International Biodeterioration & Biodegradation 116 (2017) 64–72

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Biosorption of copper from aqueous environments by *Micrococcus luteus* in cell suspension and when encapsulated

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ARTICLE INFO

Article history:
Received 29 May 2016
Received in revised form
20 September 2016
Accepted 27 September 2016

Keywords:
Copper uptake
Micrococcus luteus
Encapsulation in electrospun polymer

ABSTRACT

The ability to sequester or tolerate copper has been described for a number of bacteria. It was shown that *Micrococcus luteus* tolerates copper, and possible mechanisms of uptake for this bacterium have been proposed. Using a count live of *M. luteus* (CFU), 10% survived a copper concentration of 370 ppm. Maximum sorption capacity was 59 mg of Cu^{2+}/g of dry cells at pH 6. Several proteins of *M. luteus* with copper affinity were identified by enrichment on a metal-chelating resin followed by LC-MS/MS to identify the metallome. Use of SEM/EDX showed how copper concentrated on the bacterial surface, whereas TEM indicated that copper was found also inside the cells. The enhanced capability of *M. luteus* to bind copper was tested using three configurations: free cells on an agar surface and cells encapsulated in alginate or in electrospun polymer composites. The latter showed the highest capability to bind copper (~76 mg Cu^{2+}/g dry cells). Such polymer composites may potentially be used in various water-based applications such as treatment of wastewater with a high concentration of copper or other heavy metals.

1. Introduction

Copper is an essential trace element, like many other heavy metals, and it is also toxic in high concentrations. The interaction of heavy metals, specifically copper, has been described for many different species of microorganisms. For example, the use of copper/nickel alloys in marine environments was considered as a means to avoid corrosion; however, it was found that sulfate-reducing bacteria could attach and grow on an alloy having a high copper concentration as long as the bacteria possess transient oxygen tolerance (Chamberlain et al., 1988). The ability of bacteria to withstand high concentrations of copper was also reported for copper plumbing, where assimilable organic carbon was required for microbial-induced corrosion (Walker et al., 1991) and high concentrations of copper in drinking water was found to be related to the biofilms of *Variovorax* sp in copper plumbing systems (Reyes et al., 2008). In another study, the exopolymer accumulation was found to allow for bacterial corrosion, and a mixed culture of *Pseudomonas* bacteria conferred high resistance to copper to one of the bacteria under study, *Pseudomonas paucimobilis* (Angell and Chamberlain, 1991). Copper has also been shown to increase polymer formation by the marine bacterium *Oceanospirillum*, allowing for neutralization of copper toxicity permitting other species to become part of a biofilm on copper containing surfaces (Wagner et al., 1996). A similar response with the formation of an extracellular emulsifying agent was described when copper concentration was increased so that the fungus *Curvularia lunata* displayed an increase in total lipid saturation (Paraszkiewicz et al., 2009).

The possible environmental applications of metal uptake by using microorganisms for wastewater treatment have been reported (Wong et al., 2001b). A review of recent studies showed many microorganisms are capable of metal biosorption, in the study of Kiran et al. The cyanobacterium *Lyngbya putealis* has been shown to take up copper in the amount of 7.8 mg/g dry cells, with 40–45% taken up after just 15 min. Adsortption was optimized with respect to pH and metal concentration (Kiran and Thanasekaran, 2011). Sulfate-reducing bacteria have been shown to have high

Abbreviations: MLF, Fibers containing live *M. luteus* cells; MLA, Alginate containing live *M. luteus* cells; IMAC, Immobilized-metal affinity chromatography; EDX, Energy-dispersive X-ray spectroscopy.

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http://dx.doi.org/10.1016/j.ibiod.2016.09.029
0964-8305/

metal-reducing capabilities in the study of Chen et al. (2000) although biosorption was limited to Cu(II) 16 mg/g dry cells. Even the dead biomass of *Fusarium Flocciferum* has been shown to be a promissing agent for heavy metal uptake, and equilibrium was achieved within seconds (Delgado et al., 1996).

One group of cocci, the genus *Micrococcus*, has repeatedly been found under variuos extreme environments such as wood amber (Greenblatt et al., 2004) and hot springs (Liu et al., 2016). *Micrococcus* also can be found in grey water from treatment facilities (Keely et al., 2015). Its apparent tolerance to harsh conditions attracted research in field of bioremediation, for example, boron removal from wastewater (Lacin et al., 2015). Metal uptake ability was shown by Wong et al. (2001b) who described the isolation of *Micrococcus* sp, from sludge from a local wastewater treatment facility and the bacterium was shown to survive several cycles (at least five) of biosorption and desorption of copper (Wong et al., 2001a). *Micrococcus luteus* can also take up strontium (Faison et al., 1990) and, to a lesser degree lead, nickel and zinc. In addition, it was shown that it is possible to retrieve the metals while maintaining bacterial cell viability.

The possible role of *M. luteus* in bioremediation was the incentive for its full genomic sequencing, initiated by our laboratory (Young et al., 2010). There are different strategies of microbial metal (copper) resistance or tolerance; among them are bioaccumulation or sequestration. Others include exclusion, compartmentalization and complexation by binding proteins such as metallothioneins or phytochelatins (Mejare and Bulow, 2001). In the genomic sequence a repertoire of functions was annotated that deal with metals. Both chromosomal and plasmid genes are involved in this resistance. In *M. luteus* the genes of the MerR/ArsR proteins, which are known to act in metal resistance (Young et al., 2010), are present. Furthermore, some eight chromosomal genes related to copper transport or function are present in the *M. luteus* genome, including the 2 Cop (A and C) resistant genes and 3 chaperones. Metal sequestration could be by a different means than tolerance, and be related to the third mechanism – complexation by cell components. Nakajima et al. (2001) treated *M. luteus* with a series of chemical extractions in order to remove polysaccharides, proteins, small molecules, lipids, and lastly the more tightly bound proteins and polysaccharides. Finally, the 17% of what was left after these extractions had the greatest ability to uptake copper. Although we possess some understanding of the mechanism governing microbial heavy metal uptake, further studies are required.

The use of immobilized cells for enhanced metal adsorption is gaining interest and several studies showed promising results. In the work of Ahmad et al. (2013) biosorption of high amounts zinc ions from aqueous solution by immobilized *Candida utilis* and *Candida tropicalis* cells was shown. Sinha et al. (2012) showed that alginate immobilized *Bacillus cereus* cells are able to absorb 104 mg mercury per g dry cells. Since many studies use simple hydrogels such as alginate for the encapsultion process, polymers which will degrade in water based applications, further studies are required for improvement of polymeric formulations.

Recently, Gensheimer et al. (2011); Klein et al. (2012, 2009) and Knierim et al. (2014) have described a system of microbial encapsulation which entails incorporating bacteria within microtubes of polymer on a nano scale. The process, called electrospinning, has been used with *M. luteus*, *Pseudomonas* ADP, *Nitrobacter winogradskyi* and genetically modified *E. coli*. Modifications of the orignal process (Gensheimer et al., 2007) entail the creation of a two-layered fiber having a water-insoluble outer membrane (shell) and an inner core of a hydrogel (co-electrospinning) (Dror, 2008). This method allows for the creation of a natural environment for the immobilized cells, mainly due to the viscosity of the water-soluble core polymer. In addition, adjustment of the properties of the shell polymers allows for tuning the mechanical properties, cell attachment and cell division.

In order to create active polymer-bacteria composites an adequate candidate bacterium should be used. For this work we chose a species that has high affinity for the targeted metals, and is capable of surviving high concentrations of the metals and accompanying toxic organic substances. It is preferable that the microbes be easily separable from their substrate and either the metals can be disposed of, or if they have economic value, retrieved. It is also desirable that organisms be recyclable so the remediative process can be repeated.

Our goal in this study was to shed some light on the copper-sequestering mechanisms of planktonic and encapsulated *M. luteus* and, in addition, to create metal-absorbing bacteria/polymer water-insoluble composites.

2. Materials and methods

2.1. Organism and culture media

M. luteus DSM20030T was purchased from DSMZ and was cultured in LB medium in Erlenmeyer flasks at 30 °C, with shaking at 150 rpm. Its identity was confirmed by 16S rDNA sequencing.

2.2. Simple screening methods

Cultures were applied to a copper oxide impregnated cloth (1.0% w/v, at the surface ~30% copper was present). The cloth consists of 70% cotton and 30% polyester and is known to inactivate copper-sensitive organisms (Borkow and Gabbay, 2004). Small, 1.0 cm squares of the fabric were placed on LB culture plates and a drop (10 μL) of cell culture was inoculated on their surface. Growth was observed on the surface. Alternatively, a carpet of organisms was spread on the culture plates in semisolid medium and either the fabric or filter paper discs containing variable concentrations of $CuSO_4$ or copper gluconate were applied to the surface. The growth curve was measured at OD_{600} in a FLUOstar Omega instrument with agitation. Readings were done every hour in triplicate.

2.3. Copper sequestration

To measure copper sequestration by planktonic cells, 10^{10} *M. luteus* cells/mL were suspended in 10 mL of a 10 mM $CuSO_4$ solution and incubated in a shaker at 30 °C. Samples were taken over a 3-h period. At the end of the test period, the bacteria were centrifuged and the pellet was extracted with 7.5% TCA.

To measure copper sequestration by polymers, calcium alginate beads or polymer composite were suspended in 10 mL of 10 mM $CuSO_4$ and incubated at 30 °C. After 30 min the supernatant was removed by aspiration. The beads were washed twice with water and then rinsed with a solution of 7.5% TCA. The copper contents of the supernatant and the TCA-extracted beads were determined by the bicinchoninic acid method (Brenner and Harris, 1995).

To measure copper sequestration of the pure polymers, the polyvinyl alcohol and polyvinyl pyrrolidone were prepared as 10 mg per mL solutions and incubated in 40 μM $CuSO_4$ for 30 min. The suspension was filtered through a Microcon filter with an exclusion value of 3 kDa. The copper concentration of the initial filtrate and a second filtrate of 1.0 mL of 7.5% TCA solution were measured. The second filtrate contains the extraction of copper from the polymer.

2.4. Cell immobilization

2.4.1. Alginate

A standard method was applied (Smidsrod and Skjakbraek, 1990). A 4% alginate solution was mixed 1:1 with the *M. luteus* culture. This mixture was added as a slow drip from a syringe into a solution of 0.1 M calcium chloride, the solidified droplets were left to harden for 5 min and washed with PBS, the resulting beads had a mean size of 4 mm. Alginate beads without bacteria were prepared as a control.

2.4.2. Electrospinning

A core-shell electrospinning technique was used for immobilization of the bacteria. The system allows for fabrication of microtubes in an electrostatic field. Two separate syringes continuously ejected solutions from a spinneret with two coaxial capillaries under high voltage. The internal capillary of this spinneret facilitated the flow of the bacterial cells in an aqueous solution, while being enveloped by the external capillary containing a non-biodegradable polymer solution. The core consisted of 20 vol% cell mass in 15 wt% polyvinylpyrrolidone (PVP) Mw = 1300 k in deionized water. The shell copolymer consisted of 15% polyvinylidene fluoride-*co*-hexafluoropropylene (PVDF-HFP) Mw = 400 k and 2 wt % PEG (Mw = 6 k) in a solvent mixture of tetrahydrofuran (THF)/dimethylformamide (DMF) in a weight ratio of 6.5:3.5, as described earlier (Nardi et al., 2012). The electrospun fibers were collected on the surface of an earthed bath of PBS solution and wound onto plastic carriers. The carriers were stored in PBS solution; the fibers were not allowed to dry out at any time.

2.5. Copper determination

Copper sulfate anhydrous ($CuSO_4$) was purchased from J.T.Baker Chemicals (#32949), and copper (II) D-gluconate from Sigma-Aldrich (#344419). Copper was measured either by flame spectrophotometry using a Varian Spectra AA Zeeman graphite tube atomizer or by the bicinchoninic acid chemical method where the copper-bicinchoninic acid chelated product is oxidized by ascorbic acid to yield a colored substance (Brenner and Harris, 1995). Two methods were used to measure the amount of copper the organisms were able to sequester. $CuSO_4$ was added to a suspension of ~0.9 A_{600}/mL of the *M. luteus*, and supernatant samples were taken at different times (up to 3 days) and centrifuged. In addition copper was also measured in the pelleted organisms and was determined both before and after DDW washes.

2.6. TEM and SEM-EDX

For electron microscopy imaging an extra high resolution scanning electron microscopy (XHR-SEM) MagellanTM 400L and high resolution transmission scanning electron microscope (HR-TEM) Tecnai F20 G2 were used. The scanning electron microscopy (SEM) was combined with an Everhart-Thornley Detector (ET) and energy-dispersive X-ray spectroscopy (EDX). Samples taken from the experiment as described in section 2.3 were air dried and gold plated or were applied to silica and air dried.

For the TEM, samples were fixed with Karnovsky's fixative (2% PFA, 2,5% glutaraldehyde in 0.1 M cacodylate buffer, pH = 7.4) for 4 h at room temperature, followed by ½ diluted Karnovsky's fixative overnight at 4 °C. For the TEM-EDX, samples were washed in 0.1 M cacodylate buffer, dehydrated in a graded series of alcohols, followed by propylene oxide, and embedded in epoxy resin. Sections of 60–80 nm thickness were cut on an ultramicrotome (Reichert-Jung), and examined with the high resolution transmission scanning electron microscope Tecnai F20 G2 equipped with an EDX detector (spectral resolution 133 eV).

2.7. Protein purification

In the procedure, done at 4 °C, in 25 mM TrisHCl buffer and 100 mM NaCl at pH 8.0, the chelating resin was charged with 100 mM $CuSO_4$ and then washed extensively. The resin was equilibrated with 250 μL wash buffer (buffer + 0.02% Triton X100). Protein was extracted by resuspending the cell pellet (from 2 L culture) with 30 mL lysis buffer (buffer + 0.2% Triton X100 + DNase + lysozyme + 0.5 mM PMSF). The cells, kept on ice at all times, were sonicated 6 times for 10 s and centrifuged for 15 min at 12,000 rpm at 4 °C. The pellet was discarded, equilibrated resin was mixed with the supernatant and incubated for 60 min 4 °C. The mixed resin/supernatant was centrifuged for 4 min at 3500 rpm at 4 °C. The unbound supernatant was discarded, and the resin was washed three additional times. Elution was done with ~500 μL elution buffer (buffer + 0.02% Triton X100 + 10 mM EDTA), with incubation for 5 min with buffer, and then centrifuged for 4 min at 3500 rpm at 4 °C. 300 μL of the eluate was loaded in a Superose 12 53 × 1.6 cm (~107 mL) column in buffer + 0.1% Triton X100.

2.8. Electrophoresis

20 μg of protein was boiled for 5 min with sample buffer (2x BioRad Laemmli Sample Buffer # 1610737) and loaded on a 12% Mini-PROTEAN TGX Stain-Free™ BioRad Precast Gel (#4568043) with 5 μL marker. Running buffer was TGS × 1 (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). The biggest bands were excised and used for mass-spec analysis.

2.9. In-gel digestion and protein identification by LC-ESI-MS/MS

Protein bands were excised from an SDS gel stained with Coomassie blue. The protein bands were subsequently reduced, alkylated and in-gel digested with bovine trypsin (Promega), at a concentration of 12.5 ng/μL in 50 mM ammonium bicarbonate at 37 °C, as described (Shevchenko et al., 1996). The peptide mixtures were extracted with 80% CH_3CN/1% CF_3COOH, and the organic solvent was evaporated in a vacuum centrifuge. The resulting peptide mixtures were reconstituted in 80% formic acid and immediately diluted 1:10 with Milli-Q water before analysis. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was performed using a 15 cm reversed-phase fused-silica capillary column (inner diameter, 75 μm) made in-house and packed with 3 μm ReproSil-Pur C18AQ media (Dr. Maisch GmbH). The LC system, an UltiMate 3000 (Dionex). was used in conjunction with an LTQ Orbitrap XL (Thermo Fisher Scientific) operated in the positive ion mode and equipped with a nanoelectrospray ion source. Peptides were separated with a two hour gradient from 5 to 65% acetonitrile (buffer A, 5% acetonitrile, 0.1% formic acid and 0.005% TFA; buffer B, 90% acetonitrile, 0.2% formic acid, and 0.005%TFA). The voltage applied to produce an electrospray was 1.2 kV. The mass spectrometer was operated in the data-dependent mode. Survey mass spectrometry scans were acquired in the Orbitrap with the resolution set to a value of 60,000. The seven most intense ions per scan were fragmented and analyzed in the linear ion trap. Raw data files were searched with MASCOT (Matrix Science) against the NCBI nr database. Search parameters included a fixed modification of 57.02146 Da (carboxyamidomethylation) on Cys, and variable modifications 15.99491 Da (oxidation) on Met, and 0.984016 Da (deamidation) on Asn and Gln. The search parameters also included: maximum 2 missed cleavages, initial precursor ion mass tolerance 10 ppm, fragment ion mass tolerance 0.6 Da. Samples

were further analyzed in Scaffold (Proteome Software).

3. Results

3.1. Growth inhibition

As an easy test for copper tolerance, *M. luteus* was inoculated on agar plates containing a copper impregnated fabric made for antiseptic purposes. This material contains 1.0% w/v (at the surface, copper may be present at ~30%). This material, consisting of 70% cotton and 30% polyester, is known to inactivate copper-sensitive organisms (Borkow and Gabbay, 2004). On contact with it, the cells did not show inhibition. In addition to testing the sensitivity of the cells during the logarithmic growth phase, the cells were incubated with ascending copper concentrations. In this experiment, up to 0.05 mM copper sulfate did not show growth inhibition. The LD_{50} in suspension was found to be 0.5 mM (32 ppm) $CuSO_4$ or about 10-fold greater than the results on the agar plates (Fig. 1).

Growth Inhibition was also measured by count of CFU and this assay showed an even greater tolerance of *M. luteus* to copper. Cultures with an initial inoculum of ~1×10^8 cells, when exposed to as much as 6 mM copper (378 ppm), showed a reduction of only a little more than a log unit. There is a change from pH 6.76 to pH 6.00 in this range of copper concentrations (0–378 ppm), and this may have a slight inhibitory effect on growth.

3.2. Bulk retrieval of copper by cells in suspension

The bicinchoninic acid assay was utilized to follow copper uptake from suspensions. Copper uptake was measured both in bulk suspensions and in individual cells using EDX (next section).

Copper remaining in the supernatant was measured in 1 mM and 10 mM copper solutions. These showed that 80% and 14%, respectively, of the copper was removed from the solution by the cells at the two concentrations (Fig. 2). Our estimate of copper uptake by living cells is 59 mg copper per gram dry cells. Results from previously mentioned studies also found that the uptake was immediate, with the major uptake occurring in the first 10 min as we also can show here (Fig. 2).

For cells in suspension the molar concentration of copper in the supernatant was reduced by 25 μM and in the pellets an equal amount of copper was recovered. When atomic absorption was applied to measuring the sequestered copper, *M. luteus* took up ~ 1–2 × 10^9 copper atoms/cell. We also attempted to estimate the

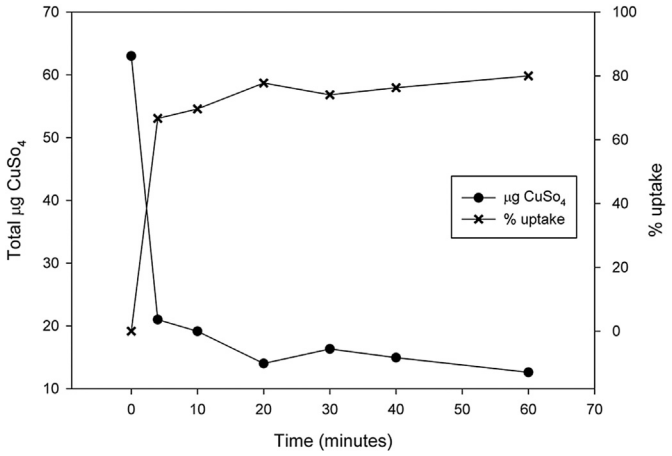

Fig. 2. Copper depletion from a *M. luteus* suspension (1 mM). Percent uptake of copper from supernatant and total amount of copper in respective cell pellet.

avidity by repeated water washes. When the pellet was washed with water three times, 25% of sequestered Cu was removed, while 0.05 M H_2SO_4 removed 50%, and 0.02 M of the chelator, Neocuproine, removed 67% in a single wash.

3.3. Copper sequestration as measured by SEM/TEM and EDX

To visualize the localization of the sequestered metal in the bacterial cells in 10 mM of $CuSO_4$, HR-SEM (Fig. 3) was performed. Fig. 3A is an image of a typical *M. luteus* tetrad; it appears having lighter shades where the heavier elements are located. Clearly, the cell area is far brighter than the background.

In the corresponding EDS spectrum (Fig. 3B) a high copper peak is seen along with other elements such as carbon, oxygen and sulfur. For a control, approximately a 1 μm spot is analyzed in an empty space on the glass surface, on to which copper treated *M. luteus* have been plated. There was no evidence of copper on the slide or in the medium. When the copper concentration is increased to 100 mM, (Fig. 3C + D), the SEM image reveals a profound effect of a high concentration of copper on a colony of *M. luteus*. Here there are surface projections, which in some cases seem to connect the outer envelopes of the cells. When the cells were washed three times the copper crystallizes. This is probably due to the rise in pH from approximately 3.5 to 5.0. Using the EDX

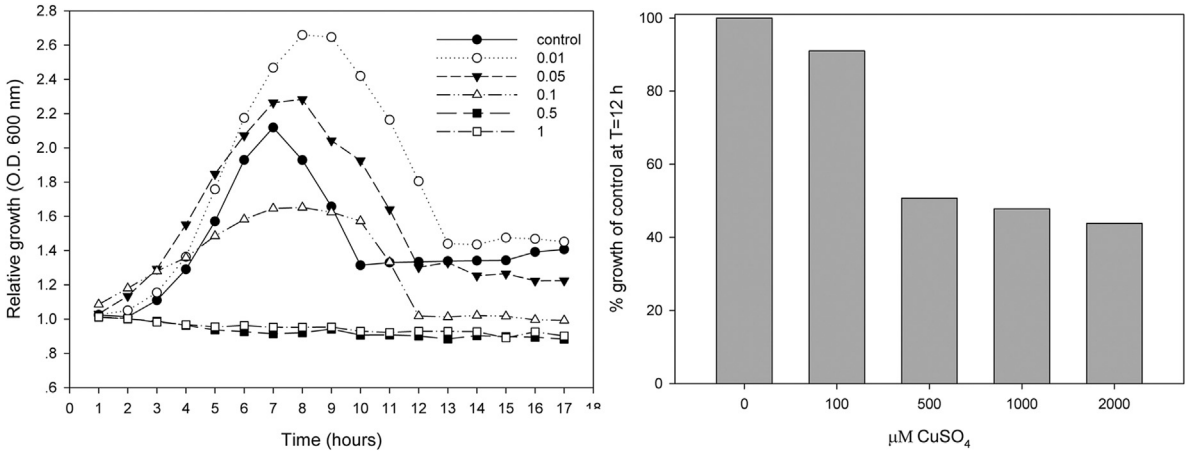

Fig. 1. *M. luteus* Growth inhibition by different copper concentrations. (A) OD_{600} as measured for 12 h. (B) Percent of control at point t = 12 h.

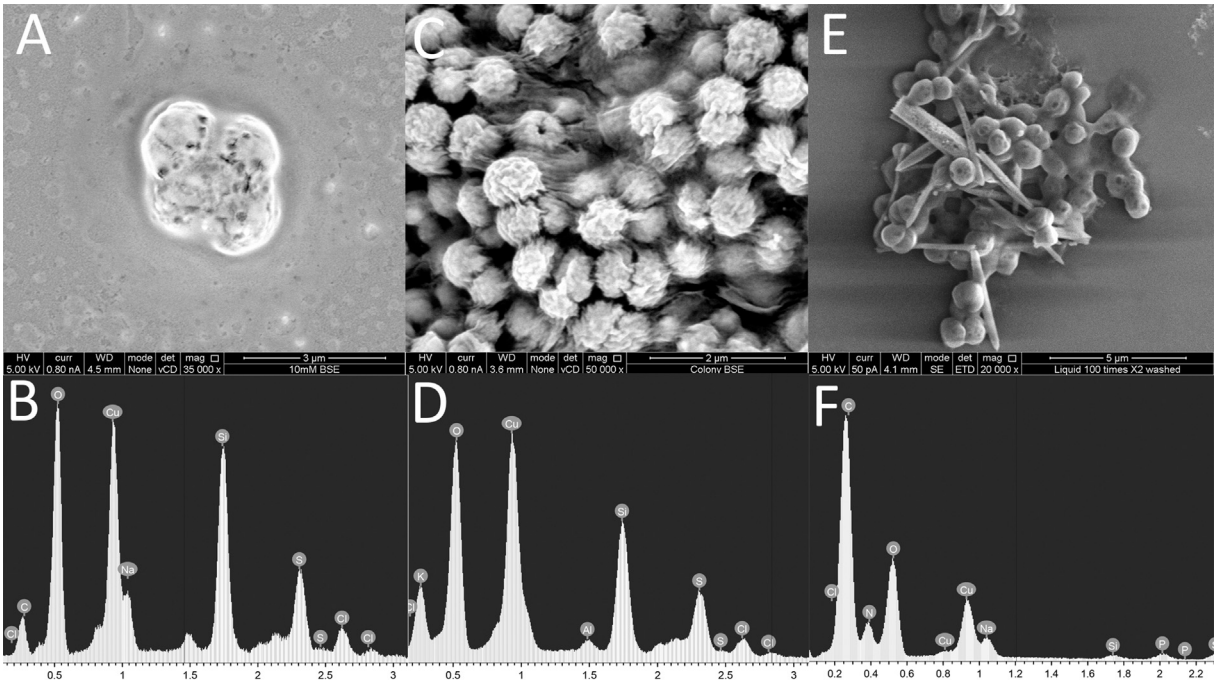

Fig. 3. X-ray backscatter analysis of copper treated *M. luteus*. (A–B) typical cell tetrad and appropriate backscatter spectrum. (C–D) A colony treated with 100 mM copper, image and spectrum showing projections on the cell surfaces. (E–F) Images and spectrum of treated cells that were washed with ddW showing crystalized copper.

mode and observing the spectra (Fig. 3F) revealed that the linear crystals contained copper and sulfur, as well as oxygen and chlorine. The crystal morphology resembles a chloride copper salt, but we cannot be certain of the nature of these crystals. One should also note that the outer calyx like material seems to have lost the surface projections. To measure the avidity of the copper to the cells, we examined the EDX spectra of washed cells. With the first wash considerable copper was retained, with a peak approximately equal to the oxygen. However with a second wash it falls to about 40% less than the oxygen. Prior to washing, the copper was nearly twice the height of the oxygen peak. The decrease of copper in relation to the carbon peak is even greater, falling from more than 6-fold geater than the carbon, to only one-third its value. The loss of copper is probably due in part to the change of pH with the washes.

From the extracellular matrix, to the membrane, to the cytoplasm, the copper increases from 0.2 to 0.6 atomic percent (Fig. 4). These numbers, that if compared to standard literature values would indicate an accumulation of ~6000 times the normal value (0.6/0.0001) of cytoplasmic copper atomic percent.

3.4. Copper-binding proteins

To identify proteins sequestering the copper we loaded the total protein cell extract on an immobilized-metal affinity chromatography (IMAC) column used to enrich the native metalloproteins (Porath et al., 1975). Since we found that most of the metal

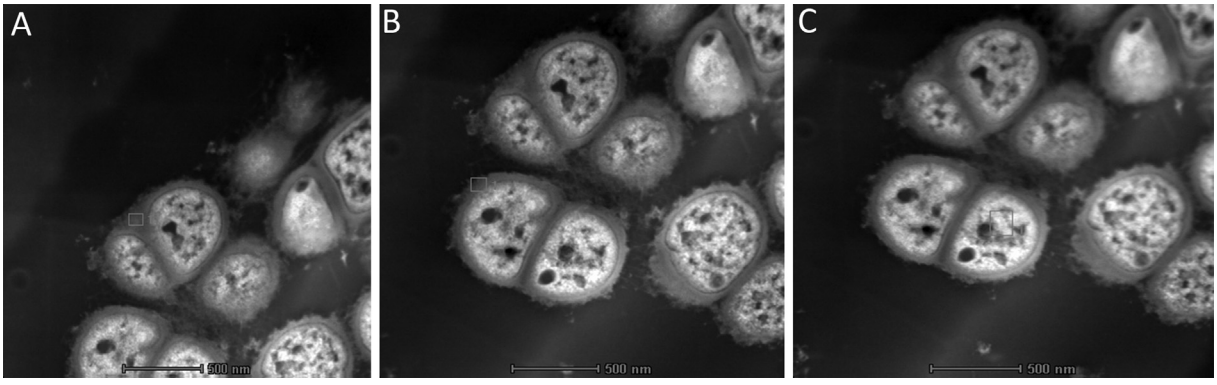

Fig. 4. EDX probe (~0.1 µm) at three areas on thin sections of *M. luteus* that have been exposed to 10 mM of copper for one hour. The dominant outer envelope has a poorly defined adherent outer calyx. The copper is measured as atomic percentage and moving inward. (A) Extra cellular matrix (B) cell envelope (C) Inner cell area.

Element	Extra cellular matrix		Envelope		Cytoplasm	
	Weight %	Atomic%	Weight %	Atomic%	Weight %	Atomic%
Carbon	67	97	58	96	58	96
Copper	0.7	0.2	1.8	0.5	1.8	0.6

Table 1
A sample from the identified proteins and thier theoretical connection to metal binding.

Protein name	Accession	Molecular weight (Da)	Comment
4-hydroxyacetophenone monooxygenase	gi|488944925	57712	Oxidoreductase family
ATP-dependent DNA helicase PcrA	gi|497765221	93113	separates the two DNA strands, and possesses a metal binding motif
malate:quinone oxidoreductase	gi|488943149	53203	oxidoreductase family
menaquinol-cytochrome c reductase cytochrome *b* subunit	gi|488943482	62796	metal ion containing active sites in respiration proteins, 4 H_H sites
molecular chaperone DnaK	gi|738364993	66564	2 H_H sites
phenol 2-monooxygenase	gi|738432093	70383	oxidoreductase family
Sirohydrochlorin cobaltochelatase	gi|751048160	24834	A cobaltochelatase that catalyzes the insertion of metal into protoporphyrin rings
pyridine nucleotide-disulfide oxidoreductase	gi 289557896	44,400	oxidoreductase family

absorption happens very quickly, probably due to biophysical ionic reactions, we focused on naturally occurring proteins that have high copper affinity instead of the induced stress proteins.

In Table 1 is the abbreviated list of the proteins identified by two independent LC-MS/MS experiments. The list contains proteins with >95% confidence (full list provided in suppl. 1).

As expected from their metal ion containing active sites, respiration proteins such as menaquinol-cytochrome c reductase cytochrome *b* subunit were found, as well as the oxidoreductase proteins phenol 2-monooxygenase and Malate:quinone oxidoreductase. On the other hand no specific copper transport/sensing proteins such as CopA, CopZ or CopY family proteins were found, most probably because their expression requires copper induction by the substrate.

Histidine rich or other specific sites are known to have copper affinity. Accordingly, some of the proteins on the list were enriched in histidine residues (<2.5% from total AA) for example sirohydrochlorin cobaltochelatase is a protein that catalyze the insertion of metal into protoporphyrin rings. Another is 4-hydroxyacetophenone monooxygenase associated with the cation diffusion facilitator CzcD. Other proteins such as succinate dehydrogenase flavoprotein subunit and ATP-dependent DNA helicase PcrA contained HMA motif (G-M-T-**C-X-X-C**) which is known for possessing a heavy metal binding sequence (Bull and Cox, 1994).

On the other hand also functionally related proteins such as the ABC transporter substrate-binding protein were observed, it contains a periplasmic component linked to metal ion transport systems known to regulate metal import to the periplasm (Nies, 2007). The DnaK/Hsp70 is a family of molecular chaperones that bind nonnative states of other proteins and assist them to reach a functional conformation and it has been shown before to assist copper binding (Hibino et al., 1999). Thioredoxin proteins have been shown to interact with copper chaperones (Abicht et al., 2014), and in our study thioredoxin region within proteins such as phenol 2-monooxygenase could be found.

3.5. Immobilized cells

Our objective in this section of the research was to explore the polymer/bacterial composite. In Fig. 5A are shown fibers containing

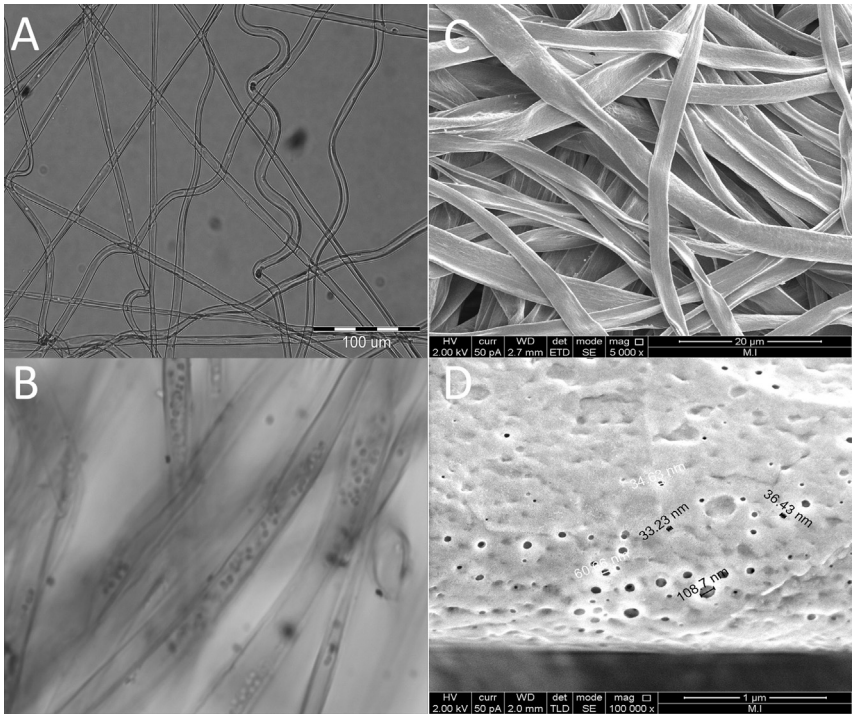

Fig. 5. Visualization of microfiber-encapsulated *M. luteus* (A) Bright Field right post processing (B) Fibers Incuabated with LB (C) HR-SEM micrograph (D) HR-SEM micrograph showing fiber pores.

live *M. luteus* cells (MLF) their uniformity of cell distribution in fibers. It should be noted that in subsequent storage cell division was observed (Fig. 5B). The fiber structure can be seen in Fig. 5C, with an average fiber diameter of 5 μm and an average of 40 nm wide pores. Due to the water soluble PVP in the fibers and nano pores on the micro tube surface (Fig. 5D) *M. luteus* cells remain inside the tubes but there is no hindrance of liquid flow.

To demonstrate the ability of the encapsulated cells to sequester copper, *M. luteus* cells were immersed in different copper concentrations and copper concentration in supernatant was measured for several days. It can be seen that the MLF succeeded in absorbing 97% (w/v) of the copper in the solution after 3 days (Fig. 6). This absorption is seen both in electrospun encapsulated cells and in alginate encapsulated cells.

4. Discussion

The development of new and improved approaches to apply microorganisms in the field of wastewater bioremediation requires greater understanding of biology, material science and other fields of study. To understand the biology we first described the microbial cell-copper interaction. We saw that the solid surface versus suspension in liquid gave very different estimates of growth inhibition. Viability as measured by CFU showed an even greater degree of copper tolerance; therefore we assumed that there are different mechanisms for copper tolerance and copper uptake.

Gram-positive bacteria are known to have high metal sorption capabilities. The ability of the cell wall of *Bacillus subtilis* to interact with different heavy metals has been studied. The maximum biosorption of the metal ions was observed at pH 3.0 ± 0.1 for Pb(II) and pH 5.0 ± 0.1 for Cu(II) ions. The maximum biosorption capacities of Pb(II) and Cu(II) ions on *Bacillus* sp. were determined to be 92.27 ± 1.17 mg g^{-1} at 250 mg l^{-1} concentration and 16.25 ± 1.64 mg g^{-1} at 200 mg l^{-1} concentration, respectively (Tunali et al., 2006). Other studies are finding new candidate strains such as strain CCNWRS33-2 that was isolated from root nodule of *Lespedeza cuneata* and shown to collect 100 μM Cu(II) in 4 h (Wei et al., 2009).

Copper uptake by *Micrococcus* sp. was studied by Wong et al. (2001a)., who reported that 36.5 mg of copper (II) per gram of dry weight is taken up at pH of 5.0, while another 15 mg is taken up when the pH is raised to 6.0. Nakajima et al.(2001) using alkaline and solvent treated *M. luteus* reported a similar value of 33.5 mg copper per gram of dried cells. Our results show similar quantities, thus verifying the high copper uptake ability of *Micrococcus* sp. in general and in *M. luteus* specifically. Comparing numerous mg g^{-1} values of different organisms one can see that *M. luteus*'s metal uptake capabilities are well above average (Veglio and Beolchini, 1997).

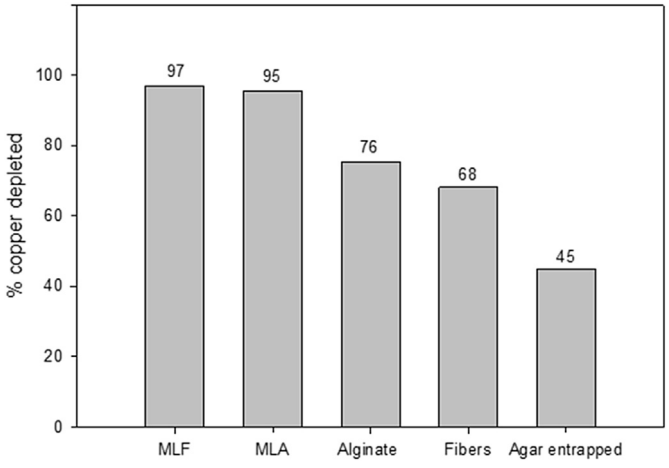

Fig. 6. Percent copper depleted from the supernatant. Incubation with 10 mM CuSO4 and appropriate treatment. MLF (*Micrococcus luteus* fibers) MLA (*Micrococcus luteus* – Alginate beads).

Since various mechanisms govern bacterial copper uptake, amongst them secreted factors (Vandenbossche et al., 2015), it was important to show that the cells themselves interact with the metal. In the SEM micrographs of a *M. luteus* colony somewhat unusual cell projections were observed in a colony which was treated with high copper concentration. Recently, Angelov et al. (2015) showed that the Flp pili in *M. luteus* are involved in horizontal gene transfer—a phenomenon which is associated with adaptation to new environments, the spread of antibiotic resistance traits and which is thought to promote rapid evolution. In their work the detection of naturally expressed pili was difficult, this is probably due to conditional expression. In accordance with our results, they propose that environmental conditions such as pH changes and other stresses can induce pili expression. Interestingly not only the cell surface interacts with the metal, it could be detected in the cytoplasm and even in higher amounts than on the surface. Copper is an essential element so it was expected to be found in the cell cytoplasm but Barton et al. (2007) in defining the bacterial "metallome" give a value of 0.0001 copper atomic percentage. Compared to this value our results indicate a 6000-fold rise in inner cell copper atomic percentage, which is highly significant.

Although the inactivated cell biomass can take up substantial amounts of copper (Butter et al., 1998), and the bacterial cell wall is also active in cation binding (Neuhaus et al., 1974), we studied the possible role of proteins in copper uptake. The main factor governing protein absorbance to the IMAC column would be by its surface number or density of exposed imidazole and thiol groups. In addition, other groups such as indole are probably important (Porath et al., 1975). The results here indicate that the basal binding ability of copper by the cell is complex and involves, expected copper containing enzymes, but also some less obvious proteins having unrelated functions.

Our research question is: Can these polymeric systems serve as an artificial habitat for bacterial functionality, as biocatalysts, as detoxifying agents of waste, in the sequestration of metals, in energy generation? However, insertion and encapsulation of functional bacteria in artificial environments is not a trivial task. This processing entails contact with solvents that can present hostile conditions for bacteria. Moreover, encapsulation itself may be stressful in terms of nutrition, metabolism and replication. Developing these bacterial-friendly polymeric environments is a serious challenge. To arrive at optimum results, we have explored encapsulation of bacteria, first in alginate and secondly in polyvinyl alcohol (PVA), only then did we move to encapsulation by coaxial electrospinning (with bacteria inside fibres), by electrospinning of bacteria encapsulated in hydrogel microparticles (Zussman, 2011).

As noted in the Introduction, only a few organisms have been electrospun and their function measured. A difficulty in understanding their viability and replication is the insoluble outer polymeric shell. With yeast, there was no question of the viability since they reproduced to the point of swelling the micro-tubes (Letnik et al., 2015). Concern with regard to the exposure of the microbes to solvent, has centred on developing an encircling hydrogel for the microbial core Encapsulation in PVA and PVP are some of the best studied methods of cell entrapment. *M. luteus* has been observed to survive storage for at least 1.5 years in PVA (Gensheimer and Greenblatt, unpublished). The high porosity of PVA allows for effective mass transport of metabolites and oxygen. Additionally,

they are biocompatible, nontoxic, and have good mechanical durability and elasticity. PVA was found to increase the tolerance of biological entities towards changes in pH, high concentrations of metabolites and the presence of toxic substances. Finally, it is an easily available and low cost hydrogel. In an earlier article (Knierim et al., 2014) it was reported on the production of bacteria containing PVA particles with a poly (methyl methacrylate) (PMMA) shell and their characterization by ATIR optical microscopy, SEM and TEM. The functionality of the PMMA shell was established by both the release of fluorescein in buffer and an altered release time of bacteria on agar plates.

Our encapsulated *M. luteus* showed similar results to work done by Wong et al. (2001a)., with cells in 10% polyacrylamide and 2% calcium alginate. In addition, both of our studies show that the "inert" encapsulating material has significant copper trapping ability on its own.

Although electrospun bacteria and those encapsulated in alginate showed similar results, actually the fibers have advantages: 1. The shell polymer is water insoluble providing the opportunity to use it in water based application such as a wastewater filter. 2. This model metal sequestering system only hints at how one may benefit from the hybrid microbe/polymer system, so one may have to broaden the vision of the bioremediation process. For electrospun *Candida tropicalis* when applied for bioremediation of phenolic compounds in olive water waste, it was found that the waste water became less toxic for *E. coli* (Letnik et al., 2015). So in principle, the microbial presence cannot only inactivate or sequester but it may also detoxify.

5. Conclusions

We have shown that *M. luteus* cells were able to absorb a large amount of copper per cell, probably by passive physical mechanism instead of an active biological one. This function of the cells involved cell wall as well as inner cell protein mechanisms. Some 5% of the un-induced cell proteome can interact with copper cations. We demonstrated that encapsulated *Micrococcus luteus* in electrospun fibers and alginate beads are able to absorb large amounts of copper, although with slower rate.

Acknowledgments

We are indebted to the Trilateral Research Project: Germany-Israel-Palestine of the Deutsche Forschungsgemeinschaft (DFG) for the funding of this research. We would like to thank Steffen Reich from University of Bayreuth for his contribution to this work.

Part of the work was done by the EM Unit of Core Research Facility of the Faculty of Medicine and the Hebrew University Center for Nanoscience & Nanotechnology.

IMAC column experiment was done by Dr. Mario Lebendiker from The Protein Purification Facility, Hebrew University. Mass-spec was done by Tevi Mehlman from The Biological Services Unit at The Weizmann Institute of Science. We would like to thank Adi Zisman and Rene Gerard Cruz Galera, Jr. for their contribution to this work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2016.09.029

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