity unit.

Study of specific features of phenotype and biochemical properties of the selected pigmented yeast strains. Shape and size of cells of the microorganisms were described and determined in cultures of different age on dense and liquid nutritive media. The first imaging and cell size measurement was performed in two–three-day cultures grown at 25–28°C. Then, the cultures were left at room temperature (17–18°C) and described again in four weeks.

To determine cell size, length and width were measured with a micrometer in at least 20 cells, and the extreme values were indicated.

RESULTS AND DISCUSSION

Today, to determine the species reference of a microorganism and the presence of a certain gene in it, the method of 16S rRNA gene sequence comparison may be used, since the gene carries both the conserved and variable regions of the nucleotide sequence. The data on nucleotide sequences of various microorganisms are contained in the international databases GenBank and EMBL-EBI.

Therefore, comparative analysis of twenty six nucleotide sequences of the *pal* gene contained in the GenBank database of genetic sequences were compared, including *Rhodotorula graminis* 83976309, *Rhodosporidium toruloides* 3293, *Rhodotorula mucilaginosa* 3284, CBS 513.88 *Aspergillus niger* 317034460, NIH2624 *Aspergillus terreus* 115437191, *Arabidopsis thaliana* 497418, ATCC_10500 *Talaromyces stipitatus* 242780352, ATCC 18224 *Penicillium marneffei* 212533750, SN15 *Phaeosphaeria nodorum* 169610841, RIB40 *Aspergillus oryzae* 317157281, NRRL3357 *Aspergillus flavus* 238493630, NRRL 1 *Aspergillus clavatus* 121698870, NRRL 181 *Neosartorya fischeri* 119480760, Af293 *Aspergillus fumigatus* 71001127, 70-15 *Magnaporthe oryzae* 389639669, CBS_148.51 *Chaetomium globosum* 116206211, OR74A *Neurospora crassa* 164422921, *Ustilago maydis* 15824530, f. sp. tritici CRL 75-36-700-3 *Puccinia graminis* 331236172, *Letharia vulpina* 507833891, *Amanita muscaria* 4127288, 7.130 *Coprinopsis cinerea okayama* 299751359, S238N-H82 *Laccaria bicolor* 170097945, NBRC 30605 *Tricholoma matsutake* 409924409, P810 *Pleurotus eryngii* 48266746, and *Agaricus bisporus* 1666264, deposited in the GenBank of the National Center for Biotechnology Information (NCBI).

Phylogenetic relationships between the microorganisms established on the basis of comparative analysis of the nucleotide sequences may be presented as a dendrogram (phylogenetic tree), an arbitrary graphical representation reflecting the affinity between the genetic macromolecules, biological species, or higher rank taxa (Fig. 1).

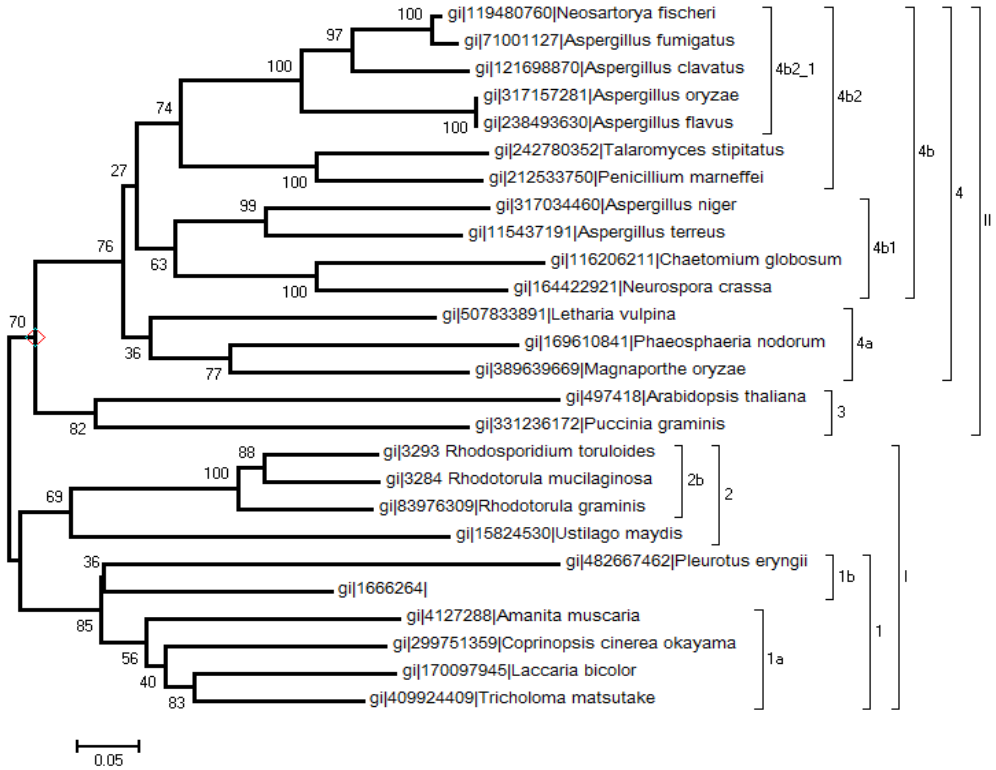

Fig. 1. Dendrogram of nucleotide sequences of the pal gene built with the NJ method using the MEGA 4.0.2 software. Figures indicate the statistical reliability of the branching order determined with bootstrap analysis.

Figure 1 presents the molecular phylogenetic tree obtained using the neighbor-joining method. The genetic distances have been calculated according to Kimura-2 method.

The performed phylogenetic analysis demonstrates rather high similarity between the *pal* gene sequences. In the phylogenetic tree, all *pal* gene sequences under study may be grouped into two big clusters, I and II.

Cluster I comprises the smaller clusters 1, with bootstrap support of 85%, and 2, with bootstrap support of 69%; *pal* gene sequences of *Agaricus bisporus* and *Pleurotus eryngii* correspond to clade 1b with bootstrap support of 36%. In clade 1a, *Amanita muscaria* forms an individual branch, and *Coprinopsis cinerea okayama, Laccaria bicolor*, and *Tricholoma matsutake* form a small clade with support of 40%, and *Laccaria bicolor* forms a separate consortium with *Tricholoma matsutake* with the support of 83%.

Sequences of *pal* gene in the representatives of the *Rhodotorula* and *Rhodosporidium* genera, *Rhodotorula graminis, Rhodosporidium toruloides,* and *Rhodotorula mucilaginosa*, form a separate clade 1b in cluster 1 with the highest bootstrap support of 100%. They are neighbored by *Ustilago maydis,* which forms an individual branch.

Cluster II may be divided into a small cluster 3 and a large cluster 4.

Cluster 3 comprises the sequences of the representtatives of the *Arabidopsis thaliana* and *Puccinia graminis* species with bootstrap support of 82%.

Representatives of the *Aspergillus* genus—*A. oryzae, A. flavus, A. clavatus, Neosartorya fischeri* (synonym with *A. fischeri),* and *A. fumigatus*—form a monophyletic clade 4b2 1 with a 100-% bootstrap support. They are neighbored by another clade with a 100-% support, which includes *pal* sequences of *Talaromyces stipitatus* and *Penicillium marneffei.*

Clade 4 b1 also comprises the representatives of *A. niger* and *A. terreus,* which form a monophyletic consortium with support of 99%, and *Chaetomium globosum* and *Neurospora crassa,* a consortium with bootstrap support of 100%.

Sequences of the *pal* gene from *Magnaporthe oryzae* and *Phaeosphaeria nodorum* form a consortium with 77-% support. The neighboring *Letharia vulpine, Letharia vulpine, Magnaporthe oryzae,* and *Phaeosphaeria* form a separate branch comprising the 4a clade with support of 33%. Clades 4a and 4b together make up cluster 4 with bootstrap support of 76%.

Multiple alignment of selected nucleotide sequences for each of the gene clusters encoding L-phenylalanine ammonia-lyase deposited in GenBank and EMBL-EBI were performed with the Clustal X V 1.75 software.

The results of the study demonstrated that the *pal* sequences in the analyzed microorganisms are poorly conserved; therefore, the area of search for universal primers was narrowed. For this purpose, *pal* gene sequences in basidiomycete yeasts *Rhodosporidium toruloides* and *Rhodotorula glutinis* were analyzed, because these very organisms possessed the highest homology with the pigmented yeast strains.

Based on the obtained conserved fragments, as well as the theoretical rules of selection, the following universal primers were selected:

– forward primer PAL1F (5'- CGC GGY CAY TCK GCK GT -3')

– and reverse primer PAL1R (5'-CAT YTC TGC CGG YTG AAC RTG -3').

Melting temperature and amplification parameters for the designed primers were calculated using the Olig 4.0 software. The results of the studies are presented in Table 3.

Analysis of the literature data demonstrated that L-phenylalanine ammonia-lyase was detected in a number of microorganisms, including the pigmented yeasts. Colorless yeasts do not contain the enzyme. In this connection, the search for culture with PAL activity was performed among the pigmented yeast from the microorganism collection (GosNIIGenetika): *Aureobasidium pullulans* Y863, *Bullera* Y1581, Y1577, *Candida* Y2813, Y242, *Cystofilobasidium* Y1573, *Debaryomyces* Y968, Y3392, *Phaffia* 1666, 1668, *Rhodosporidium* Y1567, Y1565, Y1569, *Rhodotorula* Y985, Y2770, Y2777, Y1193, *Saccharomyces* Y1127, Y2559, *Sporobolomyces* Y987, *Tilletiopsis* Y1650, *Torulopsis* Y566, and *Tremella* Y1624, Y1625.

As a result of amplification reaction, a PCR product of expected length was obtained for each of the samples. However, we failed to sequence it, since the reaction yielded additional non-specific products of various length, including the one very close to the target fragment. We decided to substitute one of the primers with another one and decrease the length of expected fragment. At the second stage the PAL2F primer (CAT YTC TGC CGG YTG AAC RTG) was used. Therefore, the pair of primers RAL2F–RAL1R flanks the *pal* gene fragment located between nucleotides 292 and 1319 of the *pal* gene in NCBI database; the size of the amplified fragment was 1027 bp.

The results of amplification were controlled with electrophoresis in 1.5% agarose gel under voltage of 5 V/cm in an SE-1 horizontal electrophoresis chamber equipped with an Elf-4 power supply (Khelikon, Russia). Artificially synthesized *pal* gene of *Rhodosporidium toruloides* was used as a positive control.

After enzymatic purification with a mixture of exonuclease I and alkaline phosphatase, the fragments were sequenced with the PAL2R primer on an ABI 3130xl (Applied Biosystems) analyzer according to standard techniques.

To prove that the obtained amplificates are indeed the *pal* gene fragments, direct sequencing of PCR fragments was performed using an ABI3730xl (Applied Biosystems) automated sequencer and BigDye® Terminator v3.1 Cycle Sequencing Kit for the following strains: *Aureobasidium pullulans* Y863, *Bullera alba* Y1581, *Bullera piricola* Y1577, *Candida glabrata* Y2813, *Candida maltosa* Y242, *Cryptococcus laurentii* Y227, *Cryptococcus macerans* Y2763, *Cystofilobasidium capitatum* Y1573, *Cystofilobasidium capitatum* Y1852, *Debaryomyces castellii* Y968, *Debaryomyces robertsiae* Y3392, *Dioszegia hungarica* Y3208, *Dioszegia sp.* Y3320, *Geotrichum klebahnii* Y3053, *Phaffia rhodozyma* Y1666, *Phaffia rhodozyma* Y1668, *Rhodosporidium capitatum* Y1567, *Rhodosporidium diobovatum* Y1565,

Rhodosporidium infirmo-miniatum Y1569, *Rhodotorula aurantiaca* Y985, *Rhodotorula glutinis* Y77, *Rhodotorula lactosa* Y2770, *Rhodotorula minuta* Y2777, *Rhodotorula rubra* Y1193, *Saccharomyces cerevisiae* Y1127, *Saccharomyces kluyveri* Y2559, *Sporobolomyces holsaticus* Y991, *Sporobolomyces roseus* Y987, *Tilletiopsis washingtonensis* Y1650, *Torulopsis apicola* Y566, *Tremella foliacea* Y1624, and *Tremella mesenterica* Y1625.

The results evidence that strains *Bullera alba* Y1581, *Cryptococcus laurentii* Y227, *Cryptococcus macerans* Y2763, *Cystofilobasidium capitatum* Y1852, *Dioszegia hungarica* Y3208, *Dioszegia sp*. Y3320, *Geotrichum klebahnii* Y3053, *Phaffia rhodozyma* Y1666, *Rhodosporidium capitatum* Y1567, *Rhodotorula aurantiaca* Y985, *Rhodotorula minuta* Y2777, *Sporobolomyces holsaticus* Y991, and *Sporobolomyces roseus* Y987 contain no *pal* gene and thus were excluded from further study.

Phylogenetic analysis of *pal* gene sequences in strains under study and reference strains from the GenBank database of genetic sequences (Table 1) was performed.

Upon multiple alignment of the sequences (CLUSTAL Omega) and their editing (intron excision), they were translated using the BioEdit 7.0.0 software. For further analysis, testing of various models of amino acid substitutions was performed with a ProtTest 3 service.

Structure of the PAL protein of *Rhodosporidium toruloides* was obtained from the PDB bank (1T6P, DOI:10.2210/pdb1t6p/pdb) in the PyMOL Molecular Graphics System, version 1.5.0.4 (Schrodinger, LLC). As follows from Fig. 2, the fragment lies in the internal part of the molecule and includes the region of a beta-sheet, two turns, and several alpha-helix regions.

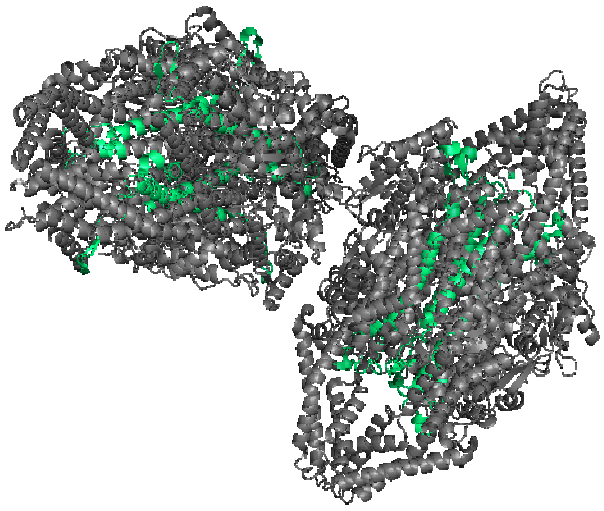

Fig. 2. Structure of PAL protein from *Rhodosporidium toruloides* (DOI:10.2210/pdb1t6p/pdb*)*. Green color indicates amino acids 398–472 (numbers are given according to the GI:3294 sequence) based on the results of alignment with the fragments under study in Clustal Omega software.

For a phylogenetic analysis in the PHYLIP 3.69 software package, 1000 bootstrap replicas of amino acid alignment were created, then the distances between the sequences (JTT substitution model, gamma = 1.78, based on a previously chosen optimal model) were calculated and the trees were built by a neighbor-joining method (NJ). The PAL sequence of *Arabidopsis thaliana* was used as an out-group. Figure 3 presents the phylogenetic consensus tree; joining was performed according to the 50% majority rule.

Also, MrBayes 3.2 software was used to built Bayes trees (based on a previously chosen model, Jones+G template of amino acid substitutions). The number of mcmc iterations was 600000. Figure 4 presents the phylogenetic tree with the possibility of node formation noted in the nodes. Again, the PAL sequence of *Arabidopsis thaliana* was used as an out-group.

Based on the analysis of phylogenetic trees one may conclude that the studied sequences are divided into two clades. Subgroup Ia includes the following families: *Aureobasidium, Candida, Cystofilobasidium, Debaryomyces, Phaffia, Puccinia, Rhodosporidium, Rhodotorula, Saccharomyces, Tilletiopsis,* and *Tremella.* Clade II includes the following families: *Aspergillus, Chaetomium, Letharia, Magnaporthe, Neosartorya, Neurospora, Penicillium, Phaeosphaeria, Talaromyces,* and *Uncinocarpus.*

Upon the analysis of the tree obtained with Bayesian statistics one may note that the sequences also rather reliably divided into two clades, and the first one (lower clade) corresponds to subgroup Ia, while the second (upper) one corresponds to subgoup Ib and clade II (Figs. 3 and 4).

The template of identity of nucleotide and amino acid sequences (Tables 6 and 7) was created for some of the investigated strains under study (all sequences obtained in the current study, as well as *Arabidopsis thaliana, Aspergillus niger, Neurospora crassa, Puccinia graminis, Rhodosporidium toruloides, Rhodotorula graminis, and Rhodotorula mucilaginosa* sequences) using the CLUSTALW software.

Analysis of the data presented in Tables 5 and 6 evidences that the rate of identity of both nucleotide and amino acid sequences inside a clade is very high and makes up to 93–97% for some species.

A logo-diagram (Fig. 5) was designed for the Ia subgroup demonstrating the fragment is rather conserved.

Therefore, using the phylogenetic analysis the investigated gene has been shown to belong to the *pal* family, and the affinity of the sequences was evaluated.

The performed comparative analysis of sequences from genomes of pigmented yeasts with completely characterized genes encoding L-phenylalanine ammonia-lyase amplified and sequenced using the developed pair of primers demonstrated undoubtedly the sequences belong to the *pal* genes.

Therefore, we developed a universal primer system revealing genes encoding L-phenylalanine ammonia-lyase.

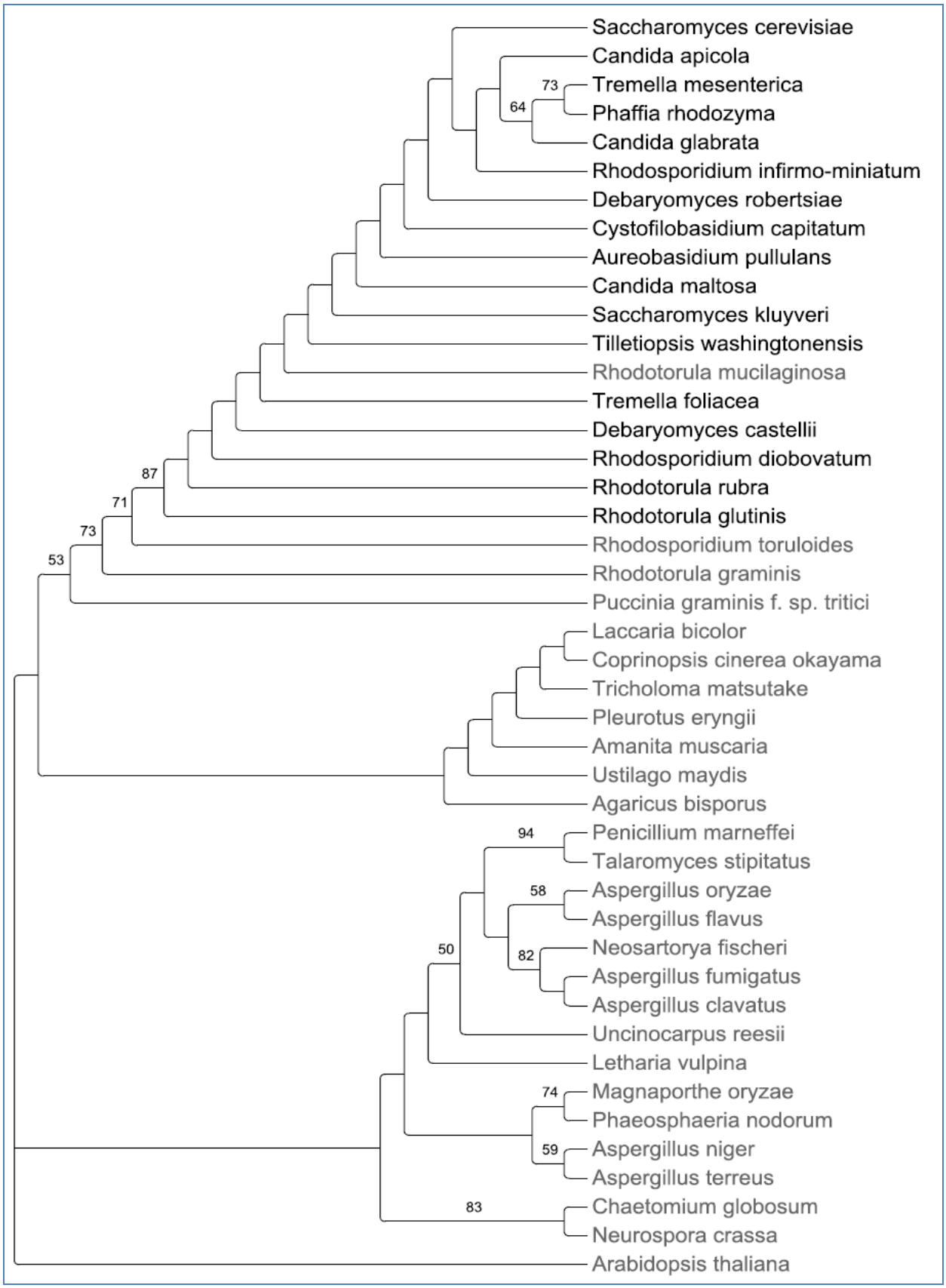

Fig. 3. Consensus tree designed with the PHYLIP 3.69 software using the data on PAL amino acid sequences obtained (trees were built using the neighbor-joining method, JTT substitution model, gamma = 1.78). In the nodes, the percent of bootstrap support is indicated for 1000 replicas. Bold font indicates the PAL sequences obtained in the work, and light font, sequences from NCBI databases.

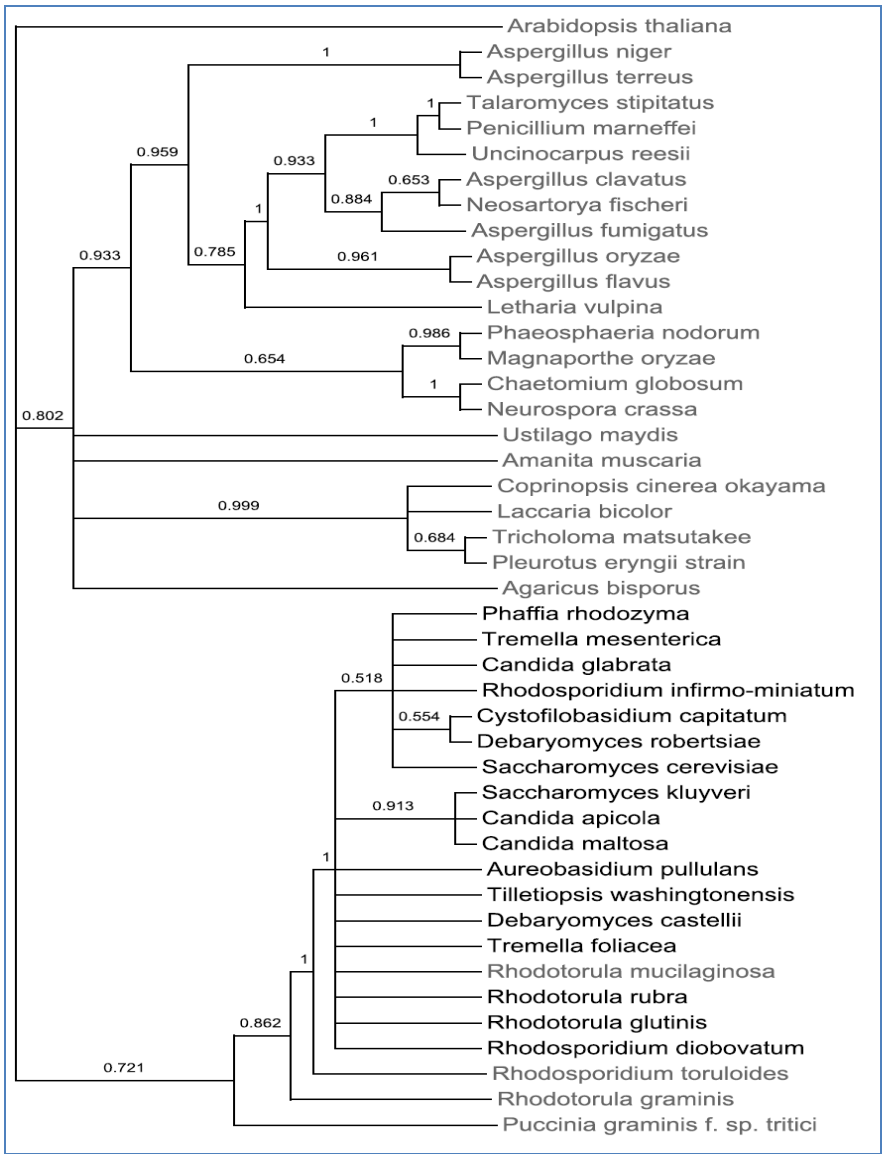

Fig. 4. Bayesian tree built using the MrBayes 3.2 software and the data on the obtained amino acid sequences of PAL (Jones+G substitution model). Node formation possibilities are indicated with the figures. Black letters indicate the sequences obtained in the work, and gray color, sequences from NCBI databases.

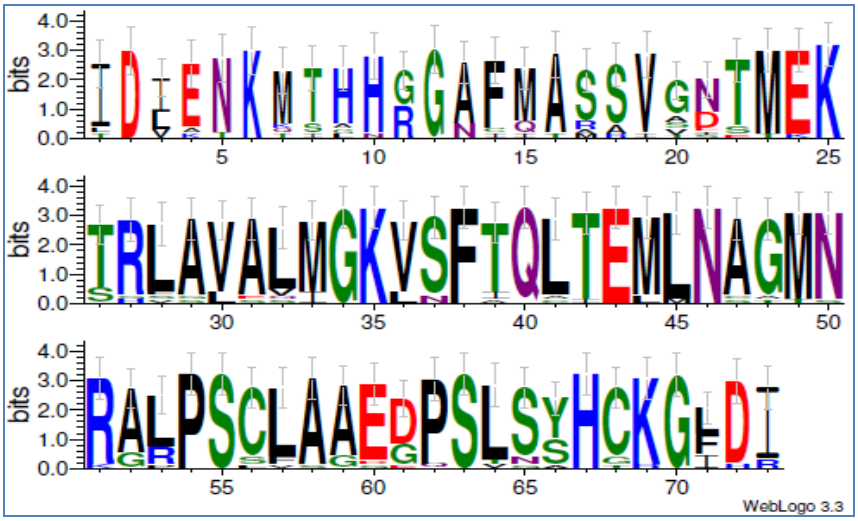

Fig. 5. Logo diagram of the PAL fragment, clade I.

Table 5. Identity percent of the *pal* gene fragment nucleotide sequences in some species

	Arabidopsis thaliana	*Aspergillus niger*	*Aureobasidium pullulans*	*Candida apicola*	*Candida glabrata*	*Candida maltosa*	*Cystofilobasidium capitatum*	*Debaryomyces castellii*	*Debaryomyces robertsiae*	*Neurospora crassa*	*Phaffia rhodozyma*	*Puccinia graminis f. sp. tritici*	*Rhodosporidium diobovatum*	*Rhodosporidium infirmo-miniatum*	*Rhodosporidium toruloides*	*Rhodotorula glutinis*	*Rhodotorula graminis*	*Rhodotorula mucilaginosa*	*Rhodotorula rubra*	*Saccharomyces cerevisiae*	*Saccharomyces kluyveri*	*Tilletiopsis washingtonensis*	*Tremella foliacea*
Aspergillus niger	60																						
Aureobasidium pullulans	23	24																					
Candida apicola	25	22	96																				
Candida glabrata	15	22	93	93																			
Candida maltosa	23	22	96	97	93																		
Cystofilobasidium capitatum	20	22	95	96	93	94																	
Debaryomyces castellii	26	25	90	86	92	93	95																
Debaryomyces robertsiae	18	22	95	96	92	94	96	95															
Neurospora crassa	52	62	21	21	16	21	18	22	18														
Phaffia rhodozyma	9	9	76	77	80	78	77	65	77	8													
Puccinia graminis f. sp. tritici	52	59	20	19	19	23	22	30	19	59	16												
Rhodosporidium diobovatum	37	40	87	84	91	92	95	97	94	33	59	43											
Rhodosporidium infirmo-miniatum	18	22	95	96	94	95	96	96	96	19	78	18	94										
Rhodosporidium toruloides	12	26	71	69	71	73	72	71	72	20	55	27	72	73									
Rhodotorula glutinis	32	35	86	83	89	90	92	94	91	31	58	38	99	92	62								
Rhodotorula graminis	43	47	35	34	32	34	34	41	34	49	21	57	58	33	43	50							
Rhodotorula mucilaginosa	23	22	88	86	83	88	86	87	86	20	65	27	89	86	75	86	38						
Rhodotorula rubra	29	34	80	77	82	84	85	88	85	27	51	35	89	85	72	87	48	97					
Saccharomyces cerevisiae	16	23	91	90	94	91	95	95	96	21	74	27	95	95	70	91	39	85	86				
Saccharomyces kluyveri	20	22	90	90	87	91	89	92	88	18	73	22	93	89	72	91	33	83	84	88			
Tilletiopsis washingtonensis	20	24	95	94	93	94	95	94	96	20	76	24	91	96	71	89	36	86	84	93	90		
Tremella foliacea	23	23	95	94	92	94	95	97	95	18	76	24	97	95	73	93	38	87	87	95	90	96	
Tremella mesenterica	17	22	84	82	82	83	84	78	82	16	67	19	79	84	62	77	32	76	71	82	80	84	83

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Table 6. Identity percent of the *pal* gene fragment amino acid sequences in some species

	Arabidopsis thaliana	*Aspergillus niger*	*Aureobasidium pullulans*	*Candida apicola*	*Candida glabrata*	*Candida maltosa*	*Cystofilobasidium capitatum*	*Debaryomyces castellii*	*Debaryomyces robertsiae*	*Neurospora crassa*	*Phaffia rhodozyma*	*Puccinia graminis f. sp. tritici*	*Rhodosporidium diobovatum*	*Rhodosporidium infirmo-miniatum*	*Rhodosporidium toruloides*	*Rhodotorula glutinis*	*Rhodotorula graminis*	*Rhodotorula mucilaginosa*	*Rhodotorula rubra*	*Saccharomyces cerevisiae*	*Saccharomyces kluyveri*	*Tilletiopsis washingtonensis*	*Tremella foliacea*
Aspergillus niger	41																						
Aureobasidium pullulans	40	41																					
Candida apicola	38	35	92																				
Candida glabrata	30	36	86	85																			
Candida maltosa	39	39	91	94	87																		
Cystofilobasidium capitatum	41	39	88	92	82	89																	
Debaryomyces castellii	43	43	92	84	84	90	87																
Debaryomyces robertsiae	38	38	90	91	86	87	93	90															
Neurospora crassa	43	53	41	40	35	41	43	44	41														
Phaffia rhodozyma	26	26	63	63	63	60	65	60	65	31													
Puccinia graminis f. sp. tritici	46	46	45	44	45	50	49	50	45	49	29												
Rhodosporidium diobovatum	45	41	91	86	86	91	93	95	93	43	53	50											
Rhodosporidium infirmo-miniatum	38	38	87	91	86	90	90	92	91	41	58	47	86										
Rhodosporidium toruloides	47	47	73	71	67	73	72	80	69	50	51	57	84	71									
Rhodotorula glutinis	47	39	91	87	85	91	93	95	91	43	53	52	100	87	85								
Rhodotorula graminis	50	47	68	65	60	67	65	70	64	46	48	58	78	63	79	79							
Rhodotorula mucilaginosa	42	42	94	92	84	94	90	96	89	43	63	52	100	89	79	100	72						
Rhodotorula rubra	44	40	87	83	81	87	88	90	87	44	60	51	100	83	81	100	77	94					
Saccharomyces cerevisiae	38	39	94	92	89	87	93	92	94	42	63	46	93	91	72	93	67	91	88				
Saccharomyces kluyveri	40	37	83	85	83	92	87	85	85	40	63	46	89	87	77	89	66	88	85	83			
Tilletiopsis washingtonensis	41	42	93	88	86	90	90	95	91	43	63	49	93	91	75	93	68	94	88	94	88		
Tremella foliacea	40	39	90	86	86	91	91	93	95	42	65	49	97	90	77	95	70	96	90	93	87	95	
Tremella mesenterica	27	28	72	69	71	66	72	69	71	31	58	33	82	69	59	81	50	71	74	75	68	72	68

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Study of the Specific Features of Phenotype and Biochemical Properties of the Selected Cultures

Phenotypic characteristics of the selected pigmented yeasts were judged by the combination of micro- and macromorphological traits (the former are studied with a microscope and the latter, visually). Micromorphology includes the features characterizing individual vegetative cells (shape, size), as well as the types of vegetative or asexual reproduction and the structures formed in the process. Macromorphology joins the culture features characterizing culture growth on dense (following the streak or in the form of a giant colony) or in liquid medium.

On a complete yeast medium (g/cm^3 distilled water: peptone, 10; yeast extract, 5; glucose, 20; agar, 20), yeasts form colonies of intermediate size and white or creamy color, colony surface is smooth, dim, elevated by a cone, colony edges are even or slightly wavy.

Description of the isolated yeast strains was performed according to a standard scheme. Shape and size of cells were described and determined in cultures of different ages on dense and liquid media. The first examination fnd measurement of cell size was performed in 2–3-day cultures grown at 25–28°C. Then, the cultures were left at room temperature (17–18°C) and described again after 4 weeks.

To determine cell size, length and width were measured with a micrometer in at least 20 cells, and the extreme values were indicated. Cells of isolated yeasts were found to be of round, round-like, oval, and cylindrical shape.

Size of mature yeast cells varied in different species from 1.0 to 8.0 μm in width and reached 17 μm and more in length in case of elongated cells. The results are presented in Table 7.

Further studies were aimed at determination of L-phenylalanine ammonia-lyase activity in the selected pigmented yeasts. The results of the investigation are presented in Table 8.

Table 7. Phenotypic traits of pigmented yeasts

Strain	Size, μm	Shape	Edge outline	Relief	Surface	Color
Candida glabrata Y2813	2.5 × 6.0	oval	uneven edges	smooth	dim	creamy
Aureobasidium pullulans Y863	8.0 × 6.0	ellipse-like	uneven edges	wrinkled	dim	creamy
Tremella foliacea Y1624	2.0 × 6.5	round	uneven edges	smooth	shiny	creamy
Phaffia rhodozyma Y1668	3.8 × 10.0	round-to-oval	uneven edges	smooth	dim	red orange
Rhodotorula lactose Y2770	2.5 × 8.0	oval-to-elongated	uneven edges	smooth	shiny	red pink
Rhodosporidium diobovatum Y1565	1.0 × 9.0	round-to-oval	uneven edges	wrinkled	dim	red pink
Rhodotorula rubra Y1193	2.3 × 6.5	oval-to-elongated	even edges	smooth	shiny	red pink
Tremella mesenterica Y1625	2.0 × 8.5	round-to-oval	uneven edges	smooth	shiny	creamy
Debaromyces castellii Y 968	3.8 × 8.5	round-to-oval	uneven edges	wrinkled	dim	white
Candida maltose Y242	2.0 × 7.0	round-to-cylindrical	even edges	smooth	shiny	creamy
Rhodosporidium capitatum Y1567	1.0 × 14.0	round-to-cylindrical	even edges	smooth	dim	pink orange
Saccharomyces kluyveri Y2559	5.0 × 12.0	round-to-cylindrical	even edges	smooth	shiny	light creamy
Bullera alba Y 1581	2.0 × 6.5	oval	uneven edges	smooth	dim	creamy
Rhodotorula glutinis Y77	2.3 × 10.0	round-to-oval	even edges	smooth	dim	red pink
Rhodosporidium infirmominiatum Y1569	1.0 × 10.0	round-to-oval	even edges	smooth	dim	red pink
Debaryomyces robertsiae Y3392	2.8 × 8.0	round-to-oval	uneven edges	wrinkled	dim	white
Saccharomyces cerevisiae Y1127	5.0 × 12	round-to-cylindrical	even edges	smooth	shiny	light creamy
Tilletiopsis washingtonensis Y1650	1.0 × 17.0	round-to-cylindrical	uneven edges	wrinkled	dim	creamy
Torulopsis apicola Y566	2.0 × 6.0	round-to-oval	even edges	smooth	shiny	yellowish-creamy

Note:. Width × length.

Table 8. L-phenylalanine ammonia-lyase activity in selected pigmented yeasts

No.	Yeast	L-phenylalanine ammonia-lyase activity, U/mg protein
1	*Aureobasidium pullulans* Y863	0.038
2	*Bullera alba* Y1581	0.010
3	*Bullera piricola* Y1577	0.008
4	*Candida glabrata* Y2813	0.007
5	*Candida maltosa* Y242	0.012
6	*Cryptococcus laurentii* Y227	0.010
7	*Cryptococcus macerans* Y2763	0.008
8	*Cystofilobasidium capitatum* Y1852	0.010
9	*Debaryomyces castellii* Y968	0.007
10	*Debaryomyces robertsiae* Y3392	0.012
11	*Dioszegia hungarica* Y3208	0.010
12	*Dioszegia sp.* Y3320	0.008
13	*Geotrichum klebahnii* Y3053	0.009
14	*Phaffia rhodozyma* Y1668	0.009
15	*Rhodosporidium diobovatum* Y1565	0.022
16	*Rhodosporidium infirmominiatum* Y1569	0.019
17	*Rhodotorula glutinis* Y77	0.008
18	*Rhodotorula lactosa* Y2770	0.049
19	*Rhodotorula rubra* Y1193	0.015
20	*Saccharomyces cerevisiae* Y1127	0.016
21	*Saccharomyces kluyveri* Y2559	0.010
22	*Sporobolomyces holsaticus* Y991	0.009
23	*Tilletiopsis washingtonensis* Y1650	0.017
24	*Torulopsis apicola* Y566	0.011
25	*Tremella foliacea* Y1624	0.019
26	*Tremella mesenterica* Y1625	0.010

The data presented in Table 8 evidence that the highest activity was exhibited by the strains *Aureobasidium pullulans* Y863, *Rhodosporidium infirmominiatum* Y1569, *Candida glabrata* Y2813, *Candida maltose* Y242, *Debaryomyces robertsiae* Y3392, *Rhodosporidium diobovatum* Y1565, *Rhodotorula lactose* Y2770, *Saccharomyces cerevisiae* Y1127, *Tilletiopsis washingtonensis* Y1650, *Torulopsis apicola* Y566, *Tremella foliacea* Y1624, *Rhodotorula rubra* Y1193, and *Debaryomyces castellii* Y968, which allows to recommend them for further studies aimed at the development of an enzyme preparation of L-phenylalanine ammonia-lyase.

Therefore, we analyzed the DNA sequence encoding the synthesis of L-phenylalanine ammonia-lyase (PAL) to create universal primers complementary to conserved regions of the *pal* gene. The pal gene fragment was amplified in the organisms under study. Nucleotide sequence of the *pal* gene in microorganisms possessing L-phenylalanine ammonia-lyase activity was determined by DNA sequencing. The sequence was compared with the relevant sequences of known species. Phenotypic features and biochemical properties of the selected cultures were investigated.

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