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

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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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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.