The potential of using plant-growth-stimulating bacteria in phytoremediation of coal dumps

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Abstract. The process of phytoremediation holds great promise for repairing soils damaged by coal mining. Due to the extreme conditions typical of coal dumps, plants face undesirable consequences, which inevitably entail a decrease in the effectiveness of soil restoration. Research in the area of enhancing plant survival has demonstrated that the use of plant growth-promoting microorganisms can be effective. These microorganisms must exhibit resistance to heavy metals in order to sustain their viability in polluted soils. The objective of this investigation was to identify growth-stimulating microorganisms that exhibit resistance to heavy metals and to examine their impact on plants under laboratory conditions. The investigation yielded the identification of 11 isolates from technogenic soils in the Kuzbass region. One of the strains demonstrated a low resistance to copper, zinc, iron, and manganese, thus excluding it from further investigation. The remaining 10 strains were examined for their growth-stimulating properties, including gibberellic acid, indole-3-acetic acid, kinetin, cytokinin, and antioxidant activity. Based on the results of biological compatibility testing, two consortium variants were formed from the five most promising isolates. The utilization of these consortiums for the treatment of coal mine waste soils resulted in a higher survival rate of the grass mixture under laboratory conditions. Consortium № 1, which had isolates 1, 4, and 6 in a ratio of 1:1:1 at a concentration of 1:50, was the most effective. The microorganisms that were included in this consortium were identified based on their cultural, morphological, and biochemical characteristics as *Enterobacter cloacae*, *Acinetobacter geminorum*, and *Lelliottia amnigena*. The strains obtained in this study have the potential to restore vegetation and soil fertility in coal mine waste areas. Further research will be conducted to investigate the impact of these strains on the accumulation of heavy metals. The consortium and the biopreparation developed on its basis will be examined under field conditions.

1 Introduction

Mining coal results in significant changes to landforms and soil depths, as well as changes in the natural cycles of nutrients and the composition of soil microbes [1, 2]. Various pollutants are released onto the soil surface during the coal mining process. The most

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prevalent of these are heavy metals [3, 4]. The purification of soil from these contaminants is accomplished through the utilization of both physicochemical and biological techniques [5]. The conventional physicochemical techniques are expeditious, straightforward, and efficient. Furthermore, they are expensive and resource-intensive. Furthermore, it should be noted that physicochemical cleaning methods are not suitable for large areas, and after their utilization, toxic waste remains [6, 7]. Also, they alter the physical and chemical properties of the soil and diminish its microbial diversity. The soil in question is unsuitable for agricultural and other purposes [5].

For soil cleaning from heavy metals, biological methods are simpler and safer than physicochemical methods. Of these, phytoremediation has received the most attention. This technique involves the absorption, transfer, or transformation of pollutants through the utilization of plants [8]. The presence of vegetation cover in areas affected by coal mining mitigates the erosion and leaching of heavy metals, enhances soil biodiversity and fertility (due to the availability of organic matter), and also aids in the fixation of CO_2 in the atmosphere [9, 10]. Simultaneously, the process of phytoremediation of soils contaminated with metals can pose a significant obstacle owing to the arduous conditions encountered in coal dumps [11]. In order to enhance the efficiency of phytoremediation, it is essential to enhance the longevity of plants in contaminated locales. Microorganisms possess the capability to infiltrate the rhizosphere of plants and exert a favorable influence on their growth and development by generating growth-stimulating substances, enhancing nutrition, and reducing oxidative stress [12–14]. In addition to their advantageous effects on plants, microorganisms also exert a beneficial influence on pollutants. They enhance the bioavailability of heavy metals by lowering soil pH, and enhance the secretion of enzymes by plant roots, thereby enhancing the rate of transformation and absorption of pollutants [15–18].

Understanding the mechanisms of growth enhancement and isolating microorganisms that are resistant to heavy metals are critical for developing strategies to optimize phytoremediation.

The objective of this investigation is to identify growth-promoting microorganisms that are resistant to heavy metals and to investigate their impact on plant tissues under controlled laboratory conditions.

2 Materials and Methods

In accordance with GOST 17.4.4.02-2017, soil samples were taken at a coal dump in the Kemerovo region-Kuzbass.

In order to isolate microorganisms, a 10 g quantity of soil was incorporated into a volume of 100 mL of sterile physiological solution and incubated on an LSI-3016A/LSI-3016R incubator shaker manufactured by Daihan Labtech in South Korea for a duration of 1.5 hours at a temperature range of 25–30 °C. Subsequently, a portion of 5 mL of the resulting mixture was incorporated into a volume of 50 mL of meat-peptone broth and cultivated at a temperature of 25–30 °C for a duration of 18 to 24 hours. After that, dilutions of 10^{-10}, 10^{-11} and 10^{-12} of the resulting culture liquid were subcultured in deep culture into cups Petri medium with the following composition, g/L: peptone – 8.0; maltose – 5.0; agar-agar – 20.0 with the addition of 1M solutions of the following salts, ml/L: monosubstituted potassium phosphate – 3; ammonium sulfate – 23; sodium citrate – 12; copper sulfate – 0.06; zinc sulfate – 0.03; iron II sulfate – 0.007; calcium chloride – 2; magnesium sulfate – 4; manganese sulfate – 0.7. For 18–24 hours, they were cultivated at 25–30 °C. In order to obtain pure cultures, they were subcultured five times using the exhaustion streak method on agar medium containing meat.

The broth dilution method was employed to determine the minimal inhibitory concentration of heavy metals [19]. Copper, zinc, iron II, and manganese were used as salts

for this purpose. The concentration at which the minimum inhibitory effect is observed was deemed to be the concentration at which the maximum inhibitory effect is observed. If growth was absent from multiple plates, the lowest concentration was utilized [20].

The determination of the quantity of indolyl-3-acetic acid that has been synthesized. A suspension of isolated strains with a titer of microorganisms of $1{,}5\times10^8$ was prepared in a meat-peptone broth medium with the addition of L-tryptophan, 0.2%. The microorganisms were grown in an incubator shaker at temperatures ranging from 25 to 30 °C and a rotation speed of 110 rpm for 48 hours. The culture liquid was centrifuged at a rotation speed of 10000 rpm for a duration of 5 minutes. The optical density of the resulting solution was measured on a UV 1800 spectrophotometer (Shimadzu, Japan) at a wavelength of 535 nm after 30 min after adding 1 mL of Salkovskys reagent to 1 mL of the supernatant. The calibration graph of a standard solution of indolyl-3-acetic acid with concentrations ranging from 10 to 200 µg/mL was used to determine the amount of indolyl-3-acetic acid produced by microorganisms.

The determination of the amount of synthesized gibberellic acid. The culture liquid of microorganisms was obtained using the method described earlier. A 10.6% solution of potassium ferrocyanide III and 280 µL of a 1 M solution of zinc acetate were added to 2 mL of cell-free culture liquid and shaken for 10 minutes. After centrifuging for 10 minutes at 4500 rpm, 1 mL of the liquid was mixed with 1 mL of 30% hydrochloric acid and left to stand for 75 minutes. The identical procedure was executed with the control sample (nutrient medium) as well as the experimental sample. The optical density was measured relative to the control using a spectrophotometer at a wavelength of 254 nm [22]. The determination of the quantity of gibberellic acid produced was made by utilizing a calibration graph of a standard solution of gibberellic acid, which ranged from 50 to 650 µg/mL.

The determination of the amount of synthesized kinetin. For this purpose, a suspension of the isolated strains was prepared (with a titer of microorganisms of $1{,}5\times10^8$ CFU/mL) in a medium of the following composition, g/L: 1-substituted potassium phosphate – 12; 2-substituted sodium phosphate – 24; ammonium chloride – 4; sodium chloride – 2; thiamine – 0.1; casamino acid – 2; biotin – 0.0002. It was maintained for 72 hours at a temperature of 25–30 °C and 110 rpm. The process of separating cells from the culture liquid was executed in accordance with the previously described methodology. The optical density was then determined using a spectrophotometer at a wavelength of 665 nm, using a reference solution of a nutrient medium [23]. The determination of the quantity of kinetin synthesized was made by utilizing a calibration graph of a standard kinetin solution containing concentrations ranging from 5 to 100 µg/mL.

The qualitative assessment of the capacity to synthesize HCN. For this purpose, a modified 4% nutrient agar medium was utilized, which was supplemented with 4.4 g/L of the amino acid L-glycine. On the inner surface of the lid of the Petri dish, filter paper soaked in 0.5% picric acid was applied. The bacterial colonies were transferred to plates containing a modified 4% nutrient agar medium for the purpose of HCN synthesis. The petri dishes were sealed with parafilm and incubated at 25–30 °C until a light, moderate, or dark brown color appeared, indicating the synthesis of HCN [24].

The determination of antioxidant activity. According to the method described in determining the amount of synthesized indolyl-3-acetic acid, a culture liquid was prepared.

The cell-free culture liquid was mixed with the DPPH solution in a ratio of 1:3, respectively, and left for 30 minutes in a dark place. The determination of optical density was carried out at a wavelength of 517 nm by means of a spectrophotometer. The control comprises a nutrient medium containing 96% alcohol (C_1) and a nutrient medium containing DPPH reagent (C_2), with a ratio of 1:3 [25]. The activity of antioxidants was determined using the first equation.

$$AOA=(1-\left(\frac{OD_{OB}\text{-}OP_{C1}}{OP_{C2}}\right)\times100 \quad (1)$$

where AOA is antioxidant activity, %;
OD_{ob} – optical density of the sample;
OP_{C1} – optical density of control 1 (nutrient medium with 96% ethanol);
OP_{C2} – optical density of control 2 (nutrient medium with DPPH reagent);
100 – conversion to percentage.

To investigate biocompatibility, pure cultures were grown in meat-peptone broth medium at 25–30 °C and a rotation speed of 110 rpm for 48 hours. The cells were then separated from the culture liquid by centrifugation. A test culture was cultivated on the meat-peptone agar medium in Petri dishes, and a cell-free culture liquid was subsequently added to wells with a diameter of 4 mm and subjected to cultivation at a temperature of 25–30 °C for a duration of 24 hours [26].

To obtain consortia, pure cultures were prepared in a meat-peptone broth medium with a turbidity coefficient of 0.5–0.7, according to McFarland, cultivated for 48 hours at 25–30 °C. Test tubes with meat-peptone broth were used to add 5% of the volume of the medium of the microorganisms included in the consortium.

Laboratory testing. To accomplish this, the biomass of the consortia was elevated for a duration of two days in a shaker-incubator at a temperature of 25–30 °C and a rotation speed of 110 rpm. After completion, a working solution of consortia was prepared with distilled water in a ratio of 1:50 and 1:100, respectively. During the process of sowing, we utilized universal soil, which is suitable for cultivating all types of plants. Additionally, we utilized technozem and technozem mixed with universal soil. The soil was divided into 170 g pots. For each sample, 1 g of grass mixture seeds (timothy grass, fescue, ryegrass, wheatgrass, bluegrass) were weighed. Seeds were sown to a depth of 1 cm in pots. The process of germination was conducted for a duration of 10 days at a temperature range of 18–25 °C. Watering was performed every other day. The control was irrigated with a volume of 50 mL of distilled water, while the samples were irrigated with the working solutions of the consortia in a volume of 50 mL.

The determination of cultural characteristics. A suspension of pure cultures with a low number of cells was prepared, immersed in meat-peptone agar, and cultured at 25–30 °C for 24 hours [27].

The determination of morphological characteristics. The Gram method was used to stain a fixed smear of bacteria. Then they were examined with a x90 or x100 objective [28].

Biochemical characteristics of isolated microorganisms were determined using an automatic microbiological analyzer Vitek 2 Compact (BioMerieux, France) using ID-GN cards (Gram-negative microorganisms). The cultures were grown on Columbia blood agar for 24 hours at a temperature of 25–30 °C, and a suspension of strains with a McFarland density of 0.50–0.63 was prepared using a Densichek plus densitometer (Sendle, Russia) [29].

All studies were conducted in five-fold repetition. The obtained values were portrayed as the average of five measurements, with a standard deviation. The statistical analysis of the data was performed using the Microsoft Office Excel 2007 (12.0.6612.1000) SP3 MSO (12.0.6607.1000) software product (Microsoft corporation, USA). For each pair of interests, statistical analysis was performed using a one-time paired Students t test. At p0.05, statistically significant differences were considered.

3 Results and Discussion

From soil samples, 11 strains of bacteria were identified. The outcomes of determining the minimum inhibitory concentration are presented in Table № 1.

Table 1. Minimum inhibitory concentration

Isolate	Minimum inhibitory concentration, $\mu g \times mL^{-1}$			
	Copper	Zinc	Iron	Manganese
1	1.5	3.0	1.0	1.5
2	1.0	2.0	1.5	2.0
3	0.8	1.0	0.8	2.0
4	2.0	0.8	0.8	3.0
5	1.5	1.5	1.0	1.0
6	0.8	0.8	1.0	1.5
7	0.2	0.2	0.1	0.4
8	0.8	1.0	0.8	1.5
9	0.4	1.5	1.5	2.5
10	1.0	2.5	2.0	0.8
11	2.0	1.5	0.6	1.0

Isolate № 7 exhibits a low resistance to the action of heavy metals, with the minimum inhibitory concentration ranging from 0.1 to 0.4 $\mu g \times mL^{-1}$. Therefore, this strain was excluded from further investigation. Table № 2 presents the outcomes of the growth-stimulating activity of the isolated microorganisms.

Table 2. Growth-stimulating activity of isolated microorganisms

Isolate	Indicators				
	Indolyl-3-acetic acid, $\mu g \times mL^{-1}$	Gibberellic acid, $\mu g \times mL^{-1}$	Kinetin, $\mu g \times mL^{-1}$	HCN	Antioxidant activity, %
1	195.31±7.81	567.56±22.70	12.51±0.35	+	91.20±3.65
2	122.86±4.91	297.01±11.88	3.06±0.10	+	85.36±3.41
3	86.42±3.48	97.00±3.88	2.56±0.08	–	82.96±3.32
4	192.57±7.70	355.55±14.22	9.45±0.25	+	91.26±3.65
5	91.80±3.67	244.58±9.78	5.36±0.12	–	72.34±2.89
6	189.99±7.59	731.03±29.24	8.42±0.16	+	90.45±3.62
8	77.16±3.08	18.57±0.74	3.89±0.11	+	77.82±3.11
9	179.66±7.18	350.09±14.00	10.05±0.31	+	86.99±3.48
10	162.15±6.48	605.52±24.22	9.34±0.28	+	86.55±3.46
11	42.51±1.70	126.59±5.06	2.25±0.08	–	62.58±2.50

The concentration of the synthesized indolyl-3-acetic acid ranged from 42.5 to 195.3 $\mu g \times mL^{-1}$. The isolates that exhibit the highest activity for this indicator are № 1, 4, 6, 9 and 10. Their activity exceeds 160 $\mu g \times mL^{-1}$. Gibberellic acid synthesized ranges from 18.5 to 731.0 $\mu g \times mL^{-1}$. The isolates that exhibit the highest activity for this indicator are № 1, 6 and 10. Their activity was greater than 500 $\mu g \times mL^{-1}$. The concentration of kinetin synthesized ranges from 2.25 to 12.51 $\mu g \times mL^{-1}$. The isolates that exhibit the highest activity for this indicator are № 1, 4, 9 and 10. Their activity was greater than 9 $\mu g \times mL^{-1}$. All isolated strains, with the exception of 3, 5, and 11, are capable of producing HCN. The antioxidant activity ranged from 62.58 to 91.26 %. The isolates № 1, 4 and 6 exhibit the highest activity in this indicator, with a percentage exceeding 90%. Therefore, the isolates № 1, 4, 6, 9 and 10 were selected for biocompatibility analysis. The analysis results are presented in Table № 3.

Table 3. Biocompatibility of isolates

Isolate	1	4	6	9	10
1	■	+	+	+	+
4	+	■	+	–	–
6	+	+	■	–	–
9	+	–	–	■	+
10	+	–	–	+	■

Isolate № 1 is biocompatible with all presented strains, and isolate № 4 is biocompatible with isolate № 6. Isolate № 9 is biocompatible with isolate № 10. Therefore, in order to conduct laboratory research on plants, consortia were established: № 1 comprised isolates 1, 4, 6, in a ratio of 1: 1:1, respectively, and № 2 comprised isolates 1, 9, 10, in a ratio of 1:1:1. The results of laboratory testing of the consortia are presented in Table № 4 and Figure № 1. utilized technozem and technozem mixed with universal soil.

Table 4. Laboratory testing

Option	Soil	Indicators		Values
Control	Universal soil	number of seedlings, pcs.		79±2
		length, mm	min	67±3
			max	155±4
	Technozem	number of seedlings, pcs.		20±1
		length, mm	min	50±2
			max	130±3
	Technozem + universal soil	number of seedlings, pcs.		65±2
		length, mm	min	70±2
			max	140±3
Consortium № 1 concentration 1:50	Universal soil	number of seedlings, pcs.		100±2
		length, mm	min	70±1
			max	175±5
	Technozem	number of seedlings, pcs.		40±2
		length, mm	min	60±2
			max	180±5
	Technozem + universal soil	number of seedlings, pcs.		85±3
		length, mm	min	80±2
			max	145±3
Consortium № 1 concentration 1:100	Universal soil	number of seedlings, pcs.		84±2
		length, mm	min	67±1
			max	170±4
	Technozem	number of seedlings, pcs.		38±1
		length, mm	min	54±1
			max	140±2
	Technozem + universal soil	number of seedlings, pcs.		68±3
		length, mm	min	75±2
			max	142±4
Consortium № 2 concentration 1:50	Universal soil	number of seedlings, pcs.		79±3
		length, mm	min	69±3
			max	167±5
	Technozem	number of seedlings, pcs.		31±1
		length, mm	min	49±2
			max	153±5
	Technozem + universal soil	number of seedlings, pcs.		65±4
		length, mm	min	72±1

			max	128±3
Consortium № 2 concentration 1:100	Universal soil	number of seedlings, pcs.		75±2
		length, mm	min	42±3
			max	158±4
	Technozem	number of seedlings, pcs.		36±1
		length, mm	min	42±1
			max	126±3
	Technozem + universal soil	number of seedlings, pcs.		62±2
		length, mm	min	57±2
			max	135±3

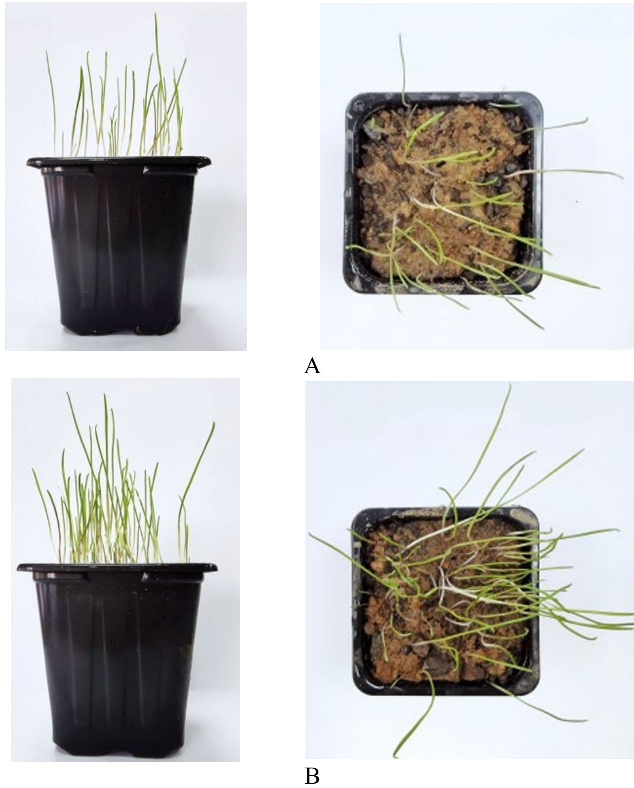


Fig. 1. Germination of grass mixture seeds, where: A – technozem + water; B – technozem + consortium № 1.

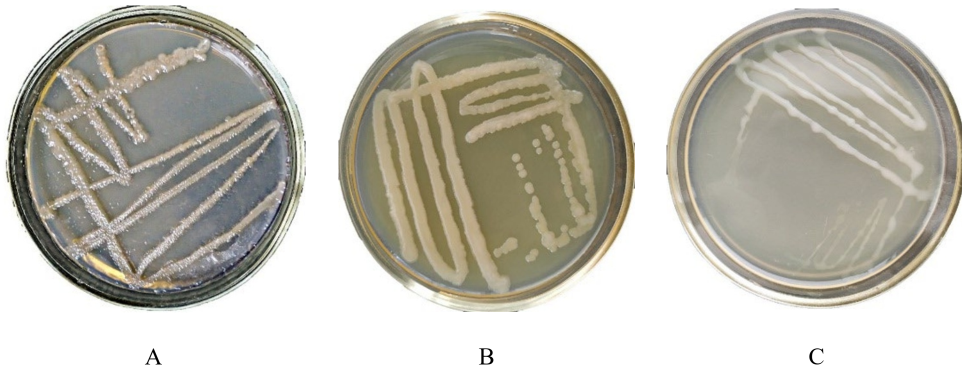
The germination and growth performance of plants grown on technozem and a mixture of universal and technozem decreased significantly when not treated with consortia. The utilization of consortia treatment enabled the enhancement of plant germination and seedling length when grown in technozem and a mixture of technozem and universal soil. The most effective outcome was observed when administered consortium № 1 at a concentration of 1:50. This treatment option enabled the grass mixture to achieve the following growth indicators:

– a technozem. Germination capacity – 40 pcs., minimum sprout length – 60 mm, maximum sprout length – 180 mm;

– mixture of technozem and universal soil. Germination capacity – 85 pcs., minimum sprout length – 80 mm, maximum sprout length – 145 mm.

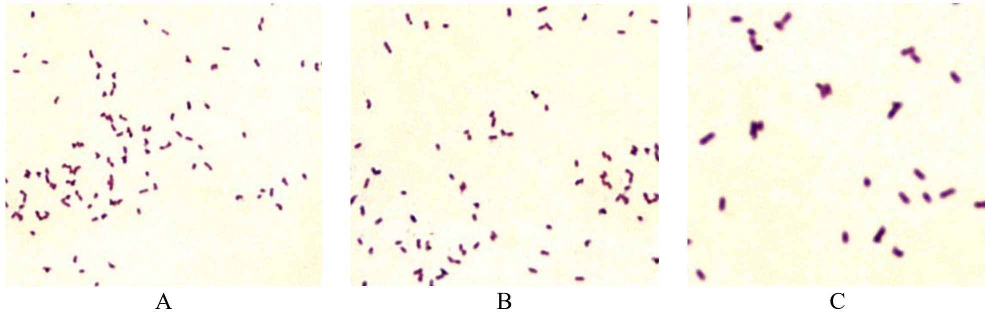
The growth-inducing effect of consortium № 1 and consortium № 2 was diminished by a 1:100 dilution.

Cultural and morphological characteristics are depicted in Figures № 2 and № 3.



A B C

Fig. 2. Growth of isolated strains in Petri dishes on MPA nutrient medium, where: A – isolate № 1; B – isolate № 4; C – isolate № 6.



A B C

Fig. 3. Microscopy of isolated strains on a ×100 objective, where: A – isolate № 1; B – isolate № 4; C – isolate № 6.

All isolates included in the consortium belong to gram-negative rods, which form round white colonies.

Table № 5 presents biochemical characteristics.

Table 5. Biochemical characteristics

Isolate	Sugars fermented by isolate
1	Adonitol, L-Arabitol, D-Cellobiose, Beta-galactosidase, Beta-N-acetyl-glucosaminidase, D-glucose, Beta-glucosidase, D-maltose, D-mannitol, D-mannose, Beta-xylosidase, Palatinose, D-sorbitol, Saccharose/sucrose, D-tagatose, D-trehalose, 5-keto-D-gluco nate, Alpha-glucosidase, Alpha-galactosidase, Phosphatase, Lysine decarboxylase, Glu-Gly-Arg-arylamidase
4	D-glucose, Beta-alanine arylamidase pNA, Arginine dihydrolase 1, L-aspartate arylamidase, Citrate (sodium), L-histidine assimilation, L-lactate alkalinisation, L-lactate assimilation, Malonate, Ornithine decarboxylase
6	L-pyrrolydonyl arylamidase, D-cellobiose, Beta-galactosidase, Beta-N-acetyl-glucosaminidase, D-glucose, Gamma-glutamyl-transferase, Fermentation/glucose, Beta-glucosidase, D-maltose, D-mannitol, D-mannose, Beta-xylosidase, Beta-alanine arylamidase pNA, L-proline arylamidase, Palatinose, Tyrosine arylamidase, D-sorbitol, D-trehalose

The results of the biochemical investigation indicate that isolates № 1, 4, and 6 have probabilities of 92, 90, and 95%, respectively, and belong to the genera *Enterobacter cloacae*, *Acinetobacter geminorum*, and *Lelliottia amnigena*.

4 Conclusion

A total of 11 strains of microorganisms were isolated from soils that have been technologically disturbed in Kuzbass, among which 10 were distinguished by a high level of resistance to copper, zinc, iron, and manganese. These microorganisms were studied for their growth-stimulating properties. The highest effectiveness was observed with Izolate № 1, 4, 6, 9 and 10. They possess the capability to produce growth-stimulating substances, including gibberellic acid, ranging from 350.09 to 731.03 $\mu g \times mL^{-1}$, indolyl-3-acetic acid, ranging from 162.15 to 192.57 $\mu g \times mL^{-1}$, kinetin, ranging from 8.42 to 10.05 $\mu g \times mL^{-1}$, and HCN. The strains have antioxidant properties, which could be useful for reducing plant damage caused by free radicals. Based on the biocompatibility of microorganisms, consortia № 1 (consisting of isolates № 1, 4, 6, in a ratio of 1:1:1, respectively) and № 2 (consisting of isolates № 1, 9, 10, in a ratio of 1:1:1, respectively) were established. A significant increase in the survival rate of the grass mixture was observed when grass was added to the soil of a technozem and a mixture of technozem and universal soil. In this particular instance, the most efficacious treatment was administered by consortium № 1 at a ratio of 1:50. Therefore, a consortium comprised of isolates № 1, 4, and 6 holds great potential for restoring vegetation in areas that have been affected by coal mining. The ortium were identified in accordance with their cultural, morphological, and biochemical characteristics. Research has demonstrated that the microorganisms under study belonged to the following species: *Enterobacter cloacae*, *Acinetobacter geminorum*, and *Lelliottia amnigena*. The strains have been sent for deposit to the All-Russian Collection of Industrial Microorganisms of the National Research Center "Kurchatov Institute". Further research is needed. Further investigations of strains and consortia will be conducted with the objective of examining their impact on the process of heavy metal absorption. The consortium will conduct comprehensive field tests and develop a biological product for the restoration of land that has been technologically disturbed.

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