ORIGINAL ARTICLE

Characterization of multiple metal resistant *Bacillus licheniformis* and its potential use in arsenic contaminated industrial wastewater

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Abstract

In the present study, the arsenic bioremediation ability of *Bacillus licheniformis* (dubbed as A6) was determined. The strain was isolated from metal polluted wastewater and was identified on the basis of 16S rRNA sequence homology with accession number of KX 785,171. The bacterium showed resistance against multiple toxic heavy metals, and MIC against arsenic was 3000 µg/ml. Resistance of the bacterium against other toxic metal ions was 3000 µg/ml (Cr), 50 µg/ml (Hg), 1000 µg/ml (Mn), 4000 µg/ml (Se), 500 µg/ml (Pb), 100 µg/ml (Co), 70 µg/ml (Cd) and 100 µg/ml (Zn). The optimum growth temperature was 37 °C while pH was 7. The strain also showed resistance against commonly used antibiotics except ceftriaxone 30 µg and amoxicillin with clavulanic acid (2:1) 3 µg. *B. licheniformis* could oxidize arsenite into arsenate 86 and 98% after 48 and 96 h from the medium at optimum growth conditions. Due to its high oxidation potential, *B. licheniformis* can be used in the biological treatment of wastewater containing arsenic.

Keywords Arsenic · Wastewater · *Bacillus licheniformis* · Bioremediation

Introduction

Arsenic (As) falls in the category of metalloid and its toxicity is very high (Xiong et al. 2006). It is a ubiquitous metal (Naureen and Rehman 2016). It is odorless and tasteless. The discovery of arsenic goes to Albertus Magnus in 1250. Its name is derived from "arsenikon" which means yellow color. There are three allotropic forms of arsenic named as yellow arsenic, gray arsenic and black arsenic. Its atomic number is 33, while the atomic mass is 74.992. As exist in two oxidation states i.e., $+3$ and $+5$. Heavy metals have a very toxic effect on human beings as well as their environment. Human beings are intoxicated by heavy metals through exposures, taking food, drinking water containing metals or metalloids (Tamás and Martinoia 2006). Having the potential role in physiological processes, living organisms require these metals as a cofactor for enzymes in biological pathways (Cummings et al. 1999).

The arsenic concentration is almost increasing in every part of the earth (Islam et al. 2004; Kumari et al. 2018; Sher et al. 2020a, b). Due to huge industries in Pakistan, the heavy metals are increasing in our environment (Sher et al. 2020a, b). As a result, As-concentration is also increasing in ground, and drinking water that is above the World Health Organization (WHO) recommended limit i.e., 10 ppb (Prasad et al. 2013). Apart from anthropogenic activities, there are also natural processes by which As come in our environment i.e., land erosion, leaching, volcanism and weathering of rocks (Naureen and Rehman 2016). It has been reported that arsenic has a lethal effect on human beings due to groundwater contamination (Mandal and Suzuki 2002). The long period of arsenic exposure causes arsenicosis. Arsenic causes skin, bladder and lung cancer. As-toxicity depends upon its form, the mobility of As^{+3} is higher than other arsenic forms that is why its toxicity is higher than others (Kumar et al. 2004). Arsenic disturbs enzyme activity because of interaction with the sulfonyl group (Patra et al. 2004). Řezanka and Sigler (2008) reported that arsenic can replace phosphorus from the DNA due to its similarity with phosphorus.

Most commonly used conventional treatment methods e.g., soil washing, land filling, flushing, physico-chemical extraction and excavation could be used but, these methods are not eco-friendly and expensive due to usage of chemical

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substances. Mechanisms of arsenic detoxification via microorganisms are oxidation (conversion of arsenite into arsenate), reduction (conversion of arsenate into arsenite) and methylation (addition of methyl group).

Eradication of metal pollutants from the environment has been a challenge for a long time. It is important to establish an efficient and low cost method for the removal of toxic pollutants including heavy metal ions (Sher et al. 2020a). As conventional cleanup strategies are being used to remove the heavy metal ions from the polluted areas but these are expensive and feasible only in small areas. Researchers have found new cost effective methods that include the use of microorganisms (Naureen and Rehman 2016; Sher et al. 2020b), biomass (Jaafari and Yaghmaeian 2019; Sultana et al. 2020) and live plants (Zargar et al. 2013; Ojuederie and Babalola 2017).

Microorganisms especially bacteria are efficient in converting arsenite into arsenate through oxidation–reduction processes. A huge variety of bacterial strains have the ability to cause arsenic oxidation including *Agrobacterium tumefaciens* (Kashyap et al. 2006), *Pseudomonas arsenitoxidans* (Matlakowska et al. 2008), *P. lubricans* (Rehman et al. 2010), *Pantoea* sp., *Enterobacter* sp., *Pseudomonas* sp. *Comamonas* sp. (Liao et al. 2011) and *Microbacterium oxydans* (Sarkar et al. 2013). The arsenate, oxidized form of arsenic, has very low toxicity due to its low mobility as compared to the arsenite, the reduced form of arsenic.

The isolation and characterization of arsenic resistant bacteria from industrial wastewater were the main objective of this study. The bacterium was optimized for its growth conditions and was also checked for its As-oxidation ability under different parameters.

Materials and methods

Wastewater sample collection

Industrial wastewater was collected from the chemical industry from Sheikhupura (Pakistan) which has a long history for metal usage. Its geographical coordinates are 31.7167° N, 73.9850° E. The samples were taken in sterile bottles and some physical parameters i.e., temperature, pH and color were also noted.

Isolation and selection of bacterial strain

Bacterial strains resistance against arsenic, isolated from wastewater samples, was determined by providing arsenic in Luria–Bertani (LB) medium (Sigma-Aldrich, USA). The industrial wastewater sample was diluted tenfold in a normal saline and 10^{-3}, 10^{-4} and 10^{-5} dilutions were used. Then, wastewater sample dilution (100 μl) was spread on LB agar plates, which were already supplemented with 400 μg sodium arsenite/ml for the isolation of arsenite resistant bacterial strains. The autoclaved LB agar plates were prepared for the spreading of samples. The following composition of LB agar was used, 10 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g agar in one liter of distilled water. The colonies of bacteria were observed after incubation at 37 °C for 24 h. The different bacteria having different morphology were selected and streaked again and again until purified bacterial strains were obtained. Quadrant streaking was performed to get pure culture of the isolates.

Morphological characterization

On the basis of shaped and appearance, different bacterial strains from mix culture were selected for further streaking to get pure bacterial strain. After getting a pure bacterial cell culture, its morphology was observed under a light microscope. The morphology of the colony was observed with naked eyes.

Biochemical characterization of isolated bacterium

Different biochemical tests i.e., gram staining, spore staining, acid fast staining, capsular staining, oxidase test, catalase test, citrate utilization test, indole test, methyl red test, Voges–Proskauer test, triple sugar iron test and motility test were performed for bacterial identification (Cappuccino and Sherman 2008).

Arsenic oxidation screening by AgNO₃ method

For As^{3+} oxidizing bacterial screening, $AgNO_3$ method was used (Simeonova et al. 2004). Arsenic containing (100 μg/ml) nutrient-agar plates were inoculated with isolated metal resistant bacteria and then incubated at 37 °C for 72 h. After the appearance of bacterial growth, 0.1 M $AgNO_3$ was flooded on the plates and then placed at room temperature for overnight.

Molecular identification

For molecular identification of bacterial strain, genomic DNA was extracted according to Carozzi et al. (1991). The 16S rRNA gene was amplified by using the universal primer through PCR. The 16S rRNA gene product was sequenced and compared with already gene bank data in NCBI.

Optimum bacterial growth conditions

The bacterial growth conditions were determined on the bases of temperature and pH.

Optimization of temperature

For determination of optimum growth temperature, 5 ml N-broth was taken in three sets of tubes for three temperatures, i.e., 28, 37 and 42 °C. Test tubes containing N-broth were autoclaved, and inoculated with overnight log phase culture and incubated at respective temperature on shaker for 24 h. After incubation, optical density (OD) was taken at 600 nm using a LAMBDA 650 UV/Vis spectrophotometer (PerkinElmer, USA).

Optimization of pH

For determination of optimum pH, six sets of nutrient broth in tubes with different pH (5, 6, 7, 8, 9, 10) were prepared. These tubes were inoculated with overnight log phase culture and incubated on shaker at 37 °C for 24 h. After incubation, OD was taken at 600 nm with the help of spectrophotometer.

Bacterial growth curves

For the isolated bacterial strain, the growth curve was formed in the presence and absence of arsenic. Autoclaved N- broth containing 100 μg/ml arsenite in 250 ml flasks was used as a stress medium. The broth was inoculated with I ml overnight bacterial culture and incubated on shaker at 37 °C. Then, 2 ml of aliquots were taken at a regular time interval (4, 8, 12, 16, 20, 24 h) from both stress and non- stress cultures. The OD was measured at 600 nm.

Bioremediation assay

The bioremediation ability of the bacterium was determined at different temperatures, pH and arsenic concentrations. For this purpose, 5 ml of autoclaved N-broth was taken in test tubes and inoculated with overnight bacterial culture. Then, these tubes were placed in a shaking incubator for 96 h. After 2 days, 1 ml broth culture was taken and centrifuged at 5000 rpm for 5 min. Then supernatant was taken and checked for arsenite concentration by Safranin O dye method. After 4 days of incubation, similar process was repeated. Finally, supernatant was used for arsenite determination by the spectrophotometer process. The As^{+3} oxidation potential of bacterium was determined at 25, 30, 37 and 42 °C after 48 and 96 h. Likewise, As^{+3} oxidation ability of strain A6 was checked at pH (3, 5, 7 and 9). The As^{+3} oxidation potential *B. licheniformis* A6 was determined at 100, 300, 500 and 1000 μg/ml. Each experiment was done in triplicate.

Arsenic estimation

Arsenic was estimated by a spectrophotometric method using the safranin O dye (Pasha and Narayana 2008). In this method, a sample (1 ml) containing As was taken into a 10 ml tube and then added KIO_3 (1 ml) solution followed by 1 M HCl. The mixture was gently shaken till the yellow color appeared and then added 0.5 ml of Safranin dye solution. Then the mixture was shaken for 2 min, and added 2 ml of acetate buffer to maintain pH 4, and diluted up to 100 ml by adding distilled water. Then 1 ml of the mixture was taken into the cuvettes, and OD was determined by a spectrophotometer at 532 nm.

Effect of sodium chloride on bacteria

The bacterium was cultured in the presence of As 100 μg/ml and NaCl (0–500 mM). NaCl was added in LB-broth according to above mentioned concentration, and OD was determined by spectrophotometer at 532 nm.

Multiple metal resistances

Multiple metal resistance of the given isolate was checked against different metal ions. The following metals were used Cr, Mn, Pb, Se, Co, Cd, Zn and Hg in the given concentrations 100, 500, 1000 μg/ml, up to 5000 μg/ml. Nutrient-agar plates supplemented with these metals were set, streaked the bacteria and incubated at 37 °C for 24 h.

Antibiotic Resistance

The given bacteria were screened for its resistance against nine regularly used antibiotics disks. Norfloxacin (30 μg), Imipenem (10 μg), amoxicillin/clavulanic acid (2:1), tetracycline (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg) and nalidixic acid (30 μg) were used. Muller Hinton-agar plates were prepared with the lawn of bacteria, and then placed the disks of antibiotics onto the MH-agar plates. Results were observed by the growth of bacterial strain on agar plates containing antibiotic disk after 24 h of incubation at 37°C.

Statistical analysis

All the experiments were performed in triplicates. Also mean and standard error was calculated for all the experiments.

Results and discussion

Isolation and metal resistance

Physico-chemical characteristics of the wastewater samples determined were color, temperature and pH. Color

of the wastewater was dark gray. The temperature and pH ranged between 25 and 32 °C and 6–7.4, respectively. Initially, 37 isolates were isolated from 3 different wastewater samples, but on the basis of minimum inhibitory concentration regarding arsenic 9 strains were selected named as (IT6, A6, S1, S4, S9, S12, P6, A15$_a$ and A15$_b$) which were resistant more than 500 μg/ml arsenic. While the other strains showed low MICs in the range of (100–400 μg/ml). While in the nine selected strains, strain A6 was selected because of higher MIC which was 3000 μg/ml and strong arsenic oxidizing ability.

B. licheniformis was found resistant against arsenite upto 3000 μg/ml. The bacterium also showed resistance against multiple metals ion, Cr (3000 μg/ml), Hg (50 μg/ml), Mn (1000 μg/ml), Se (4000 μg/ml), Pb (500 μg/ml), Co (100 μg/ml), Cd (70 μg/ml) and against Zn the resistance was (100 μg/ml). In the present investigation, nine antibiotics disks were used, against the arsenic resistant bacterial strain. The strain A6 showed weak resistance against cefuroxime sodium and nalidixic acid but A6 showed strong resistance against norfloxacin, amikacin, imipenem, tetracycline and ciprofloxacin. The isolated strain was sensitive against amoxicillin/clavulanic acid (2:1) 30 μg and ceftriaxone 30 μg disks.

The diversity of arsenic resistance bacteria is high in such an environment where arsenic level is high or medium (Cai et al. 2009). In one of the studies, 12 strains of bacteria with an arsenite MICs greater than 20 mM were obtained from the high arsenic contaminated soil sample (Cai et al. 2009). Selective pressure due to high arsenic concentration leads to low diversity of metal resistant organisms (Achour-Rokbani et al. 2007; Jackson et al. 2005). Microorganisms take some time to build resistance against metals (Pennanen et al. 1996). Turpeinen also ascertained that a variety of arsenic resistance bacteria is high in complex contaminated soil samples from different metals compared to dry soil (Turpeinen et al. 2004).

In the present study, cross metal resistance of bacterium was checked against heavy metals i.e., chromium, zinc, manganese, and it was found that strain AS6 has resistance against most of the metals. In one of the other study, it was found that the bacteria named as *B. safensis* MS11 has high resistance regarding arsenite and arsenate (40, 400 mM) and for boron (200 mM) along with the high concentration of salt 15% in LB-medium. *B. safensis* MS11 also has resistance against Cd, Cr, Cu, Ni, Pb and Zn (Raja and Omine 2012). *B. safensis* can be used in decontamination of soil having high salt concentration for the removal of arsenic. The arsenic resistant bacteria belong to genera (*Actinobacteria, Microbacterium, Pseudomonas* and *Rhizobium)*, apart from arsenic other metals resistance mechanisms are also found in these genera (Paul et al. 2014).

Characterization of metal resistant bacterial strain

The bacterial colony shape was circular, medium size and color was light white, margin was entire, elevation was flat and transparency was opaque, and texture was smooth. The organism was gram positive rod, catalase negative and oxidation test was positive (Table 1). The 16S rRNA gene was sequenced partially and submitted to NCBI to check the similarity and the blast query indicated that this gene has highest homology to the *Bacillus licheniformis*. The nucleotide sequences were deposited to Genbank in FASTA format under the accession number of KX 785,171.

Arsenic presence in the industrial wastewater is lethal for human beings as well as for life in water bodies and terrestrial environments (Mateos et al. 2006). These kinds of heavy metal can easily be detoxified by the way of bioremediation. The biggest problem of arsenic is its carcinogenic effect due to its toxicity (Shakoori et al. 2010). In many studies, mostly the arsenic oxidizing bacteria were recognized from *Acinetobacter, Comamonas, Pseudomonas, Stenotrophomonas, Delftia, Agrobacterium* and *Bacillus* as the major genera (Cai et al. 2009).

The optimum pH for the isolated *B. lichenifromis* was 7 while optimum temperature was 37 °C. The growth curves were studied under As-stress and non-stress condition. In the presence of As, the lag phase of the given strain was

Table 1 Morphological and biochemical characteristics of *Bacillus licheniformis*

| Morphological characteristics	*Bacillus licheniformis*
Motility	+ ve
Shape	Rod
Texture	Smooth
Color	Light white
Elevation	Flat
Size	2 μm
Margin	Entire
Transparency	Opaque
Gram's staining	Gram positive
Spore staining	Spore former
Capsular staining	+ ve
Acid fast staining	− ve
Biochemical characteristics	
Indole production	− ve
Methl red reaction	− ve
Voges–Proskauer reaction	+ ve
Citrate use	+ ve
Catalase activity	+ ve
Oxidase activity	+ ve
Triple sugar iron test	+ ve

+ *ve* Positive, − *ve* negative
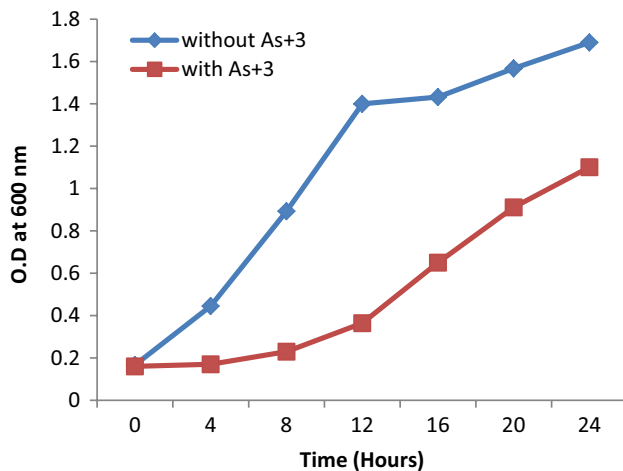
Fig. 1 Growth pattern of *B. licheniformis* A6 in the absence and presence of arsenite (100 µg/ml) in N-broth at optimum conditions

Fig. 2 Brown precipitates (right plate) showing the arsenite oxidizing ability of *B. licheniformis* A6. Left plate represents control (with out arsenite)

extended for a bit of time (Fig. 1). Isolated strain was grown in arsenic presence (100 µg/ml) and NaCl (0–500 mM). It was interpreted that NaCl concentration was inversely proportional to the growth of bacteria after a certain limit. Maximum growth was observed at concentration of 100 mM NaCl and minimum when the NaCl was 500 mM.

Arsenite oxidizing potential of the bacterial strain

The brownish precipitate appearance after the use of 0.1 M AgNO$_3$ on the streak growth plates indicates that the isolated strain has arsenic oxidation potential. The AgNO$_3$ reaction depends upon the arsenic ion either arsenite or arsenate. While the reaction among AgNO$_3$ and As (V) generate yellow precipitate (Fig. 2).

Arsenic bioremediation was determined at 25, 30, 37 and 42 °C with the 100 µg/ml. As^{+3} concentration after 48 and 96 h. Strain A6 showed maximum arsenite oxidation potential 86% after 48 and 98% after 96 h at 37 °C. At 25°C, 56% bioremediation activity was observed while at 30°C, 75% and 42 °C, it was 75 and 17%, respectively. After 96 h incubation, the bacterium showed 64, 83 and 23%. As^{+3} oxidation potential at 25, 30, 37 and 42 °C, respectively (Fig. 3a).

As^{+3} oxidation potential was determined at pH 3, 5, 7 and 9 with 100 µg As/ml after 48 and 96 h, Strain A6 had As^{+3} oxidation potential of 86 and 98% after 48 and 96 h at pH 7. At pH 3 the strain showed 9% oxidation potential, at pH 5, 71%, while at pH 9 the oxidation ability was about 82%. The oxidation potential of the bacterium A6 was 14% (pH3), 85% (pH5), 98% (pH7) and 92% (pH9) after 96 h incubation at 37 °C (Fig. 3b).

As^{+3} oxidation potential of the bacterium was also checked at various arsenite concentrations i.e., 100, 300, 500 and 1000 µg/ml. After 48 and 96 h. Strain A6 had As^{+3}

oxidation potential of 86% after 48 h and 98% after 96 h at 100 µg/ml. At concentration 300 µg/ml, *B. licheniformis* showed oxidation ability of 81%, at 500 µg /ml, 56% and at 1000 µg/ ml, the oxidation ability was 19% after 48 h of incubation. The oxidation ability of the bacterium after 96 h of incubation was 98% (100 µg/ml), 86% (300 µg/ml), 56% (500 µg/ml) and 30% (1000 µg/ml) (Fig. 3c).

Bacteria and other organisms play a very important role in different biogeochemical cycles including arsenic cycle, to convert different metal ions into their different oxidation states having different solubility, mobility and toxicity (Silver and Phung 2005). Microorganisms like bacteria, fungi, ciliates, algae, mosses, macrophytes used different ways for detoxification or removal of metals from different environments (Volesky and Holan 1995). Gadd (1990) and Lovley and Coates (1997) reported that microorganisms response to metals present in them by many ways such as Bio sorption of metals, Adsorption, interaction of metal with the molecules in cytosol, entrance into the capsule of cell, precipitation, oxidation of metal (arsenite into arsenate), reduction of metal (arsenate into arsenite) as well as protein-DNA adduct formation (Zhitkovich and Costa 1992) and production of some protein due to metal stress (Ballatori 2002).

The highest arsenic oxidizing bacteria are *Pseudomonas*, *Agrobacterium* and *Corynebacterium glutamicum,* the later one is industrially important for the amino acid manufacturing having MIC (up to 12 mM arsenite and > 400 mM arsenite) (Lovley and Coates 1997). In the present study, the strain A6 showed resistance against As up to 40 mM.

In one of the studies, it was examined that the arsenite oxidation potential was 92% for *B. cereus* and 88% for *A. junii* after 6 days of incubation in industrial wastewater (Naureen and Rehman 2016). While the above isolated strain *Bacillus licheniformis* showed arsenite oxidation potential 86 and 98% after 48 and 96 h, respectively. Due to its strong
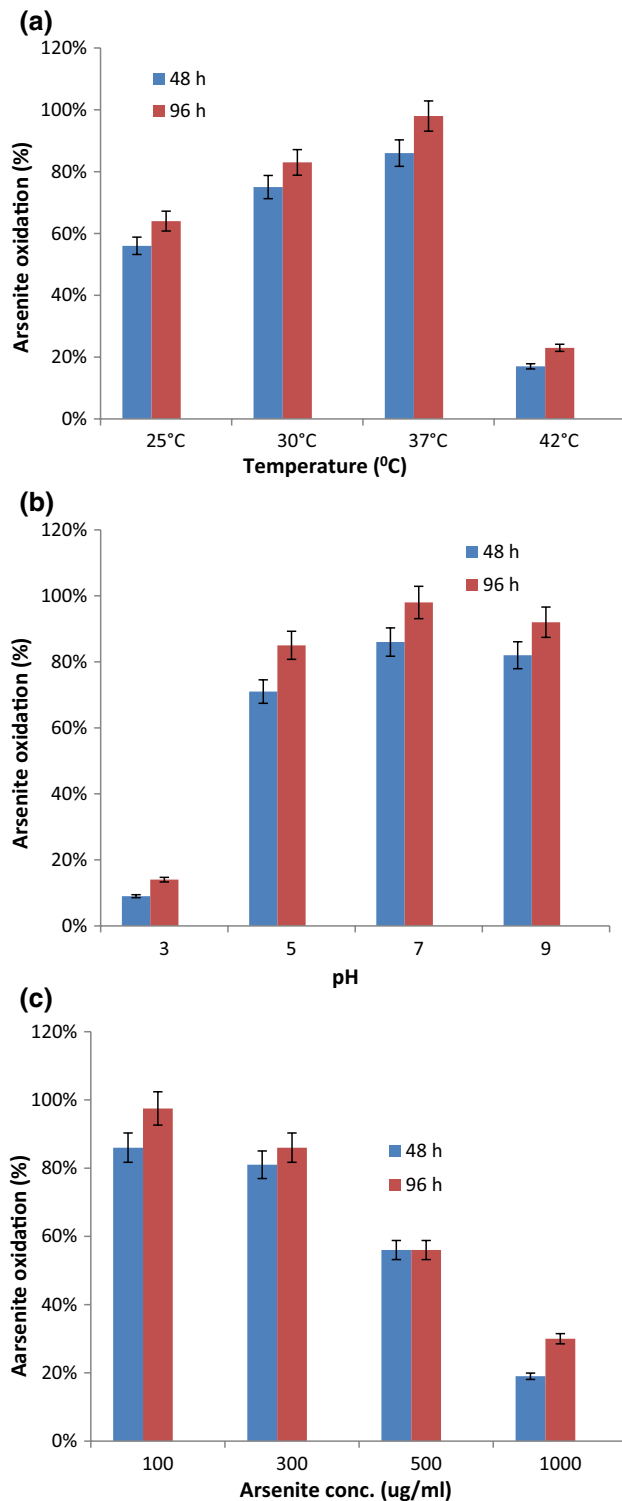
Conclusion

In the present investigation, bacterial strain, isolated from industrial wastewater, showed MIC value upto 40 mM against arsenite. The bacterium was also able to resist other toxic metal ions e.g., Cd, Cr, Mn, Zn, Se, Hg, Pb, Co. The arsenite oxidizing potential of *B. licheniformis* was 86 and 98% after 48 and 96 h at pH 7. This bacterial strain could be used to treat the industrial waste to ameliorate the toxic metal ions. For practical use, further research work is required to investigate arsenite oxidizing potential of this bacterial strain with experiments using original industrial wastewater in batch and continuous bioreactors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The current study does not contain experiments involving animals/Humans.

Fig. 3 Arsenite oxidation potential of *B. licheniformis* A6 at temperature (**a**), pH (**b**) and arsenite concentration (**c**)

oxidation potential, this isolated strain can be used to purify wastewater containing As and other heavy metals containing wastewater.

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