



Plant growth-promoting and antifungal activity of yeasts from dark chestnut soil



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ABSTRACT

538 yeast strains were isolated from dark chestnut soil collected from under the plants of the legume family (*Fabaceae*). The greatest number of microorganisms is found at soil depth 10–20 cm. Among the 538 strains of yeast 77 (14.3%) strains demonstrated the ability to synthesize IAA. 15 strains were attributed to high IAA-producing yeasts (above 10 µg/ml). The most active strains were YA05 with 51.7 ± 2.1 µg/ml of IAA and YR07 with 45.3 ± 1.5 µg/ml. In the study of effect of incubation time on IAA production the maximum accumulation of IAA coincided with maximum rates of biomass: at 120 h for YR07 and at 144 h for strain YA05. IAA production increased when medium was supplemented with the L-tryptophan. 400 µg/ml of L-tryptophan showed maximum IAA production. 10 strains demonstrated the ability to inhibit the growth and development of phytopathogenic fungi. YA05 and YR07 strains formed the largest zones of inhibition compared to the other strains – from 21.6 ± 0.3 to 30.6 ± 0.5 mm. Maximum zone of inhibition was observed for YA05 against *Phytophthora infestans* and YR07 strains against *Fusarium graminearum*. YA05 and YR07 strains were identified as *Aureobasidium pullulans* YA05 (GenBank accession No JF160955) and *Rhodotorula mucilaginosa* YR07 (GenBank accession No JF160956).

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1. Introduction

The PGPM (plant growth-promoting microorganisms) have a number (direct and indirect) beneficial effects on plants, such as: nitrogen fixation, production of plant hormones like auxins, gibberellins, cytokins, synthesis of vitamins, antifungal and antibiotic compounds, ability to solubilize minerals like phosphorus and other nutrients, capability to degrade numerous toxic chemicals, etc. (Pérez-Montañó et al. 2014; Yang et al. 2009; Amprayn et al. 2012; Martínez-Viveros et al. 2010; Kim et al. 2011; Vessey 2003; Nutaratat et al. 2014).

The most attention is paid to the role of auxin in the stimulation of plant growth and nutrition, because the ability to produce indole-3-acetic acid (IAA) is widespread among soil organisms (Rao et al. 2010; Limtong and Koowadjanakul 2012; Limtong et al. 2014; Nassar et al. 2005; El-Tarabily 2004; Bilkay et al. 2010). The positive effect of microbial auxins on the initiation and elongation of roots and stems (Nassar et al. 2005; El-Tarabily and Sivasithamparam

2006), on the development of lateral roots and root hairs is shown, that may be important for plant growth promotion, the uptake of nutrients and the formation of plant resistance to stress (Glick et al. 1999; Halliday et al. 2009; Moller and Weijers 2009). The synthesis of auxins by soil and rhizosphere microorganisms is largely determined by the composition of root exudates containing the major metabolic precursor L-tryptophan (Lynch 1985; Bharucha et al. 2013; Maslov et al. 2011).

It is noteworthy that effects of PGPM are preserved not only in natural communities, but also after plants treatment with microorganisms in laboratory and field conditions (Agamy et al. 2013; Bennett and Whipps 2008; Ji et al. 2014). Many studies have noted the increase in germination, length and biomass of seedlings after seed inoculation with yeast strains, enhance plant growth, increase of photosynthesis productivity (Hu and Qi 2013; Amprayn et al. 2012; Agamy et al. 2013; Nassar et al. 2005). The positive effect of biologically active compounds produced by microorganisms on agricultural plants begins at the earliest stages of plant development and further is expressed in the suppression phytopathogens and increase productivity.

Biocontrol of plant pathogenic organisms by soil yeast is carried out, on the one hand, by improving the uptake of water and mineral elements nitrogen, phosphorus, potassium by the plant, on the

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Fig. 1. Soil profiles.

other hand – through the production of antifungal agents and the displacement of pathogenic bacteria and fungi in the rhizosphere by inhibiting their growth (Santos et al. 2004; Compant et al. 2005; Nutaratat et al. 2014; Vero et al. 2002; Chanchaichaovivat et al. 2007; Hatoum et al. 2012; Golubev 2006).

2. Materials and methods

2.1. Sampling

Samples of dark chestnut soil were collected in the foothill areas of Almaty region of Kazakhstan in the period from August to September 2013. The soil samples were collected from under the plants of the legume family (*Fabaceae*): alfalfa (*Medicago sativa*), sainfoin (*Onobrychis viciifolia*), clover (*Trifolium pratense*), sweet clover (*Melilotus officinalis*), and soybean (*Glycine max*). Material was taken from 0 to 10 cm, 10 to 20 cm and from 20 to 40 cm depth (Fig. 1).

The soil samples were analyzed for a variety of physical and chemical characteristics (Table 1).

2.2. Isolation of yeasts

Yeasts were isolated from soil samples using serial dilution and pour plate method. Sabouraud dextrose agar (SDA), Chapek-Dox agar, Ashby-sucrose agar were used for the isolation.

2.3. Screening of indole-3-acetic acid (IAA) producing yeasts

Yeast isolates were grown in Sabouraud dextrose broth supplemented with 0.1% (1000 µg/ml) L-tryptophan. Strains were incubated on a shaker (180 rpm) at 26 °C. At the end of the

incubation cultures were centrifuged at 8000 g for 10 min and the supernatants were collected. One ml of supernatant was mixed with 2 ml of the Salkowski reagent (1 ml of 0.5 M FeCl₃ in 50 ml of 35% HClO₄). The mixture was allowed to stand for 30 min for color development. The intensity of the color developed was measured at a wavelength of 530 nm using a spectrophotometer (Gordon and Weber 1951). Calibration curve using authentic IAA was established for calculation of IAA concentration. The high IAA-producing strains were selected for further studies.

2.4. Study of effect of incubation time and L-tryptophan concentration on IAA production

2.4.1. Influence of incubation time on IAA production

The effect of incubation time on IAA production by yeast strains was studied in Sabouraud dextrose broth amended with 1% (1000 µg/ml) L-tryptophan. Samples were drawn every 12 h up to 192 h. Yeast biomass was determined by dry weight. Yeast culture samples (10 ml) were filtered over preweighed filters. The filters were dried at 85 °C for 2 h and weighed. IAA production was determined as described previously in Section 2.3.

2.4.2. Influence of L-tryptophan concentration on IAA production

Tryptophan is a main metabolic precursor for indole-3-acetic acid biosynthesis pathways in microorganisms (Martens and Frankenberger 1993; Mano and Nemoto 2012; Spaepen et al. 2007). L-Tryptophan in an amount of 50, 200, 400, 600, 800 and 1000 µg/ml was added into the medium. IAA production was also detected in the absence of tryptophan. IAA production was determined as described previously in Section 2.3.

2.5. Study of antifungal activity

Phytopathogenic fungi from the collection of the Institute of Microbiology and Virology of the MES RK *Fusarium graminearum*, *Cladosporium* sp., *Phytophthora infestans* and *Botrytis cinerea* were used as test objects. Yeast strains were grown on SDA medium in tubes for 5 days. 0.1 ml of aqueous suspensions of yeasts with concentration of 10⁶ cells/ml was plated on the surface of SDA plates. Yeasts were grown as a lawn on a Sabouraud agar surface for 5–7 days and then disks of 8 mm in diameter were cut. Phytopathogenic fungi were cultured for 4–5 days. Suspensions containing 10⁴ conidia/ml were prepared from grown cultures and then lawns of phytopathogenic fungi were made on plates. On the surface of the Petri dishes with phytopathogenic fungi disks with yeasts cultures were placed. Antifungal activity was determined by measuring zone of inhibition produce by yeasts against phytopathogenic fungi.

2.6. Molecular identification of strains

Molecular identification of yeast strains was performed in National Scientific Laboratory of Biotechnology in National Biotechnology Center of the MES RK (Astana, Kazakhstan). Yeast cells were grown under aerobic conditions at 24 °C for 1 day. After extraction and purification of genomic DNA the DNA concentration was measured using a NanoDrop spectrophotometer at a wavelength of 260 nm. The DNA concentration was normalized

Table 1
General physical and chemical properties of soil.

Sampling depth (cm)	pH	Organic C (%)	Total N (%)	Moisture content (%)	Horizon	Texture
0–10	8.40	2.84	0.20	3.9	A	Light loam
10–20	8.36	2.65	0.18	6.1	A	Light loam
20–40	8.24	2.47	0.17	7.6	B	Light loam

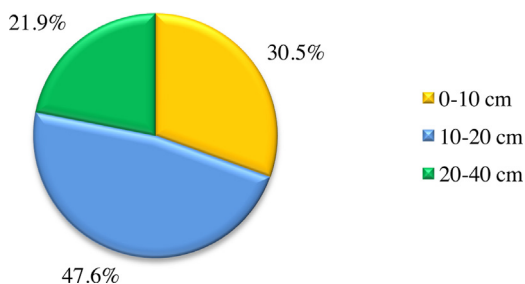


Fig. 2. The distribution of yeast strains based on depth of soil.

to 30 ng/ml. Amplification of ITS region fragment was performed with universal primers ITS4-5'-TCCTCCGCTTATTGATATGC-3', ITS5 5'-GGAAGTAAAGTCGTAACAAGG-3' in a total reaction volume of 30 μ l consisting of 2 μ l of DNA template, 1.0 U of *Taq* DNA polymerase, PCR buffer (10 $\times$), 0.8 mM dNTP (0.2 mM each), 25 mM $MgCl_2$, 10.0 pmol each of forward and reverse primers and autoclaved deionised water. PCR was carried out using the following conditions: initial denaturation at 95 $^{\circ}C$ for 7 min; 35 cycles of denaturation (95 $^{\circ}C$ for 15 s), annealing (52 $^{\circ}C$ for 30 s), and extension (72 $^{\circ}C$ for 30 s); and a final extension step at 72 $^{\circ}C$ for 7 min.

Purification of PCR products from unbound primers was performed by the enzymatic method using Exonuclease I and alkaline phosphatase. Sequencing reaction was performed using BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. Fragments were separated on an automated Genetic Analyzer 3730xl DNA Analyzer (Applied Biosystems). The alignment of nucleotide sequences was performed using the ClustalW algorithm. The selection of nucleotide sequences for construction of phylogenetic trees was done by comparing the target sequences with sequences in the GenBank using the BLAST algorithm. Construction of phylogenetic trees was performed using the Mega 3.1 software. The method of neighbor-joining was used to build phylogenetic trees.

3. Results

3.1. Plant growth-promoting activity of yeasts

538 yeast strains were isolated from rhizosphere soil collected from under the plants of the legume family (Fabaceae): alfalfa (*Medicago sativa*), sainfoin (*Onobrychis viciifolia*), clover (*Trifolium pratense*), sweet clover (*Melilotus officinalis*), soybean (*Glycine max*).

The distribution of yeast strains based on depth of soil was as follows: 0–10 cm – 164 strains (30.5%) 10–20 cm – 256 (47.6%) 20–40 cm – 118 (21.9%) (Fig. 2).

Plant growth-promoting activity was investigated by the ability of yeast to synthesize IAA and by the influence of seed treatment with yeast strains on germination and seedling growth.

Soil yeasts were tested for the capability of producing IAA using colorimetric method. Among the 538 strains of yeast 77 (14.3%) strains demonstrated the ability to synthesize IAA and 461 (85.7%) isolates did not able to produce IAA. It is known that it is possible to classify strains according to IAA production as low and high IAA-producing yeast (Nutarat et al. 2014). In accordance with this 62 strains were attributed to producers of low levels (up to 10 μ g/ml), 15 strains – to high levels (above 10 μ g/ml). Most of the active strains were isolated from the depth of 10–20 cm. Among the strains possessing high levels of IAA production 9 strains produced up to 20 μ g/ml, 3 strains up to 30 μ g/ml and 3 – above 30 μ g/ml. The most active strains were YA05 with 51.7 ± 2.1 μ g/ml of IAA and YR07 with 45.3 ± 1.5 μ g/ml (Fig. 3).

15 yeast strains possessing high levels of IAA production were selected for further studies. The task of the research included

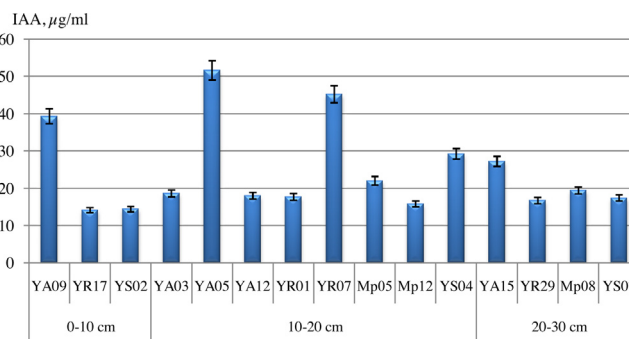


Fig. 3. Indolyl-3-acetic acid production by soil yeasts.

a comparative study of plant growth-promoting activity of cell-associated (biomass) and extracellular (supernatant) biologically active compounds of yeast strains. It was observed that the stimulating effect of yeast could be detected at the earliest stages of plant development, starting from seed germination.

3.2. Effect of incubation time and L-tryptophan concentration on IAA production

The most active plant growth-promoting yeast strains YA05 and YR07 were taken to investigate the influence of incubation time and L-tryptophan concentration on IAA production.

Determination of the IAA concentration in the dynamics revealed that the phytohormone IAA is not detected to 12 h cultivation of yeast strains. At 24 h yeasts produced IAA in trace amounts. Maximum IAA achieved at 120 h for isolate YR07 (45.3 ± 1.5 μ g/ml) and at 144 h for strain YA05 (51.7 ± 2.1 μ g/ml). With further cultivation of strains the concentration of IAA did not increase. It is noted that the maximum accumulation of IAA coincided with maximum rates of biomass: at 120 h for YR07 (96.2 ± 2.5 mg/ml) and at 144 h for strain YA05 (85.1 ± 3.2 mg/ml). The data is presented in Fig. 4.

Concentration of L-tryptophan as a precursor greatly influences the microbial synthesis of the phytohormone IAA (Martens and Frankenberger 1993; Mano and Nemoto 2012; Spaepen et al. 2007). L-Tryptophan in the range of 50–1000 μ g/ml (50, 200, 400, 600, 800 and 1000 μ g/ml) was added into the medium. The results of this study showed that accumulation of IAA in medium without tryptophan was negligible. Tryptophan in concentration of 50 μ g/ml had the perceptible stimulating effect. The increasing of tryptophan concentration in the medium from 50 to 200 μ g/ml improved the IAA production 2.7–2.9 fold. 400 μ g/ml of L-tryptophan showed maximum IAA production. With further increase of L-tryptophan concentration (up to 1000 μ g/ml) response of yeast isolates to tryptophan reduced (Fig. 5).

Thus, the investigated yeast strains are able to utilize L-tryptophan as a precursor and transform it to IAA during their

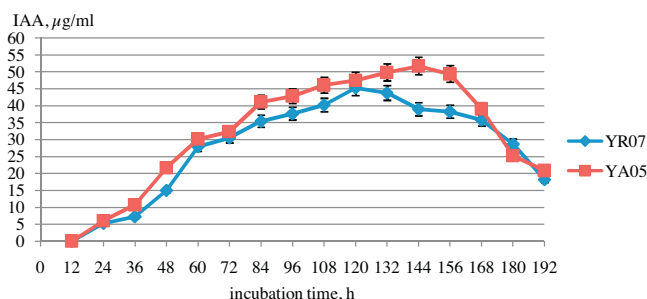
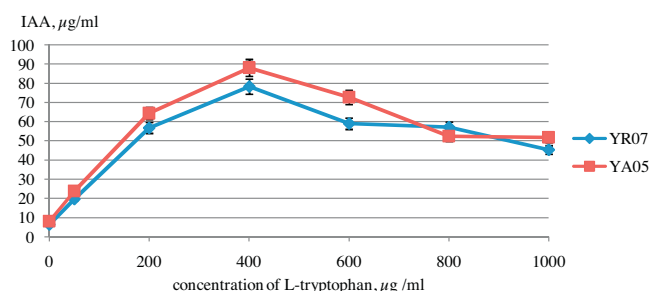


Fig. 4. Effect of incubation period on IAA production by yeast strains.

Table 2
Antifungal activity of yeast strains.

Name of the isolate	Zone of inhibition (mm)			
	<i>Fusarium graminearum</i>	<i>Cladosporium</i> sp.	<i>Phytophthora infestans</i>	<i>Botrytis cinerea</i>
YA03	20.4 ± 0.6	0	0	0
YA05	22.1 ± 0.5	0	30.6 ± 0.5	26.1 ± 0.8
YA12	0	0	10.5 ± 0.2	0
YA15	20.8 ± 0.4	0	0	0
YR01	17.8 ± 0.6	19.5 ± 0.5	0	0
YR07	27.3 ± 1.1	21.6 ± 0.3	0	24.3 ± 0.6
YR17	10.9 ± 0.2	0	0	0
Mp05	0	16.7 ± 0.5	0	13.5 ± 0.3
Mp08	0	0	18.2 ± 0.5	0
YS02	12.4 ± 0.5	0	17.5 ± 0.2	0

**Fig. 5.** Effect of L-tryptophan concentration on IAA production by yeast strains.

growth in the medium. The studies showed that the optimal concentration of tryptophan is 400 µg/ml.

3.3. Antifungal activity of yeasts

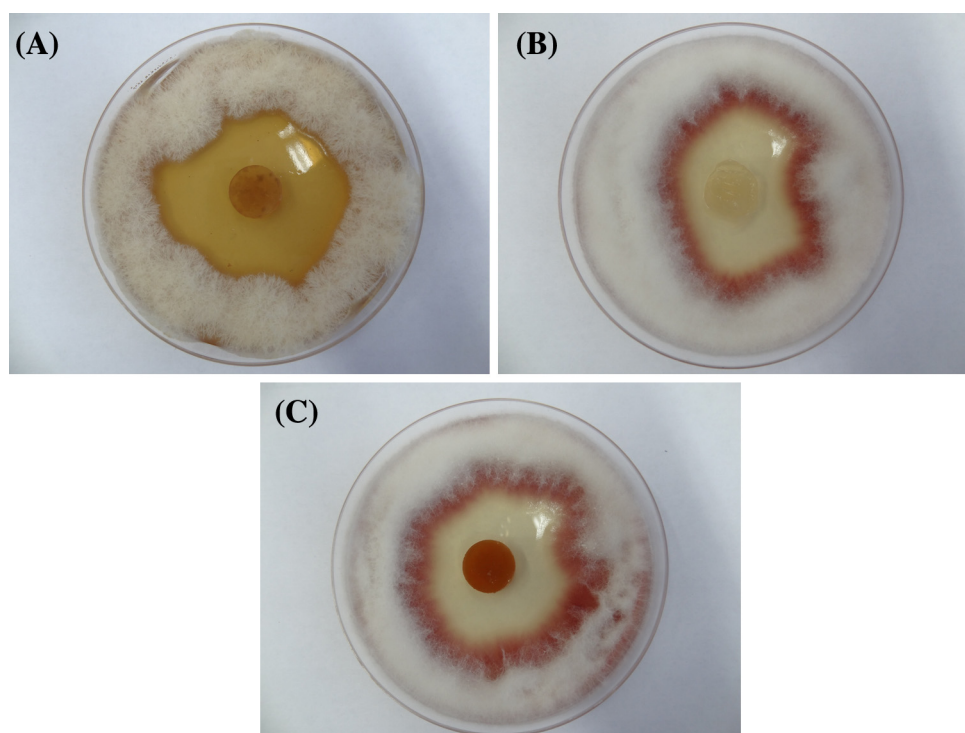
Antifungal activity of selected 15 strains was studied. The ability to inhibit the growth and development of phytopathogenic fungi was demonstrated by 10 strains. Among them 5 strains were with a limited spectrum activity against test pathogenic fungi. Thus, YA03,

YA15 and YR17 strains were active only against *F. graminearum*, YA12 and Mp08 strains were active only against *P. infestans*. Five strains had a relatively broader spectrum of action; they showed antagonistic activity against 2–3 pathogens (Table 2).

Yeast strains inhibited the growth of fungi in varying degrees. Zones of inhibition were in the range of 10.5 ± 0.2 to 30.6 ± 0.5 mm. Maximum zone of inhibition was observed for YA05 against *P. infestans* and YR07 strains against *F. graminearum*. It was noted that the phytopathogen was depressed strongly, that was visually observed in the formation of a rare mycelium pressed to the substrate. Minimal activity was observed in YA12 and YR17 strains – in this case the zone of growth inhibition did not exceed 10.9 ± 0.2 mm. Of particular interest were 2 strains YA05 and YR07, which showed antifungal activity against 3 pathogens (Fig. 6). It should be noted that these strains formed large zones of inhibition – from 21.6 ± 0.3 to 30.6 ± 0.5 mm (Table 2).

3.4. Molecular identification of yeast strains

Two selected yeast strains YA05 and YR07 were identified by the ITS-region sequencing. The isolates were identified as *Aureobasidium pullulans* YA05 and *Rhodotorula mucilaginosa* YR07. The

**Fig. 6.** Antifungal activity of YA05 and YR07 strains. (A) YA05 against *Phytophthora infestans*. (B) YA05 against *Fusarium graminearum*. (C) YR07 against *Fusarium graminearum*.

ITS-region sequences were deposited in GenBank: *A. pullulans* YA05 Accession No. JF160955; *R. mucilaginosa* YR07 Accession No. JF160956.

4. Discussion

Soil – the most favorable environment for the development of different groups of microorganisms, including yeasts. Yeast strains were isolated from different soil depths (0–10, 10–20, 20–40 cm). The greatest number of microorganisms is found at soil depth 10–20 cm.

Soil yeast and yeast-like fungi produce a variety of biologically active compounds (phytohormones, vitamins, amino acids, enzymes etc.) that have active stimulating effect on the plant growth and development and help to increase their productivity. In addition, yeasts produce antimicrobial substances helping to reduce phytopathogenic infection (Botha 2011; Pérez-Montañó et al. 2014; Compant et al. 2005; Schulz et al. 2013; Dixon and Tilston 2010). One of the most widespread phytohormones and the most active at auxin group is indole-3-acetic acid (IAA) (Moller and Weijers 2009; Scarpella et al. 2010; Sundberg and Ostergaard 2009; McSteen 2010). A diverse range of studies indicate that plant growth may be directly or indirectly promoted by yeasts (Xin et al. 2009; Botha 2011; Medina et al. 2004; Nassar et al. 2005; El-Tarabily and Sivasithamparam 2006).

After screening of yeasts for the ability to synthesize IAA, it was noted that the largest number of active strains were isolated from the soil depth of 10–20 cm. It was found that the studied isolates differ in the amount of IAA and they can be divided into low and high IAA-producing yeasts. Among the 15 high-level producers 2 strains demonstrated the highest results – YA05 ($51.7 \pm 2.1 \mu\text{g IAA/ml}$) and YR07 ($45.3 \pm 1.5 \mu\text{g IAA/ml}$). IAA production by strains YA05 and YR07 was comparably higher than that reported for the some other analyzed yeast strains such as *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* (29.5, 24.1 and $31.7 \mu\text{g/ml}$ respectively) (El-Tarabily 2004); *Williopsis saturnus* ($22.5 \mu\text{g IAA/ml}$) (Nassar et al. 2005); *Hannaella sinensis*, *Cryptococcus flavus*, *Rhodospiridium paludigenum* and *Torulaspora glabosa* (up to 29.3 mg/g) (Nutarat et al. 2014).

The study of effect of incubation time and L-tryptophan concentration was performed to select conditions for enhancing IAA production. The results indicated that IAA production increased linearly from 1 to 5–6 days, depending on the strain and decreased later with a decrease in the growth of organisms. According to this data further cultivation of strains is impractical. A comparison of the dynamics of yeast biomass accumulation with the synthesis of IAA showed that the maximum amount of IAA coincided with the stationary phase of growth of the yeasts – at 5 d for YR07 and at 6 d for YA05. Our results are in agreement with other studies. The accumulation of IAA by *Saccharomyces cerevisiae* reached its highest level after cultures entered stationary phase (Rao et al. 2010). It was shown that endophytic yeasts genus *Rhodotorula* produced maximum IAA at 7 d (Xin et al. 2009).

Investigation of effect L-tryptophan concentration indicated that IAA production reached maximum amount in the presence of $400 \mu\text{g}$ tryptophan per ml of the media. It is known that auxin production by microorganisms of different taxonomic groups enhanced when culture medium supplemented with tryptophan which confirm the results of other studies (Mohite 2013; Bharucha et al. 2013; Idris et al. 2007; Maor et al. 2004; Nassar et al. 2005).

Currently of particular relevance is a search for microorganisms with multifunctional biological activity. In this regard selected yeast isolates with IAA-producing ability were tested for their antifungal ability against phytopathogenic fungi *F. graminearum*, *Cladosporium* sp., *P. infestans* and *B. cinerea*. These phytopathogens

cause diseases of economically important plants, contributing significant economic damage. Representatives of the genera *Fusarium* and *Cladosporium* cause diseases of cereal crops and some fruit (Popovski and Celar 2013; Goswami and Kistler 2004; Tasic and Tasic 2007); *B. cinerea* infects onion, potato, strawberry, apple, table grapes (Santos et al. 2004; De Curtis et al. 1996; Janisiewicz 1994); *P. infestans* infects tomato and potato leaves, stems and tubers (Dowley et al. 2008; Nelson 2008; Nowicki et al. 2012).

It was shown that 10 strains had the antifungal activity against phytopathogenic fungi. Among them strains with a limited and a relatively broader spectrum activity were found. YA05 and YR07 strains are the most promising, because showed antifungal activity against 3 pathogens and formed the largest zones of inhibition. These results are in agreement with other studies. It is known that representatives of genera *Aureobasidium* and *Rhodotorula* have antagonistic activity. *R. glutinis* is active against *Penicillium expansum* and *B. cinerea* (Lima et al. 1998). *A. pullulans* is active against various pathogens of apples, peaches, pears – *B. cinerea*, *Monilinia fructigena*, *Monilinia laxa*, *P. expansum*, *Pezizula malicorticis* (Zhang et al. 2010; Wagner et al. 2013; Vero et al. 2009). The representatives of *A. pullulans* produce water-soluble exopolysaccharides, particularly pullulan and aubazidan which are also able to inhibit the growth of various microorganisms (Chlebowska-Smigielska and Gniewosz 2009).

After the study of biological activity two selected yeast strains YA05 and YR07 were identified by the ITS-region analysis as *A. pullulans* YA05 and *R. mucilaginosa* YR07.

As a result of research among the 538 yeast isolates 2 strains *A. pullulans* YA05 and *R. mucilaginosa* YR07 with high plant growth-promoting and antifungal activities were selected. Conducted studies are of interest in the development of multi-functional biological preparations for agriculture, in which microorganisms have different biological activities. Strains *A. pullulans* YA05 and *R. mucilaginosa* YR07 are promising for inclusion into commercial PGP fertilizers for sustainable agriculture.

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