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ALTERNATIVE METHOD FOR DETERMINING METAL UPTAKE FROM WATER BY ESCHERICHIA COLI K12 CD3

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Abstract

Bacteria are usually applied in biotechnology in areas such as element adsorptions due to extracellular/intracellular accumulation. Electron microscopes and spectrophotometry have shown proximate/accurate methods for observing and detecting these accumulated elements. In contrast, X-ray fluorescence (XRF) validates the examination of elements, and direct investigation for studying these collected elements. This research assessed the prospects for applying XRF in examining samples of bacterial-accumulated cadmium, *Escherichia coli* K12 Cd3, from artificial wastewater (AWW) by comparing the commonly used techniques of transmission electron microscopy (TEM-EDX) and inductively-coupled plasma mass spectrometry (ICP-MS). The results document a new means for using the XRF device in determining accurate quantities of cadmium accumulated in bacterial cells. By comparison, usual TEM-EDX analyzes show that the concentration was not qualitatively determined. At the same time, the ICP-M device was

not as efficient in measuring cadmium concentrations. Finally, the findings show that an XRF device directly measures the element within bacterial cells with greater accuracy than those analyzed by TEM-EDX and ICP-MS devices. This demonstrates that XRF can be applied in various areas of biotechnology to investigate the absorption of accumulated elements and to explain the procedures underlying the biotechnology process.

Keywords: Accumulation of elements, Bacteria, Bioremediation, Cadmium, ICP-MS, TEM, XRF.

طريقة اخرى لتقدير المعدن المزال من الماء بواسطة ESCHERICHIA COLI K12 CD3

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الخلاصة

تُستخدم البكتيريا بشكل شائع في مجالات التكنولوجيا الحيوية مثل امتزاز العناصر بسبب التراكم خارج الخلية/ داخل الخلايا. أثبتت المجاهر الإلكترونية والقياس الطيفي أنها طريقة تقريبية/ دقيقة لرصد هذه العناصر المتراكمة والكشف عنها. في المقابل، يقوم مضان الأشعة السينية (XRF) بتوثيق تحليل العناصر، والفحص المباشر، والذي قد يؤدي إلى تحليل هذه العناصر المتراكمة. هنا، قامت هذه الدراسة بتقييم إمكانية استخدام XRF للتحليل المباشر لأمثلة الكادميوم البكتيري المتراكم *Escherichia coli* K12 Cd3 من مياه الصرف الصناعي (AWW). تمت مقارنة هذه الدراسة بالتقنيات المستخدمة عادةً، مثل المجهر الإلكتروني النافذ (TEM–EDX) ومطياف كتلة البلازما المقترنة حثيًا (ICP–MS). توثق هذه الدراسة اختراعاً جديداً لممارسة جهاز XRF بكميات دقيقة من الكادميوم المتراكم في الخلايا البكتيرية. بالمقارنة، أظهرت تحليلات TEM–EDX المعتادة أن التركيز لم يتم

تحديده نوعيًا، بينما وجد أن جهاز ICP–M ليس فعالاً في قياس تركيز الكادميوم. وأخيرا، يوضح هذا الاكتشاف أن استخدام جهاز XRF في القياس المباشر للعنصر داخل الخلايا البكتيرية يتمتع بكفاءة متطورة مقارنة بأجهزة TEM–EDX وICP–MS. توضح هذه النتيجة أنه يمكن استخدام XRF في مجالات مختلفة في التكنولوجيا الحيوية للتحقيق في التركيزات المتعلقة بامتزاز العناصر والتحقق من صحة إمكانية XRF لعرض العمليات في قطاعات التكنولوجيا الحيوية.

كلمات مفتاحية: تراكم العناصر، بكتيريا، المعالجة الحيوية، الكادميوم، ICP–MS، TEM، XRF.

Introduction

Bacteria are commonly applied in biotechnology in the bioremediation process (3 and 10). Measuring bioremediation elements to assess the capability of the process has been consistently applied in element-peak detection using electron microscopes of TEM-EDX (7). TEM-EDX is able to provide rough element detection that appear in altered peaks, meaning the proportion of the elements is present in the particular section. However, it is difficult to apply EDX analysis for bacteria and further processes must be applied and modified which consumes additional time and costs (1 and 17). ICP-MS or ICP-OES analyses are currently used in validating bioremediation research, and involves the sample digestion process before the analyses. This could be less accurate as digestion is not enough to release all the elements from the samples. On the other hand, XRF has not been directly applied in examining elements usually through the bioremediation process (6). This applies to either metal accumulated bacterial cells, for example, and does not specifically involve determining elements by XRF technique for bacterial cells in the biotechnology sector or the bioremediation process (4, 6, 8, 9, 11 and 13).

This research used *E. coli* K12 Cd3 for evaluating cadmium accumulation in AWW for additional determinations of the element within the cells through the XRF method that has been previously used for the purpose (2). This use of XRF in determining cadmium accumulation in bacterial cells is based on applying it (5) for direct examination of the elements in algae instead of applying ICP-MS or ICP-OES. Accumulation assays for cadmium using *E. coli* K12 Cd3 were demonstrated in the batch tests. Initially, analyzes of bacterial cells after the accumulation experiment were carried out by TEM-EDX. Then, ICP-MS examination was done on the accumulated cells. The XRF investigation was verified in a bench stand, and the possibility of applying it for examining elements within the bacteria was confirmed for direct checking of the elements through the bioremediation process. Furthermore, in a previous study (11), *E. coli* K12 Cd3 was investigated for its ability to remove and its cells were viewed under an electron microscope. This study investigated the possibility of producing H_2S by this strain in addition to the UV-generated strain, mutant *E. coli* K12 $Cd3^R$. This is because in the previous study, yellow colored precipitation was seen through during the process of removing cadmium from water.

Materials and Methods

Bacterial cell preparation and cadmium accumulation experiments: To detect cadmium accumulation in bacteria before investigating this novel use of direct analysis by XRF, *E. coli* K12 Cd3 (sourced from 11) from earlier experiments were kept in a batch flask. Briefly, 50 mL of suspension cells were inoculated into AWW, 500 mL (10), in Erlenmeyer flasks (2000 mL) in triplicates, incubated at 37 °C for four days and shaken at 150 rpm. These bacterial cells are described as 'absorbent' i.e., able to uptake cadmium or any elements such as those found in AWW (N, H, Cl, K, H, P, O, Na, and C). The designated concentration of Cd added into AWW and accumulated in bacteria was equal to the MIC or 3 mM of the Cd strain (4). Cadmium was added as a $CdCl_2$ solution and, at the same time, control cultures without the cadmium were made for evaluation. During and after incubation, the AWW was separated from the bacterial cells by centrifugation and analyzed by ICP-MS. The part of cadmium accumulation in the bacterial cells was sprayed with NaCl (0.9 *w*/*v*) and kept at -20 °C preceding examination for both XRF analysis or fixation for TEM examination. The formula for testing the uptake of cadmium by the bacterial cells in AWW (*Q*) is as follows:

$Q\ (\%) = (C_0\text{-}C_i/C_0)/100$ (10 and 11)

Where, C_0 is the nominated cadmium concentration and C_i is the remaining cadmium concentration after incubation.

Investigating H_2S production by *E. coli* K12 Cd3 and mutant *E. coli K*12 $Cd3^R$: The bacteria were grown on EBS agar plates containing 20 mM pyruvate (as the source of carbon) supplemented with cadmium, with their MIC concentrations of 3 mM Cd and 9 mM Cd, respectively. Then the confirmation of the orange or yellow color precipitation was investigated for H_2S production. Briefly, lead-acetate paper was placed inside the lid of a sterilized glass tube (15 mL) containing bacterial inoculum inoculated in 2 mL of nutrient broth. For validation of the media broth, other independent experiments were conducted using 2 mL EBS broth containing 20 mM pyruvate (as the carbon source) instead of nutrient broth. The uninoculated controls were considered. The evaluation of the H_2S production was determined by observing its precipitation on the lead-acetate paper, as a grey color.

ICP-MS analysis of cadmium: The accumulated amounts of cadmium on the cells (P), and designated and residual concentrations of cadmium in the resultant supernatant (S, AWW) were determined. The P1 was resuspended in saline in a volumetric flask (5 mL) and the dry biomass determined. Suspension pellets (P) amounting to 0.5 mL were transferred into a volumetric flask (10 mL) and aqua regia solution (5 mL, prepared from mixing 1V of HNO_3 (>68%) with 3V of HCl (<37%). The color of the solution which changed to golden after a few mins (meaning the solution was ready for use) was added for acid digestion for 48 h in a fume hood at constant room temperature (20 °C). After digestion, the volume was completed with HNO_3 (2%) to 10 mL, containing indium (as an external standard, 0.43 µM in the total volume). On the other hand, S1 was collected and transferred immediately into a clean Falcon tube (50 mL). 10 mL from the aqueous phase was transferred into a volumetric flask (25 mL) using a glass analytical pipette (10 mL). Then, the volume of the volumetric flask was filled with HNO_3 (2%) containing indium as an external standard and 0.43 µM in the total

volume to be analyzed. The accuracy of this process was assessed using a spike recovery test (15).

The element analyses of total concentrations of cadmium in the S and P were determined using ICP-MS (Thermo Scientific, X Series 2). Yttrium was added before measurement in a concentration of 0.56 µM for use as an external standard. Procedure blanks were used comprising AWW without cadmium. The instrument was certified by estimating some method limitations: linearity, limit of detection (LOD), limit of quantities (LOQ), accuracy, and precision. Linearity was based on the systematic reaction from the standard solution used in the analyses. Standard solutions of cadmium were prepared in HNO_3 (2%) in amounts of 0, 1, 2, 3, 4, and 5 mM. As shown in Figure 1 and Table 1, the measurement of standard solutions in the instrument, linear regression (R^2=0.93), interceptions, and percentage interrupt (0.02) were achieved, indicating no systematic error.

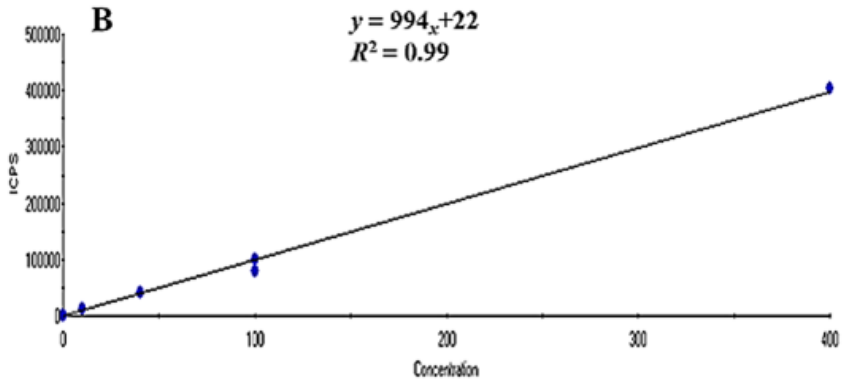

Figure 1: Relationship between concentration data and the ICPS values for cadmium.

Table 1: Linear regression data of cadmium.

Parameter	Cd
Linear range	10 – 400 µM
R^2	0.99
Slope	994
Intercept	22
Intercept %	0.02

Fixation of bacterial cells: For comparative investigation in the purposeless analysis (TEM) of accumulated cadmium by bacterial cells, the cells from the control culture and from cadmium accumulation experiments were stabilized according to (10) prior for TEM analysis. The fixative bacterial cells were analyzed using TEM (JEM-1400).

XRF analysis of cadmium accumulated in bacterial cells: Cadmium accumulated in bacterial cells was analyzed by XRF (Niton XRF analyzer, model XL3T). The pelts were dehydrated at 80 °C in an oven for 24 hours, placed in an XRF cup and covered with polypropylene reedy film to be placed over the XRF window to analyse cadmium (180 seconds, 20 KV). Certified Reference Materials (CRM) of cadmium (clay loam MLS) was applied to confirm accuracy and exactness of the instrument, as no market CRM for cadmium-loaded bacterial cells is available. The recovery rate was 92%.

Statistical analysis: Data for this study, namely the mean and standard error of the mean, and Sigma Plot 13 were used to illustrate them. *t*-test and one-way ANOVA were performed on the data of Cd concentrations to determine the differences between the ICP-MS and XRF techniques.

Results and Discussion

Cadmium accumulation experiment by *E. coli* K12 Cd3: In this research, Cd-accumulated bacterium *E. coli* K12 Cd3 with the capability to resist against cadmium was used to remediate cadmium from AWW. The investigation of accumulation tests is not only valuable for amplification strategies and quantities of uptake but also for assessing the best technique for examining cadmium accumulated in bacterial cells. The experiment revealed an acceptable percentage of cadmium uptakes (1.2 mmol Cd, Figure 2A). This finding is comparable to a previous study (11) on cadmium removing of 5.2 mg Cd g^{-1} dry biomass cell of 17 mg Cd g^{-1}. The TEM micrograph of cadmium accumulated cells at the end of the experiments showed morphological changes of the strain compared to cells grown without Cd. Under Cd-free conditions, cells had a thin cell wall and the presence of cell organelles (Figure 2 (B1). After Cd uptake, the cells showed electron-dense distribution within cells (Figure 2 (B2). In addition, the EDS spectra (Figure 2C) demonstrate the characteristic elemental peak of Cd, which confirms the possible uptake of Cd by *E. coli* K12 Cd3. According to the TEM micrographs, cells that grew without Cd had a thin cell wall and the presence of cell organelles in comparison to the electron-dense distributed within accumulated cadmium cells shown as the peaks of Cd (2 and 3).

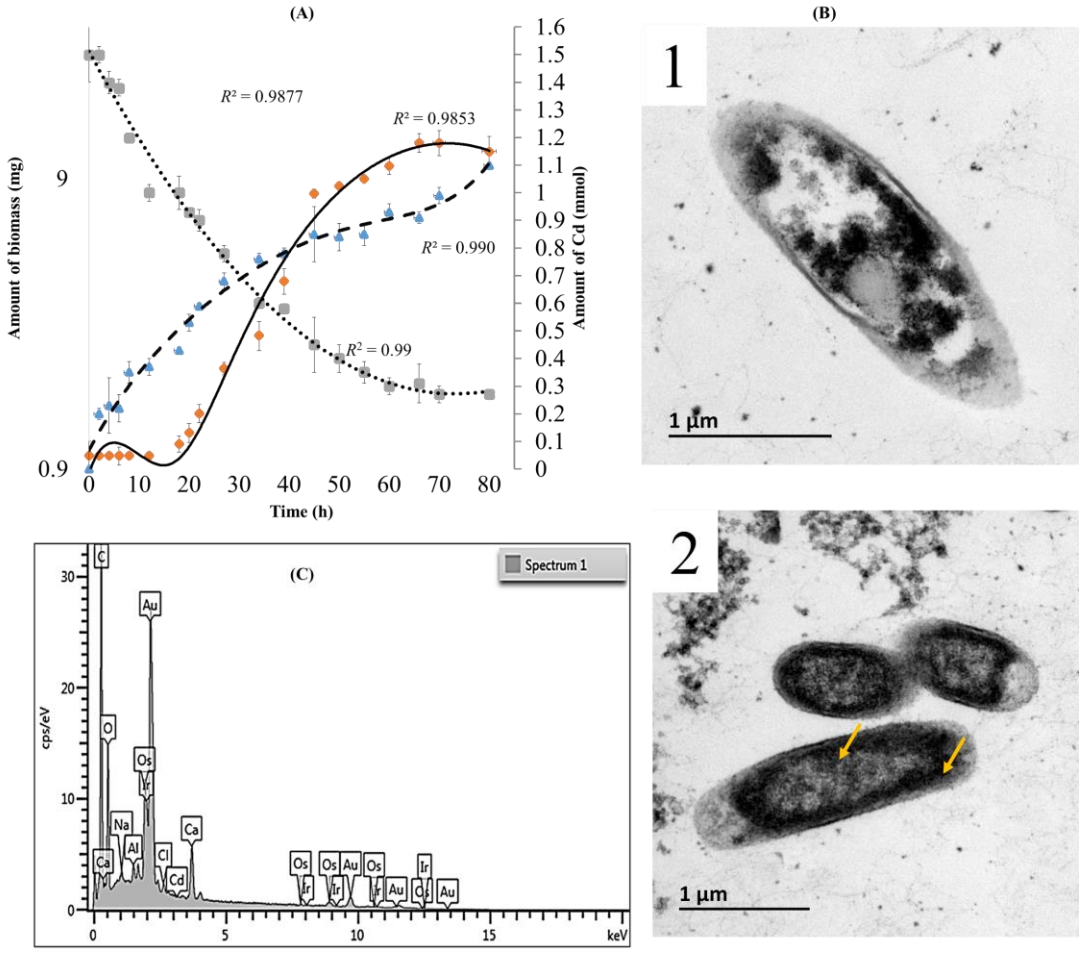

Figure 2: (A) Accumulation amounts of Cd by *E. coli* K12 Cd3 in batch cultures, AWW (500 mL), and 1.5 mmol (3 mM) Cd. The dry bacterial mass was determined (♦). The accumulated amounts of Cd on the cells (▲) and residual Cd in the resultant supernatant (■) were determined after separating by centrifugation and each point through the graph is respectable between them. The error bars symbolize the standard error of the mean (*n = 3*). (B) TEM images of the harvested cells of *E. coli* K12 Cd3 after the accumulation times, showing the electron-dense (yellow arrow, micrograph 2) compared to electron-lucent in grown cells without Cd (micrograph 1). (C) Elemental spectrum of the cadmium accumulated cells showing the presence of Cd.

H_2S production by *E. coli* K12 Cd3 and mutant *E. coli K*12 $Cd3^R$: A relevant mechanism for cadmium uptake by a bacterium requires the production of H_2S. Based on this, the examination revealed the presence of orange or yellow color precipitation of bacteria colonies growing in the media containing cadmium (Figure 3 B and C). Though this study did not concern the production of H_2S by the mutant *E. coli K*12 $Cd3^R$, some investigation on it can provide the basis for further research on this strain. The *E. coli K*12 $Cd3^R$ also appears to induce some color precipitation (Figure 3 A and D). Color cadmium precipitation has been seen to occur in different microorganisms through 2 alternative means, i.e., producing peptides to cap off CdS or due to the cysteine desulfhydrase enzyme (16). Such color precipitation is not apropos H_2S production as *E. coli* K12 Cd3 did not form the grey color on the lead-acetate paper (Figure 4 A2 and B2), while the mutant *E. coli K*12 $Cd3^R$ does in EBS broth/pyruvate

(Figure 4 B3). It is well known that mutagenesis of a bacterium can induce genes that either produce peptides or proposed enzyme (8).

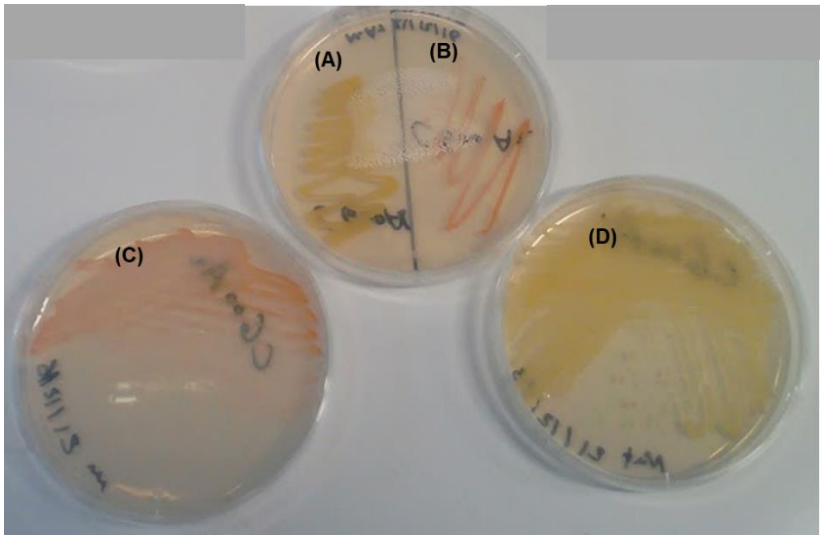

Figure 3: *E. coli* K12 Cd3 and mutant *E. coli K*12 Cd3^{R} on EBS agar plates containing 20 mM pyruvate (as the source of carbon) supplemented with cadmium (10 mM). (A) and (D) yellowish colonies represent the *E. coli K*12 Cd3^{R}; (B) and (C) orange colonies represent the *E. coli* K12 Cd3.

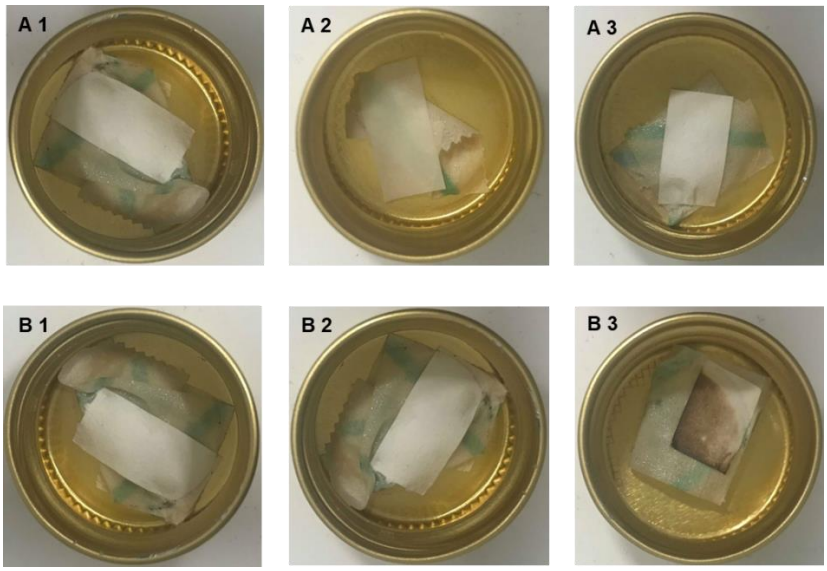

Figure 4: H2S production by E. coli K12 Cd3 and mutant E. coli K12 Cd3R. (Row A) A1: Uninoculated control; A2: E. coli K12 Cd3, and A3: mutant E. coli K12 Cd3R inoculated in 2 mL of nutrient broth in a sterilized glass tube (15 mL) with fixing lead-acetate paper inside the tube`s lid. (Row B) B1: Uninoculated control; B2: E. coli K12 Cd3, and B3: mutant E. coli K12 Cd3R inoculated in 2 mL EBS broth containing 20 mM pyruvate (as the source of carbon) in a sterilized glass tube (15 mL) with fixing lead-acetate paper inside the tube`s lid. The control, E. coli K12 Cd3, and mutant E. coli K12 Cd3R were incubated at 37 °C for 48 h.

ICP-MS and XRF Analysis: Three techniques were used in this study to detect the accumulated cadmium. First, the two available and common TEM-EDX and ICP-MS methods were used followed by novel XRF method. The ICP-MS and XRF analysis was carried out of the cadmium-accumulated cells at the end of the experiments. The result of the ICP-MS analysis showed the total concentrations of cadmium at 37.8 ± 0.6 µg Cd g^{-1} cells, a significant difference from those analyzed by XRF (52.5 ± 0.3 µg Cd g^{-1} cells, Figure 5).

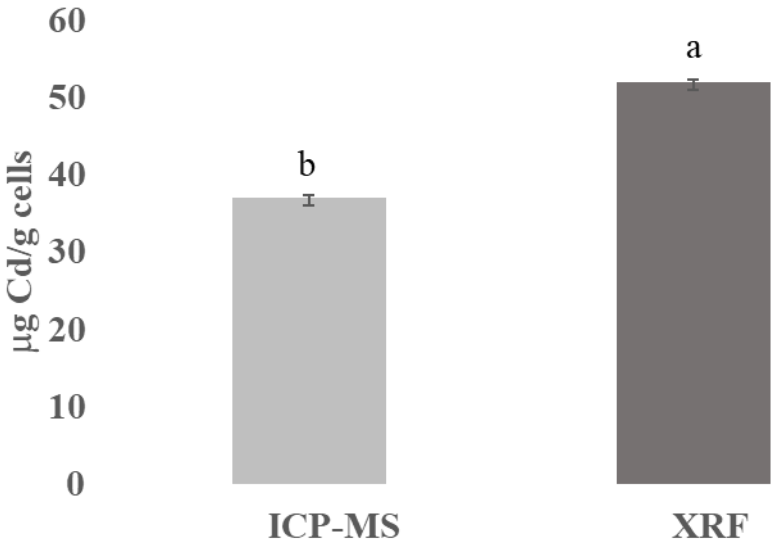

Figure 5: Comparison of Cd concentrations in accumulated cells of E. coli K12 Cd3 measured by ICP-MS and XRF techniques. The (a) and (b) letters on the bars represent the major variance in the measured devices.

All the procedures detect the accumulated cadmium due to the associations in the results of the cadmium uptake (10, 12, 14 and 17) and absorption in the cells and the TEM-EDX detections, ICP-MS and XRF, though they vary in their ability. The detection of cadmium by TEM-EDX was by observing the peak Cd in cps/EV (10). On the other hand, the cadmium concentrations within the cells were in µg Cd g^{-1} cells; this µg/g is the specific unit for determining and absorptions of cadmium rather than calculating the electron per second by TEM−EDX (11). Also the absence of techniques prior to TEM-EDX observation that deals with toxic chemicals is time consuming in fixing the samples. However, between the ICP-MS and XRF techniques for determining cadmium levels, the latter showed a significant concentration over the other. The ingestion of the accumulated cells before analysis by ICP-MS is occasionally not adequate to release the elements; therefore, the recorded concentration was lower than that by XRF.

Conclusions

This research investigated the use of XRF for the direct examination of elements, such as cadmium accumulations within the *E. coli* K12 Cd3 bacterial cells compared to the ineffective, simple, and difficult technique of the peak detection-based SEM-EDX. This detection of the detailed concentrations of cadmium in bacterial cells through direct examination by XRF allows it to be used in examining other elements. It enables the bioremediation process to be directly associated with other additional

chemistry approaches. On the other hand, mutant *E. coli K*12 $Cd3^R$ do produce color precipitation of cadmium and H_2S production. As such, further study can be conducted on this strain.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Both authors contributed in designing the methodology and conducting the experiments and in writing the manuscript.

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Informed Consent Statement:

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Data Availability Statement:

No Data Availability Statement.

Conflicts of Interest:

The authors declare no conflict of interest.

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