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ADAPTATION OF NATURAL YEAST STRAINS TO HEAVY METAL AND RADIONUCLIDES SALTS[*]

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Ability of natural yeast strains to grow in conditions of high concentrations of heavy metal and radionuclides salts was studied. More than 500 strains were tested for resistance to salts of heavy metals (U, Cs, Sr, Ni, Ar, Cu, Cd, and Co) and to elevated temperature (t) (+37…+52 °C). Most of the strains tested were resistant to one or more selective factors. Combinations of (t, Cd, Cu, Co) and (Cd, Cu, Co) occurred with the highest frequencies: 36 and 26 %, respectively. Ability of isolated strains to grow in the presence of high concentrations of radioactive isotopes Cs and Ni and to bind them with high efficiency was established. The results showed the possibility of potential using of libraries of natural microorganisms for disposal of both radionuclides and heavy metals, which are the main pollutants of natural and anthropogenic objects, as well as the possibility of using of isolated and tested strains of microorganisms for concentrating metals from low-grade ores or mining industry waste. Phenotypes diversity revealed indicates probable existence of several mechanisms of resistance to high heavy metals concentrations.

Keywords: natural yeast strains, adaptation, heavy metals, radioisotopes ^{137}Cs and ^{63}Ni, bioremediation

Activity of industrial enterprises, especially metallurgical, mining, and nuclear ones, inevitably results in discharge of pollutants into the environment and in deterioration of ecosystems due to heavy metals accumulation in them [24]. For this reason, increasing attention has to be paid to potential health hazards caused by the presence of this type of pollutants in the environment. Their removal requires applying of economical and effective methods, and this leads to development of new technologies [24]. Disposal, ion exchange, and electrochemical and/or membrane processes are widely used for industrial wastewater treatment. However, application of these processes in some cases is impossible due to technical or economic reasons [12]. The search for new technologies for the removal of toxic metals from liquid waste leads to biosorption methods based on ability of various biosorbents, including microorganisms, to bind metals. Microorganisms in the environment play the main role both in elements circulation in nature and in formation of sedimentary rocks. They also affect geochemical properties of groundwater due to modification and transport of organic and inorganic pollutants [11]. While organic pollutants

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can be decomposed to CO_2 and water [17 ; 25], radionuclides can be only immobilized. When developing bioremediation methods, it has to be taken into account that at low concentrations many metals can play an important role in metabolic processes, but at high concentrations they often become toxic.

Biosorption is considered a way not only to remove toxic metals from solutions but also to obtain precious metals. Thus, the binding of heavy metals, including radionuclides, requires the search for microorganisms that both sorb metals selectively and remain viable in the presence of radioactive contamination with a level of volumetric activity up to 370 $kBq \cdot mL^{-1}$, as in technological waste [8]. It can be assumed that microorganisms with such properties can primarily live in natural and technogenic environments under conditions being extreme for their existence, as in the Kamchatka Peninsula and the Kuril Islands areas, as well as in technological waters of reactors, in water treatment systems of industrial enterprises, and in storage lakes of liquid technological waste from radiochemical plants.

The aim of this work was to study the possibility of using the collection of microorganisms for sorption of various heavy metals and radionuclides. The microorganisms were collected by the employees of the Petersburg Nuclear Physics Institute named by B. P. Konstantinov in the Kamchatka Peninsula and the Kuril Islands.

MATERIAL AND METHODS

The study was carried out using a collection of microorganisms (over 2000 yeast and yeast-like fungi strains) collected by the employees of the Petersburg Nuclear Physics Institute named by B. P. Konstantinov during expeditions to areas with geothermal activity (Kamchatka Peninsula; Kunashir and Iturup islands of the Kuril Chain). Initial substrate used for isolation of microorganisms was living plants (flowers, fruits, bark, leaves, and roots) and their fallen parts, as well as soil and insects. Samples were collected on volcanoes slopes, in valleys along rivers and streams, near hydrothermal vents, and inside active zones.

To identify natural strains from material canned, filters were transferred to Petri dishes with a dense enriched medium D (YPD; 2 % glucose; 1 % peptone; and 0.5 % yeast extract); from there, as the colonies grew at room temperature, the cells were inoculated again onto the dense D-medium by a streaking technique. Dishes with yeast-like fungi colonies were tested under a binocular magnifier and identified by morphological characteristics: color, shape, size, and surface character. Taxonomic determination was carried out using the method described in species guides [7 ; 13]. Not only the species name but also the numbers according to the collection catalog are given (indicated in the parentheses). The collection consists mainly of unicellular fungi that can exist in the medium containing fairly high levels of ions of almost all metals.

Sensitivity of fungi strains to high uranium concentrations was determined by the replica method [2] on dishes with agar and the nutrient D-medium containing 10, 120, and 150 $mmol \cdot L^{-1}$ of $^{238}UO_2(CH_3COO)_2$.

Selection of strains being resistant to high Ni content was carried out on dishes with a concentration gradient of nickel chloride. Nutrient agar was poured into sterile Petri dishes and left to cool down at an angle. After cooling, agar with the metal tested was inoculated (in this case, it was 10 $mmol \cdot L^{-1}$ of Ni). To evaluate nickel binding efficiency by the strains isolated, radioactive isotope ^{63}Ni was subsequently used.

Selection of fungi strains capable of growing in the presence of Cs^+ ions was carried out at various concentrations of stable cesium in the form of chloride (CsCl). So, a solution of 1 $mol \cdot L^{-1}$ of CsCl was prepared and added to the nutrient D-medium with agar so that final cesium concentration was 10, 50, or 100 mmol·L^{-1}. Cell cultures were inoculated onto agar surface by a streaking technique; dishes were placed in a thermostat at +30 °C. Only the strains, being well-growing at maximum Cs^+ ions content, were used. In addition, from the strains selected, we used only those, that could grow in dishes containing CsCl and $SrCl_2$ at concentrations of 100 mmol·L^{-1}, *i. e.* were tolerant to several metals. Resistance was determined by the replica method.

Sensitivity of the strains selected to gamma irradiation inactivating effect was determined using an "Issledovatel" setup (^{60}Co) at a dose rate 100 Gy·min^{-1}.

To study the ability of free cells to bind radioactive ^{137}Cs or ^{63}Ni, cells from a culture, grown to a stationary phase in the D-medium, were inoculated onto fresh D-medium in a ratio 1:100; then either ^{137}Cs was added to 110 kBq·mL^{-1} or ^{63}Ni was added to 111 kBq·mL^{-1}. As they grew, two samples of equal volume (0.5 mL) were taken in parallel from each culture, transferred to 1.5-mL Eppendorf tubes, and deposited at 10,000 rpm for 10 minutes.

Amount of ^{137}Cs or ^{63}Ni in deposit and supernatant was determined using a Beckman LS 6500 counter (USA).

Efficiency of cesium or nickel radioactive isotopes binding by cells was determined as a percentage of the ratio of radioactivity in the deposit to the total sample radioactivity.

Efficiency of nickel binding by cells was optimized by changing glucose concentration in the growth medium.

For each strain, at least three independent experiments were made, according to which the mean value (see Fig. 3, Fig. 4, Fig. 5, and Table 1) and the mean standard error of a small sample were determined (Table 1).

RESULTS

Totally 2107 strains were isolated and microbiologically purified from the material collected. About 100 strains were identified. Among them, there were representatives of 21 species: *Candida haemulonii*, *Candida sake*, *Candida sorbosivorans*, *Cryptococcus albidus*, *Cryptococcus hungaricus*, *Cryptococcus laurentii*, *Debaryomyces hansenii*, *Pichia farinosa*, *Rhodotorula aurantiaca*, *Rhodotorula glutinis*, *Rhodotorula minuta*, *Rhodotorula mucilaginosa*, *Phaffia rhodozyma*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Tremella foliacea*, *Sporobolomyces roseus*, *Metschnikowia reukaufii*, *Sporidiobolus salmonicolor*, one representative of the genus *Bullera*, and one – of *Trichosporon*. Representatives of three classes were identified: Ascomycota, Basidiomycota, and imperfect yeast. The most common species were *Cryptococcus albidus* (20 strains) and *Debaryomyces hansenii* (7 strains). There are candidates for new species. About 100 yeast lines were isolated from a usual habitat on the Sakhalin. Species diversity revealed mainly corresponds to the data obtained when studying species composition of yeast living in the northern latitudes of Western Siberia and Alaska [21].

More than 500 strains were tested for resistance to salts of heavy metals (U, Cs, Sr, Ni, Ar, Cu, Cd, and Co) and to elevated temperature (t) (+37…+52 °C). Testing strain sensitivity to uranium salts is shown in Fig. 1. Strains being sensitive to low uranium concentrations were neither used nor identified further. The greatest resistance was shown by representatives of the genus *Rhodotorula*. Clones of *Rhodotorula minuta* (KI-20-1a) were also nickel-resistant. Totally 72 % of the strains tested were resistant to one or more selective factors. The most frequent combinations were (t, Cd, Cu, Co) and (Cd, Cu, Co): 36 and 26 %, respectively.

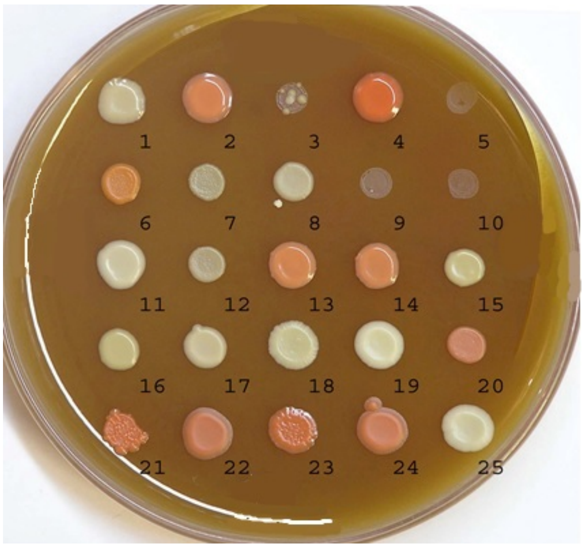

Fig. 1. Testing yeast strains sensitivity to uranium salts (10 mmol·L^{-1}):
1 – unidentified strain;
2 – *Rhodotorula mucilaginosa* (KI-20-4) strain;
3–5 – unidentified strains;
6 – *Phaffia rhodozyma* (KI-54-1) strain;
7–11 – unidentified strains;
12 – *Candida sake* (KI-38-2) strain;
13–17 – unidentified strains;
18–19 – separate clones of *Candida haemulonii* (KII-29-2a) strain;
20–24 – *Rhodotorula minuta* (KI-20-1a) strains;
25 – clone of *Candida haemulonii* (KII-29-2a) strain

Totally 30 strains with various resistance signs were tested for their ability to grow in the presence of non-radioactive ^{87}Sr (as oxide) and ^{133}Cs (as cesium chloride). Strains capable of growing at high cesium concentrations (up to 100 mmol·L^{-1}) were isolated. The representatives of the species *Rhodotorula minuta* (KI-17-5-1) and *Rhodotorula mucilaginosa* (KI-215-4) were the most resistant ones.

Nickel-resistant strains were selected among strains being resistant to several metals (Fig. 2). Clones of *Sporobolomyces roseus* (C26-2-1) and *Candida haemulonii* (KII-29-2a) strains showed high resistance to nickel (up to 5 mmol·L^{-1}). They were also resistant to uranium salts.

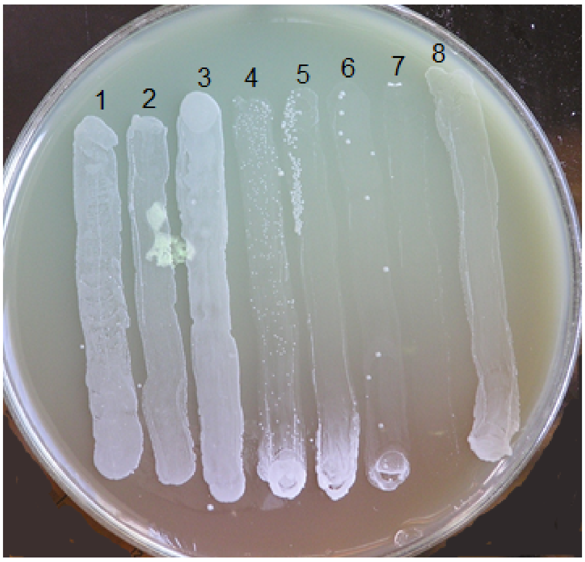

Fig. 2. Testing yeast strains sensitivity to nickel salts:
1–3 – clones of *Sporobolomyces roseus* (C26-2-1) strain;
4 – *Pichia farinosa* (KI-174-4a) strain;
5 – *Pichia farinosa* (KI-6-7a) strain;
6 – *Debaryomyces hansenii* (KI-126-1a) strain;
7 – *Saccharomyces cerevisiae* XII$_7$ strain, diploid;
8 – *Candida haemulonii* (KII-29-2a) strain

Sensitivity of the strains selected to “acute” ^{60}Co γ-irradiation is shown in Fig. 3 in comparison with that of *Saccharomyces cerevisiae* XII$_7$ strain. Among them, there are strains both more and less sensitive to γ-irradiation, being characterized with DMF (dose modification factor at the level of D$_{37}$) 0.7 to 1.15. *Rhodotorula minuta* (KI-17-2) isolate appeared to be the most radioresistant one.

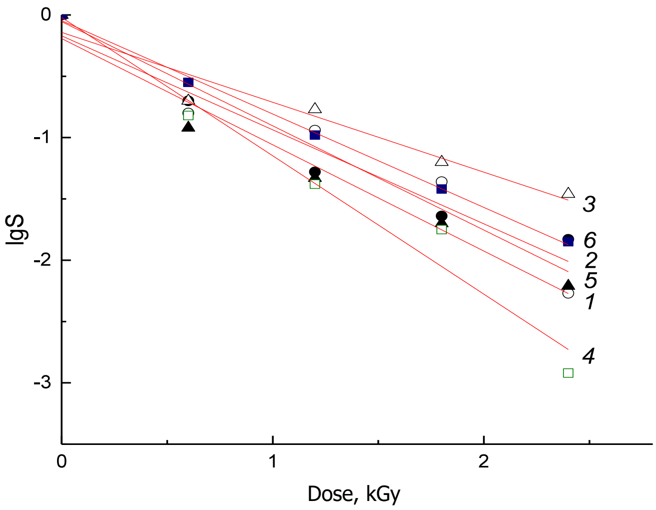

Fig. 3. Survival (lgS) of strains, selected for testing ^{137}Cs binding effectiveness, after "acute" ^{60}Co γ-irradiation:
1 – *Rhodotorula minuta* (KII-110-3) strain;
2 – *Rhodotorula minuta* (KI-20-1a) strain;
3 – *Rhodotorula minuta* (KI-17-2) strain;
4 – *Rhodotorula minuta* (KI-17-5-1) strain;
5 – *Rhodotorula mucilaginosa* (KI-215-4) strain;
6 – *Saccharomyces cerevisiae* XII$_7$ strain

The strains obtained were tested for their ability to grow in a medium with radioactive isotopes, in particular ^{137}Cs at a concentration up to 110 kBq·mL^{-1}, at different temperature and pH values, and when grown in different growth media. Several strains of the genus *Rhodotorula* have shown the ability to bind ^{137}Cs with efficiency 80–90 % (Fig. 4).

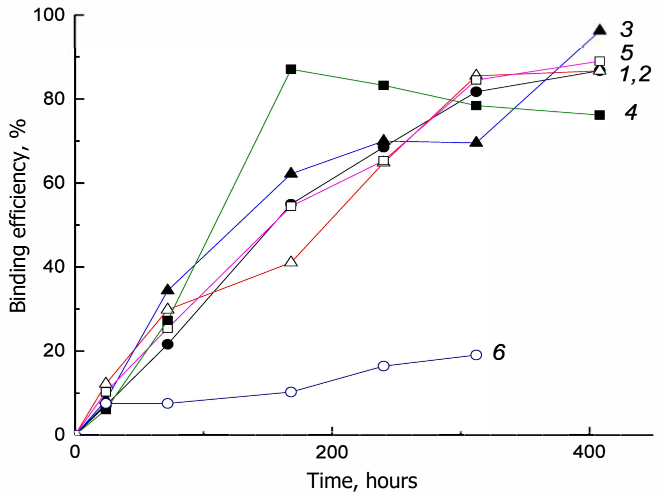

Fig. 4. Efficiency of ^{137}Cs binding by free cells from growth medium at pH ~ 6 during growth:
1 – *Rhodotorula minuta* (KII-110-3) strain;
2 – *Rhodotorula minuta* (KI-20-1a) strain;
3 – *Rhodotorula minuta* (KI-17-2) strain;
4 – *Rhodotorula minuta* (KI-17-5-1) strain;
5 – *Rhodotorula mucilaginosa* (KI-215-4) strain;
6 – *Escherichia coli* AB1157 strain

It follows from the results obtained (see Fig. 4) that when grown for 400 h, yeast-like fungi *Rhodotorula minuta* and *Rhodotorula mucilaginosa* bind ^{137}Cs much more efficiently than bacteria *Escherichia coli*.

Efficiency of ^{63}Ni binding, as that of ^{137}Cs before, was determined in percent – as a ratio of the radioactivity of cells deposited by centrifugation to the total activity of deposit and supernatant (Fig. 5).

It was found that *Rhodotorula glutinis* and *Sporobolomyces roseus* strains show fast kinetics of ^{63}Ni accumulation, while *Rhodotorula mucilaginosa* and *Rhodotorula minuta* after a long growth are characterized with a maximum binding percentage (~ 95 %), significantly exceeding this of *Saccharomyces cerevisiae*. To increase nickel binding efficiency by the strains tested, glucose was added to the growth medium with a concentration varying from 2 to 10 % (Table 1). *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* responded positively to the additional energy source, binding up to 96–99 % of the metal.

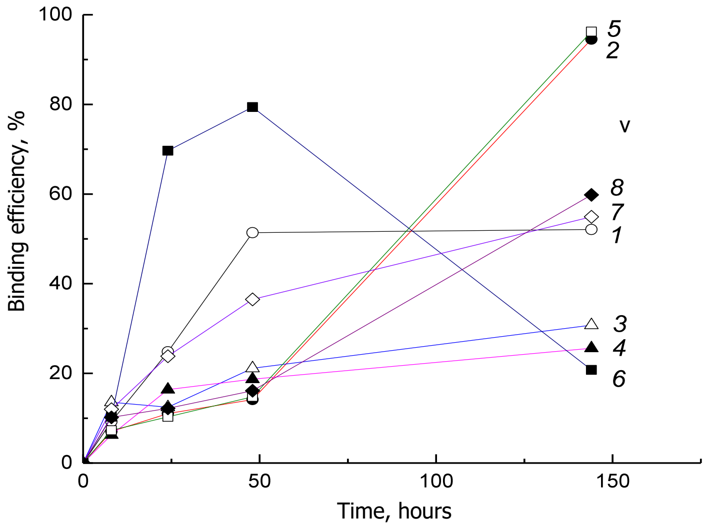

Fig. 5. Efficiency of ^{63}Ni binding (%) by free cells from growth medium at pH ~ 6 during growth:
1 – *Rhodotorula glutinis* (KI-216-4) strain;
2 – *Rhodotorula mucilaginosa* (KI-20-4) strain;
3 – *Cryptococcus albidus* (KII-III-24) strain;
4 – *Cryptococcus albidus* (KII-III-19) strain;
5 – *Rhodotorula minuta* (KII-110-3) strain;
6 – *Sporobolomyces roseus* (C-26-2-1) strain;
7 – *Saccharomyces cerevisiae* (C-20-2) strain;
8 – *Candida haemulonii* (KII-29-2a) strain

Table 1. Efficiency of ^{63}Ni binding (%) by free cells from growth medium at different glucose concentration

Strain	Time, h	Glucose concentration, %		
		2	5	10
Rhodotorula glutinis (KI-216-4)	0	0	0	0
	8	5.1 ± 0.5	9.6 ± 0.9	13.9 ± 0.5
	24	22.4 ± 0.2	28.2 ± 0.8	36.4 ± 0.4
	48	28.7 ± 0.4	40.6 ± 0.4	92.2 ± 0.6
	72	24.9 ± 0.2	50.6 ± 0.2	96.4 ± 0.7
	144	17.9 ± 0.2	86.5 ± 0.4	96.2 ± 0.3
Rhodotorula mucilaginosa (KI-20-4)	0	0	0	0
	8	6.8 ± 0.4	4.6 ± 0.9	14.8 ± 0.6
	24	8.7 ± 0.5	17.6 ± 0.2	16.2 ± 0.5
	48	36.1 ± 0.7	25.5 ± 0.1	23.0 ± 0.5
	72	24.4 ± 0.5	84.5 ± 1.0	45.7 ± 0.5
	144	83.8 ± 0.3	97.7 ± 0.5	99.3 ± 1.6
Rhodotorula minuta (KII-110-3)	0	0	0	0
	8	3.6 ± 0.7	7.4 ± 0.5	9.7 ± 0.4
	24	10.8 ± 0.4	19.8 ± 0.3	26.8 ± 0.4
	48	21.7 ± 0.2	27.9 ± 0.6	24.5 ± 0.3
	72	20.7 ± 0.6	30.1 ± 0.5	37.9 ± 0.6
	144	76.1 ± 0.5	65.8 ± 0.2	48.2 ± 0.3
Sporobolomyces roseus (C-26-2-1)	0	0	0	0
	8	10.0 ± 0.7	10.2 ± 0.3	13.5 ± 0.5
	24	67.6 ± 0.7	77.9 ± 0.6	63.9 ± 0.6
	48	26.4 ± 0.3	45.4 ± 0.4	31.7 ± 0.2
	72	28.5 ± 0.7	29.2 ± 0.3	40.6 ± 0.1
	144	32.0 ± 0.8	31.9 ± 0.3	32.0 ± 0.6

DISCUSSION

The uniqueness of yeast strains collection of the Petersburg Nuclear Physics Institute named by B. P. Konstantinov is determined by geographical peculiarity of the sampling sites. On the Kamchatka Peninsula, practically isolated from the mainland, there are about 60 volcanoes. Only half of them are active; however, hydrothermal and mud emissions of different temperature, containing various natural inorganic compounds, continue functioning on the slopes of extinct volcanoes. The Kuril Islands also abound in areas being characterized with manifestation of various emissions. Therefore, yeast and yeast-like fungi collected there, *i. e.* under conditions of constant strong environmental pressure, have to be highly resistant both to physical factors (elevated temperature, increased radioactive background, or increased intensity of UV radiation) and chemical ones (increased concentration of heavy metals salts and extreme pH values). Among these microorganisms, there might be those capable of efficiently accumulating ions of highly toxic heavy metals and radionuclides. *Rhodotorula* strains isolated by us showed the ability to grow at high concentrations of heavy metals and high radioactive background. Moreover, they were able to effectively bind these metals, in particular cesium and nickel.

To date, the main pollutants of liquid low-level waste (hereinafter LLW) are long-lived radionuclides ^{137}Cs ($T_{1/2} = 30.2$ years) and ^{90}Sr ($T_{1/2} = 28.8$ years). Only now, the problem of environmental protection has become the main one in the sphere of fissile material production. Obviously, level of costs for solving waste disposal problems might turn out to be almost the same as for main production processes. At the same time, to ensure radiochemical plants environmental safety, it is very important to prevent the risk of radionuclides entering groundwater and their outlet to soil surface. The approaches, currently being developed in the sphere of bioremediation, include both *ex situ* and *in situ* methods [6 ; 22 ; 23 ; 25], which makes it possible to find comprehensive solutions for both open storage reservoirs and pools for storing nuclear fuel.

Any methods of liquid LLW purification from ^{137}Cs are costly; all is determined by requirements to water and air quality. Liquid LLW are accumulated in large volumes, and this stimulates the search for cheap methods of purification from radionuclides and metals. Bioremediation methods, *i. e.* using microorganisms for this purpose, are today considered the most promising ones. The strains of natural yeast of the genus *Rhodotorula*, isolated by us, showed high ^{137}Cs binding efficiency with its activity up to 110 $\text{kBq}\cdot\text{mL}^{-1}$.

Besides radionuclides, heavy metals, such as Ni, pose a great environmental hazard. Nickel is the most common industrial pollutant. Its level in the soil is 3–100 $\text{mg}\cdot\text{kg}^{-1}$; in tailings from gold mining operations it is 580 $\text{mg}\cdot\text{kg}^{-1}$, and in Tanzania it is 11,200 $\text{mg}\cdot\text{kg}^{-1}$. In unpolluted and slightly polluted river waters, nickel concentration usually ranges from 0.8 to 10 $\mu\text{g}\cdot\text{dm}^{-3}$; in polluted ones, Ni content amounts to several tens of mg per 1 dm^3. In seawater, average nickel concentration is 2 $\mu\text{g}\cdot\text{dm}^{-3}$, in groundwater it is ~ 10^3 $\mu\text{g}\cdot\text{dm}^{-3}$. In groundwater, washing nickel-containing rocks, Ni concentration sometimes increases to 20 $\text{mg}\cdot\text{dm}^{-3}$ [1 ; 3 ; 5].

The most toxic and carcinogenic nickel state is +4. Being catalysts, Ni compounds play an important role in blood-forming processes. Its increased concentration has a specific effect on the cardiovascular system. Nickel is a carcinogenic element capable of causing respiratory illnesses. It is believed that free nickel ions (Ni^{2+}) are approximately 2 times more toxic than its complex compounds [4]. Nickel excess causes hypoglycemia, asthma, nausea, headache, and nasal and lung cancer. The mechanisms of Ni toxicity are diverse; their action ultimately leads to cell membranes destruction [9 ; 10 ; 14 ; 15 ; 16]. Again, the strains of natural yeast of the genus *Rhodotorula* binding nickel, that were isolated from the ecological niche of extremophiles, seem to be promising for bioremediation and for production processes.

Using both native and genetically modified organisms is on biotechnologists' agenda. The results of this work show the possibility of using natural strains but isolated from extreme living conditions (they already attract much attention [20]). Further study of extremophilic microorganisms involving omics technologies [18] will help in optimizing binding mechanisms [19] and stepping towards efficient cell-free systems.

Conclusions:

1. The results obtained show the possibility of potential using a library of natural microorganisms for disposal of both radionuclides and heavy metals, being the main pollutants of natural and technogenic objects, as well as the possibility of using isolated and tested strains of microorganisms for concentrating a number of metals from low-grade ores or mining industry waste.
2. The diversity of phenotypes revealed, namely multiple resistance, indicates that most likely there are several tolerance mechanisms.

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ВЫЯВЛЕНИЕ АДАПТИВНОСТИ ПРИРОДНЫХ ШТАММОВ ДРОЖЖЕЙ К СОЛЯМ ТЯЖЁЛЫХ МЕТАЛЛОВ И РАДИОНУКЛИДОВ[*]

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Изучена способность природных штаммов дрожжей расти в условиях высоких концентраций солей тяжёлых металлов и радионуклидов. Свыше 500 штаммов проверены на устойчивость к солям тяжёлых металлов (U, Cs, Sr, Ni, Ar, Cu, Cd, Co) и к повышенной температуре (t) (+37…+52 °C). Бóльшая часть изученных штаммов оказалась устойчива к одному или нескольким селективным факторам. С максимальной частотой — 36 и 26 % — возникают комбинации (t, Cd, Cu, Co) и (Cd, Cu, Co) соответственно. Установлена способность отобранных штаммов расти в условиях высокой концентрации радиоактивных изотопов Cs и Ni и связывать их с высокой эффективностью. Полученные результаты показали потенциальную возможность использования библиотеки природных микроорганизмов для осаждения как радионуклидов, так и тяжёлых металлов (основных загрязнителей природных и техногенных объектов), а также возможность применения выделенных и изученных штаммов микроорганизмов для концентрирования металлов из малообогащённых руд или из отходов добывающей промышленности. Обнаруженное разнообразие фенотипов свидетельствует о том, что существует, скорее всего, несколько механизмов устойчивости к высоким концентрациям тяжёлых металлов.

Ключевые слова: природные штаммы дрожжей, адаптация, тяжёлые металлы, радиоизотопы ^{137}Cs и ^{63}Ni, биоремедиация

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