# Indirect implication of bacterial proteins in the biouptake of metals from aqueous solution

E. Fosso-Kankeu[1,2], A. F. Mulaba-Bafubiandi[1], T. G. Barnard[3], P. T. Campana[4]

[1]*Minerals Processing and Technology Research Center, Department of Metallurgy, School of Mining, Metallurgy and Chemical Engineering, Faculty of Engineering and the Built Environment, University of Johannesburg, PO Box 17011, Doornfontein 2028, Johannesburg, South Africa*
[2]*School of Chemical and Minerals Engineering, Faculty of Engineering, North West University, Potchefstroom Campus, Potchefstroom, South Africa, e-mail: 24838616@nwu.ac.za*
[3]*Water and Health Research Unit, Faculty of Health Sciences, University of Johannesburg, PO Box 17011, Doornfontein 2028, Johannesburg, South Africa*
[4]*Escola de Artes, Ciências e Humanidades, Universidade de São Paulo, Brazil*

**Abstract** To investigate the adsorptive role of proteins on the cell walls of four bacteria (*Bacillaceae bacteria, Bacillus subtilus, Escherichia coli* and *Pseudomonas aeruginosa*), they were mechanically disrupted and the cell residues used for adsorption of nickel. The effect of mechanical treatment on the biomasses was assessed using SDS-PAGE, specific protein assay kit and Fourier Transform Infrared Spectroscopy (FTIR). A decrease of the adsorption capacities of all the biomasses was observed following treatment. FTIR spectra showed slight shift in the signals of active groups on all the cells after treatment. Analysis confirmed removal of proteins from cells during treatment.

**Keywords** Biosorption, metal binding proteins, Gram-negative bacteria, Gram-positive bacteria, bioremediation, nickel

## Introduction

Metal biosorption processes are exploited in the remediation of polluted water or recovery of values from mine solutions. Biosorbents often used are agricultural products, plants and, microorganisms reported to be more effective. Metal uptake by microorganisms occurs through two mechanisms namely passive and active mechanisms; in the active mechanism metal ions are transported across the membrane yielding to intracellular accumulation. This mechanism is often associated with an active defence system of microorganisms whereby metal binding proteins are induced in response to metals. Some metal ions (*e.g.* $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$ and $Zn^{2+}$) have a role in a variety of important functions of proteins in microorganisms, however at higher concentrations even essential metals can become toxic (Roane and Pepper 2000). The control of intracellular metal ions is achieved by families of proteins including transmembrane transporters, metalloregulatory sensors and diffusible cytoplasmic metallochaperone proteins (Finney and Ohalloran 2003). Typical example of metalloproteins often found in yeast, fungi and algae are low molecular weight metallothioneins with a high cysteine content (Mejare and Bulow 2001; Ibrahim *et al.* 2001). The functions of metalloprotein in microbial cells are distinguishable, but all contribute in ensuring metal homeostasis in the cell, by influencing uptake, efflux, intracellular trafficking within compartments and storage (Tottey *et al.* 2008; Waldron and Robinson 2009).

Although the affinity for a metal ion in a metalloprotein is high if the function of the protein requires keeping the metal ion bound, conserved histidines, cysteines regions as well as other sites can be implicated in the binding

(Mejare and Bulow 2001; Pastore *et al.* 2007; Maret 2010; Passerini *et al.* 2006; Passerini *et al.* 2007; Shu *et al.* 2008). It is said that metalloproteins have affinity for specific metals to a certain extent, allowing them to bind the metals and move them across the cell (Ma *et al.* 2009).

Metalloproteins as part of the microbial cell membrane can affect its adsorption capacity and specificity. Acquisition of essential metal ions from the extracellular environment by the bacteria systems is made possible by cell membrane. The structure of bacterial cell membrane determines the ability of bacteria to uptake metal ions and meets cellular metal demands. Proteins, peptide, lipoproteins, polysaccharides and other extracellular polymeric substances of the cell wall can adsorb metals. It has been reported that proteins associated to microorganisms cell membranes play a crucial role in the removal of metals from solution (Fukushi *et al.* 1996; Bupp and Ghosh 1991; Ghosh and Bupp 1992). However the nature of the cell wall considerably varies between Gram-positive and Gram-negative bacteria. The cell wall of Gram-positive bacteria is mainly composed of peptidoglycan, while this polymer is thinner in Gram-negative bacteria but supplemented with lipopolysaccharide (Beveridge 1999; Vijayaraghavan and Yun 2008; Wang and Chen 2009).

For a better understanding of the influence of proteins on the adsorption capacity of microorganisms two types of cells, Gram-positive (*Bacillus subtilis* and *Bacillaceae bacterium*) and Gram-negative (P*seudomonas aeruginosa* and *Escherichia coli*) bacteria were studied.

Determination of the level of implication of membrane proteins in metal uptake by microorganisms will facilitate understanding of the mechanism of the process and also set a basis for the development of metal biosensors.

## Methodology

### *Preparation of metal solutions*

Analytical salts of copper and nickel sulphate were dissolved in distilled water to make stock solutions of 1000 mg/L.

### *Proteins extraction and characterization*

To induce the production of metal binding proteins, cells of *Bacillaceae bacterium, Escherichia coli, Bacillus subtilis* and *Pseudomonas aeruginosa* were grown to early log phase and exposed to nickel (0, 40, 100 and 200 mg/L) in an aqueous solution at 37 °C for approximately four hours. Control and induced cells were both centrifuged at 8867 g for 5 min at 4°C and the pellets recovered. The cells were suspended in 1 mL phosphate buffer saline (NaCl: 0.138 M, KCl: 0.0027 M, pH 7.4) and a 3 mm bead was added. The cells were then lyzed by vortexing the mixture intermittently (1 min vortex and 1 min in ice) for 5 min using a Disruptor Genie machine (Scientific Industries, USA). Cell debris were then separated from the supernatant by centrifuging the mixture at 15600 g for 5 min. The supernatant was then collected and stored at 4 °C for the next experiment.

### *SDS-PAGE*

Reagents were prepared according to manufacturers' specification. Prior to electrophoresis, presumptive protein fractions were added to equal volume of laemli buffer and a quarter of volume of 2-mercapto ethanol; the mixture was then heated at 95 °C for 5 min. The samples and SDS-PAGE pre-stained standard were loaded on pre-packed gels from Biorad and ran at 120 V for 45 min.

### *Quantification of proteins*

For quantification of proteins present in the lysis extract, the Pierce BCA Protein Assay Kit (Thermo Scientific, SA) was used. Experiment was conducted in test-tubes according to the protocol provided by the manufacturer. The absorbance of all the samples was measured within 10 min at 562 nm. The standard curve was used to determine the protein concentration of each lysis extract.

### *FT-IR experiment*

Freshly grown and lysed cells of *Bacillaceae bacteria, E. coli, B. subtilis* and *P. aeruginosa* were dried for 24 h in the oven at 50°C and

then crushed in the mortar. Spectra of cell pellets were recorded within the wavenumber range of 400–4000 $cm^{-1}$ with a Nicolet iS10 spectrometer (Thermo Fisher Scientific, SA).

### *Metal adsorption experiment*

Cells of *Bacillaceae bacterium, Escherichia coli, Bacillus subtilis* and *Pseudomonas aeruginosa* were inoculated in nutrient broth ('Lab-Lemco' powder: 1.0 g/L; yeast extract 2.0 g/L; peptone 5.0 g/L; sodium chloride 5.0 g/L; pH 7.4 ± 0.2 at 25 °C; Merck Chemicals, SA) and incubated in incubator with shaker at 37 °C for 20 h. Cells were then harvested using a centrifuge at 8867 g for 5 min at 4°C.

Both freshly grown and lysed cells (0.1 g) were used for adsorption of nickel (10, 20, 30 and 40 mg/L) at 37 °C in an incubator with shaker (160 rpm, Labcon). Aliquot (5 mL) of the mixture was collected every 30 min of the duration of experiment (two hours). Collected samples were centrifuged at 15600 g for 5 min and the residual metal in the supernatant was measured using the Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

The adsorption capacity at equilibrium was determined using the following equation:

$$q_e = \frac{(C_o - C_e)V}{m} \quad (1)$$

where: $q_e$ is the absorption capacity in mg/g, $C_o$ is the initial concentration of metal ions in solution (mg/L), $C_e$ is the equilibrium of metal ions (mg/L), $m$ is the biomass (g), $V$ is the volume of the solution (L)

## Results and discussion

### *Characterization and quantification of proteins*

### *Visualization of isolated proteins on SDS-PAGE*

This experiment was carried out in order to identify some of the proteins naturally produced by the cells but mostly to determine if other proteins were produced by bacteria in response to the presence of nickel. The Fig. 1 shows (head of arrows) that some additional proteins were produced during the exposure of cells to nickel. It could also be observed that the size of metal induced proteins varied per cells. This certainly implies the specificity of response with regard to the defence mechanism.

### *Relative quantity of specific proteins in the extract*

The technique used for proteins' quantification was specific to proteins rich in four particular amino acids (cysteine, cystine, tryptophan and tyrosine). According to Passerini *et al.* (2012), high-throughput experimental techniques based on X-ray adsorption spectroscopy are effective in identifying metalloproteins, but do not allow detection of ligands involved in binding the metals. Metal binding proteins including metallothioneins and phytochelatins are reported to be rich in cysteine (Mejare and Bulow 2001; Passerini *et al.* 2012). It was therefore expected that cysteine bearing proteins will constitute the major part of proteins quantified in the extract isolated from bacteria cells. Results (not shown here) indicate that such proteins were mostly isolated from *E. coli* cell membrane (0.25 µg/mL). In

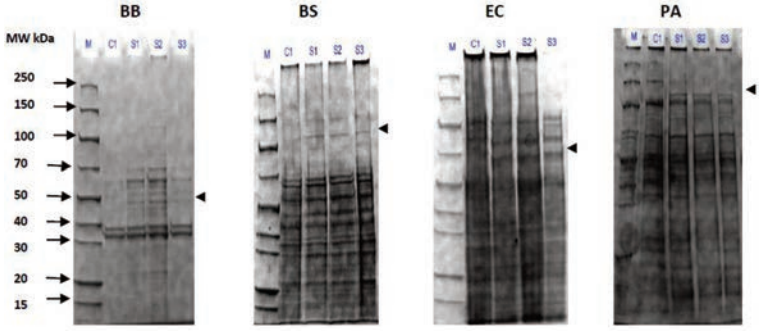


***Fig. 1*** *Isolated proteins observed on SDS-PAGE (M: marker, C1: cells non exposed to Ni, S1: Cells exposed to 40 mg/L Ni, S2: Cells exposed to 100 mg/L Ni, S3: cells exposed to 200 mg/L Ni). BB:* Bacillaceae bacterium, *BS:* B. subtilis, *EC:* E. coli, *PA:* P. aeruginosa.

general higher amount of proteins were removed from Gram-negative bacteria than Gram-positive bacteria. It has been reported that the proteins or peptides easily removed by mechanical disruptions are mostly part of the extracellular polymeric substances (EPS; Yee and Fein 2001; Vijayaraghavan and Yun 2008).

### *Active groups on treated and untreated cells (FTIR)*

Discrepancies in the spectra of treated and untreated biomasses of Gram-negative bacteria were observed both in the region of 3300 – 2800 and 1700 – 750 $cm^{-1}$ (Fig. 2) corresponding to the effect of mechanical disruption to the active groups. Such treatment affected the signals of active groups from E. coli (3265 $cm^{-1}$, 3065 $cm^{-1}$, 1300 $cm^{-1}$ and 850 $cm^{-1}$) and mostly from *P. aeruginosa* (2900 $cm^{-1}$, 1600 $cm^{-1}$, 1100 $cm^{-1}$ and 950 $cm^{-1}$).

For the Gram-positive bacteria, mechanical disruption also resulted in the bands' shift of signals in *Bacillaceae bacterium* (2870 $cm^{-1}$, 1470 $cm^{-1}$ and 1300 $cm^{-1}$) as well as *B. subtilis* (3290 $cm^{-1}$ and 3070 $cm^{-1}$) biomasses spectra (Fig. 3).

The disappearance or reduction of signals mainly corresponding to the carboxylic and amine functional groups in the treated biomasses, implies that mechanical disruption certainly affected the peptide or proteins content of cells. It was observed that bands' shift of signals were more pronounced with *P. aeruginosa* biomasses while *B. subtilis* biomasses were less affected.

### *Metal adsorption behaviour of treated and untreated biomasses*

According to previous works an estimation of one-quarter to one-third of all proteins require metals, the exploitation of elements varying from cell to cell (Ferrer *et al.* 2007; Bertini and Cavallaro 2008; Waldron and Robinson 2009).

### *Adsorption behaviour as a function of metal concentration*

The determination of adsorption behaviour of treated and untreated biomasses was carried out by plotting the adsorption capacity in a Langmuir model ($C_e/q_e$ *vs.* $C_e$). It was observed (Fig. 4) that the adsorption behaviour of treated and untreated biomasses was similar; for both types of biomasses the adsorption capacity increased with an increase of equilibrium concentration, mainly due to mass transfer. The adsorption data showed acceptable fit with the Langmuir isotherm as indicated by the correlation coefficient for *Bacillaceae bacteria* (0.9594 and 0.9482), *B. subtilis* (0.9512 and 0.9648), *E. coli* (0.9805 and 0.9954) and *P. aeruginosa* (0.9724 and 0.9889) for untreated and treated biomasses respectively. However better adsorption of nickel by untreated bio-

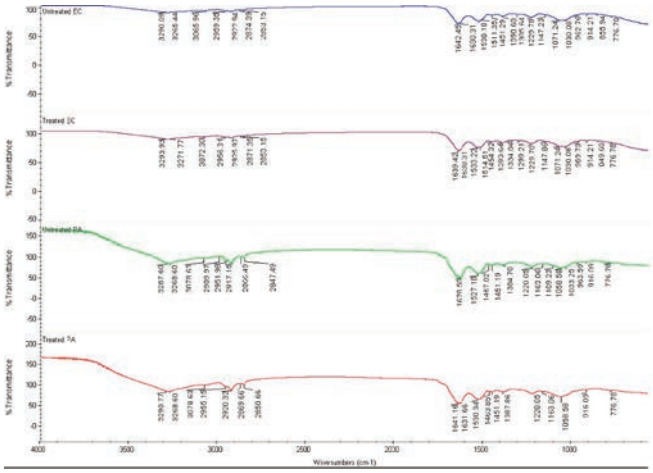

***Fig. 2*** *FTIR spectra of the treated and untreated Gram-negative bacteria biomasses; EC:* E. coli*, PA:* P. aeruginosa

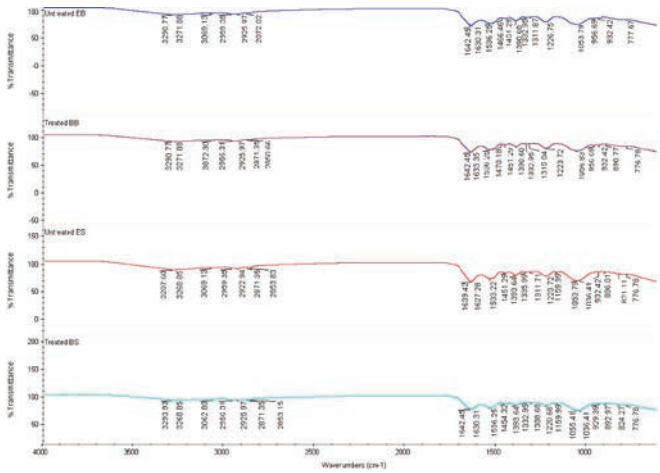

***Fig. 3*** *FTIR spectra of treated and untreated Gram-positive bacteria biomasses; BB:* Bacillaceae bacterium*, BS:* B. subtilis

masses compared to treated biomasses could be observed.

In previous study, Wei *et al.* (2011) also observed that removal of EPS from *B. subtilis* and *P. aeruginosa* reduced their affinity towards cadmium. In general, although the treatment affected mostly the adsorption capacity of Gram-positive biomasses, no significant difference was observed among the adsorption capacities of Gram-negative and -positive bacteria after treatment. According to Kulczycki *et al.* (2002), the difference in metal adsorption capacity between Gram-positive and -negative bacteria could not only ascribed to the differences between the sorptive functional groups of these bacteria, but also from variation in cell wall architecture.

## Conclusion

Mechanical disruption of biomasses, has allowed to determine the indirect implication of proteins or peptides of the cell wall of Gram-negative (*E. coli* and *P. aeruginosa*) and -positive (*Bacillaceae bacterium* and *B. subtilis*) during nickel's adsorption; removal of proteins from cell walls led to reduction bacteria affin-

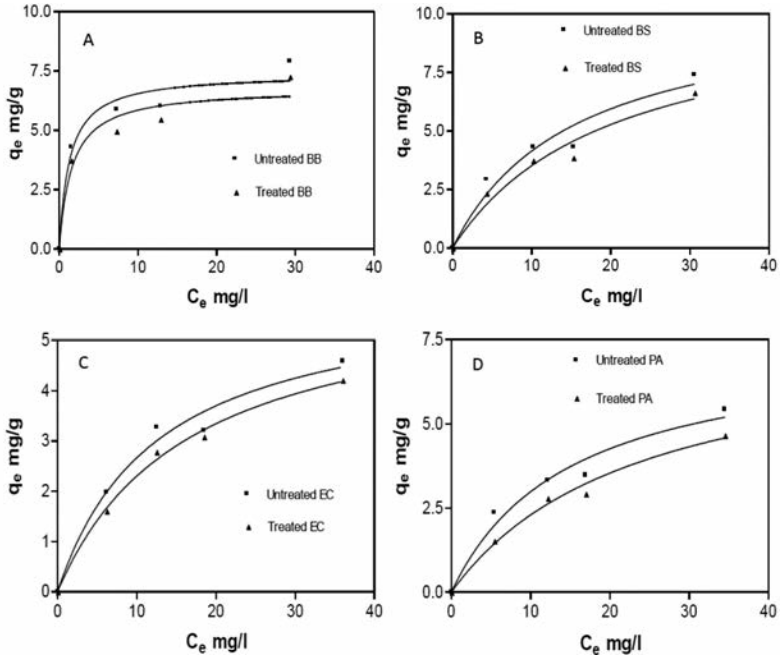


***Fig. 4*** *Adsorption behaviour of treated and untreated biomasses during uptake of nickel. (A)* Bacillaceae bacteria *"BB"; (B)* B. subtilis *"BS"; (C)* E. coli *"EC"; (D)* P. aeruginosa *"PA".*

ity towards nickel. Some of these proteins content a certain level of cysteine, which is an amino acid abundant in most of the metal binding proteins. Despite the chemical and physical differences among the cell walls of Gram-negative and -positive bacteria, no significant difference in the absorptive role of proteins on their cell wall was established in this study.

## References


Beveridge TJ. 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. J Bacteriol. 181: 4725–4733

Bupp S, Ghosh S. 1991. Heavy metal uptake by microbial protein complexation. Paper presented at the Research Symp., Annual WPCF Conf., Toronto, Canada, October.

Finney L.A., T.V. O'Halloran. 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science, 300: 931–935.

Fukushi K, Chang D, Ghosh S. 1996. Enhanced heavy metal uptake by activated sludge cultures grown in the presence of biopolymer stimulators. Wat. Sci. Tech., 34(5–6): 267–272.

Ghosh S, Bupp S. 1991. Uptake of heavy metals by unacclimated aerobic culture. Prof. Conf. On Hazardous Waste Research, L. Erickson (ed.) Engineering Extension, Kansas State University, Manhattan, KS, pp 30.

Ibrahim Z., Azlina W., Baba A.B. 2001. Bioaccumulation of silver and the isolation of metal-binding protein from *P. diminuta*. Brazilian Archives of Biology and Technology, 44(3): 223–225.

Kulczycki E, Ferris FG, Fortin D. 2002. Impact of cell wall structure on the behaviour of bacterial cells as biosorbents of cadmium and lead. Geomicrobiology Journal. 19(6): 553–565.

Ma Z, Jacobsen FE, Giedroc DP. 2009. Metal Transporters and Metal Sensors: How Coordination Chemistry Controls Bacterial Metal Homeostasis. Chem. Rev. 109(10): 4644–4681.

Maret W. 2010. Metalloproteomics, metalloproteomes and the annotation of metalloproteins. Metallomics, 2: 117 – 125.

Mejare M, Bulow L. 2001. Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends in Biotechnology. 19(2): 67–73.

Passerini A, Lippi M, Frasconi P. 2012. Predicting Metal Binding Sites from Protein Sequence. IEEE/ACM Transactions on Computational Biology and Bioinformatics. 9(1): 203–213.

Passerini A., Andreini C., Menchetti S., Rosato A., Frasconi P. 2007. Predicting zinc binding at the proteome level. BMC Bioinformatics, 8: p 39.

Passerini A., Punta M. Ceroni A., Rost B., Frasconi P. 2006. Identifying cysteines and histidines in transition-metal-binding sites using support vector machines and neural networks. Proteins, 65(2): 305 – 316.

Pastore C., Franzese M., Sica F., Temussi P., Pastore A. 2007. Understanding the binding properties of an unusual metal-binding protein – a study of bacterial frataxin. FEBS Journal, 274: 4199 – 4210.

Roane TM, Pepper IL. 2000. Microorganisms and metal pollutants. In: Maier, R.M., Pepper, I.L.,Gerba, C.P. (Eds.), Environmental Microbiology. Academic Press. San Diego, USA, 403–423.

Shu N, Zhou T, Hovmoller S. 2008. Prediction of zinc-binding sites in proteins from sequence. Bioinformatics, 24(6): 775 – 782.

Tottey S, Waldron KJ, Firbank SJ, Reale B, Bessant C, Sato K, Cheek TR, Gray J, Banfield MJ, Dennison C, Robinson NJ. 2008. Nature, 455–1138.

Vijayaraghavan K, Yun YS. 2008. Bacterial biosorbents and biosorption. Biotechnol. Adv. 26, 266–291.

Waldron KJ, Robinson NJ. 2009. How do bacterial cells ensure that metalloproteins get the correct metal? Nature Reviews. 6: 25–35.

Wang J, Chen C. 2009. Biosorbents for heavy metal removal and their future. Biotechnology Advances. 27, 195–226.

Wei X, Fang L, Cai P, Huang Q, Chen H, Liang W, Rong X. 2011. Influence of extracellular polymeric substances (EPS) on Cd adsorption by bacteria. Environmental Pollution. 159: 1369–1374.

Yee N, Fein J. 2001. Cd adsorption onto bacterial surfaces: a universal adsorption edge? Geochim Cosmochim Acta. 65: 2037–2042.