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Accumulation of cadmium by immobilized *Zoogloea ramigera* 115

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SUMMARY

Zoogloea ramigera 115 was immobilized into beads of calcium-alginate and placed into batch air-bubbled column reactors. In the absence of any added nutrients the immobilized bacterium adsorbed Cd from solutions containing levels of 2 and 20 $\mu g\ ml^{-1}$ per day, over a period of 21 and 20 days, respectively. Adsorption of Cd from solutions containing 20 $\mu g\ ml^{-1}$ Cd was better than 90% for 16 days. Beads treated with Cd at 2 $\mu g\ ml^{-1}$ never adsorbed less than 95% of the metal. Alginate adsorbed Cd as well, but inclusion of cells changed the effectiveness of adsorption. Of a 250 $\mu g\ ml^{-1}$ Cd solution, alginate adsorbed 70.4% Cd in 60 min whereas alginate plus cells adsorbed 90.5% in the same time span. Temperature had no effect on adsorption by immobilized cells at levels of 2 and 10 $\mu g\ ml^{-1}$ Cd. However at higher concentrations, binding was enhanced as temperature increased. *Z. ramigera* beads were stable during all treatments and for prolonged periods of time (21 days).

INTRODUCTION

Zoogloea ramigera 115 is a Gram-negative bacterium which forms zoogloeae or finger-like projections due to production of an extracellular anionic polysaccharide [5,18,19]. The bacterial polysaccharide acts as a polyelectrolyte and adsorbs such metal ions as Co^{2+}, Cu^{2+}, Cd^{2+}, UO_2^{2+}, and Fe^{3+} [11,12]. Adsorption of metal cations is possible through phosphoryl, carboxyl, sulfhydryl and hydroxyl groups, which are found on cell wall components, proteins, and lipids [7,14,17]. Metal adsorption by *Zoogloea ramigera* is attributed mainly to its extracellular polymer. The extent of binding is determined by the number of sites available on the extracellular polymer. The zoogloeal polymer, which is composed of D-glucose, D-galactose and pyruvic acid should allow abundant binding due to its slight negative charge and its many hydroxyl groups [6]. Cadmium is of interest because it has been implicated as a mutagen, carcinogen and teratogen [3]. This heavy metal is naturally present in the earth's crust at $<0.15\ \mu g\ ml^{-1}$, but continues to increase in the environment [4,15,16].

Other authors have attempted to use the metal-binding capacities of microorganisms for decontamination of industrial wastes. Dead *Zoogloea* have been used to remove Cd^{2+}, Cu^{2+} and UO_2^{2+} from solutions [11]. *Citrobacter* sp. has been studied extensively in Cd removal. When the bacterium is immobilized in polyacrylamide it removes Cd when glycerol 2-phosphate is included in the metal-containing solutions. The substrate induces a cell-bound phosphatase which precipitates the metal on the bacterial cell surface as metal phosphate [9,10]. A patented process, called AMT-BIOCLAIM, uses treated, granulated *Bacillus subtilis* to remove metals from industrial effluents [2]. The purpose of this study was to develop a way in which to use *Zoogloea ramigera* to detoxify liquid wastes. In order for a biological metal removal system to work on an industrial scale, the organism used has to be readily contained and the set-up has to be reusable. *Zoogloea ramigera* was immobilized in calcium-alginate to take advantage of the metal-binding properties of its extracellular polymer and the metal-binding capacity of the suspending alginate. This study considered immobilized *Zoogloea ramigera* and characterized the optimum pH and temperature conditions for binding or adsorption of cadmium to the cells, their exopolymer and the alginate.

MATERIALS AND METHODS

Strains and Media. Zoogloea ramigera 115 (ATCC 25935) was obtained from The Ohio State University

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Culture Collection, Columbus, Ohio. *Z. ramigera* was grown in a defined medium (DM) [12] containing 0.5 g/l arginine-HCl (Sigma Chemical Co., St. Louis, MO), 1.0 g/l alanine (Sigma), 0.2 g/l $MgSO_4 \cdot 7H_2O$, 2.0 g/l K_2HPO_4, 1.0 g/l KH_2PO_4, 20 g/l glucose (Cerelose-Dextrose 2001 (CPC International Inc., Englewood Cliffs, NJ)) and 0.002% yeast extract (instead of vitamin B_{12} used by Parsons and Dugan [12]). The organism was grown on a rotary shaker at 28 °C, and at 150 rpm. Media were inoculated with 0.1 ml from stock cultures (approximately 10^9 cells per ml) stored in liquid nitrogen.

Atomic absorption spectrophotometry. Cd was quantified using a Perkin-Elmer atomic absorption spectrophotometer Model 403 equipped with an air-acetylene flame. Liquid samples were assayed to determine the amount of Cd which had not been adsorbed by the immobilized cells. Samples were not pretreated since the pH of liquids was always below the range at which Cd solubility changed. Liquid rather than bead samples were used to maintain a constant bead volume in the reactors.

Immobilization of Zoogloea ramigera. Zoogloea ramigera was cultured overnight in 500 ml DM medium, harvested by centrifugation at 10400 × *g* for 10 min and the pellet suspended in 20 ml 0.85% NaCl. The resuspended cells were added to 250 ml 2% high viscosity sodium alginate (Sigma, final concentration: 1.85%) in 0.85% NaCl and mixed. *Z. ramigera* was immobilized by forcing the cell-alginate mixture through 25 gauge needles into 250 ml of a 1.47% $CaCl_2$ solution using a variable speed pump. Once immobilization was complete, the cell-containing alginate beads were allowed to harden for 2 h before they were stabilized with 250 ml 1% polyethyleneimine, pH 5.0 (PEI (Polysciences, Warrington, PA), in double-distilled H_2O), for 5 min and washed with 3 l of double-distilled H_2O. Beads of alginate were formed as those containing cells, except that 20 ml 0.85% NaCl without cells was added to 2% alginate. Beads with or without cells were approximately 2.5–3.0 mm in diameter. After stabilization the immobilized cells were placed into a bubble column reactor in DM medium for 18–24 h, before exposure to solutions containing metals. Unless specifically mentioned, solutions were not buffered and were made from concentrated stock solutions of Cd in the form $CdCl_2 \cdot 2H_2O$. All experiments were run as batch processes and not as continuous flow experiments.

Bubble column reactor. A glass tube (Dimensions: diameter 9 cm, height 29 cm, sampling port 8.5 cm from the bottom), containing a ground glass filter at the bottom and sealed with a rubber stopper at the top, was used for all experiments. A hollow rod, covered with nylon netting, was inserted through the stopper to reach the bottom of the column. This tube was used to pump liquid in and out of the reactor, and to obtain samples for Cd assays. The sampling port was used only to remove solution samples during temperature experiments. The port was covered with glass wool on the inside of the column to prevent loss of beads during rapid sampling. The column was aerated, at 250 to 350 cc/min, with compressed air forced through the ground glass filter at the bottom of the reactor. All volumes for metal exposure and wash were arbitrarily set at 500 ml. Although the capacity of each column was approximately 2 l, the working volume was generally 750 ml, 500 ml of liquid and 250 ml of beads.

Cd adsorption by Ca-alginate beads and alginate-immobilized bacteria. Beads containing *Z. ramigera* and alginate beads were generated and treated as described above. Both sets of beads were exposed at room temperature to solutions containing 2, 20, 50 and 250 $\mu g\ ml^{-1}$ Cd in bubble column reactors.

Fill/draw studies. Three different sets of immobilized *Z. ramigera* were exposed daily to 500 ml solutions containing 0.5, 2, or 20 $\mu g\ ml^{-1}$ Cd. No nutrients were supplied to the immobilized cells during the experiments which lasted up to 21 days.

The effect of temperature on Cd adsorption. Immobilized cells were placed into batch reactors at 10 °C, 25 °C and 37 °C. Temperature was achieved by placing the columns into a water bath, so that the internal reactor temperature was as desired. All solutions added to the reactor were prewarmed or precooled. The immobilized cells were pretreated twice with 2 $\mu g\ ml^{-1}$ Cd solutions for 20 min each.

The effect of pH on Cd adsorption. Optimum pH was determined using double-distilled H_2O, 15 mM 2-*N*-[*N*-morpholono]ethanesulfonic acid (MES), pH 6.0 (Sigma), 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.0 (Calbiochem-Behring, La Jolla, CA), and 15 mM Tris, pH 8.0 (Sigma). Solutions containing 10, 20 and 50 μg Cd ml^{-1} were made with these buffers.

RESULTS AND DISCUSSION

Many authors have used bacteria for the removal of Cd and other metals from polluted waters [9–12]. The experiments described here were designed to make use of the metal-binding properties of *Z. ramigera* by immobilizing the organism in Ca-alginate beads. Immobilization did

TABLE 1

Cadmium adsorption by Ca-alginate beads and alginate-immobilized bacteria

Time (min)	Metal adsorption (%) from solutions containing Cd at levels of:		
	20 μg ml^{-1}	50 μg ml^{-1}	250 μg ml^{-1}
Alginate			
10	24.1	32.4	24.6
20	28.1	49.4	29.5
30	35.4	62.2	47.9
40	ND	70.3	55.1
50	ND	73.6	58.4
60	ND	79.7	70.4
Alginate + cells			
10	85.5	88.5	66.7
20	94.1	92.4	78.3
30	96.8	94.6	85.1
40	96.8	95.0	88.1
50	97.1	95.3	89.7
60	97.6	95.4	90.8

ND, not determined.

away with the need for centrifugation or flocculation since the beads settled quickly. Beads were stabilized using polyethyleneimine (PEI) as a crosslinking agent agent.

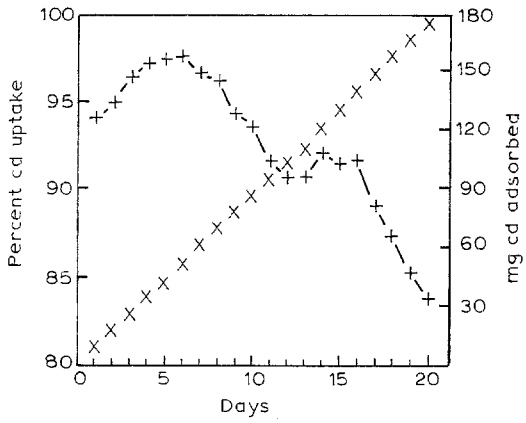

Fig. 1. Cadmium adsorption by immobilized *Z. ramigera* exposed to 20 μg ml^{-1} Cd daily for 20 days. Three-ml samples were taken every 10 min for 60 min after Cd solution was first added. After initial overnight growth in DM medium no nutrients were supplied. +, percent Cd adsorbed; ×, mg Cd adsorbed.

After earlier experiments indicated that *Z. ramigera* immobilized into Ca-alginate beads would adsorb Cd, we set out to characterize these *Z. ramigera*-containing beads.

Cadmium adsorption by Ca-alginate beads and alginate-immobilized bacteria. Alginate, a mixture of polyguluronic and polymannuronic acid, has abundant hydroxyl groups [13]. Since alginate was chosen as the immobilization matrix it was important to determine its metal-binding capacity. Experiments showed that, after exposure to a solution containing 2 μg ml^{-1} Cd for 30 min, alginate adsorbed 54% of the Cd, whereas Ca-alginate immobilized *Z. ramigera* adsorbed at least 97% of the metal (data not shown). Exposure of alginate beads to solutions containing 20, 50 or 250 μg ml^{-1} Cd compared to Ca-alginate immobilized *Z. ramigera* (Table 1) clearly showed that the bacterium contributed to metal adsorption. After 30 min *Z. ramigera*-containing beads adsorbed at least 30% more Cd than alginate alone. Over the next 30 min beads containing cells adsorbed only about 14% more Cd than beads alone. The amount of Cd adsorbed by alginate alone increased with time, but did not reach the levels achieved with Ca-alginate immobilized *Z. ramigera*, indicating that the addition of *Z. ramigera* made the alginate beads more efficient. Alginate contributed less to the overall metal binding efficiency when Cd concentrations were less than or equal to 20 μg ml^{-1}. At any concentration, the inclusion of *Z. ramigera* reduced metal solution contact time, which will be advantageous when large volumes need to be processed. Bacteria-containing beads were exposed to growth medium prior to initial exposure to Cd. The bacterium, perhaps due to oxygen limitations, grew near the inside surface of the alginate bead. So in addition to the alginate matrix there was a layer of bacteria which had produced metal-adsorbing polymer for 24 h. In alginate beads Cd had to diffuse inward to reach more metal binding sites thus generating a more linear response to Cd than cell-containing beads which have more binding sites at their surface. This may explain the more rapid binding of Cd by beads containing *Z. ramigera*.

Fill/draw studies. Ca-alginate immobilized *Z. ramigera* repeatedly exposed to 20 μg ml^{-1} Cd-containing solutions, adsorbed >90% Cd for 16 days, and after 20 days they were still 83.7% efficient (Fig. 1). At that point the beads in the reactor had been exposed to 376.3 μg ml^{-1} Cd (total: 0.188 g) and of that they removed 347.8 μg ml^{-1} (total: 0.174 g). Immobilized cells in the reactor were

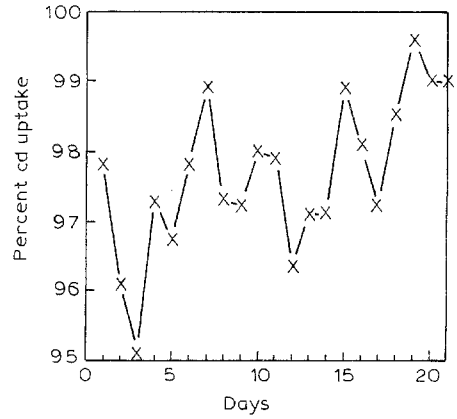

Fig. 2. Cadmium adsorption by immobilized *Z. ramigera* exposed to 2 μg ml^{-1} Cd daily for 21 days. Three-ml samples were taken every 10 min for 30 min after Cd solution was first added. After initial overnight growth in DM medium no nutrients were supplied.

exposed to 2 μg ml^{-1} Cd 21 times (Fig. 2). The binding efficiency was never lower than 95%. At termination the beads' maximum Cd-binding capacity had not been reached. Similarly, Fig. 3 demonstrates adsorption of 0.5 μg ml^{-1} Cd each day for 4 days by *Z. ramigera* immobilized into beads. This experiment was not continued for a longer period of time, since at 0.5 μg ml^{-1} the immobilized cells could have been exposed to Cd for at least 600 days (or a total volume of 300 l of 0.5 μg ml^{-1} Cd-containing solutions (based on values obtained for the beads exposed to 20 μg ml^{-1})). The problem of prolonged exposure times at low concentrations could have been solved through the use of a continuous flow system. Such studies were done and showed the same efficient metal adsorption (data not shown). The present experiments were done in batch bubble column reactors to test the longevity of the cell-containing beads, in addition to Cd adsorption. The advantage of using PEI-crosslinked beads was their stability.

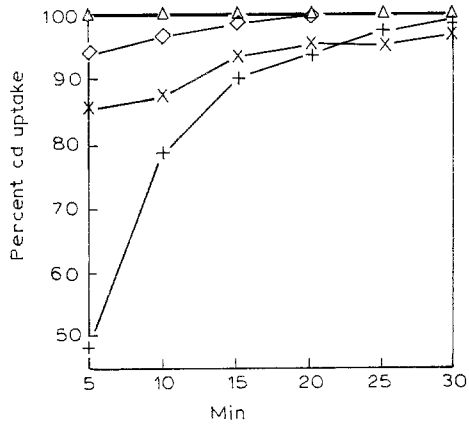

Fig. 3. Cadmium adsorption by immobilized *Z. ramigera* exposed to 0.5 μg ml^{-1} Cd daily for 4 days. Three-ml samples were taken every 5 min for 30 min after Cd solution was first added. After initial overnight growth in DM medium no nutrients were supplied. +, 1st day; ×, 2nd day; ◇, 3rd day; △, 4th day.

The effect of temperature on Cd adsorption. Ca-alginate immobilized *Z. ramigera* were placed in columns incubated at 10, 25 and 37 °C. Cd adsorption proceeded very rapidly at 2 (data not shown) and 10 μg ml^{-1} and was temperature independent. Fig. 4 shows adsorption of Cd by immobilized *Z. ramigera* when exposed to solutions containing 10, 50, 100, 250 μg ml^{-1} Cd at 10, 25, and 37 °C. There was a difference in the amount of metal adsorbed after 20 min (Fig. 4b–d) at 50, 100, and 250 μg ml^{-1} Cd. At 37 °C the time necessary to adsorb a specific amount of Cd was decreased. There was a bigger difference in the percentage of metal bound between 37 and 25 °C than between 25 and 10 °C. This variation was not readily explained. The differences in adsorption at 25 and 10 °C were expected to follow the results seen for 37 and 25 °C. As temperature increased, the amount of Cd

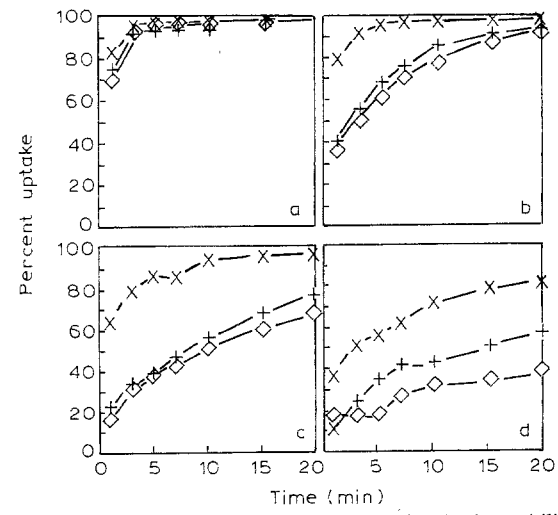

Fig. 4. Effect of temperature on Cd adsorption by immobilized *Z. ramigera* exposed to solutions containing 10 (a), 50 (b), 100 (c) and 250 (d) μg ml^{-1} Cd at 10, 25 and 37 °C. Three-ml samples were taken at 1, 3, 5, 7, 10, 15, and 20 min after addition of solution. ◇, 10 °C; +, 25 °C; ×, 37 °C.

adsorbed was expected to increase since diffusion increases at higher temperatures. However, it was expected that the increase seen between 10 and 25 °C would be closer to the increase seen from 25 to 37 °C. Aside from the temperature variations all beads were treated identically. There was no direct relationship between the amount of Cd adsorbed and the temperature. Kinetic rate calculations could not be made, since the experimental set-up did not permit more rapid sampling. The experiments did show, that optimum Cd adsorption could be achieved at 37 °C in the shortest period of time, when Cd concentrations were $> 10\ \mu g\ ml^{-1}$. Immobilized cells at all temperatures were preexposed twice to solutions containing $2\ \mu g\ ml^{-1}$ Cd for 20 min each. This was a direct result of experimental observations, in which it was noticed that at low concentrations of Cd ($< 2\ \mu g\ ml^{-1}$), initial adsorption was lower than expected. Fig. 3 shows this phenomenon with $0.5\ \mu g\ ml^{-1}$ Cd. Binding of Cd to the zoogloeal polymer and to alginate appeared to occur sequentially. At low concentrations the results obtained indicated coordinated binding. Pretreatment with a total of $4\ \mu g\ ml^{-1}$ Cd assured a uniform response to subsequent metal exposure.

The effect of pH on Cd adsorption. Attempts to buffer the metal solutions are shown in Table 2. Hepes buffer provided the best conditions, but, at 15 mM, Hepes did not maintain the pH at 7.0. It dropped to between 6.4 and 6.8. The conditions in Hepes approached those of Cd in double-distilled H_2O. Cd solutions were between pH 6 and 7 before addition to the reactors and after contact with the beads the pH of the solutions was approximately pH 6.0. The ability to remove Cd was not hampered at pH 8.0, but was reduced at pH 6.0 (Table 2). This may have been due in part to interference by MES, since the solutions without buffer performed well at this pH. Attempts to control the pH were deemed unnecessary, since there was no significant increase in Cd adsorption when the Cd solutions were buffered. At pH 8.0 most of the Cd is present as Cd^{2+}. Significant speciation to $Cd(OH)^+$ does not occur until a higher pH [1]. The ionic strength of Cl has an effect on Cd speciation [1]. At most concentrations used Cd was present in its free form (Cd^{2+}). At the high concentrations some of the Cd was probably present as $CdCl^+$, but this did not have an effect on adsorption of Cd by immobilized cells.

TABLE 2

The effect of pH on cadmium adsorption

Time (min)	Metal adsorption (%) in buffers (pH):			
	MES (6.0)	Hepes (7.0)	Tris (8.0)	Double-distilled H_2O (6.5)
$10\ \mu g\ ml^{-1}$				
10	81.1	96.0	82.0	97.1
20	90.6	97.1	94.5	97.7
30	91.4	98.5	95.5	98.8
$20\ \mu g\ ml^{-1}$				
10	84.9	97.4	87.2	85.5
20	92.4	98.0	96.7	94.1
30	93.8	98.6	98.3	96.8
$50\ \mu g\ ml^{-1}$				
10	83.0	93.2	75.6	85.6
20	91.1	97.2	97.3	94.4
30	94.0	97.2	97.9	95.7

While results presented are not averages, they are typical of experiments with the immobilized *Z. ramigera*. The cell-containing beads did not conform to conventional methods for metal adsorption analysis. The amount of Cd adsorbed per gram of cells or per gram of beads was not determined. The cell-containing beads did not conform to such analyses. Cell number was difficult to determine due to the zoogloeal extracellular polymer. The bacteria-containing alginate beads were in a dynamic state. Cell number and polymer/bead increased after overnight growth, yet bead dry weight actually decreased. However, addition of *Z. ramigera* to alginate increased metal adsorption and their use should be exploited even if they did not conform to standard procedures to record metal removal. The immobilized cell system has potential industrial application value and is in the process of further evaluation.

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