Int. J. Electrochem. Sci.,6 **(2011)** 2171 - 2191

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Influence of Zinc(II) and Copper(II) Ions on *Streptomyces* Bacteria Revealed by Electrochemistry

Petr Majzlik[1], Andrej Strasky[2], Vojtech Adam[1,7,8], Miroslav Nemec[2], Libuse Trnkova[3,4], Josef Zehnalek[1], Jaromir Hubalek[5,7], Ivo Provaznik[6], Rene Kizek[*1,7,8]*

[1]Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic-European Union
[2]Department of Microbiology, and [3]Department of Chemistry, and [4]Research Centre for Environmental Chemistry and Ecotoxicology, Faculty of Science, Masaryk University, Kotlarska 2, CZ-611 37 Brno, Czech Republic-European Union
[5]Department of Microelectronics, Faculty of Electrical Engineering and Communication, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic-European Union
[7] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic-European Union
[8] Lead and Cadmium Inicitatives, United Nation Environment Program, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic-European Union
[*]E-mail: kizek@sci.muni.cz

Received: 25 April 2011 / Accepted: 15 May 2011 / Published: 1 June 2011

Heavy metals are toxic and can be harmful for organisms. For this reason, a number of organisms including bacteria develop processes which are able to withstand the effects of these pollutants. An accumulation of heavy metals in different bacterial strains in soil has been observed and could be used in the process of remediation of soils contaminated by heavy-metal-pollutants. In this study, *Streptomyces*, well known for their capacity to accumulate heavy metals from the environment, were selected. The ability to accumulate heavy metals in biomass was determined for three strains both collection strain (CCM3243) and two strains isolated from soil exposed to heavy traffic emissions in the centre of Brno (ON3 and M4). The aim was to observe the effect of zinc(II) and copper(II) ions on growth characteristics and content the metal ions in selected bacterial strains using an automated electrochemical detection. Primarily, the common characteristic as growth and minimum inhibitory concentration (MIC, the lowest concentration of metal that completely stops the growth of target microorganisms) was determined. Based on the results of the MICs on solid medium (agar) can be studied organisms of the genus *P. flavovirens* out in order with respect to their sensitivity to zinc(II) and copper(II) ions as follows: ON3 >> CCM3243 > M4. The results also show that the collection strain is more sensitive to copper(II) and zinc(II) ions in comparison with ON3 and M4 strains. Further, we aimed at determination of zinc(II) and copper(II) ions in three selected strains, which were cultivated in the presence of different concentrations of zinc(II) (0, 50 and 100 μM) and copper(II) ions

(0, 100, 200 and 500 µM). Limit of detection estimated as 3 S/N was 1 nM for zinc(II) ions and limit of quantification as 10 S/N was 5 nM. The accumulation data obtained correlate very well with the results of biological experiments, especially ON3 strain with the highest resistance also has the ability to accumulate highest concentration of metal ions in the cell wall.

Keywords: automated electrochemical detection; bioremediation; differential pulse voltammetry; heavy metal; bacteria.

1. INTRODUCTION

The anthropogenic sources of metal contamination can be divided into five main groups: metalliferous mining and smelting, industry, atmospheric deposition, agriculture, and waste disposal[1,2]containing several heavy metals of health and environmental concern, such as cadmium, copper, chromium, zinc and nickel [3]. Worldwide, there is an increasing market for raw materials causing intensified minin gactivities. Use and dispersion of metals has assumed enormous proportions during the last century, and the behaviour of metals in the environment is therefore a matter of rising concern [4,5]. Based on the estimation of the European Environment Agency, 1.4 million metal-contaminated sites occurs worldwide [6]. In contrast to organic pollution, it is impossible to reduce the extent of metal contamination by degradation. Thus, either they can be removed from arable land with subsequent safe depositing or the negative effects can be minimized by immobilization at the spot [7,8].

Conventional methods for the removal of the heavy metals ions from environment include mainly soil excavation and disposal to landfill, chemical precipitation, electro flotation, ion exchange and reverse osmosis, adsorption onto activated carbon[9]. However, there have been suggesting and testing new methods for remediation of polluted environment based on the organisms, which include bioremediation[10-16] and/or phytoremediation [10-13,17-28]. Bioremediation is the use of microorganisms for removing pollutants. Technologies can be generally classified as in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site, while ex situ involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are bioventing, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation [29].

The mechanisms of metal resistance of microbes can be summarized as (1) Metal resistance of microbes is accomplished by intra- and extracellula rmechanisms; (2) Metals can be excreted via efflux transport systems; (3) Sequestering compounds of the cytosol can bind and detoxify metals inside the cell; (4) The release of chelators into the extracellular milieu leads to bound and fixed metals; (5) The structure of the cell envelope is prone to bind large amounts of metals by sorption thus preventing influx [4].A great number of heavy metal resistant bacteria such as, e.g., Cupriavidus metallidurans is known to possess efflux transporters that excrete toxic or overconcentrated metals [30,31]. This type of transporters is characterized by a high substrate affinity and can therefore keep low the cytosolic metal concentration. Other groups of soil microbes, e.g. the widespread fungus Aspergillus niger, solubilize metals by the release of organic acids, while others – or even the same

microorganisms – immobilize metals through the excretion of compounds as, e.g., oxalates [32,33]. If toxic metals have entered the cell and can not be excreted by efflux transporters several organisms have developed a cytosolic sequestration mechanism for protection. It has been shown for many metal resistant organisms that internal inclusion bodies, like, e.g., polyphosphate granules (volutin) bind large amounts of metal cations [34].

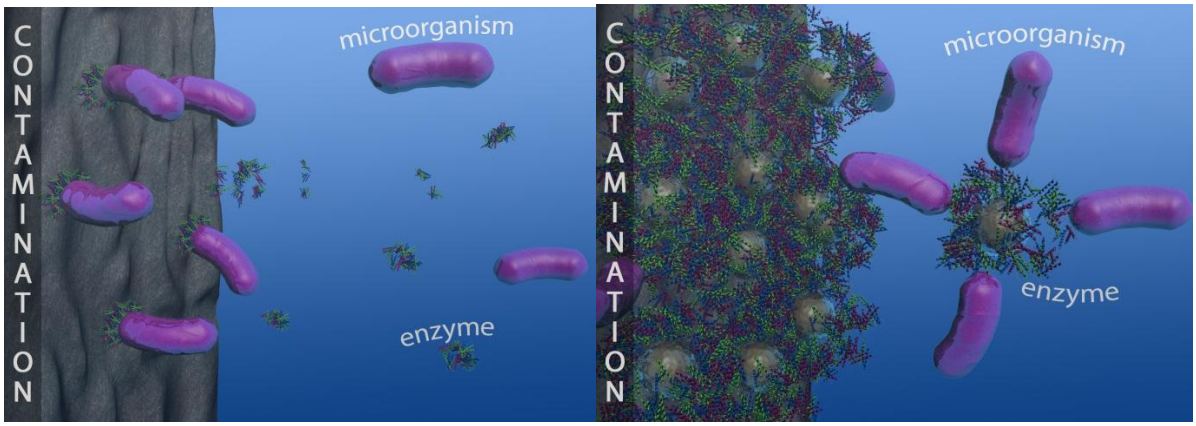

Figure 1. Bioaccumulation of heavy metals by microorganisms using metabolites. Firstly, the metabolites of microorganisms bind contaminants from the environment. The contaminants are then surrounded by other enzymes. Thus, the immobilized contaminant is dispersed from the original location and binds on the surface of the microbial cell, which completes the process of remediation (the enzyme catalyzed cleavage of organic molecules to the molecules of water and carbon dioxide).

The genus *Streptomyces* is known to be the largest antibiotic-producing group and is still of central importance in the identification of medically relevant natural compounds. With the discovery of streptothricin and streptomycin in the forties of the last century, a systematic screening of antibiotics on isolates of this genus and related taxa was initiated [35]. Interestingly, it was calculated that only a tiny fraction of the antimicrobial compounds the genus is capable to produce has been discovered so far. A total number in the order of 100,000 antibiotics was calculated using a conservative estimate [36]. Metals facilitate secondary metabolism not only in actinobacteria, but in some other prokaryotictaxa and fungal groups as well [4]. The complex effect of metals on secondary metabolism can be seen best with results of product research: A *Streptomyces galbus* strain producing an antifungal antibiotic is enhanced in production if the fermentation medium is supplemented with copper, zinc or iron, whereas nickel and cadmium addition lead to a reduction of antibiotic concentration in the same strain [37]. Some other experimental *Streptomyces* strains showed the considerable activity to bioaccumulate zinc, copper and cadmium, chromium [38,39,], nickel, strontium and uranium [7].

To consider whether the microbial specie is able to or is not able to remediate the polluted environment, level of metals in bacteria belongs to the one of the main criterion. Analytical methods and instruments for detection of zinc(II) [40-44]and copper(II) [45-49]ions have been reviewed several times. Electrochemical ones are among the very sensitive analytical methods available for detection of heavy metal ions [48,50-64]. The classic instrument consists of a potentiostat/galvanostat with an electrochemical cell including three electrodes (working, reference and auxiliary). However the current

trend of analytical techniques is to miniaturize the whole instrument due to the many advantages of small devices including portability, low costs and less demands on service and operations, sufficient sensitivity and selectivity [65,66]. As the working electrode a hanging mercury drop electrode (HMDE) is commonly used [67].The HMDE can be also modified with biologically active substances to improve the sensitivity or selectivity of heavy metal ion detection [61-63,68-70].

In this study genus *Streptomyces* was used for investigation of the ability of these bacteria to accumulate heavy metals into their biomass. As bacterial strains suitable for this type of study both collection strain and two strains isolated from soil exposed to heavy traffic emissions in the centre of Brno were used. The aim was to observe the effect of zinc(II) and copper(II) ions on growth characteristics and content the metal ions in selected bacterial strains using an automated electrochemical detection.

2. EXPERIMENTAL PART

2.1 Biological materials

2.1.1 Selected bacterial strains

To test the bioaccumulation of zinc and copper in the biomass three strains of *Streptomycetes* were selected [12]. The first two strains ON3 and M4 were previously isolated from anthropogenically contaminated soils from the centre of the city Brno, Czech Republic. These strains were identified based on the profile of fatty acids (FAME) and by sequencing the 16S RNA gene. As a control strain *Streptomyces flavovirens* CCM3243 strain was used. Strains were stored as spore suspension in 20% (*v*/*v*) glycerol at -20 °C. Prior to use in this study, the strains were thawed and the glycerol was removed by washing with distilled water.

2.1.2 Cultivation of bacterial strains

For the production of spores, Streptomycetes strains were cultivated in the presence of Mannitol Soya Flour Medium (MSFA: mannitol 20 g, soy flour 20 g, agar 15 g, tap water 1,000 ml, pH = 7.2 ± 0.2) in Petri dishes. For physiological studies and for obtaining relatively dispersed growing mycelia, it is appropriate to inoculate culture media with sub-cultivated streptomycetes spores. Pre-germination was carried out in the Complex germination medium containing casaminoacids 2 g, yeast extract 10 g, KH_2PO_4 6.8 g $(NH_4)_2SO_4$ 2 g, $MgSO_4$ 0.2 g, $CaCl_2$ 0.01 g, deionised water 1,000 ml (pH 7.0) according to [71]. To determine growth curves and bioaccumulation of metals Minimal medium with low phosphate content (hereinafter referred to as MM: 0.5 g asparagine, 0.5 g K_2HPO_4, 10 g glucose, 0.2 g $MgSO_4$, 0.01 g $FeSO_4$) was used. After thawing, the required amount of spore suspension was washed 3 times with deionised water as described above to remove the glycerol. Spores were pre-germinated in 30 ml of Complex germination medium for 6 hours at 30 °C. After this time the mixture of media and spores was centrifuged in sterile centrifuge tube for 10 min at 8,000 g and then washed 3 times with sterile Tris-HCl buffer (0.05 M, pH = 7.2). MSFA contained petri dish were inoculated with spores across the surface to obtain a continuous increase mycelia. After 14 days long cultivation at 28

°C the spores were harvested from the surface of media using a spatula and resuspended in sterile deionised water. To disperse of spores clusters and to homogenize the obtained suspension ten-second pulse of ultrasound of 50 W was applied. Subsequently, the spore suspension was centrifuged at 8,000 g for 10 minutes. The supernatant was obtained and the sediment washed with sterile dionised water to remove remaining nutrients. This washing procedure was repeated 3 times. After the final wash spores were resuspended using 20% (v/v) glycerol and divided into aliquots, which were kept at -20 °C prior to other experiments.

2.1.3 Growth curves

Growth curves of the bacterial strains were determined by the amount of biomass produced in relation to time. One hundred and fifty ml of MM in 500 ml Erlenmeyer flask fitted with a coil (baffled spring) were inoculated with suspensions of pre-germinated spores of the certain strain to obtain a final optical density (OD) as 0.1 measured using spectrometer (SPECORD, Germany). Inoculated cultures were cultured on a rotary shaker (200 rpm orbital shaker, BIOSAN, Latvia) at 25 °C for 96 hours. At defined time intervals 10 ml of the culture were collected. This part of growing culture was filtered under vacuum through a weighed nitrocellulose filters with a pore size 0.45 μm. The filter-captured bacterial biomass was washed with 2×25 ml of Tris-HCl buffer (0.05 M, pH = 7.2) and then 1×25 ml of deionised water. After drying for 1 h at 60 °C (BMT, Czech Republic) the biomass was weighed. Minimum inhibition concentration was determined in liquid MM using turbidity measurements on automatic device BIOSCREEN C.S. (USA), using the "honeycomb" micro-plates.

2.2 Bioaccumulation

One hundred of MM containing pre-germinated spores was inoculated into Erlenmayer flask with baffled spring containing Zn(II) in concentrations of 0, 50, 100 and 150 μM and/or Cu(II) at concentrations of 0, 100, 200 and 500 μM. The amount of bacterial culture was determined by OD = 0.1 (SPECORD, Germany) at the beginning of the experiment. Experimental group cultivated at 0 concentration of metal ions was used as a control culture. The cultivation was carried at 25 °C for 72 hours on the Orbital Shaker (BIOSAN, Latvia) at 200 rpm. Samples of bacterial suspensions were collected after 48 and 72 hours. Forty ml of the medium, which was filtered through weighed membrane with pore size 0.45 μm, was collected. The obtained filtrate was used for determination of the residual concentration of heavy metals. Biomass captured on the filter was washed with 3×25 ml of Tris-HCl buffer (0.05 M, pH = 7.2). The filter was dried biomass for 1 h at 60 °C (BMT, Czech Republic) and the amount of biomass produced was found weighed. The dried bacterial cells were then subjected to further study with regard to the amount and distribution of Zn(II) and Cu(II).

2.3 Sample preparation

To prepare the samples microwave digestion were used according to recently published paper [72]. Briefly, the mineralization of samples took place in a microwave system Multiwave3000 (Anton-Paar GmbH, Austria). Cell lysate (100 μl), respectively, 100 mg (cell wall) of the sample was placed into

glass vials MG5 and 900 µl of nitric acid (w/w, 65 %) was added. Prepared samples were sealed and placed into the rotor 64MG5 (Anton-Paar GmbH, Austria). Rotor with the samples was inserted into the microwave system and the microwave digestion was carried out under the following conditions: power 100 W, ramp 10 min, hold 99 min, cooling 10 min, maximum temperature 80 °C. Sample preparation for subsequent electrochemical measurements was as follows: 15 µl mineralized sample was pipetted into Eppendorf tubes with 985 µl acetate buffer (pH = 5.00). A blank digestion was simultaneously carried out in the same way.

2.4 Determination of zinc and copper by differential pulse voltammetry

Electrochemical analyser (Metrohm AG, Switzerland) was used for determination of Zn(II) and/or Cu(II) [73]. The analyser (757 VA Computrace from Metrohm, Herisau, Switzerland) employs a conventional three-electrode configuration with a hanging mercury drop electrode (HMDE) working electrode: 0.4 mm^2, Ag/AgCl/3MKCl as reference electrode, and a platinum auxiliary electrode. The following setup assembled of automated voltammetric analysis is supplied by Metrohm (Fig. 2). A sample changer (Metrohm 813 Compact Autosampler) performs the sequential analysis of up to 18 samples in plastic test tubes. For the addition of standard solutions and reagents, two automatic dispensers (Metrohm 765 Dosimat) are used, while two peristaltic pumps (Metrohm 772 Pump Unit, controlled by Metrohm 731 Relay Box) are employed for transferring the rinsing solution in the cell and for removing solutions from the voltammetric cell (Fig. 2). Differential pulse voltammetric measurements were carried out under the following parameters: deoxygenating with argon 60 s; deposition potential -1.3 V; time of deposition 240 s; start potential -1.3 V; end potential 0.15V; pulse amplitude 0.025V; pulse time 0.04s; step potential 5.035 mV; time of step potential 0.3 s.

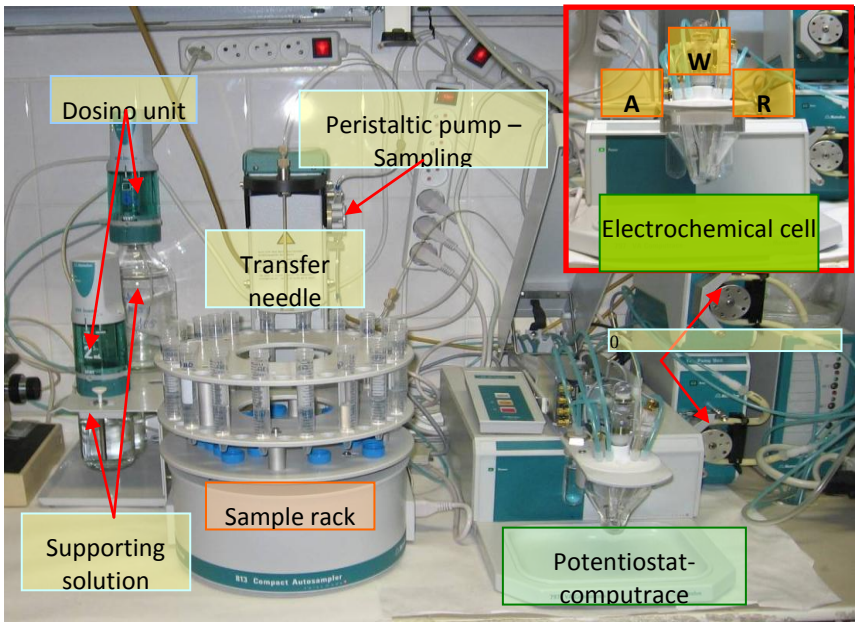

Figure 2. Automatic electrochemical system was used for **determination of metal ions**. The sample is transported using a peristaltic pump. Two **dosine** units provide transport **of** electrolyte **and** solution of known concentration, which is used for standard addition method. The measuring cell is rinsed with two independent peristaltic pumps.

2.5 Chemicals

Working standard solutions were prepared daily by diluting the stock solutions. All other chemicals used were purchased from Sigma Aldrich unless noted otherwise. Stock solutions of 1mg/ml of each Zn, and Cu were prepared by dissolving appropriate amount of copper nitrate, and zinc chloride (Sigma Aldrich, USA) in water and diluted to 1000 ml volumetric flask. Acetate buffer of pH 5 was prepared with 0.2 M acetic acid and 0.2 M sodium acetate and diluted with water and used as a supporting electrolyte. High purity deionised water (Milli-Q Millipore 18.2 MΩ/cm, Bedford, MA, USA) was used throughout the study.The IAEA-413 Algae reference material was prepared by the IAEA Terrestrial Environment Laboratory in Austria in co-operation with the Institute of Microbiology, Academy of Sciences of the Czech Republic in Trebon. The IAEA-413 algae material (type: Chlorella Boehm) was produced under standard outdoor culture conditions (reference material IAEA-413, IAEA Environment Laboratories, Vienna, Austria).

2.6 Preparation of deionised water and pH measurement

The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.7 Mathematical treatment of data and estimation of detection limits

Mathematical analysis of the data and their graphical interpretation was realized by software Matlab (version 7.11.). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [74], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

2.7.1 Accuracy, precision and recovery

Accuracy, precision and recovery of heavy metals were evaluated with homogenates (plant tissues) spiked with standards. Before extraction, 100 µl heavy metals compounds standards and 100 µl water were added to bacterial samples. Homogenates were assayed blindly and heavy metals concentrations were derived from the calibration curves. Accuracy was evaluated by comparing estimated concentrations with known concentrations of heavy metals compounds. Calculation of accuracy (%Bias), precision (%C.V.), root mean square error (RMS error) and recovery was carried out as indicated by Causon [75].

2.7.2 MEDUSA program

Make equilibrium diagrams using sophisticated algorithms (MEDUSA program) was used for the construction of a distribution diagram of different cadmium chemical forms present in the basic electrolyte. The basic parameters, including equilibrium constants that are necessary for the calculation of distribution diagrams are in the program database [76,77]. The program author is Ignasi Puigdomenech from the Inorganic Chemistry of Royal Institute of Technology, Stockholm, Sweden. The MEDUSA program is the freeware and is available on http:\\www.kemi.kth.se/medusa.

3. RESULTS AND DISCUSSION

Study of bio-remediation potential is one of the major directions of research in the early 21st century, which is mainly applied to the sites with the major contamination with organic or inorganic pollutants. Heavy metals (lead, cadmium, mercury) can be included among the major inorganic pollutants, which can be accumulated from the environment using a variety of organisms and then easily removed. For the purpose of accumulation of pollutants plants, fungi and bacteria are used [10-14,17,20,24,25,27,28,70,78-82].

3.1 Characteristics of Streptomyces bacteria growth in the presence of zinc(II) and copper(II) ions

It was found that the bioaccumulation is process of depositing of heavy metals in the biomass of organisms under consumption energy. Increased concentrations of heavy metals have inhibitory effects on growth and metabolism of microorganisms. Therefore, bacteria have developed molecular biological mechanisms for their survival in such polluted environment.

3.1.1 Growth and minimal inhibition concentration on solid medium

In our experiments the effect of zinc(II) and copper(II) ions on *Streptomyces* bacteria strains was investigated. Primarily, the common characteristic as growth and minimum inhibitory concentration (MIC, the lowest concentration of metal that completely stops the growth of target microorganisms) was determined. However, both tested metals in higher concentrations hamper sporulation and aerial mycelium formation although the vegetative mycelium is still capable of growth. The colonies lacking aerial mycelia and spores display „bald phenotype“. Another common phenomenon is reduction in number and dimensions of the colonies with increasing concentrations of both tested metals. Similar effects as described above were observed for environmental strains of *Streptomyces* on solid media in the presence of various heavy metals by Abbas and Edwards [83]. MICs for zinc(II) and copper(II) ions determined in ON3, M4 and CCM 3243 strains on liquid and solid media are shown in Tab. 1.

ON3 strain was able to grow in the agar in concentrations of zinc ions (1.1 ± 0.1 mM), which was 10 % higher concentrations compared with the collection strain CCM 3243. In the case of liquid medium, the sensitivity of the studied strains was considerably higher. In the strain ON3, MIC 0.6±0.1 mM was found, which was 42 % higher concentrations compared with the collection strain. For

determination of MIC for copper(II) ions in ON3 strain, MIC was 1.5±0.2 mM in the agar and 0.8±0.2 mM in liquid medium, which was 27 % and 38 % higher concentrations compared with the collection strain CCM 3243. The interesting results were obtained for the MIC value of strain M4 obtained from contaminated area. MIC for zinc(II)/copper(II) ions was 0.55±0.05/1.4±0.2 mM in agar and 0.25±0.02/0.50±0.05 mM in liquid medium. Differences in values of the MIC on agar and liquid medium are likely to be caused by completely different environment. In liquid medium, bacteria are able to create a suitable environment around the cells (synthesis of peptides, proteins that are released into their immediate surroundings) and internal defence mechanisms are altered even at concentrations of about 50 % lower compared to the agar environment. Another hypothesis is based on the nature of the agar as a complex organic molecule contains binding sites reacting with heavy metals. Chelation of heavy metal ions or the formation of complexes with components of the medium reduces the effective concentration of free metal.

Table 1. Minimal inhibition concentrations defined on agar (solid) and liquid media.

metal	media	**ON3**[1]	**M4**[1]	**CCM 3243**[1]
Zn(II)	agar	1.80	0.95	1.00
	liquid	0.60	0.45	0.45
Cu(II)	agar	1.50	1.40	1.10
	liquid	0.80	0.80	0.50

n = 5 (number of independent repetions); R.S.D. (relative standard deviation) = 8.5 %; [1] expressed as mM

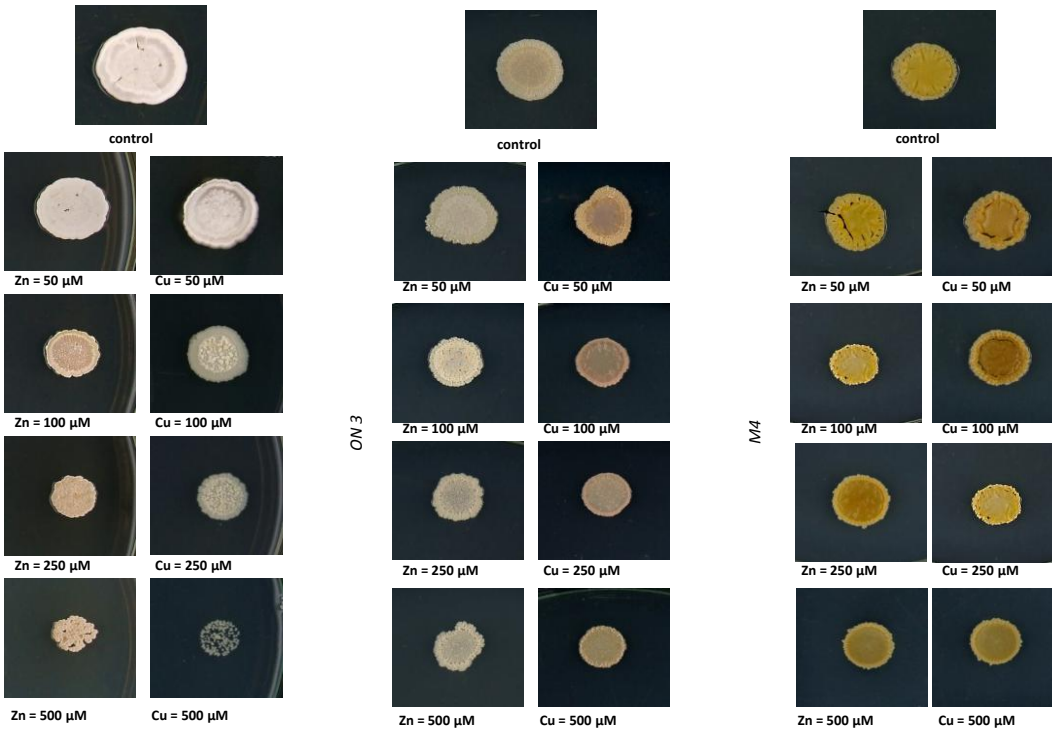

Figure 3. Changes in the shape of *S. flavovirens* strains (CCM 3243, ON3 and M4) during their growth on agar medium with addition of zinc(II) (0, 100, 350, 600 and 800 µM) and copper(II) ions (0, 100, 400, 700 and 1,000 µM).

Typical photos of growth of different strains studied on agar medium are shown in Fig. 3. The collection strain CCM 3243 is a noticeable changed in colour pigmentation of colonies, depending on the metal studied. Moreover, it is clearly observable formation of separate groups of colonies. Strains ON 3 and M4 showed appearance of separate colonies. Significant pigmentary changes were observed in strain M4. Based on the results of the MICs on solid medium (agar) can be studied organisms of the genus *P. flavovirens* out in order with respect to their sensitivity to zinc(II) and copper(II) ions as follows: **ON3 >> CCM3243 > M4**.

3.1.2 Growth and minimal inhibition concentration on liquid medium

It is known that bacteria of the *Streptomyces* genus because of its nature to form mycelia do not grow as a homogeneous suspension in liquid medium, but in the form of relatively compact clusters.

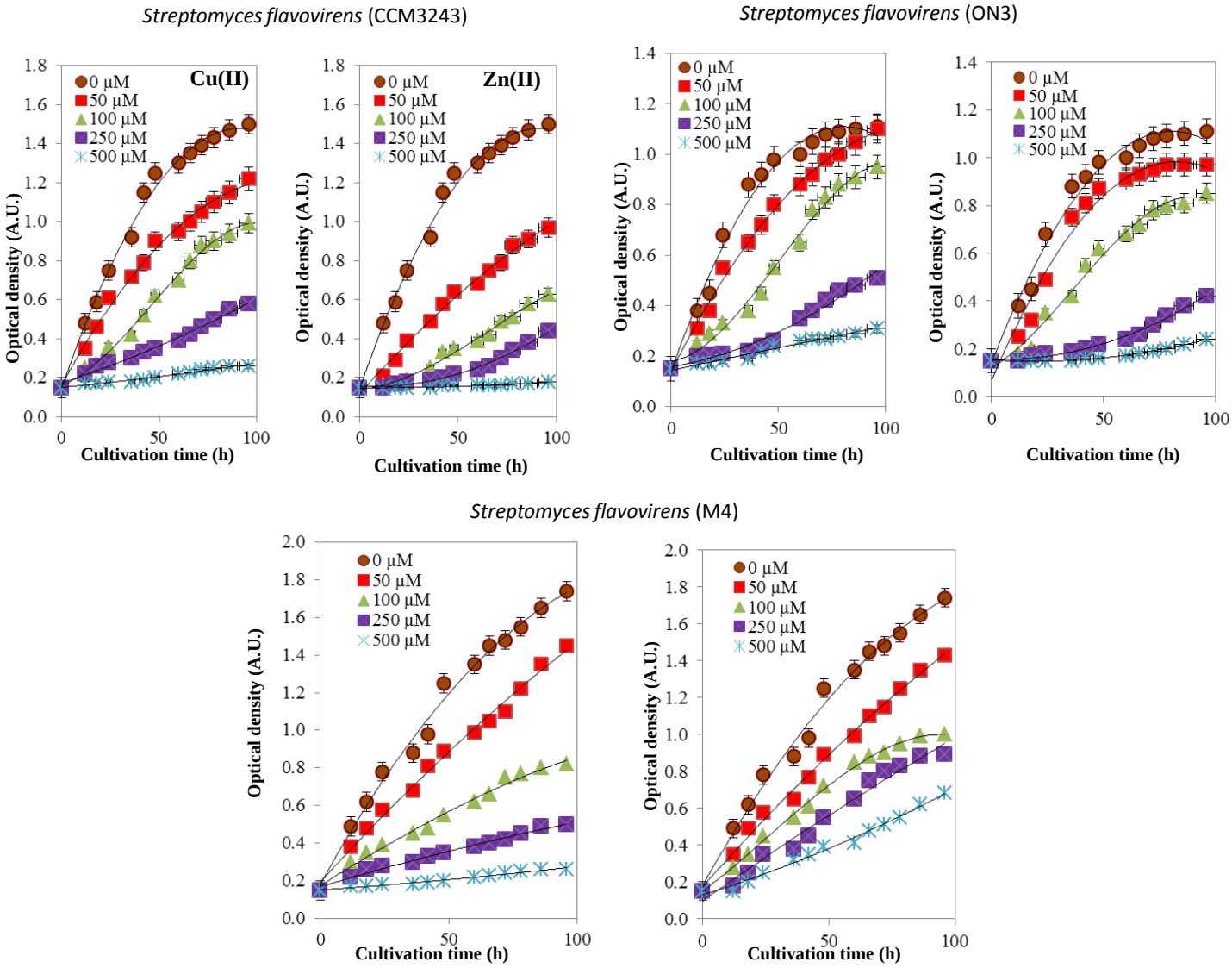

Figure 4. Growth curves of bacterial strains CCM3243, ON3 and M4 in liquid cultivation medium. The curves are expressed as weight of fresh mass from 0 to 90 hours long cultivation (shaking 200 rpm, 37 °C).

Such manifestations of physiological behaviour significantly affect turbidimetric measurements (optical density). This disadvantage, we tried to minimize turbidity measurements in the vertical direction and before analyzing the sample was shaken. Under this optimized procedure, the relative error of determination of turbidity of bacterial suspension was app. 20 % (n = 6). In addition, growth curve was determined by weighing of bacterial masses.

Growth curves determined by weighing for CCM3243, ON3 and M4 cultivated without addition of metal ions are shown in Fig. 4 . Based on the growth characteristics its is follows that M4 strain reached stationary growth phase in 78±5 h (n = 3; CI 95%; mathematical model of curve: $y = 1E\text{-}06x^4 - 0.0002x^3 + 0.0131x^2 + 0.0097x$, $R^2 = 0.9938$) a amount of biomass was 200 % hogher compared to ON3 strain (mathematical model of curve: $y = -3E\text{-}08x^5 + 7E\text{-}06x^4 - 0.0006x^3 + 0.0137x^2 + 0.1596x$, $R^2 = 0.9852$) and to collection strain CCM3243 (mathematical model of curve: $y = 3E\text{-}07x^4 - 7E\text{-}05x^3 + 0.0042x^2 + 0.0268x$, $R^2 = 0.990$). Moreover, ON3 strain reached stationary growth phase in 86±5 h (n = 3; CI 95%).

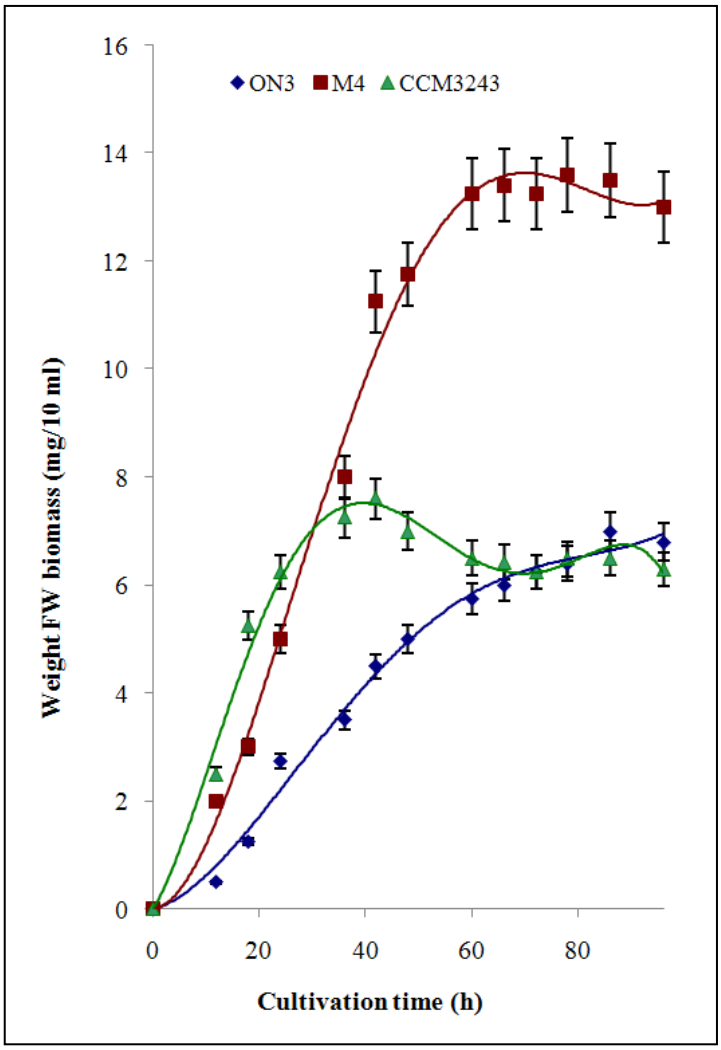

Figure 5. Growth curves of CCM3243, ON3 and M4 bacterial strains in liquid cultivation medium. The curves are expressed as weight of fresh mass from 0 to 90 hours long cultivation (shaking 200 rpm, 37 °C).

ON3 and M4 strains can be characterized by a shift in the growth rate up to 40 h compared with the collection strain. In the case of adding of zinc(II) or copper(II) ions inhibitory effects on the growth characteristics were observed. The obtained data are shown in Fig. 5. Acquired growth characteristics for each variant of the experiment showed a similar pattern. All bacterial strains showed inhibition of growth (about 60-90%) in the presence of heavy metals compared to control groups. The results also show that the collection strain is more sensitive to copper(II) and zinc(II) ions in comparison with ON3 and M4 strains.

3.2 Automated electroanalytical determination of metal ions

Electrochemical determination of heavy metals ions at HMDE belongs to the ultrasensitive analytical tools [68,76-78] and can be utilized for studying their reactions with complexes [79,80]. We have recently shown appropriate procedure for such purpose. The suggested technology enables fully automated analysis of real sample. In the case optimization of measurement parameters there are not observed any major differences between the published papers. The achieved results can be summarized as: the most suitable electrolyte acetate buffer pH 5, the deposition time in the interval 120-240 s and deposition potential in the interval from -1.0 to -1.2 V [68,69,81]. In this study, determination of metal ions concentration was carried out using both standard addition method and calibration curve. For this purpose, there is available very accurate dosing instrument (dose of 5 ± 1 µl), see in Fig. 1.

3.3 Electroanalytical study of zinc(II) and copper(II) ions behaviour

It is known that heavy metal ions form with other molecules many complexes. Formed complexes may subsequently react with working electrodes made of different materials. Distribution diagrams of zinc(II) and copper(II) ions in the acetate buffer pH 5 are shown in Fig 6. Based on the diagrams, Zn_2OH^{3+}, $ZnCl_4^{2-}$, $ZnCl^{3-}$, $ZnCl_2$ and $Zn(CH_3COOH)^+$ belong to the most occurred zinc(II) complexes and $CuCl_2$, $CuCl^+$, $Cu(CH_3COO)_4^{2-}$, $Cu(CH_3COO)^{3-}$, $Cu(CH_3COO)^+$ and $Cu(CH_3COO)_2$ belong to the most occurred copper(II) complexes.

In many previous works, it is clear that the determination of zinc(II) and copper(II) ions at HMDE is very convenient. In our fully automated arrangement, we tested the influence of zinc(II) ions concentration on its signal height (concentration range 10 nM to 100 µM, time of accumulation 240 s, accumulation potential -1.3 V). In Fig 7, there are shown typical voltammograms. At low concentrations up to 40 µM very well defined peaks were detected. Relative error was calculated as 1.8 % for interday assay (n = 10) and 2.9 % for intraday assay (n = 10). The calibration curve was linear with R^2 = 0.9972. At HMDE, amalgam is probably formed during pre-concetration step under adsorptive stripping, which structure may not be fully clear (there probably originates following complex Zn/Hg; Zn(Hg)/Zn(II)). In the reductive scan the following reaction takes place at the electrode: $Zn(Hg) + 2\ OH^- \rightarrow ZnO + H_2O + 2\ e^-$. Under concentration higher than 50 µM we observe distortion of signals at potential app. -1.05 V (Fig. 7). These changes may be related to the fomration of various types of complexes (Fig. 6), which creates much more complex amalgam-based compounds

at the surface of an electrode. The resulting complexes may exhibit different thermodynamic properties and very complicated redox reactions take place at a working electrode.

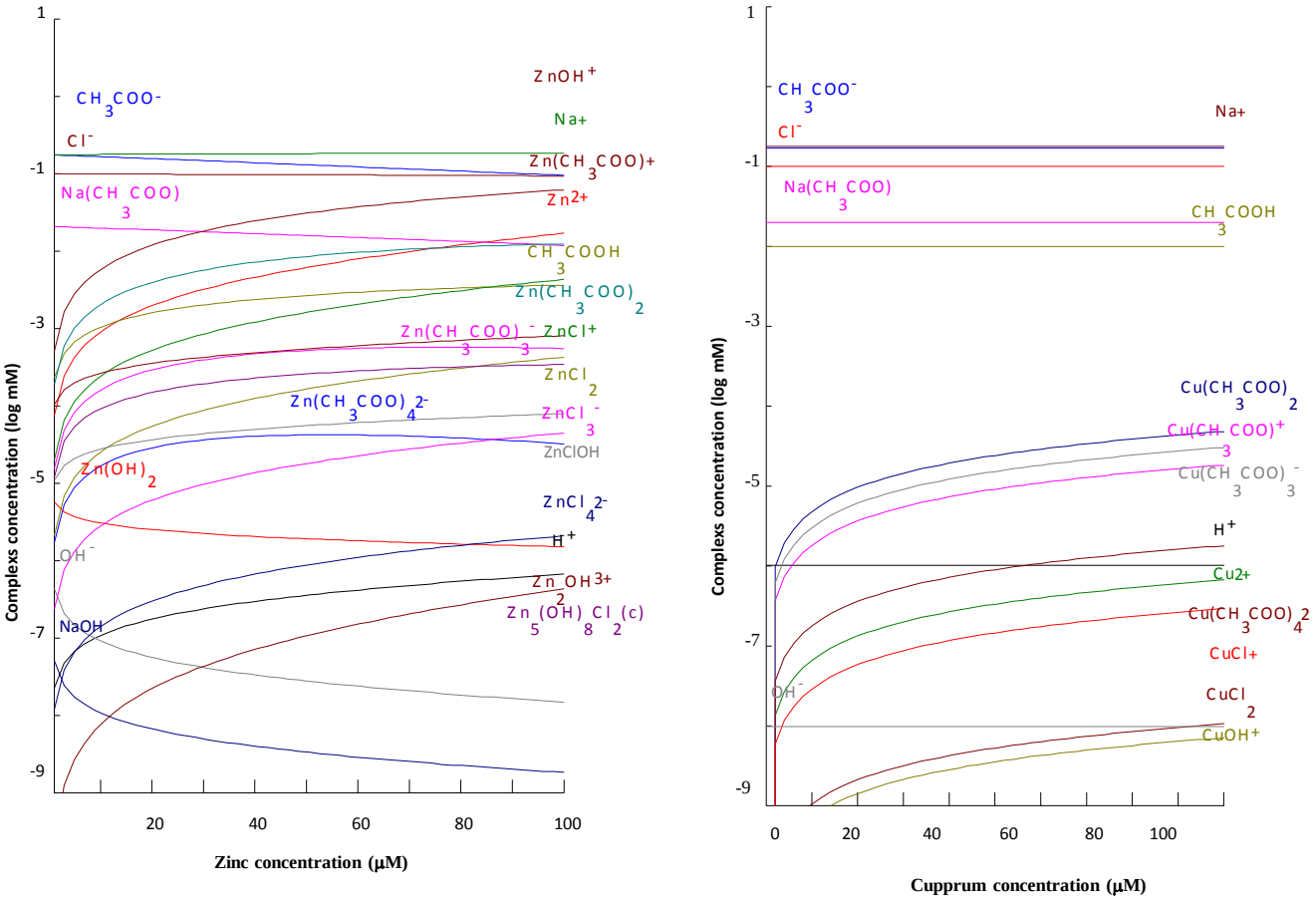

Figure 6. MEDUSA diagrams for zinc(II) and copper(II) ions (metal ions concentration from 0 to 100 µM) in sodium acetate buffer (pH 5.0); Cl^-_{TOT} = 100 mM; H^+_{TOT} = 10 mM; Na^+_{TOT} = 200 mM; $CH_3COOH^-_{TOT}$ = 200 mM.

The complexity of the electrochemical behaviour of the resulting complexes at HMDE electrode also indicates an increase of R.S.D. up to 12 %. Despite these apparent difficulties in the detection of zinc(II) ions very good linearity was achieved in the studied concentration range (y = 75.8x + 104.6, 188.8 RMS error). Limit of detection estimated as 3 S/N was 1 nM for zinc(II) ions and limit of quantification as 10 S/N was 5 nM.

For electroanalytical studies of copper(II) ions behaviour there were not observed any changes in the voltammograms (Fig. 8). Copper(II) ions gave very well-defined symmetrical peaks. Relative error was calculated as 1.1 % for interday assay (n = 10) and 1.9 % for intraday assay (n = 10). The calibration curve was linear with R_2 = 0.9992. At HMDE, amalgam is probably formed during pre-concentration step under adsorptive stripping, which structure may not be fully clear (there probably originates following complex Cu/Hg/; Cu(Hg)/Cu(II)). In the reductive scan the following reaction takes place at the electrode: $Cu(Hg) + 2\ OH^- \rightarrow CuO + H_2O + 2\ e^-$. Equation of calibration curve was

y = 76.8x - 14.3, 120.0 RMS error, R.S.D. 3.6 %). Limit of detection estimated as 3 S/N was 2.5 nM for zinc(II) ions and limit of quantification as 10 S/N was 8.5 nM. The experimental results show that the complexes of copper(II) ions are less stable and much less affected the current responses.

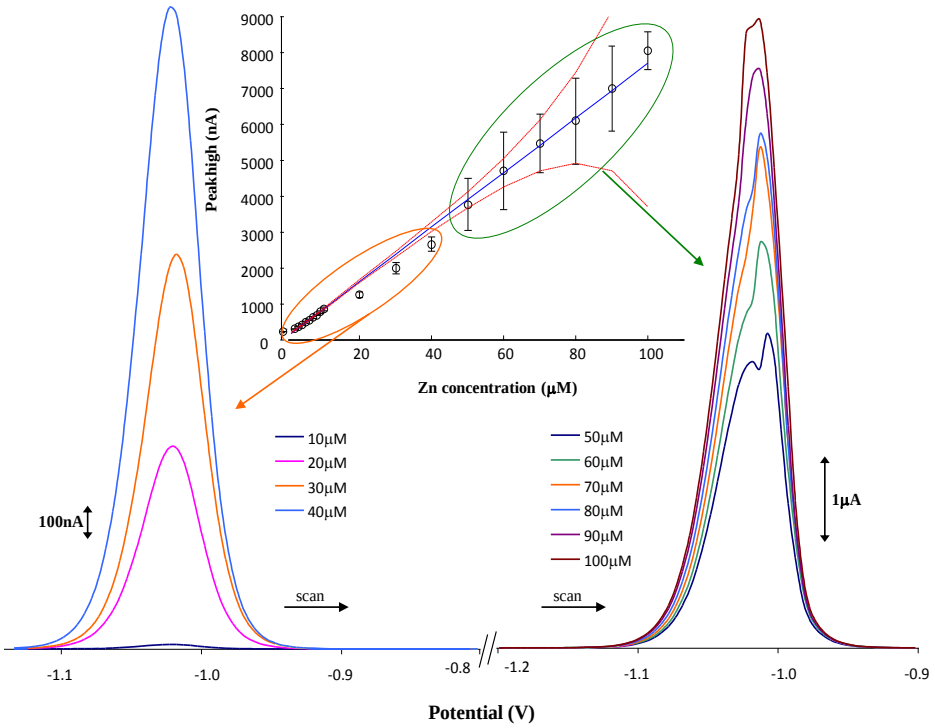

Figure 7. Typical AdS DP voltammograms of various concentrations of zinc(II) ions measured at HMDE in 0.2 M acetate buffer pH 5.0. Voltammograms of 10, 20, 30 and 40 µM are on the left;, 50, 60, 70, 80, 90 and 100 µM are on the right. Calibration dependence is shown in the middle of the figure (n = 10). Accumulation potential -1.3 V, time of accumulation 240 s.

3.3 Electrochemical determination of zinc(II) and copper(II) ions in bacterial extracts

Bacterial strains (CCM3243, ON3 and M4) were cultivated in the presence of different concentrations of zinc(II) (0, 50 and 100 µM) and copper(II) ions (0, 100, 200 and 500 µM). Individual samples were collected after 24 and 48 hours. Immediately after the collection cultivation medium was separated by centrifugation (which was immediately subjected to electroanalytical analysis) and bacterial cells. Bacterial cells were homogenized in acetate buffer using liquid nitrogen and disintegration by ultrasound (100 W, 2 min.). Thus, two fractions were obtained (the first containing biomembranes and the second cytoplasmic). For the analysis of samples from bacterial strains, modified open-vessel method using smaller polystyrene liners in an 80-postion rotor with a small amount of sample applications at low reaction temperature was used [72].

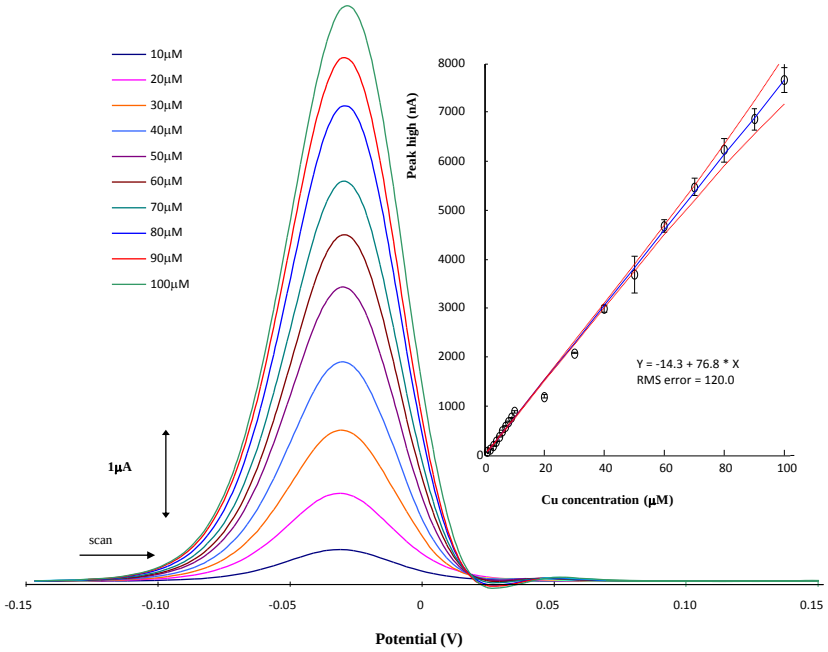

Figure 8. Typical AdS DP voltammograms of various concentrations of copper(II) ions (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM) measured at HMDE in 0.2 M acetate buffer pH 5.0. Calibration dependence is shown (n = 10, R.S.D. = 3.6 %). Accumulation potential -1.3 V, time of accumulation 240 s.

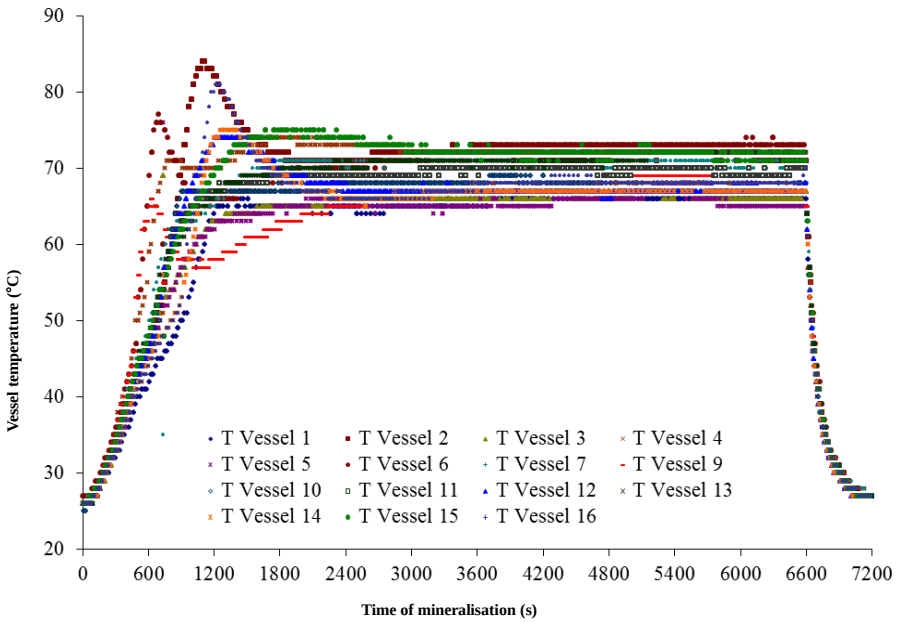

Figure 9. Typical records of the mineralization in glass vials. To 600 s there is a rapid rise in temperature inside the vials. From the time of 1,000 to 6,600 s temperature varies slightly and cooling phase is followed to 7,200 s. After this, a Teflon lid is perforated and steams of nitric acid are extracted. Digest is then processed and prepared for electrochemical analysis.

In this study, a mixture of 70 % of nitric acid (*v*/*v*) and 30 % of hydrogen peroxide (*v*/*v*) was used for mineralization. A sample app. 20 mg was weighed into glass vials at a temperature of 80 °C. To prevent explosions, Teflon cap was double layered. Many biological materials exhibit a high reactivity during mineralization (decarboxylation etc.), which often leads to a sharp increase in pressure in the glass vessel and damage the Teflon cap. Dried excrement or parts of plants belong to very complex samples. A more gradual increase in pressure in the glass vessel is observed during mineralization of fresh biological samples. In our experiments, the maximum pressure of around 10 bar (other experimental detail will be published elsewhere) was determined. Accuracy (% certified reference concentration) of heavy metals concentration in certified reference material from the sheet of material measured after the micro-scaled digestion and subsequent electrochemical detection was 78 ± 5 % (n = 3) for zinc(II) ions and 81 ± 3 % (n = 3) copper(II) ions.

The amount of zinc(II) ions in the culture medium ranged between 10-40% of the total added amount. Among various strains were observed no significant differences. The results indicate a slight increase of zinc(II) ions in the medium with increasing concentrations of added metal ions (from 16 to 33 % after 24 h, from 7 to 30 % after 48 h), see in Fig. 10. Similar courses of changes in levels of free copper(II) ions in cultivation medium were observed. The amount of copper(II) ions ranged from 2 to 42% of the total applied concentration. Unlike zinc(II) ions there was higher concentration of copper(II) ions in cultivation medium after 24 h (10 to 39 %) compared to medium after 48 h (from 2 to 42 %), see in Fig. 11. Great variation in the studied heavy metals is likely associated with the synthesis of many biologically important compounds (organic acids, aromatic compounds, amino acids, low molecular weight thiols). This fact confirms a significant proportion of metals bound in the medium from 60 to 98 % (Figs. 10 and 11).

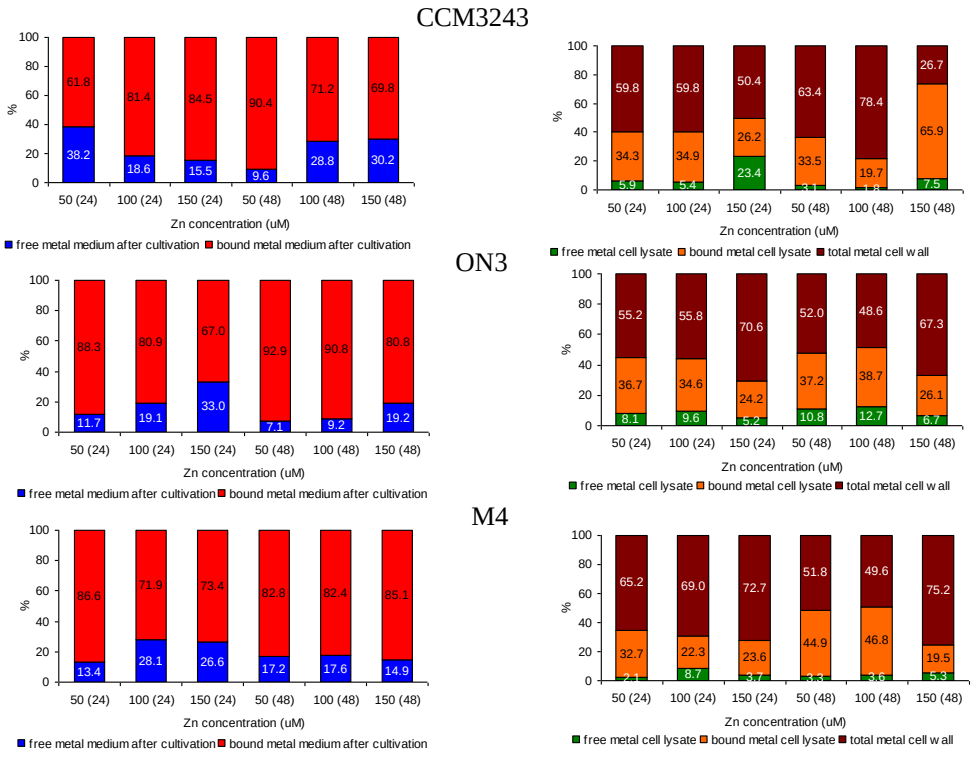

Figure 10. Electrochemical determination of zinc(II) ions in cultivation medium and bacterial cells.

In other experiments, free metal ions in the cytoplasm, amount of metal ions bound to cytoplasm components and the total amount of metal ions in the cell wall were determined. Cytoplasmic extracts were analyzed directly by our electroanalytical technique. Recovery of zinc(II) and copper(II) ions added to cytoplasmic extracts ranged between 80-90 %. The results indicate that the amount of free cytoplasmic zinc is trace and ranged from 2 to 10 % (in average 7 %).

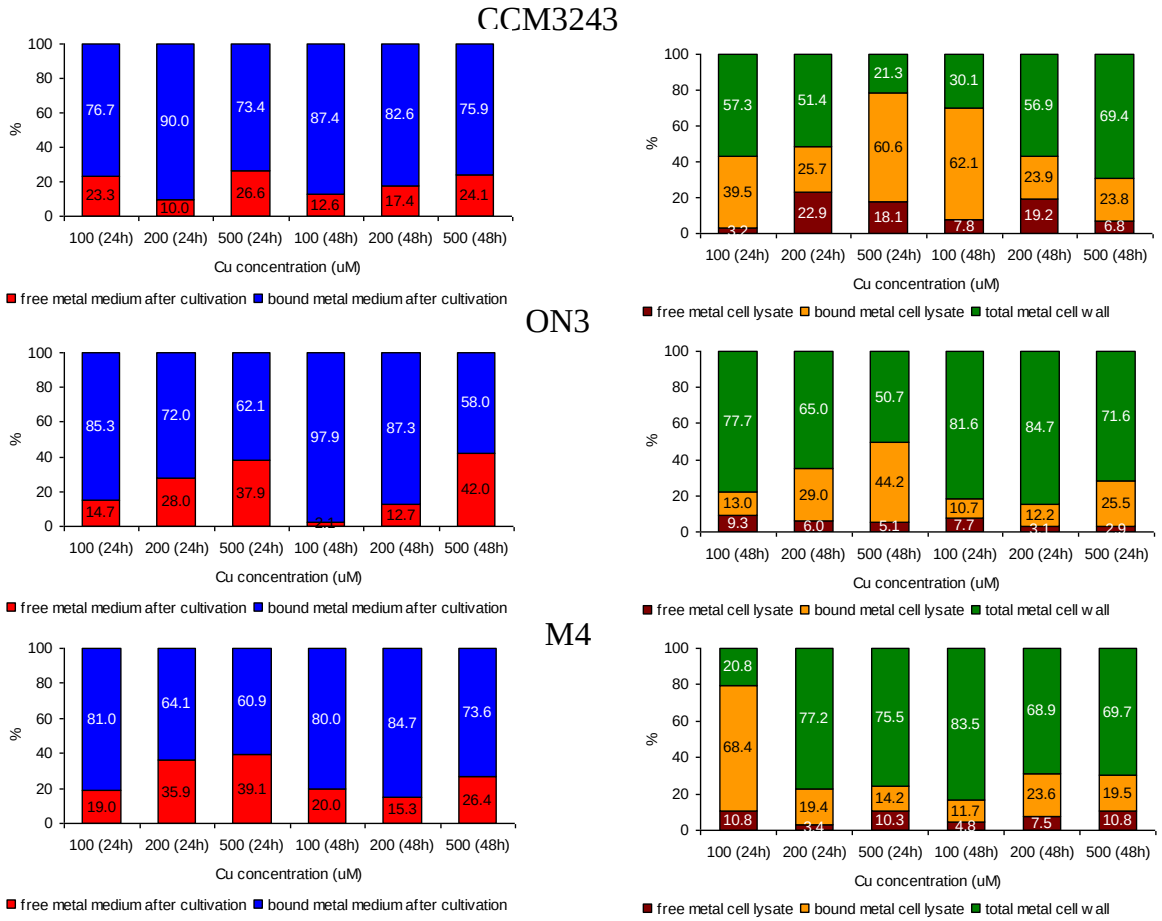

Figure 11. Electrochemical determination of copper(II) ions in cultivation medium and bacterial cells.

Content of zinc(II) ions was from 2 times to 6 times higher compared to content of free zinc(II) ions in all studied cases. The similar results were achieved for copper(II) ions, of which content of free copper(II) ions was from 3 to 20 % (in average 9 %). Zinc(II) ions are bound on molecules in cytoplasm according to strains as follows: M4 (23.7 %) < ON3 (24.7 %) < CCM 3243 (26.8 %). According to our expectations, the highest contents of zinc(II) ions were determined in cell walls, which contain many biomolecules with higher sorption activity as saccharides. Content of zinc(II) ions in cell walls was 47.9 % (M4) > 43.7 % (ON3) > 42.3 % (CCM 3243). We found that content of free copper(II) ions was relatively low and varied from 6 to 7 %. The contents for the single tested strains were as follows: ON3 (4.3%) > M4 (6.0%) > CCM 3243 (9.8%). The contents of bound copper(II)

ions varied from 17 to 30 % (ON3: 16.8 % > M4: 19.5 % > CCM 3243: 29.5 %). Similar to zinc(II) ions, 40-50 % of copper ions were bound to cell wall as follows: ON3 (53.9 %) > M4 (49.5 %) > CCM 3243 (35.8 %), see Figs. 9 and 10. The data obtained correlate very well with the results of biological experiments, especially ON3 strain with the highest resistance also has the ability to accumulate highest concentration of metal ions in the cell wall.

4. CONCLUSIONS

Based on the obtained data we can conclude that the isolated strains of bacteria of the genus *Streptomyces* are suitable organisms for accumulation of zinc(II) and copper(II) from contaminated environment. It was found that not only the collection strain, but strains isolated from contaminated soil have bioremediation ability. It can be also concluded that the strains isolated from contaminated soils are able to accumulate a larger amount of metals in their biomass. It is obvious that ignorance of the profile of soil organisms and their precise classification of the biochemical cycles is a major issue in the future. Their exact destination will be a major step toward new possibilities for decontamination of the environment polluted by heavy metals.

ACKNOWLEDGEMENTS

Financial support from REMEDTECH GACR 522/07/0692, INCHEMBIOL MSM0021622402 and NANIMEL GACR 102/08/1546 and CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. We would like to thank Petr Koudelka for help with writing of this article.

References

1. N. T. Basta, J. A. Ryan and R. L. Chaney, *J. Environ. Qual.*, 34 (2005) 49.
2. M. J. McLaughlin, K. G. Tiller, R. Naidu and D. P. Stevens, *Aust. J. Soil Res.*, 34 (1996) 1.
3. V. B. H. Dang, H. D. Doan, T. Dang-Vu and A. Lohi, *Bioresour. Technol.*, 100 (2009) 211.
4. G. Haferburg and E. Kothe, *J. Basic Microbiol.*, 47 (2007) 453.
5. J. O. Nriagu, *Environment*, 32 (1990) 6.
6. M. N. V. Prasad, H. Freitas, S. Fraenzle, S. Wuenschmann and B. Markert, *Environ. Pollut.*, 158 (2010) 18.
7. G. Haferburg and E. Kothe, *Appl. Microbiol. Biotechnol.*, 87 (2010) 1271.
8. A. A. Juwarkar, S. K. Singh and A. Mudhoo, *Rev. Environ. Sci. Bio-Technol.*, 9 (2010) 215.
9. G. Cimino, R. M. Cappello, C. Caristi and G. Toscano, *Chemosphere*, 61 (2005) 947.
10. P. Babula, M. Beklova, Z. Svobodova, P. Majzlik, V. Adam and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 398.
11. O. Krystofova, P. Babula, J. Kaiser, K. Novotny, J. Zehnalek, V. Adam and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 402.
12. P. Majzlik, A. Strasky, M. Nemec, L. Trnkova, L. Havel, J. Zehnalek, P. Babula and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 417.
13. J. Sochor, P. Majzlik, P. Salas, V. Adam, L. Trnkova, J. Hubalek and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 414.
14. I. Soukupova, O. Krystofova, P. Sobrova, P. Majzlik, J. Zehnalek, V. Adam, M. Beklova and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 415.
15. S. Clemens, E. J. Kim, D. Neumann and J. I. Schroeder, *Embo Journal*, 18 (1999) 3325.
16. C. Leyval, K. Turnau and K. Haselwandter, *Mycorrhiza*, 7 (1997) 139.

17. P. Babula, R. Opatrilova, O. Krystofova, J. Zehnalek, V. Adam, L. Havel and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 397.
18. J. Baloun, V. Adam, L. Trnkova, M. Beklova, Z. Svobodova, L. Zeman and R. Kizek, *Environ. Toxicol. Chem.*, 29 (2010) 497.
19. V. Diopan, V. Shestivska, O. Zitka, M. Galiova, V. Adam, J. Kaiser, A. Horna, K. Novotny, M. Liska, L. Havel, J. Zehnalek and R. Kizek, *Electroanalysis*, 22 (2010) 1248.
20. D. Huska, V. Adam, L. Havel, J. Zehnalek, J. Hubalek and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 405.
21. D. Huska, O. Zitka, O. Krystofova, V. Adam, P. Babula, J. Zehnalek, K. Bartusek, M. Beklova, L. Havel and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 1535.
22. O. Krystofova, P. Majzlik, J. Zehnalek, L. Havel, V. Adam and R. Kizek, *Listy Cukrovarnicke a Reparske*, 126 (2010) 409.
23. O. Krystofova, L. Trnkova, V. Adam, J. Zehnalek, J. Hubalek, P. Babula and R. Kizek, *Sensors*, 10 (2010) 5308.
24. O. Krystofova, J. Zehnalek, V. Adam, L. Trnkova, P. Babula and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 408.
25. P. Majzlik, J. Prasek, L. Trnkova, J. Zehnalek, V. Adam, L. Havel, J. Hubalek and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 413.
26. O. Zitka, D. Huska, V. Adam, A. Horna, M. Beklova, Z. Svobodova and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 1082.
27. O. Zitka, O. Krystofova, N. Cernei, V. Adam, J. Hubalek, L. Trnkova, M. Beklova and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 418.
28. O. Zitka, J. Najmanova, N. Cernei, V. Adam, M. Mackova, T. Macek, J. Zehnalek, L. Havel, A. Horna and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 423.
29. A. Malik, *Environ. Int.*, 30 (2004) 261.
30. D. H. Nies, *J. Bacteriol.*, 177 (1995) 2707.
31. D. H. Nies, *Fems Microbiol. Rev.*, 27 (2003) 313.
32. G. M. Gadd, in Advances in Microbial Physiology, Vol 41, Academic Press Ltd, London, 1999, p. 47.
33. C. White, J. A. Sayer and G. M. Gadd, *Fems Microbiol. Rev.*, 20 (1997) 503.
34. H. Gonzalez and T. E. Jensen, *Microbios*, 93 (1998) 179.
35. L. Hilbrich and P. Sleight, *Eur. Heart J.*, 27 (2006) 2158.
36. M. G. Watve, R. Tickoo, M. M. Jog and B. D. Bhole, *Arch. Microbiol.*, 176 (2001) 386.
37. A. K. Paul and A. K. Banerjee, *Folia Microbiologica*, 28 (1983) 397.
38. M. J. Amoroso, G. R. Castro, A. Duran, O. Peraud, G. Oliver and R. T. Hill, *J. Ind. Microbiol. Biotechnol.*, 26 (2001) 210.
39. M. A. Polti, M. J. Amoroso and C. M. Abate, *Chemosphere*, 67 (2007) 660.
40. N. Burham, S. M. Abdel-Azeem and M. F. El-Shahat, *Anal. Chim. Acta*, 579 (2006) 193.
41. E. Tomat and S. J. Lippard, *Curr. Opin. Chem. Biol.*, 14 (2010) 225.
42. P. J. Jiang and Z. J. Guo, *Coord. Chem. Rev.*, 248 (2004) 205.
43. J. S. Cavet, G. P. M. Borrelly and N. J. Robinson, *Fems Microbiol. Rev.*, 27 (2003) 165.
44. S. Ressalan and C. S. P. Iyer, *Rev. Anal. Chem.*, 23 (2004) 159.
45. N. Shao, J. Y. Jin, H. Wang, Y. Zhang, R. H. Yang and W. H. Chan, *Anal. Chem.*, 80 (2008) 3466.
46. Y. Zhao, X. B. Zhang, Z. X. Han, L. Qiao, C. Y. Li, L. X. Jian, G. L. Shen and R. Q. Yu, *Anal. Chem.*, 81 (2009) 7022.
47. D. A. Cremers and R. C. Chinni, *Appl. Spectrosc. Rev.*, 44 (2009) 457.
48. R. De Marco, G. Clarke and B. Pejcic, *Electroanalysis*, 19 (2007) 1987.
49. J. Sardans, F. Montes and J. Penuelas, *Spectroc. Acta Pt. B-Atom. Spectr.*, 65 (2010) 97.

50. N. Lewen, S. Mathew, M. Schenkenberger and T. Raglione, *J. Pharm. Biomed. Anal.*, 35 (2004) 739.
51. E. Szlyk and A. Szydlowska-Czerniak, *J. Agric. Food Chem.*, 52 (2004) 4064.
52. O. Mikkelsen and K. H. Schroder, *Electroanalysis*, 15 (2003) 679.
53. F. Faridbod, M. R. Ganjali, B. Larijani, M. Hosseini, K. Alizadeh and P. Norouzi, *Int. J. Electrochem. Sci.*, 4 (2009) 1528.
54. E. Khaled, H. N. A. Hassan, I. H. I. Habib and R. Metelka, *Int. J. Electrochem. Sci.*, 5 (2010) 158.
55. M. R. Ganjali, S. Aghabalazadeh, M. Khoobi, A. Ramazani, A. Foroumadi, A. Shafiee and P. Norouzi, *Int. J. Electrochem. Sci.*, 6 (2011) 52.
56. S. K. Mittal, P. Kumar, S. K. A. Kumar and L. F. Lindoy, *Int. J. Electrochem. Sci.*, 5 (2010) 1984.
57. J. Petrek, J. Vitecek, H. Vlasinova, R. Kizek, K. J. Kramer, V. Adam, B. Klejdus and L. Havel, *Anal. Bioanal. Chem.*, 383 (2005) 576.
58. J. Vacek, J. Petrek, R. Kizek, L. Havel, B. Klejdus, L. Trnkova and F. Jelen, *Bioelectrochemistry*, 63 (2004) 347.
59. V. Adam, J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, L. Trnkova, F. Jelen and R. Kizek, *Electroanalysis*, 17 (2005) 1649.
60. V. Adam, J. Zehnalek, J. Petrlova, D. Potesil, B. Sures, L. Trnkova, F. Jelen, J. Vitecek and R. Kizek, *Sensors*, 5 (2005) 70.
61. J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, V. Adam, L. Trnkova and R. Kizek, *Electrochim. Acta*, 51 (2006) 5169.
62. V. Adam, P. Hanustiak, S. Krizkova, M. Beklova, J. Zehnalek, L. Trnkova, A. Horna, B. Sures and R. Kizek, *Electroanalysis*, 19 (2007) 1909.
63. S. Krizkova, V. Adam, J. Petrlova, O. Zitka, K. Stejskal, J. Zehnalek, B. Sures, L. Trnkova, M. Beklova and R. Kizek, *Electroanalysis*, 19 (2007) 331.
64. J. Petrlova, S. Krizkova, O. Zitka, J. Hubalek, R. Prusa, V. Adam, J. Wang, M. Beklova, B. Sures and R. Kizek, *Sens. Actuator B-Chem.*, 127 (2007) 112.
65. J. H. Pei, M. L. Tercier-Waeber and J. Buffle, *Anal. Chem.*, 72 (2000) 161.
66. S. Krizkova, D. Huska, M. Beklova, J. Hubalek, V. Adam, L. Trnkova and R. Kizek, *Environ. Toxicol. Chem.*, 29 (2010) 492.
67. C. Fernandez-Bobes, M. T. Fernandez-Abedul and A. Costa-Garcia, *Electroanalysis*, 10 (1998) 701.
68. V. Adam, J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, L. Trnkova, F. Jelen and R. Kizek, *Electroanalysis*, 17 (2005) 1649.
69. L. Trnkova, S. Krizkova, V. Adam, J. Hubalek and R. Kizek, *Biosens. Bioelectron.*, 26 (2011) 2201.
70. O. Zitka, J. Sochor, N. Cernei, V. Adam, J. Zehnalek, A. Horna, J. Hubalek, L. Trnkova, L. Havel and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 422.
71. K. Mikulik, I. Janda, J. Weiser, J. Stastna and A. Jiranova, *Eur. J. Biochem.*, 145 (1984) 381.
72. T. H. Hansen, K. H. Laursen, D. P. Persson, P. Pedas, S. Husted and J. K. Schjoerring, *Plant Met.*, 5 (2009).
73. V. Adam, I. Fabrik, V. Kohoutkova, P. Babula, J. Hubalek, R. Vrba, L. Trnkova and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 429.
74. G. L. Long and J. D. Winefordner, *Anal. Chem.*, 55 (1983) A712.
75. R. Causon, *J. Chrom. B*, 689 (1997) 175.
76. M. M. Abou-Krisha and A. M. Abushoffa, *Int. J. Electrochem. Sci.*, 2 (2007) 418.
77. D. Jagner, E. Sahlin and L. Renman, *Talanta*, 42 (1995) 1447.
78. P. Babula, R. Opatrilova, V. Kohoutkova, I. Dankova, J. Hubalek, J. Zehnalek, V. Adam and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 395.
79. O. Krystofova, P. Majzlik, J. Zehnalek, L. Havel, V. Adam and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 409.

80. V. Shestivska, S. Krizkova, O. Zitka, M. Mackova, T. Macek and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 403.
81. J. Zehnalek, V. Adam and R. Kizek, *Lis. Cukrov. Repar.*, 120 (2004) 222.
82. J. Zehnalek, J. Vacek and R. Kizek, *Lis. Cukrov. Repar.*, 120 (2004) 220.
83. A. Abbas and C. Edwards, *Appl. Environ. Microbiol.*, 55 (1989) 2030.