



Assessment of cadmium tolerance and biosorptive potential of *Bacillus Cereus* GCFSD01 isolated from cadmium contaminated soil

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(With 4 figures)

Abstract

Continuous occurrence of heavy metals is a major cause of environmental pollution due to its toxic effects. At minimum concentrations, these metals are highly reactive and can gather in the food chains and food web, causing major dangers to public health concerns. Soil samples were collected from Paharang drain, Faisalabad. Cadmium tolerant bacteria were isolated and evaluated for their MIC against Cd. The isolated bacterial strain GCFSD01 showed MIC value upto 30 mM/L. The bacterial strain with the highest resistance against Cd was selected for further study. Molecular characterization of bacterial isolate GCFSD01 was performed by 16S rRNA which confirmed it as *Bacillus cereus*. Optimum growth conditions of bacterial strain were also evaluated. Strain GCFSD01 showed optimum growth at pH 7 and 37 °C temperature. Our result revealed that *B. cereus* strain GCFSD01 reduced 61.3% Cd after 48 hrs. Multiple metal tolerance and Cd reduction by *B. cereus* indicate its potential for further use for decontamination of polluted soil.

Keywords: textile effluents, industrial hub, metal contaminated soil, heavy metals, *Bacillus cereus*, biosorption, biodegradation.

Avaliação da tolerância ao cádmio e do potencial biosorptivo de *Bacillus Cereus* GCFSD01 isolado de solo contaminado com cádmio

Resumo

A ocorrência contínua de metais pesados é uma das principais causas de poluição ambiental devido aos seus efeitos tóxicos. A contaminação por metais pesados representa um grande risco para todas as formas de vida encontradas no meio ambiente. Em concentrações mínimas, esses metais são altamente reativos e podem se acumular nas cadeias alimentares e na cadeia alimentar, causando grandes perigos às preocupações com a saúde pública. Amostras de solo foram coletadas no esgoto de Paharang, Faisalabad. Bactérias tolerantes ao cádmio foram isoladas da amostra coletada pelo método da placa de ágar. As colônias separadas individuais selecionadas foram avaliadas quanto às suas concentrações inibitórias mínimas contra Cd. A cepa bacteriana isolada GCFSD01 apresentou valores de CIM de 30 mM/L. A colônia bacteriana que apresentou maior resistência contra o Cd foi selecionada para identificação. Após seleção da maior colônia bacteriana resistente ao Cd, coloração de Gram e diferentes testes bioquímicos foram realizados para a caracterização da bactéria isolada. A caracterização molecular do isolado bacteriano GCFSD01 foi realizada por PCR 16S rRNA confirmando a presença de *Bacillus cereus*. Após a identificação molecular, as condições ótimas de crescimento da cepa bacteriana também foram verificadas. A cepa GCFSD01 apresentou crescimento ótimo em pH 7 e temperatura de 37 °C. Nosso resultado revelou que a cepa de *B. cereus* GCFSD01 reduziu 61,3% de Cd após 48 horas. A tolerância a múltiplos metais e a redução de Cd por *B. cereus* indicam seu potencial para uso posterior na descontaminação do solo poluído.

Palavras-chave: efluentes têxteis, cubo industrial; solo contaminado por metais, metais pesados, *Bacillus cereus*, biossorção, biodegradação.

1. Introduction

Metal pollution is one of the most persistent and complex environmental issues, causing threat to the ecosystem and human health (Das et al., 2016). The discharge of untreated

wastewater containing heavy metals in the ecosystem is one of the most important environmental and health challenges in our society (Igiri et al., 2018; Phillips and

Rainbow, 2013). Toxic metals are highly reactive even at present in low concentrations. Cadmium, a highly toxic, non-biodegradable and non-essential heavy metal that is harmful even at relatively low concentrations (0.001-0.1 mg/L) to organisms and has been suggested to be human carcinogen (Wu et al., 2016). Prolonged and persistent use of polluted water for irrigation of agricultural lands and as feeding source for animals and humans might have toxic effects (Sharaf et al., 2019). In literature, different methods has been used to remediate Cd pollution like chemical and physical but these approaches are costly and often generate toxic secondary waste products that are also harmful to the soil (Das et al., 2016). This situation emphasizes to find out eco-friendly and cost effective methods for remediation of heavy metals (Yasar et al., 2013). Therefore, among the alternatives, the use of microbes for the removal of heavy metals has gained great attention due to many advantages including cheap production, their ability to treat large area of soil and ecofriendly nature (Congeevaram et al., 2007).

Microorganisms that are able to remove heavy metals are of great interest as bioremediation agents because they can achieve it due to their high surface to volume ratios (Vijayaraghavan and Yun, 2008). Gram-negative bacterial cell wall composed of phospholipids, lipopolysaccharides and polypeptidoglycan of which highly charging nature and anionic character provide metal binding (Silhavy et al., 2010; Das et al., 2016). Some bacteria having above can also detoxify heavy metals and produce chelating agents that can bind metal ions while some microorganisms have the ability to transform toxic forms into less toxic forms. Normally indigenous microorganisms adopt themselves under changing environmental conditions and could be a better option to cope these changes (Ndi and Barton, 2012).

Faisalabad being the 3rd biggest city of Pakistan is the industrial hub and known as “Manchester of Pakistan” (Jaffrelot, 2002). Heavy industries especially textile industry producing different types of waste are directly discharging their waste into Paharang drain passes through Faisalabad. Therefore, the aim of present study was to isolate, identify and characterize heavy metal tolerant indigenous bacteria and to investigate their biosorptive potential against heavy metals.

2. Material and Methods

2.1. Study area and sample collection

Samples were collected from Faisalabad area (31°25'0"N/73°5'0"E). Industrial effluents are major source of heavy metal contamination in this area. Industries are directly discharging these wastes in to river Chenab through Paharang drain that originates from Chak Jhumra and passes through Faisalabad with collection of these effluents from industrial hub areas. Samples of soil were collected in pre-cleaned plastic bottles. Samples were collected from 0-10 cm depth of ground surface from approximately 500 meters distance intervals. Total 25 soil samples were collected in triplicate.

2.2. Heavy metals analysis of collected samples

After collection, heavy metals were determined in the samples using the method previously described by Zeiner et al. (2007). Cadmium (Cd), cobalt (Co), nickel (Ni), chromium (Cr), zinc (Zn) and lead (Pb) were included for analysis. Samples were digested and metal analysis was performed using Atomic Absorption Spectrophotometer (AAS) (Zeeman Atomic Absorption Spectrophotometer, ZA3000 Series). On the basis of the results, cadmium (Cd) was selected for further study.

2.3. Selection and isolation of cadmium resistant bacteria

Soil samples were taken in sterile water and to isolate bacteria from soil, samples were shaken at 150 rpm for 2 hr. After that, samples were settled for 5 min and one mL of the suspension was serially diluted upto 10^{-6} . Each dilution was spread onto an LB plate supplemented with 1mM Cd²⁺ and incubated at 37 °C. The growing colonies were observed and again inoculated onto LB plates containing Cd to get pure culture.

2.4. Determination of Minimum Inhibitory Concentration (MIC)

To evaluate the minimum inhibitory concentration (MIC) of the isolated bacteria, MS agar (Minimal Salt agar), (0.1% K₂HPO₄, 0.1% MgSO₄, 1% (NH₄)₂SO₄, 0.02% FeSO₄, 0.086% CaCl₂ and 0.05% Glucose) was prepared supplemented with Cd ranging from 1mM Cd²⁺ to 30mM. Each isolate was inoculated on these plates and incubated at 37 °C for 24h. The minimum Cd²⁺ concentrations at which bacterial isolate did not show growth was referred as its MIC.

2.5. Identification of bacterial isolate

After selecting Cd resistant bacterial isolate, identification was done on the basis of cultural characteristics, gram's staining and biochemical characteristics like oxidase reaction, catalase reaction, starch hydrolysis, fermentation tests and glucose utilization.

2.6. 16S ribosomal RNA (rRNA) sequencing

For the molecular characterization of bacterial isolate, two primers 27F 5-AGAGTTTGATCCTGGCTCAG-3 and 1522R 5-AAGGAGGTGTGCCARCCGCA-3 were used (Balashov and Humayun 2003). Total genomic DNA was extracted by CTAB method (Wilson, 2001). To attain a final volume of 25 mL, a reaction mixture contained template DNA, nuclease-free water, 12.5 mL 2X PCR master mix (Ferments) and primers was prepared. PCR was carried out with the help of thermocycler (Advance Primus 96) by using initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 45 sec, primer annealing at 50 °C for 60 sec, DNA extension at 72 °C for 90 sec and final extension at 72 °C for 10 min.

For ribotyping, PCR product was sent for commercial sequencing to Macrogen (Korea). The sequences obtained were checked for base calling using FinchTV and contigs made using NCBI BLAST (two sequence alignments). After amplification, 16S rRNA sequence was aligned

using ClustalW and compared with known sequences in the GenBank database.

2.7. Determination of optimum growth conditions for GCFSD01 strain

Optimum growth conditions of bacterial isolate were determined with respect to pH and temperature. Bacterial isolate was grown in LB broth at different incubating temperatures viz, 20, 30, 37, 45 and 55 °C for the determination of optimum temperature in the presence and absence of Cd. Their absorbance was determined at 600 nm after 24 h. For pH optimization, pH of the LB broth was adjusted at 5, 6, 7, 8, 9 and 10. OD was measured at 600 nm after 24 h incubation.

2.8. Effect of temperature and pH on Cd²⁺ uptake ability of bacterial isolate

Effect of pH and temperature on the Cd²⁺ uptake ability of the bacterial isolate was determined. Bacteria was grown in LB broth supplemented with 1mM Cd²⁺ at different incubating temperatures ranging from 25 °C to 42 °C and pH ranging from 5 to 9. After 24h of incubation, samples were collected and centrifuged for 5 min at 3000 rpm. Supernatants and pellets were used to evaluate the quantity of Cd²⁺ by atomic absorption spectrophotometer (Abbas et al., 2014).

2.9. Multi Metal Resistance (MMR) and antibiotic resistance

Multi metal resistance of isolated strain was determined as described by (Saini and Pant, 2016). LB agar medium was prepared, autoclaved and filtered sterilized solution of Cd, Cr, Pb and Ni in equal concentration i.e. 1:1:1:1 incorporated in the medium. Antibiotic susceptibility testing of isolated strain GCFSD01 was carried out using disc diffusion method. Antibiotic discs used were: ceftazidime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), Amikacin (30 µg), piperacillin-tazobactam (100/10 µg), meropenem (10 µg), erythromycin (15 µg) chloramphenicol (30 µg), streptomycin (10 µg), ampicilline (10 µg), rifampicine (5 µg), kanamycin (30 µg) and tetracyclin (30 µg). The interpretation of susceptibility results was done according to the guidelines of Clinical Laboratory Standards Institute (CLSI, 2016).

2.10. Evaluation of Cd reduction potential

Reduction potential of strain GCFSD01 (LT985360) was determined against Cd. For this, LB broth was prepared supplemented with 100 mg/L of Cd. After autoclave one flask was inoculated with 2 mL of 24h bacterial culture having turbidity equal to 0.5 McFarland. Flasks were incubated on a shaker at 28 °C for 24, 48 and 72 hr then centrifuged at 8,000 rpm for 15 min and supernatants were collected and Cd concentration was determined through atomic absorption spectrophotometer (Kumar et al., 2010).

2.11. Fourier Transform Infrared Spectroscopy (FTIR)

Isolate cultured in presence and absence of Cd were harvested by centrifugation at 4000×G for 10 minutes and washed three times with 1xPBS (phosphate buffer saline). The cell pellets were placed in bench top freeze

drier (Labconco™, Missouri, United States of America) overnight. The dried cell pellets were used for infrared spectroscopic analysis and spectra was recorded on FTIR spectrometer (Bruker, Massachusetts, United States of America). The FTIR spectrum was recorded in the region 4000 to 500 cm⁻¹ wave number (Khan, 2013).

3. Results

3.1. Heavy metals analysis in collected soil samples

Heavy metal analysis of soil samples through atomic absorption spectrophotometer revealed the presence of different heavy metals in the samples. Concentrations of different heavy metals are: Zn 121.7 ± 0.30, Cr 80.3 ± 0.13, Pb 57. ± 0.07, Cd 11.0 ± 0.03, Cu 36.8 ± 0.04 and Ni 36.5 ± 0.09. Due to higher concentration of Cd in soil, it was selected for further study.

3.2. Isolation of Cd resistant bacterial species from soil samples

Out of 25 samples, only 15 samples exhibited positive growth on medium containing Cd. 15 isolated bacteria using spread plate procedure labeled as GCFSD-01 to GCFSD-15 showed resistance to cadmium were selected for minimum inhibitory concentrations (MIC).

3.3. Determination of minimum inhibitory concentrations

MIC of selected isolates was determined. All selected bacterial isolates showed growth up to 10 mM of Cd+2. However, only five isolates, GCFSD-01, GCFSD-05, GCFSD-08, GCFSD-09 and GCFSD-13 showed growth upto 20 mM of Cd+2 while only one isolate GCFSD-01 showed growth at at 30 mM concentrations. From MIC results, GCFSD-01 isolate has been selected for further studies.

3.4. Identification of bacterial isolates GCFSD01

Initial identification up to genus level was done by using standard microbiological techniques. Results revealed G+ve, non-motile rods (as shown in Table 1).

3.5. 16S ribosomal RNA (rRNA) sequencing

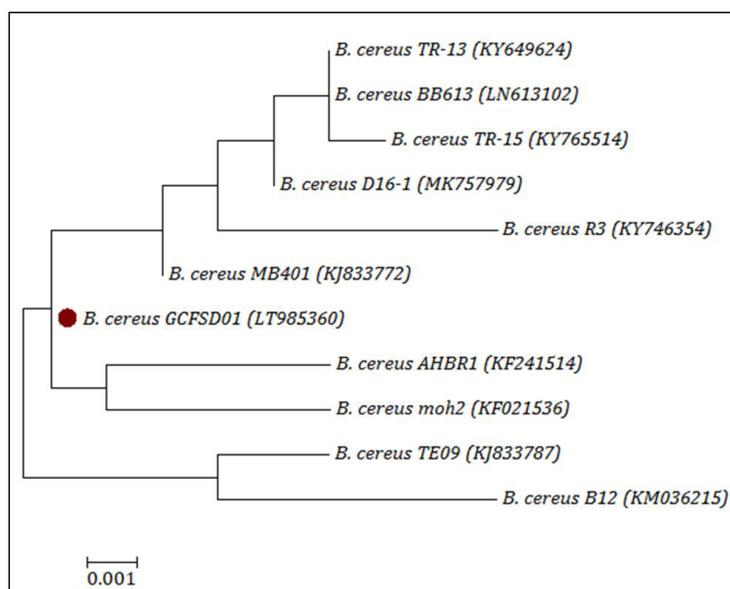
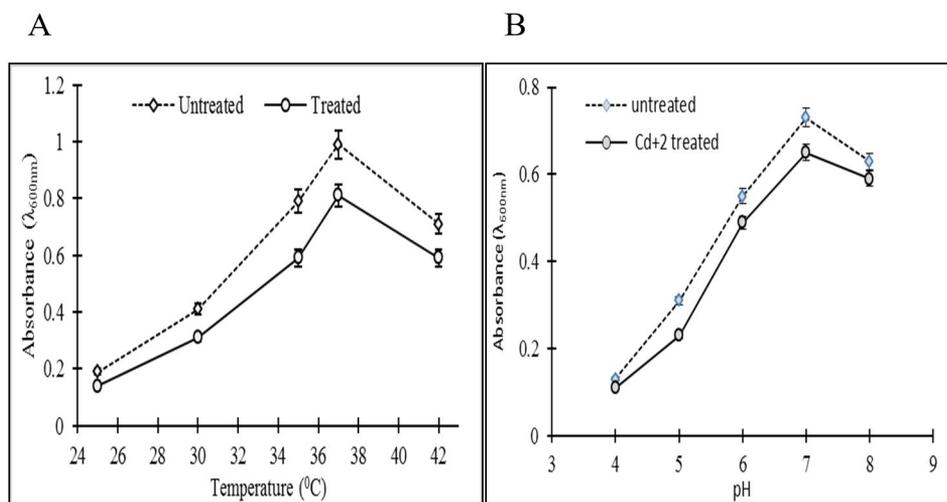
Molecular characterization through ribotyping confirmed bacterial strain GCFSD01 as *Bacillus cereus* and submitted to GenBank with accession number LT98536. The strain displayed highest level of identity 98.44% (query coverage 97%) with *B. cereus* MB401 (Accession number KJ833772) isolated from food products of Punjab, Pakistan. Second closest relative of our isolate was found *B. cereus* AHBR1 (KF241514) isolated from household dust samples from Singapore. Finally, evolutionary status of our isolate was confirmed by constructing phylogenetic tree using maximum likelihood method (See Figure 1).

3.6. Determination of optimum growth conditions for GCFSD01 strain

Bacillus cereus GCFSD01 was found to be grown best at 37 °C and optimum pH for its growth was found to be 7 (as shown in Figure 2A and 2B).

Table 1. Morphological and biochemical characteristics of bacterial isolate GCFSD01.

Morphological Characteristics		Biochemical Characteristics	
Colour	White	Catalase	+
Cell morphology	Rod	Oxidase	-
Motility	+	Indole test	-
Flagella	+	Methyl red test	-
Gram staining	Gram +ve	Voges-Proskauer (VP) test	+
Endospore staining	+	Nitrate reduction	+
Capsule staining	-	H ₂ S production	-
Aerobic growth	+	Citrate utilization	+
Fluorescent pigment	+	Starch hydrolysis	+
Optimum temperature	37 °C	Tyrosine hydrolysis	+
Optimum pH	7	Gelatin hydrolysis	-
Resistant to bacteriolyisin	+		

**Figure 1.** 16S rRNA sequence-based phylogenetic tree of *Bacillus cereus* isolated from Cd contaminated soil.**Figure 2.** Graph showing effect of Cd vs. control on growth rate of *Bacillus cereus* strain GCFSD01: (A) at different temperature; (B) at different pH.

3.7. Effect of temperature and pH on Cd²⁺ uptake ability of *Bacillus cereus* GCFSD01

B. cereus GCFSD01 showed variations in its Cd²⁺ uptake ability with the changes in pH and temperature. At 25 °C *B. cereus* GCFSD01 was able to remove 50% Cd²⁺ from the medium. At 37 °C, *B. cereus* exhibited maximum uptake ability of 77%. While at 42 °C, it removed only 40% Cd²⁺ from the medium. At pH 5, *B. cereus* was able to remove 40% Cd²⁺ from the medium. With the increased pH, Cd²⁺ uptakes ability of *B. cereus* also increased. At pH 6, it was able to remove 75% Cd²⁺ from the medium while further increase in pH caused a decrease in removing ability of Cd²⁺. At pH 7, it removed 68% Cd²⁺ while at pH 8, it was able to remove 59% Cd²⁺ from the medium. At pH 9, it removed only 35% Cd²⁺ from the medium, respectively.

3.8. Multi Metal Resistance (MMR) and antibiotic resistance

The result of multi metal resistance is presented in Table 2. *B. cereus* strain GCFSD01 was found to be sensitive to amikacin (30 µg), chloramphenicol (30 µg), kanamycin (30 µg), gentamicin (10 µg), tetracycline (30 µg), ampicillin (10 µg) (as shown in Table 2).

3.9. Evaluation of Cd reduction potential

Reduction of Cd by of *B. cereus* GCFSD01 was evaluated using atomic absorption spectrophotometer (AAS). For this, percentage reduction was measured after 24, 48 h and 72 hrs. Our data suggested that strain GCFSD01 reduced 40.2%, 61.3% and 57.6% of Cd (100 mg/L) in 24, 48 h and 72 hrs respectively (as shown in Figure 3).

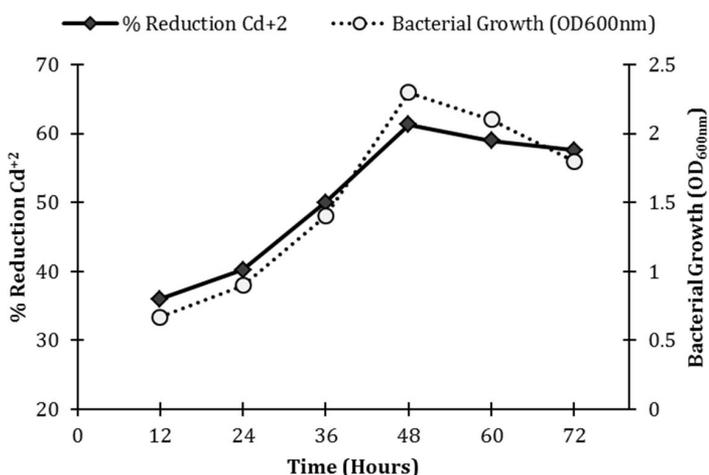


Figure 3. Percentage reduction of Cd after inoculation of bacterial strain GCFSD01.

Table 2. Multi Metal Resistance (MMR) against heavy metals and antibiotics sensitivity profiling of strain GCFSD01.

Multi Metal Resistance (MMR) concentration of Cd, Cr, Pb and Ni (mM) at 1:1:1:1		Antibiotics susceptibility testing	
1	+	ceftazidime (30 µg)	R
1	+	ciprofloxacin (5 µg)	R
2	+	gentamicin (10 µg)	S
2.5	+	amikacin (30 µg)	S
3	+	piperacillin-tazobactam (100/10 µg)	R
3.5	+	meropenem (10 µg)	R
4	-	chloramphenicol (30 µg)	S
4.5	-	streptomycin (10 µg)	R
5	-	ampicilline (10 µg)	S
5.5	-	rifampicine (5 µg)	R
6	-	kanamycin (30 µg)	S
6.5	-	tetracyclin (30 µg)	S
7	-	erythromycin (15 µg)	R
7.5	-		
8	-		
8.5	-		
9	-		

S = Sensitive; R = Resistance.

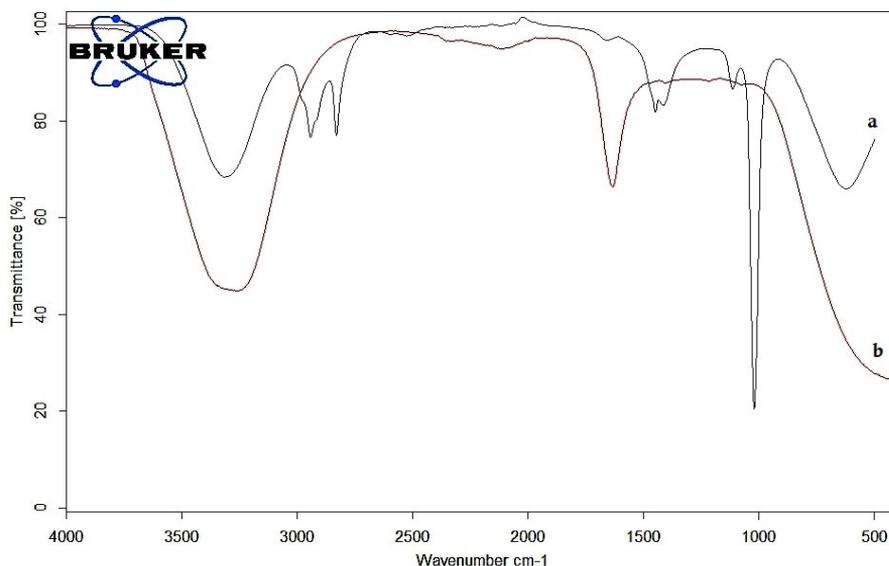


Figure 4. FTIR spectrum of *Bacillus cereus* strain GCFSD01 (a) without metal loading (b) with Cd.

3.10. FTIR spectrum

The shifts in IR spectrum peaks were noticed upon binding of Cd. Furthermore, attachment of Cd significantly decreased the transmittance (%) and position of multiple peaks. According to our results, carboxyl, amino and hydroxyl groups played important role in binding of Cd by electrostatic interactions. Spectra indicated contribution of amide group (1362.50 cm^{-1} and 1635.83 cm^{-1}) and hydroxyl group (3261.87 cm^{-1}) in Cd attachment. After binding of Cd, 1635.83 cm^{-1} shifted to 1635.84 cm^{-1} , 2125.23 cm^{-1} to 2120.084 cm^{-1} , 2360.04 cm^{-1} to 2359.98 cm^{-1} , 3261.87 cm^{-1} to 3263.81 cm^{-1} due to NH_2 scissoring, amide vibration and OH stretching, respectively (as shown in Figure 4).

4. Discussion

Applications of heavy metals resistant bacterial isolates play an important role in the reduction of toxic metals from the environment. Effluents discharged from industries harbor bacterial growth by adopted heavy metals resistant mechanisms which making them more resistant to high concentrations of these toxic metals. Modifications in bacterial structures presented their adaptations in high concentrations of heavy metals (Ghani et al., 2012). Heavy metal-resistant bacterial strains shared common resistant adaptations among them due to the same toxic mechanisms of heavy metals (Ianeva, 2009). Contamination due to high concentrations of heavy metals in the environment posing a serious threat to all life forms living either in aquatic bodies and on land.

In the present study, Cd^{+2} resistant bacteria was isolated from the soil contaminated with high concentrations of toxic metals from Paharnag main drain, Bawa chak, Faisalabad, Pakistan. It has been reported that 0.001 to 0.21 mg/L of Cd present in ground water samples in different areas

of Pakistan. Highest value of 0.21 mg/L was reported in Khyber Pakhtunkhwa (KPK) province industrial area (Waseem et al., 2014). Cd^{+2} ions removal by the applications of bacterial biomass has been reported in many studies (Vijayaraghavan and Yun, 2008; He et al., 2011; Huang et al., 2014). Bacterial strain has high capacity for the reduction of Cd^{+2} from the water polluted with toxic metals and has earned great deal in the field of bioremediation. After initial screening, it was observed that 15 out of total 25 soil samples exhibited bacterial growth on LB agar containing 1mM of Cd. MIC of selected bacterial strains were determined and one strain GCFSD01 was able to grow on minimal salt agar incorporated with 30mM of Cd. It was then screened for MMR against Cd, Cr and Pb. Results showed the tolerance of bacteria to Cd upto 30mM and MMR (Cd: Cr: Pb: Ni, 1:1:1:1) up to 5.5 mM. Molecular characterization confirmed the strain as *Bacillus* sp/ showing 98.44% similarity with *B. cereus* MB401 (KJ833772). Presented results in this study are accordance to Shamim and Rehman (2012) who performed the similar work and isolated the *Klebsiella pneumoniae* strain CBL-1 and found the Cd tolerance upto 550 $\mu\text{g}/\text{mL}$. Heavy metal tolerant bacteria have been isolated from tannery effluents and found that *Bacillus* spp exhibited tolerance in this order $\text{Pb} > \text{Cu}$ and $\text{Zn} > \text{Cr} > \text{Hg}$. They found the Cd removal upto 93% at 96 hrs but in this study we found the maximum capacity of Cd removal at 48 hrs and after that removal % declined. Pagananelli et al., 2010, reported the difference between Cd removal by bioprecipitation (23%) and biosorption (77%) by the use of sulphate reducing bacteria, thus concluding biosorption method more efficient. Kao et al. (2009) reported 82.7% Cd removal by *E. coli* cells with 10 mg/L initial Cd concentration. Shamim and Rehman (2012) found that *K. pneumoniae* capable of decreasing 46%, 68%, 83% and 93% of Cd ($100\mu\text{g}/\text{mL}$) from the medium after 24, 48, 72 and 96 hrs. Our data about Cd

removal clearly related with bacterial growth as maximum Cd removal was observed after 48 hrs (61.3% reduction) that is maximum growth phase of our strain as predicted by optimum growth condition of the strain GCFSD01. After that percentage reduction of Cd decreases as shown in our data. Some scientists believed that bacterial growth decreases after maximum bioaccumulation of metal ions (Abbas et al., 2014).

FTIR analysis was performed to observe the difference between functional groups present in bacterial cell wall in relation to biosorption of Cd. Results revealed the presence of a number of absorption peaks in control as compared to sample with metal stress. It was observed that there was a change in peak at 3261 and 2125 cm^{-1} showing the involvement of amino and hydroxyl group in Cd biosorption. Afzal et al. (2017) also reported amino and hydroxyl group role for the Ni and Co absorption. Antibiotic susceptibility testing of *B. cereus* GCFSD01 against different antibiotics has been exhibited. The results present in this study are in accordance with the Abbas et al. (2017) who demonstrated that Cd resistant *Bacillus* also harbored the antibiotic resistance. In literature, correlation between metal resistance and antibiotic resistance has been reported. This is due to the result of contaminated environments causing coincidental selection of resistance factors both for heavy metal and antibiotics (Chellaiah, 2018). Similar observations on metal and antibiotics resistance have been reported in different bacterial strains (Thacker et al., 2007). To detoxify the heavy metals effects, bioremediation approach has gained much attention due to environment friendly, safe and cost effective nature.

On the basis of this study, it was concluded that native bacterial strain GCFSD01 *B. cereus* isolated from industrial soil of Faisalabad Pakistan possessed Cd tolerance ability and biosorptive potential. However, exact resistance mechanisms needs further investigation but, it may be a potential candidate as bioremediation agents development in future to detoxify contaminated soil at industrial surroundings within natural environments in Pakistan.

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