



Multiple Metal Resistant *Bacillus cereus* 3.1S Isolated from Industrial Effluent has Promising Arsenite Oxidizing Potential

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ABSTRACT

The arsenite resistant *Bacillus cereus* 3.1S, isolated from pesticide industry effluent, showed maximum growth at pH 7 at 37 °C in LB medium after 24 h of incubation. The strain tolerated As³⁺ up to 40 mM and also showed resistance against Pb²⁺ (8mM), Cd²⁺ (6mM), Cr⁶⁺ (6mM), and Cu²⁺ (10mM). The arsenite oxidase is responsible for the conversion of arsenite (As³⁺) into arsenate (As⁵⁺) and the predominant form of arsenite oxidase was intracellular and its optimum activity recorded was determined as 730 and 750 μM/min (calculated by both Safranine O spectrophotometric and molybdene blue methods) at pH 7 and 37°C in the presence of Zn²⁺ as cofactor. The protein profile of *B. cereus* 3.1S, showed two bands of approximately 14 and 70 kDa, which had their possible role in arsenite oxidation. This was confirmed by transforming *E. coli* DH5α with plasmid DNA of *B. cereus* 3.1S. This arsenite resistant bacterial strain oxidized 76 and 86.5% As³⁺ from the original industrial wastewater after 3 and 6 days of incubation, respectively. This bacterially treated wastewater, when used for plant growth, revealed an improved growth of *Vigna radiata* as compared to the original (untreated) wastewater. This multiple metal resistant bacterium's ability to convert toxic arsenite into relatively less toxic form may find potential application in environmental biotechnology.

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Authors' Contribution

AN performed the experiments and wrote the manuscript. AE helped in experiments and analyzed the results. DAB helped in analysis of results. AR supervised the study. DAB and AR helped in manuscript preparation.

Key words

Arsenite, *B. cereus* 3.1S, Arsenite oxidase, Glutathione, Metallothioneins, Bioremediation

INTRODUCTION

Huge quantity of untreated industrial waste water is being discharged from industries such as metal processing, mining, textile, tanneries, pigment production, pharmaceuticals, pesticides, smelting, organic chemicals, alloy industries and storage batteries, rubber and plastics manufacturing industries, severely pollute the environment (Ghosh *et al.*, 2018; Elahi and Rehman, 2019). Heavy metals containing lumber and wood products aggravate water pollution even more day by day when they get entered into the fresh water bodies (Mohammadi *et al.*, 2005). Arsenic due to its non-degradability and persistent nature accumulates in the water bodies thus decreasing the water potential to sustain life.

Arsenic entry into the water system is the first key step of arsenicosis that seriously affects the health of human beings (Oremland and Stolz, 2005). Arsenic exists mainly in two forms; arsenite (As³⁺) and arsenate (As⁵⁺), the former is more toxic and is present in high concentration in the wastewater as compared to the later one which is less toxic and less available in the environment. Arsenic and its compounds are mutagenic, teratogenic, and carcinogenic in

nature (Mead, 2005). It is a major cause of skin, liver, lung, and lymphatic cancer, and also highly toxic for the kidneys (Tseng *et al.*, 2002). Arsenic exacerbates human health by negatively affecting mental capabilities, decreasing the levels of red and white blood cells' production, and also causes abdominal cramps, weakness, diarrhea, headaches, anemia (Kohnhorst *et al.*, 2002; USEPA, 2004). Moreover, it has also been linked to be a cause of type II diabetes in human beings (Walton *et al.*, 2004). The continuous exposure of high concentration of arsenic compounds causes infertility and miscarriages in women as well as weak immune system in neonates. Only 0.05 mg l⁻¹ arsenic quantity is permissible in drinking water, as recommended by the World Health Organization (WHO).

Arsenic is being removed from the contaminated sites through commonly used techniques such as isolation, physical separation, immobilization, extraction of metal, and toxicity reduction (Lim *et al.*, 2014). These methods are not eco-friendly; they are also expensive due to use of chemicals in order to detoxify arsenic impurities. Microorganisms have the ability to oxidize, reduce, and adsorb arsenic. They are capable of detoxifying arsenic by changing arsenite into arsenate (oxidation), and by adding methyl group to the arsenic (methylation). Thus, arsenite oxidizing bacteria could play a vital role in the bioremediation process (Simeonova *et al.*, 2005; Dey *et al.*, 2016) utilizing their ability to detoxify more toxic and

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mobile arsenite into less toxic and slowly mobile arsenate. Therefore, they are suggested to be used in environmental clean-up operations (Satyapal *et al.*, 2016, 2018; Mu *et al.*, 2019).

In the present study, a multiple metal resistant bacterium isolated from industrial effluents has been found to have arsenite oxidizing potential. Arsenite oxidase activities, an enzyme responsible to convert arsenite into arsenate, were also evaluated at different pH, temperature, and metal ions. Impact of As^{3+} on the growth pattern of bacterial strain and changes in proteomics and cell physiology were also determined.

MATERIALS AND METHODS

Samples collection and isolation of arsenite resistant bacteria

Samples of industrial wastewater were collected in the sterile screw capped bottles, from different industrial sites of Lahore, Pakistan. Then, 100 μl of wastewater specimen was spread on acetate minimal salt medium (MSM) agar plates augmented with 100 $\mu\text{g}/\text{ml}$ of arsenite for bacterial isolation. The minimum inhibitory concentration (MIC) was determined by preparing MSM-agar plates (Pattanapitpaisal *et al.*, 2001) supplemented with increasing concentrations of sodium dihydrogen arsenite (NaH_2AsO_3) up to 3000 $\mu\text{g}/\text{ml}$. The 13 isolated bacteria were evaluated for their tolerance to As^{3+} at different concentrations and the bacterium 3.1S showing highest resistance (40 mM) was selected for further study.

Bacterial characterization

Arsenite resistant bacterial isolate was characterized morphologically and biochemically by using standard methods (Cappuccino and Sherman, 2001). The molecular characterization of the bacterium was done by isolating DNA (Supplementary Fig. S1a) according to Sambrook *et al.* (2001), and 16S rRNA gene amplification (Supplementary Fig. S1b) was done according to procedure described in Rehman *et al.* (2007) using universal primer pair RS1 and RS3. Fermentas purification kit (# K0513) was used to clean PCR products, and sequenced with Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The data obtained after sequencing was submitted to GenBank to get the accession number.

Heavy metal resistance

Resistance pattern of the bacterial isolate against other heavy metals e.g. as lead (PbNO_3), cadmium (CdCl_2), copper ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), nickel ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), chromium ($\text{K}_2\text{Cr}_2\text{O}_7$), and arsenite (NaH_2AsO_3) were also checked.

MSM broth medium with different concentrations of heavy metals was prepared *i.e.* 100, 300, 500, 1000, 1500, 2000, and 2500 $\mu\text{g}/\text{ml}$ (Pattanapitpaisal *et al.*, 2001), and inoculated with log phase culture of bacterium. Culture was allowed to incubate at 37 °C for 24 h, and then growth (O.D_{600}) was determined.

Optimization of growth conditions

The optimum cultivation parameters of the bacterium were determined *i.e.*, temperature and pH, by cultivating it at various temperatures (25°C, 30°C, 37°C, and 42°C) and pH (5, 6, 7, 8, and 9) in 100 ml Luria broth, inoculated (100 μl) with log phase culture (inoculum $\text{O.D}_{600\text{nm}}$ was maintained at 0.5). Cell density was determined by taking absorbance at $\text{O.D}_{600\text{nm}}$ after 24 h of incubation.

Effect of arsenite on bacterial growth

Growth curves of bacterial strain were determined by growing the bacterial isolate in LB broth augmented with 100 $\mu\text{g}/\text{ml}$ NaH_2AsO_3 (treated) and 1% glucose (control), inoculated with log phase bacterial culture (1 ml). Growth was measured by taking optical densities at $\text{O.D}_{600\text{nm}}$ at 0 h (immediately after inoculation), and after a regular interval of four hours up to 36 h.

Determination of arsenite oxidase activity

Qualitative method (AgNO₃ assay)

Acetate minimal agar plate augmented with NaH_2AsO_3 (100 $\mu\text{g}/\text{ml}$) was streaked with the loop full of bacterial culture, incubated at 30°C for 48 h and was flooded with 0.1 M silver nitrate solution. Arsenate produces brownish precipitates when combined with AgNO_3 and these observations were made as mentioned by Simeonova *et al.* (2004).

Quantitative methods

Intracellular enzyme: Acetate minimal medium containing NaH_2AsO_3 (100 $\mu\text{g}/\text{ml}$) was inoculated with bacterial isolate and without inoculation (control), and incubated for up to four days. Culture was centrifuged, and the pellet was washed with a 50 mM phosphate buffer (pH 7) twice before sonication. Cells were sonicated at 4°C (on ice) for 15 sec with an interval of 1 minute, centrifuged at 14,000 $\times g$ for 10 min and supernatant thus obtained was shifted to new eppendorf. This supernatant was used to assay the intracellular enzyme activity.

Extracellular enzyme: MSM, augmented with 100 $\mu\text{g}/\text{ml}$ NaH_2AsO_3 , was prepared and inoculated with the bacterial culture and without inoculation (Control). Flasks were incubated at 28 °C for 24, 48, 72, and 96 h, and after completion of incubation, cells were harvested by

centrifugation at 14000 xg for 10 min, and supernatant was separated. This supernatant was used for the estimation of extracellular enzyme activity.

Enzyme activity reaction mixture contained each sample supplemented with As^{3+} (100 $\mu\text{g}/\text{ml}$) along with 1 ml of 2% potassium iodate, and 1 ml of 1 M hydrochloric acid. The mixture was mixed gently until the color turned bright yellow. Then, 0.02% safranin O (0.5 ml) was added, and volume was made with distilled water up to 100 ml, and mixed gently for 2-3 min. The pH of the solution was adjusted at 4 with the help of 2 ml acetate buffer, and the flask was shaken well. $\text{OD}_{532\text{nm}}$ of the reactions was measured against reagent blank (Pasha and Narayana, 2008).

Arsenate determination by molybdene blue

Arsenate was estimated as reported by Lenoble *et al.* (2003). In this, the reaction mixture contained 4 ml enzyme (intracellular or extracellular), 2 ml of reagent A (dissolving 1390.5 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ in 9 M H_2SO_4), 1 ml of reagent B (dissolving 0.5 g of ascorbic acid in 100 ml sterilized distilled water) and 50 ml of deionized water. $\text{OD}_{870\text{nm}}$ was determined after regular time intervals (0, 5, 10, 15, 20, 25 and 30 min). Control was also treated in the same manner and observations were made.

For the determination of arsenite oxidation potential, 4 ml of bacterial enzyme (extracellular or intracellular) was mixed with ascorbic acid solution (100 μl) and reagent A (200 μl), and deionized water was added to make the volume up to 5 ml. Reaction mixture was incubated at room temperature for 30 min and $\text{OD}_{870\text{nm}}$ was measured. Arsenite oxidation potential of the bacterial strain was calculated from arsenate standard curve (Lenoble *et al.*, 2003).

Enzyme assay

The bacterium was cultivated in MSM with and without 100 $\mu\text{g}/\text{ml}$ NaH_2AsO_3 for three days. Culture was harvested by centrifugation for 10 min at 6,000 rpm and the cells obtained were washed with 50mM phosphate buffer (pH 7) twice. The cell pellet was sonicated three times, for 15 sec at 4°C with a time interval of 1 minute between each sonication cycle. The sonicated pellet was centrifuged for 10 min at 14,000 xg, and the supernatant obtained was shifted to a fresh Eppendorf, regarded as the soluble fraction.

Two (50 ml) sterilized flasks, labeled as “treated” and “control”, were taken. The reaction system consist of 0.1 ml bacterial sample (supernatant or resuspended pellet), along with 0.9 ml of reaction mixture containing 20 μl of 1 M phenazine methosulfate (PMS), 2 μl of 1 M 2,4-dichlorophenolindophenol (DCPIP), 2 μl of 1 M NaH_2AsO_3 , and volume of the reaction mixture was made up to 10 ml in properly labeled flask, and incubated for

30 min at 37°C. Similar procedure was performed for the treated as well as control samples. Finally $\text{O.D}_{600\text{nm}}$ was determined (Noreen and Rehman, 2016).

Effect of temperature, pH and metal ions on enzyme activity

Optimal conditions for the maximum activity of arsenite oxidase of the *B. cereus* 3.1S were determined. For the optimal temperature, crude extract (100 μl) was incubated at various temperatures (30, 37, 42, 55, 70 and 90 °C) for 30 min then crude extract (100 μl) was mixed with 0.9 ml reaction mixture [20 μl of 1 M phenazine methosulfate (PMS), 2 μl of 1 M 2,4-dichlorophenolindophenol (DCPIP), 2 μl of 1 M NaH_2AsO_3 , and 9.76 ml of H_2O]. To check the enzyme thermostability, the reaction was carried out by incubating assay mixture at 37°C for 30 min.

For pH optimization, the enzyme extract was incubated at pH values 4, 5, 6, 7, 8, and 9 in different buffers. Buffers used were acetate buffer (pH 4), sodium acetate (pH 5-6), Na_2HPO_4 (pH 7-8), and Tris HCl (pH 9). Then 200 μl from each buffer and 200 μl of the crude extract mixed and incubated for 60 min at 37°C. After that 200 μl from the enzyme-buffer mixture was mixed with 0.8 ml of reaction mixture [20 μl of 1 M PMS, 2 μl of 1 M DCPIP, 2 μl of 1 M NaH_2AsO_3 , and 9.76 ml of H_2O] and incubated at 37°C for 30 min.

The metal ions effect on the enzyme activity was determined. For this, 100 μl crude enzyme was mixed with metal ion solution *i.e.*, Zn^{2+} , Ca^{2+} , Cu^{2+} , Mg^{2+} and was incubated at 37°C for 30 min. Then 0.9 ml of reaction mixture [20 μl of 1 M PMS, 2 μl of 1 M DCPIP, 2 μl of 1 M NaH_2AsO_3 , and 9.76 ml of H_2O] was added and incubated again for 30 min at 37°C. All the enzyme activities were determined by measuring absorbance at 600 nm.

Estimation of glutathione and non-protein thiols

Under arsenite stress, the altered levels of glutathione (GSH) and other non-protein thiols (NPSHs) were measured according to Khan *et al.* (2015).

SDS-polyacrylamide electrophoresis

The bacterium was cultivated in MSM broth with and without 100 $\mu\text{gAs}^{3+}/\text{ml}$ and SDS- PAGE was done according to Laemmli (1970).

Arsenite oxidase gene amplification and transformation

Amplification of arsenite oxidase gene was done by using degenerate primers as reported by Que'me'neur *et al.* (2008). Briefly, the reaction mixture consisted of 50 μL of distilled water containing 6 μl of genomic DNA and 5 μl of each primer (10 pmol), (Amersham Pharmacia, Piscataway, NJ, USA). The polymerase chain reaction

(PCR) was performed according to Jinbo *et al.* (2007). Plasmid from bacterial isolate was isolated by using mini-preparation protocol according to Sambrook *et al.* (2001). Extracted plasmid DNA (550bp) was analyzed by gel electrophoresis and *E. coli* DH5 α was transformed with the isolated plasmid DNA (Sambrook *et al.*, 2001). Arsenite-oxidizing ability of transformants was checked by using the method of Simeonova *et al.* (2004).

Oxidation of As^{3+} in industrial wastewater

The bacterial strain potential to oxidize As^{3+} was determined by inoculating 300 ml bacterial culture (24 h old) in 1 liter of industrial wastewater. Also as control samples, autoclaved and un-autoclaved wastewater was placed under the same experimental conditions. In the wastewater, As^{3+} initial concentration was determined according to Noreen and Rehman (2016) and final As^{3+} concentration (100 μ g/ml) was adjusted with NaH_2AsO_3 . Changes in As^{3+} concentration were calculated with the help of a calibration curve prepared under the same experimental conditions.

Microbial treated wastewater use for plant growth

To check the effect of treated wastewater on the plant growth system, small pots were filled with autoclaved soil and seeds of *Vigna radiata* (mung beans) were cultivated in them. At least three experimental pots were employed; each pot differs from one another in watering scenario. Two pots served as controls; one was watered with original wastewater and the other with tap water, while the third one was watered with bacterially treated wastewater (experimental). The plants were cultivated under 1:1 light and dark for 10 days at room temperature. Finally, the growth in control, tap and untreated wastewater was compared.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS

As^{3+} resistant bacterium

Wastewater samples were taken from leather, pesticide and steel industry near Lahore, Pakistan. The temperature of wastewater samples ranged between 23-35°C, pH ranged between 5.5 to 8, and the sample color was muddy. Thirteen different colonies, appeared on MS-agar plates (100 μ g As^{3+} /ml), were selected and evaluated for their

ability to tolerate maximum As^{3+} concentration. Only one (3.1S) showed growth on MS-agar plates containing up to 40 mM As^{3+} (3000 μ g/ml) which also showed resistance against Pb^{2+} (8 mM), Cd^{2+} (6 mM), Cr^{6+} (6 mM) and Cu^{2+} (10 mM). This bacterial isolate 3.1S showed maximum homology with the genus of *Bacillus* (Table I). The data obtained from 16S rRNA gene sequencing for *Bacillus cereus* (3.1S) was submitted to GenBank database under accession number of KF003020. The dendrogram on the basis of homology was also created (Fig. 1).

Table I. Morphological and biochemical characteristics of *B. cereus* 3.1S.

Morphological and biochemical characteristics	<i>B. cereus</i> 3.1S
Gram staining form	+ve
Spore staining	+ve
Starch hydrolysis	-ve
Triple sugar iron	-ve
Mannitol salt agar	-ve
Methyl red	+ve
Motility	+ve
Indole	-ve
MacConkey agar	-ve
Catalase	+ve
Oxidase	-ve
Pigment production	-ve
Citrate utilization	+ve

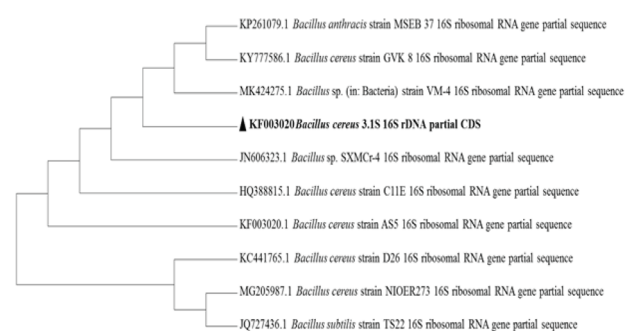


Fig. 1. Neighbor-joining methods based tree of selected *B. cereus* 3.1S strains. *B. anthracis* strain MSEB 37 (KP261079.1), *B. cereus* strain GVK 8 (KY777586.1), *Bacillus* sp. strain VM-4 (MK424275.1), *Bacillus* sp. strain SXMCR-4 (JN606323.1), *B. cereus* strain C11E (HQ388815.1), *B. cereus* strain D26 (KC441765.1), *B. cereus* strain NIOER273 (MG205987.1), and *B. cereus* strain TS22 (JQ727436.1).

Figure 2 shows effect of As^{3+} on the bacterial growth at 37°C and pH of 7. The growth of bacterium slowed down and the maximum growth was obtained after 24 h in the presence of $100\ \mu\text{g}\ \text{As}^{3+}/\text{ml}$ as against 10 h in the control.

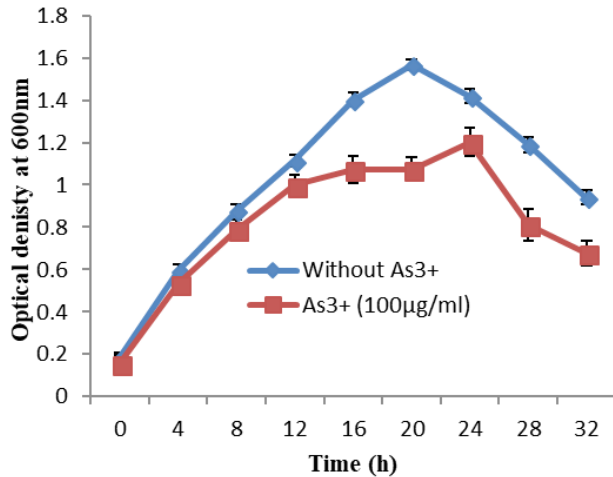


Fig. 2. Growth pattern of *B. cereus* 3.1S growing in $100\ \mu\text{g}\ \text{As}^{3+}/\text{ml}$ and without As^{3+} in minimal salt medium.

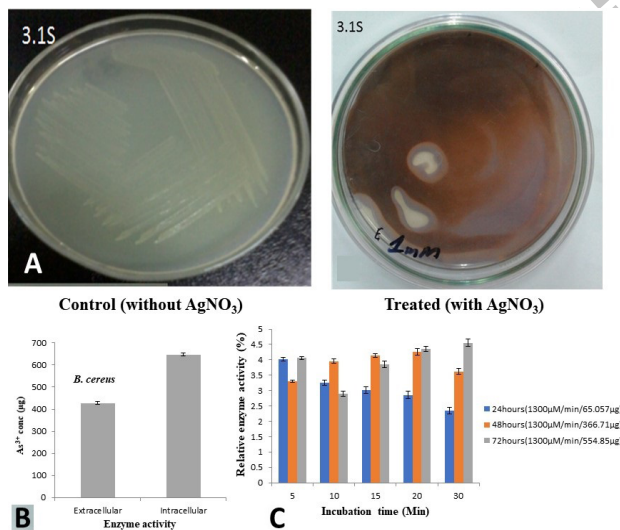


Fig. 3. Appearance of brown precipitates on plate, containing $100\ \mu\text{g}\ \text{As}^{3+}/\text{ml}$, is showing the arsenite oxidizing ability of *B. cereus* 3.1S as compared to the control in AgNO_3 assay (A), Concentration of As^{5+} transformed during extra and intra-cellular enzyme activity (B), Enzyme assay for soluble fraction of bacterial isolate 3.1S (C).

Arsenite oxidase activity in *B. cereus* 3.1S

Figure 3A shows production of brownish precipitates after treatment AgNO_3 in culture plates where bacteria

were grown in the preserve the arsenite. On the other hand, control plates/ bright showed yellow precipitates in which no bacteria were present. *B. cereus* 3.1S assay results clearly demonstrated the presence of arsenite oxidase activity that converts As^{3+} (arsenite) into less toxic form As^{5+} (arsenate).

Moreover, Safranin O spectrophotometric method was used to determine any change in the concentration of As^{3+} present in the bacterial culture medium after a specific incubation time period. Assay results revealed that extra cellular and intracellular arsenite oxidase of 3.1S oxidized 63.8% and 71%, respectively As^{3+} to As^{5+} after 96 h of growth. Figure 3B shows that the predominant form of the enzyme was intracellular as compared to the extracellular (OD 0.846nm).

Arsenite oxidase assay was performed after 24, 48, and 72 h of bacterial growth for soluble fraction to determine enzyme activity in $\mu\text{M}/\text{min}/\mu\text{g}$ in different time intervals. Arsenite oxidase revealed the maximum activity after 30 min of incubation of reaction mixture of 72 h grown bacterial culture. This assay was performed to confirm that enzyme activity was increased with the increase in time of growth and reaction mixture (Fig. 3C).

Characterization of arsenite oxidase

Bacterial enzyme showed maximum activity at 37°C i.e. 66.5% (Fig. 4A) and at pH 7 i.e. 124.5% (Fig. 4B). All the tested heavy metal ions enhanced the arsenite oxidase activity but the maximum enzyme activity was determined in the presence of Zn^{2+} (29.6%) when compared with the control containing no metal ions (Fig. 4C).

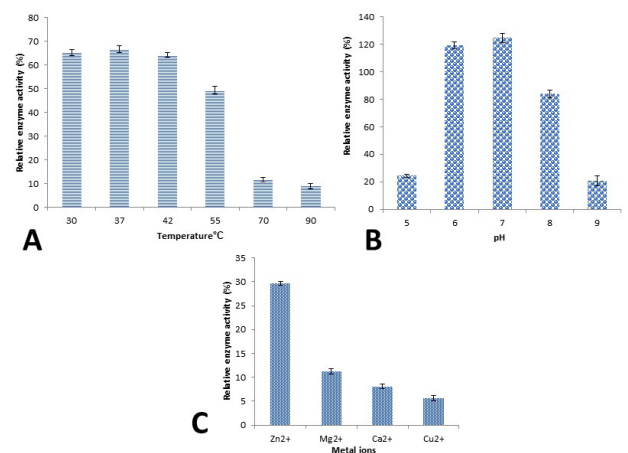


Fig. 4. Effect of temperature (A), pH (B) and metal ions (C) on arsenite oxidase activity.

Effect of As^{3+} on antioxidant molecules

It was found that As^{3+} stress stimulates GSH and

NPSHs levels in *B. cereus* 3.1S (Table II). In the presence of 100 $\mu\text{g As}^{3+}/\text{ml}$, 1597% and 86% increase in GSH and NPSHs was determined as compared to the control. This increase in antioxidants molecules indicates that bacterial cells try to maintain homeostasis by neutralizing the reactive oxygen species which are produced during metal stress.

Table II. Effect of As^{3+} (100 $\mu\text{g}/\text{ml}$) on the glutathione level (GSH, GSSG) and non-protein thiols (NPT) of *B. cereus* 3.1S.

	Control	As^{3+} treated	% increase
GSH (mMg^{-1}FW)	10.50	26.47	1597
GSSG (mMg^{-1}FW)	9.02	7.88	-
Total glutathione (mMg^{-1}FW)	19.52	34.35	-
GSH/GSSG ratio	1.16	3.35	-
NPT	1.21	2.07	86

Effect of As^{3+} on protein profile

A significant increase in intracellular protein content was shown by the bacterium in the presence of As^{3+} , presumptively arsenite oxidase (Fig. 5A). The enzyme has a large subunit of 60kDa protein and smaller subunit with low molecular weight 14kDa protein in the bacterial protein sample under As^{3+} stress. The enzyme concentration was very low in a control sample containing no As^{3+} stress.

Arsenite oxidase gene is responsible for oxidation of As^{3+}

A 550 bp product was obtained, after amplifying the arsenite oxidase gene, by using degenerate primers which presumably indicates the presence of enzyme protein in the bacterial culture under metal stress (Fig. 5B). The plasmid DNA containing arsenite oxidase gene isolated from *B. cereus* 3.1S (Supplementary Fig. S2) was transformed into *E. coli* DH5 α (Supplementary Fig. S3A). *E. coli* strain containing plasmid DNA showed the colonies on minimal acetate medium with arsenite stress (Supplementary Fig. S3B).

Arsenite oxidizing ability of the transformants was observed by brownish precipitation appearing on agar plates when AgNO_3 reacts with arsenate. Otherwise bright yellow precipitation takes place when AgNO_3 reacts with arsenite. The assay results reveal arsenite oxidase activity that converts arsenite into less toxic arsenate (Supplementary Fig. S4).

Oxidation of As^{3+} in industrial wastewater by *B. cereus* 3.1S

The As^{3+} oxidation in industrial wastewater was clearly observed by observing change in color of the medium supplemented with bacterial culture as compared to the control containing no bacterial cells. As^{3+} was

oxidized 76 and 86.5% by the bacterial strain after 3 and 6 days of incubation at room temperature (Fig. 6) showing the promising potential of the bacterial strain to detoxify the wastewater containing arsenic.

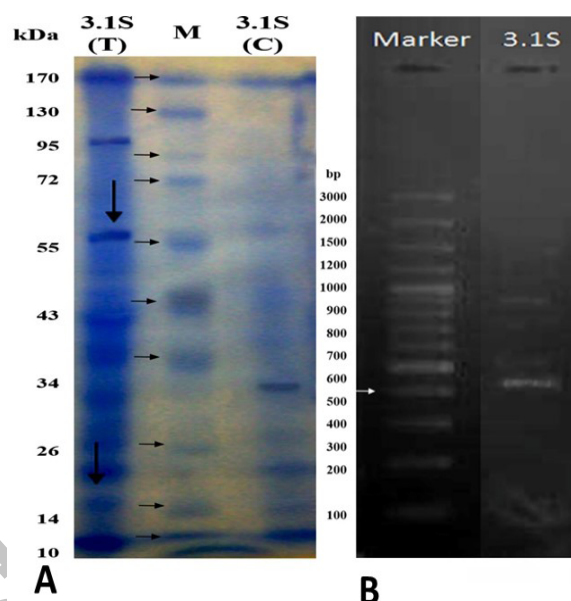


Fig. 5. A: SDS-PAGE of bacterial isolates intracellularly with arsenite and without arsenite in the culturing medium. On right hand side is marker a line (M), next to it, control sample of 3.1S (C) and 3.1S (T). Treated sample have the arsenite in the medium and represented with (T) while control does not have arsenite in the medium and marked with (C). B: Agarose gel (1%) is showing the amplified product of arsenite oxidase gene from *B. cereus* 3.1S. Lane marker and 3.1S is showing DNA marker and *B. cereus*, respectively.

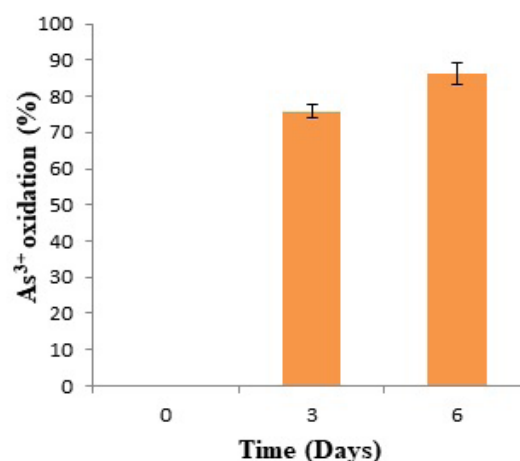


Fig. 6. As^{3+} oxidation (%) by *B. cereus* 3.1S from industrial wastewater after 3 and 6 days of incubation at room temperature.

B. cereus 3.1S treated wastewater enhances plant growth

Vigna radiata (mung beans) seeds irrigated with *B. cereus* 3.1S treated wastewater germinated normally and attained good growth size within 10 days as compared to the seeds irrigated with untreated wastewater. Seeds watered with untreated industrial wastewater showed delayed germination and poor growth (Fig. 7). From this experiment it can be deduced easily that microbially-treated industrial wastewater can be used safely for irrigation purposes.

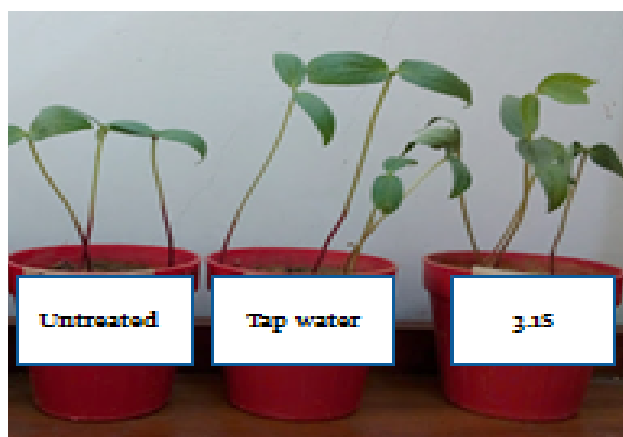


Fig. 7. Growth of *Vigna radiata* (mung beans) in microbially treated wastewater (*B. cereus* 3.1S), tap water and untreated wastewater after 10 days of incubation under 1:1 light and dark period.

DISCUSSION

Microorganisms have evolved survival strategies to thrive in the metal increasing environment. In the current investigation, wastewater samples were collected from pesticide industry near Lahore, Pakistan and the bacterium isolated has the potential to resist As^{3+} contamination up to 3000 $\mu\text{g}/\text{ml}$ (40 mM). This research lab is embarked upon isolating the heavy metals resistant microorganisms including arsenic resistant bacteria from the tannery and industrial effluents (Butt and Rehman, 2011; Noreen and Rehman, 2016; Ilyas and Rehman, 2018).

Besides As^{3+} , *B. cereus* 3.1S also developed resistance against other heavy metals e.g. Cu^{2+} , Cd^{2+} , Cr^{6+} , and Pb^{2+} at varying concentration. The arsenite oxidizing bacteria possess the property to resist other toxic metal ions (Rehman *et al.*, 2010). Similarly, Muller *et al.* (2003), Drewniak *et al.* (2008), Bachate *et al.* (2013), and Das and Barooah (2018) also reported that arsenite tolerating bacteria were able to resist other heavy metals including Se, Mn, Cr, Sb, Cd, Ni, Zn, Pb and Cu.

Silver and Phung (2005) reported that arsenite oxidase

is located both on membrane and within the periplasmic space. Microorganisms produce arsenite oxidase both intra and extracellularly, which is involved in converting more toxic As^{3+} to less toxic As^{5+} . The oxidation ability of *B. cereus* 3.1S was visualized by AgNO_3 assay by producing brown precipitates. Our findings are in good agreement with other researchers (Krumova *et al.*, 2008; Heinrich-Salmeron *et al.*, 2011; Raja and Omine, 2012). Liao *et al.* (2011) found that arsenite oxidase transformed 65% As^{3+} to As^{5+} within 30 min of incubation and complete As^{3+} oxidation was achieved within 50 min. In this study, arsenite oxidase of soluble fraction from *B. cereus*, 72 h grown culture, was able to transform 43% As^{3+} to As^{5+} after 30 min of incubation.

The temperature effect on the enzyme activity was evaluated by pre-incubation of enzyme at a temperature range of 30-90°C. *B. cereus* arsenite oxidase showed maximum activity at 37 °C as reported by Bachate *et al.* (2013). Many studies have been reported that the optimum temperature for bacterial arsenite oxidase activity is 30°C (Rehman *et al.*, 2010; Butt and Rehman, 2011; Raja and Omine, 2012). However, the arsenite oxidase isolated from bacteria showed the substantially decreased activity at 90 °C and in case of *B. cereus* 3.1S it was 9%. Enzyme activity is markedly affected by pH. The optimum pH for arsenite oxidase activity (124.5%) from *B. cereus* 3.1S was 7 and it was decreased markedly over both sides of optimum pH being 119% at pH 6 and 84% at pH 8 for *B. cereus*. Our findings are similar with other studies that reported the same results from other bacterial species (Rehman *et al.*, 2010; Butt and Rehman, 2011; Raja and Omine, 2012; Marzan *et al.*, 2017). Generally, pH optimum for the enzyme is in the range of 6 to 7. Enzyme activity was increased in the presence of each metal; however, the maximum activity was obtained in the presence of Zn^{2+} .

In the current study, contents of GSH and NPSHs were increased when grown under metal stress which is indicative of the fact that this bacterium is able to tolerate arsenic induced ROS stress. The level of GSH and NPSHs was increased up to 1597 and 86% in *B. cereus* 3.1S as compared to the cells grown without metal stress. It has been reported that GSH has reducing potential when arsenate enters into the bacterial cell (*E. coli*), As^{5+} reacts with ATP and GSH and converts into As^{3+} (Tsai *et al.*, 2009). As^{3+} reduction in plants through GSH has also been documented by Singh *et al.* (2006). The oxidative stress resulting from the formation of ROS is combated by antioxidant molecules including GSH and NPSHs. Metallothioneins (MTs) are involved in the metal scavenging process (Fig. 7).

The enzyme presence was also confirmed by SDS-PAGE and results showed that approximately 60 and

14kDa protein bands were present in the As^{3+} treated sample (Fig. 5A). Exactly the similar findings were reported by other researchers (Conrads *et al.*, 2002; Stolz *et al.*, 2006). It has been documented that presence of As^{3+} induces the enzyme synthesis which is composed of two polypeptides; large catalytic subunit has molecular weight of 98 and smaller has 14kDa (Santini and Hoven, 2004). The same results were reported by Ellis *et al.* (2001) and Yang and Rosen (2016). Koechler *et al.* (2010) reported that *Herminiimonas arsenicoxydans* contains aoxB protein with molecular mass of 92kDa.

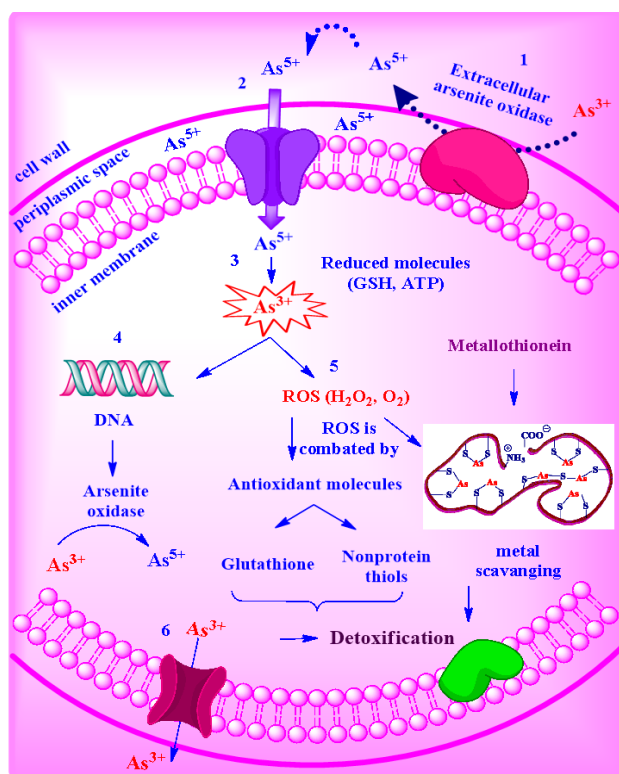


Fig. 8. Proposed As^{3+} resistance and oxidation mechanism in gram positive bacterium, *B. cereus* 3.1S. (1) As^{3+} oxidizes into As^{5+} extracellularly by arsenite oxidase. (2) As^{5+} enters into cell cytoplasm. (3) As^{5+} reduces into As^{3+} by reduced molecules e.g., GSH and ATP. (4) As^{3+} induces DNA to make arsenite oxidase which converts As^{3+} into As^{5+} . (5) As^{3+} is also involved in the generation of ROS and the oxidative stress resulted by the formation of ROS is combated by antioxidant molecules including glutathione and non-protein thiols. Metallothioneins are involved in metal scavenging process. (6) After certain level of accumulation, As^{3+} expel out through the efflux system.

The impact of microbial purified wastewater on the growth of *V. radiata* was also determined. Mung beans plants showed efficient growth in *B. cereus* 3.1S purified

wastewater as compared to the growth in untreated wastewater which indicates the efficiency of bacterial arsenite oxidase. Noreen and Rehman (2016) reported that *B. cereus* and *A. junii* treated industrial wastewater was safe for the growth of mung beans plants.

CONCLUSIONS

In conclusion, *B. cereus* showed maximum growth at 37°C and pH of 7. The multiple metal resistant bacterium tolerated As^{3+} up to 40 mM due to employing various mechanisms including antioxidant molecules and metallothioneins. The arsenite oxidase, which transforms toxic As^{3+} into less toxic As^{5+} , showed maximum activity at 37°C, pH 7 and in the presence of Zn^{2+} . This inevitable role of arsenite oxidase was also confirmed by transformation. The bacterial strain oxidized 76 and 86.5% As^{3+} after 3 and 6 days of incubation, respectively from the real industrial wastewater. This microbially treated wastewater is safe for the growth of plants. This metal resistant bacterium with good oxidizing ability, may find potential applications in environmental biotechnology.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: <https://dx.doi.org/10.17582/journal.pjz/20190620050601>

Statement of conflict of interest

The authors have declared no conflict of interest.

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Online First Article



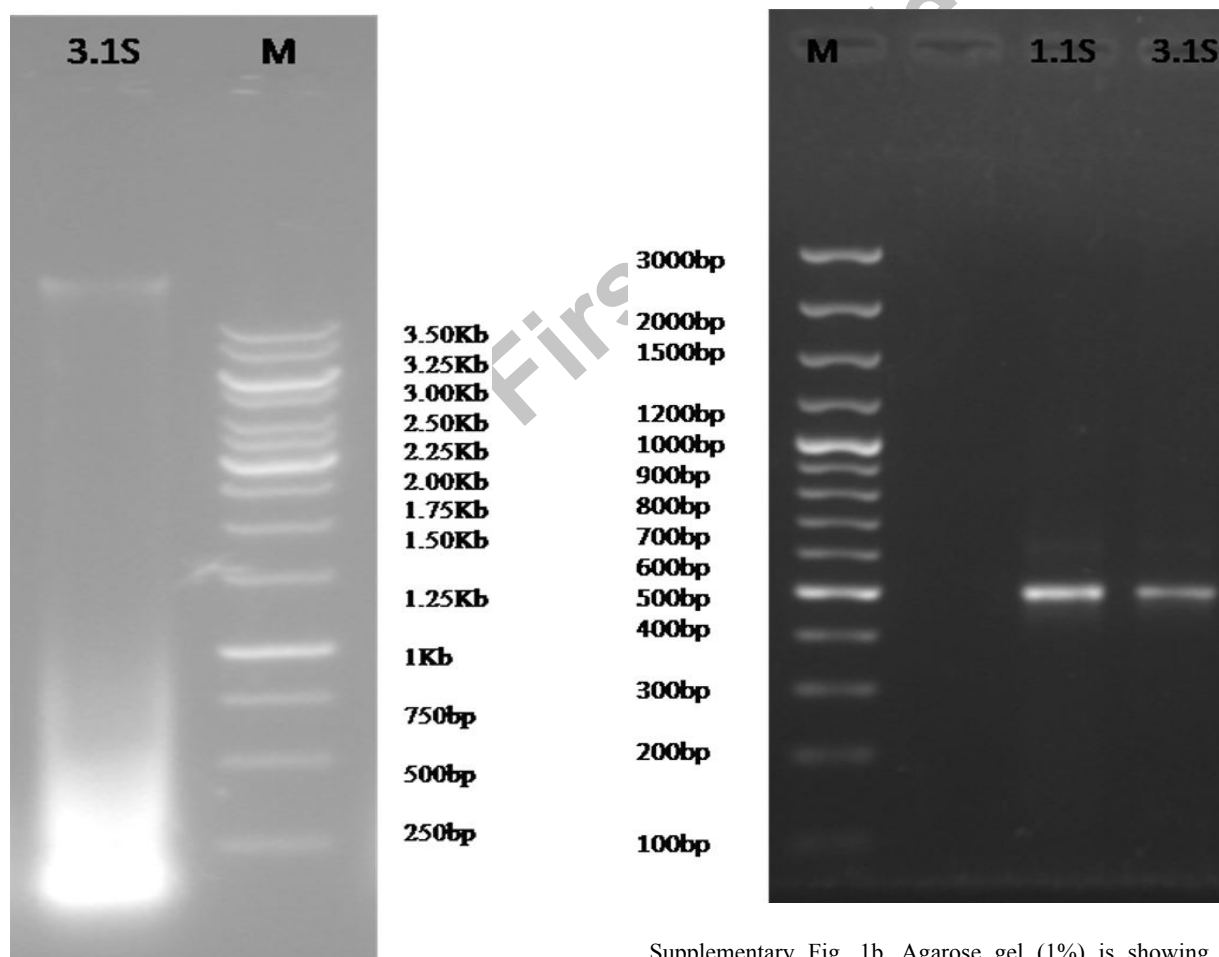
Supplementary Material

Multiple Metal Resistant *Bacillus cereus* 3.1S Isolated from Industrial Effluent has Promising Arsenite Oxidizing Potential

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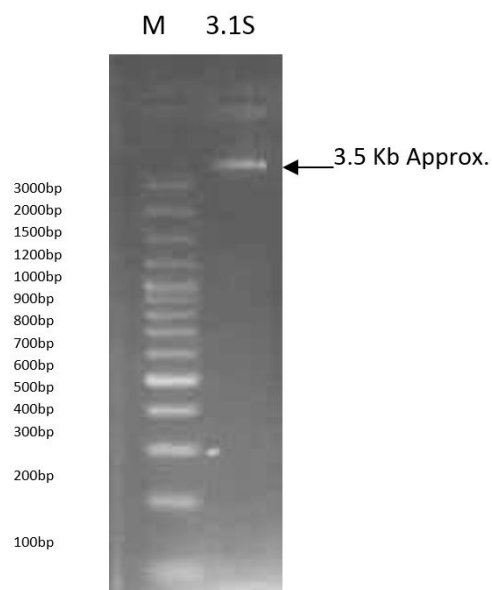
Supplementary Fig. 1a. Agarose gel (1%) is showing genomic DNA of 3.1S isolate and M represents DNA marker.

Supplementary Fig. 1b. Agarose gel (1%) is showing the amplified products of 16S rRNA gene from bacterial isolates. Lane 1 on left hand side is showing marker (M) while lane 2 and 3 showing isolate 1.1S and 3.1S, respectively.

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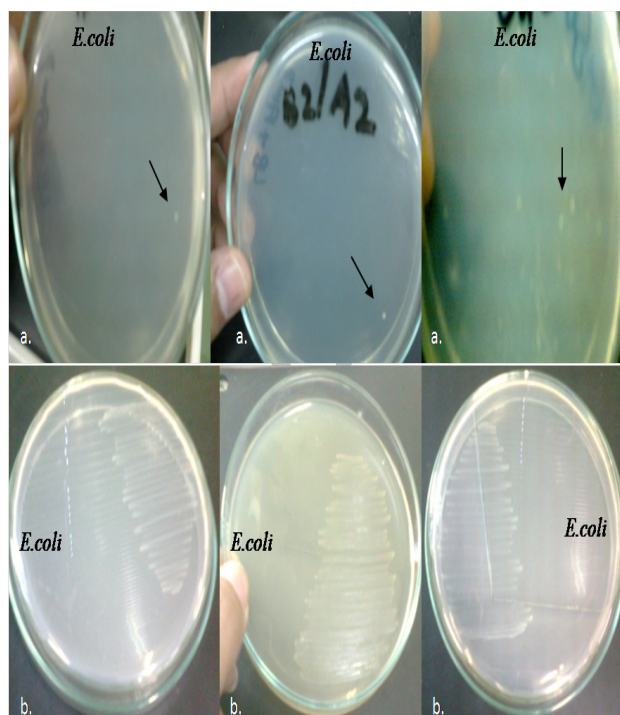
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Supplementary Fig. 2. Plasmid DNA band (3.5 Kb approx.) isolated from *Bacillus cereus* 3.1S and lane M represents DNA marker.



Supplementary Fig. 4. AgNO_3 assay of the transformants for their ability to oxidize arsenite into arsenate.



Supplementary Fig. 3. (a) The *E. coli* DH5 α transformed colonies with plasmid DNA containing arsenite oxidase gene as shown by arrow. (b) *E. coli* strain containing plasmid DNA showed the colonies on minimal acetate medium with arsenite stress indicating their ability to resist metal stress.