Journal homepage
www.ajas.uoanbar.edu.iq
Anbar Journal of Agricultural Sciences
(University of Anbar – College of Agriculture)

BIOREMEDIATION OF CADMIUM CONTAMINATED WATER: BACTERIAL ACCUMULATION AND SURFACE COMPLEXITY BY *REVIBACILLUS AGRI* C15 AND *BREVIBACILLUS AGRI* C15 CD^R

N. M. T. Jebril[1]* **A. K. Murad[2]** **R. Boden[3]**

[1] Department of Biology, College of Sciences for Women, University of Babylon.
[2] Department of Sciences, College of Basic Education, University of Babylon.
[3] School of Biological and Marine Sciences, University of Plymouth, United Kingdom.

***Correspondence to:** Nadia Mahmoud Tawfiq Jebril, Department of Biology, College of Sciences for Women, University of Babylon, Iraq.
Email: nadia.tawfiq@uobabylon.edu.iq

Article info

Received: 2024-04-20
Accepted: 2024-05-19
Published: 2024-06-30

DOI-Crossref:
10.32649/ajas.2024.147810.1166

Cite as:
Jebril, N. M. T., Murad, A. K., and Boden, R. (2024). Bioremediation of cadmium contaminated water: bacterial accumulation and surface complexity by *revibacillus agri* c15 and *brevibacillus agri* c15 cd^r. Anbar Journal of Agricultural Sciences, 22(1): 217-233.

Abstract

Brevibacillus agri C15 and *Brevibacillus agri* C15 Cd^R have been reported as a tool for do uptake of cadmium (Cd). Nevertheless, it is unclear whether these strains contrast in their adsorption behaviors and surface complexation could be one of the possible mechanisms that occurred. Therefore, this research aimed to investigate the mechanisms of cadmium uptake by *B. agri* C15 and *B.agri* C15 Cd^R in batch cultures and under TEM detection based on the maximum ATP or NADH production to energy metabolism using a resting cell experiment. In addition, surface complexation of cadmium in strains was studied. The results showed that the accumulation of Cd under TEM detection predicts that Cd bioaccumulation is the main mechanism in *B. agri* C15 and that adsorption is the main mechanism in *B.agri* C15 Cd^R. The observation of vesicles and granules, mainly poly-β-hydroxybutyrate under Cd-sublethal concentration may contribute to the adsorption and intercellular sequestration of Cd. There were differences in the cell compartments through the uptake process of Cd by the bacterial cells and the examinations of the contribution of surface complexation mechanisms in the uptake of Cd

were obtained. Therefore, determining the thermodynamic stability constants of both strains from the available obtained parameters from this study was necessary.

Keywords: ATP, Brevibacillus agri, Cadmium, NADH, Surface complexation.

المعالجة الحيوية للمياه الملوثة بالكادميوم: تراكم البكتيريا وتعقيد السطح بواسطة *BREVIBACILLUS AGRI* C15 CD^R و*REVIBACILLUS AGRI* C15

نادية محمود توفيق جبريل[1]* أمنة كاظم مراد[2] ريتش بودن[3]

[1] قسم علوم الحياة، كلية العلوم للبنات، جامعة بابل

[2] قسم العلوم، كلية التربية الأساسية، جامعة بابل

[3] كلية العلوم البيولوجية والبحرية، جامعة بليموث، المملكة المتحدة

***المراسلة الى:** نادية محمود توفيق جبريل، قسم علوم الحياة، كلية العلوم للبنات، جامعة بابل، العراق.

البريد الالكتروني: nadia.tawfiq@uobabylon.edu.iq

الخلاصة

تم الإبلاغ عن *Brevibacillus agri* C15 و*Brevibacillus agri* C15 CdR كأداة لاستيعاب الكادميوم (Cd). ومع ذلك، فمن غير الواضح ما إذا كانت هذه السلالات تتباين في سلوكيات الامتزاز، وقد يكون التعقيد السطحي أحد الآليات المحتملة التي حدثت. لذلك، هدف هذا البحث إلى دراسة آليات امتصاص الكادميوم بواسطة *B. agri* C15 و*B. agri* C15 CdR في التجارب المختبرية وتحت اكتشاف TEM بناءً على الحد الأقصى لإنتاج ATP أو NADH لتحرير الطاقة باستخدام تجربة خلية الراحة. بالإضافة إلى ذلك، تمت دراسة التعقيد السطحي للكادميوم في البكتريا. أظهرت النتائج أن تراكم الكادميوم تحت اكتشاف TEM يتنبأ بأن التراكم الحيوي للكادميوم هو الآلية الرئيسية في *B. agri* C15 وأن الامتزاز هو الآلية الرئيسية في *B. agri* C15 CdR. ملاحظة الحويصلات والحبيبات، وخاصة بولي-بيتا-هيدروكسي بوتيرات تحت تركيز الكادميوم تحت التركيز القاتل قد ساهم في امتزاز الكادميوم وعزله بين الخلايا. كانت هناك اختلافات في المكونات الخلوية من خلال عملية امتصاص الكادميوم بواسطة الخلايا البكتيرية وتم الحصول على فحوصات مساهمة آليات التعقيد السطحي في امتصاص الكادميوم. ولذلك، كان من الضروري تحديد ثوابت الاستقرار الديناميكي الحراري لكلا السلالتين من المعلومات المتاحة التي تم الحصول عليها في هذه الدراسة.

كلمات مفتاحية: ATP، Brevibacillus agri، الكادميوم، NADH، تعقيد السطح.

Introduction

There is an urgent need to develop effective methods for removing cadmium from contaminated water to the standard limit in drinking water (9). Bioremediation is an eco-friendly process that can be used to uptake cadmium by using Cd-resistant microbes (17). Microbial resistance to cadmium is mainly carried out through bioaccumulation, efflux pump, biosorption, or precipitation mechanisms (18). Most of these mechanisms can be used in the Cd-bioremediation process, mainly bioaccumulation, and biosorption of precipitation. Cadmium is actively accumulated on components of the cell as a result of the reaction of a variety of bacterial functional groups, which are produced by bacterial cells, forming immobile and insoluble Cd compounds. This accumulation rarely contributes to the cadmium uptake in cells as it needs the elements to be produced (13). On the other hand, bioaccumulation is a complex process of intracellular accumulation of cadmium in specific organelles; therefore, it is more possible for cadmium uptake by biosorption mechanisms. Biosorption is a physicochemical process in which cadmium is passively exchanged, chelated, complexed, or precipitated on the surface of bacteria. Precipitation is the metabolic or non-metabolic dependent process in which cadmium is passively accumulated on components of the cell as a result of the adhering of cadmium on the functional groups of the cell wall, using several different biosorption mechanisms (12). The functional groups include such as –OH (hydroxyl), -COOH (carboxyl), $-PO_3$ (phosphate), $-NH_2$ (ammonia), and –SH (sulfhydryl), which mostly have a negative charge, and simultaneously an extraordinary binding magnetism to cadmium cation via van der Waals force or covalent bonding (chemisorption) (16). One of the possible mechanisms of biosorption of cadmium is surface complexation. This research aimed to identify the mechanisms of cadmium uptake by *B. agri* C15 and *B. agri* C15 Cd^R. The identification was first evaluated based on uptake assay, which was carried out in batch cultures, to investigate cadmium bioaccumulated within the cells and under TEM detection as well. The effect of cadmium on cell morphology was estimated based on the maximum ATP or NADH production to energy metabolism using a resting cell experiment. In addition, as (25) reviewed that surface complexation can also enhance the removal of metal when the biosorption materials immobilised in calcium alginate, therefore, the bacterial cells were immobilised in calcium alginate and cadmium bioaccumulation experiments were carried out in constructed bench lab-column reactor. Surface complexation assay was used for determining possible cadmium adsorption in immobilised biosorption materials as surface complexation can also enhance the removal of metal when the biosorption materials immobilised in calcium alginate.

Materials and Methods

Batch experiments for cadmium bioaccumulation: The accumulation of cadmium by free bacterial cells, *B. agri* C15 and *B. agri* C15 Cd^R (18) was estimated after collecting samples from batch experiments (aqueous phase and digested cell pellets using ICP-MS) and under TEM detection after performing the uptake in batch culture, according to (13) with modification. Briefly, suspension of bacterial cells (50

mL) was inoculated into EBS growth medium (500 mL), containing pyruvate (20 mM, as carbon source) in Erlenmeyer-flasks (2000 mL) in triplicates and incubated at 37°C for 100 h shaken at 150 rpm. The initial concentration of Cd to be accumulated was chosen according to data on the sublethal concentration of Cd obtained from *B. agri* C15 (15), which was 13 mM Cd (6.5 mmol). For distinguishing the accumulation of Cd within cells in TEM images, a control culture was carried out without adding Cd. At regular time intervals, samples (20 mL) of each of the three replicates were collected in Falcon tube (50 mL), and the dry bacterial biomass was determined, as designated in (15), and plotted as a logarithmic graph against time. The accumulated amounts of Cd on the cells and residual Cd in the resultant supernatant were determined after the separating by centrifugation using Avanti J-26XP centrifuge (Beckman Coulter) for 15 min at 16000 rpm. Cells were separated from the cultures, and the aqueous phases were transferred immediately into a clean Falcon tube (50 mL). A glass analytical pipette (10 mL) was used to transfer 10 mL from the aqueous phase into a volumetric flask (25 mL), and the volume was concluded with HNO_3 (2%) containing indium (as an external standard, 0.43 µM in the total volume of 25 mL) to be analysed. The cell pellets were washed and centrifuged twice with cooled ice physiological normal saline. Then, the pellets were resuspended in the saline in a volumetric flask (5 mL), and the dry biomass was determined. 0.5 from the suspension pellets was transferred into a volumetric flask (10 mL) and 5 mL of the *aqua regia* solution (14) was added for acid digestion for 48 h in a fume hood at room constant temperature (20 °C). The rest of the suspension pellets were cautiously prepared for TEM analysis. After digestion, the volume was completed with HNO_3 (2%) containing indium (as an external standard, 0.43 µM in the total volume of 10 mL) for analyses of cadmium using ICP-MS.

Effect of cadmium on cell morphology: The response of cells to cadmium was estimated based on the morphological observation under transmission electron microscopy. The response assay was studied using a resting cell experiment using different concentrations of Cd provided as $Cd(NO_3)_2 \cdot 4H_2O$ in 5 mL EBS growth medium containing pyruvate as carbon source. From the Gibbs energy (ΔG) change for the oxidation of pyruvate:

$C_3H_4O_2 + 3O_2 \rightarrow 3CO_2 + H_2O \quad \Delta G^o = -237.1$ kJ/mol pyruvate

We can estimate the maximum ATP or NADH production (assuming perfect coupling of substrate oxidation to energy metabolism) based on the ΔG^o for formation of ATP from ADP and orthophosphate (+46.1 kJ/mol ADP) and of NADH and NAD^+ and protein (+83.4 kJ/mol NAD^+) -5.1 mol ATP/mol pyruvate or 2.8 mol NADH/mol pyruvate. If Cd(II) detoxified by reduction (NADH-consuming) to Cd^o then:

$Cd^{2+} + 2NADH \rightarrow Cd^o + 2NAD^+ + 2H^+$

Which would be analogous to the detoxification of Hg (II) (2). Thus, for a 1 L culture with 20 mmol pyruvate, at most 56 mmol NADH could be formed; therefore, 56 mmol Cd (II) detoxified based on thermodynamic maximum only. The concentrations of Cd on the effects of cells were studied in the range from 1 to above 150 mM as logarithmic scale numbers: 1, 2, 5, 10, 20, 50, 100, 125 and 150 mM. From the exponential phase, cells were harvested, washed and resuspended with EBS

without a carbon source. 200 L of cell suspension (1.5 mg dry biomass) was added to each tube. Positive and negative controls EBS without cadmium, inoculated cells and EBS supplemented with Cd without cells, respectively. After 24 h of incubation, cells were harvested, washed and resuspended with 1mL Dulbecco buffer for fixation before the TEM observation, as described in (10). The possible toxicity of Cd on the morphology of cells was assumed choosing the control cells, and the cells are grown under 150 mM and backward concentrations until the observation of the toxicity effects of Cd on the cells was observed.

Laboratory bench-scale column reactors set-up for cadmium bioaccumulation: The set-up of the laboratory reactor is showed in (11). In the experiments, EBS/F with a nominal concentration of 13 mM Cd, was used to be cleaned by five reactors process, one reactor with three columns:

1. Ca-alginate beads holding, *B. agri* C15.
2. Ca-alginate beads holding *B. agri* C15 Cd^R.
3. Control Ca-alginate beads without any of bacterial cells.

The immobilising of bacterial cells in the Ca-alginate matrix was carried out according to (13). Reactor were operated for 100 hours, and eluanta samples were collected every 10 hours to determine the total concentration of cadmium.

The amount of adsorbed cadmium were regularised (i) per dry biomass (mg) (except Ca-alginate beads without bacterial cells [per beads]), and (ii) per mass of beads in a column. Percentage removal of cadmium (% adsorption was as below:

% adsorption= $\frac{Ci-C}{Ci}\ x100$

Where C_i is the initial nominal concentration of cadmium in solution and C is the concentration of cadmium in the eluanta samples through/after the adsorption experiments.

Estimation of cell surface area: The surface area of cells was required for complexation experiments. The bacterial cell was approximated to a right cylinder capped with domes; therefore, the surface area was determined by calculating the areas of the cylinder (A_1) and domes (A_2 and A_3) using std geometric methods.

Area of a right cylinder (ignoring top/bottom):

A= $\pi\ \delta\ \lambda$

Area of a dome (spherical cap):

$$A\ =\ 2\ \pi(\frac{\delta}{2})$$

Lines were drawn along the bacterial cell on TEM to determine the starting point of each dome in both sides as shown in Figure 1.

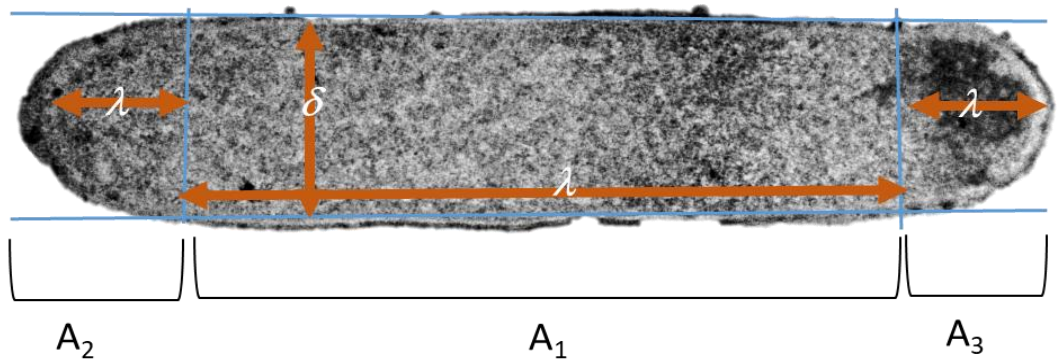


Figure 1: Photo of *B. agri* C15 on TEM showing the starting point of each dome in both sides.

Estimation of cell concentrations: For estimation, the concentrations of the measured optical density (*OD*) of the cell suspension (OD_{440}=4.0), which used in the complexation experiment, a calibration curve between the cell concentrations and *OD* was used (6). For obtaining these values, serial dilutions combined with direct cell count was performed. The initial *OD* of the cell suspensions to be diluted was chosen to be higher than the *OD* that needs to count its cell concentration. Cell suspensions were diluted with Norris-Powell diluent (19) resulted in 4 dilution steps, each in 4 replicates. The diluent was prepared from 5 mL of 40% *v/v* formaldehyde solution containing 10% *v/v* methanol in ddH_2O, adjusted to pH 7.00 by adding solid Na_2HPO_4, then 0.02 g sodium dodecyl sulfate was added. To avoid aggregation of cells during counting, 50 mM HCl was added to the diluent for dilution to be counted (19). The serial dilution was carried out in a volumetric flask (2 mL). A Euler's square (5) was used during the counting of cells to minimise systematic error owing to cell settling or aggregation or systematic pipetting error from fatigue *etc*. Counting of cells was carried out in using an improved cytometer, Gallenkamp (0.1 mm depth, 0.0025 mm^2) under a light microscope at power 400X, and if cells touched the edges of a grid square, only cells on the top and right were counted and cells in the left and bottom ignored. Based on a counting area, 1 mm x 1 mm and a cell height of 0.1 mm, the volume of the counting area is 0.1 µL.

Surface complexation of cadmium in *B. agri* C15 and *B. agri* C15 Cd^R: The experiment was conducting by taking suspensions of *B. agri* C15 and *B. agri* C15 Cd^R in 0.1 M $NaClO_4$ according to (6). Briefly, cells of *B. agri* C15 and *B. agri* C15 Cd^R were spin down and then rinsed for 2x in fresh 0.1 M $NaClO_4$.Then, the pellets were resuspended in 30 mM HNO_3 for an hour, rinsed 5x in 0.1 M $NaClO_4$ and finally, resuspended in 0.1 M $NaClO_4$ to OD_{440}=4.0 (cell g/L). to do the actual experiment, class test tube (10 ml, acid washed for 48 h) filled with 5 mL of suspension cells (measured by volumetric pipette). Cadmium from ICP std (0.01mM, in 2% HNO_3) was added and the pH was adjusted to 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 using HNO_3 or NaOH. After two hours of equilibrated time 25 °C, the liquid was centrifuged to be ready for measuring the concentration of cadmium.

Elements analyses: The total concentrations of Cd in the aqueous phase and digested cell pellets were determined using inductively coupled plasma mass

spectrophotometry (ICP-MS, Thermo Scientific, X Series 2). Standard solutions of cadmium were prepared in HNO_3 (2%) in ranging 0, 2.5, 5, 7.5,10, 12.5,15,17.5 and 20 mM. Yttrium was added before the measuring in a concentration of 0.56 µM. Procedure blanks were used. Blanks consisted of EBS media, containing 20 mM pyruvate (as carbon source) without Cd. The blanks were run for each digestion procedure to check all procedure for interferences and cross-contamination. The complex of cadmium from sodium protectorate with surface functional groups on bacterial cells were analysed by determining the concentrations of the elements remaining in the eluants using inductively coupled plasma optical emission (ICP-OES, Thermo Scientific, iCAP 700 Series). The instrument-limited detection (LOD) of cadmium was 0.0003±0.0002 mM which established from the five times of the standard deviation (S.D.) of measuring the lowest standard (n=10). The quality control for the consistency of the measurement was evaluated by the analysis of the procedural blank samples (without suspension cells, n=3 blanks).

Statistical analysis: All the data of the experiments mentioned above were the mean and standard error of the mean. Statistical analyses, least significant difference (LSD) test was used to find the variance. SigmaPlot 13 was used to illustrate the data.

Results and Discussion

Accumulation of cadmium in *B. agri* C15 and *B. agri* C15 Cd^R: The Cd uptake by strains *B. agri* C15 and *B. agri* C15 Cd^R was investigated based on the accumulation and surface complexation methods. The accumulations of cadmium in both strains were determined from the resultant supernatants and observed under TEM detection after performing the Cd uptake in batch culture and separating the cells from resultant supernatant. The appearance of electrondense of intercellular compartments showed that both cells confirmed Cd uptake. As shown in figure 2 A and B from an initial 6.5 mmol Cd, strains *B. agri* C15 and *B. agri* C15 Cd^R removed 1.34 and 1.52 mmol Cd, respectively. The TEM micrographs of harvested cells at the end of the experiments showed morphological changes of both strains compared to their cells that grown without cadmium. Under Cd-free condition, cells had a thin cell wall and the presence of cell organelles (Figure 2 (A(A) and B(A)). After the experiments, strain *B. agri* C15 showed electrondense distributed within cells (Figure 2 (A(B)), while the electrondense distribution was allocated on the edge of strain *B. agri* C15 Cd^R (Figure 2 (B(B)).

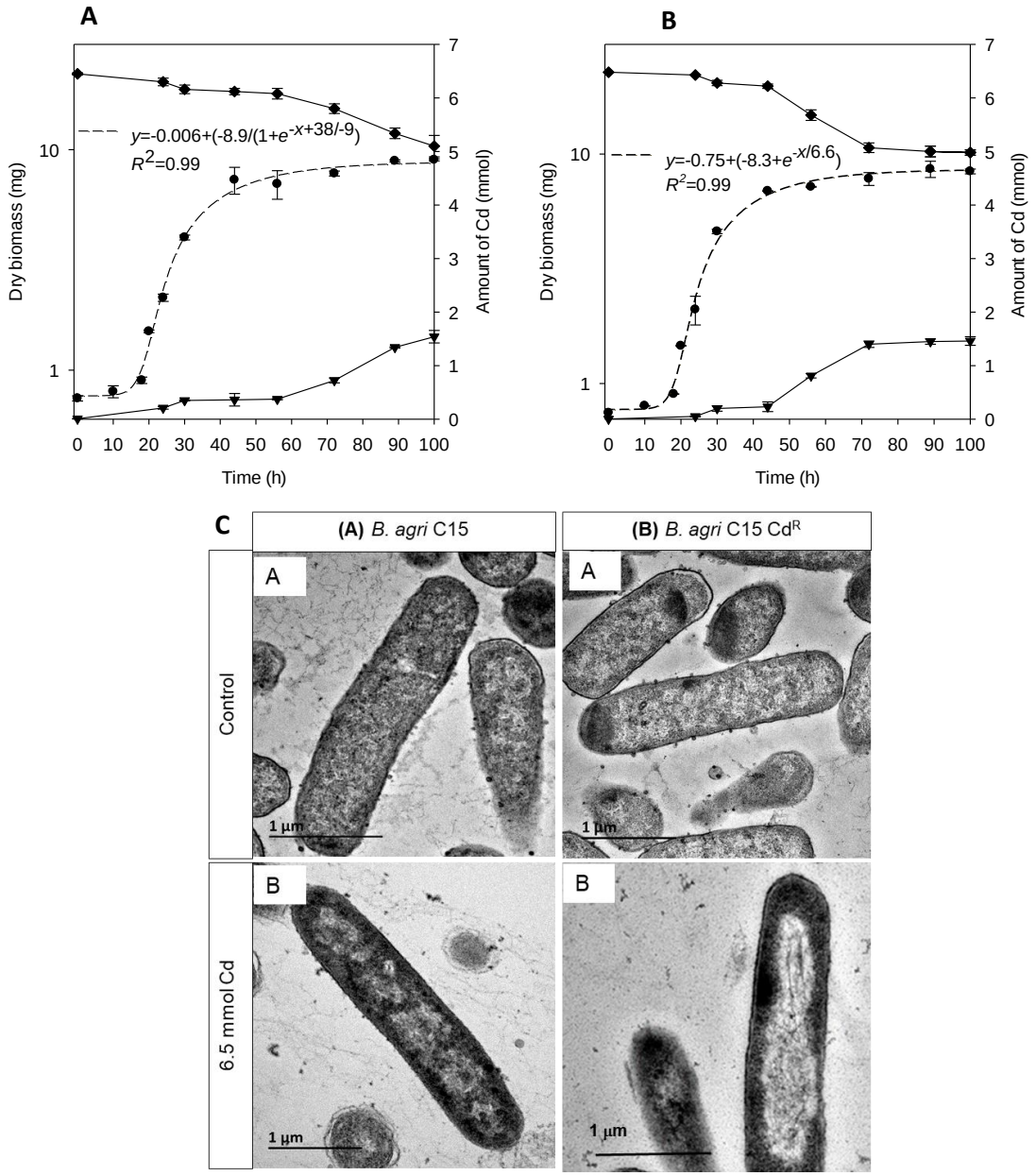


Figure 2: Accumulation amounts of cadmium by (A) *B. agri* C15 and (B) *B. agri* C15 Cd^R in batch cultures, EBS growth medium (500 mL), containing pyruvate (20 mM as carbon source) and nominated with 6.5 mmol Cd. The dry bacterial mass was determined (●). The accumulated amounts of Cd on the cells (▼) and residual Cd in the resultant supernatant (♦) were determined after separating by centrifugation and each point through the graph is respective between them. The error bars represent the standard error of the mean (*n* = 3). (C) TEM micrographs of the harvested cells of (A) *B. agri* C15 and (B) *B. agri* C15 Cd^R after the accumulation times (100 h), showing the electrondense (row B) compared to electronlucent in grown without Cd (row A).

It was reported that the detection of electrondense of intercellular compartments after a Cd-uptake experiment is responsible in the metal-accumulation (22, 23 and 30). The observed electrondense was higher in the wild type than in the mutant. This accumulation explains why the of *B. agri* C15 was less resistant to Cd as it seems its

efflux system not active as much as in the mutant. The mutant *B. agri* C15 Cd^R was more resistance to Cd by preventing of entering Cd into the cells. This prevention could be due to the presence of active or different efflux system than that in the wild type. Furthermore, the possible mechanism of the prevention was effective of its surface complexation, and this showed why the electrondense of sections were on the wall of its cell.

Effect of cadmium on cell morphology: The observation of the effects of cadmium on the bacterial cells was estimated under TEM analysis. For the observations of the effect, bacterial cells from the resting experiments were chosen from under the effects of two cadmium concentrations, sublethal (10 mM) and lethal (150 mM). The results showed that under the unexposed condition, the cell walls of both strains were appeared as an electronlucent fibrillary layer (Figure 3 (A(A) and B(A)) compared to their growth under cadmium effect in batch cultures, as shown in the previous Figure 2. Under 10 mM exposure, the effects of Cd was evident as the cell morphology changed to unwell-cells with the presence of several of granules and spores (Figure 3 (A(B) and B(B)). There were no cell lysis or destruction. Where the exposure to 150 mM Cd showed that both strains could not survive (Figure 3 (A(C) and B(C)). It has been observed that cell morphology is expressed differently in response to heavy metal stress. The results from the effect of Cd on cell morphology in this study showed that both strains had been identified to be differentially. Firstly, in terms of using the resting experiment and under Cd-free condition, electronlucent fibrillary layer of the cell walls was observed. This observation was different from that obtained when the cells were grown in batch culture and harvested after 100 h. The incubation period in the rest experience (24 h) appears to contain a period to adapt the cells under stress conditions; hence, the shape of the cell wall appears as at the time of adaptation. The observed electronlucent fibrillary layer was detected in *Burkholderia cepacia* GYP1 by another study (22) however the exposure time was for 7 days and under Cd stress. Similarly, in this study also The observed electronlucent fibrillary layer was observed in both strains under Cd stress (10 mM). Under this exposure, endospores and vesicles were observed in both strain in addition to the electrondense granules were detected in strain *B. agri* C15 Cd^R. Bacteria sporulation endospore to tolerate extreme environmental conditions which are unsuitable for the vegetative cellular phase. Therefore, both strains under 10 mM Cd formed endospores as a mechanism for protecting from Cd toxicity. The shape and the position of the endospores in both strains were similar to the endospores in *Brevibacillus laterosporus* (21). (4) concluded that these vesicles and granules were responsible in the detoxification system for Pb in *Penicillium simplicissimum*. The mechanisms in which these components involved are adsorption and intercellular sequestration.

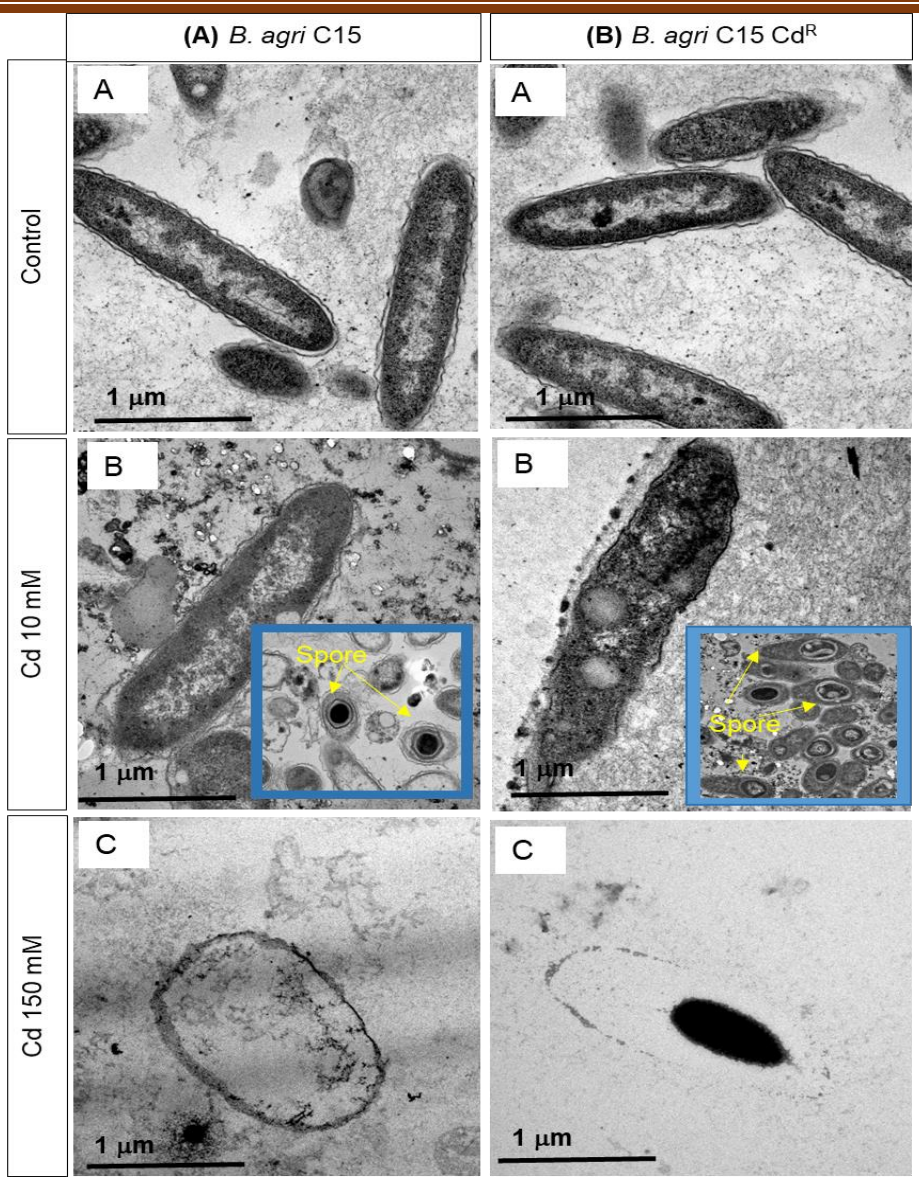


Figure 3: The effect of cadmium at: Row A control, Row B 10 mM and Row C 150 mM on the cell morphology of (A) *B. agri* C15 and (B) *B. agri* C15 Cd^R after the resting experiment under TEM analyses. Row B shows the effects of Cd on cell phenotypes under 10 mM Cd and spore-forming (insect picture) and row C shows the unsurvived cells under 150 mM Cd.

The higher effect of Cd at a lethal concentration (150 mM) on both strains were observed by the lack of cytoplasm components and texture the cell walls were lost. (29) reported that the Cd effects on *Penicillium chrysogenum* XJ was by oxidative stress led to the production of reactive oxygen species. Therefore after observing the endospores, vesicles and granules within the cells under TEM detection, it was essential to confirm the endospore-forming and quantity if these were poly-β-hydroxybutyrate (PβHB) and polyphosphate granules. The staining of cells with Malachite green solution showed endospores formed in both strains (18). The detection of the poly-β-hydroxybutyrate granules (PβHB) was confirmed in both strains while polyphosphate granules were not shown. Therefore, endospores (black ellipse) and granules (white) observed under TEM images revealed that the forming of endospores and PβHB. (28) found that there was not a relationship between

producing PβHB granules and sporulation in the genus *Bacillus*. Strains were produced poly-β-hydroxybutyrate as carbon storage granule. Bacteria produce PβHB to protect itself from heavy metal stress. (20) found that the production of poly-β-hydroxybutyrate(PβHB) was increased in *Rhizobium tropici* LBMP-C01 under cooper and chromium exposure. It is known that polyphosphate granule is a starvation response in bacteria and it is vital for survival and resistance to toxic stresses. Copper tolerance in *Escherichia coli* was mediated by polyphosphate (7). In this study, both strains did not produce polyphosphate granule and may be this due to the low concentration of phosphate in EBS growth media as (8) obtained polyphosphate in *E. coli* when grown with 37 mM phosphate.

Estimation of cell surface area: Area of both strains was similar (Table 1). *B. agri* C15 has a mean area of 6.00±0.06 μm^2 and *B. agri* C15 Cd^R has a mean area of 6.07±0.02 μm^2.

Table 1: Calculation of the areas of strains *B. agri* C15 and *B. agri* C15 Cd^R. Three of TEM micrographs for each strain were used to obtain the area. A$_1$, A$_2$ and A$_3$ are respective regions of *B. agri* C15 on TEM as shown in Figure 1. LSD between cell area = 20.0.

Parameters / Strains	Cylindrical region (A$_1$) (μm^2)			Dome (A$_2$) (μm^2)			Dome (A$_3$) (μm^2)			Cell area μm^2
	δ	λ	A$_1$	$\frac{\delta}{2}$	λ	A$_2$	$\frac{\delta}{2}$	λ	A$_3$	ΣA
B. agri C15	0.62	2.10	4.52	0.31	0.35	0.68	0.31	0.30	0.58	5.78
B. agri C15	0.61	2.15	4.79	0.31	0.40	0.77	0.31	0.35	0.68	6.24
B. agri C15	0.61	2.20	4.80	0.31	0.30	0.58	0.31	0.32	0.62	5.99
B. agri C15 Cd^R	0.61	2.20	4.80	0.31	0.40	0.77	0.31	0.30	0.58	6.15
B. agri C15 Cd^R	0.62	2.10	4.52	0.31	0.40	0.77	0.31	0.35	0.68	5.97
B. agri C15 Cd^R	0.62	2.15	4.79	0.31	0.35	0.68	0.31	0.32	0.62	6.08

In the surface complexation experiments, it was essential to calculate the surface area of each strain to provide the total areas that used for acquiring the complexation. The standard geometric methods were used to calculate the surface area of *B. agri* C15 and *B. agri* C15 Cd^R strains. Any bacterial surface area is different from one to others based on their dimensions. In this study, the surface area of both strains was similar to the surface area of *Salmonella typhimurium* (5.82 μm^2) (27). The cell diameters and length of this strain is 0.7 -1.5 μm and 2 -5 μm, respectively, which are similar to the *B. agri* dimensions. *Lactobacillus plantarum* has range diameters 0.9-1.2 μm and range length 3-8 μm showed surface area 3.0 μm^2 (26).

Estimation of cell concentrations: A calibration curve was used to estimate the concentrations of cells by counting the cells under serial dilutions, and the cells count in the combination of using a Euler's square in counting. This estimation of the cells density based on their growth that obtained from the (16). Table 2 and 3 show the number of cells in Euler's squares for both strains. The concentrations of cells used in

the complexation experiment in OD_{440} 4.0 were 180323 (cells/mL) for *B. agri* C15 (Figure 4) and 193040 (cells/mL) *B. agri* C15 Cd^R (Figure 5).

Table 2: The counted number of cells of *B. agri* C15 in an improved cytometer in using a Euler's square. A, B, C and D are the serial dilutions, respectively and numbers 1, 2, 3 and 4 are the replicates of each dilution step.

Dilution (number of cells)	Dilution (number of cells)	Dilution (number of cells)	Dilution (number of cells)
A1 (4423000)	B3 (201600)	C4 (9204)	D2 (352)
B2 (2400032)	A4 (4231000)	D3 (315)	C1 (9177)
C3 (9890)	D1 (336)	A2 (4173000)	B4 (2080060)
D4 (327)	C2 (9162)	B1 (200393)	A3 (4060005)

Table 3: The counted number of cells of *B. agri* C15 Cd^R in an improved cytometer in using a Euler's square. A, B, C and D are the serial dilutions, respectively and numbers 1, 2, 3 and 4 are the replicates of each dilution step.

A1 (6012000)	B3 (462100)	C4 (310)	D2 (151)
B2 (464300)	A4 (5925000)	D3 (178)	C1 (3111)
C3 (2845)	D1 (143)	A2 (5807100)	B4 (403000)
D4 (167)	C2 (2976)	B1 (400393)	A3 (57000703)

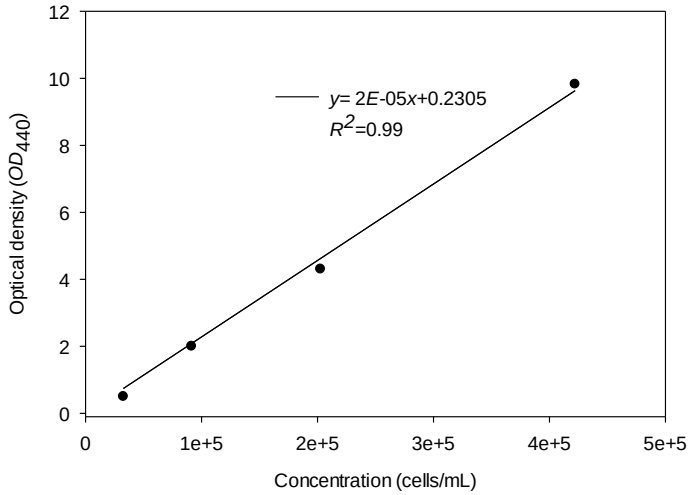


Figure 4: Calibration curve showing the relationship between OD_{440} and concentrations of *B. agri* C15 was used in the estimations of its concentration of OD_{440} 4.0 that used in surface complexation experiment.

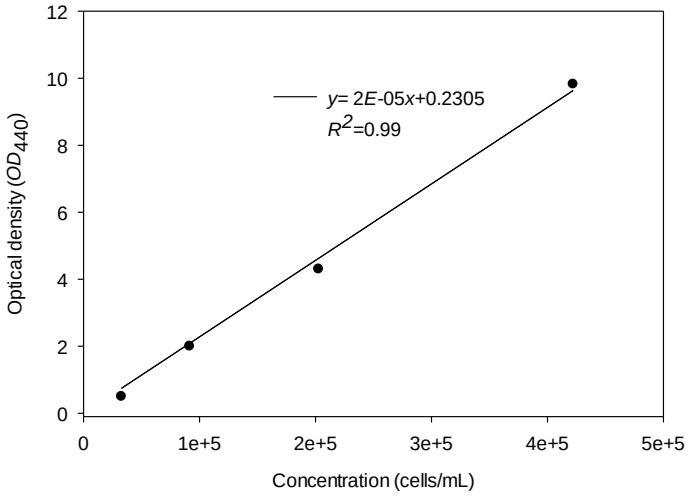
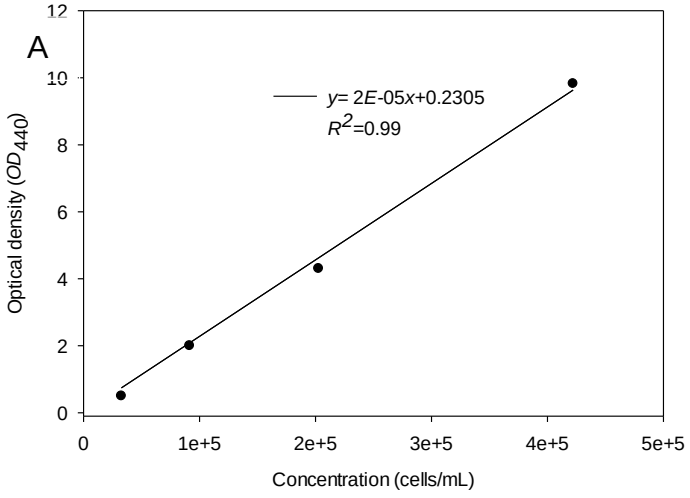


Figure 5: Calibration curve showing the relationship between OD_{440} and concentrations of *B. agri* C15 Cd^R was used in the estimations of its concentration of OD_{440} 4.0 that used in surface complexation experiment.

Surface complexation of cadmium in *B. agri* C15 and *B. agri* C15 Cd^R: From surface complexation of cadmium in *B. agri* C15 and *B. agri* C15 Cd^R, strains do cadmium adsorption over the pHs. At pH 8.5, both strains reveal uptake of <80% (Figure 6 A and B). The result confirmed that the adsorption capacity was not related to the differences in surface area of both strains during the adsorption as both strains had a similar area. Both strains, *B. agri* C15 and *B. agri* C15 Cd^R showed similar cadmium uptake and the highest value at pH 8.5. The cell-wall ligands of amino, carboxyl, phosphoryl and hydroxyl could be reason for the binding of cadmium in the pH above 7.5 (3). On the other hand, the primary functional groups of *B. agri* C15 are N, P, or S as stated previously (13). On the other hand, autotrophic and heterotrophic bacteria determined as tolerant to110 mg Cd/L and 60 mg Cd/L, respectively (1).



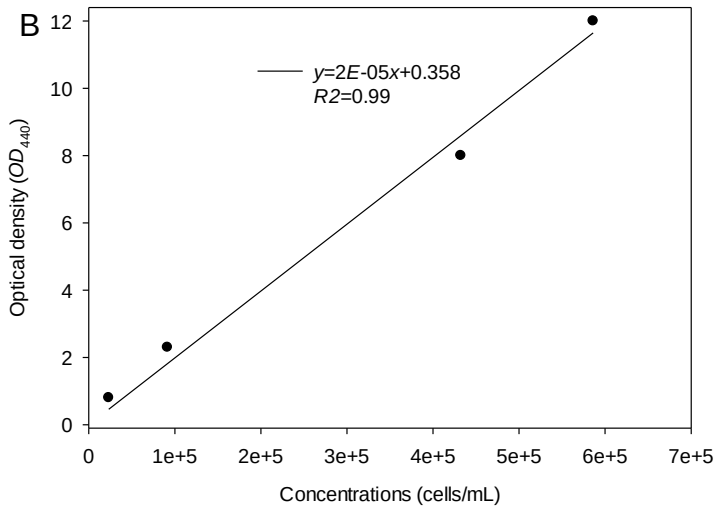


Figure 6: The cadmium adsorption onto (A) *B. agri* C15 and (B) *B. agri* C15 Cd^R using 0.01 Mm Cd and 180323 (cells/mL) for *B. agri* C15 and 193040 (cells/mL) *B. agri* C15 Cd^R (wet weight) in a suspension of 0.1 mM $NaClO_4$.

Conclusions

The accumulation of Cd under TEM detection predicts that Cd bioaccumulation is the main mechanism in wild type and that adsorption is the main mechanism in the mutant. The observation of vesicles and granules, mainly poly-β-hydroxybutyrate under Cd-sublethal concentration may contribute to the adsorption and intercellular sequestration of Cd. There were differences in the cell compartments through the uptake process of Cd by the bacterial cells. Therefore the examinations of the contribution of surface complexation and applying software model in the uptake of Cd to determine the thermodynamic stability constants of both strains from the available obtained parameters was necessary.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Author N. M. T. Jebril; methodology, writing—original draft preparation, Author A. K. Murad and R. Boden writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding:

This research received no external funding.

Institutional Review Board Statement:

Non.

Informed Consent Statement:

No Informed Consent Statement.

Data Availability Statement:

No Data Availability Statement.

Conflicts of Interest:

The authors declare no conflict of interest.

Acknowledgments:

We would like to express our deep gratitude and appreciation to the Department of Biology, College of Sciences for Women, University of Babylon and Department of Sciences, College of Basic Education, University of Babylon and School of Biological and Marine Sciences, University of Plymouth, United Kingdom for their significant support and provision of resources necessary for the completion of this research. Their valuable contributions were essential in achieving the objectives of this study.

References

1- Abed, I. A., Marzoog, A., Addaheri, A. M. S., and Al-IssawI, M. H. (2022). Isolation and diagnosis of cadmiumresistant bacteria and its potential phytoremediation with the broad bean plant. SABRAO Journal of Breeding and Genetics, 54(2): 416-425.

2- Boden, R., and Murrell, J. C. (2011). Response to mercury (II) ions in Methylococcus capsulatus (Bath). FEMS microbiology letters, 324(2): 106-110. https://doi.org/10.1111/j.1574-6968.2011.02395.x.

3- Boyanov, M. I., Kelly, S. D., Kemner, K. M., Bunker, B. A., Fein, J. B., and Fowle, D. A. (2003). Adsorption of cadmium to Bacillus subtilis bacterial cell walls: a pH-dependent X-ray absorption fine structure spectroscopy study. Geochimica et Cosmochimica Acta, 67(18): 3299-3311. https://doi.org/10.1016/S0016-7037(02)01343-1.

4- Chen, S. H., Cheow, Y. L., Ng, S. L., and Ting, A. S. Y. (2019). Mechanisms for metal removal established via electron microscopy and spectroscopy: a case study on metal tolerant fungi Penicillium simplicissimum. Journal of hazardous materials, 362: 394-402. https://doi.org/10.1016/j.jhazmat.2018.08.077.

5- Euler, L. (1782). Recherches sur un nouvelle espéce de quarrés magiques. Verhandelingen uitgegeven door het zeeuwsch Genootschap der Wetenschappen te Vlissingen, 85-239.

6- Fein, J. B., Boily, J. F., Yee, N., Gorman-Lewis, D., and Turner, B. F. (2005). Potentiometric titrations of Bacillus subtilis cells to low pH and a comparison of modeling approaches. Geochimica et Cosmochimica Acta, 69(5): 1123-1132. https://doi.org/10.1016/j.gca.2004.07.033.

7- Ghanem, S. A., and G. Ibrahim, A. (2023). Variation In Parent Material and Its Effect on The Properties and Development of Soil Profiles in The Northern Part of The Coastal Region (Lattakia - Syria). Anbar Journal of Agricultural Sciences, 21(2): 480-493. doi: 10.32649/ajas.2024.144257.1095.

8- Owaid, M.N., Al-Saeedi, S.S.S., Abed, I.A. (2018). Cultivation performance of pleurotus salmoneostramineus mushroom on wastes of date-palm trunk, phoenix dactylifera L., and woodworking sawdust. Walailak Journal of Science and Technology, 15 (12), pp. 831-839.

9- Jebril, N. (2020). Lab-scale bioremediation technology: the development of environmental biotechnology for the ex situ bioremediation of cadmium-contaminated freshwater (Doctoral dissertation, University of Plymouth).
10- Jebril, N. M. T. (2020). Evaluation of two fixation techniques for direct observation of biofilm formation of Bacillus subtilis in situ, on Congo red agar, using scanning electron microscopy. Veterinary World, 13(6): 1133. https://doi.org/10.14202%2Fvetworld.2020.1133-1137.
11- Jebril, N. M. T. (2023). Nanobiopolymer: Potential Applications in Bioremediation of Cadmium Contaminated Water. Iraqi Journal of Industrial Research, 10(2): 117-125. https://doi.org/10.53523/ijoirVol10I2ID290.
12- Jebril, N. M. T., Boden, R., and Braungardt, C. (2023). Remediation technique for cadmium contaminated groundwater: a systematic review. Anbar Journal of Agricultural Sciences, 21(1): 1-18.
13- Jebril, N., Boden, R., and Braungardt, C. (2021). Cadmium removal with mutant Brevibacillus agri C15 CdR entrapped in calcium alginate gel: a new process. In IOP Conference Series: Earth and Environmental Science, 761(1): 012028. DOI 10.1088/1755-1315/761/1/012028.
14- Jebril, N., Boden, R., and Braungardt, C. (2021). The isolation and identification of cadmium-resistant *Brevibacillus agri* C15. In *Journal of Physics: Conference Series*, 1879(2): 022015. DOI 10.1088/1742-6596/1879/2/022015.
15- Jebril, N., Boden, R., and Braungardt, C. (2022). Cadmium resistant bacteria mediated cadmium removal: a systematic review on resistance, mechanism and bioremediation approaches. In IOP Conference Series: Earth and Environmental Science, 1002(1): 012006. DOI 10.1088/1755-1315/1002/1/012006.
16- Qadir, B., and Sheikh-Abdullah, S. (2023). Response of Wheat (Triticum aestivum L.) to Organic Manure and Manganese Levels Applications in Two Different Soil Orders. Tikrit Journal for Agricultural Sciences, 23(1): 103–111. https://doi.org/10.25130/tjas.23.1.13.
17- Krieg, N. R., Gerhardt, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., and Phillips, G. B. (1981). Manual of methods for general bacteriology. Washington, DC.
18- Leonel, T. F., Moretto, C., Castellane, T. C. L., da Costa, P. I., and Lemos, E. G. D. M. (2019). The influence of cooper and chromium ions on the production of exopolysaccharide and polyhydroxybutyrate by Rhizobium tropici LBMP-C01. Journal of Polymers and the Environment, 27: 445-455. https://doi.org/10.1007/s10924-018-1359-4.
19- Marche, M. G., Mura, M. E., Falchi, G., and Ruiu, L. (2017). Spore surface proteins of Brevibacillus laterosporus are involved in insect pathogenesis. Scientific Reports, 7(1): 43805. https://doi.org/10.1038/srep43805.
20- Marusak, K. E., Feng, Y., Eben, C. F., Payne, S. T., Cao, Y., You, L., and Zauscher, S. (2016). Cadmium sulphide quantum dots with tunable electronic properties by bacterial precipitation. RSC advances, 6(80): 76158-76166. https://doi.org/10.1039/C6RA13835G.

21- Naji, H. F., and Hassan, A. A. (2023). Determining The Occurrence of Some Virulence Genes in Proteus Species Isolates. Journal of Life Science and Applied Research, 4(2): 75–87. https://doi.org/10.59807/jlsar.v4i2.88.

22- Naji, H. F., and AL-Jabber, M. A. (2024). Genetic Diversity and Antibiotic Resistance Patterns of Pseudomonas aeruginosa Isolates from Iraqi Hospitals. Journal of Life Science and Applied Research, 5(1): 8–15. https://doi.org/10.59807/jlsar.v5i1.93 .

23- Norris, K. P., and Powell, E. O. (1961). Improvements in determining total counts of bacteria. Journal of the Royal Microscopical Society, 80(2): 107-119. https://doi.org/10.1111/j.1365-2818.1961.tb00475.x.

24- Qin, H., Hu, T., Zhai, Y., Lu, N., and Aliyeva, J. (2020). The improved methods of heavy metals removal by biosorbents: A review. Environmental pollution, 258: 113777. https://doi.org/10.1016/j.envpol.2019.113777.

25- Shen, Y., Zhao, S., Zhao, X., Sun, H., Shao, M., and Xu, H. (2019). In vitro adsorption mechanism of acrylamide by lactic acid bacteria. Lwt, 100: 119-125. https://doi.org/10.1016/j.lwt.2018.10.058.

26- Smit, J., Kamio, Y., and Nikaido, H. (1975). Outer membrane of Salmonella typhimurium: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. Journal of bacteriology, 124(2): 942-958. https://doi.org/10.1128/jb.124.2.942-958.1975.

27- Wang, X., Li, Z., Li, X., Qian, H., Cai, X., Li, X., and He, J. (2016). Poly-β-hydroxybutyrate metabolism is unrelated to the sporulation and parasporal crystal protein formation in Bacillus thuringiensis. Frontiers in Microbiology, 7: 836. https://doi.org/10.3389/fmicb.2016.00836.

28- Xu, X., Xia, L., Zhu, W., Zhang, Z., Huang, Q., and Chen, W. (2015). Role of Penicillium chrysogenum XJ-1 in the detoxification and bioremediation of cadmium. Frontiers in Microbiology, 6: 1422. https://doi.org/10.3389/fmicb.2015.01422.

29- Zhang, J., Li, Q., Zeng, Y., Zhang, J., Lu, G., Dang, Z., and Guo, C. (2019). Bioaccumulation and distribution of cadmium by Burkholderia cepacia GYP1 under oligotrophic condition and mechanism analysis at proteome level. Ecotoxicology and environmental safety, 176: 162-169. https://doi.org/10.1016/j.ecoenv.2019.03.091.