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TITLE: REMOVAL OF DISSOLVED HEAVY METALS AND
RADIONUCLIDES BY MICROBIAL SPORES.

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ABSTRACT: Microbial systems have been shown to
remove specific heavy metals from
contaminated aqueous waste to levels
acceptable to EPA for environmental
release. However, systems capable of
removing a variety of heavy metals from
aqueous waste to environmentally
acceptable levels remain to be reported.
The present studies were performed to
determine the specificity of spores of the
bacterium Bacillus megaterium for the
adsorption of dissolved metals and
radionuclides from aqueous waste. The
spores effectively adsorbed eight heavy
metals from a prepared metal mix and from
a plating rinse waste to EPA acceptable
levels for waste water. These results
suggest that spores have multiple binding
sites for the adsorption of heavy metals.
Spores were also effective in adsorbing
the radionuclides ⁹⁰strontium and
¹³⁷cesium. The presence of multiple sites
in spores for the adsorption of heavy
metals and radionuclides makes this
biosorbent a good candidate for the
treatment of aqueous wastes associated
with the plating and nuclear industries.

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Introduction

The removal of dissolved metals from aqueous waste by microbes has been reported by several investigators. The microbial removal mechanisms include, (1) adsorption to cell surface, (2) bioaccumulation, (3) metal reduction and/or, (4) conversion to insoluble metal sulphides (1, 2, 3, 4, 5, 6 and 7). The mechanisms associated with the various microbial species appear to be specific for the different metals. For example, mercury (Hg^{2+}) and cadmium (Cd^{2+}) are associated with bioaccumulation, lead (Pb^{2+}), selenium (Se^{2+}), silver (Ag^{+}) and Hg^{2+} with metal reduction, uranium (UO_2^{2+}) and arsenic (As^{3+}) with cell surface adsorption and Hg^{2+} , Pb^{2+} and Cd^{2+} with the formation of metal sulphides. Based on this information it would appear that when selecting microbial systems for aqueous waste treatment careful consideration must be given to the microbial species and the type of metal to be removed.

We have recently investigated microbial strains with broad specificity for removing metals from aqueous waste. Studies were also performed to determine the removal efficiency for radionuclides by these strains. Of the strains studied, microbial spores showed broad specificity for removing heavy metals and the isotopes ^{90}Sr and ^{137}Cs from aqueous waste. Results showing the application of spores for treating aqueous waste containing toxic metals and radionuclides are described below.

Materials and Methods

Isolation

Bacillus megaterium was isolated from sediments contaminated with Hg, Pb, ^{90}Sr and ^{137}Cs . The pure isolate was maintained at 4°C on agar plates containing nutrient broth made up at 1/10 strength.

Preparation of Spore Suspensions

A loop of surface growth from a slant was used to inoculate "seed" 400 ml flasks containing 100 ml of 50 mM KCl, 1 mM alanine, and 1 mM inosine for 2-12 h. A 3 liter flask containing 2 liters of medium (ingredients in g/liter: glucose, 2; MgCl_2 , 0.1; NaCl, 3; NH_4Cl , 2; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 6; KH_2PO_4 , 3; Na_2SO_4 , 0.11) was inoculated from the seed flask at 10% by volume and incubated at room temperature with aeration for 36 h. Nearly complete sporulation was achieved. The spores were harvested by centrifugation at 10,000 rpm at a flow rate of 0.8 liters per min in a continuous flow centrifuge. The harvested spores were stored in deionized water at 4°C until used. For adsorption studies spores

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were added to the metal mix or plating rinse waste to give a weight ratio of 1/10 (i.e., 100 grams of spores (wet weight) per 1000 grams (or ml) of rinse waste). Spore dry weight was determined by heating a known volume of a spore suspension for 24 h at 105°C on watch glasses of known weight. After heating, the watch glasses were cooled and then re-weighed. The spore dry weight was approximately 20% of the spore wet weight.

Adsorption of Metals by Spores

A solution of metals was prepared containing the following compounds in mg/L: $\text{Hg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$, 20; CdCl_2 , 20; $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, 25; AsO_3 , 20; Na_2SeO_3 , 20; $\text{Pb}(\text{NO}_3)_2$, 25; $\text{Sr}(\text{NO}_3)_2$, 40; and BaCl_2 , 40. The pH of this solution was adjusted to 7.1 with 5N ultra-pure HCl and the solution was filtered. Spores were incubated with the filtered metal mix at room temperature for various time intervals. Aliquots of the culture were removed, centrifuged at 30,000 rpm for 30 min or filtered, and the concentration of the above metals determined in the supernatant (or filtrate) and pellet by inductively coupled plasma spectrometry (ICP). The pellet (or filter) was digested in concentrated ultra pure nitric acid prior to metal analysis by ICP.

Desorption of Metals from Spores

Desorption of metals from spores was accomplished as described by Bender and Marquis (8). Briefly, spores from the adsorption studies were suspended in deionized water and titrated to pH 2 with 5N ultra-pure HCl. They were maintained at this pH for 3 h at room temperature, heat shocked at 60°C for 30 min, centrifuged at 10,000 rpm for 10 min and washed (3x) by centrifugation with deionized water. The supernatant after each wash was collected and pooled, and the metal concentration determined by ICP analysis.

Adsorption of Radioisotopes by Spores

In some studies, the radioisotopes of strontium (^{89}Sr , specific activity 10.2 mCi/mg) and cesium (^{137}Cs , specific activity 87 mCi/mg) were added to a suspension of spores in deionized water or metal mix. The suspension was incubated at room temperature for various time intervals and aliquots were removed, filtered and the radioactivity determined in the filter and filtrate by gamma spectroscopy.

Adsorption Studies of Plating Rinse Waste

The pH of plating rinse waste was adjusted to 7.1

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with ultra pure HCl and the resulting solution was filtered before use. The filtered plating rinse waste was added to a suspension of spores, and this mixture was incubated at room temperature for 24 hours. Aliquots of the mixture were filtered and the concentrations of metals in the filtrates and filters were determined by ICP analysis. The filters were digested as described above.

Results

In previous experiments we have observed that spores added to metal mix in a weight ratio of 1/10 (i.e., 100 grams of spores (wet weight) per 1000 grams (or mls) of rinse waste) and incubated for 240 min gave maximum adsorption for the metals tested in this study. Thus this ratio and incubation time were used in the adsorption studies discussed below. The adsorption by spores of heavy metals frequently associated with environmental pollution is shown in Table 1. The adsorption ranged from 43 to 92% for the metals shown. Spores adsorbed more Hg, Pb and Cr than As or Cd. The cumulative amount of metals adsorbed per gram of spores (dry weight) was 2.5 mg or 2.5% of the total dry weight of spores. Adsorption by spores of the metals listed in Table 1 was not affected by pH in the range of 4.1 to 9.4. Furthermore, the treated spores subsequently germinated on nutrient agar, which would suggest that the spores are resistant to the toxic effects of these metals.

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Table 1 The Adsorption of Dissolved Metals by Spores of *Bacillus megaterium*

Compounds	Initial Concentration (ppm)	Adsorbed μ g metal per gram spore (dry weight)	% of Initial Metal Remaining in Filtrate
HgCl ₂	8.0	370	8
CdCl ₂	20.0	560	44
As ₂ O ₃	16.6	360	57
PbCl ₂	10.0	450	10
CrCl ₃	19.8	760	23

A metal mix was prepared containing the compounds shown above and added to a suspension of spores (weight ratio of spores (wet weight) to metal mix 1/10). This suspension was incubated at room temperature for 240 min and filtered through a 0.45 μ m filter. Metals were determined in the filter and filtrate by ICP analysis. Results are the mean of two experiments.

Results in Table 1 suggested that spores have multiple binding sites for heavy metals. In an attempt to determine the presence of other binding sites in spores, adsorption studies were performed using 8 different metals. These metals were added to deionized water and the pH adjusted to 7.1 with HCl. After filtering this mixture (0.45 μ m filter), the metal concentrations were determined. Based on the amount of metal added to the deionized water (see Methods) several metals in this mixture had limited solubility at this pH (Table 2). Nevertheless, the observed metal concentrations after filtration were sufficient to determine the available binding sites in spores for the metals shown in Table 2. The percentage of the initial metal concentration adsorbed by spores ranged from 15 to 100 for the 8 metals studied. Relative to the initial concentrations, Hg, Pb and Cr showed the highest percentage adsorption. In contrast, Sr and Ba showed the lowest adsorption. However, the absolute amount of these metals adsorbed (in μ g/gram dry weight of spores) was similar to that seen for the other metals (i.e. Hg, 510; Pb, 40; Cr, 100; As, 180; Cd, 610; Se, 720; Sr, 350; and Ba, 280). When the filtrate obtained after spore treatment was re-treated with new spores, the final

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concentrations observed in the filtrate for most metals were below the detection limits.

Table 2 The Adsorption of Dissolved Metals by Spores of Bacillus megaterium

Compounds	Initial Metal Concentration (ppm)	Metal Concentration After Incubation		% Adsorbed
		Spores (mg/100 g spores (wet weight))	Filtrate (mg/liter)	
HgCl ₂	13.3	10.1	1.7	84
As ₂ O ₃	7.0	3.6	3.1	51
PbCl ₂	0.72	0.77	0	100
CdCl ₂	18.0	12.2	7.7	64
CrCl ₂	2.2	2.0	0	100
BaCl ₂	38.1	5.5	29.0	15
SrCl ₂	31.0	7.0	24.4	23
Na ₂ SeO ₃	19.0	14.4	5.0	74

A metal mixture was prepared (as shown in Methods) and added to spores to give a weight ratio of 1/10 (spores to metal mix). The suspension was incubated at room temperature for 240 min, filtered and the above metals determined in the filter and filtrate by ICP. Results are the mean of 3 experiments.

The radioactive isotopes of both strontium and cesium are associated with waste from the nuclear power industry. Their removal from aqueous waste is desirable because of the cost associated with storing radioactive waste. Results shown in Table 2 indicate that spores were effective in adsorbing strontium. In an attempt to expand this observation, the adsorption efficiency of spores for strontium and cesium was determined. As shown in Table 3, spores were more effective in adsorbing ⁸⁹Sr than ¹³⁷Cs. Maximum adsorption for both isotopes was achieved after 240 min of incubation. At 240 min of incubation spores had adsorbed 99% of the available ⁸⁹Sr (specific adsorption was 63 ng ⁸⁹Sr/gram dry weight of spores). The total capacity for spore adsorption of strontium in the absence of other

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competing metals (i.e., Ca and Ba) was 609 μg Sr/gram dry weight of spores. The total capacity for spore adsorption of cesium was 2.7 ng Cs/gram dry weight of spores. We have no evidence that other metals compete for cesium adsorption sites. We have observed that adsorbed strontium can be desorbed with acid (HCl) or molar concentrations of calcium chloride.

Table 3 The Adsorption of Radioactive Strontium and Cesium by Spores of Bacillus megaterium

Compounds	Initial concentration (dpm/10ml)	Exposure time (min)	Spores dpm/gram of spore (wet weight)	Filtrate dpm
$^{89}\text{SrCl}_2$	280,000	60	168,000 (60)	113,000 (40)
		120	244,000 (87)	33,000 (12)
		240	277,000 (99)	1,500 (1)
		480	274,000 (98)	2,700 (1)
$^{137}\text{CsCl}$	320,000	60	80,000 (25)	237,000 (74)
		120	76,000 (24)	229,000 (72)
		240	88,000 (28)	223,000 (70)
		480	82,500 (26)	244,000 (76)

Spores were added to a solution containing ^{89}Sr and ^{137}Cs (1 gram of spores (wet weight)/10ml of radioactive solution), incubated at room temperature, and aliquots removed at various time intervals. After filtering the mixture, radioactivity in the filtrate and filter was determined by gamma spectroscopy. Results are the mean of two experiments. Percent of added metal is shown in the parentheses.

Results shown in Table 4 indicated that spores have a broad specificity for adsorbing dissolved metals

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in aqueous waste. For example, in addition to adsorbing the metals shown in Tables 1 and 2, spores adsorbed zinc, copper, aluminum, cobalt, nickel and uranium from plating rinse waste. Complete adsorption of the metals aluminum, chromium, nickel and copper was observed following an additional treatment with fresh spores. Table 4 also shows the extent to which the adsorbed metals could be desorbed from spores with HCl. Furthermore, we have observed that these acid treated spores can be reused in metal adsorption studies.

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Table 4 Adsorption of Dissolved Metals from Plating Rinse Waste by Spores of Bacillus megaterium

Metal	Initial metal concentration (ppm)	metal adsorbed (μ g metal/g dry weight spore)	metal desorbed
Cr	60	1140 (38)	490 (43)
Zn	0.99	50 (100)	28 (56)
Cd	0.41	210 (100)	84 (40)
Pb	0.38	190 (100)	146 (77)
Cu	3.9	195 (78)	103 (53)
Ni	3.9	195 (66)	80 (41)
Al	60.6	1520 (50)	1064 (70)
Hg	0.15	8 (100)	4.8 (60)
U	0.83	42 (100)	39 (93)
Co	0.15	8 (100)	6.6 (83)

The pH of a plating rinse waste was adjusted to 7.1 with HCl and the solution was filtered. For adsorption studies, the filtrate was added to spores to give a weight ratio of 1/10 (spores (wet weight) to plating rinse waste). After incubating this mixture at room temperature for 24 hours, aliquots were removed and filtered. The desorption of metals from the spores was determined by taking the spores used in the adsorption studies and acid stripping the adsorbed metals. The concentration of metal in spores (after acid digestion) and in the acid leachate was determined by ICP analysis. Percent of metal adsorbed or desorbed is shown in parentheses.

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Discussion

Microbial systems have been and are currently being considered for treating aqueous waste containing a variety of toxic metals. However, unlike chemical and physical systems, microbial systems require careful consideration of the potential toxicity of the metals in the waste to the microbes and of the physiological needs (i.e., nutrients) of the microbe(s) used in the treatment system. Consideration must also be given to type of metal to be treated because microbes frequently develop specific mechanisms for detoxifying heavy metals. For example, variations in the microbial mechanisms of removing heavy metals from aqueous waste have been reported. Various microbial species can effectively remove heavy metals from aqueous media by, (1) cell surface adsorption, (2) chelation by thiol and carboxyl groups (contained in low molecular weight molecules, peptides and/or proteins), (3) reduction of heavy metals, or (4) the conversion of heavy metals to metal sulphides. Since these mechanisms do not all exist in a single microbe, it has been proposed that several microbial strains be combined to treat aqueous waste contaminated with heavy metals. We have studied a bacterial strain that appears capable of removing a variety of heavy metals from aqueous waste. Furthermore, the form of the organism active in our metal adsorption studies is the dormant spore, which does not require nutrients, and appears resistant to the toxic effects of heavy metals.

The removal of heavy metals and radionuclides from aqueous waste by the spores of the bacterium Bacillus megaterium was investigated because spores have been shown to be relatively resistant to a variety of conditions that are frequently encountered in treating aqueous industrial waste (i.e. radiation, temperature, and extremes of pH) (9, 10 and 11). However, the adsorption efficiency of heavy metals and radionuclides in aqueous waste by spores remains to be reported. Results of the present studies show that spores effectively adsorbed those metals frequently associated with industrial aqueous waste, such as Hg, Pb, As, Cd, Se, Cr, Ba and Sr. However, spores were more effective in adsorbing Hg, Pb, Se and Cr than As, Cd, Ba and Sr. The metal adsorption efficiency depended on the concentrations and types of metals present in the mixture. For example, in solutions containing only As, Cd, Ba or Sr, spore adsorption of these metals was complete at concentrations of 20 ppm or less. If Ba was added to a solution containing Sr, the adsorption of Ba

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and Sr was correspondingly decreased. Similar observations of apparent competition for binding sites were made with the groups Hg, As, and Cd, Hg and As, Hg and Cd, and As and Cd. These results suggest that similar adsorption sites in spores exist for Ba and Sr and for Hg, As and Cd. Results likewise suggest that Pb, Se, Cs and Cr have adsorption sites which are different from those for Hg, As, Cd, Ba, and Sr.

Several investigators have observed that spores accumulate Sr and Ba (12 and 13). These metals accumulate in the absence of calcium, which is the metal that normally accumulates in sporulating bacteria. It has been shown that these metals accumulate in sporulating bacteria in conjunction with the synthesis of the spore coat constituent dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA). Furthermore, studies have shown that Ca, Ba and Sr bind to the carboxyl groups of DPA (14). Studies have also shown that Sr will displace Ca bound to DPA. Thus, the relative concentration of Ca in aqueous waste may not affect Sr adsorption. However, we have observed that when the concentration of Ca is 10 times greater than that of Sr, spore adsorption of Sr is reduced by 50%. Thus, the aqueous levels of Ca and the concentration of DPA in spores will affect adsorption of Sr. Several investigators have reported that the thiol concentration in spores is relatively high, which may explain the efficiency of spores for adsorbing Hg, As and Cd (15 and 16). Increasing the concentrations of thiol-containing macromolecules in spores would increase the amounts of Hg, Cd and As adsorbed, because thiol groups are associated with the chelation of these metals (17). We are presently investigating nutritional methods that increase the spore concentration of DPA and thiol-containing macromolecules.

Results from the present studies also showed that the adsorbed metals could be partially or completely desorbed by acid treatment. Furthermore, acid treated spores could be reused for adsorbing metals after neutralization. It was also observed in this study that the spores were resistant to the toxic effect of the metals. Thus spores, unlike other microbial systems, are highly resistant to the toxic effects of heavy metals and do not require nutrients for normal maintenance in an aqueous waste treatment system.

Ion exchange resins are the most frequently used system for treating aqueous waste containing heavy metals. Because of the metal specificity of resins, a variety of resins may be necessary to treat a mixed metal waste. Thus, the adsorption specificity for the heavy metals and radionuclides will in part determine

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whether spores will be more effective than the various ion exchange resins. Studies are currently in progress to determine the specificity and efficiency of spores versus resins for the adsorption of heavy metals and radionuclides from aqueous waste. Operational costs for aqueous waste treatment using spores versus ion exchange resins are also being considered. Based on information of ion exchange resins and the results described above, it would appear that spores have a broader specificity for adsorbing heavy metals and the radionuclides of strontium and cesium.

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