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EVALUATION OF THE TOXICITY OF SOME HEAVY METALS ON YEASTS USING A DIFFUSIMETRIC METHOD OF ANALYSIS

BY

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Abstract. This study reports the influence of cadmium, chromium and nickel ions on yeast strains using the diffusimetric method, on agar plate culture media. The tested yeast species show a different sensitivity depending on the metal ion in their culture medium and its concentration. *S. cerevisiae* proved to be the least sensitive to the presence of metal ions. Chromium ions have been shown to be the most toxic to the three yeast strains (*R. rubra*, *T. cutaneum* and *S. cerevisiae*).

Keywords: heavy metal; diffusimetric methods; yeast; inhibition; toxicity.

1. Introduction

Pollution with heavy metals represents an important environmental problem both for the abiotic and biotic compartments. Due to industrialization and modern agriculture practices, in the past twenty years, there was an increase in environmental pollution with heavy metals, so large amounts of these metals

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are released into environment and accumulate in living organisms causing growth inhibition and the emergence of toxicity symptoms. Heavy metals generally exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active conformations of biological molecules (Idrees *et al*., 2020; Doelman *et al*., 1994; Gadd and Griffiths, 1978; Wood and Wang, 1983). In order to survive in heavy-metal polluted environments, many microorganisms have developed means of resistance to toxic metal ions (Nies, 2000). These mechanisms include: metal exclusion by permeability barriers, active transport of the metal away from the cell organism, intracellular sequestration of the metal by protein binding, extracellular sequestration, enzymatic detoxification of the metal to a less toxic form and reduction in metal sensitivity of cellular targets (Bruins *et al*., 2000). The detoxification mechanisms may be directed against one metal or a group of chemically related metals. Furthermore, the detoxification mechanisms may vary depending on the type of microorganism (Nies and Silver, 1995). Most microorganisms are known to have specific genes for resistance to toxic ions of heavy metals. Mostly, the resistance genes are found on plasmids or on chromosomes (Nies, 1999). Plasmid-encoded metal resistance determinants have been reported to be inducible (Rosen, 2002). The intake and subsequent efflux of heavy metal ions by microbes normally includes a redox reaction involving the metal.Also, since the oxidation state of a metal ion may determine its solubility, many scientists have been attempting to use microbes that are able to oxidize or reduce metals in order to remediate metal contaminated sites (Spain, 2003). Cadmium is considered highly toxic to plants, animals and microorganisms (Oliveira *et al*., 2020, Raab and Fieldman, 2003). The cadmium cycle in nature has widened considerably as a consequence of interferences with human activities generating environmental pollution and, as a consequence, large amounts of this metal accumulate in the environment and in living organisms (Singh and Cameotra, 2004). The manifestation of cadmium toxicity depends on the chemical species in which it is found. The toxicity of cadmium also depends on the route of exposure and as a consequence a different impact on ecosystems depends on its absorption in living organisms. The toxic effects of chromium are mainly dependent on the metal species, which determines its absorption, translocation and accumulation. The Cr (VI) transport pathway is through an active mechanism involving carriers of essential anions, such as, for example, sulphate (Cervantes *et al*., 2001). Fe, S and P are also known to compete with chromium ions in transport mechanisms. Cr (III) intracellular cationic complexes also interact electrostatically with negatively charged phosphate groups of DNA, which could affect replication, transcription, and mutagenesis (Zinicovscaia *et al*., 2020; Cervantes *et al*., 2001). Furthermore, Cr (III) interferes with DNA replication to produce an increased rate of transcription errors in the cell's DNA. In addition, Cr (III) can alter the structure and activity of enzymes by reacting with their carboxyl and thiol groups (Cervantes *et al*., 2001). Many harmful

effects of nickel are due to its interference with the metabolism of essential metals, such as Fe (II), Mn (II), Cu (II), Zn (II), Cu (II) or Mg (II). The toxicity of nickel probably results from its ability to replace other metal ions in enzymes and proteins or to bind to cellular compounds containing O-, S-, and N groups, such as some enzymes and nucleic acids, which are then inhibited (Bleackley and MacGillivray, 2011).The purpose of this study was to develop a toxicity assay for heavy metals, using some yeast strains as the test microorganisms. This rapid and simple procedure is based on the property of toxic compounds to diffuse into the culture medium over a certain distance, achieving a concentration gradient that decreases in proportion to the distance, thus inhibiting the growth of microorganisms.

2. Materials and Methods

2.1. Microorganisms and Growth Conditions

In the experiments we used three yeast species belonging to the genera Rhodotorula (*Rhodotorula rubra*), Trichosporon (*Trichosporoncutaneum*) and Saccharomyces (*Saccharomyces cerevisiae*) from the collection of the Laboratory of Microbiology. The yeast strains were isolated and maintained on a solidified medium with the following composition: glucose - 20 g / L; yeast extract - 10 g / L; peptone - 20 g / L; agar - 20 g / L, distilled water - 1000 mL; pH - 6.5, sterilization at 0.8 atmosphere, for 20 minutes. At the beginning of the experiments, the yeast strains were passed on a liquid culture medium, with the following formula: glucose - 30 g; peptone - 10 g; yeast extract - 5 g; NaCl - 5; distilled water - 1000 mL; pH - 6.5; sterilization 20 minutes at 0.8 atm. The culture medium was inoculated with 5 x 10^5 CFU / mL for each strain studied and placed under aeration and stirring at 30±.2^{0}C to develop and obtain cell suspension.

2.2. Metal Ions and Reagents

All chemicals used in this work were of analytical reagent grade and were used without further purification. The nickel, chromium and cadmium ions were used as $NiSO_4.6H_2O$, $K_2Cr_2O_7$ and $CdCl_2$ Stock solution of 1000 mg/L of the individual metal ion were prepared in deionized distilled water. The solutions were diluted for different metal concentrations (60, 150 and 300 ppm) by distilled water as required by the working procedure.

2.3. The Toxicity Bioassay Procedure

The sensitivity of microorganisms to certain toxic substances is tested "in vitro" by placing them in optimal and standardized culture conditions (culture medium, inoculum, incubation time, temperature, etc.) in the presence of toxic

substance solutions (Diaconu, 2016). The diffusimetric method is simple, fast, economical and easy to perform. The diffusimetric method is performed on solid culture media and is based on the property of toxic compounds to diffuse into the culture medium over a certain distance, achieving a concentration gradient that decreases in proportion to the distance. The toxic substance is applied in niches created in the culture medium, called wells. The growth of the microorganism will be inhibited in the area where the toxic substance exceeds or has reached the MIC, *i.e*. the smallest amount of toxic substance that inhibits the development of the tested microorganism. Place about 20 - 25 mL of culture medium cooled to 40 - 45^0C, in Petri dishes placed on a perfectly horizontal surface. After solidification of the medium, its surface is inoculated by flooding, with 3 ml of standardized cell suspension, obtained from the culture in 18-hour broth, of the tested microorganism. The suspension is uniform by tilting the Petri dish in different directions, carefully absorbing all excess and allowing the surface of the culture medium to dry well for 30 minutes. On the dry medium, the wells are made with a sterile tubular instrument. Place the test substance solution (1 mL) in each well. In parallel, a control is performed that will contain sterile saline in the well. The control is necessary to compare the effect of toxic compounds on the tested microorganism. The Petri dishes thus prepared are incubated in a thermostat at a temperature of 30^0C for 24-48 hours, for the development of the microorganism. After this time, the obtained results are read.

2.4. Data Analysis

The reading is done with the naked eye, measuring the diameter of the inhibition area in mm, 2-3 times in different directions, using a graduated ruler. The toxicity of the substances used in the test is expressed as the degree of inhibition of the development (%) of living cells and is calculated using the formula (Eq. (1)):

$$I\% = \frac{A-B}{A} x100 \quad (1)$$

in which: *I* - degree of inhibition of cell development (%); *A* - diameter of the cell development area in control (mm); *B* - diameter of the cell development area in the sample (mm).

3. Results and Discussions

The aim of this paper was to investigate the sensitivity of some yeast strains to the action of three heavy metals used at various concentrations. Yeast strains were selected for experiments because they are easy to grow, developing very well in laboratory conditions. The results obtained by using the diffusimetric method are shown in Tables 1 - 3 and illustrated in Figs. 1-4.

3.1. Sensitivity of Yeast Strains to the Action of Cadmium Ions

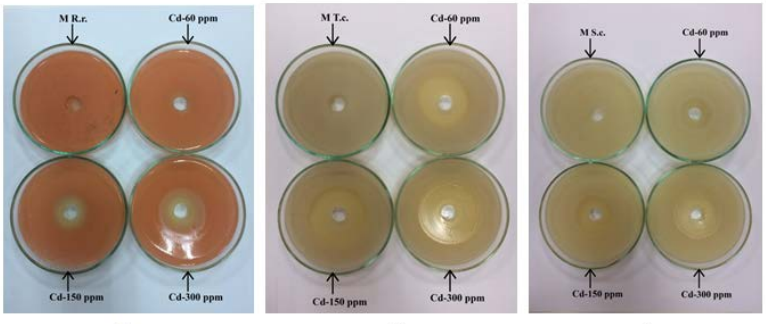
The data obtained by measuring the diameter of the inhibition zone, in the development of yeast cells of the three strains studied, in the presence of cadmium ions, presented in Table 1 and illustrated in Fig. 1, show that the cadmium solution diffused into the culture medium has an inhibitor effect on yeast cells (Munir *et al*., 2021; Oliveira *et al*., 2020). The degree of inhibition varies depending on the yeast strain and the concentration of the solution.

Table 1

The Influence of Cadmium Ions on the Grow of Yeast Cells

The yeaststrain	Diameter of inhibition zone (mm) Cadmium ion concentration (ppm)			
	Control	60	150	300
Rhodotorularubra	0	16	24.5	37.7
Trichosporoncutaneum	0	46	50.5	56
Saccharomycescerevisiae	0	30	36.2	40

Analyzing the results obtained in this experiment we find the following aspects. The diameter of the inhibition zone, of the development of the yeast strains varies both depending on the tested species and depending on the metal concentration used (Pozgajova *et al*., 2019). The most sensitive to the action of cadmium ions is the *T. cutaneum* strain, where the largest areas of growth inhibition were recorded, such as 46 mm at a concentration of 60 ppm / L; 50.5 mm at a concentration of 150 ppm, respectively 56 mm at a concentration of 300 ppm. *S. cerevisiae* showed an intermediate sensitivity to the action of cadmium ions which increases with increasing metal concentration. *R. rubra* is the least affected by the presence of cadmium ions in the culture medium, because in this species the smallest areas of growth inhibition were registered. Controls showed a uniform development over the entire surface of the culture medium distributed in the Petri dishes (Fig. 1).



a) b) c)

Fig. 1 – Aspect of the areas of inhibition of yeast grows under the influence of cadmium ions a) *R. rubra*; b) *T. cutaneum*; c) *S. cerevisiae*.

3.2. Sensitivity of Yeast Strains to the Action of Chromium Ions

The results obtained after incubating the samples for 48 hours at 30^0 C are shown in Table 2 and photographically illustrated in Fig. 2.

Table 2
The influence of Chromium Ions on the Grow of Yeast Cells

The yeaststrain	Diameter of inhibition zone (mm)			
	Chromium ion concentration (ppm)			
	Martor	60	150	300
Rhodotorularubra	0	55.2	58.3	60.2
Trichosporoncutaneum	0	59.5	64.2	69.7
Saccharomycescerevisiae	0	37.2	45.5	48.7

Analyzing the obtained data, it is observed that the diameter of the zone of inhibition of the growth of yeast strains varies both on the species tested and on the metal concentration used. The most sensitive chromium yeast is also *T. cutaneum*, as the highest zones of inhibition were obtained, and the least affected in development was proved to be *S. cerevisiae*, where the lowest zones of inhibition were highlighted. *R. rubra* showed an intermediate sensitivity to chromium ions that increases with increasing metal concentration. Also, the control showed a uniform development on the whole surface of the culture medium (Fig. 2).

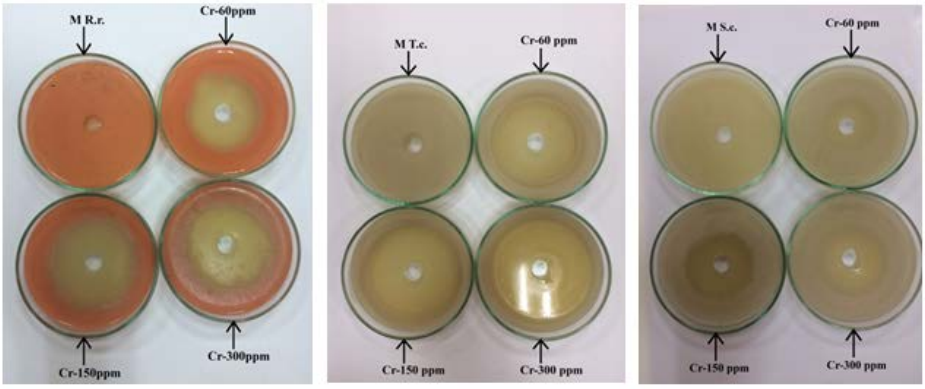


Fig. 2 – Aspect of the areas of inhibition of yeast grow under chromium ions influence a) *R. rubra*; b) *T. cutaneum*; c) *S. cerevisiae.*

3.3. Sensitivity of Yeast Strains to the Action of Nickel Ions

Although nickel is considered an essential element for cell metabolism, at high concentrations it becomes toxic producing various effects. The data

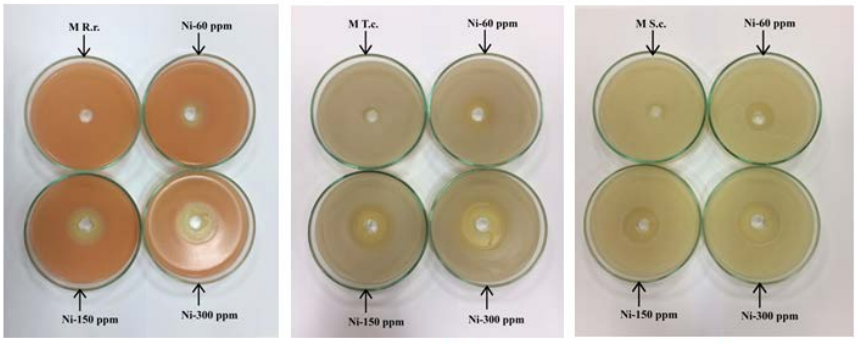
obtained by measuring the diameter of the inhibition zone, in the development of yeast cells, in the presence of nickel ions, presented in Table 3 and illustrated in Figure 3, demonstrate that nickel solutions diffused into the culture medium have inhibitory effect on yeast cells. The degree of inhibition varies depending on the yeast strain tested and the concentration of the metal solution. Analyzing the obtained data, we find that at a concentration of 60 ppm nickel, the diameters of the yeast growth inhibition zones are almost the same; the least affected being the species *T. cutaneum. R.rubra* and *S. cerevisiae* showed a similar sensitivity at concentrations of 150 and 300 ppm nickel, respectively, because the areas of development inhibition were very close as values.

Table 3

The influence of nickel ions on the grow of yeast cells

The yeaststrain	Diameter of inhibition zone (mm)			
	Nickel ion concentration (ppm)			
	Martor	60	150	300
Rhodotorularubra	0	24	29	34.5
Trichosporoncutaneum	0	20	34	45.5
Saccharomycescerevisiae	0	23	29	35.5

T. cutaneum proved to be very sensitive with increasing nickel ion concentration, so that at the concentration of 300 ppm the largest diameter of the growth inhibition zone of 45.5 mm was recorded. Also, the control showed a uniform development on the whole surface of the culture medium (Fig. 3).



a) b) c)

Fig. 3 – Aspect of the areas of inhibition of yeast grow under nickel ions influence
a) *R. rubra*; b) *T. cutaneum*; c) *S. cerevisiae.*

3.4. Percentage Assessment of the Degree of Inhibition of Yeast Growth Under the Action of Metal Ions (Cadmium, Chromium, Nickel)

To determine the degree of inhibition of the development of the three yeast strains under the action of metal ions we took into account the total surface

of the Petri dish (90 mm diameter). The diameter of the growth inhibition zones was decreased and then to the obtained results we applied the formula (1). The results obtained are shown graphically in Figs. 4 - 6. The recorded data show that the three yeast strains studied have the highest sensitivity to chromium ions. The degree of growth inhibition varies depending on the yeast species, but increases with increasing concentration of chromium ions diffused in the culture medium, so that at concentrations of 150 and 300 ppm, respectively, it determines the inhibition of development by over 50% - 70%. Analyzing the data from Fig. 4 we notice that *R. rubra* proved to be very sensitive to the action of chromium ions which led to the inhibition of yeast development by over 60% even from the first concentration used (60 ppm).

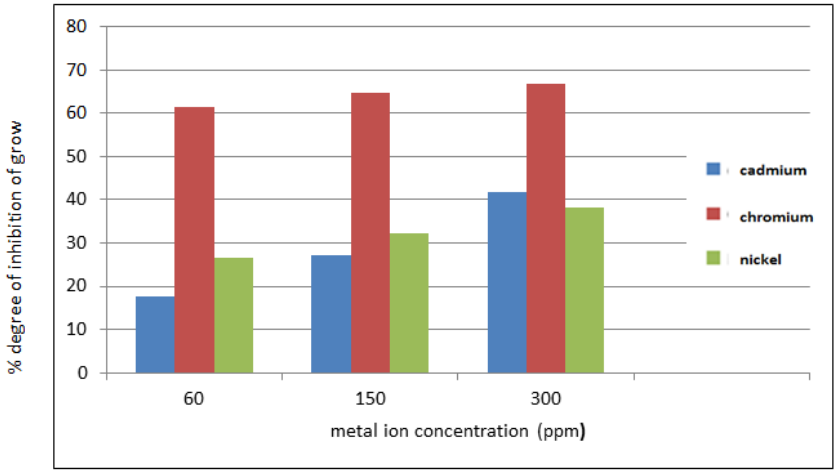


Fig. 4 – The effect of metal ions on R. rubra - degree of grow inhibition (%).

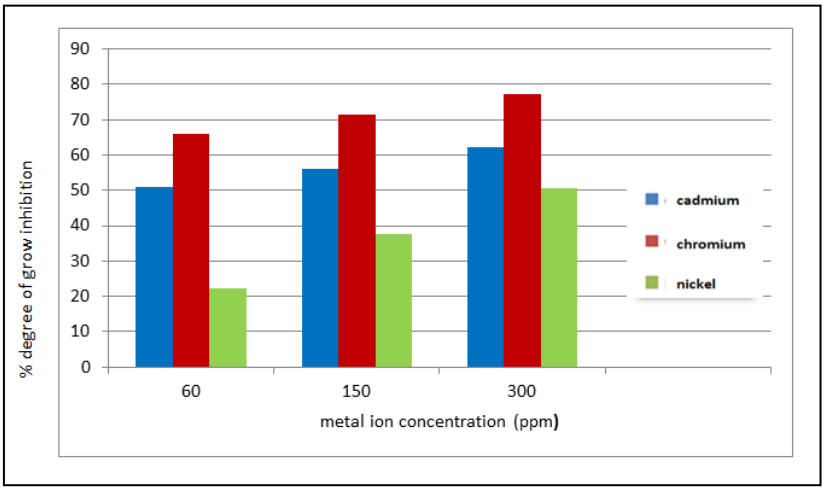


Fig. 5 – The effect of metal ions on *T. cutaneum* - degree of grow inhibition (%).

Cadmium ions are less toxic at concentrations of 60 and 150 ppm, respectively, compared to nickel ions at the same concentrations. But at 300 ppm the situation is reversed, as it has been found that at this concentration cadmium causes an inhibition of yeast cell growth by about 3% higher compared to nickel.

The responses of the organism to the toxicity of the metal ions were dose-dependent (Fig. 5). *T. cutaneum* is very sensitive to both chromium and cadmium ions present in the culture medium, which lead to growth inhibition by over 50% even at a concentration of 60 ppm (Pandey and Sudheesh, 2019). Nickel ions have a moderate effect on yeast, which is more pronounced as their concentration increases, so that at a concentration of 300 ppm there was an inhibition of microorganism growth of about 50%.

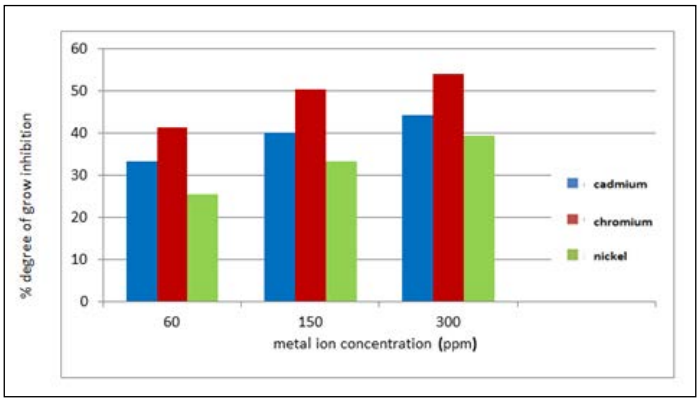


Fig. 6 – The effect of metal ions on *S. cerevisiae* - degree of grow inhibition (%).

The data plotted in Fig. 6 show that *S. cerevisiae* proved to be the least sensitive to the presence of metal ions in the culture medium. Chromium and cadmium ions are more toxic at all concentrations used leading to inhibition of growth by about 30-55%, depending on the concentration (Munir *et al*., 2021). Nickel ions have a moderate toxicity at the concentrations used, with a growth inhibition of about 40% at a concentration of 300 ppm.

4. Conclusions

The experiments were designed to expose the yeast strains to variable doses of metal ions, in order to follow their effect depending on the concentration. Analysis of experimental data indicated that metal ions have an inhibitory effect on yeasts. The degree of toxicity is dependent on both the yeast strain and the metal ion and its concentration. The diffusimetric method used in this study allows the rapid assessment of the toxicity of heavy metals on microorganisms and may be a preliminary step in the studies of biosorption and bioaccumulation of toxic compounds.

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EVALUAREA TOXICITĂȚII UNOR METALE GRELE PENTRU DROJDII FOLOSIND METODA DIFUSIMETRICĂ DE ANALIZĂ

(Rezumat)

Acest studiu prezintă influența ionilor de cadmiu, crom și nichel asupra tulpinilor de drojdie, dezvoltate pe mediul de cultură agarizat repartizat în plăci, utilizând metoda difuzimetrică. Speciile de drojdie testate prezintă o sensibilitate diferită în funcție de ionul metalic din mediul lor de cultură și de concentrația acestuia. *S. cerevisiae* s-a dovedit a fi cea mai puțin sensibilă la prezența ionilor metalici. A rezultat că ionii de crom sunt cei mai toxici pentru cele trei tulpini de drojdie (*R. rubra*, *T. cutaneum* și *S. cerevisiae*).