Cadmium Removal with Mutant *Brevibacillus Agri* C15 Cd<sup>r</sup> Entrapped in Calcium Alginate Gel: a New Process

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**Abstract.** With large-scale mining and industrial use of cadmium (Cd), contamination with this metal increased steadily, concerns due to its toxicity and potential damage to the environment and human health have led to the introduction of legislation that regulates acceptable environmental concentrations in different contexts. Therefore, many treatment methods for water and soils have been developed to limit the concentration of Cd in the environment and comply with regulations. In this study, a small laboratory bench-scale column reactor was constructed, optimised, and used to evaluate the removal of Cd from artificial groundwater (AGW) with mutant *Brevibacillus agri* C15 Cd<sup>r</sup> and its wild type *B. agri* C15 entrapped in calcium alginate beads at different Cd concentrations. The morphological properties of the beads were studied by scanning electron microscopy (SEM), and energy-dispersive X-ray (EDX) spectroscopy and the location of Cd adsorbed in the beads was detected using a dithizone histochemical method. The experimental results showed that the mutant had significantly higher removal rates than its wild type *B. agri* C15 (9 and 5 nmol per day per gram of biomass, respectively) due to the presence of the Cd-dithizone complexes on the bead containing the mutant, compared with the less presence of these complexes on the beads containing the wild type. In conclusion, a new process was developed that achieved higher Cd removal rate from AGW by the mutant *B. agri* C15 Cd<sup>r</sup>. In addition, an alternative detection method of Cd (Cd-dithizone complexes) was introduced that showed that Cd was distributed throughout the Cd-loaded beads.

1. Introduction:
Cd is considered as a serious environmental issue because of its toxicity, non-biodegradability and bioaccumulation [1]. The contamination of groundwater with Cd has become a global challenge, and more effective methods for removing Cd are required. Several treatment technologies, such as precipitation, adsorption, ion exchange, and bioremediation, are used for this purpose [2]. Among these technologies, the bioremediation—the use of bacteria to remove Cd from water—is known as one of the processes applied for removing Cd from solutions and water, and this can be achieved in both batch and reactor experiments. Microbial mass, on its own, is not a mechanically stable material to be used for an effective process within a reactor. Therefore, to protect the bacterial cells against washing out, caused by the circulating fluids in the reactor or associated turbulence and shear force, the immobilisation of bacterial cells is required [3]. In recent times, bioremediation processes have employed immobilisation methods...
to achieve highly efficient pollutant removal, a reduction in the risk of cell mutations, an increase in the resistance of biocatalysts to toxic compounds, and an increase in the survival rate of the biocatalyst during the treatment [4]. Although the immobilisation of the cells is an additional processing step, it is a cost-effective preparation method for high-stability laboratory operations. In recent times, bioremediation processes have employed immobilisation methods to achieve highly efficient pollutant removal, a reduction in the risk of cell mutations, an increase in the resistance of biocatalysts to toxic compounds, and an increase in the survival rate of the biocatalyst during the treatment [4]. These carrier materials provide functional groups in their structure, mainly hydroxyl and amine groups, which allow for direct interaction between the immobilised cells and metal cations, thus, facilitating the vital uptake of metals [5]. These materials are renewable and easy to obtain; in many cases, they are by-products of various algae, making them low-cost, reducing the expenses associated with the immobilisation process. Sodium alginate is one of the most common matrices for the entrapment of cells for the accumulation of toxic materials by microbial cells. The alginate is less toxic than other polymers; it is an anionic polysaccharide that provides attractive properties for gel formation, concentration, and stabilisation [6]. Sodium alginate, with an acid dissociation constant (pKa) of 1.5 – 3.5, has been used to treat metal and other substances from water and wastewater as reviewed by Wang [7]. Therefore, the main purpose of this study was to investigate the Cd removal by Ca-alginate beads containing bacterial cells that are resistant to Cd and sodium alginate as a bacterial support matrix. The active cells of mutant B. agri C15 CdR [8] were subjected to the process of Cd removal along with its wild type B. agri C15 [9] in addition to their boiled-cells (called killed-control). Since the Ca-alginate beads, without bacterial cells, were identified as a good Cd-adsorbent [10], their ability to remove Cd in this reactor was investigated first, functioning also as an experimental control that evaluates the contribution of Cd removal by the Ca-alginate beads versus by the bacterial cells. The investigation was carried out with the objectives: setting up and operation of the column reactor for the removal of Cd for 28 days, followed by the evaluation of the Cd removal rates at higher concentrations of Cd (8.8, 13.4, and 17.3 μM) for several days. The morphology of the Ca-alginate beads containing bacterial cells before and after the removal was observed with SEM, while the distribution of Cd in the beads was observed under a light microscope, using a dithizone histochemical method.

2. Materials and Methods:
2.1 Removal of cadmium from AGW in a small laboratory bench-scale column reactor
The reactor system was consisted of three columns (10 mL), which had a 1.60 cm inner diameter and was 8.63 cm long (high-grade polypropylene); it was run by the up-flow pump-fed system, using a peristaltic pump (model MCP 3 channel, Pharmacia Fine Chemical Company) to pump Cd-AGW from one bioprocess bag. The eluants to be collected from each column separately. AGW was used as a source of Cd removal with a similar real groundwater composition to the study of removal under controlled composition. AGW was used as a source of Cd removal that has the same groundwater composition to that studied in the removal under a controlled composition. AGW was prepared in ddH₂O. The weight method was used to measure the total volume of the column reactor by fitting the adapter caps firmly on the top of the three columns and pumping the AGW into the columns. The column head (adapter) was removed, and the AGW was transferred into a beaker. The mass of AGW was determined by measuring the mass of holding AGW based on density (ρ) of AGW, which was determined using a pycnometer (50.329 mL at 22 °C). The pump flow rates were calibrated using the column reactor (Error! Reference source not found.).
The flow rate curve at different settings of the peristaltic pump pumped through the column reactor filled with Ca-alginate beads. The error bar represents the standard error of the mean (n = 1, one reactor with three columns).

The hydrodynamic residence time (HRT) of the reactor system was calculated based on the volume occupied by the beads. Two methods were used to determine the volume of the selected beads. The first method was determined by measuring the mass of each column by filling them with AGW and beads. The difference between the volumes of the total liquid of an empty column and the volume of the filled column is the volume of the beads. The precise method of measuring the volume of the beads was determined by measuring the total areas of all beads that were placed in the column after initially measuring the diameter and the volume of each bead. After the volumes of the beads were determined, AGW liquid holdup was determined from the differences between the total volume of AGW and the volume of the number of beads that fitted into the columns. Then, the HRT was determined by dividing the value of the liquid held with the value of the flow rate.

In Cd removal experiments, AGW (pH 7.00) with a nominal concentration of 4.4 µM Cd, chosen from the mean levels of Cd-contaminated freshwater reported in the literature, was pumped into five column-packing materials (n = 1), one reactor with three columns):

1. Ca-alginate beads containing live cells of B. agri C15,
2. Ca-alginate beads containing live cells of B. agri C15 Cd⁸,
3. Ca-alginate beads containing killed-control cells of B. agri C15,
4. Ca-alginate beads containing killed-control cells of B. agri C15 Cd⁸, and
5. Control Ca-alginate beads without bacterial cells.

Reactor systems were in operation for 28 days, and samples were collected daily from eluants to measure the total concentration of Cd.

The embedding of live or killed cells in the Ca-alginate matrix was carried out. Cells of B. agri C15 and B. agri C15 Cd⁸ were immobilised using an entrapment method with Ca-alginate gel (Li et al., 2018). Sodium alginate powder (3 g) was dissolved in 100 mL of sterilised ddH₂O by stirring for one h at 60 °C and was added to 100 mL of mixed cell suspensions-AGW, without CaCl₂. The cell suspension was obtained by growing strains in EBS/pyruvate (20 mM), with a nominal concentration of Cd (13 mM Cd for B. agri C15 and 18 mM for B. agri C15 Cd⁸) and incubated shaking at 100 rpm, 37 °C. From the late exponential phase, the cells were cooled in ice for an hour, centrifuged, washed with, and suspended in AGW without CaCl₂. The obtained
sodium alginate cells were mixed using a sterilised glass rod for 10 mins, loaded into a syringe (size 1mL) without the needle and dropped into CaCl₂ solution (100 mL, 2% w/v). The dropped mixture turned into beads within a few seconds. After an hour, the formed beads were hard, removed using a sieve, and washed to remove any CaCl₂ with sterilised ddH₂O. Finally, the beads were dried, transferred into the tube (50 mL, Falcon), stored at 4 °C and used within 48 h. Boiled-cells (killed-control cells) were used as adsorbents for Cd. To embed killed-control cells in calcium alginate matrix, estimates of the optimal killing times of both strains, which caused 100% of killing the cells, were obtained. The killed-control cells were obtained through the incubation of suspension cells at 100 °C in a water bath for 15, 30, 45, 60, and 90 mins, and a mercury-thermometer in a glass was used to measure the temperature of the cell suspensions. The cells from each incubation were spread on Reasoner's 2A (R2A) agar, incubated at 37 °C for 16 h, along with controlled cells, which were incubated at constant room temperature (22 °C). The CFU per plate was counted, and the percentage of CFU against the times of killed-curve was plotted. After the determination of the time that caused 100% of killing cells, the cell suspension, which boiled at 100 °C for the optimal boiled time, was mixed with AGW, without CaCl₂ and with alginate solution (100 mL) in a 250 mL beaker. To obtain Ca-alginate beads without bacterial cells, AGW without CaCl₂ was used instead of bacterial cell suspension, and the preparation of the alginate beads in CaCl₂ was performed as explained above. The mean volume and the mass of the beads were determined, and the diameter of all types of beads was measured using a micrometer. The bead diameters were recorded and presented in diameter distribution curves (Figure 2 (a, b, c, d, e)). The diameter values that have 50% in the distribution curve were chosen as the best bead diameters to be filled in the column reactor. The mean of the selected diameters was determined within the standard deviation (SD) of 200 beads. The diameters ranged from 0.5 mm to 5.0 mm, and a mean diameter of 2.9 mm (SD = 0.8266 mm) was obtained. Most beads (23%) showed a mean diameter ranging from 3.2 – 3.4 mm, and 50% of beads between 2.5 mm and 3.5 mm. The masses of the selected beads (50% of diameter values in the distribution curve) to be used in the column reactor were measured to normalise the recorded concentrations of cadmium. A sensitive analytical balance (Pioneer, Ohaus Company) was used to measure bead masses, and sterile forceps were used to hold and measure each bead. In addition, the different bead masses were recorded and presented in histogram charts, combined with the distribution curves. The distribution of bead diameters (Figure 2 (f, g, h, I, j)) showed a mean mass of 53 mg (SD = 64 mg) with a smaller mass distribution compared to diameter distribution. The bead mass ranged from 35 to 65 mg, and 30% of beads showed an average mass of 55 mg, with 50% of beads between 48 mg and 55 mg. After the beads were selected, they were stored in Falcon tubes, kept at 4 °C, and used in the column reactor within 48 h.
Figure (2) Characterisation of typical Ca-alginate beads containing live and killed-control cells of *B. agri* C15 and *B. agri* C15 Cd\textsuperscript{5}, and Ca-alginate without bacterial cells. The mean of the measured diameters and masses of the beads were determined within the standard deviation (SD) of the total number of 200 beads. a, b, c, d and e histograms of bead diameters measured by a micrometer and a mean of diameter was 2.9333 mm (SD = 0.8266 mm). The respective histograms of bead masses measured by a sensitive analytical balance and a mean mass was 53 mg (SD = 0.64 mg) as displayed in f, g, h, i and j.
The Ca-alginate beads containing killed-control cells of *B. agri* C15 and *B. agri* C15 Cd^R^ were obtained after incubating the strain cells at 100 °C in a water bath for 15, 30, 45, 60, and 90 mins. These incubations at different temperatures were carried out to estimate the optimal kill times of both strains, which caused 100% cell death. The results showed that 30 mins of boiling were enough time to kill 100% of the cells (Error! Reference source not found.). Therefore, this time was chosen as the boiling period for both strains at 100 °C to obtain inactive cells (killed-control).

Figure (3) The lethal time curves of (♦) *B. agri* C15 and (◊) *B. agri* C15 Cd^R^. 100 mL of a diluted sample of a mixed bacterial cell-AGW was boiled in a water bath at 100°C for 15, 30, 45, 60, and 90 mins. Control of a mixed bacterial cell-AGW was incubated at constant room temperature (22 °C) for 90 mins. The boiled and controlled cell-AGW were spread on R2A agar and incubated at 37 °C for 16 h. The total viable count (CFU/mL) was counted to determine the percentages of CFU. The percentages were the mean, and error bars indicate the standard error of the mean of three independent experiments (n = 3). After optimising the reactor system, as shown in s were identified and conducted.

Table, reactor experiments were identified and conducted.

Table (1) The conditions of the experimental setup and operating of the study reactor.

| Parameter                  | Value                        |
|----------------------------|------------------------------|
| AGW density                | 1.0                          |
| Diameter of bead           | Range: 3.2 – 3.4 mm          |
| Mass of bead               | Range: 40 – 50 mg            |
| The volume of liquid holdup| Mean: 4.5 ± 0.6 mL           |
| The total volume of bead   | Mean: 3.7 ± 0.25 mL          |
| Flow rate                  | Mean: 40 ± 0.32 µL/min       |
| Hydraulic residence time   | Mean: 112.5 ± 0.9 min        |

Under the same optimised process, the effects of different Cd concentrations (8.8, 13.4, and 17.4 µM Cd) in AGW on Cd removal were investigated by all types of Ca-alginate beads for 10
days, including the collection of samples from eluants every day. Following collection, samples were acidified immediately with 1% nitric acid, and indium (in final concentration, 0.43 µM) was added to be used as an internal standard for ICP-MS. The total concentrations of Cd in samples were determined as described in ‘Elemental Analyses’. These concentrations were normalised per dry biomass (mg) according to He and Chen (2014) by firstly determining the amount of dry biomass (mg) of cells in the suspension per mass of beads formed by the suspension of cells (mg dry biomass/mg bead) (except Ca-alginate beads without bacterial cells [per beads]). Then, the concentration of Cd was normalised, per mass of beads in a column, to determine the amount per the volume of water holdup. The tangent lines were applied to the curves to give approximate rates of Cd removals during the initial time of operating the reactor.

2.2 SEM bead analyses

The beads, loaded with Cd-AGW, were drained and washed with ice-cold physiological normal saline. After draining water from the column, the column was closed from the top using dental wax, and from the bottom using a blue Tack and kept at −20 °C for the fixation process before SEM observations. Single beads (six for each type and treatment) were selected and cut in half with a sterilised blade. The beads were then subjected to DEC fixation (n-(3-dimethyl aminopropyl)-n’-ethyl carbodiimide), as described in [11].

2.3 Distribution of cadmium in the beads using a dithizone histochemical method

It is well known that dithizone is used for Zn histochemistry and chelates Mg, Fe, Co, Pb, Hg, and Cd [12]; therefore, dithizone was used for the localisation of Cd within the beads.

To prepare beads for histochemistry, exposure of the beads to Cd was carried out as part of a batch of adsorption tests. For this, Ca-alginate beads containing live cells of B. agric C15 and B. agric C15 CdR and control Ca-alginate beads not containing bacterial cells were subjected to this assay. The Ca-alginate beads were prepared using the entrapment method in Ca-alginate gel, as described above.

Cd uptake into Ca-alginate beads was obtained from AGW with a nominal concentration of pyruvate (20 mM) and 10 mM Cd. The adsorption experiments were carried out in flasks (250 mL Erlenmeyer flask), incubated at 22 °C for 48 h, which were stirred regularly using a glass rod. After two days of the adsorption, the beads were collected from each batch using a sieve, rinsed quickly with physiological normal saline, and transferred into a Falcon tube for the histochemical procedure. The histochemical method for Cd localisation in the beads involved the dehydration of the beads using a tissue processor (Leica, TP 1020), which usually takes 12 to 24 h. The beads were held in mesh biopsy cassettes to be placed on the tissue processor in graded percentages of ethanol: 50%, 70%, 90%, and 100%. The beads were embedded in paraffin using an embedder (Leica EG 1150H). A microtome sectioner (Leica RM 2235) was used to cut the embedded beads to a thickness of 20 µm. After the sectioning, the paraffin sections were transferred onto slides, without being flattened on water, and the sections were fixed onto the slides using a hot plate before clearing in xylene over three changes for 10 mins. The staining of Zn and Cd in the beads was carried out, according to Kiernan (2008), using mixed dithizone solutions. For the staining of Zn, the reagent consisted of 24 mL of solution A (100 mg dithizone and 100 mL anhydrous acetone), 5.8 mL of solution B (55 g sodium thiosulphate, 5.9 g sodium acetate, potassium cyanide 1.0 g and 100 ddH2O), 2 mL of solution C (2 mL of acetic acid (1.0 M), 0.2 mL of solution D (sodium potassium tartrate solution, 2%w/v), and 18 mL ddH2O. CCl4 was used to extract traces of Zn, which occurred in solution B, by using a separatory funnel. The dithizone reagent used for the staining of Cd consisted of 24 mL of solution A and 14 mL ddH2O. During the staining procedure, the mixed dithizone solution was applied to the slide for 10 mins, followed by rinsing in two changes of chloroform for 30 s. The slides were then washed in water and left to dry. Before staining for Cd, Zn was removed by applying 1% acetic acid to the beads. Observations of Zn or Cd in the beads were carried out under a light microscope (Leica).

2.4 Elements analyses
The uptake of Cd from AGW by the beads was analysed by determining the concentrations of Cd remaining in the eluants after operating the reactors using inductively coupled plasma mass spectrophotometry (ICP-MS, Thermo Scientific, X Series 2). The instrument’s limit of detection (LOD) of Cd was established from the five times the standard deviation (SD) of measuring the lowest standard \( n = 10 \) at 0.02 \( \mu \text{M} \). Certified Reference Material (CRM, drinking water, EnviroMAT, Cat 140-025-031) was analysed to verify the recovery of the Cd analysis; the percentage recovery of ICP-MS via the use of CRM was \( 100 \pm 0.03 \%), which was calculated from the recorded \( 2.34 \pm 0.03 \mu \text{M} \text{Cd} \) and certified \( 1.7 \pm 0.01 \mu \text{M} \text{Cd} \) values of the reference material.

2.5 Statistical analysis
A two-way ANOVA, followed by a Tukey post hoc test (IBM SPSS statistics 22 software) was used to determine whether the removal rates of absorbents or the different Cd concentrations in the AGW, returned results that were significantly different from each other. SigmaPlot (version 13) was used to graphically express the data as the mean concentrations, and the error bars represented the standard error of the mean \( n = 3 \). The curves were fitted with nonlinear regressions (exponential decay). The tangent lines were applied to the curves to give approximate rates of Cd removals during the initial time of operating the reactor.

3. Results:
3.1 Removal of cadmium from AGW in a small laboratory bench-scale column reactor
During the operation of the reactors, the samples were collected every day to measure the total concentrations of Cd remaining in the eluants. HRT values were varied in the single experiment, mainly due to the slight differences in the bead masses, resulting from the changes in the volume of the liquid holdup and flow rate. The average HRT was measured, which depended on the flow rate and the liquid hold up of 112.5 \( \pm 0.9 \text{ min} \). Therefore, the extent of Cd removal in the column reactor systems depends on the biomass (mg) within the number and mass of the filled beads in the reactor per column.

Figure 4A, using Ca-alginate beads without bacterial cells, showed that the amount of Cd remaining in the eluant from the column reactor involves three stages over 28 days. First, there was a rapid reduction in the amount of Cd in the eluent, indicating an increase in Cd removal during the first five days \( 9.4 \text{ nmol/g of wet beads} \). Second, the removal has reached a maximum within 8 to 9 days, resulting in low Cd amounts in the eluent \( 1.6 \text{ nmol/g of wet beads} \). Finally, the Cd removal decreased gradually from day 10 to day 28 and recorded a higher amount of Cd in the eluant \( 15 \text{ nmol/g of wet of beads} \) at the end of the experiment. Surface complexation and electrostatic attractions played an important role in Cd removal from a solution containing non-growing bacterial cells. It is clear from Figure 4B and C that the Ca-alginate bead containing killed-control cells of either \( B. \text{ agri C15} \) or \( B. \text{ agri C15 Cd}^8 \) had similar patterns in the removal amounts. In the Ca-alginate beads containing killed-control cells of \( B. \text{ agri C15} \), the removal amounts reached \( 10.3 \text{ nmol/g of dry biomass} \) on the second day of operating the reactor (Figure 4B). Similarly, the equivalent amount was removed \( 10.6 \text{ nmol/g of dry biomass} \) by Ca-alginate beads containing killed-control cells of \( B. \text{ agri C15 Cd}^8 \) on the second days of reactor operation (Figure 4B).
The highest Cd removal amount was obtained within 5 to 8 days (20 nmol/g of dry biomass), leaving similar amounts of Cd recorded in the eluants. Between days 8 and 10 of the reactor operation, both killed-control cells of B. agri C15 or B. agri C15 CdR showed a reduction in their abilities to remove Cd from AGW and reached a plateau of high amounts Cd (13 – 14 nmol per g of dry biomass) in the eluants and these amounts were similar to the amounts recorded by the Ca-alginate beads without bacterial cells.

The removal amount of Ca-alginate beads containing live cells of B. agri C15 was 12.8 nmol/g dry of biomass after one day of operating the reactor, and this was analogous to the amount removed by the killed-control cells (Figure 4D, B, respectively). However, the live cells of B. agri C15 showed increasing amounts of Cd removal over the next few days, out-performing both Ca-alginate beads without bacterial cells and the killed-control cells in their abilities to remove Cd. The initial Cd removal rate for live cells of B. agri C15 CdR (Figure 4E), was similar to that of live cells of B. agri C15. However, the Cd amounts recorded in the eluants on the second day (3.6 nmol/g of dry biomass) was substantially lower, compared to the live cells of B. agri C15 (8.7 nmol/g of dry biomass). The Ca-alginate beads containing live cells of B. agri C15 showed lower and a somewhat unstable removal amount over 28 days. Thus, Ca-alginate beads containing live cells of B. agri C15 exhibited a much slower Cd removal, 21 nmol/g of dry biomass within 16 days, compared to the Ca-alginate beads containing live cells of B. agri C15 CdR that achieved the same amount of removal (21 nmol/g of dry biomass) within just four days of operating the reactor.
3.2 Evaluation of the cadmium removal from AGW with higher concentrations of cadmium

The Cd removal from AGW at pH 7.00 was further evaluated with concentrations of 8.8, 13.4, and 17.4 µM Cd in the column reactors, operated under the same conditions as the control concentration (4.4 µM), except for a shorter operating time (10 days). As before, the study was undertaken with five sets of Ca-alginate beads: without bacterial cells, containing killed-control cells of B. agri C15 or B. agri C15 CdR, and live cells of B. agri C15 or B. agri C15 CdR. The results in columns not containing bacterial cells (Figure 5) show a slight increase in the initial Cd removal rates (tangent slopes; -4.8 to -6.0) of the experiments. This indicates that during the early days, the removal rates of Cd from AGW were slightly more at higher initial Cd concentrations. Around days 8 – 10, the amount of Cd in the eluent plateaued at approximately the maximum removal of 14, 15, 22, and 27 nmol Cd per g of beads for the starting concentrations of 4.4, 8.8, 13.4, and 17.4 µM Cd, respectively. This shows that, although the proportion of Cd removed from AGW is higher (66%) at lowest initial Cd concentrations than it is at highest ones (28%), the capacity for Cd removal by Ca-alginate beads without cells has not been exhausted by day 10, even at 17.4 M Cd in AGW. The increase in the amount of Cd in the eluent observed at AGW with 4.4 mM Cd in the previous section (the final stage in Figure 4A) was not reached in this experiment, as it concluded on day 10. However, there is an indication of a slight upturn in Figure 5(C, D) on day 10.

In contrast to Ca-alginate beads without cells, reactors containing Ca-alginate beads with killed-control cells showed a higher initial removal rate at the lowest AGW Cd concentrations (slope = -7), compared to higher Cd concentrations (slope = -3.5 to -5.3) (Figures 6, 7). The minimum amount of Cd in the eluant was lower (1.5 to 3 nmol per g of dry biomass) at AGW 4.4 µM Cd with killed-controlled than without killed-controlled cells (Figure 5A). The overall reduction in the amount of Cd in the eluant by the end of the experiment was higher or similar for reactors containing killed-control cells than no cells (22, 15, 30, and ~30 nmol per g biomass for AGW concentrations of 4.4, 8.8, 13.4, and 17.4 µM Cd, respectively).

\[
y = -10.7 + (21.7e^{-t}) + (-2.1t) \\
R^2 = 0.91
\]

\[
y = -4.8t + 23 \\
R^2 = 0.93
\]

\[
y = -t + -3.7 \\
R^2 = 0.83
\]

\[
y = -5.5t + 44 \\
R^2 = 1
\]
Figure (5) The evaluation of Cd removal by the Ca-alginate beads without bacterial cells from AGW at pH 7.00 with nominal concentrations of Cd: (A) 4.4 µM (control), (B) 8.8 µM, (C) 13.4 µM, and (D) 17.4 µM. The amounts were the mean, and the error bars indicate a standard error of the mean SEM (n = 1, one reactor with three parallel columns). The amounts of Cd were obtained, and tangent lines were applied, as described in Figure 4.
Figure (6) The evaluation of Cd removal by the Ca-alginate beads containing killed-control cells of *B. agri* C15 from AGW at pH 7.00 with nominal concentrations of Cd: (A) 4.4 µM (control), (B) 8.8 µM, (C) 13.4 µM and (D) 17.4 µM. The amounts were the mean, and error bars indicate standard error of the mean S.E.M (*n* = 1, one reactor with three parallel columns). The amounts of Cd were obtained, and tangent lines were applied, as described in Figure 4.
Cd removal from AGW took place at slightly lower initial rates in reactors containing live cells of *B. agri* C15 (slope = -2 to -5; Error! Reference source not found.), compared with killed-control cells (slope = -3.5 to -7; Figures 6, 7) and the amount of Cd in eluant remained relatively high at high AGW Cd concentrations (Error! Reference source not found. C, D) throughout the experiment. In contrast, initial removal rates (slope = -8 to -9.3; Figure 9) of reactors containing live cells of *B. agri* C15 CdR was substantially higher than in any other reactor in this experiment and displayed a weak trend towards increased initial removal rates with increasing Cd concentrations in AGW. In addition, the employment of *B. agri* C15 CdR in the reactor resulted in the lowest amounts of Cd in the eluant. The absence of a plateau for Cd in the eluant at higher Cd AGW concentrations (Figure 9C, D) indicates a further capacity for Cd removal in the reactors at the end of the experiment. Overall, approximately 22, 42, 70, and 85 nmol Cd per g of dry biomass were removed from AGW with initial concentrations of 4.4, 8.8, 13.4, and 17.4 µM Cd, respectively.
Figure (8) The evaluation of Cd removal by the Ca-alginate beads containing live cells of B. agri C15 from AGW at pH 7.00 with nominal concentrations of Cd: (A) 4.4 µM (control), (B) 8.8 µM, (C) 13.4 µM, and (D) 17.4 µM. The amounts were the mean, and the error bars indicate a standard error of the mean SEM (n = 1, one reactor with three parallel columns). The amounts of Cd were obtained, and tangent lines were applied, as described in Figure 4.
Figure (9) The evaluation of Cd removal by the Ca-alginate beads containing live cells of *B. agri* C15 Cd from AGW at pH 7.00 with nominal concentrations of Cd: (A) 4.4 µM (control), (B) 8.8 µM, (C) 13.4 µM, and (D) 17.4 µM. The amounts were the mean, and the error bars indicate a standard error of the mean SEM (n = 1, one reactor with three parallel columns). The amounts of Cd were obtained, and tangent lines were applied, as described in Figure 4.

### 3.3 Determination of maximum initial rates of cadmium removals

The effect of initial Cd concentrations on the Cd removal was obtained by applying the tangent lines to the curves (Figures 5, 6, 7, 8, 9) to give approximate rates of Cd removals during the initial time of operating the reactor.

Figure 10 shows that the rates of Cd removal decreased in the Ca-alginate beads containing live cells of *B. agri* C15 with increasing Cd-AGW concentrations from 4.4 to 13.4 µM. The Ca-alginate beads, containing live cells of *B. agri* C15 Cd displayed significantly higher Cd removal rates (9 nmol per day per gram of biomass) than its *B. agri* C15 (5 nmol per day per gram of biomass) or than other beads (*p*<0.05) at different initial Cd-AGW concentrations, with no differences observed between the concentrations of the mutant.
Figure (10) The respective maximum initial rates of Cd removal by the Ca-alginate beads (●) without bacterial cells, containing live cells of (○) B. agri C15 and (□) B. agri C15 CdR, killed-control cells of (♦) B. agri C15 and (◂) B. agri C15 CdR that were used as adsorbents in the reactor system operated with nominal concentrations of Cd: 4.4, 8.8, 13.4, and 17.4 µM in AGW (pH 7.00). The rates were subjected to a two-way ANOVA, Tukey post hoc test, and a different letter indicates a significant difference between groups at each Cd concentration.

3.4 Observation of SEM and SEM-EDX of Cd-loaded beads after Cd removal
The Cd-loaded beads from the reactors were used for Cd removal from AGW (17.4 µM Cd), as shown in Figure 8E of B. agri C15 and Figure 9E of B. agri C15 CdR, were examined with SEM analyses. These analyses were also carried out with control beads (beads filled within the column reactor, containing AGW without Cd) for each bacteria strain for comparison between the control and Cd-loaded beads. Furthermore, the control beads (Ca-alginate beads without bacterial cells from the experiment shown in Figure 6E) were examined, as well as the control beads (filled within the column reactor, containing AGW without Cd). The images showed that all types of beads had ample pores and showed a heterogeneous structure (Figures 11, 12). The Cd-loaded beads, containing live cells of B. agri C15 (Figure 11 b1, b3), which seemed to be a cavity or Ca-alginate beads containing live cells of B. agri C15 CdR (Figure 12 b3) appeared to have wrinkles, compared to the structure of the control beads (Ca-alginate beads without bacterial cells (Figure 11 a1, a3). The heterogeneous composition of the Ca-loaded beads, as shown in Figure 11 a3 and Figure 34 a3, indicated that their surfaces were covered with more impurities. The higher magnification of these impurities showed the filamentous structures in the Ca-alginate beads without bacterial cells (Figure 13 A1) or containing live bacterial cells (Figure 13 B1). The EDX spectra of the beads acquired from SEM images assessed the difference of the elements before and after the removal of Cd. EDX spectra for all the beads showed the distribution of different element peaks of Ca, Na, C, P, Au, U, Fe, and Os in addition to Cd peak, which was rarely detected in Cd-loaded beads (Figure 11 b4).
Figure (11) The SEM micrographs and EDX spectra of the beads obtained after Cd removal from (a) AGW or (b) AGW with a concentration of 17.4 µM Cd. Three replicates of the beads, using DEC fixation, were employed for the pictures and representative images from three replicates are shown. SEM image of Ca-alginate beads without bacterial cells (a1 and a3), and respective EDX spectra (a2 and a4), SEM image of Ca-alginate beads containing live cells of *B. agr* C15 (b1 and b3), and respective EDX spectra (b2 and b4).
Figure (12) The SEM micrographs and EDX spectra of the beads obtained after Cd removal from (a) AGW or (b) AGW with a concentration of 17.4 µM Cd. Three replicates of the beads, using DEC fixation, were employed for the pictures, and representative images from three replicates are shown. SEM image of Ca-alginate beads without bacterial cells (a1 and a3), and respective EDX spectra (a2 and a4), SEM image of Ca-alginate beads containing live cells of \textit{B. agri} C15 Cd\textsuperscript{3+} (b1 and b3), and respective EDX spectra (b2 and b4).
3.5 Distribution of cadmium in the beads using a dithizone histochemical method

Sometimes it is impossible to detect the EDX spectra of Cd in the Cd-loaded beads under SEM-EDX analyses; therefore, alternative detection technique was investigated using a histochemical method. The beads were treated as a tissue, and the localisation of Cd was observed after the staining with a mixed dithizone solution. The detection of Zn was carried out for comparison. The unstained beads were observed to find the effects of staining on the beads. The main observations were differences in bead colour. Figure 14A(A1), B(A1), C(A1) showed that the unstained beads seemed to have alginate (observed as dark brown), with differences between the beads with and without bacterial cells. As shown in Figure 14A(B2), B(B2), C(B2), the beads loaded with Cd and stained with Zn stain, appeared to have lost some alginate (transparent colour of the beads) without detecting Zn. In contrast, Cd-loaded beads, stained with Cd stain, showed a red colour (Cd-dithizone complexes), locating Cd. However, Ca-alginate beads that contained bacterial cells and were exposed to Cd showed a much darker colour.

![SEM microphotographs and EDX spectra of Cd-loaded beads](image1)

Figure (13) The SEM microphotographs and EDX spectra of Cd-loaded beads (A1 and A2, respectively) Ca-alginate beads without bacterial cells and (B1 and B2, respectively) Ca-alginate beads with bacterial cells.
Figure (14) The light microscopy observations of (A) Ca-alginate beads without bacterial cells, and containing live cells of (B) \textit{B. agri} C15 and (C) \textit{B. agri} C15 Cd\textsuperscript{R} after the exposures to AGW (containing 20 mM pyruvate) or AGW with Cd (containing 20 mM of pyruvate) and incubation for 48 h. Three sections of the beads were employed for the pictures of staining Zn and Cd. Two independent experiments for each bead are shown in which the unexposed beads are in the left panels, and the exposed beads are in the right panels. 

Row \textbf{A} comprises unstained beads. \textbf{Row B} exhibits beads stained with Zn stain. \textbf{Row C} shows beads stained with Cd stain. \textbf{A(A1, A2)}, \textbf{B(A1, A2)}, and \textbf{C(A1, A2)} unstained beads are showing different colour in response to Cd. \textbf{A(B1, B2)}, \textbf{B(B1, B2)} and \textbf{C(B1, B2)} showing no Zn presence. \textbf{A(C2), B(C2), and C(C2)} showing Cd-dithizone complexes (green circles) compared to unexposed beads \textbf{A(C1), B(C1), and C(C1)}. 

4. Discussion

The ability to remove Cd from AGW by the mutant \textit{B. agri} C15 Cd\textsuperscript{R}, compared to the wild type \textit{B. agri} C15 entrapped in Ca-alginate gel, was investigated. Experiments were performed in an up-flow column reactor, using a slow flow rate (40 µL/min) in accordance with the optimisation experiment, which aimed at using a stable flow rate and hydraulic residence time (1.5 h) and achieved the rapid removal of Cd from AGW during the operation of the reactor. 

In this study, the reactor was operating for approximately a month, treating AGW with a concentration of 4.4 µM Cd at a pH 7.00. The results showed that Ca-alginate beads containing live cells of \textit{B. agri} C15 or \textit{B. agri} C15 Cd\textsuperscript{R} removed higher amounts of Cd than Ca-alginate beads without bacterial cells. Similar observations have been recorded in previous studies [10, 13], who reported that the Ca-alginate beads containing bacterial cells are more able to remove Cd due to having more functional groups.

According to SEM images of the beads’ morphological characterisations, Ca-alginate beads without bacterial cells had larger cavities than those of the Ca-alginate beads containing bacterial cells. This characteristic may provide effective adsorption by allowing Cd to move through the pores. However, this is only applicable in the initial time of the removal experiments, as described by Duan and Su [14]. The long-term performance (>10 to 28 days) of reactors containing no cells or killed-control cells reached a plateau that indicated the establishment of an equilibrium between the supply of Cd with AGW and its amount of removal. The primary mechanism of Cd removal by Ca-alginate beads alone is the ion exchange between Ca in the beads with Cd from AGW. Besides, the possible Cd binding sites of the functional groups in Ca-alginate beads are carboxyl and hydroxyl groups. However, when Ca-alginate beads are combined with bacterial cells, additional, more diverse, functional groups are available on the cell wall structure of the bacteria.

It has been reported that the carboxyl, amino, and phosphate groups are the main functional Gram-stain-positive group [15, 16]. Therefore, the role of surface interaction on Ca-alginate beads and Ca-alginate beads containing cells in the mechanisms of Cd removal can be proposed by drawing framework structures of Ca-alginate bead and bacterial cells, showing functional groups (Figure 15) that are analogous to the published
literature [17, 18]. The possible Cd binding sites on the functional groups in Ca-alginate beads are carboxyl and hydroxyl groups (Figure 15A). The cell wall of Gram-stain-positive bacteria, such as B. agri C15, consists of peptidoglycan, which provides carboxyl, hydroxyl, and amide functional groups. The phosphate functional group is added by other constituents of teichoic acid and teichuronic acid. The primary functional groups of B. agri C15 are illustrated in Figure 15B. Stability constants between Cd and functional groups containing N, P, or S are at least one order of magnitude higher compared to stability constant or Cd complexes with -OH functional groups [19, 20], and hence, binding between Cd and the functional groups in Ca-alginate beads containing cells was more than in the Ca-alginate beads without bacterial cells. In contrast, the mutant B. agri C15 Cd<sup>R</sup> could provide absorbency with different characterisation. As we stated in the previous study [8], the better ability to resist Cd by the mutant could be analogous with conclusions of other studies [21, 22], due to the introduction of –SH (sulphydryl) compounds. Therefore, the role of the surface interaction on Ca-alginate beads containing cells of the mutant B. agri C15 Cd<sup>R</sup> can be proposed (Figure 15C). This biosorbent, with additional –SH compounds or with other functional groups, produced a new biosorbent with more ability to adsorb Cd.

![Framework structures](image)

Figure (15) Framework structures of (A) Ca-alginate without bacterial cells, (B) cell of B. agri C15 and (C) cell of B. agri C15 Cd<sup>R</sup> (TEM micrographs), showing different functional groups. The structures were adopted and redrawn, according to Zou et al. [17] and Shim et al. [18].

Most of the studies have been amid to develop the uptake process of Cd by finding a novel component of alginate beads [23, 18]. All these studies have been succeeded by introducing new absorbents with new functional groups to coordinate with Cd. In this study, two different biosorbents produced, one Ca-alginate
bead containing live cells of *B. agri* C15 and other Ca-alginate beads containing live cells of *B. agri* C15 Cd⁸. Both biosorbents are new, but they are different in their absorption capacities due to the differences in their bead composition. The combination of the functional groups of the Ca-alginate beads and bacterial cell wall structure is the primary mechanism that contributed to the uptake of Cd by biosurface adsorption in killed-control cells as the bioaccumulation inside the cell is absent due to the inactivation of the cell. Interestingly, the uptake by killed-control cells gave a lower uptake amount than the live cells, as an intracellular accumulation of Cd is active in live cells [24]. Recently, Mohapatra *et al.* [25] reported the equivalent relationship in *Bacillus xiamenensis* PbRPSD202 as an electron dense particle was observed inside the live bacterial biomass.

The metal concentration is essential to factors of the removal experiments; therefore, the removal in the reactor was also evaluated with higher concentrations of Cd (8.8, 13.4, and 17.4 µM). High removal rates were achieved by mutant *B. agri* C15 Cd⁸, providing a new development Cd process. No observed differences in their removal rates were evaluated based on the effect of the Cd concentrations, which were the same. It is reported previously that the UV mutant of *B. subtilis* 38 could uptake Cd higher than the wild type [26].

The structure of the observed bead was similar to the construction of Ca-alginate beads reported by Zazzali *et al.* [27]. The heterogeneous composition of the beads provides effective adsorption [28]. The more varied structure of the bead surface after Cd loading showed surface impurities, which could demonstrate that Cd was deposited onto the surface of the beads by one of the previously discussed mechanisms, such as the electrostatic interaction with the functional groups. SEM analyses were employed to detect the Cd-loaded beads to detect Cd and resulted in the detection of only a few peaks of some elements. The presence of Ca and Na peaks originate from the composition of the bead matrix, and C or P peaks from the AGW composition. U and Os are the main elements used in fixing the beads for SEM analysis, while Au peaks were from the gold used for coating the blocks. Other elemental peaks could be present due to contamination. SEM-EDX analyses suggest that the percentages of the elemental compositional detection (EDX data) resulting from Cd loading were impossible in the beads. This obstacle is comparable with previous studies, which excluded EDX data due to the nature of absorbents and the presence of Cd in low concentrations [14, 29, 30]. Alternatively, the detection of the distribution of cadmium on the absorbents using EDX mapping [31] or polished samples to produce a flat section [32] can support the adsorption mechanism of Cd.

The dithizone histochemical method employed in this study to determine the distribution of Cd on the Cd-loaded beads showed that the stain revealed information on the beads’ compositions as the colour changed from brown (related to alginate) to transparent (loss of alginate). Unstained beads showed a visible structure, and the brown colour mainly appeared in Cd-exposed beads. Furthermore, a denser, or more intense colour was observed in Ca-alginate beads containing bacterial cells. Zn was not detected when beads were stained with a Zn-specific mixed dithizone solution, which confirmed that the method could differentiate between Zn and Cd within the beads.

In contrast, the staining of Cd-loaded beads with Cd-specific mixed dithizone solution showed red pigments, and their distributions through the beads indicated removal mechanisms of Cd from AGW. The pigment distribution on the Ca-alginate beads without bacterial cells (control beads) illustrated that Cd just deposited on the beads’ surface, suggesting that removal relied on surface interactions, such as ion exchange. The pigment distribution on Ca-alginate beads containing live bacterial cells showed Cd present inside the beads, suggesting that active Cd uptake by bacterial cells (i.e., bioaccumulation) played a role in Cd removal from AGW. The presence of Cd on edge could explain the ion exchange process that occurred as well, while the presence of Cd inside the beads could support the bacterial cell activity, especially in these experiments; pyruvate was added as a source of carbon, which could support their activities.

5. Conclusion:
The Ca-alginate beads containing live cells of the mutant *B. agri* C15 Cd⁸ could uptake Cd from AGW with the maximum rate. *B. agri* C15 could also be used for Cd removal, but its removal rate was low compared to the mutant. The uptake mechanism of this process is that the Cd was making contact with the Ca-alginate beads, firstly due to the ion exchange with Ca or the combination of carboxyl, hydroxyl, amide, phosphate, and sulfhydryl (for mutant) functional groups and the bioaccumulation with bacterial cells. Thus, it is concluded that the localisation of Cd in Cd-load beads, showed that the Cd localised through the beads, giving evidence that two mechanisms of the bioremediation occurred during the removal of Cd:
bioaccumulation and biosorption by bacterial cells. During the removal experiments of Cd in the column reactors, the mutant B. agri C15 CdR was able to absorb more Cd. It was not understood, whether the absorption was due to the exchange of ions, precipitation or complexation of Cd with the composition of Ca-alginate beads or due to the bioremediation of the biomass, bioaccumulation of Cd inside the cells (intracellular) or adsorption outside of the cell (extracellular). FTIR [33] of the Cd-loaded Ca-alginate beads without bacterial cells, containing live cells of B. agri C15 or live cells of B. agri C15 CdR and the determination of the functional groups present on the surface of the beads will explain the binding mechanisms of Cd. It is possible to investigate the surface complexation of Cd in B. agri C15 and B. agri C15 CdR for determining the model of the Cd adsorption reaction [34]. Staining of Cd-loaded Ca-alginate beads with dithizone stain showed the distribution of Cd-dithizone complexes within the beads. Alternatively, using SEM imaging, EDX mapping, and X-ray photoelectron spectroscopy (XPS) [31] could observe the distribution of Cd and predict the mechanism of absorbing Cd. It is possible to run the reactor for an extended period [35] and depending on the removal rate during the operation time, the flow rate and the concentration of Cd could be altered. Therefore, the rate decreased at a specific time; the flow rate and the concentration of Cd must be reduced to less. In this study, a constant room temperature of 22 °C was used for all the Cd removal experiments. This temperature allows the process to be implemented in an area with the same temperature. This process could be applied at groundwater temperature after investigating the process at temperatures ranging from 4 to 15 °C [36]. It would be possible to determine the absorption mechanism of Cd by the Ca-alginate beads, even if the ion exchange process is performed X-ray diffraction (XRD) or co-precipitation using FTIR [37]. Hence, the uptake may be affected by the existence of groundwater compounds. The effect remaining unclear requires further investigation. The investigation can be potentially studied by using a multi-reactor set up instead of a single reactor set up, as used in this study. These reactors can maximise the effects of the existence of groundwater compounds on uptake efficiency due to the differences in the operation parameter between the reactors.

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