The isolation and identification of cadmium-resistant
*Brevibacillus agri*C15

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Abstract. Eight Cd-resistant bacteria were isolated from the soil. Based on the resistance and specific growth rate, one isolate from these was chosen. The 16S rRNA (rrs) gene sequence was used to assert this isolate belongs to the genus of Brevibacillus and closely related to Brevibacillus agri (B.agri) DSM 6348T (AB112716). The isolate was termed B.agri C15. The specific growth yields and the Cd-dependent fall in specific growth yield of B.agri C15 was determined under different concentrations of Cd. The current study provides a basis for isolation Cd resistant bacteria with maximum tolerable concentration (MTC) 15 mM Cd from a contaminated soil 490±50 µmolal Cd that could be suitable for a new Cd-bioremediation process; therefore, further studies are needed to investigate this possibility.

Keywords. Brevibacillus agri, cadmium, growth rate, isolation, soil.

1. Introduction

The contamination of environments with metals has become a global challenge in the relation to human health. Cadmium is a non-essential element for life, and it is toxic to Homo Sapiens. The United States Environmental Protection Agency (US EPA) and the International Agency for Research on Cancer (IARC) classified some elements, including Cd, As, Hg, and Pb, as human carcinogens [1]. Furthermore, chronic exposure to low levels of Cd is associated with several diseases, such as deranged blood pressure regulation, osteoporosis, early onset of diabetic renal complications, and end-stage renal failure. The Cd contamination, exceeding the US EPA limit of 3 µg/L, has become a major concern, especially in the relation to increasing groundwater withdrawals for human use and agricultural irrigation. This contamination may be subject to regulatory requirements for treatment, and more effective methods for removing Cd are required. Techniques, such as physiochemical methods (precipitation, adsorption and ion exchange) biological methods (bioremediation) are used to remove Cd from groundwater [2]. Bioremediation is the process of transfer, forming contaminants to less harmful substances, and depending on the type of pollutant, different approaches can be employed. One approach is the isolation of bacterial resistance to the pollutant with the aim of
enhancing remediation efficiency. The first use of bacteria for Cd removal was investigated in species of Citrobacter in the batch experiment [3, 4]. Following these studies, this Citrobacter was also studied for cadmium removal in reactor experiments [5, 6]. And later, more modification methods were applied for using Citrobacter sp. in cadmium removal such as in solutions supplemented with phosphatase substrate [7, 8]. Also, Citrobacter MCM B-181 was used for removal of cadmium by [9]. The main aim of the present study was to isolate Cd-resistant bacteria from well-known contaminated soil, identify the strain, optimise its growth, and determine its tolerance to Cd.

2. Materials and Methods

2.1. Soil sampling from the contaminated site and isolation of bacteria to resist cadmium

2.1.1. Soil sampling

In the current study, for the isolation of Cd-resistant bacteria, HayTor quarry, Dartmoor, England was chosen as a source for the isolation of the strains, as this soil has been contaminated with cadmium copper, tin, silver, and lead due to historic mining activities [10]. The global positioning system (GPS) coordinates of HayTor are 50°57’054” N latitude and 3.75549” W longitude. One kg of clayey soil from the surface was collected in a sterilised plastic box, and transferred to the laboratory, then stored in a refrigerator at 4 ºC until required at 24 hrs.

2.1.2. Soil analysis

For elemental analysis, a sub-sample of the soil was dried at 50°C–60°C for 72 hrs., then disaggregated and ground using a mortar and sieved by 180-micrometer particle sizes. Cadmium was extracted from the soil via aqua regia digestion [11], and the concentrations in the extract were determined using inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS analysis was conducted according to work carried out by [12]. Briefly, 1.0 g of sieved soil (n = 3 samples) was processed following aqua regia digestion using a Tecator digestion at 60°C for 2hrs. After digestion, the solution was cooled, filtered (Whatman 541) into a clean volumetric flask, and the volume was completed with 2% HNO₃. The limit of detection (LOD) for the ICP-MS analysis for Cd (0.02 µM) was established from the five standard deviations (SDs) of measuring the lowest standard (n = 10). As no certified reference material (CRM) containing appropriately high Cd concentrations was available and hence, an in-house reference material, contaminated soil SS-2, was used to indicate recovery. The percentage recovery of ICP-MS via the use of contaminated soil- SS-2 was Cd (50%), which was calculated from the recorded (4.0±0.3 µmolal Cd) and certified (8.0±0.06 µmolal Cd) values of the reference material. However, the procedural blank (n = 3 samples) was of a similar magnitude (3.0±0.03 µmolal Cd) as this reference material, explaining the poor recovery obtained.

2.1.3. Isolation of Cd resistant bacteria

For the enrichment of bacteria, 0.5 g of the soil sample was transferred into 250 mL of Erlenmeyer flask containing 50 mL of E-Basal salts media (EBS) supplemented with D-fructose (10 mM) as a carbon source [13]. The flask was incubated at 37°C for five days, and the turbidity was monitored regularly. After incubation, 5 mL of enriched bacterial culture was transferred to 45 mL of EBS/D-fructose. The serial subculture was repeated for four-time cycles until an enriched bacterial growth was obtained, at which point isolation was attempted. The isolation was preliminarily done in terms of MTC to Cd [14]. Five mL of the bacterial culture was inoculated into 45 mL of EBS/D-fructose in Erlenmeyer-flasks (250 mL) in triplicate, amended with Cd and incubated at 37°C for 48 h. The nominal concentration of Cd (supplied as Cd(NO₃)₂·4H₂O) started at 1 µM and increased by 2 µM at each transference of the screening process. A control without Cd was used to determine whether there
was growth at each step. The concentration at that below no growth occurred, and was accepted as the MTC. After the determination of MTC values, three cultures were grown with Cd at their MTC values, which is 10 mM for two cultures and 15 mM for the third. To obtain single colonies from each bacterial culture (the MTCs of 10 mM and 15 mM), 100 µL of the culture was spread onto EBS/ D-fructose agar plates supplemented with Cd (in triplicate). The nominal concentrations of Cd (mM) were below and above MTC values of the bacterial culture. The plates were incubated at 37°C until the colonies were visible. After growing the colonies, each one was purified, and considered as a Cd-resistant isolate.

2.2. Optimising growth conditions and the selection of isolates

Different bacterial growth conditions, including temperature, carbon, and nitrogen sources, were studied in 50 mL of EBS in the Erlenmeyer-flasks (250 mL) in triplicate, and the amount of biomass was determined. The optimum temperature for the isolate was first studied by growing of the culture in EBS/D-fructose and incubation at different temperatures of 4°C, 18°C, 25°C, 30°C, 37°C, 42°C, 44°C, 50°C, and 60°C for 4 days. The most suitable carbon source for the growth of isolates was studied using glucose and fructose (10 mM concentration), acetate, pyruvate, and sucrose (20 mM concentration), separately. The experiments were carried out at an optimised temperature of 37°C for seven days and incubated at a shaking speed of 100 rpm. Finally, the suitable nitrogen source for the growth was studied by the addition of ammonia, urea, thiocyanate, cyanate, nitrate, or glycine (3 mM concentration), in addition to the dinitrogen in the air. The experiments were performed at 37°C, and the carbon sources were either fructose for some isolates or pyruvate for other isolates. Based on their Cd MTC value, four out of eight Cd-resistant isolates with the highest MTC ability (C15-1, C15-2, C15-3 and C15-4), were chosen for further study.

2.3. Estimation of the specific growth rate and selection of one isolate

The specific growth rate was determined for isolates C15-1, C15-2, C15-3, and C15-4 under their respective optimised conditions. One mL of the growing culture of each isolate was inoculated into 50 mL of EBS/ pyruvate (20 mM), or D-fructose (10 mM) for isolate C15-4. During the incubation at 37°C, samples were collected at regular time intervals, and the amount of biomass was determined. The growth of cells in the EBS was determined in terms of dry biomass. From the batch cultures, samples were collected at regular times, and the dry biomass was determined. The growth of bacterial strains was determined by measuring the optical density (OD) at 440 nm using a UV visible spectrophotometer against a cell-free EBS medium as a blank. The OD should be within the range of 0.2 to 0.9. It was assumed that 0.1 of OD was equivalent to 23 mg/L of dry cell biomass of strains. After determining the dry biomass of the growth culture at regular times, the logarithmic dry biomass against the times was plotted to obtain the bacterial growth curve as a semi-logarithmic graph. The curve was fitted with the nonlinear regression of the biomass mean data using the SigmaPlot (version 13). The growth curve was used to measure the specific growth rate by plotting one point of the exponential phase of the amount of biomass of bacterial growth (axis y) and its double point against time (axis x). Monod’s equation (1949) was used to calculate the growth rate. Based on the specific growth rates of four isolates, the isolate with the highest growth (re-coded as ‘C15’), was chosen to achieve the objectives of the present study.

2.4. Characterisation of B.agri C15

The morphology isolate of B.agri C15 was observed after being stained with Gram stain and observed under a light microscope, according to the work of [15]. Biochemical tests, including indole, methyl red, Voges-Proskauer, and citrate tests (IMVIC) were performed in triplicate, with control. The genomic DNA (gDNA) isolate of B.agri C15 was extracted according to the JGI (Doe Joint Genome
Institute) method [16]. Quality and quantity of gDNA, agarose gel electrophoresis, gDNA quantification using Nanodrop spectrophotometer, gDNA quantification using the Qubit 2.0 Fluorometer were used. The electrophoresis was run at 60 V for 60 mins and then the gel was illuminated with a UV transilluminator to be photographed using the gel-imaging system. The quantity of pure gDNA was determined using a NanodropTM 1000 spectrophotometer (Thermo Scientific Ltd., DE, USA) at 260 nm based on the measurement ratios of A260/A230 and A260/A280. A260/A230 ratio indicates residual phenol and protein contaminations, whereas the A260/A280 ratio indicates isopropanol or phenol contaminations. The accurate method for the quantity of pure gDNA was determined using the dsDNA BR Assay Kit (catalogue number Q32850, Q32853, Life Technologies) and the Qubit 2.0 Fluorometer (Life Technologies). The Qubit 2.0 fluorimeter was turned on for three h, before starting the measurements, to stable its signal. Two µL of the gDNA sample was diluted with 199 µL of Qubit dsDNA BR buffer and with 1 µL of Qubit dsDNA BR reagent (200X concentrate in DMSO) in an Eppendorf tube. A calibration curve, zero ng/µL DNA (Qubit® dsDNA BR standard #1) and 100 ng/µL DNA (Qubit dsDNA BR standard #2) were diluted with 190 µL of Qubit dsDNA BR buffer and with 1 µL of Qubit dsDNA BR reagent (200 concentrates in DMSO) in an Eppendorf tube. All samples were incubated for 2 mins, and the concentrations of gDNA were measured using the fluorimeter by sequentially putting in 2 µL from each sample and recording the gDNA concentrations. The acceptable gDNA samples for sequencing required a concentration of 10 – 30 ng gDNA /µL Tris-HCl. The gDNA was subjected to 16S rRNA (rrs) gene sequence. MUSCLE (Multiple Sequence Comparison by Log-Expectation) was used to make the multiple sequence alignment to be tested for the best fit of models, based on the Bayesian Information Coefficient (BIC). The Phylogenetic analysis was done using MEGA software (Version7.0.26); the trees were reconstructed accordingly (with a percentage of 5,000 bootstrap replications) with a discrete gamma distribution to model rate differences across the sites (5 categories and gamma parameter 0.1466) allowance for some (40.39 %) evolutionarily invariant sites.

3. Results

3.1. Isolation of bacteria resistant to cadmium

The isolation was carried out from the soil of HayTor quarry. For the soil, a concentration of 490±50 µmolal Cd was determined after aqua regia acid extraction and analysis by ICP-MS. Although the reference material used for quality control showed poor recovery (50%), this concentration is two orders of magnitude higher than Cd in the reference material. Therefore, the Cd concentration in the soil clan is seen as a reasonable, probably low estimate. Compared to soil contamination assessment criteria in the UK (EA, 2015; CLEA: e.g., for residential lifetime exposure: 98 µmolal Cd or allotment soil: 9.38 µmolal Cd, both at pH 7) and the US EPA [17] guidelines for soil (recommendation: Cd < 44 µmolal). This soil is considered as contaminated with Cd concentrations greater than one order of magnitude higher than the guidelines levels. Based on the MTC values of bacterial cultures, eight Cd-resistant bacteria were isolated from three different Cd-tolerant bacterial cultures. These isolates were named depending on their MTC abilities as C10-1, C10-2, C10-3, C10-4, C15-1, C15-2, C15-3, and C15-4 (Table 1). The isolates C15-1, C15-2, C15-3, and C15-4 showed the highest MTC (15 mM Cd), followed by the isolates C10-1, C10-2, C10-3, and C10-4 (10 mM Cd).
Table 1. MTC values of Cd-resistant bacterial isolates. The isolate names were coded depending on the MTC values; 10: Means the isolate resist to 10 Cd mM, 15: Means the isolate resist to 15 Cd mM, and 1, 2, 3, and 4: Mean the number of the isolate from each resistant culture.

| Isolate | MTC (mM Cd) |
|---------|-------------|
| C10-1   | 10          |
| C10-2   | 10          |
| C10-3   | 10          |
| C10-4   | 10          |
| C15-1   | 15          |
| C15-2   | 15          |
| C15-3   | 15          |
| C15-4   | 15          |

3.2. Optimising growth conditions and selection of isolates

The growth conditions were optimised. The amount of the biomass of eight Cd-resistant isolates at different temperatures (4 °C, 18 °C, 25 °C, 30 °C, 37 °C, 42 °C, 44 °C, 50 °C, and 60 °C), as summarised in Figure 1. The optimum temperature for the growth of these isolates was 37°C, which showed the significant highest dry biomass (Figure 1, $^aP<0.05$). At 37°C, the lowest growth was demonstrated by the isolates C10-1 and C10-2, compared to isolates C10-3 and C10-4, respectively. Similarly, the isolates C15-1, C15-2, C15-3, and C15-4 demonstrated the highest dry biomass at 37°C, and there were no differences observed among them (Figure 8, $^aP<0.05$). Eight isolates showed no growth at temperatures, 4°C, 18°C, 50°C, and 60°C compared to other temperatures. With the temperature increase to 25°C and 30°C, all the isolates displayed a significant increase in biomass amounts compared to lower temperatures. However, the increase in the temperatures to 42°C and 44°C significantly decreased the biomass amounts.
Figure 1. Determination of suitable temperature for the growth of eight Cd-resistant isolates. (A) Isolate C10-1, (B) isolate C10-2, (C) isolate C10-3, (D) isolate C10-4, (E) isolate C15-1, (F) isolate C15-2, (G) isolate C15-3, and (H) isolate C10-4. These isolates were grown in the EBS/D-fructose, incubated for 48 h at 37 °C and shaken at 100 rpm. The error bars represent the standard errors of the mean (n = 3, batch experiments) and a different letter on the bars indicates a significant difference among temperatures within isolates.

The determination of a suitable carbon source was identified by measuring the growth of each isolate in batch cultures, supplemented with glucose or fructose (10 mM), acetate, sucrose, or pyruvate (20 mM). The suitable carbon source for the growth of eight isolates demonstrated that the isolates C10-1, C10-2, C10-3, C10-4, and C15-4 had the highest biomass when growing with 10 mM of fructose (Figure 2, A, B, C, D and H, *P<0.05) compared to other isolates, C15-1 (Figure 2, E *P<0.05), C15-2 (Figure 9, F *P<0.05) and C15-3 (Figure 2, G *P<0.05). At the same time, the isolates; C15-1, C15-2, and C15-3, as outlined in the Figures 2E 2F 2G, respectively. Figure (2) showed the highest biomass (*P<0.05) in growing within 20 mM pyruvate compared to their growth within other carbon sources.
The suitable nitrogen sources for growing the eight isolates were optimised under different nitrogen sources at an optimised temperature (37°C), and carbon sources, either with fructose (10 mM) for isolates C10-1, C10-2, C10-3, C10-4, and C15-4 or pyruvate (20 mM) for isolates C15-1, C15-2, and C15-3. All isolates illustrated the highest growth when grown with ammonia (Figure 3, *P*<0.05) that was initially used in the composition of EBS medium, compared to other nitrogen sources. Based on the MTC abilities of the eight Cd-resistant isolates, four isolates i.e. C15-1, C15-2, C15-3, and C15-4 demonstrated the highest MTC abilities, and were then chosen for further study.

**Figure 2.** Determination of suitable carbon sources for the growth of eight Cd-resistant isolates. (A) Isolate C10-1, (B) isolate C10-2, (C) isolate C10-3, (D) isolate C10-4, (E) isolate C15-1, (F) isolate C15-2, (G) isolate C15-3, and (H) isolate C10-4. The isolates were grown in EBS supplemented with different carbon sources, incubated for seven days at 37°C and shaken at 100 rpm. The error bars represent the standard errors of the mean (*n* = 3, batch experiments) and a different letter on the bars indicates a significant difference among carbon sources within isolates.
Figure 3. Determination of nitrogen sources for the growth of eight Cd-resistant isolates. (A) Isolate C10-1, (B) isolate C10-2, (C) isolate C10-3, (D) isolate C10-4; (E) isolate C15-1, (F) isolate C15-2, (G) isolate C15-3, and (H) isolate C10-4. The isolates were grown in EBS supplemented with fructose (10 mM) for isolates C10-1, C10-2, C10-3, C10-4, and C15-4 or with pyruvate (20 mM) for isolates C15-1, C15-2, and C15-3, incubated for seven days at 37°C and shaken at 100 rpm. The error bars represent the standard errors of the mean (n = 3, batch experiments) and a different letter on the bars indicates a significant difference among nitrogen sources within isolates.
3.3. Estimation of the specific growth rate and selection of one isolate

The selection of the potential isolate was based on its specific growth rate in addition to its MTC ability. The dry biomass of isolates C15-1, C15-2, C15-3, and C15-4 were determined, and their growth curves were plotted to identify a specific growth rate (Figure 4). The values of the specific growth rate ranged from 0.09 to 0.12 h⁻¹. Among the four isolates, C15-1 and C15-2 significantly showed (Figure 4A, B, *P<0.05) the lowest specific growth rate (0.09 ± 0.004 h⁻¹ and 0.09 ± 0.005 h⁻¹, respectively). The isolate C15-3 displayed the highest specific growth rate (0.12 ± 0.007 h⁻¹), as summarised in the Figure 4C, *P<0.05, followed by the isolate C15-4 (0.11 ± 0.02 h⁻¹), as outlined in the Figure 4D, *P<0.05. Therefore, the isolate C15-3 was re-coded as ‘C15’ and chosen for further experiments.

![Figure 4. Growth curves of four Cd-resistant isolates. (A) Isolate C15-1, (B) Isolate C15-2, (C) Isolate C15-3, and (D) Isolate C15-4. The isolates were grown in EBS supplemented with D-fructose (10 mM) for isolate C15-4 or with pyruvate (20 mM) for isolates C15-1, C15-2, and C15-3, incubated at 37°C and shaken at 100 rpm for 72 h. The error bars represent the standard errors of the mean (n = 3, batch experiments). The specific growth rate (μ) is shown and a different letter on the μ indicates a significant difference among isolates.](image)

3.4. Morphological, biochemical, and 16S rRNA sequence analysis

Morphological observation of isolate C15 showed Gram-stain-positive bacilli (Figure 5A). The biochemical characterisation (IMVIC test) of the isolate C15 was performed. The isolate was negative and did not produce indole from tryptophan; the acid formed from glucose but produced acetone from the glucose and did not use the citrate as a carbon source. The 16S rRNA (rrs) gene sequence of the isolate C15 identified that the isolate belongs to the genus of *Brevibacillus*, closely related to *B. agric* DSM 6348T (AB112716) with 100% identification. The isolate C15 was named *B.agri* C15, as outlined in the Figure 5E.

3.5. Assessment of the inhibition effects of cadmium on *B. agric* C15

The inhibition effects of Cd on *B. agric* C15 under 2.5, 5, 10, and 15 mM Cd were studied. An increase in the Cd concentration led to an increase in the amounts of unconsumed pyruvate. The exposure to 2.5 mM Cd decreased the utilised pyruvate (mM) from 14.1 (Figure 6A) to 12.3 (Figure 6B). The amounts of unconsumed pyruvate were inhibited with the nominal concentrations of 5 mM Cd (Figure 6C) and 10 mM Cd (Figure 6D). In contrast, the amount of unconsumed pyruvate under 15 mM Cd was reduced to 9.76 mM (Figure 6E). Nevertheless, the amounts of the biomass under the inhibited effect of Cd were recorded as small differences, and the specific growth rates under these toxic concentrations were significantly inhibited at the nominal concentration of 15 mM Cd (Figure 6E, *P<0.05). All of these results indicated that the toxic effect of Cd controlled the consumed amounts of pyruvate.
Figure 5. Figure 5. *Brevibacillus agri* C15. (A) Light microscopy observation Gram-stain-positive. The stains were analysed simultaneously in triplicates of three separately grown on nutrient agar plates. Control strains (B ) *Bacillus subtilis*, showing Gram-stain-positive, (C) *Cupriavidus metallidurans* DSM 2839T, showing Gram-stain-negative and (D) uninoculated slides. (E) Phylogenetic analysis. The maximum likelihood tree produced based on the 16S rRNA (rrs) gene showing the positions of isolates C15 versus *Brevibacillus* species with validly published names. The tree shown is the optimal tree with the lowest log-likelihood (-4773.56), with numbers at nodes indicating the percentage of 5,000 bootstrap replications in which the topology was preserved (values <70 % omitted for clarity). All of the positions at which there was less than 95 % coverage were omitted from the final analysis, in which 1,342 nucleotides were used. The branch lengths are to scale, and they indicate the number of substitutions per site, and the bar represents 0.05 substitutions per site. The outgroup is the same gene from *Paenibacillus polymyx* IAM 13419T, type genus of the *Paenibacillaceae*, in which all members of the in-group are circumscribed. The accession numbers are given in parentheses and refer to the GenBank or Integrated Microbial Genomes (IMG) databases (the latter contain an underscore "_").
Figure 6. The effects of Cd on the concentration of biomass (●) and pyruvate utilisation (▼) of *B. agri* C15. The strain was grown in EBS/pyruvate, (A) control and with nominal concentrations of Cd (B) 2.5 mM, (C) 5 mM, (D) 10 mM, and (E) 15 mM. The specific growth rate (♂) is shown. Inhibition effects of Cd on (F) their respective values of the $Y_S$ formed. The error bars represent the standard errors of the mean of three independent cultures at each Cd concentration. b, c, d, and e are significantly different compared to the value of control without Cd.

The results observed that the specific growth produces were intensely inhibited under the Cd concentrations (Figure 6F); the highest yield was obtained in grown without Cd; this showed statistical differences (Figure 6F, $^a p \leq 0.05$) compared to growth under other Cd exposures (2.5 mM, $^b p \leq 0.05$), (5 mM, $^c p \leq 0.05$), (10 mM, $^d p \leq 0.05$), and (15 mM, $^e p \leq 0.05$).

4. Discussion

In the current study, soil from Hay Tor quarry was chosen for the isolation of Cd-resistant bacteria, due to its history of mining, where metal ores have been observed in the soil. This soil is known to be contaminated with Cd (this study), and with other metals [10]. Therefore, it was assumed that it could be possible to isolate Cd-resistant bacteria from it. The isolation of Cd-resistant bacteria was screened sequentially by increasing the Cd concentration in the cultures. Moreover, the isolates were obtained from the highest concentrations that permitted culture survival. [18] reported that increases in Cd concentrations affect the properties of bacterial cells, such as protein activity and DNA stability, leading to evolutionary adaptations in phenotype (adaptation) and acclimatisation (changes in gene expression). Also, eight Cd-resistant isolates observed Cd-tolerant abilities, and their growth was optimised under different conditions. The best incubation temperature was established at 37°C. It is common to assume that different bacteria will prefer different carbon sources for the enrichment and that structurally similar carbon sources, which are metabolised by relevant biochemical pathways, are selected by the same microbial strains. About 55% of the carbon taken by the bacteria serve as
substrates of the metabolic network after being broken down to supply pools of amino acids and other components of a cell [19]. The isolated bacteria in the present study were enriched in EBS medium on five different carbon sources. The biomass of the isolates generated from enrichment cultures containing different carbon sources showed that pyruvate or fructose were the preferred sources of different strains. Fructose was added as the carbon source in the first steps of the enrichment culture of the soil sample; therefore, some isolates choose this source. Other strains preferred pyruvate as a carbon source. This could be as a result of using more than one carbon source for the enriched isolates and the changes in the composition of the growth medium over time will be reflected by changes in the behaviour of the isolates[20]. As well as, [21] observed that bacteria chose fructose as the sole carbon source and did not use glucose at all. Ammonia, which was used in the original EBS medium, was preferred among the nitrogen sources added. The specific growth rate of the selected four isolates (C15-1, C15-2, C15-3 and C15-4) from the eight isolates showed that isolate C15-3 grew faster than other isolates. So it was chosen for further experiments and re-coded as ‘C15’. Isolate C15 was tolerant to 15 mM of Cd (supplied as Cd (NO₃)₂•4H₂O); it was considered as a potential isolate. Isolate C15 was identified as Brevibacillus agri C15 that used pyruvate as a carbon source, showing a new characteristic compared to other Brevibacillus species, which was reported by [15]. It has been published in a recent study that several species of Brevibacillus have been isolated from diverse habitats and geographical locations. Brevibacillus sp. has been previously demonstrated to have the ability to grow in the presence of Cd (85 mg/kg or ~760 µmolal) [22]. The mechanisms of resistant bacteria to resist Cd have been described and involve an efflux pump, adsorption, precipitation, and intracellular accumulation processes [23]. The bioaccumulation process consists of the accumulation and localisation of Cd in specific organelles; Cd-resistant bacteria can resist Cd through other mechanisms as well. The proposed Cd resistance of B. agri C15 may be due to a mechanism as mentioned above. The isolate B. agri C15, which had a natural resistance in Cd-contaminated soil, may harbour one or several ATP, RND, or CDF transporter responsible for the efflux system [24]. In addition, [25] mentioned that a PIB-type ATPase gene in a uranium-resistant bacterium isolated from uranium-contaminated soil. Similar efflux systems were reported with Cd resistance in Acidithiobacillus ferrooxidans [26]. Another mechanism of effective resistance is the precipitation or the adsorption of Cd on the cell surface. The extracellular polymeric substance (EPS) produced by B. agri C15 could allow the binding of Cd to anionic carboxyl groups (COO⁻) on the EPS. When exposed to Cd, the growth of B. agri C15 was adversely affected. The efflux of Cd outside the cells was likely responsible for the higher concentrations of Cd in the growth medium that changed the efflux process, increasing the intracellular uptake of Cd. [27] reported that Cd might cause damage to the cells through the accumulation of reactive oxygen species (ROS). The cell numbers of Acidithiobacillus ferrooxidans were reduced when exposed to CdSO₄ [26]. It has also been reported that the growth of Pseudomonas fluorescens H₂ was affected by Cd exposure [28]. The results in the current study agreed with results as found [29], and [30], and reporting that Cd damages bacteria and leads to growth inhibition. The inhibition effect of Cd on the specific growth yield of B. agri C15 could also be due to the presence of other cations in the EBS growth medium. [28] found that Cd toxicity in cells decreased with the presence of Zn in the medium, a result of the inhibition of Cd uptake due to competing effects. In the present study, the EBS growth medium was used, and the possible competing effects were EDTA and Zn, both with lower concentrations compared to the Cd concentrations used in the exposure test. The high concentration of Cd suggests that the Cd were the cause of inhibited bacterial growth.

5. Conclusion

The B. agri C15 was isolated from a contaminated soil based on the MTC, and this strain showed tolerance to Cd and had a MTC value of 15 mM to Cd, which is considered as Cd-resistant bacteria. Thus, it is concluded that this strain could be suitable for a new Cd-bioremediation process; therefore, further studies are needed to investigate this possibility..
6. References

[1] Agency for Toxic Substances and Disease Registry (ATSDR) 2012 Toxicological profile for Cadmium. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

[2] Hashim MA, Mukhopadhyay S, Sahu JN and Sengupta B 2011 Remediation technologies for heavy metal contaminated groundwater J. Environ. Manag. 92 2355.

[3] Macaskie LE and Dean A 1984 Cadmium accumulation by a Citrobacter sp. Microbiology 130 53.

[4] Macaskie LE and Dean A 1984 Cadmium accumulation by immobilized cells of a Citrobacter sp Environ. Technol. 5 71.

[5] Macaskie LE and Dean A 1984 Heavy metal accumulation by immobilized cells of a Citrobacter sp Biotechnol. Lett. 6 71.

[6] Macaskie L, Wates J and Dean A 1987 Cadmium accumulation by a Citrobacter sp. immobilized on gel and solid supports: applicability to the treatment of liquid wastes containing heavy metal cations Biotechnol. Bioeng. 30 66.

[7] Michel L, Macaskie L and Dean A 1984 Cadmium accumulation by immobilized cells of a Citrobacter sp. using various phosphate donors Biotechnol. Bioeng. 28 1358.

[8] Macaskie L, Bonthrone K and Rouch D 1994 Phosphatase-mediated heavy metal accumulation by a Citrobacter sp. and related enterobacteria FEMS Microbial. Lett. 121 141.

[9] Puranik P and Paknikar K 1999 Biosorption of lead, cadmium, and zinc by Citrobacter strain MCM B-181; Characterization Studies Biotechnol. Prog. 15 228.

[10] Howard AJ, Kincey M and Carey C 2015 Preserving the legacy of historic metal-mining industries in light of the water framework directive and future environmental change in Mainland Britain: challenges for the heritage community Historic Environ. Policy Pract. 6 3.

[11] Jebril NMT 2020 Novel use of XRF in the adsorption processes for the direct analysis of cadmium and silver in absorbent Na-alginate beads Baghdad Sci. J. 17 1139.

[12] McBride MB and Spiers G 2001 Trace element content of selected fertilizers and dairy manures as determined by icp–ms Comm. Soil Sci. Plant Anal. 32 139.

[13] Boden R and Hutt LP 2018 Determination of Kinetic Parameters and Metabolic Modes Using the Chemostat In: Steffan R (ed.) Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation. Handbook of Hydrocarbon and Lipid Microbiology Springer, Cham 1.

[14] Schmidt T and Schlegel HG 1994 Combined nickel-cobalt-cadmium resistance encoded by the ncc locus of Alcaligenes xylosoxidans 31A. JB 176 7045.

[15] Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH and Whitman WB 2011 Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes. vol. 3. Springer Science and Business Media.

[16] William S, Feil H and Copeland A 2012 Bacterial genomic DNA isolation using CTAB Sigma 50 6876.

[17] United States Environmental Protection Agency (US EPA) 2011 Edition of the drinking water standards and health advisories. Washington, DC: U.S. Environmental Protection Agency, Office of Water. EPA822R06013. PB2007101258.

[18] Podrabsky JE and Somero GN 2004 Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish Austrofundulus limnaeus J. Exp. Biol. 207 2237.

[19] Lehninger AL, Nelson DL and Cox MM 2008 Lehninger principles of biochemistry W.H. Freeman, New York, 5th Ed.

[20] Wawrik B, Kerkhof L, Kukor J and Zylstra G 2005 Effect of different carbon sources on community composition of bacterial enrichments from soil. Appl. Environ. Microbiol. 71 6776.
[21] Costa E, Teixido N, Usall J, Atares E and Vinas I 2002 The effect of nitrogen and carbon sources on growth of the biocontrol agent Pantoea agglomerans strain CPA-2 Lett. Appl. Microbiol. 35 117.

[22] Ruiz-Lozano JM and Azcón R 2011 Brevibacillus, arbuscular mycorrhizae and remediation of metal toxicity in agricultural soils. Endospore-forming Soil Bacteria Springer: 235-258.

[23] Bruins MR, Kapil S, and Oehme FW 2000 Microbial resistance to metals in the environment Ecotoxicol. Environ. Safe 45 198.

[24] Leedjärv A, Ivask A and Virta M 2008 Interplay of different transporters in the mediation of divalent heavy metal resistance in Pseudomonas putida KT2440 JB 190 2680.

[25] Nongkhlaw M, Kumar R, Acharya C and Joshi SR 2012 Occurrence of horizontal gene transfer of PIB-type ATPase genes among bacteria isolated from the uranium rich deposit of Domiasiat in North East India PloSone 7: e48199.

[26] Ramos-Zúñiga J, Gallardo S, Martinez-Bussenius C, Norambuena R, Navarro CA, Paradela A, and Jerez CA 2019 Response of the biomining Acidithiobacillus ferrooxidans to high cadmium concentrations J. Proteom. 198 132.

[27] Stohs SJ and Bagchi D 1995 Oxidative mechanisms in the toxicity of metal ions Free Radic. Biol. Med. 18 321.

[28] McEldowney S 2000 The impact of surface attachment on cadmium accumulation by Pseudomonas fluorescens H2 FEMS Microbiol. Ecol. 33 121.

[29] Peptides P 2009 A Common Highly Conserved Cadmium Detoxification Mechanism from Bacteria to Humans J. Biol. Chem. 284 4936.

[30] Chudobova, D, Dostalova S, Ruttkay-Nedecky B, Guran R, Rodrigo MAM and Tmejova K 2015 The effect of metal ions on Staphylococcus aureus revealed by biochemical and mass spectrometric analyses Microbiol. Res. 170 147.