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YEAST-BASED MICROBIOLOGICAL DECONTAMINATION OF HEAVY METAL CONTAMINATED SOILS OF TARNIŢA

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Abstract: Heavy-metal pollution of forest soils represents an important environmental concern due to the toxic effects of metals, and their accumulation throughout the food chain. In this paper, the yeast-based decontamination of soil containing high amounts of heavy metals and arsenic, originated from a sterile dump of Tarniţa-Suceava, Romania, was investigated. The sterile dump and soil around it contain increased concentrations of Cu, Fe, Pb, Zn, Ba, and As, that affected the germination of wheat seeds used here as an indicator of toxicity. The germination of wheat seeds (*Triticum aestivum*) was interrupted by small amounts of sterile dump material, but recovered after soil washing with distilled water, and removing the supernatant. Furthermore, seed germination was strongly inhibited when the wheat seeds were treated with the toxic supernatant, which was extracted from the sample. In conclusion, yeast (*Saccharomyces cerevisiae*) succeeded to partly retain heavy metals and arsenic from soil extracts.

Keywords: yeast, arsenic, heavy metals, wheat, germination

1. INTRODUCTION

Heavy metal pollution of soils around closed mines is a major comprehensive problem worldwide. Heavy metals and arsenic have been identified as a primary research task for the polluted forest areas in Romania (Voica et al., 2012). Tarniţa site was chosen for investigation and decontamination since it is a strongly polluted area, especially with heavy metals and arsenic and due to a persistent ecological disaster (Stumbea, 2010).

Heavy metals such as Cu, Zn, Fe, Mn, Mo, Ni, Co are essential for the metabolic processes of vegetable and animal organisms. Nevertheless, higher concentrations of such metals have toxic effects on plants (Nagajyoti et al., 2010). The main heavy metal ions which have harmful effects on human health are cadmium, copper, lead, mercury, arsenic and their combinations (Järup, 2003).

A large number of methods have been developed for the removal of heavy metals from environment such as precipitation, evaporation, electroplating, ion exchange, membrane processes, etc. (Das et al., 2008). However, these methods have several disadvantages such as unpredictable metal ion removal, high reagent requirement, generation of toxic sludge, etc. Metal remediation by common physico-chemical techniques is expensive and unsuitable in case of low metal contamination (Malik, 2004). Biotechnological approaches have received a great deal of attention in the last years (Dary et al., 2010; Alkorta et al., 2010; Wang & Chen, 2006).

Microbiological decontamination of soil is generally based on the ability of microorganisms to retain toxic substances from their environment through their interaction with molecules from the surface of their cells ("biosorption") or by entering their cells ("bioaccumulation") (Soares & Soares, 2012; Gavrilescu, 2004). The first is a passive process, and the last is the active one, a metabolism-mediated process.

Both living and dead yeast cells can be effective metal accumulators and there is evidence that some biomass-based clean-up processes are economically viable (Gadd, 1990; Cojocaru et al., 2009). The tolerance of some yeast species to cadmium, copper and zinc as well as the physiological response to metals have been also determined (Balsalobre et al., 2003). The removal of heavy metal ions, Ni^{2+}, Cu^{2+} and Pb^{2+} using yeast (*Saccharomyces cerevisiae*) as carriers in a crossflow microfiltration was previously investigated (Bayhan et al., 2001). Studies in yeast are providing a large body of information about the regulation and metabolism of heavy metals (Singh et al., 2003).

This work aims firstly to investigate the soil toxicity originated from Tarniţa sterile dumps on wheat seed germination and the growth of the resulted seedlings. Secondly, the effect of washing water of this contaminated soil is to be investigated. Lastly, the treatment of waste waters with suspensions of alive or dead yeast will be done and the decontamination degree will be studied within wheat seed germination experiments.

2. METHODS AND MATERIALS

Equipment. The content of heavy metals and arsenic in soil samples from Tarniţa area was measured by Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES). An Optima 5300DV spectrometer (PerkinElmer, Waltham, MA, USA) coupled with an ultrasonic nebulizer CETAC 6000AT+ (CETAC, Omaha, NE, USA) was employed. Operation conditions: pump rate 1.2 mL/min, RF power 1.2 kW, carrier gas 18 L/min, auxiliary gas 0.5 L/min, nebulizer gas 34 psi, sample uptake time 30 sec, integration time 40 sec. Argon (99.999% Ar) was the carrier gas to sustain plasma (Murariu et al., 2016). Three replicates were taken to measure the analytical signal. Limits of quantification (LQ), measured and reported with 99% confidence that the analyte concentration is greater than zero according to EPA Method 2007, are measured by spiking a reagent blank with analytes at a concentration level 3 times more than the instrument detection limits found in water samples. Limits of quantification for solid samples in mg/kg were calculated from LQs obtained in 100 mL of aqueous solution of 1 g soil extract. Centrifugation was performed using a Hettich centrifuge Mikro 22R (from Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). An Ultrasons ultrasonic water bath (from JP Selecta its Barcelona, Spain) was used for ultrasonic extraction of contaminants from sterile dump material.

Chemical reagents. The reagents used were of analytic purity (Merck, Sigma, Chimopar) and, when necessary, the solutions and the water slurries were prepared by using MiliQ grade water (18.2 MΩ·cm). The degradation solution for analyzing samples of collected sterile dump material or soil was a mixture of HNO_3 and HCl prepared 1:3 v/v.

Biological material. Commercially distributed "active" wet pressed biomass of *Saccharomyces cerevisiae*, containing living cells, was supplied from the company S.C. ROMPAK S.R.L., Paşcani, România. The inactivation of some amount of such yeast resulted in inactive or dead yeast. The wheat samples (*Triticum aestivum*), Putna variety, were taken from the Agricultural Research Station of Suceava, kept at 4 °C before use. The 1000-seed mass of the wheat was 51.42 g.

Sampling. Samples of sterile dump material and soil were taken from Tarniţa mine site, Suceava region situated along the county road 177A, between this road and Brăteasa River (the geographic coordinates of 47° 21' 32" N latitude and 25° 42' 51" E longitude).

Supernatants used in treating seeds. Triplicate samples of 1 g of homogenized material collected from the sterile dump of Tarniţa site, were extracted three times with 10 ml of distilled water on the ultrasonic bath for 15 min. Then, the centrifuge tubes were centrifuged at 5,000 rpm to obtain clear supernatants. Volumes of 1-5 mL of toxic supernatant collected from the first washing of the material being analyzed were treated with 4-0 mL of water to obtain various concentrations of heavy metals. These concentrations of contaminants can be calculated from the soil or sterile content, as measured by ICP-OES.

Yeast-based decontamination procedure. Triplicate samples of 5 g of active yeast were treated with a mixture of 6 mL of toxic supernatant and 4 mL of distilled water for 1 hour. Then, the centrifuge tubes were centrifuged at 5,000 rpm, for 5 min to obtain a clear supernatant, of which 5 mL was taken in the germination experiments. The procedure with inactivated yeast samples was the same. The yeast cells were inactivated on boiling in distilled water and

keeping them on a water bath at 100°C for 1 hour.

Seed germination. Triplicate lots of 50 seeds were laid as uniformly as possible together with the treatment supernatants or soil samples on double filter paper, in 9-cm diameter Petri dishes, according to ISTA recommendations (Seed Science and Technology, 1993). The germination process in the presence of treatment materials lasted for 7 days. The seeds were daily sprayed with 5 ml of redistilled water. The germination rate (GR, expressed as percent) was determined on the 7th day. At the end of the 7-day experiments, the plantlets were cut off at the level of the seeds, measured (height, *H*, in cm) and weighed (mass, *m*, in milligrams). We investigated the resulted seedlings as well as the germinated, abnormal and dead seeds.

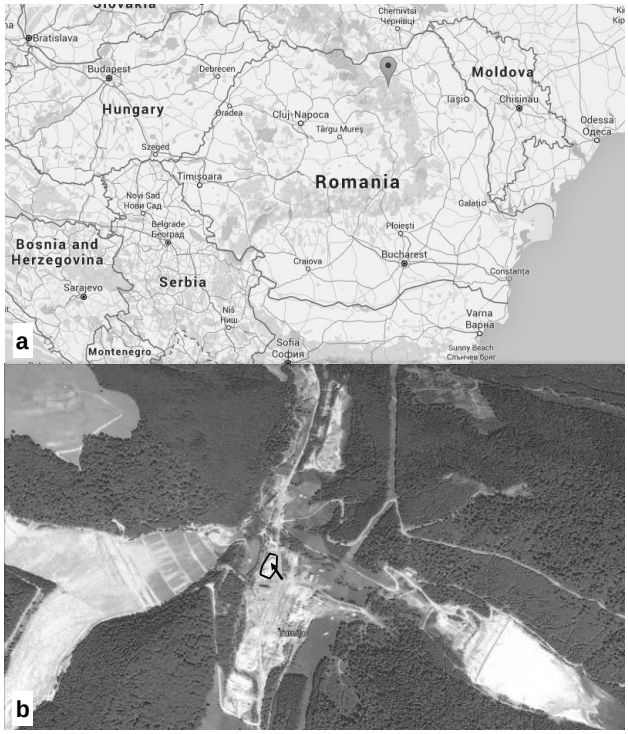

Figure 1. The location of Tarniţa mining complex in Suceava County, Nord-Est Romania (**a**), and the sampling area (**b**), which is marked with arrow (from https://www.google.ro/maps).

Metal determination. The three samples were collected from sterile deposit, 30-m distance far from sterile dump, whereas the third from the Brăteasa River bank (Fig. 1). Triplicate samples of 0.2-0.3 g of sterile dump material or soil collected from the vicinity of sterile dump were treated at boiling temperature, in a beaker covered with glass watch, with a mixture of HNO_3 and HCl (1:3 v/v); 15 mL) for 25 min. The clear solution thus obtained was filtered and diluted up to 50.0 mL with milliQ grade water, and analyzed using a dual viewing inductively coupled plasma optical emission spectrometer. Axial view was used for metals determination, while 2-point background correction and six replicates were used to measure the analytical signal.

Calibration standards (concentration range 5 - 100 µg/L or 1 - 10 mg/L) were prepared by appropriate dilution of: (1) multi element standard ("Ultra scientific", Lot: P00332) with 24 elements in 5% HNO_3 (Al, Ba, Be, Bi, B, Cd, Ca, Cr, Co, Cu, Ga, Fe, Pb, Li, Mg, Mn, Ni, K, Se, Na, Sr, Te, Tl, Zn; 100 ± 5 mg/L of each element), and (2) As in 5% HNO_3 (1000 ± 3 mg/l). Each standard was scanned at least three times and a mean analytical signal for each component was calculated. The wavelengths used for direct determination of target metals were chosen to avoid the spectral interferences at the studied concentration range.

Statistics. For each treatment the standard deviation of triplicate values was calculated.

3. RESULTS AND DISCUSSION

Contaminants. Table 1 shows the high content of heavy metal ions and arsenic of the sterile dump and the soil around it. While the most present metal was iron, this element was spread toward the dump neighborhood, becoming even more evident than in the dump. However, the most toxic elements found in the sterile dump were arsenic (676 mg/kg), copper (3,119 mg/kg), and lead (2,672 mg/kg), whereas the other elements are either less toxic (Ba, Zn) or were present as trace elements. Table 1 also shows an active and effective dissemination of poisoning elements in the surroundings. For example, Cr and Ni were found left from the sterile dump, which suggests that the dumps in Tarniţa area are a powerful source of contamination.

Table 1. Arsenic and heavy metal content in sterile dump (T1) and soil samples (T2) collected 30 m far from the sterile dump (mg/kg)

Element	**(As)**	**(Cu)**	**(Fe)**
T1	676.00	3119.00	357869.00
T2	96.25	334.00	493500.00
Element	**(Pb)**	**(Zn)**	**(Ba)**
T1	2672.00	432.00	13.86
T2	137.00	421.00	537.00
Element	**(Cr)**	**(Mn)**	**(Ni)**
T1	<0.002	<0.002	0.00
T2	19.25	661.00	29.00

Soil toxicity. The first experiments conducted aimed to determine the influence of soil from Tarniţa on wheat germination and growth of seedlings (Table 2). During the 1-week treatment,

the unwashed sample of 1 g of soil completely inhibited wheat seed germination, as an effect of the high amount of copper, arsenic, lead, and iron. However, the seed germination recovered after washing the soil two or three times, because the toxic elements penetrated into the waste waters.

Germination rate (GR) of wheat seeds treated only with distilled water (blank) was 95%. In the presence of 1 g of contaminated soil from Tarniţa sterile dump, GR has decreased to 3%. The average weight and height of the resulted seedlings also drastically decreased. However, following the second and third extraction of contaminants from soil samples, GR returned to 94% and 97%, respectively (Table 2). Such data indicated that toxic substances in the soil were removed during washing and they concentrated in the supernatant. The average weight and height of the seedlings partly recovered after the contaminants extraction in distilled water.

Table 2. The biological effect of soil samples from Tarniţa site on wheat germination and the growth of the resulted seedlings.

Treatment*)	G.R.**)	Average weight of the seedlings (*m*, mg)	Average height of the seedlings (*H*, cm)
Blank, H_2O	95 ± 1.2	63.5 ± 2.3	11.3 ± 0.4
Soil	3 ± 1.2	10.3 ± 5.6	1.6 ± 0.5
Twice washed soil	98 ± 2.0	58.6 ± 5.7	10.4 ± 0.7
Three times washed soil	97 ± 2.3	65.5 ± 5.1	10.9 ± 0.5

*) Average of three independent values
**) Germination rate

Table 3. The inhibitory effect of increasing volumes of toxic supernatant on wheat germination and the growth of the resulted seedlings.

Treatment*)	G.R.**)	Average weight of the seedlings (*m*, mg)
Blank, H_2O	97 ± 4.6	62.1 ± 0.4
1 mL toxic supernatant + 4 mL H_2O	95 ± 3.1	41.3 ± 2.0
2 mL toxic supernatant + 3 mL H_2O	91 ± 2.3	37.2 ± 3.8
3 mL toxic supernatant + 2 mL H_2O	67 ± 12.2	31.2 ± 5.6
4 mL toxic supernatant + 1 mL H_2O	51 ± 7.9	28.8 ± 2.1
5 mL toxic supernatant	48 ± 7.7	18.9 ± 2.1

*) Average of three independent values
**) Germination rate

In another experiment, we determined the toxicity of the supernatants obtained from the first extraction of the contaminated soil, using solution of various concentrations of toxic elements. The measurements (Table 3) showed a decrease in the germination rate from 97% (control or blank sample) to 67% in the case of 3 mL of the toxic supernatant and to 48% when 5 mL of the toxic supernatant was added to the three lots of 50 seeds. The average weight of plantlets decreased progressively with increasing volumes of toxic supernatant on wheat seeds. As a result, we selected the experiment using 3 mL of the supernatant solution (diluted to 5 mL) as a starting point for yeast decontamination experiments.

In the next experiment, we investigated the role of active and inactive (living and dead) yeast in the removal of contaminants from supernatants (Table 4). Consequently, 6 mL of toxic supernatant were mixed with 4 mL of distilled water and 5 g of yeast biomass. A decrease in the germination parameters (percent of germinated seeds, average weight and length of plantlets) was noticed when the seeds were treated with the supernatant obtained from the incubation of yeast with water followed by centrifugation. Such decrease was probably induced by the organic substances released by yeast in supernatant. However, living yeast cells released less organic material in the supernatant, which had a lower influence on wheat seed germination as compared with the inactive yeast. While living yeast reduced the germination rate of wheat seeds by only 3%, the inactivate yeast decreased GR by 12.4%. Important effects were noticed when the masses and heights of the resulted seedlings had been considered. These parameters decreased by 9.3% and 34.2%, respectively, in the case of average mass (active and inactive yeast) and 22.1% and even 52.9%, when considered the seedling height.

Table 4. The biological effect of active and inactive yeast on wheat germination and the growth of the resulted seedlings.

Treatment*	G.R.**)	Average weight of the seedlings (*m*, mg)	Average height of the seedlings (*H*, cm)
Blank, H_2O	97 ± 1.2	61.3 ± 3.8	10.4 ± 0.3
Active yeast	94 ± 3.4	55.6 ± 1.7	8.1 ± 0.2
Inactive yeast	85 ± 9.9	40.3 ± 8.2	4.9 ± 1.5

*) Average of three independent values.
**) Germination rate.

The biological effect of the supernatants resulted in the decontamination process which used active yeast on wheat seeds germination was then investigated (Fig. 2, Table 5). The germination rate thus increased from 71% to 93% as a result of decontamination by yeast suspensions. Moreover, the average weight and height of the resulted seedlings increased by 46.6% and 70.0%, respectively, when compared with the effect of toxic supernatant. However, probably due to the high concentrations of the supernatant resulted in sterile material washing, the total recovery of seed germination was not possible. The average weight and height of seedlings treated with the decontaminated supernatant were only 76.4% and 60.5% of the treatment with yeast supernatant.

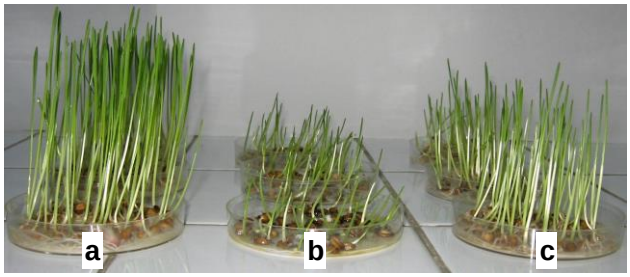

Figure 2. Decontaminant effect of the active yeast on toxic supernatant. Seeds treatments: active yeast; b) toxic supernatant; c) toxic supernatant treated wih active yeast.

Table 5. The effect of supernatants from active yeast suspensions, toxic supernatant from sterile dump, as well as their mixture on wheat germination and the growth of seedlings.

Treatment*)	G.R.**)	Average weight of seedlings (*m*, mg)	Average height of seedlings (*H*, cm)
Active yeast	94 ± 3.5	55.6 ± 1.7	8.1 ± 0.2
Toxic supernatant	71 ± 13.3	29.0 ± 2.4	2.9 ± 0.4
Toxic supernatant and active yeast mixture	93 ± 4.2	42.5 ± 1.3	4.9 ± 0.3

*) Average of three independent values.
**) Germination rate

The inactive yeast, in an experiment consisting in mixing the suspension of dead yeast cells with the toxic supernatant containing heavy metal ions and arsenic from sterile dump of Tarniţa, also demonstrated decontamination properties (Table 6). The inactivation of yeast by boiling resulted in the diminished germination rate of wheat seeds from 94% to 85%. Besides, the weight and height of the resulted seedlings were found diminished from 55.6 mg and 8.1 cm, respectively, when living yeast cells were used, to only 40.3 mg and 4.9 cm, in the case of dead yeast cells. After incubating the inactive yeast suspension with the toxic supernatant, an increase in the germination rate (92%), and average weight and length of the seedlings (33.3 mg; 3.6 cm) was noticed. Although these results showed significant decontamination effect of dead yeast cells, the average height of seedlings from the seeds treated with the toxic supernatant and inactive yeast mixture was only 73.5% of that of yeast supernatant and 34.6% of control with distilled water.

Table 6. The effect of supernatants from inactive yeast suspensions, toxic supernatant from sterile dump, as well as their mixture on wheat germination and the growth of seedlings.

Treatment*)	G.R.**)	Average weight of seedlings (*m*, mg)	Average height of seedlings (*H*, cm)
Inactive yeast	85 ± 9.9	40.3 ± 8.2	4.9 ± 1.6
Toxic supernatant	71 ± 13.3	29.0 ± 2.4	2.9 ± 0.4
Toxic supernatant and inactive yeast mixture	92 ± 1.9	33.3 ± 1.8	3.6 ± 0.2

*) Average of three independent values
**) Germination rate

In this study, the results show that the decontamination of the heavy metals from the toxic supernatant of the extracted soil was more effective when used living yeast than dead yeast. Thus, the first yeast proved to be the best sorbent for heavy metal and arsenic. However, we did not measure the exact amount of such toxic elements. In the case of dead yeast cells, large amounts of organic substances might be released, which can be toxic to wheat seeds. However, they may be used in the coming years for microbiological decontamination and soil cultivation, because of the possibility of total degradation (mineralization) of these substances released by yeast.

Decontamination would not be installed directly in the ground because dead micro-organisms may remain in the ground together with the metal retained and the process cannot be controlled effectively. Therefore, the microbiological remediation of heavy metals can take place in two stages. In a first stage, heavy metals can be extracted with water, and in the second phase, the water containing heavy metals may be passed over the

mass of yeast in the reactor to be thus retained. The yeast resulted in the decontamination process should be dried and incinerated, and then their content of heavy metals chemically recovered, such as by electrolysis or precipitation with suitable reagents outside the natural environment.

This paper provides a biosorption study carried out on yeast, a promising natural biosorbent, which could serve as an economical means of treating effluents charged with toxic metallic ions. Our experiments demonstrated that yeast can be used to remove heavy metal contaminants from washing waters of sterile dump material and that wheat germination tests may be used to follow the decontamination process. After metal extraction from ore, large amounts of heavy metals remain in sterile dumps. Rainwater and wind take some of these heavy metals in soil around the mine enters contaminating it. Therefore, the waste tailings containing waste have to be decontaminated from heavy metals using a method that does not contaminate the soil with other chemicals. Decontamination by yeast *Saccharomyces cerevisiae* could be a useful method as it has been extensively studied for the property of retaining heavy metals such as Ag, Au, Cd, Co, Cr, Cu, Hg, Ni, Pb, U, Th and Zn (Wang & Chen, 2006; 2009). When pure biosorptive metal removal is not feasible (Malik, 2004) the application of a judicious consortium of growing metal-resistant organisms can ensure better removal through a combination of bioprecipitation, biosorption and continuous metabolic uptake of metals after physical adsorption. Nevertheless, we recommend here metal removing from metal-concentrated soils or sterile dumps by chemical way, for example by precipitation with alkaline solutions (NaOH, $Ca(OH)_2$, etc), followed by biosorption at neutral pH values.

On the other hand, improvement of the capacity of plants to tolerate and accumulate metals by genetic engineering should open up new possibilities for phytoremediation (Alkorta et al., 2004). Herein we used wheat seeds to demonstrate the toxicity of soil extracts and the opportunity to use yeast for the remediation of metal-contaminated soils.

Saccharomyces cerevisiae is known to remove toxic metals and recover precious metals from aqueous solutions to various extents, being not only a by-product of the fermentation processes, but also a low-cost biomass, easily obtained in high quantities (Goksungur et al., 2005; Humelnicu et al., 2004; Murariu et al., 2011; Murariu et al., 2009; Popa et al., 2003). Our experiments also show that the toxic supernatants containing heavy metal ions and arsenic decreased wheat germination, but it increased in the sample incubated with active yeast. We suspect that glutathione, a thiol-containing tripeptide, which is an essential metabolite in yeast, may play an important role in metal binding; its intracellular levels have been reported to lie between 1 – 10 mM (Zaguła et al., 2014).

Germination tests proved to be a reliable tool in the investigation of plant responses to noxious herbicide (Weiss et al., 2006). These authors elaborated the dose–response assays based on seed germination and analyses of shoot and root elongation on various materials such as paper, sand or agar-agar. Our described protocol is as simple and reproducible as that recommended by Weiss et al. (2006), and can be easily adopted for some other plant species and for various heavy metals contaminated media.

Herein, a decontamination facility was proposed. The most abundant heavy metals in the studied samples were iron, copper, and lead. However, arsenic is also extremely high both in the sterile dump and around it. Germination tests proved to be a strong proof for the high level of toxicity of sterile dump material and soils from Tarniţa area. The wheat seeds incubated with contaminated Tarniţa soil and water failed to germinate. Besides, the toxicity decreased as the soils were repeatedly washed with distilled water. Yeast organisms were found to be efficient decontaminants for toxic supernatant containing heavy metals and arsenic, favoring seeds germination and seedling development.

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