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Electrochemical study of heavy metals and metallothionein in yeast *Yarrowia lipolytica*

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Abstract

The bioaccumulation of heavy metals (cadmium, nickel, cobalt and zinc) and the effect of these metals on the production of metallothionein and metallothionein-like proteins (MT) in *Yarrowia lipolytica* was studied by electrochemical methods. The concentrations of heavy metals were determined by differential pulse voltammetry (DPV). A combination of the constant current chronopotentiometric stripping analysis (CPSA) and adsorptive transfer stripping technique (AdTS) was used to determine the content of MT in cells. Both the bioaccumulation of heavy metals and the production of MT in different cell compartments of *Y. lipolytica* exposed to heavy metals were monitored. The LD_{50} of each metal was determined from the number of viable cells in yeast cultures: LD_{50}Cd (37.5 μM), LD_{50}Ni (570 μM), LD_{50}Co (700 μM), and LD_{50}Zn (1800 μM). The highest concentrations of heavy metals were found in the cell wall and membrane debris while the lowest concentrations were detected in the cytoplasm. Cadmium and nickel showed the most significant effect on the production of MT. This study provides new insights into the ecophysiology of microorganisms and demonstrates the potential use of these electrochemical methods in biotechnology.
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Keywords: Yeast; *Yarrowia lipolytica*; Differential pulse voltammetry; Adsorptive transfer stripping technique; Constant current chronopotentiometric stripping analysis; Metallothionein; Cadmium, nickel, cobalt and zinc; Bioaccumulation

1. Introduction

Among a number of biotechnological applications, *Yarrowia lipolytica* is used in the bioremediation of soils contaminated by petroleum products. Industrialization and domestic activities have accelerated biogeochemical cycles of a large number of elements, including heavy metals. This has contributed to the increasing deposition of heavy metals in natural ecosystems. Moreover, heavy metals occur in petroleum products and therefore represent a serious problem for the environment and living organisms [1,2]. If the environment is severally polluted by petroleum products, it is necessary to prevent pollution spreading and to restore the contaminated environment to previous conditions as soon as possible. The possible approach to tackle this problem consists of the microbiological degradation of petroleum products. For bioremediation of contaminated soils and waters, the yeast *Y. lipolytica* can be used [3]. Yeast cells defend themselves against heavy metals [1,4] by production of specific proteins [5], which bind metals in cytoplasm or transport them into vacuoles [6]. For example, metallothioneins (MTs) are proteins binding heavy metals [7–9].

We attempted to find the resistance of *Y. lipolytica* CCM 4510 to heavy metals. The aim of this paper is to study the impact of cadmium, nickel, cobalt and zinc on yeast growth, the bioaccumulation of heavy metals in different cell compartments, and the level of metallothionein-like proteins (especially metallothionein) in cytoplasm. The results of this paper will contribute to the potential application of electrochemistry in the study of the effects of heavy metals on MT production.

2. Materials and methods

2.1. Organisms and their growth conditions

The strain CCM 4510 of *Y. lipolytica* was isolated from soil which had been exposed to crude petroleum for long

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time (Czech Collection of Microorganisms, Faculty of Science, Masaryk University, in Brno Czech Republic). Malt Extract Agar (MEA) and Malt Extract Broth (MEB, Hi-Media, India) were used for storing and cultivation of yeast cells [10] and contained (in g/l deionized water): malt extract (17), peptone (5), MEA contained yet agar (20), pH 5.4. Yeast cells were taken from slant MEA at 4 °C and cultivated in MEB. The starter culture was transferred from the MEA medium into 20 ml of the MEB medium and cultivated for 24 h at 25 °C under intensive shaking. The yeast was grown in 100 ml of MEB in a conical bottle inoculated with 0.5 ml of the starter culture. For possible experiments with bacteria, we tested universal medium Nutrient Broth (NB, Hi-Media) contained (in g/l deionized water): peptone from animal tissue (5.0), sodium chloride (5.0), beef extract (1.5), yeast extract (1.5), pH 7.4. The media components were mixed in boiling water and autoclaved 121 °C for 15 min. The pH value of the media were adjusted using a pH meter Praecitronic MV870 (Germany) at room temperature.

Salts of heavy metals ($CdCl_2$, $NiCl_2$, $ZnCl_2$, $CoCl_2$; ACS Reagent, Sigma Aldrich, Chemical, St. Louis, MO, USA) were added to the MEB medium containing yeast cells. Concentration ranges (μmol/l) of Cd 0–37.5, Ni 0–1000, Co 0–1200 and Zn 0–2400 were chosen. During cultivation, the solution was shaken intensively: 150 rpm, amplitude 3.5 cm, at 25 °C for 16 h using water bath shaker (Elpan 357, Poland). The growth of yeast cells was followed by means of optical density (OD_{620}) of solutions carried out using a diode array spectrometer (Hewlett Packard, Model 8452A, USA) with temperature-controlled cells and by an amount of viable cells which were determined by plate colony count method on the MEA. The amount of total soluble proteins were determined by Bradford's method [11].

Samples of yeast cells were prepared using previously used procedures adopted from the literature [12,13]:

(a) After cultivation (16 h), the cells were centrifuged for 10 min, at 4 °C at 10,000 × *g* (Hettich RF 32, Germany). The measurement of amount of heavy metals in MEB medium was made in the supernatant. The amount of adsorbed heavy metals on the surfaces of cultivation bottles was monitored in used bottles without cells.
(b) The amount of heavy metals on the cell surface was determined as follows: the cells were washed twice with 0.2 M acetic buffer (pH 4.8) containing 0.5 M EDTA, then stirred (Scientific Industries, Vortex-2 Genie, USA) for 5 min at room temperature. Then the suspension of cell was centrifuged for 10 min at 10,000 × *g*. The possible leaking of the cytoplasm was determined by measuring the activity of lactate dehydrogenase (cytoplasmic marker). The method was described by Appleby and Morton [14].
(c) The yeast cells were mixed with 0.2 M acetic buffer (pH 4.8) and disintegrated by ultrasonic waves at 4 °C (Bandelin 200 W, Germany) for 10 min. Prior to and after the sonication, the portion of the intact cells was determined microscopically. The sonicated cells were centrifuged at 4 °C for 30 min at 16,000 × *g* (Beckman, Avanti 30I, USA). The resultant pellet consisted of isolated particles of cell walls and membranes, while the supernatant contained cytoplasm. The supernatant was analysed for heavy metals and metallothionein-like proteins (MT). The samples for the determination of the MT were prepared according the procedure reported by Kizek et al. [15]. The sample was kept at 99 °C in a thermomixer compact (Eppendorf 5430, USA) for 15 min with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C and at 15,000 × *g* for 30 min (Eppendorf 5402). The heat treatment and the solvent precipitation effectively removed the high molecular weight proteins from the samples [16].
(d) The samples were subjected to microwave mineralization in 0.5 ml H_2O_2 (31%) + 2.5 ml HNO_3 (65%) for 16 min and 280 W (MLS-1200 PYRO Rapid Microwave Ashing System, USA) and amount of heavy metals bonded to the cell wall and membrane debris were measured. The mineralizate was neutralized with NaOH (13 M) and measured by differential pulse voltammetry (DPV).

2.2. Electrochemical measurements

Electrochemical measurements were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl/3 M KCl electrode and the auxiliary electrode was a graphite electrode. The supporting electrolyte was prepared by mixing buffer components and its pH value was measured (pH meter Praecitronic MV870).

2.2.1. Differential pulse voltammetry

The amount of the heavy metal was measured using DPV. The components of the supporting electrolyte (acetate buffer: 0.1 M CH_3COOH + 0.1 M CH_3COONa, pH 5) from Sigma Aldrich in ACS purity were purchased. DPV parameters were as follows: an initial potential of − 0.1 V, an end potential − 1.4 V, a scan rate of 10 mV/s, a step potential of 5 mV/s, a pulse amplitude of 50 mV. All experiments were carried out at room temperature. For smoothing and baseline correction, the software GPES 4.4 supplied by EcoChemie was employed [17–19].

2.2.2. Adsorptive transfer stripping technique and chronopotentiometric stripping analysis

Rabbit liver MT, containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (MW 7143). Standard sol-

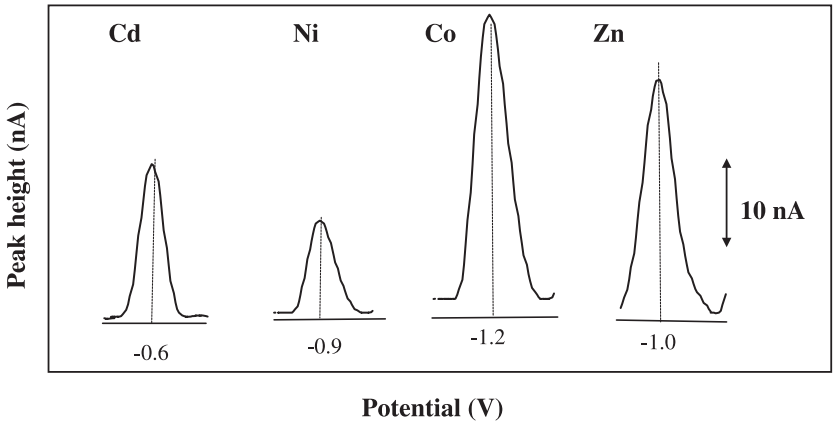

Fig. 1. Differential pulse voltammograms of heavy metals (500 nM) in 0.1 M acetate buffer (pH 5). Row data treated by baseline correction. Step potential 5 mV/s, scan rate 10 mV/s, pulse amplitude 50 mV, initial potential − 0.1 V, end potential − 1.4 V.

utions of MT were prepared by diluting a stock solution (1.0 mg MT/ml) with water (Sigma Aldrich, ACS). For the determination of the MT content, an adsorptive transfer stripping technique (AdTS) [20] in connection with chronopotentiometric stripping analysis (CPSA) were used [15] to record the inverted time derivation of potential $(dE/dt)^{-1}$ as a function potential E [20,21]. CPSA parameters were as follows: time of accumulation of 120 s, I_{str} of − 1 μA. After

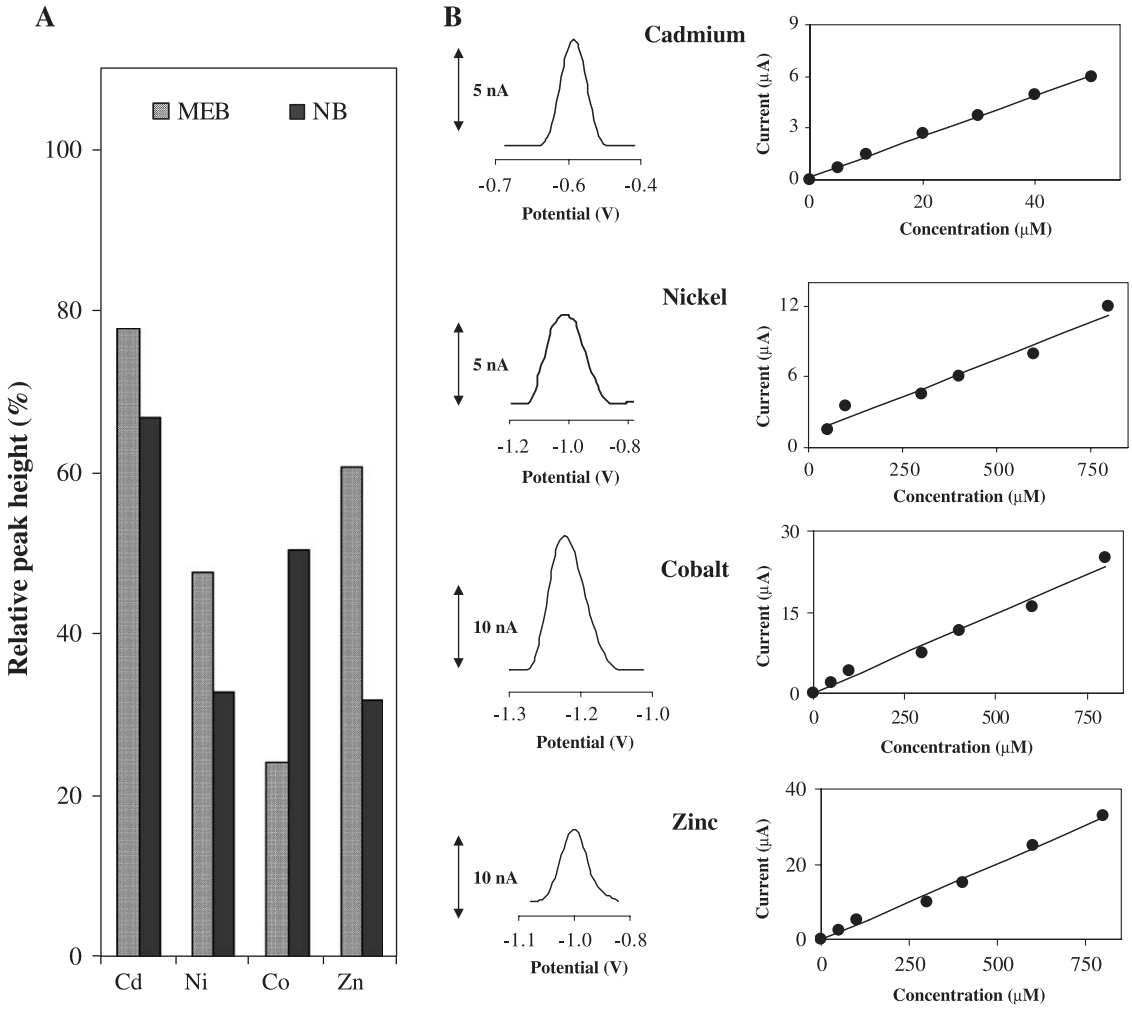

Fig. 2. (A) Relative peak heights of heavy metals in different growth media (MEB, NB; see Organisms and their growth conditions). Peak height obtained in acetate buffer was taken as 100%. (B) Differential pulse voltammograms of Cd, Ni, Zn, Co (5 μM) in medium (left) and calibration curves in MEB (right).

adsorption of MT, the HMDE was washed and transferred in an electrolytic cell with 0.1 M H_3BO_3 + 0.05 M $Na_2B_4O_7$ (Sigma Aldrich, ACS) where the CPSA was performed (see Fig. 3A).

3. Results and discussion

3.1. Detection of heavy metals and MT

The reduction signals of cadmium, nickel, cobalt and zinc in the acetate buffer (without MEB) recorded by means of DPV are shown in Fig. 1. The peak potentials (E_p) of individual metals are as follows: − 0.6 V for cadmium, − 0.9 V for nickel, − 1.0 V for zinc, and − 1.2 V for cobalt vs. Ag/AgCl/3 M KCl reference electrode. Electrochemical methods for monitoring metal ion concentrations in cultures of living bacteria and cells without separation of biomass and medium were described recently using mercury [22] and solid electrodes [23]. The effect of the medium on the DPV heavy metal signals is shown for MEB and NB media in Fig. 2A. Peak heights are presented in relative values, and peak height, obtained in the acetate buffer without medium, was taken as 100%. It is obvious that the media affect the values of the detection limits for heavy metals and the visible decrease of peak heights were observed in all cases. Therefore, a calibration graph should be measured for every medium. Fig. 2B shows the signals of metals at a concentration of 5 μM in MEB. Linear calibration curves were measured for micromolar concentrations of Cd, Ni, Co and Zn (Fig. 2B). The detection limit of heavy metals in MEB was 1 μM (relative standard deviation 5%; n = 5).

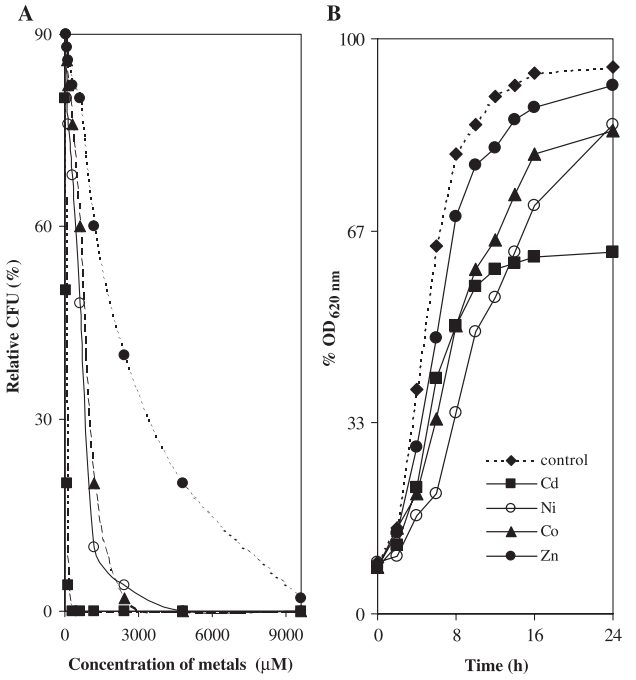

Fig. 4. Effect of (□) cadmium, (○) nickel, (▲) cobalt and (□) zinc dosage on the growth of *Y. lipolytica* during 16 h cultivation. (A) The dependence of the number of viable cells on the concentration of heavy metal; (B) spectrophotometrical dependence (OD_{620}) of the cell growth on cultivation time (♦ control without metals). Solutions of 30 μM Cd and 600 μM Ni, Co, Zn were added to the MEB at the start of cultivation. Total growth CFU obtained in the absence of heavy metals was taken as 100%. Number of measurements n = 5. Other details are provided in Materials and methods.

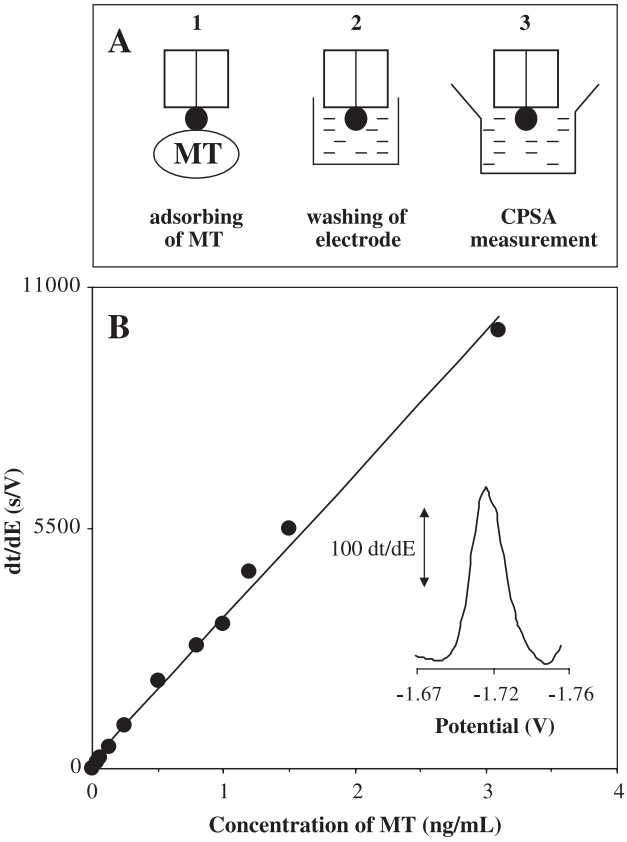

Fig. 3. (A) Scheme of adsorptive transfer technique used for the detection of MT; (1) adsorbing of MT in a drop solution onto HMDE; (2) washing electrode in borate buffer (pH 8); (3) measurement of MT by AdTS CPSA. (B) Dependence of the peak height on the content of MT using AdTS CPSA. Inset: signal of 1 ng MT/ml borate buffer adsorbed in 5 μl drop solution. Time of accumulation 120 s of MT at HMDE, I_{str}—1 μA. The HMDE was washed and transferred into the 0.1 M H_3BO_3 + 0.05 M $Na_2B_4O_7$ in a common electrolytic cell where the CPSA was performed.

After the adsorptive transfer procedure, the catalytic signal of MT [15,24] was measured by CPSA. The individual steps of the procedure are illustrated in Fig. 3A. This procedure is based on immobilization of MT at the HMDE surface (Fig. 3A(1)) from the drop of solution (~ 5 μl), which is followed by washing (Fig. 3A(2)).

Electrochemical measurements were carried out in a cell containing buffer solution (Fig. 3A(3)). The calibration curve of MT in borate buffer (the dependence of peak height on the content of MT Fig. 3B) was obtained with the period of accumulation t_A of 120 s. The AdTS CPSA signal of MT at a concentration of 1 ng/ml (200 pM) is shown in the inset of Fig. 3B.

3.2. Effect of heavy metals on the growth of the yeast

We studied the growth of the yeast at different concentrations of cadmium, nickel, cobalt and zinc. The half inhibition concentrations of metal (LD_{50}—50% dosis lethalis), i.e. the metal concentrations killing the yeast cells at 50%, were determined from the number of viable cells (CFU—colony forming units) of yeast cultures. The highest inhibition effect of all applied metals was observed for cadmium. Nickel and cobalt provided similar inhibition effects, while zinc showed the lowest inhibition activity. *Y. lipolytica* CCM 4510 was able to grow in a medium containing cadmium (0–75 μM), nickel (0–2400 μM), cobalt (0–2400 μM) and zinc (0–9600 μM), see Fig. 4A. This means that the sensitivity of the yeast to heavy metals can be classified as follows: the first class—zinc, exhibited a weak effect on living cells; the second class—nickel and cobalt, showed a medium effect on yeast cells; the third class—cadmium, is very toxic for cells. Therefore, the tolerance of the yeast to heavy metals decreases as follows: Zn>Co ~ Ni≫Cd. A similar inhibition effect on the growth was observed for filamentous fungus *Bispora* sp. [25].

The growth of the yeast culture can be detected by measurement of optical density (OD) at 620 nm. The dependence of the yeast growth (OD_{620}) on time in MEB with and without metals (30 μM Cd, 600 μM Ni, Co, and Zn) is shown in Fig. 4B. These experimental conditions were used as the starting experimental conditions for monitoring of the MT production.

3.3. Bioaccumulation of cadmium, nickel, cobalt and zinc in cell compartments

We suppose that the mechanism of metal ion binding to the cell wall in bacteria [26] is similar to the one in yeast. Metals were accumulated in cell compartments at different rates. For the study of bioaccumulation of heavy metals in cell compartments, we applied concentrations of Cd: 4.8, 9.3, 18.7, and 37.5; Ni and Co: 75, 150, 600, 1200; Zn: 300, 600, 1200, 2400 μmol/l. Accumulation of cadmium and nickel in *Y. lipolytica* CCM 4510 was higher than that of cobalt and zinc, i.e. cobalt and zinc were less bonded on yeast biomass in comparison with cadmium or nickel. Moreover, cadmium and nickel were not detected on the surface of the cell wall, but mainly they interacted with the cell wall and membrane debris (Fig. 5A). The high level of cadmium and nickel in the cell wall and membrane debris is probably due to the interaction of heavy metals with carboxylic groups, which are dominant functional groups in the cell wall. These carboxylic groups descended from peptides would be the potential sides for binding of heavy metals, while the binding process is not exactly described

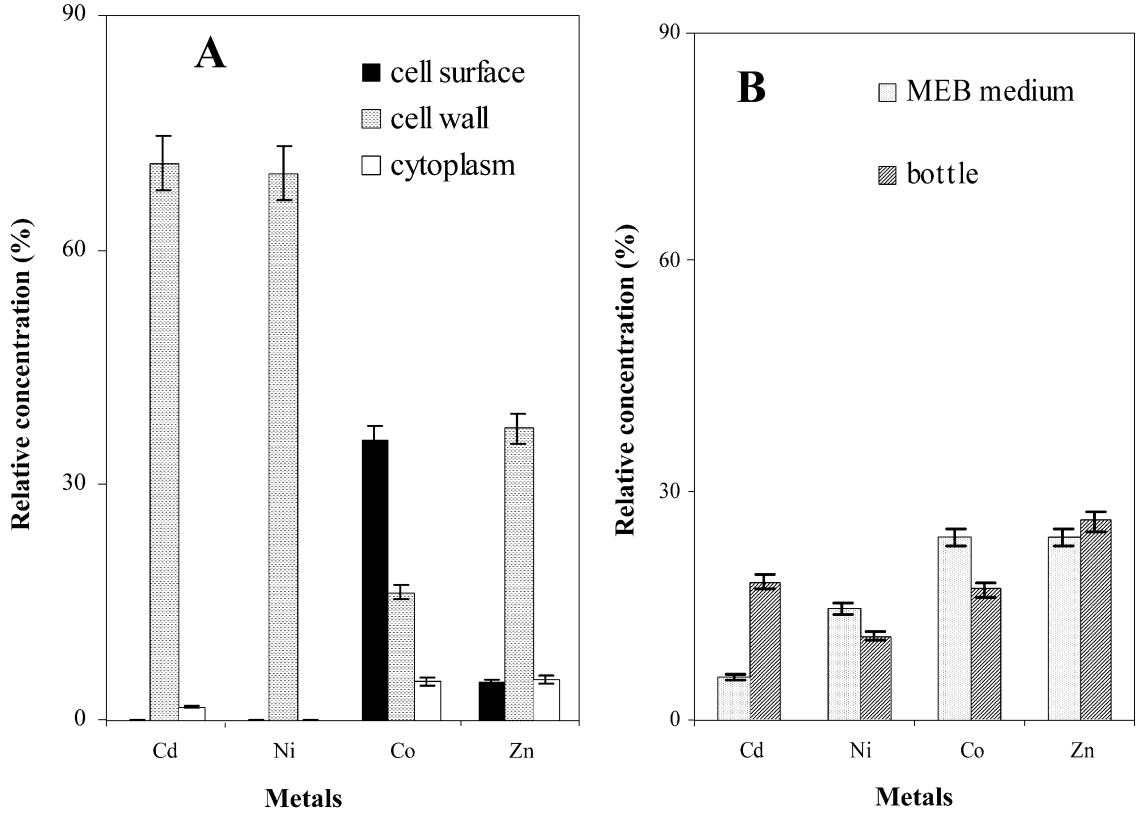

Fig. 5. Uptake of cadmium, nickel, cobalt and zinc to (A) cell surface, wall and membrane debris and cytoplasm of *Y. lipolytica* and the concentration of heavy metals in (B) MEB and on the surface of cultivation bottles after finishing of cultivation. The concentration of metal added to the cultivation medium were taken as 100%; ($n = 5$). Other details in Fig. 4 and Materials and methods.

[26]. Only a small amount of cadmium (1.6%) passed through the cell wall and membrane into the cytoplasm (see the first column in Fig. 5A). The quantity of nickel in the cytoplasm and on the surface of the cell wall was below 1 μM in MEB (Fig. 5A). Interestingly, cobalt was largely adsorbed on the cell surface (36%) and on the cell wall and membrane debris (16%). Cobalt and zinc passed through the plasma membrane into the cell. In the cytoplasm, 5% of cobalt and 5% of zinc of the total applied metals were found. On the contrary, zinc (37%) was adsorbed on the cell wall and membrane debris and 5% of zinc was detected on the cell surface (Fig. 5A). The remainder of the used metal (a complement to 100%) was in the medium or on the surface of the cultivation bottle in the adsorbed state (Fig. 5B).

The sorption of heavy metals by microbial biomass has been observed to varying extents. Yeast biosorption largely depends on parameters such as pH, metal ion and biomass concentration, physical or chemical pre-treatment of biomass, presence of various ligands in solution, and to a limited extent on temperature (in Ref. [27]). Muter et al. [28] published the metal uptake by yeast *Candida utilis*: Zn>Cd>Pb>Cu>Cr. In our study, the incorporation of heavy metals into individual cell compartments of *Y. lipolytica* decreased in the following order: by (a) cell surface, Co>Zn≫Cd, Ni; (b) cell wall and membrane debris, Cd, Ni≫Zn>Co; and (c) cytoplasm of yeast cell, Zn, Co>Cd≫Ni (Fig. 5A). Furthermore, not only yeasts, but also prokaryotic organisms have been used to study microorganism biosorption. In comparison, the metal sorption of cations by the bacteria *Citrobacter* MCM B-181 biomass exhibited the following order of metal sorption as: Zn>Cu>Cd>Ni>Co [29] has been recently published. According to references, metal uptake by microbial cells probably depends on various processes [30]; in fact, for *Pseudomonas cepacia*, the metal sorption sequence was different: Cu>Ni>Cd>Zn [22].

3.4. Effect of heavy metals on the production of yeast metallothionein

Recently, the CUP1 locus in *Saccharomyces cerevisiae* was determined. The CUP1 locus has an open reading frame capable of encoding a 61-amino acid polypeptide that resembles mammalian metallothionein in its high proportion of cysteine residues [7]. This yeast metallothionein thus exhibits two distinct binding configurations for Cu and Cd, as does the mammalian MT [7]. This screen led to the identification of the CRS5 (copper-resistant suppressor) gene. By sequence analyses, CRS5 encodes the small molecular weight cysteine-rich protein with an amino acid sequence bearing all the features of an eukaryotic metallothionein. Cysteines represent nearly 30% of the total CRS5 polypeptide, and many are present in the Cys–X–Cys, Cys–X–X–Cys, and Cys–Cys in *S. cerevisiae* [31] and *Y. lipolytica* [32]. Therefore, we studied the MT production in *Y. lipolytica* at different concentrations of applied heavy metals using AdTS CPSA (Fig. 6). Since electrochemical quantitation of MT is not a specific procedure as RIA [33], it is crucial to eliminate the contribution of other interfering cysteine-containing proteins from complex samples before making determinations. In most cases, the interfering compound is eliminated by heat denaturation, leaving the heat-stable MT in solution [16,34,35]. Prior to each CPSA measurement, MT in cytoplasm samples was adsorbed on the electrode surface for an accumulation time of 120 s. The physiological content of MT (1 ng MT/g protein) in *Y. lipolytica* was that produced by yeast in the absence of heavy metals (Fig. 6, column 1). From the results obtained on the effect of heavy metals on the growth of yeast (Fig. 4), several heavy metal concentrations were chosen for monitoring MT production. When cadmium was present at a concentration of 9 or 30 μM ($CdCl_2$) the amount of MT increased from physiological levels to 260 and 660 ng MT/g protein, respectively (Fig. 6 columns 2 and 3). It is interesting that the strong effect of nickel (300 and 600 μM) on MT production was observed (Fig. 6, columns 4 and 5), where the concentration of MT was 530

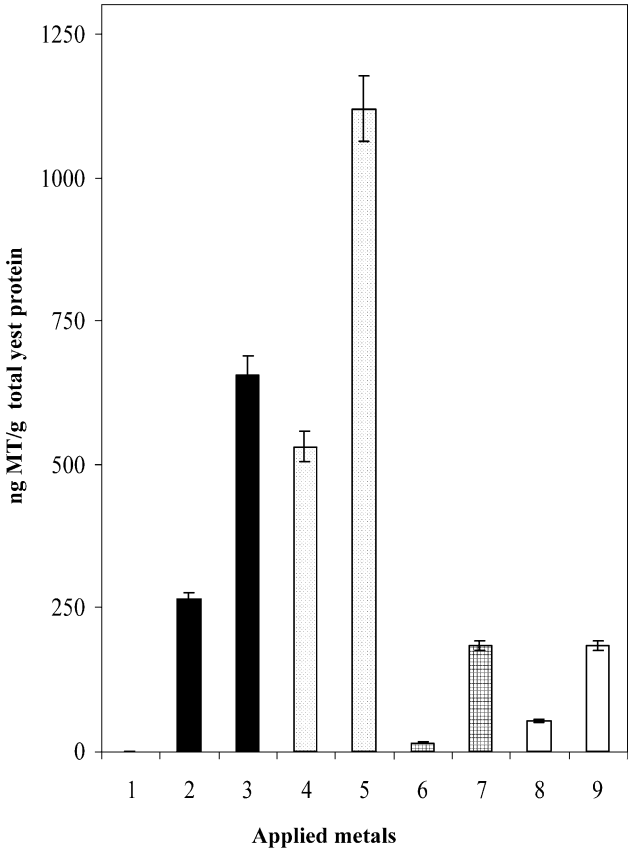

Fig. 6. Content of MT in *Y. lipolytica* at different concentrations of heavy metals. (1) Without metals—physiological content of MT was 1 ng/g protein, (2) 9 μM Cd, (3) 30 μM Cd, (4) 300 μM Ni, (5) 600 μM Ni, (6) 600 μM Zn, (7) 1200 μM Zn, (8) 300 μM Co, (9) 600 μM Co; ($n=3$). Other conditions in Fig. 3.

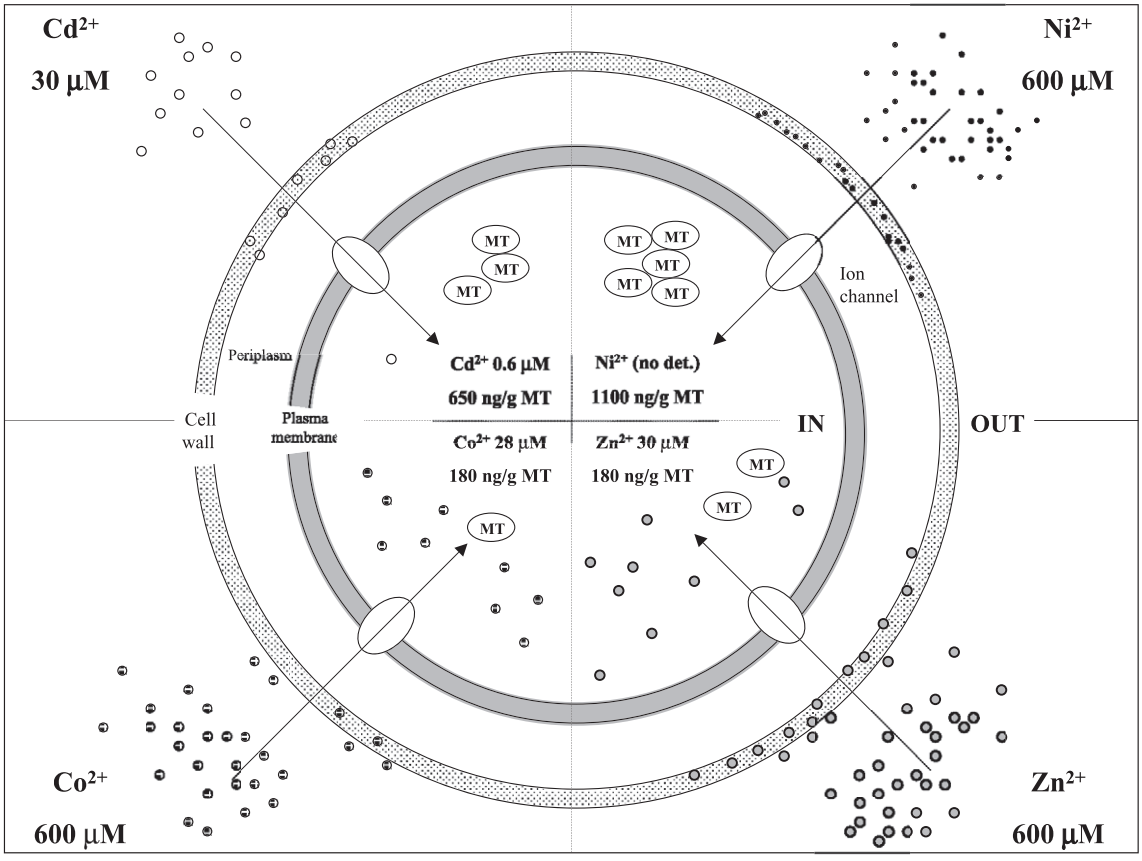

Fig. 7. Incorporation of cadmium, nickel, cobalt and zinc in the cell surface, wall and cytoplasm of yeast *Y. lipolytica* and effect of metallothionein-like proteins. Other details in Figs. 3 and 6.

and 1120 ng MT/g protein, respectively. For zinc (600 and 1200 μM $ZnCl_2$) and cobalt (300 and 600 μM $CoCl_2$), the effect on MT production was similar (Fig. 6, columns 6 and 7; 8 and 9, respectively).

The differences in MT levels in the yeast cell treated with heavy metals may arise due to the variety of physiological–biochemical changes that take place during the growth process and the ageing of cells. Though the toxic metals (Cd, Ni) induce biosynthesis of MT more significantly than essential metals (Co, Zn). Our results, obtained by using electrochemical methods, support the hypothesis that the intracellular concentrations of heavy metals and the contents of MT are possibly mutually dependent. The production of MT is induced by increasing intracellular concentrations of heavy metals.

A comprehensive scheme of heavy metal incorporation into yeast cell compartments and their effect on the MT production was formed in order to explain these processes. This scheme (Fig. 7) is based on experimental data presented in Figs. 5 and 6. For the assessment of the metal effect on the yeast cells, we chose the same concentrations of nickel, cobalt and zinc (600 μM). With respect to the toxicity of cadmium to yeast, the concentration of cadmium was 30 μM. Metal ions were probably transported into the cytoplasm through ion channels in plasma membrane [36]. Low concentrations of toxic metals (cadmium and nickel) and the relatively high content of MT were detected in the cytoplasm. On the contrary, cytoplasmic concentrations of essential metals (zinc and cobalt) were higher and the amount of MT was lower than that for toxic metals. On the basis of our results, we suppose that the cell wall is the first protective barrier to prevent penetration of heavy metals into the cell.

4. Conclusion

The yeast *Yarrowia* can be used for remediation of the environment contaminated by various pollutants, including heavy metals [37–39], and can grow in the presence of hydrocarbons [10]. The yeast strain CCM 4510 was grown in the medium containing various carbohydrates and showed considerable resistance to applied heavy metals as demonstrated by our results. The *Y. lipolytica* cells can bind large amounts of heavy metals and produce specific protein metallothionein as protective agent. This approach offers the possibility to apply the yeast in bioremediation of soil and water contaminated by petroleum products in the connection with heavy metals.

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References

[1] M. Mullen, D. Wolf, F. Ferris, T. Beveridge, C. Flemming, G. Bailey, Bacterial sorption of heavy metals, Appl. Environ. Microbiol. 55 (1989) 3143–3149.
[2] G. Gadd, A. Griffiths, Microorganism and heavy metal toxicity, Microb. Ecol. 4 (1978) 303–317.
[3] M. Sinigaglia, R. Lanciotti, M. Guerzoni, Biochemical and physiological characteristics of *Yarrowia lipolytica* strains in relation to isolation source, Can. J. Microbiol. 40 (1994) 54–59.
[4] J. Trevors, G. Stratton, G. Gadd, Cadmium transport, resistance, and toxicity in bacteria, algae, and fungi, Can. J. Microbiol. 32 (1986) 447–464.
[5] Z.-S. Li, Y.-P. Lu, R.-G. Zhen, M. Szczypka, D. Thiele, P. Rea, A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 42–47.
[6] Z.-S. Li, M. Szczypka, Y.-P. Lu, D. Thiele, P. Rea, The yeast cadmium factor protein (YCP1) is a vacuolar glutathione S-conjugate pump, J. Biol. Chem. 271 (1996) 6509–6517.
[7] D. Winge, K. Nielson, W. Gray, D. Hamer, Yeast metallothionein sequence and metal-binding properties, J. Biol. Chem. 260 (1985) 14464–14470.
[8] R. Palmiter, The elusive function of metallothioneins, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8428–8430.
[9] A. Rodriguez, Characterisation of mammalian Cd, Zn metallothioneins using differential pulse polarography, Methallothionein IV (1999) 85–91.
[10] G. Barth, C. Gaillardin, Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*, FEMS Microbiol. Rev. 19 (1997) 219–237.
[11] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[12] A. Murray, D. Kidby, Sub-cellular location of mercury in yeast grown in the presence of mercuric chloride, J. Gen. Microbiol. 86 (1975) 66–76.
[13] P. Magnelli, J. Cipollo, C. Abeijon, A refined method for the determination of *Saccharomyces cerevisiae* cell wall composition and b-1,6-glucan fine structure, Anal. Biochem. 301 (2002) 136–150.
[14] C. Appleby, R. Morton, Lactic dehydrogenase and cytochrome b2 from yeast. Purification and crystallization, Biochem. J. 71 (1959) 492–496.
[15] R. Kizek, L. Trnková, E. Paleček, Determination of metallothionein at the femtomole level by constant current stripping chronopotentiometry, Anal. Chem. 73 (2001) 4801–4807.
[16] M. Erk, D. Ivankovic, B. Raspor, J. Pavičc, Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins, Talanta 57 (2002) 1211–1218.
[17] M.U.A. Bromba, H. Ziegler, Application hints for Savitzky–Golay digital smoothing filtres, Anal. Chem. 53 (1981) 1583–1586.
[18] M. Ryan, J. Chambers, Dynamic electrochemistry: method and application, Anal. Chem. 64 (1992) 79R–116R.
[19] J. Wang, Analytical Electrochemistry, 2nd ed., John Wiley and Sons Ltd., 2000.
[20] E. Paleček, I. Postbieglová, Adsorptive stripping voltammetry of bimacromolecules with transfer of the adsorbed layer, J. Electroanal. Chem. 214 (1986) 359–371.
[21] E. Palecek, From polarography of DNA to microanalysis with nucleic acids-modified electrodes, Electroanalysis 8 (1996) 7–14.
[22] I. Savvaidis, M. Hughes, R. Poole, Differential pulse polarography: a method of directly measuring uptake of metal ions by live bacteria without separation of biomass and medium, FEMS Microbiol. Lett. 92 (1992) 186–1981.
[23] R. Kizek, L. Trnková, S. Ševčíková, J. Šmarda, F. Jelen, Silver electrode as a sensor for determination of zinc in cell cultivation medium, Anal. Biochem. 301 (2002) 8–13.
[24] L. Trnková, R. Kizek, J. Vacek, Catalytic signal of rabbit liver metallothionein on a mercury electrode: combination of derivative chronopotentiometry with adsorptive transfer stripping, Bioelectrochemistry 56 (2002) 57–61.
[25] H. Gimmler, J. Jesus, A. Greiser, Heavy metal resistance of the extreme acidotolerant filamentous fungus *Bispora* sp., Microb. Ecol. 42 (2001) 87–98.
[26] A. Plette, M. Beneditti, V. Riemsdijk, Competitive binding of protons calcium, kadmium and zinc to isolated cell walls of a gram-positive soil bacterium, Environ. Sci. Technol. 30 (1996) 1902–1910.
[27] A. Kapoor, T. Viraraghavan, Fungal biosorption—an alternative treatment option for heavy metal bearing wastewaters: a review, Bioresour. Technol. 53 (1995) 195–206.
[28] O. Muter, I. Lubinya, D. Millers, L. Grigorjeva, E. Ventinya, A. Rapoport, Cr(VI) sorption by intact and dehydrated *Candida utilis* cells in the presence of other metals, Process Biochem. 38 (2002) 123–131.
[29] P. Puranik, K. Paknikar, Biosorption of lead, cadmium, and zinc by *Citrobacter* strain MCM B-181: characterization studies, Biotechnol. Prog. 15 (1999) 228–237.
[30] H. Kambe-Honjoh, A. Sugawara, K. Yoda, K. Kitamoto, M. Yamasaki, Isolation and characterization of nickel-accumulating yeasts, Appl. Microbiol. Biotechnol. 48 (1997) 373–378.
[31] C.V. Culotta, W.R. Howard, X.F. Liu, CRS5 encodes a metallothionein-like protein in *Saccharomyces cerevisiae*, J. Biol. Chem. 269 (1994) 25295–25302.
[32] S. Garcia, M. Prado, R. Degano, A. Dominguez, A copper-responsive transcription factor, CFR1, mediates copper and cadmium resistance in *Yarrowia lipolytica*, J. Biol. Chem. (2002) (in press, July 11, 2002 as M201091200).
[33] C. Hogstrand, C. Haux, Evaluation of differential pulse polarography for the quantification of metallothionein—a comparison with RIA, Anal. Biochem. 200 (1992) 388–392.
[34] R.W. Olafson, P.-R. Olsson, Electrochemical detection of metallothionein, Methods Enzymol. 205 (1991) 205–213.
[35] P.R. Olsson, Thesis, 1987.
[36] D. Nies, Microbial heavy-metal resistance, Appl. Microbiol. Biotechnol. 51 (1999) 730–750.
[37] L. Diels, S. Roy, K. Somers, I. Willems, W. Doyen, M. Mergeay, D. Springael, R. Leysen, The use of bacteria immobilized in tubular membrane reactors for heavy metal recovery and degradation of chlorinated aromatics, J. Membr. Sci. 100 (1995) 248–258.
[38] D. Nies, Heavy metal-resistant bacteria as Extremophiles: molecular physiology and biotechnological use of *Ralstonia* sp. CH34, Extremophiles 4 (2000) 77–82.
[39] S. Dahlin, E. Witter, A. Martensson, A. Turner, E. Baath, Where's the limit? Changes in the microbiological properties of agricultural soils at low levels of metal contamination, Soil Biol. Biochem. 29 (1997) 1405–1415.