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Characterization of a Multi-Metal Resistant *Bacillus cereus* Strain TWSL_5 and it's Bioremediation Potential for Cr, Pb, Zn and Fe

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Abstract: An effluent sample collected from a textile dyeing factory was screened to isolate metal resistant endogenous bacteria using standard microbiology techniques. A bacterium with the highest metal resistance was characterized by 16S rRNA gene sequencing and the bacterial genome was screened for any possible metal resistant genes using the Polymerase Chain Reaction (PCR). The bioremoval ability of the isolate was evaluated using a multi-metal contaminated industrial effluent. Metal immobilisation was assayed using Scanning Electron Microscopy and Energy-Dispersive X-ray (SEM-EDX) analysis. An isolate (strain TWSL_5) was characterized as a *Bacillus cereus* strain. MICs of the strain TWSL_5 were found to be 75 mg/L (copper), 50 mg/L (cadmium) and 1000 mg/L (lead). The MIC for lead is among the highest reported for the species, *B. cereus*. The strain TWSL_5 showed a higher metal removal activity ($p<0.001$) for copper (93.13±0.17%) and lead (85±1.4%) in spiked cultures. The bacterium could successfully remove lead (100±0.00%), iron (68.22±0.07%), zinc (88.71±0.62%) and chromium (100±0.00%) from an effluent in 7 days of incubation at pH 6 and an insoluble black solid formed was detected as iron by SEM-EDX. The presence of *copA* in the genome of TWSL_5 was confirmed by PCR followed by sequence analysis. The presence of a gene involved in heavy metal resistance (*copA*), metal immobilization ability and the multi-metal removal activity of TWSL_5 in both spiked cultures and in an industrial effluent, indicates its potential to be used in metal bioremediation of industrial wastewater.

Keywords: Metal Bioremediation, Heavy Metal Resistance, *copA*, *Bacillus cereus*, Wastewater

Introduction

Heavy metals found in the natural environment are considered hazardous. The increase in industrial activities, including agricultural and pharmaceutical, in the recent past has led to the pollution of the environment by metals in numerous ways. Several disasters have been experienced as a result of large-scale contamination of land and aquatic habitats by disposal of waste without treatment. Therefore, industries have been urged to address the production of toxic waste matter and remediate or recycle them. Soil erosion, electroplating, natural weathering, mining, urban runoff and sewage discharge are some of the sources of heavy metal contamination (Jaishankar *et al*., 2014).

Some heavy metals are essential for biochemical and physiological functions at low concentrations. However, beyond a certain threshold they are lethal to organisms (Jaishankar *et al*., 2014). Uptake of some metal ions into an organism and detoxification of metal ions taken into the organisms are controlled by several mechanisms and has influenced some organisms to be self-resistant to these metal ions (Hall, 2002). Metal ions can interact with many biomolecules such as proteins and DNA, resulting in membrane damage, DNA damage, alteration of enzyme activity and apoptosis leading to an adverse impact on health of living organisms and plants (Martelli *et al*., 2006; Beyersmann and Hartwig, 2008; Tchounwou *et al*., 2012; Wang and Shi, 2001; Booth *et al*., 2015; Igiri *et al*., 2018; Cervantes *et al*., 2001; Bruins *et al*., 2000). Therefore, regulations have been introduced and the

tolerance limits for Cd, Cu and Pb ions in industrial waste, discharged to inland surface water for Sri Lanka under the authority of a license issued by the Central Environmental Authority, should be less than or equal to 0.1, 3.0 and 0.1 mg/L respectively (National environmental act, No. 47 of 1980, 2008).

With increasing health effects due to large scale heavy metal contamination, a greater attention has been paid to the treatment of wastewater to minimize pollution and thereby health issues. Metal decontamination using bacteria offers several advantages over conventional methods and they can be used for metabolic conversion of toxicants in industrial effluents into nontoxic or less toxic products, thereby minimizing their adverse impacts (Rajendran *et al*., 2003; Brim *et al*., 2000; Malik, 2004). Cellular components and reactions in microbes are involved in bioremediation of heavy metals (Ojuederie and Babalola, 2017). Some bacteria have evolved resistant mechanisms such as efflux systems, uptake systems and extracellular or intracellular precipitation and accumulation, which help withstand high concentrations of heavy metals (Gutiérrez-Barranquero *et al*., 2013). Reducing toxicity via valence change is also considered as a detox mechanism expressed by microbes (Igiri *et al*., 2018). The metal removal ability of bacteria depends on the intracellular cysteine rich proteins such as Metallothioneins (MT) via intracellular sequestration too (Gadd, 1990; Kaur *et al*., 2018; Murthy *et al*., 2011). It has been reported that ionizable groups or metal binding ligands such as amino, carboxyl, phosphate and hydroxyl groups in the cell walls of cells and spores play a vital role in passive absorption of positively charged metal ions and preventing their entry into cells or spores (Syed and Chinthala, 2015; Ayangbenro and Babalola, 2017). Precipitation of metal ions as carbonates, phosphates and sulphates in bacteria has also been reported (De *et al*., 2008; Ayangbenro *et al*., 2018; Luptakova and Kusnierova 2005; Zhang and Wang 2016; Sharma *et al*., 2000; Wang *et al*., 2002). Copper is found to be precipitated in the form of sulphides by Luptakova and Kusnierova (2005).

Genes *copA* and *cadA* encode proteins of efflux pumps which efflux copper and cadmium/ lead metal ions, respectively, from the cytosol to the periplasm of bacteria (Rensing *et al*., 1997; Tsai *et al*., 1992; Odermatt *et al*., 1992; Solioz *et al*., 1994; Nucifora *et al*., 1989). These are found in many eubacteria belonging to the gram positive Firmicutes and the Gram negative Proteobacteria (Rensing *et al*.,1997; Tsai *et al*., 1992; Rensing *et al*., 2000). Bacteria which produce metal induced metal binding proteins such as *copA*, CopB, ZiaA and CadA expel metal ions from the cytoplasm to periplasm or accumulate in everted membrane vesicles to maintain metal homoeostasis (Igiri *et al*., 2018; Rensing *et al*., 2000; Thelwell *et al*., 1998). Silver and Phung proposed a mechanism for Cd^{2+} uptake by Cadmium ATPase (CadA) with the help of inside-out membrane vesicles formed by the organism (Silver and Phung, 1996).

Bacteria can live all over the biosphere because of their remarkable adaptability to environmental changes, stress conditions (such as toxic metal stress) and evolving many resistant mechanisms to maintain homeostasis (Ayangbenro *et al*., 2018). Therefore, bacteria in metal contaminated sites (such as in industrial effluents), have evolved tolerant systems which have a higher potential to be used in metal bioremediation (Kapahi *et al*., 2019; Kumari *et al*., 2021). Isolation of heavy metal resistant microbes and the identification of genes involved would benefit further research into bioremediation and biomonitoring. The bacterium isolated in the study was assayed *in vitro* for its bioremediation capacity in metal spiked cultures and for an industrial effluent. Interestingly, this isolated bacterium (strain TWSL_5) was found to be capable of withstanding elevated levels of copper, cadmium and lead and has shown high multi-metal removal capacity in both spiked and effluent samples, producing an insoluble compound in the presence of metals indicating its capacity for metal immobilization. PCR was performed to ascertain the presence of metal resistance genes, *copA* and *cadA*, of the isolated bacterial strain to determine the genetic basis for metal resistivity. The long-term goal of the project was to identify metal remediation or removal mechanisms of the bacterium to be used in wastewater treatment and/or use as a biosensor development.

Materials and Methods

Bacterial Strains, Growth Media and Growth Conditions

Several bacterial strains with bioremoval ability were isolated and the bacterial strain TWSL_5 was further characterized. It was grown on Luria-Bertani agar/broth media (HiMedia, India) at 37°C. McFarland standard (0.05 mL of 1% W/V anhydrous $BaCl_2$ and 9.95 mL of 1% w/v H_2SO_4) was used as the reference to determine the cell number. For metal bioremediation and resistance analysis, bacteria were grown in 1 mg/L metal (Cd^{2+}, Pb^{2+} and Cu^{2+}) spiked LB media (spiked cultures) and the metal concentrations of the media were varied (1-1000 mg/L) in the Minimum Inhibitory Concentration (MIC) determination experiments.

Sample Collection

A textile effluent was collected into sterile screw-capped bottles from the biological stage of a wastewater treatment plant in the Colombo district, Sri Lanka. The sample was collected at a depth of ~3-7 cm from the surface to avoid surface bacteria in the effluent. The temperature, pH, colour and odour of the sample were noted. The presence of metals and their concentrations were determined using a GCB 932

Plus Atomic Absorption Spectrophotometer (AAS). The collected effluent sample was stored at 4°C until use.

Isolation of Bacterial Strains

The effluent samples were serially diluted and a 50 µL aliquot of each dilution was spread on LB agar plates. All spread plates were incubated overnight at 37°C and well isolated colonies were streaked on LB agar plates to obtain pure colonies. The isolated pure colonies were stored at -80°C as 15% (v/v) glycerol stocks for future use and the culture used this study was deposited in the NBRC culture collection.

Screening of Heavy Metal Resistant Bacteria

Several isolated bacterial strains were screened for heavy metal resistivity by observing their growth on LB agar plates enriched with a mixture of 20 mg/L concentrations of Cd^{2+}, Pb^{2+} and Cu^{2+} for two days. A bacterial culture designated TWSL_5 which grew well was selected as one of the most promising strains for further analysis.

The growth of bacterial strain TWSL_5, with time, in the presence of heavy metals (Cd^{2+}, Pb^{2+} and Cu^{2+}) was determined by measuring the Optical Density at 600 nm (OD_{600}) using a scanning UV-VIS spectrophotometer (Spectro UV-VIS Dual Beam (Split), Helios Alpha Thermo Fisher Scientific) at predefined time intervals (1, 3, 5 and 7 days). All experiments were carried out in triplicate and the sample without an inoculum but with the metal ion was used as the abiotic Control (C1). Minimum Inhibitory Concentration (MIC) of the strain TWSL_5 was determined by spreading 10 µL of an overnight grown culture on LB agar plates supplemented with metal ions (Cd^{2+}, Pb^{2+} and Cu^{2+}) at different concentrations (1- 1000 mg/L). In these experiments, concentrations were initially increased in increments of 100 mg/L and whenever a growth inhibition was observed, the growth inhibition in that range was further tested in increments of 5 and 10 mg/L. The plates were incubated for three days (72 h) at 37°C. Experiments were carried out in triplicate and the concentration at which no growth was observed on the third day was considered as the MIC for the metal ion.

Bioremoval Capacity and Growth Analysis (Metal Resistivity) of the Bacterial Strain (for Spiked Cultures)

LB broth (100 mL) in a 250 mL flask was separately spiked with heavy metals (1 mg/L of Cd^{2+}, Pb^{2+} and Cu^{2+}) and then inoculated with 50 µL (~2 $\times 10^7$ CFU/mL) of a bacterial culture of TWSL_5 grown overnight. Concentrations of metals in the spiked cultures were determined at predefined intervals (1, 3, 5 and 7 days) using a GCB 932 Plus Atomic Absorption Spectrophotometer (AAS). Aliquots from above samples were separately centrifuged at 3500 rpm for 30 min and the supernatant was filtered (0.25 µm) before AAS analysis. The bioremoval of metal ions by the bacteria was calculated by determining the difference between the initial and final concentrations of heavy metals in the media (Radhika *et al*., 2006). Half-life ($t_{1/2}$) was defined as the time taken for the metal concentration of the medium to reduce to 50% of its initial concentration (Durrani *et al*., 2014). The Bioremoval percentage (BR%) of metal ions and the metal ion Removal Rate (RR) by the strain were calculated using the following equations (Kumari *et al*., 2021):

$$BR\% = \left[\left(C_0 - C_t\right) / C_0\right] \times 100 \quad (1)$$

$$RR = \ln\left(C_t - C_0\right) / t \quad (2)$$

C_0 and C_t are metal ion concentrations at the time, 0 min and at time interval t, respectively. All experiments were carried out in triplicate (n = 3) where, the sample without inoculum was used as the abiotic Control (C). The results were statistically analysed using GraphPad Prism 8.0.1 software.

Biochemical and Physiological Characterization of the Bacterial Strain

The colony morphology of the bacteria was observed using a simple microscope (hand lens). Cellular morphology, gram staining, motility and formation of endospores were determined using a compound light microscope (Olympus CKX41) according to the procedure described in the Benson Manual (Benson, 2001). Tests, including the methyl red test, starch hydrolysis test, urease test, catalase test, Simmon citrate agar test, coagulase test, gelatine hydrolysis test and MacConkey agar test, were also carried out to determine the biochemical characteristics of the bacterial strain as described previously (Benson, 2001; Gordon *et al*., 1973).

Analysis of the Black Precipitate

The colour of the media turned dark brown after few days (3-7 days) when TWSL_5 was grown in Pb^{2+} and Cu^{2+} supplemented media. Spores/remaining cells were washed with sterile Phosphate Buffer Solution (PBS) and observed under a light microscope (200 x). Then it was analysed by AAS after acid digestion to determine the accumulated or adsorbed metals in the precipitate.

Molecular Characterization of the Bacterial Strain

Genomic DNA was extracted as previously described (Pitcher *et al*., 1986) and the 16S rRNA gene was amplified using the primer pair fD1/rD1 (Weisburg *et al*., 1991). The amplified product (~1500 bp) was sequenced (Macrogen, Rep. of Korea) and analysed. The sequence

was compared with DNA sequences available in the NCBI database using BLAST. Multiple sequence alignment was carried out using Clustal W software and a phylogenetic tree was constructed using the Neighbour-joining method based on the Kimura 2-parameter model, using the MEGA X software (Saitou *et al*., 1987; Kimura, 1980; Kumar *et al*., 2018).

Antibiotic Susceptibility of the Bacterial Strain

The antibiotic susceptibility of the isolated strain was determined with selected antibiotics ampicillin (25 µg/disc), amoxicillin (10 µg/disc), ciprofloxacin (5 µg/disc), vancomycin (5 µg/disc), cefotaxime (30 µg disc), erythromycin (15 µg/disc) and gentamycin (10 µg/disc) using the Kirby Bauer method (Bauer *et al*., 1959; 1966). Well isolated colonies (grown overnight) were picked and re suspended thoroughly in sterile saline solution (0.9% w/v NaCl) using a sterilized loop until the solution become milky and equal to the freshly prepared 0.5 McFarland standard (~1.5 X 108 CFU/mL). This was used in disc diffusion assays and the zone of inhibition (diameter) was measured using a ruler. The mean value of three replicates was considered in evaluating antibiotic susceptibility.

Identification of Genes Responsible for Heavy Metal Resistance

The presence of possible genes (*copA* and *cadA*) involved in heavy metal tolerance of cadmium, lead and copper was investigated using the Polymerase Chain Reaction (PCR). Genomic DNA was amplified using specific primers designed in this study (Table 3). The PCR program was optimized with an initial denaturation at 95°C (2 min) followed by 35 cycles of denaturation at 94°C (30 s), annealing at the specific optimized temperature for 1 min, extension at 72°C (1 min), followed by a final extension at 72°C (3 min). The amplified products were verified and quantified using gel electrophoresis. PCR products were then gel purified and sequenced (Macrogen, Rep of Korea) using the Dideoxy method. Partial nucleotide sequences were first analysed using BioEdit software. Basic Local Alignment Search Tool (BLAST), at the National Center for Biotechnology Information (NCBI) was used to identify the organisms to which the sequences were belonged. Sequence homology was further confirmed by constructing a phylogenetic tree using the Maximum Likelihood method and the Jukes-Cantor model (Jukes and Cantor, 1969). The tree was generated using MEGA X software (Kumar *et al*., 2018).

Bioremediation Assay with an Industrial Effluent

The industrial effluent sample (collected from a textile dyeing industry) was centrifuged (3500 rpm/30 min) and the supernatant was sterilized by filtering through an autoclaved 0.25 µm membrane filter. The filtrate was collected into sterilized 250 mL reagent bottles (150 mL/bottle). An aliquot was analysed by AAS to determine the presence and concentrations of metal ions (Cr, Fe, Zn, Cu, Cd and Pb). Both the supernatant of the centrifuged effluent and filtrate were examined for the presence of any indigenous bacterial strains by spread plating on LB agar. The absence of colonies after incubation of the plate at 37°C for 48 h was considered as the devoid of any bacteria. The bacteria free effluent was then inoculated with an overnight grown TWSL_5 culture (50 µL; ~2 $\times 10^7$ CFU/mL). The growth and bioremoval capacity of the TWSL_5 was evaluated using the same method followed for spiked cultures.

Scanning Electron Microscopy and Energy-Dispersive X-ray (SEM-EDX) analysis

Industrial effluent (above) was transferred onto an EM stub and the sample was examined under a scanning electron microscope (SU6602) and subjected to energy-dispersive X-ray analysis.

Statistical Analysis

All data sets [means of replicates, Standard Deviation (SD)] were statistically analyzed using Graph Pad Prism 8.0.1 software. Group analysis (Alpha = 0.05) was carried out using "Two-way ANOVA-multiple comparison" and $p<0.05$ was considered statistically significant. The graphs were plotted and error bars indicate ± Standard Deviation (SD).

Results

Isolation and Screening of Heavy Metal Resistant Bacteria

The wastewater sample collected from the biological treatment stage was positive for the metal ion Cu^{2+} (0.050 mg/L) and free of Pb^{2+} and Cd^{2+}. The temperature and pH of the sample collected were 38°C and 8.9, respectively. It was dark blue-black in colour with a pungent and foul odour. From the pool of bacterial strains grown on the LB agar plates, 10 isolates were recovered as pure colonies. Among them one bacterial strain which could grow in the multi-metal enriched medium (20 mg/L) was selected and was designated as TWSL_5. The NBRC culture collection number obtained for the bacterial strain TWSL_5 is NBRC 114812.

Growth Analysis (Metal Resistivity) of the Bacterial Strain (for Spiked Cultures)

The strain TWSL_5 was found to be resistant to all the metal ions (Cd^{2+}, Pb^{2+} and Cu^{2+}) tested. The strain TWSL_5 showed a high resistance for Pb [MIC: 1000 mg/L (0.0048 mol/L)] where MIC values of Cd and Cu were 50 and 75 mg/L respectively. The growth of the bacteria in the presence of different metal ions was significantly different ($p<0.05$) and showed a steady

growth with time in the presence of three metals tested (Fig. 1). The highest growth rate of TWSL_5 was observed in the presence of Cu at the beginning of growth, while the lowest was with Pb. On the seventh day, the highest growth was observed in the presence of Pb, while a lower growth rate for Cd and Cu (Fig. 1). Two-way ANOVA test with multiple comparison option (see Additional file 1) revealed the growth of the bacterium with metal ions after inoculation to be statistically significantly (p<0.05) different with time and no significant differences were observed with the control sample (C) where no inoculum was present (p>0.99).

Bioremoval Capacity of the Bacterial Strain (Spiked Cultures)

The strain TWSL_5 could reduce an initial metal ion concentration (1 mg/L) by over 50% on the 7th day after inoculation (p<0.05) and it was 0.98±0.01 to 0.36±0.01 mg/L (p<0.05), 1.07±0.01 to 0.16±0.02 mg/L (p<0.05) and 0.97±0.01 to 0.13±0.01 mg/L (p<0.05) for Cd, Pb and Cu respectively (Fig. 2a). Maximum removal of Cu (93.13±0.17%) could be achieved by the 5th day whereas for other metals it was the 7th day. The lowest metal removal percentage was observed for Cd (63.16±0.17%) and for the other metal ions it was over 80% (Pb = 84.98±1.37% and Cu = 86.75±0.42%) on the seventh day of the study. The bioremoval (BR) percentage of heavy metals with time is shown in (Fig. 2b). Multiple comparison of data with two-way ANOVA testing revealed the metal reduction after inoculation of the bacterium to be statistically significantly different with time (p<0.05) but not the control (see Additional file 2 and 3). This implies that the significant reductions in metal ion concentrations were solely due to the inoculated bacterium, TWSL_5.

It was interesting to note that the colour of the media turned dark brown after a few days (3-7 days) only when TWSL_5 was grown in Pb^{2+} and Cu^{2+} supplemented media. Further, observations with a light microscope revealed black and red brown coloured spores (Figs. 3a and 3b). *Bacillus cereus* is a spore producing bacterial strain and strain TWSL_5 was also confirmed to have endospores in microscopic observations. The cellular morphology of the bacterial strain at the beginning of the growth (day 1) was observed and found to be rod shaped and subsequently the shape turned oval. Change in the cellular shape from rod to oval and production of spores under stress conditions (presence of heavy metals) has been reported recently (Barros *et al*., 2019). Black (Pb) and red (Cu) coloured spores are considered as evidence of pigmentation with the respective metal ions as shown in (Fig. 3). AAS analysis of the acid digested precipitate confirmed that it contains Cu and Pb.

Identification of the Bacterial Strain TWSL_5

Microscopic analysis of TWSL_5 revealed it to be rod shaped, motile and Gram-positive. Colonies were white in colour, flat and irregular shaped, with a shiny/wet surface. Morphological and biochemical characteristics are similar to those of the family Bacillaceae. Biochemical characteristics of the bacterial strain TWSL_5 are summarised in Table 1.

BLAST analysis of the PCR amplified 16S rRNA sequence (1406 bp) of strain TWSL_5 showed it to be identical (100% sequence identity) to the 16S rRNA sequence of *Bacillus cereus* strain ATCC 14579 (Accession number: NR_074540.1) in the NCBI GenBank database. Phylogenetic analysis (Fig. 4) revealed the strain TWSL_5 to belong to the genus *Bacillus* and in the tree, the species *Bacillus cereus* was clearly differentiated from other *Bacillus* sp., by positioning it in a different branch. *Bacillus anthracis* (NR_074453.1) and *Bacillus thuringiensis* (NR_112780.1) showed a sequence identity of 99% in BLASTn analysis to the strain TWSL_5 while the *Bacillus cereus* strain (NR_074540.1) showed a sequence identity of 100% and this is clearly reflected in the phylogenetic tree (Fig. 4). Other *Bacillus* sp., *Bacillus flexus* (NR_113800.1), *Bacillus megaterium* (NR_043401.1) and *Bacillus subtilis* (NR_104873.1) were less similar with sequence identities of <96% to *Bacillus cereus*. *Staphylococcus aureus* (NR_118997.1) was used as the out-group to draw the phylogenetic tree. With phylogenetic and BLAST analysis of the amplified 16S rRNA sequence, the strain TWSL_5 was confirmed as "*Bacillus cereus*". Hence, the strain TWSL_5 was authenticated as "*Bacillus cereus* strain TWSL_5" and the 16S rRNA sequence was deposited in the NCBI repository under accession number KR027923.1.

Antibiotic Susceptibility

The zone of inhibition per trial was obtained by computing the average diameter of the zone. Calculated mean for three trials and the standard deviation of each replicate using GraphPad Prism software are shown in Table 2. Antibiotic sensitivity assays revealed the strain TWSL_5 to be resistant to vancomycin (5 µg/disc) and erythromycin (30 µg/disc).

Identification of Genes Responsible for Heavy Metal Resistance

Forward and reverse primer pairs, Cop1 and Cad1 were designed to amplify partial gene sequences of *copA* and *cadA* genes belonging to *Bacillus* sp., respectively (Table 3). The Cop1 primer pair successfully amplified a ~900 bp fragment. The fragment was sequenced and analysed using BLASTn. The highest degree of sequence identity (99.65%) was observed with the *copA* gene of *Bacillus cereus* strain D17 (Accession No. CP009300.1) which codes for copper translocating P-Type ATPase

(*copA*). Further, BLASTn analysis of the partial *copA* sequence revealed a sequence identity of 99.65 and 99.41% to sequences from *B. thuringiensis* strain HD571 and *B. anthracis* strain Tyrol 4675, respectively. However, a lower degree of sequence identity was seen with other *Bacillus* species. The evolutionary relationship of this gene to other species of the genus *Bacillus* is shown in Fig. 5. To confirm the identity further, the translated nucleotide sequences were queried using protein databases at NCBI and UniProt, https://www.uniprot.org/blast/. The translated partial gene sequence (deduced partial amino acid sequence) was identical to the "ATPase P" protein of *Bacillus cereus* species. The relationship of *copA* protein to closely related species (*B. cereus*) and evolutionary divergent species to the deduced amino acid sequence is shown in the phylogenetic tree (Fig. 6). GenBank accession number of the deposited partial *copA* gene sequence (846 bp), is KT032152.1 and protein ID of the deduced protein sequence is AKQ06254.1.

Bioremediation Assay with the Industrial Effluent and Formation of an Insoluble Material

The sample received from the textile dyeing factory was khaki coloured, slightly turbid (opaque) and had a pungent, foul odour. The temperature and pH of the sample were 37°C and 7.9, respectively. After centrifugation and filtering, the colour of the effluent was significantly reduced and became grey and transparent. AAS analysis revealed the presence of four metal ions, Cr (~0.189 mg/L, Pb (~1.320 mg/L), Zn (~1.322 mg/L) and Fe (~11.7 mg/ L). Inoculum grew significantly in the effluent and showed a high bioremoval capacity for Pb, Zn, Fe and Cr (see Additional file 4 and 5). The effluent became turbid during the growth of the bacterium. By the third day, the culture (effluent with strain TWSL_5) became significantly dark and insoluble black particles were observed attached to the wall of the bottle. The insoluble particles were scraped and collected into a fresh microcentrifuge tube (1.5 mL) for further analysis by SEM-EDX. Immobilization of metal ions (Fe) by extracellular sequestration via biofilm formation by the bacterial strain TWSL_5 is shown in Fig. 7. Oval shaped spores produced by *Bacillus cereus* strain TWSL_5 were visible and appeared to be entrapped in the biofilm or the insoluble materials formed (Fig. 7a). The presence of Fe in the biofilm was confirmed by the SEM-EDX layered image (Fig. 7b) and the chromatogram (Fig. 7c). The Fe peak appeared between 6 and 8 keV on the x-axis as shown in the chromatogram (Fig. 7c) and amounted to 16.9±1.2% (Wt. %) of the total elements.

Table 1: Biochemical characteristics of TWSL_5

Biochemical test	Result
Catalase test	+
Urease test	+
Methyl red	+
Coagulase	-
MacConkey agar test	-
Starch hydrolysis test	+
Simon citrate agar test	-
Gelatin hydrolysis	+

+ = Positive, - = Negative

Table 2: Antibiotic susceptibility of strain TWSL_5

Antibiotic	Concentration (µg/disc)	Zone of the inhibition (cm) (Mean ± SD)
Amoxicillin	25	3.43±0.08
Ampicillin	10	3.12±0.13
Cephalexin	30	3.77±0.06
Ciprofloxacin	05	3.00±0.44
Erythromycin	15	1.37±0.08
Gentamycin	10	2.10±0.09
Vancomycin	05	1.45±0.09

SD = Standard deviation, $n = 3$

Table 3: Primers designed to amplify metal resistance genes

Target gene	Primer set		Oligonucleotide sequence (5' ---> 3')	Annealing Temperature (optimized) (°C)	Complete gene size (bp)	Expected size of the PCR product (bp)
copA	Cop1	Forward	AGCTCCTATTCAAAGGGTAG	55	2418	967
		Reverse	CTTCCATCGCTACATCCGTTC			
cadA	Cad1	Forward	ACAGTTCCTGCTGTCAAAGATGTACG	52	2562	588
		Reverse	CTTTACGACAATCGTAGTGCCGATTG			

The growth of the bacterial strain in heavy metals enriched (spiked; 1 mg/L) LB broth. LB broth without inoculum was used as the abiotic control, C ($n = 3$).

Fig. 1: Growth curves of the bacterial strain TWSL-5 (in spiked cultures)

Heavy metal removal with time is shown in Fig. 2a. Removal % of metals with time is shown in Fig. 2b. Cu, Cd and Pb enriched media (1 mg/ L) were used for the assay and abiotic controls for the respective metal ions (labelled as Cu, Cd and Pb) are labelled as C-Cu, C-Cd and C-Pb. Error bars represent standard deviations (n = 3). Significance of mean differences was set at $p \geq 0.05$.

Fig. 2: Bioremoval capacity in metal spiked cultures by TWSL_5

During the growth of bacterial strain TWSL_5 in the presence of (a) Pb^{2+} and (b) Cu^{2+} metal spiked cultures, a colour change in the cell pellets were observed. Further it was noted that the colour was associated with the spores produced by the strain TWSL_5.

Fig. 3: Observation of black (Pb) and red (Cu) coloured spores using a light microscope (200 x)

The tree was constructed using the Neighbor-joining method (Saitou and Nei, 1987 **)** based on the Kimura 2-parameter model (Kimura, 1980) and the variation among sites was modeled with a gamma distribution (shape parameter = 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Accession numbers of the sequences used in this study are shown in parenthesis next to the taxon name. Evolutionary analyses were performed using MEGA X software (Kumar *et al*., 2018).

Fig. 4: Molecular phylogenetic tree of strain TWSL_5 and other *Bacillus* strains based on 16S rRNA sequences

a) The evolutionary history was inferred using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were performed using MEGA X software (Kumar *et al*., 2018).
b) The expanded sub tree of the cluster of genes circled in Fig. 5a. The tree is drawn to scale, with branch lengths measured in number of substitutions per site and are shown below the tree and sub tree.

Fig. 5: Molecular phylogenetic tree of *copA* genes constructed by using the Maximum Likelihood method

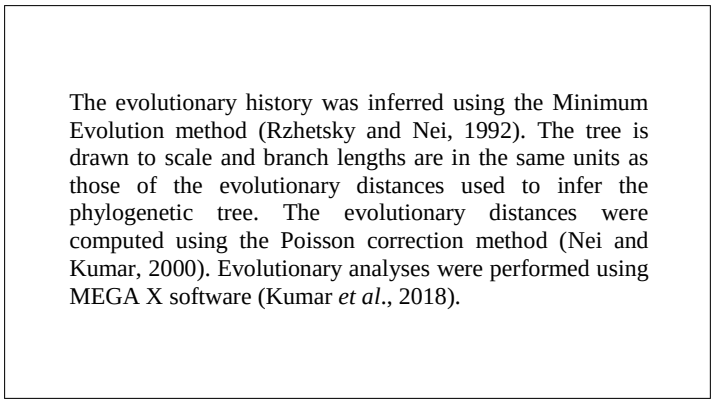

Fig. 6: Molecular phylogenetic tree of *copA* partial sequence of *Bacillus cereus* strain TWSL_5

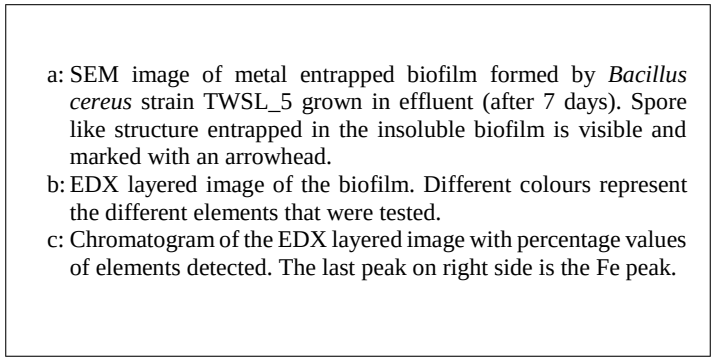

Fig. 7: SEM-EDX image of the biofilm/insoluble materials formed by strain TWSL_5

Discussion

Use of bacteria for bioremoval of metals in contaminated wastewater is a promising tool in bioremediation and requires a metal resistant bacterium having metal removing capacity. Microbes inhabiting industrial effluents containing toxic metals should be metal resistant. Therefore, an industrial effluent obtained from a dyeing factory was screened and a bacterial strain designated TWSL_5 was recovered from a pool of bacterial community.

Sequence of the 16S rRNA gene is used to accurately distinguish most Gram-positive and Gram-negative bacteria up to species level if the query shows >99% similarity to sequences available in genome databases (Drancourt *et al*., 2000). Isolated bacterial strain TWSL_5 was identified as *B. cereus* and confirmed by phylogenetic analysis. Several strains of *B. cereus* with metal resistance and bioremediation ability have been reported previously (Igiri *et al*., 2018; Syed and Chinthala, 2015; Chen *et al*., 2016). A transcription regulatory protein (CadC) and ATPase type protein (CadA) from a *Bacillus megaterium*strain NBRC 114811 and its resistance to Zn, Cu, Cd and Pb has also been reported (Kumari *et al*., 2021).

As the environment from which the bacterium was isolated was rich in Cu, it was expected to have a high degree of resistance to Cu but was found to have the highest resistance for Pb [MIC: 1000 mg/L (0.0048 mol/L)] with a steady growth with time (Fig. 1). This MIC was much higher than that reported for *Providencia alcalifaciens* strain 2EA [MIC: ~310 mg/L (0.0015 mol/L)] with Pb^{2+} bioprecipitation ability (Naik *et al*., 2012). Observation of growth of the bacterial strain TWSL_5 in the presence of heavy metals (Pb^{2+}, Cd^{2+} and Cu^{2+}) supplemented LB medium (more than 3 days) indicates that the strain TWSL_5 is resistant to metal ions tested in the order Pb>Cu≥Cd. Metal resistance of TWSL_5 is unique as it shows resistance to different metal ions and it varied with time (Fig. 1). This could be due to the activation of different resistance mechanisms in the bacterium TWSL_5 to different metals at different times. Competition to a common regulatory element in the corresponding gene could be another reason and it implies that the tolerance mechanisms for copper and cadmium are being activated from the beginning whereas those for lead are getting activated gradually with time. This indicates the presence of P-type ATPases (*copA*, *cadA*), which are being induced by the metals themselves within

a short time period. Strains expressing *copA* have been reported to show resistance to copper (Bayle *et al*., 1998; Teitzel *et al*., 2006). The similar behaviour of strain TWSL_5 confirms the resistance to Cu via *copA* which was later confirmed by sequence analysis. However, the presence of a *cadA* gene (*cadA* provides resistance against Pb and Cd) could not be confirmed with the primers designed in this study probably due to the variations in sequences in the primer annealing region. The genes involved in metal resistance have been successfully used for the construction of biosensors, such as the reported use of *copA* in Cu biomonitoring (Raja and Selvam, 2011).

The relationship between growth rates and metal removal rates (Fig. 1 and 2, Pb>Cu≥Cd) indicates a possible competition of metal ions (Pb>Cd) to chelators/chaperons or to metal binding sites of transporters such as *copA* or CadA (Rensing *et al*., 1997; 2000; 1998; Naik and Dubey, 2013; Kang *et al*., 2016). Non-specific mechanisms such as biosorption, immobilization of lead as sulphides or phosphates through the precipitation of lead, enhanced siderophore production and intracellular lead bioaccumulation are the possible lead resistance mechanisms which have been reported previously (Naik *et al*., 2012; Naik *et al*., 2013). Furthermore, formation of colonies with dark pigments on LB agar plates containing Cu^{2+} and Pb^{2+} salts (spiked cultures) likely indicate the immobilization of copper and lead ions by the bacterial strain TWSL_5 around pH 6-7. It is a well-known fact that Pb and Cu form insoluble sulphides, oxides, or phosphates around this pH range (De *et al*., 2008; Chen *et al*., 2016; Naik *et al*., 2012; Gramp *et al*., 2006; Glass *et al*., 2018; Fowle and Fein, 2001; Si *et al*., 2009; Arceo-Gómez *et al*., 2016; Massey *et al*., 1973; Ansari and Nematollahi, 2018). Bacterial spore ligands are likely to chelate divalent metal ions to stabilize spores and depending on the metal concentration of the medium, they can accumulate metal ions (Chung *et al*., 1971). Insoluble compound formation has been observed in many instances, such as immobilizing copper in the form of Cu_2O (reddish-brown) (Arceo-Gómez *et al*., 2016) or $CuFeS_2$ (brass-yellow) (Massey *et al*., 1973) and lead in the form of PbS (black) (Nucifora *et al*., 1989), PbO_2 (β - black/α - brown) (Ansari and Nematollahi, 2018) or any other formula (Chen *et al*., 2016) on the coat of the spores (exospore). For Example, the reported transformation of Pb into rod-shaped $Ca_2.5Pb_7.5(OH)_2(PO_4)_6$ nanocrystals by *Bacillus cereus* 12-2 (Chen *et al*., 2016). The strain TWSL_5 also produced an insoluble biofilm in the presence of Cu^{2+} and Pb^{2+} in spiked cultures as well as in the industrial effluent. Hence, the TWSL_5 strain may also be able to biomineralize metal ions as previously reported for *Bacillus cereus* strains (Syed and Chinthala, 2015; Ayangbenro and Babalola, 2017). SEM-EDX analysis confirmed the presence of Fe (16.9±1.2%) in the biofilm (Fig. 7c) together with other elements Ca (4.1±0.4%), Al (0.4±0.1%), S (1.0±0.2%), K (1.7±0.3%), Cl (5.6±0.4%), C (60.3±1.8%) and O (10.0±1.0%). The original source of these elements is the effluent itself or compounds produced by the organism. Compounds formed by the organism such as Extracellular Polymeric Substances (EPS), siderophores and other extracellular compounds or metabolic by-products could have facilitated Fe immobilization. Other metals Cr, Pb and Zn could also have been trapped in the biofilm or immobilised in a different form but not detected due to low abundance compared to the other elements detected. Precipitation of metal ions into an insoluble form is considered as a microbe metal resistance and metal bioremediation mechanism (Naik *et al*., 2012; Guo *et al*., 2010; Moore and Kaplan, 1992; Shanab *et al*., 2012). Therefore, this strain could potentially be used for *in-vivo/ex-vivo* metal bioremediation of Fe, Cu, Cr, Zn and Pb in industrial effluents.

Conclusion

The strain isolated in this study was identified as *Bacillus cereus* (Accession No: KR027923) and was deposited in the NBRC culture collection repository using a culture collection number NBRC 114812. Previous studies have revealed the bioremediation capabilities of *Bacillus cereus* strains found from metal contaminated sites (Pugazhendhi *et al*., 2018). This strain showed significant metal tolerance capacity for Cd, Pb and Cu ions and the order of resistance was found to be Pb>Cu>Cd. Copper tolerance is achieved through *copA* which is involved in copper resistance via effluxing of copper from the cytoplasm to the periplasm or to everted membrane vesicles (Rensing *et al*., 2000; Migocka, 2015). It has also been reported that metal resistant bacteria could develop resistance to many antibiotic drugs in addition to their cross tolerance to other metal ions such as cadmium, zinc and mercury (Naik and Dubey, 2011; Naik *et al*., 2013; Naik *et al*., 2012). TWSL_5 is also resistant for several antibiotics.

Furthermore, the strain TWSL_5 was positive for urease activity, where it could be involved in copper immobilization (Kang *et al*., 2016) and dark coloured precipitates observed in cultures are evidence for the bio-precipitation of copper and lead (De *et al*., 2008; Chen *et al*., 2016; Naik *et al*., 2012; Gramp *et al*., 2006; Glass *et al*., 2018; Fowle and Fein, 2001; Si *et al*., 2009; Arceo-Gómez *et al*., 2016; Massey *et al*., 1973; Ansari and Nematollahi, 2018). SEM-EDX analysis confirmed the immobilization of Fe^{2+} indicating the metal bioremediation capacity of this strain. The multi-metal removal capacity of this bacterium and its multi-metal resistance makes it a potential tool to be used in *in-vivo/ex-vivo* metal bioremediation of Fe, Cu, Cr, Zn and Pb ions in industrial wastewater and perhaps for the construction of a biosensor for Cu biomonitoring.

Additional Files

Additional file 1: Results of the statistical analysis of the growth of the bacterium in the spiked (Cu, Cd and Pb) samples. Data were analysed using GraphPad Prism 8.1.1 (330) software. Two-way ANOVA (multiple comparisons) was used to analyse data. Growth was analysed compared to day1 and the abiotic control C (CVS 4 kb).

Additional file 2: Results of the statistical analysis of removal of metal ions by the bacterium in the spiked (Cu, Cd and Pb) samples. Data were analysed using GraphPad Prism 8.1.1 (330) software. Two-way ANOVA (Multiple comparisons) was used to compare, metal removal with the concentration on day1 (PDF 206 kb).

Additional file 3: Results of the statistical analysis of removal of metal ions by the bacterium in the spiked (Cu, Cd and Pb) samples. Data were analysed using GraphPad Prism 8.1.1 (330) software. Two-way ANOVA (Multiple comparisons) was used to analyse the removal of metal ions respective to the abiotic controls (C-Cu, C-Cd and C-Pb) (PDF 209 kb).

Additional file 4: Graphical representation of the growth of strain TWSL_5 and removal of metal ions in the effluent. Growth and bioremoval of Cr (a), Fe (b), Pb (c) and Zn (d) were measured for a 7-day period. Bioremoval percentage is graphically illustrated as a bar graph (e). Graphs were plotted and statistically analysed using GraphPad prism 8.1. (330) software. The error of the replication (n) is given as ± SD (standard deviation for 3 replications) (PDF 357 kb).

Additional file 5: Data and results of removal of metal ions by the bacterium in the effluent sample. Bioremoval (BR), Bioremoval percentage (BR%), Removal Rate (RR) and half-life of the metal ions are shown in the spreadsheet (XLSX 13 kb).

Declarations

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Availability of Data and Materials

The dataset(s) supporting the conclusions of this article are available in the NCBI repository, GenBank accession no: KR027923.1, https://www.ncbi.nlm.nih.gov/nuccore/KR027923.1 [16S rRNA gene]; KT032152.1 [*copA* gene], https://www.ncbi.nlm.nih.gov/nuccore/KT032152.1. Results of statistical analysis supporting the conclusions of this article are included as Additional Files 1, 2, 3, 4 and 5. Other datasets generated during and/or analyzed during the current study are available with the corresponding author and can be obtained on reasonable request. Bacterial (wild-type) strain used in this study (*Bacillus megaterium* TWSL_4) is deposited in the NBRC culture collection repository using a culture collection number NBRC 114812.

Author's Contributions

Weerasingha Mudiyanselage Nilmini Hasintha Kumari: Carried out the experimental work and contributed to manuscript preparation and editing.

Naduviladath Vishvanath Chandrasekharan: Conceptualized and designed the study and contributed to manuscript preparation and editing.

Champika Dilrukshi Wijayarathna: Conceptualized and designed the study. Involved in interpretation of results and contributed to manuscript preparation and editing.

Ethics Approval and Consent to Participate

Prior approval was obtained from the textile factory to collect the effluent sample for research purposes.

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Abbreviations

BLAST: Basic Local Alignment Search Tool
LB: Luria-Bertani
AAS: Atomic Absorption Spectrophotometer
EPS: Extracellular Polymeric Substances
PCR: Polymerase Chain Reaction
MH: Mueller Hinton
MIC: Minimum Inhibitory Concentration
NCBI: National Center for Biotechnology Information
UniProt: The Universal Protein Resource
SEM: Scanning Electron Microscopy
EDX: Energy-Dispersive X-ray
DNA: Deoxyribonucleic Acid
rDNA: Ribosomal DNA