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EXPERIMENTAL ARTICLES

Massive Isolation of Anamorphous Ascomycete Yeasts *Candida oleophila* from Plant Phyllosphere

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Abstract—Many years of research has confirmed a wide distribution of anamorphous ascomycete yeasts in the phyllosphere of diverse plants of Moscow and the Moscow oblast. Based on the standard morphological and physiological criteria, on the results of restriction analysis of the 5.8S-ITS rDNA region, and on the sequencing of the D1D2 region of 26S rDNA, these yeasts were identified as *Candida oleophila* Montrocher. Previous isolation of this species has been rare, possibly due to its incorrect identification. This species, together with phytobiotic basidiomycete yeasts, was shown to be dominant in the yeast epiphytic communities on the surface parts of plants. The relative abundance of *C. oleophila* is highest on plant fruits and increases significantly by the end of the vegetation period. Wide occurrence of this yeast species on fruits and in the phyllosphere may be related to its ability to compete with rapidly growing phytopathogenic fungi.

Key words: yeasts, *Candida oleophila*, phyllosphere, epiphytic microorganisms.

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Plants and plant debris are the main natural habitat for the majority of yeast species. The yeast communities formed on the surface of living plant parts are the most numerous and diverse [1]. Exudations (secretions of living plants, which contain simple sugars, organic acids and other easily utilized compounds) are the main nutrient source for such epiphytic yeasts. Numerous works on epiphytic yeasts dealt mostly in yeast biodiversity [2] and development of the methods of phytopathogen control [3]. The yeasts from plant leaves belong both to ascomycete and basidiomycete species; anamorphous stages of yeastlike basidiomycete fungi are, however, the most typical epiphytes. They belong to such species as *Cryptococcus albidus, Cr. magnus, Cr. laurentii, Rhodotorula glutinis, Rh. mucilaginosa*, and *Sporobolomyces roseus*. A number of basidiomycete yeast species produce carotenoid pigments, capsulae, and actively removable ballistospores; these features facilitate their existence as epiphytes.

Ascomycete yeasts are less common on plant leaves. They usually inhabit substrates with high concentrations of simple sugars (nectar-containing entomophilous flowers, outflows of tree juice, or sweet fruits). However, numerous investigations have confirmed the presence of ascomycete yeasts in phyllosphere communities as minor components. They relatively infrequently occur in the phyllosphere; they do not inhabit plant leaves permanently or in high amounts. Only a few species are exceptions, including *Metschnikowia pulcherrima*, usually inhabiting flower nectar, and eurybiotic *Debaryomyces hansenii*, which can be recovered with an almost equal probability from a wide range of habitats. Apart from these species, anamorphous yeasts are found relatively often in the phyllosphere; they are classified within the formal genus *Candida.* Different *Candida* species have been mentioned, however, by different authors. This may be a result of the difficulty of phenotypic identification of the species within this genus, which is due to the absence of characteristic morphology and a usually narrow spectrum of assimilated substrates. Apart from incorrect identification, the inconsistencies of the ascomycete yeast composition may be the result of the specific character of their distribution in plant and soil substrates. Since many ascomycete yeasts are known to be closely associated with invertebrates [4], their isolation from leaves is random.

Our research of several years involving modern molecular genetic identification methods revealed that, separate from various randomly occurring species, one species of anamorphous ascomycete yeasts is constantly present in the epiphytic yeast communities. The goal of the present work was identification of this species, as well as description of its distribution patterns.

MATERIALS AND METHODS

The research was carried out from 2001 to 2005 in Moscow (Losinyi Ostrov National park and Izmailovo

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Origin of the investigated *C. oleophila* strains

Strain	Substrate	Plant species
AY C5	Green leaves	*Betula verucosa*
BR 129-1	Green leaves	*Vaccinium vitisidea*
CB 26-2	Fruits	*Thelycrania alba*
ZhVCh-98-1	Green leaves	*Ajuga reptans*
ODV 141-1	Flowers	*Taraxacum officinale*
BER 6-2	Decomposing leaves	*Betula verucosa*

Park) and in the vicinity of the village of Burtsevo, Shakhovskoi region, Moscow oblast. The samples were collected in two types of biogeocenoses: a mixed birch–spruce forest and a secondary afterforest meadow on mid-loamy sod–podzolic soil. Twenty-five plant species, representing various life forms, ecological groups, and durations of the phyllosphere vegetation period, were chosen as the objects of this investigation. These species have been previously characterized by us in detail [5].

The leaves were sampled two to three times a week throughout the vegetation period, from the residual bud leaves to the decomposing leaves in the litterfall. In winter the samples of evergreen plant species were collected from under the snow cover. The flowers were sampled from buds, including the periods of active blossoming, wilting, and fruit formation. The fruits were also analyzed from formation to complete decomposition. The samples were analyzed either in the day of sampling or in the course of two to three days.

For yeast quantification, leaves, flowers, or fruits were homogenized with scissors; 5–10 portions of 0.1–0.5 g were diluted with sterile water to 1 : 50 and vortexed for three min. Malt agar acidified with lactic acid to prevent bacterial growth was used as a nutrient medium. From each dilution, inoculations were repeated twice. The plates were incubated at room temperature for five to seven days. The morphological type of the colonies was determined under a dissection microscope, and the number of each colony type was determined. The representatives of each colony type were isolated and identified by standard methods, using the manual [6] and more recent identification keys. These data were used to determine the total number of yeasts in CFU per 1 g of dry matter, and the ratio of each species.

The molecular biological methods of identification used were restriction analysis of the 5.8S-ITS rDNA region (RFLP) and sequencing of the D1D2 region of the rDNA big subunit.

Amplification of the 5.8S-ITS DNA region was carried out using yeast biomass according to the modified procedure [7, 8]. The culture was grown on a complete yeast medium (glucose, 20 g/l; yeast extract, 5 g/l; peptone, 10 g/l; agar, 10 g/l) for one to two days. DNA extraction was carried out on 30 μl of the reaction mixture containing PCR buffer, 0.25 mM dNTP, and primers ITS1 and ITS4 (30 μM each). The amplifier Tertsik (DNA Technologiya, Russia) was used according to the following program: 95°C, 15 min. Each sample was then supplemented with 1.25 U of *Taq* polymerase (Sintol, Russia) and the fragment was amplified: initial denaturing, 3 min at 94°C; then 30 cycles of denaturing, 2 min at 94°C, annealing, 1 min at 60°C, and DNA synthesis, 2.5 min at 72°C; and final extension, 10 min at 72°C.

Analysis of restriction fragment lengths was carried out as described previously [9]. Endonucleases *Hae*III, *Hin*fI, and *Cfo*I were used (Fermentas, Lithuania). The products were separated in 1.5% agarose gel at 140–150 V in 0.5× TAE buffer for 1–1.5 h. The BioRad GelDoc imaging system (BioRad, Richmond, United States) was used for the visualization and treatment of the fragments. The profile images were aligned according to the 100 bp DNA ladder marker (Fermentas, Lithuania) using Quantity One software (BioRad, Richmond, United States) and analyzed. The tables mentioned in [7, 8] were used for the identification of the analyzed isolates.

Analysis of the nucleotide sequences of the D1D2 region of 26S rDNA was carried out as described previously [10]. The sequencing was carried out on an ALF Express II DNA analyzer semiautomatic sequencer (Amersham Pharmacia Biotech, Sweden). The results were corrected manually. The identification was carried out by comparison of the obtained nucleotide sequences with those in online databases.

RESULTS

Multiphase identification. Over 300 strains of anamorphous yeasts of ascomycete affinity were isolated from plant leaves, flowers and fruits during the period of investigation; according to their morphological and physiological characteristics, the isolates certainly belonged to one species. The yeasts exhibited a characteristic creamy white dry streak, which became uneven in the course of incubation. On malt agar, the cells were mostly of rounded oval shape, 2.5 × 4.5 μm, although elongated forms (up to 10 μm) were often present (Fig. 1). A complex pseudomycelium with oval blastoconidia was formed.

The standard physiological spectrum was determined for 105 strains. All the strains assimilated glucose, galactose, sorbose, xylose, sucrose, maltose, trehalose, salicin, arbutin, melezitose, sorbitol, mannitol, 2-keto-gluconate, malic, succinic, and citric acids, glycerol, and ethanol. Ribose, L-arabinose, D-arabinose, rhamnose, α-methyl-D-glucoside, melibiose, lactose, raffinose, inulin, starch, erythritol, dulcitol, inositol, and 5-keto-gluconate were not assimilated. Nitrate was not assimilated. Glucose and galactose were fermented; sucrose, maltose, and trehalose were weakly fermented; lactose and raffinose were not fermented.

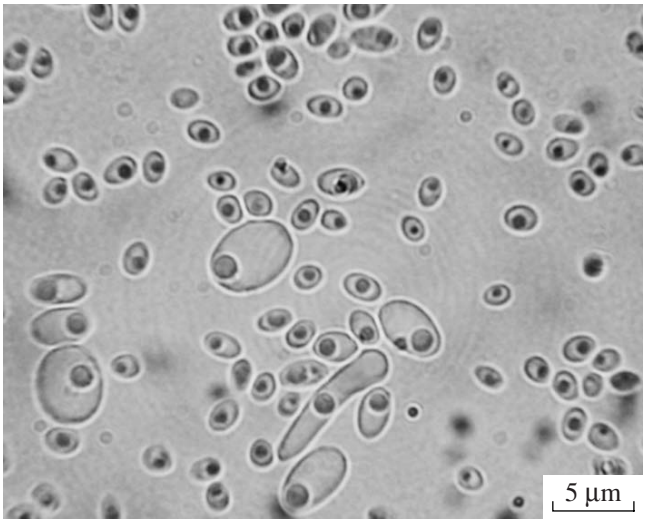

Fig. 1. *Candida oleophila* cells in a malt agar 7-day culture.

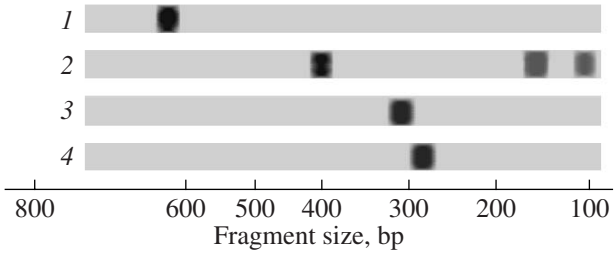

Fig. 2. Results of analysis of the 5.8S-ITS rDNA region. Schematic representation of the fragment isolation. PCR product (*1*); restriction with the *Hae*III endonuclease (*2*); restriction with the *Hin*fI endonuclease (*3*); restriction with the *Cfo*I endonuclease (*4*).

This set of morphological and physiological characteristics corresponds most closely to the following species: *Candida oleophila, C. sake, C. kunwiensis, C. railenensis*, and *C. shehatae* var. *lignosa*.

Six strains were chosen for more precise identification and confirmation of the specificity by analysis of DNA restriction fragment length and rDNA sequencing (table).

Restriction fragments length polymorphism analysis of the smaller ribosomal subunit and of two internal transcribed spacers has been successfully used for rapid and reliable identification of many ascomycete yeasts, including the genus *Saccharomyces* [11], *Kluyveromyces lactis* [12], the species responsible for fruit juice spoilage [13], wine yeasts [14], and clinical isolates [15]. In all six strains investigated, the size of the PCR product of the 5.8*S*-ITS region was ca. 630 bp; restriction profile 420, 140, and 80 bp with *Hae*III, twin fragments with *Cfo*I (295 bp) and *Hin*fI (320 bp) (Fig. 2). This restriction profile is known for several *Candida* species: *C. membranifaciensis, C. santamariae, C. conglobata, C. atlantica, C. atmosphaerica, C. zeylanoides*, and *C. oleophila* [8]. However, according to the results of the physiological tests, all these species, apart from *C. oleophila*, differ significantly from the investigated strains.

Due to the small scale of the differences, the method of restriction fragments length polymorphism analysis did not segregate the aforementioned species reliably; the same authors found, however, that this task can be achieved by sequence analysis of the D1D2 region of 26S rDNA. Analysis of these sequences confirmed that the investigated strains belong to the species *C. oleophila.* In compliance with the requirements for species identification based on genetic criteria [16], no differences were revealed between the sequences of these isolates (GenBank accession no. EF452234) and the *C. oleophila* type strain (CBS 2219, NRRL Y-2317, U45793).

Thus, according to the results of combined phenotypic and molecular genetic identification, the strains chosen for analysis belong to the species *Candida oleophila* Montrocher.

Ecological features. The average content of *C. oleophila* in all the analyzed samples of plant substrates is 3.7% of the total yeast number. At the same time, the ratio of phylobasidial cryptococci of the *Cryptococcus albidus* group on plant leaves was ca. 20%; of red-pigmented yeasts *Rhodotorula glutinis*, ca. 13%; of eurybiont ascomycete yeasts *Debaryomyces hansenii*, 4%; of *Sporobolomyces roseus*, 3%; and of tremellan cryptococci close to *Cryptococcus laurentii* and *Cr. victoriae*, less than 3%. *Rhodotorula mucilaginosa* and *Metschnikowia* species mostly associated with entomophilous nectar-containing flowers (*M. pulcherrima* and *M. reukaufii*) constituted ca. 1–2% each. The average ratio of the other 60 species found on plant leaves did not exceed 1%; in the phyllosphere of the plants of the moderate zone, it was on average 0.05–0.5%.

Significant differentiation was observed in *C. oleophila* distribution in different types of plant substrates. On vestigial leaves, its average abundance was 0.2%; on mature leaves, 2.8%; on flowers, 2.5%; in the decomposing litterfall, 2.5%; and on fleshy fruits, 14.3%.

The relative abundance of *C. oleophila* varied significantly throughout a year (Fig. 3). In spring its relative abundance was minimal (ca. 1% on all plant leaves. The ratio of this species among the phyllosphere yeasts increased noticeably during summer; continued to increase in autumn, and peaked (over 20%) in November in the litterfall and on the leaves of evergreen plants. The highest relative abundance of *C. oleophila* (up to 30%) was revealed during late autumn on dry and fleshy fruits, especially on the following plant species: *Physocarpus opulifolious, Thelycrania alba, Euonymus verrucosa, Symphoricarpos albus*, and *Tanacetum vulgare.* The relative abundance then began to decrease and reached its minimal values by next spring. Such

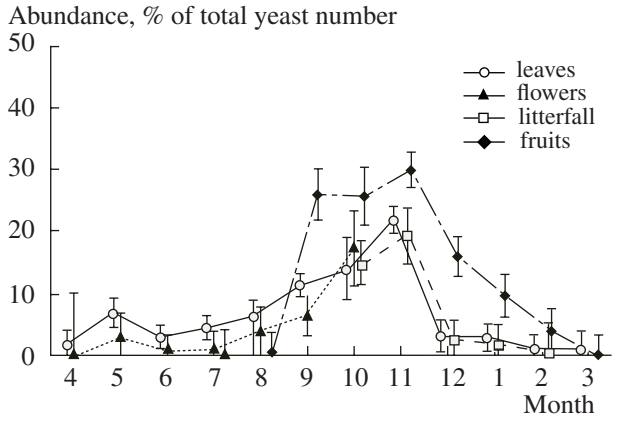

Fig. 3. Average monthly relative abundance of *C. oleophila* in various plant substrates: leaves (*1*), flowers (*2*), litterfall (*3*), and fruits (*4*).

seasonal dynamics are typical of most of the epiphytic species of basidiomycete affinity; the ratio of these species increased in autumn, when the numbers and diversity of epiphytic yeasts peak into moderate zone [5]. The ratio of *C. oleophila* in the epiphytic yeast community varied according to the same pattern on different plant species, independent on their ontogenetic characteristics. Thus these variations are primarily related to the seasonal changes in temperature and humidity throughout the year.

DISCUSSION

Ascomycete anamorphous yeasts identified as *Candida oleophila* have been repeatedly isolated from various natural and anthropogenic substrates (leaves and fleshy fruits, decomposing wood, and soft drinks) [6]. However, similar to the overwhelming majority of *Candida* species, these isolations have been rare and did not reveal any distributional patterns. Our research of many years, involving analysis of thousands of plant samples, has demonstrated that *C. oleophila* is among the most widespread yeast species always present on leaves of various plant species in various phytocenoses. It belongs to the group of the dominant species of the phyllosphere of the plants of the temperate zone, together with typical dimorphous basidiomycetes, a eurybiont ascomycete *D. hansenii*, and *Metschnikowia* species. Since mass isolation of this species from leaves of diverse plants was achieved, its previous isolations have probably resulted in its multiple misidentification among the *Candida* species. By means of phenotypic identification based on the standard spectrum of physiological characteristics, it is difficult to differentiate between this species and *C. sake, C. shehatae*, or anamorphous *Metschnikowia* species. Moreover, such a wide-scale work, resulting in the isolation of over 300 strains of one species from the phyllosphere of diverse plants of the temperate zone has never before been performed.

This species has recently attracted attention due to the ability of some of its strains to suppress fast-growing fungi causing berry and fruit spoilage (*Botrytis cinerea, Penicillium digitatum*, etc.) A number of studies revealed that *C. oleophila* can be successfully used for biocontrol of these phytopathogens [17–19]. The commercial preparation Aspire based on these yeasts is used as a biopesticide [20]. The mechanism of the *C. oleophila* antagonistic effect is not yet clear. Apart from the competition with phytopathogenic fungi, it may be the result of secretion of certain lytic enzymes (1,3-β-glucanase, chitinase, and protease) [21]. This is probably the reason for wide *C. oleophila* distribution in the phyllosphere and on fruits. Moreover, *C. oleophila* has diverse assimilatory capacities. It can utilize more than half of the compounds included in the standard identification spectrum, a feature unusual for most ascomycete species.

We have previously demonstrated that in certain brief periods the numbers of some ascomycete yeast, including saccharomycetes, in the phyllosphere can increase significantly [22]. The present research revealed that, apart from the ubiquitous basidiomycete phytobionts, ascomycete yeasts are present among the constantly dominant yeast species. In spite of the absence of such characteristic adaptations as carotenoid pigments and polysaccharide capsulae, they can successfully develop on plant leaves. This information may be useful for the microbiological monitoring of biodiversity and for the development of biocontrol methods against phytopathogenic microorganisms.

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REFERENCES

1. Bab'eva, I.P. and Chernov, I.Yu., *Biologiya drozhzhei* (Biology of Yeasts), Moscow: Tovarishchestvo nauchnykh izdanii KMK, 2004.
2. Fonseca, A and Inacio, J, Phylloplane Yeasts, *Biodiversity and Ecophysiology of Yeasts. The Yeast Handbook*, Rosa, C.A. and Peter, G., Eds., Springer, 2006, pp. 263–301.
3. El-Tarabily, K.A. and Sivasithamparam, K., Potential of Yeasts as Biocontrol Agents of Soil-Borne Fungal Plant Pathogens and as Plant Growth Promoters, *Mycoscience*, 2006, vol. 47, no. 1, pp. 25–35.
4. Terenina, E.E. and Chernov, I.Yu., Taxonomic Structure of the Yeast Communities Associated with Invertebrates, *Mikol. Fitopatol.*, 2001, vol. 35, no. 4, pp. 65–73.

5. Glushakova, A.M. and Chernov, I.Yu., Seasonal Dynamic of the Numbers of Epiphytic Yeasts, *Mikrobiologiya*, 2007, vol. 76, no. 5, pp. [*Microbiology* (Engl. Transl.), vol. 76, no. 5, pp. 590–595].

6. *The Yeasts, a Taxonomic Study. 4th Revised and Enlarged Ed.*, Kurtzman, C.P. and Fell, J.W., Eds, Amsterdam: Elsevier Sci. Publ, 1998.

7. Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A., Identification of Yeasts by RFLP Analysis of the 5.8S rRNA Gene and the Two Ribosomal Internal Transcribed Spacers, *Int. J. Syst. Bacteriol.*, 1999, vol. 49, no. 1, pp. 329–337.

8. Frutos, R.L., Fernández-Espinar, M.T., and Querol, A., Identification of Species of the Genus *Candida* by Analysis of the 5.8S rRNA Gene and the Two Ribosomal Internal Transcribed Spacers, *Antonie van Leeuwenhoek*, 2004, vol. 85, no. 3, pp. 175–185.

9. Yurkov, A.M. and Chernov, I.Yu., Geographical Races of Certain Species of Ascomycetous Yeasts in the Moscow and Novosibirsk Regions, *Mikrobiologiya*, 2005, vol. 74, no. 5, pp. 687–692 [*Microbiology* (Engl. Transl.), vol. 74, no. 5, pp. 597–601].

10. Inacio, J., Portugal, L., Spencer-Martins, I., and Fonseca, A., Phylloplane Yeasts from Portugal: Seven Novel Anamorphic Species in the *Tremellales* Lineage of the *Hymenomycetes* (*Basidiomycota*) Producing Orange-Coloured Colonies, *FEMS Yeast Res.*, 2005, vol. 5, pp. 1167–1183.

11. Fernández-Espinar, M.T., Esteve-Zarzoso, B., Querol, A., and Barrio, E., RFLP Analysis of the Ribosomal Internal Transcribed Spacers and the 5.8S rRNA Gene Region of the Genus *Saccharomyces:* a Fast Method for Species Identification and the Differentiation of Flor Yeasts, *Antonie van Leeuwenhoek*, 2000, vol. 78, no. 1, pp. 87–97.

12. Naumova, E.S., Sukhotina, N.N., and Naumov, G.I., Molecular-Genetic Differentiation of the Dairy Yeast *Kluyveromyces lactis* and Its Closest Wild Relatives, *FEMS Yeast Res.*, 2004, vol. 5, no. 3, pp. 263–269.

13. Heras-Vazquez, F.J.L., Mingorance-Cazorla, L., Clemente-Jimenez, J.M., and Rodriguez-Vico, F., Identification of Yeast Species from Orange Fruit and Juice by RFLP and Sequence Analysis of the 5.8S rRNA Gene and the Two Internal Transcribed Spacers, *FEMS Yeast Res.*, 2003, vol. 3, no. 1, pp. 3–9.

14. Guillamón, J.M., Sabate, J., Barrio, E., Cano, J., and Querol, A., Rapid Identification of Wine Yeast Species Based on RFLP Analysis of the Ribosomal Internal Transcribed Spacer (ITS Region), *Arch. Microbiol.*, 1998, vol. 169, no. 5, pp. 387–392.

15. Chen, Y.C., Eisner, J.D., Kattar, M.M., Rassoulian-Barrett, S.L., Lafe, K., Bui, U., Limaye, A.P., and Cookson, B.T., Polymorphic Internal Transcribed Spacer Region 1 DNA Sequences Identify Medically Important Yeasts, *J. Clin. Microbiol.*, 2001, vol. 39, no. 11, pp. 4042–4051.

16. Kurtzman, C.P. and Robnett, C.J., Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences, *Antonie van Leeuwenhoek*, 1998, vol. 73, no. 4, pp. 331–371.

17. Mercier, J. and Wilson, C.L., Colonization of Apple Wounds by Naturally Occurring Microflora and Introduced *Candida oleophila* and Their Effect on Infection by *Botrytis cinerea* during Storage, *Biological Control*, 1994, vol. 4, no. 2, pp. 138–144.

18. Lima, G., Ippolito, A., Nigro, F., and Salerno, M., Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* Against Postharvest Strawberry Rots, *Postharvest Biol. Technol.*, 1997, vol. 10, no. 2, pp. 169–178.

19. Lahlali, R., Serrhini, M.N., and Jijakli, M.H., Efficacy Assessment of *Candida oleophila* (Strain O) and *Pichia anomala* (Strain K) Against Major Postharvest Diseases of Citrus Fruits in Morocco, *Commun. Agric. Appl. Biol. Sci.*, 2004, vol. 69, no. 4, pp. 601–609.

20. Droby, S., Cohen, L., Daus, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M., and Shachnai, A., Commercial Testing of Aspire: a Yeast Preparation for the Biological Control of Postharvest Decay of Citrus, *Biological Control*, 1998, vol. 12, no. 2, pp. 97–101.

21. Bar-Shimon, M., Yehuda, H., Cohen, L., Weiss, B., Kobeshnikov, A., Daus, A., Goldway, M., Wisniewski, M., and Droby, S., Characterization of Extracellular Lytic Enzymes Produced by the Yeast Biocontrol Agent *Candida oleophila, Curr. Genet.*, 2004, vol. 45, no. 3, pp. 140–148.

22. Glushakova, A.M., Ivannikova, Yu.V., Naumova, E.S., Chernov, I.Yu., and Naumov, G.I., Massive Isolation and Identification of *Saccharomyces paradoxus* Yeasts from Plant Phyllosphere, *Mikrobiologiya*, 2007, vol. 76, no. 2, pp. 236–242 [*Microbiology* (Engl. Transl.), vol. 76, no. 2, pp. 205–210].