



## Original article

## MALDI-TOF-MS and 16S rRNA characterization of lead tolerant metallophilic bacteria isolated from saffron soils of Kashmir for their sequestration potential

Baba uqab<sup>a</sup>, Ruqeya Nazir<sup>b,\*\*</sup>, Bashir Ahmad Ganai<sup>b</sup>, Praveen Rahi<sup>c</sup>, Sabeedah Rehman<sup>b</sup>, Saleem Farooq<sup>a</sup>, Rubiya Dar<sup>b</sup>, Javid A. Parray<sup>b,d</sup>, Al-Bandari Fahad Al-Arjani Al-Arjani<sup>e</sup>, Baby Tabassum<sup>f</sup>, Elsayed Fathi Abd\_Allah<sup>g,\*</sup>

<sup>a</sup> Department of Environmental Science, University of Kashmir, 190006 Jammu & Kashmir, India

<sup>b</sup> Centre of Research for Development (CORD), University of Kashmir, 190006 Jammu & Kashmir, India

<sup>c</sup> National Centre For Microbial Research (NCMR), Pune, India

<sup>d</sup> Govt SAM Degree College Budgam, JK 191111, India

<sup>e</sup> Botany and Microbiology Department, College of Science, King Saud University, P.O. Box. 2460, Riyadh 11451, Saudi Arabia

<sup>f</sup> Toxicology Laboratory, Department of Zoology, Govt. Raza P.G. College Rampur, 244901 U.P., India

<sup>g</sup> Plant Production Department, College of Food and Agricultural Sciences, King Saud University, P.O. Box. 2460, Riyadh 11451, Saudi Arabia



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## ABSTRACT

Toxic metal contamination in soils due industrialization is nowadays a concern to the scientists worldwide. The current study deals with the evaluation of response and tolerance by isolated metallophilic bacteria in different lead concentrations (100 ppm to 1000 ppm). By taking optical densities of the isolates, the minimum inhibitory concentration (MIC) of Pb<sup>2+</sup> were determined. 16S rRNA and MALDI-TOF MS were used for the identification of the bacteria. Total of 37 isolates were observed, among them 04 (*Staphylococcus equorum*, *Staphylococcus warneri*, *Bacillus safensis* and *Bacillus thuringiensis*), isolated were detected having efficacy of Pb<sup>2+</sup> tolerance and sequestration at varying MIC. Furthermore, *B. thuringiensis* was observed to have highest (900 ppm) tolerance for lead and lowest (500 ppm) for *Staphylococcus warneri*. Moreover, the highest (65.3%) sequestration potential has been observed for *B. thuringiensis* and least (52.8%) for *S. warneri*. The tolerance and sequestration potential properties of these isolated species can be utilised to exterminate heavy metals and reduce their toxicity from the contaminated environment.

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## 1. Introduction

Developing countries around the globe are facing the problem of the heavy metal pollution. Heavy metal contamination in the environment is attributed to industrial processes and technologies (Dynarski and Houlton, 2018; Parray et al., 2020). The non-degradable and persistent nature of these metals poses a serious

threat to human health and accumulation of these metals at different levels of the food chain also adds threat to humans and other organisms. Heavy metals cause significant environmental problems in soil and water by their presence, which is further aggravated by different anthropogenic activities (Förstner and Wittmann, 2012). Bacterial heavy metal resistance results from main contacts in the setting with natural metals again linked to intensive human interference and natural resource exploitation (Noreen et al., 2019). Of the different metals and metalloids occurring in nature, lead (Pb) being toxic even at minute concentrations is regarded to be one of the most toxic pollutant with primary sources from metal smelting industries (Chen et al., 2009) and manufacturing of insecticides or plumbing pipes (Oves et al., 2013). Some natural processes like those of soil erosion, volcanic emissions and mineral mobilization also cause environmental contamination of lead (Parray et al., 2020). Many environmental risks have direct relationship with lead toxicity that include nucleic acid

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [ruqeya.ku@gmail.com](mailto:ruqeya.ku@gmail.com) (R. Nazir), [efabdallah@gmail.com](mailto:efabdallah@gmail.com) (E. Fathi Abd\_Allah).

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modifications, conformation of proteins, disruption in activity of enzymes, cell function interference as well as alterations in oxidative phosphorylation and osmotic balance. In addition,  $Pb^{2+}$  is more closely related to thiol and oxygen groups relative to vital metals such as calcium and zinc (Hashim et al., 2018). Despite its elevated toxicity, however, many microorganisms have acquired survival mechanisms like metal and antibiotic efflux, intracellular bioaccumulation, precipitation, extracellular sequestration and biosorption (Jarosławiecka and Piotrowska-Seget, 2014; Dynarski and Houlton, 2018; Uqab et al., 2016) for their survival. The environment of Kashmir valley is most suitable for the growth and propagation of saffron. Saffron produced here is known to be the finest qualities all over the world (Jan 2009). The major concern of the state is gradual decline in the average yield of saffron in Kashmir from last few decades. According to Husaini et al., (2010), pollution is the greatest threat for the quality and quantity of saffron. Saffron soil of Kashmir is surrounded by large number of cement industries due to which the land is under tremendous pressure of heavy metals. This provides us the opportunity to isolate the heavy metals resistant bacteria for evaluation of their sequestration potential.

## 2. Materials and methods

### 2.1. Sites description

The saffron plateaus of Pampore located in district Pulwama of Jammu and Kashmir were selected for collection of soil samples, the reason being that the vicinity of the said area is dominated by a large number of cement industries, which has resulted in elevated level of heavy metal accumulation. Three different locations (Fig. 1) determined in this area according to the degree of influence were selected for the collection of composite soil sample taken at a depth of 15 cm to undertake the characterization work of the heavy metal (Pb) resistant bacteria.

### 2.2. Processing of soil samples

Soil samples were seasonally collected from the chosen locations for 2 ½ years. Replicates of the soil samples were obtained from different locations in equal proportions (at 10 g) thoroughly mixed and pooled for isolation of lead resistant bacteria using standard methodology (Jiang et al., 2008; APHA, 2005).

### 2.3. Bacterial isolation and enumeration

Bacterial isolation from the soil samples was done using serial dilution technique accompanied by immediate direct plate technique (Chen et al., 2005) and pure culture technique (Olofsson et al., 2007). Inoculums of 0.1 ml was then dispersed from dilution tubes (Agarwal et al., 2014) on petri plates containing nutrient agar and were incubated for 24 h in an incubator at a temperature of 37 °C (APHA, 2005). For the viable count of bacteria, the calculation of colony forming units (CFU / g) per gram of soil was used.

## 3. Identification of the isolated species

### 3.1. Morphological approach

For the identification of bacteria, grams staining procedure was applied using direct microscopy (at 100× Olympus IX 71 fluorescence microscope). For studying the macro and micro morphological features of bacteria, media plates were subjected to incubation at 37 °C for 1–3 days. The plates were then observed for different culture parameters like size, shape, margin, elevation, surface texture, opacity and colony color on media plates.

### 3.2. MALDI-TOF MS and molecular approach

MALDI-TOF-MS (Matrix- assisted laser desorption ionization time-of- flight mass spectrometry) technique was used for identi-

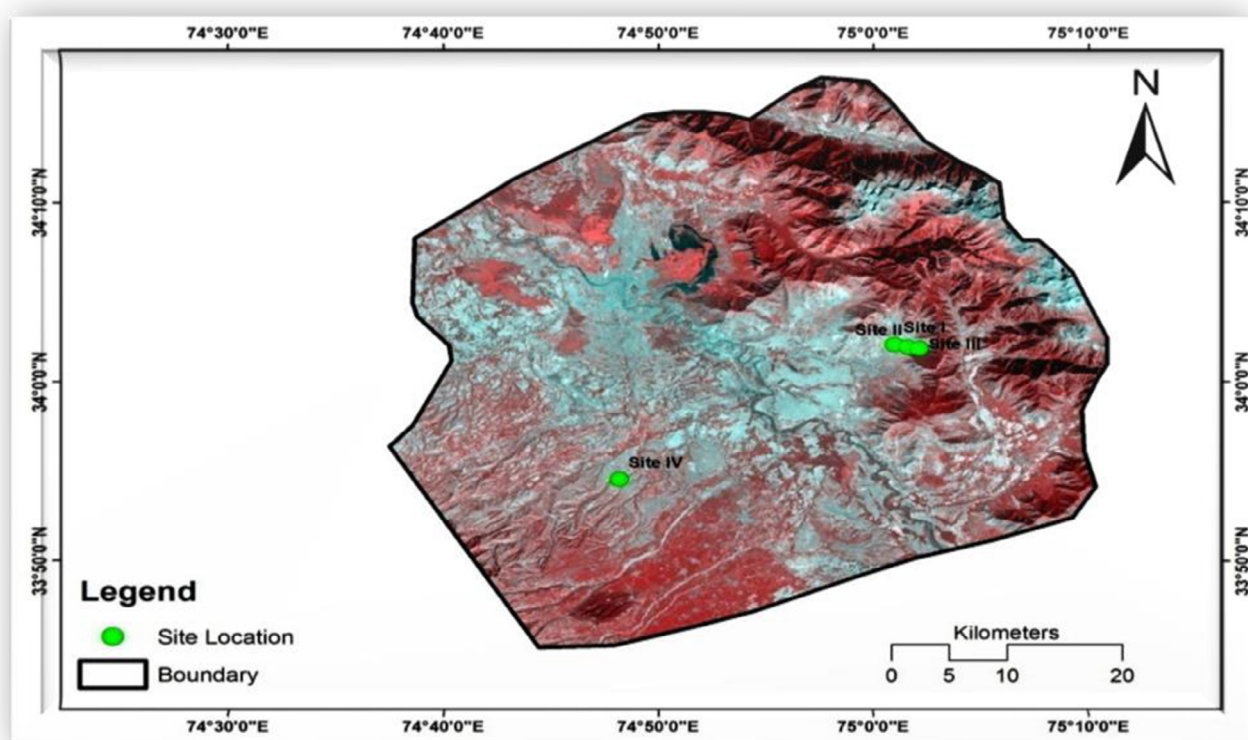


Fig. 1. Geographical positions of sampling sites.

fication of bacteria using Ethanol-forming acid extraction method (Rahi et al., 2016). DNA extraction and purification was done using GenElute bacterial Genomic DNA kit (Sigma, USA) followed by amplification of 16S rRNA gene using universal bacterial primers (27F) 5'-AGAGTTGATCTGGCTCAG-3' and (1429R) 5'-GGTACCTT GTTACGACTT-3' (Chatterjee et al., 2012). Amplification was carried out in a thermal cycler (Eppendorf, Germany), which was operated at 95 °C for 5 min followed by 30 cycles of amplification at 94 °C for 60 s, 57 °C for 60 s, and 72 °C for 90 s and a final extension at 72 °C for 5 min. Running an aliquot on 1% agarose tested the quality and quantity of the amplified products. PCR products obtained were sequenced by Sanger method (AgriGenome, Kerala and NCMR, Pune) and the sequences thus obtained were further evaluated by BLAST analysis to infer their taxonomic relatedness. Further, the phylogenetic analysis of the sequences was inferred by constructing a phylogenetic tree based on Neighbor Joining by using Tamura-Nei model in MEGA 7 (Tamura and Nei, 1993) (Fig. 2).

### 3.3. Evaluation of heavy metal tolerance

Metal tolerance was determined by preparing the stock solution (1000 ppm) of lead acetate, (Himedia) filtered through 0.45 µm

pore size syringe filter to remove any bacterial contamination. From the stock solution, different concentrations were prepared to determine the MIC according to the available protocols (Wiegand et al., 2008) using Muller Hinton Broth. Before the broth dilution, all the isolates were re-cultured and put in 1X PBS solution to maintain McFarland standard of 1 which comprises of  $3.0 \times 10^8$  colonies (McFarland, 1907) and the lowest growth inhibiting concentration was taken as MIC.

### 3.4. Sequestration potential of metal tolerant isolates.

Sequestration potential of lead tolerant isolates was performed using the standard methods (Shin et al., 2012; Alboghobeish et al., 2014) and analysis of heavy metal according to standard methods given by APHA (2005). For determining the metal sequestration Muller Hinton Broth (with 100 ppm lead) was placed in shaking incubator (180 rpm) at 37° C for 48 h. A sample of 5 ml was taken from the flask regularly after every 6 h and centrifuged at 10,000 rpm for 5 min. Nitric acid and sulphuric acid was used for acid digestion of the supernatant and concentration of the metal in percentages was determined using Atomic Absorption Spectrometry (AAS).

## 4. Results

### 4.1. Recovery of the bacteria

Digital colony counter enumerated the bacterial colonies established on the plates and evaluated the bacterial load in terms of colony forming units (CFU / g). Compared to the winter seasons the bacterial load was found to be higher in the summer seasons. The overall increase in bacterial load in successive years of study was higher in all seasons of the second year of study compared to the first year of study. The CFU/g in the three sampling stations varied between  $0.8 \times 10^4$  to  $9.5 \times 10^4$  CFU/g. The percentage of Gram negative out of total 37 bacterial isolates was 35.5% while for Gram positive the percentage was 64.5%. The taxonomical classification of the isolated bacteria revealed that family Bacillaceae

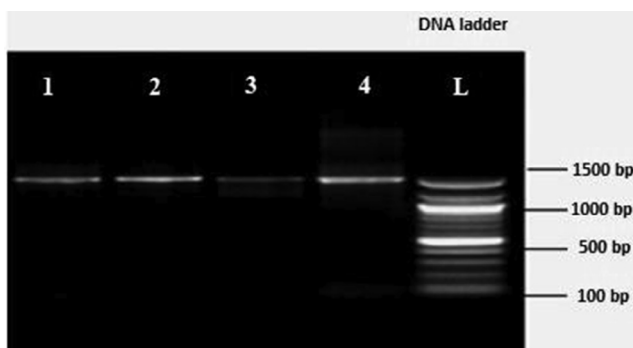


Fig. 2. Image of amplified 16s-rDNA region of DNA of lead tolerant species.

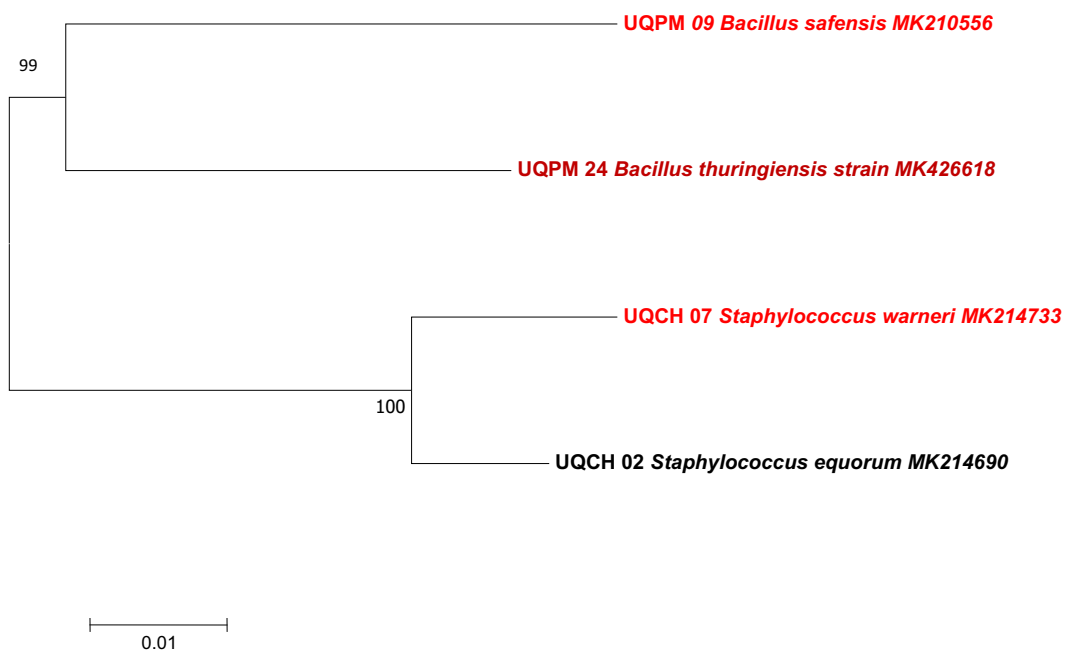


Fig. 3. Phylogenetic tree constructed from 16S rRNA gene sequences. The tree was constructed by Neighbor joining method in MEGA 7.

was most dominant (43.24%) followed by the representation of family Enterobacteriaceae (21.62%) and Pseudomonadaceae (5.40%), Staphylococeaceae (5.40%), Planococcaceae (8.10%), Microbacteriaceae (8.10%) and Aeromonadaceae (2.70%). Four bacterial species were tolerant to lead and were identified by MALDI-TOF-MS and 16 s rRNA gene sequencing which include be *Staphylococcus equorum* (MK214690) *Staphylococcus warneri* (MK214733), *Bacillus safensis* (MK210556) and *Bacillus thuringiensis* (MK426618) (Fig. 3). Among the four metal tolerant species *Bacillus safensis* was the only gram negative strain with rest of them as gram positive.

#### 4.2. Maldi-tof ms

Library and software of Bruker Biotype was used (version 2.0,3740 entries) and all lead resistant isolates were analyzed using a linear MALDI-TOF-MS mass spectrometer (Bruker Daltonics, Germany). Criteria score used for the identification used was same as recommended by the manufacturer Isolates with score of  $\geq 2.000$  were identified up to species level while as isolates with score between 1.700 and 1.999 were identified upto genus level. The MS spectra generated (Fig. 4) and protein extracted identified the isolates as *Staphylococcus equorum*, *Staphylococcus warneri*, *Bacillus safensis* and *Bacillus thuringiensis*.

#### 4.3. Metal tolerance

The tolerance of all isolates against lead and their subjective minimum inhibitory concentration (Fig. 5.) determined for the tolerant isolates showed that *B. thuringiensis* has the highest tolerance limit of 900 ppm of lead followed by *Staphylococcus equorum* with a tolerance level that of 700 ppm, *Bacillus safensis* with a tolerance limit of 600 ppm and *Staphylococcus warneri* with a tolerance limit of 500 ppm.

#### 4.4. Sequestration potential

The sequestration potential of all the lead tolerant isolates (Fig. 6) varied with respect to each isolate after 48 h and among

the lead tolerant species. *B. thuringiensis* showed highest sequestration potential of 65.3%, while as *S. warneri* showed the least sequestration potential of 52.8%.

## 5. Discussion

Due to the extreme importance of microorganisms in energy and material transformation, microbial diversity has become an important issue (Vos et al., 2013 Rangasamy et al. 2018a). Knowledge of the structure and diversity of bacterial communities is vital for recognizing the association between environmental factors and ecosystem functions (Parray et al., 2015). Such knowledge may be used to measure the impact on ecosystems of environmental stress and anomalies like pollution, agricultural exploitation, and climate change (McGuire and Treseder, 2010). Soil harbors various microbial communities and their structure varies greatly between distinct settings (Macdonald and Singh, 2014). In determining soil functions like carbon turnover, nitrogen mineralization (N) and pest, microbial communities play a significant role (Dynarski and Houlton, 2018). In this study, a maximum of 37 bacterial species were isolated from saffron fields, the existence of such varied species in the soil can be ascribed to the existence of soil nutrients that promotes growth circumstances for microbes in the soil (Pommier et al., 2018). The microbial isolates were firstly morphologically identified followed by MALDI TOF and 16S sequence. Morphological identification enables to comprehend certain characteristics of isolates, but at the same time these phenotypic profiles, colony morphologies, gram stain outcomes, metabolic characteristics and growth requirements are not static and may alter with pressure and evolution (Parray et al., 2013). Hence, use of more reliable and up to date methods (MALDI TOF and 16S sequence) was adopted.

Heavy metal pollution in recent years has become a Global problem, as heavy metals unlike other pollutants, are non-biodegradable and bioaccumulate in the system (Macdonald and Singh, 2014). In many microorganisms metal resistance is a common process which posses different mechanisms like extended delay stage, for adapting to heavy metal ions (Rangasamy et al., 2018b). In metal contaminated environments, heavy metal -

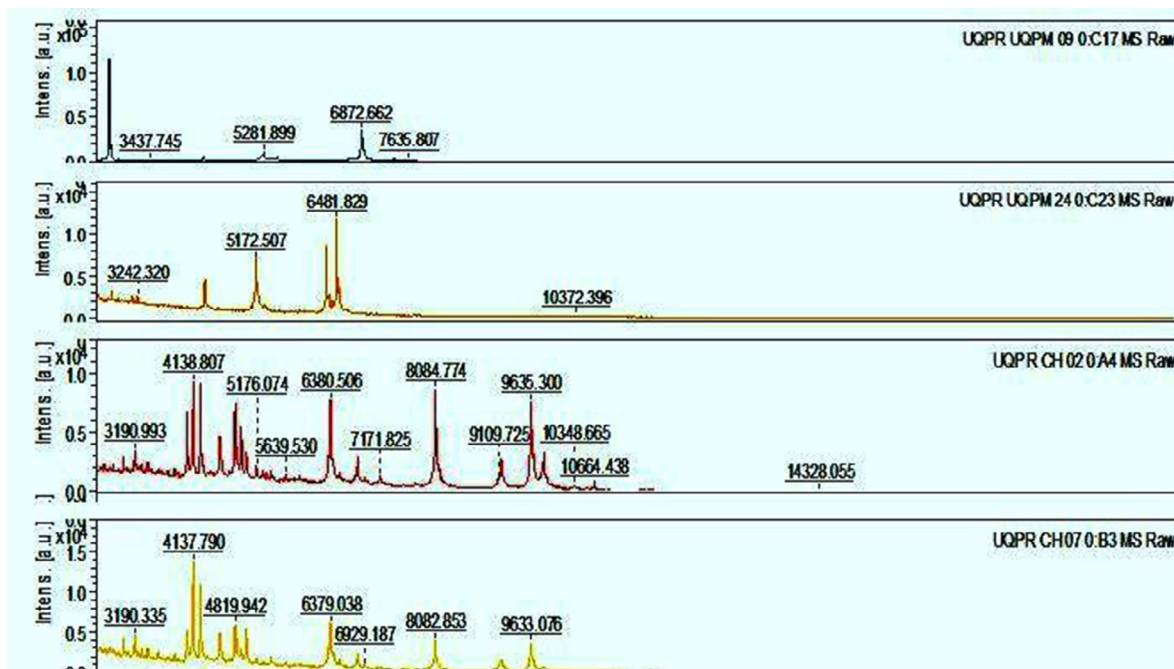


Fig. 4. MALDI-TOF MS spectra of lead tolerant isolates.

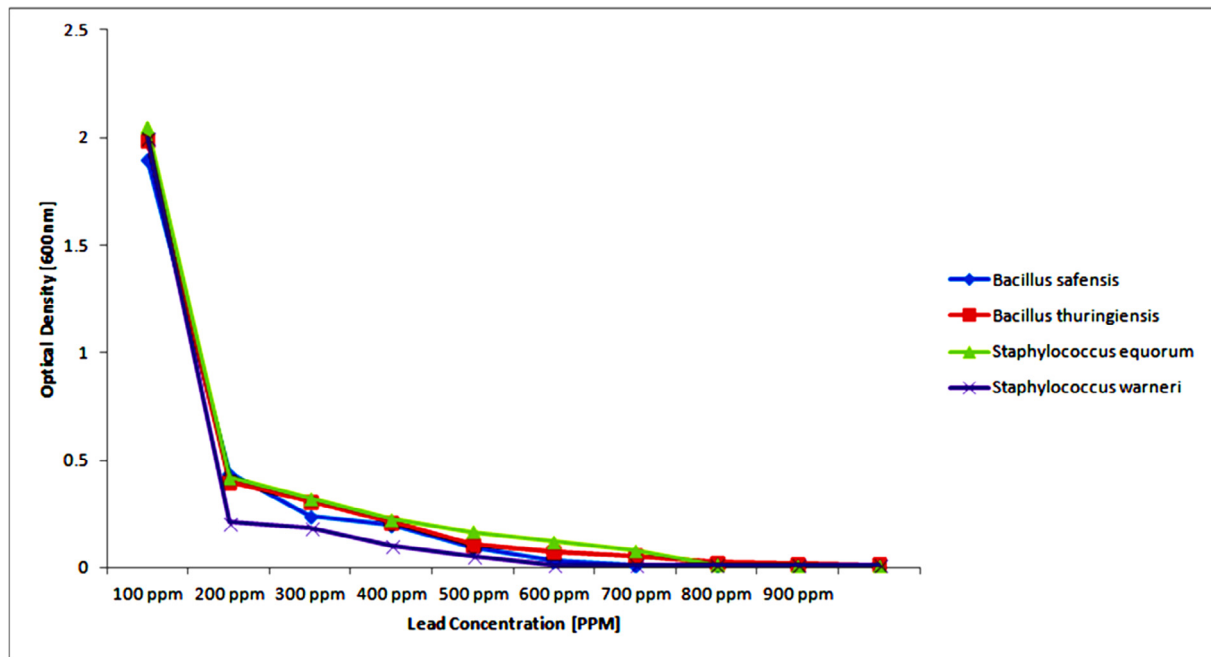


Fig. 5. Optical density (OD) values showing MICs of lead tolerant isolates.

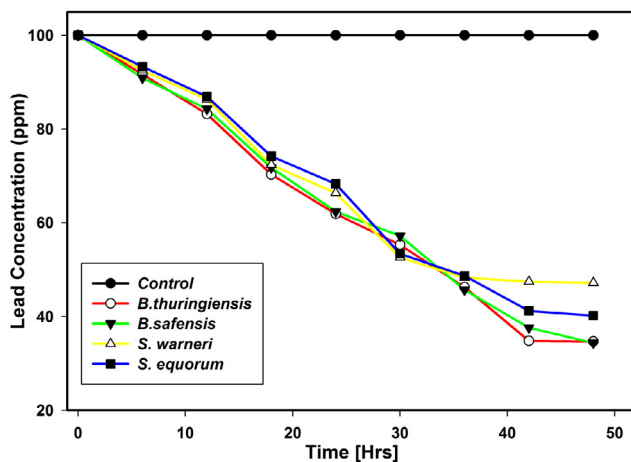


Fig. 6. Sequestration potential of metal tolerant isolates for lead in ppm.

resistant microbial species occur naturally and these metals generate selection pressures to enable this proliferation and survival (Rahman and Singh, 2018). Saffron soils in pampore area of Kashmir, that are under a serious impact of heavy metals because of the existence of many cement industries along with the intensive use of fertilizers (Husaini et al. 2010). We isolated four lead tolerant bacteria including *Staphylococcus equorum*, *Staphylococcus warneri*, *Bacillus safensis* and *Bacillus thuringiensis* considered as “metallophiles” attributed to their production of Metallothioneins (MTs) concerned in protecting these cells from toxic metals as reported by (Blindauer, 2011). Metallothioneins (MTs) which safeguard cells against toxic metals are often linked to as proteins, but they play a vital role in zinc homeostasis (Blindauer, 2011). The presence of *smt* genes and MT biosynthesis was also reported by (Naik et al., 2012) in *Streptomyces* sp., *Bacillus cereus*, *Salmonella choleraesuis* 4A and *Proteus penneri* GM-10 in presence of  $Pb^{2+}$ . In

addition, it was suggested that a metallothioneine-like protein bind  $Pb^{2+}$  in *Bacillus megaterium* (Arora et al., 2017) and it could be referred to the present study. Microorganism present in soil and water has the ability to produce volatile compounds that are less toxic from organic and inorganic leading compounds (Kohli et al., 2019, Abd\_Allah et al., 2019) and it can be attributed to the present study. For the proper growth in presence of metals these species may have created a protective system as an additional component in relation to cytochrome c in the ultimate part of the respiratory chain, which is not inactivated by the metal (Wang et al., 2010; Lima e Silva et al., 2012). Lead resistance in the microorganisms is also acquired by limiting their movement across the cell envelope (Rahman et al., 2019). The cell wall acts as natural obstacle to  $Pb^{2+}$ , as several important macromolecules bind this metal. In case of the gram negative bacteria this important role is played by lipopolysaccharide, while as in Gram positive bacteria peptidoglyca is responsible for binding Pb with teichoic and teichuronic acids (Parray et al., 2015). This can be the feasible mechanism for the lead tolerance in gram +ve and gram -ve bacterial species in our study. Synthesis of extracellular polymers (EPs) is a characteristic feature of many microorganisms that bind toxic metal cations and thus protect delicate and essential cellular components (Guibaud et al., 2003). The EPs composition is very complex that includes polysaccharides, proteins, nucleic acids and humic acid, which has the property of chelation of metals with diverse affinity and specificity (Hashem et al., 2016) and same can be attribute to this study.

Sequestration potential of the isolates clearly indicated that *B. thuringiensis* showed highest sequestration potential of 65.3% after 48 h, while as *S. warneri* showed least sequestration potential of 52.8%. This sequestration capacity can be ascribed to a number of anions recognized to respond with  $Pb^{2+}$ , including phosphates, chlorides and hydroxyl ions to create non soluble precipitates. To decrease the free  $Pb^{2+}$  concentrations several microorganisms use  $Pb^{2+}$  precipitation by sequestering it outside and inside the cell in the form of phosphate salts. In addition, intracellular lead mechanisms have been grouped into three phases viz., binding,

metabolism and precipitation of lead phosphates (Naik et al., 2018). Such protection mechanism allows bacterial strains to withstand more than 600 times in  $Pb^{2+}$  dose when compared to a sensitive strain (Herr et al., 2018). Thus lead resistant property of the isolated species can be beneficial for detoxifying and eliminating lead from affected sites. Bacteria such as *Arthrobacter sp.*, *P. marginalis*, *B. megaterium*, *S. aureus* and *C. freundii* are also reported to be lead resistant (Sun and Shao, 2007; Hashim et al., 2018; Parray and Shameem 2019; Hashem et al., 2019). With regards to *Bacillus safensis*, in this study, it is the first report of lead tolerance, hence for this bacterial species a metabolism of resistance to heavy metals must be taken up for future studies.

## 6. Conclusion

MALDI-TOF-MS and 16s rRNA sequencing identified four lead tolerant bacterial species *S. equorum*, *S. warneri*, *B. safensis*, and *B. thuringiensis*. The highest (65.3%), sequestration potential has been observed for *B. thuringiensis* and least (52.8%) for *S. warneri*. The primary conclusion depicts that lead tolerant bacteria isolated from saffron soils of Kashmir Valley significantly highlights their sequestration role. Furthermore, lead resistant property of these isolates can be beneficial for detoxifying and eliminating heavy metals from the Lead contaminated soils, there by providing viable option for bioremediation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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