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Biosorption and Sequestration of Lead by Stutzerimonas Stutzeri Strain Isolated from Industrial Effluent

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KEYWORDS

Lead, Biotransformation; reduction; sequestration; Biosorption.

ABSTRACT:

Background: This study was carried out to isolate and characterize the bacterial strains from industrial effluent for their biosorption and sequestration potential.

Methods: In this study we have done with the enrichment of the cultures for the isolation of bacterial strains, in which the tryptone soya broth (TSB) and mineral salt medium (MSM) supplemented with different concentrations of lead metal. Further, screening was done on the basis of tolerance where turbidity act as an indicator which corelates with the tolerance towards varying concentrations of lead. Selected isolates were studied for their growth kinetics and mass balance profiling studies. AS-3 strain showed the best sorption and sequestration potential that was further subjected to biochemical and molecular characterization including 16S rDNA sequencing was performed and analysis was done. Phylogenetic tree was generated for maximum likelihood using MEGA11 software. One isolate from this study was finally characterized as *Stutzerimonas Stutzeri*.

Results: Out of the 10 isolates, AS-3 exhibited 99% of the sequestration for the metal (a.i. 50 mg/l Pb) among other isolates which accumulated variable amounts of Pb. After attaining the required growth in shake flasks, mass balance studies further confirmed the maximum sequestration. Characterization of AS-3 revealed its phylogenetic relationship with the family Pseudomonadaceae (ɤ-Proteobacteria) and 99% similarity with *Stutzerimonas sp*., a facultative anaerobe.

1. Introduction

The air, water, and soil's suitability for human habitation are all part of a terrible global issue. Soil supports the global ecosystem in addition to yielding food and fibre. Any modifications made to the soil may have an impact on the hydrosphere, biosphere, pedosphere, and atmosphere [1]. Due to the human-caused effects of inadequate treatment, soil pollution with heavy metals (HMs) has recently become a major worldwide ecological trouble. This problem has negative effects on the ecology as well as the community, economics, and health of the species. There are generally two categories of Heavy Metals (HMs) crucial for biological processes: essential and non-essential [2]. Two distinct ecosystems exhibit minimal prerequisites for indispensable elements vital to physiological and biochemical functions. The presence or absence of certain micronutrients, encompassing both excess and deficiency, can unveil various disorders within these biomes. It is noteworthy that for physiological and metabolic functions, ecosystems do not necessitate heavy metals that are either toxic or non-essential [3].

Lead

Lead (Pb), a heavy metal with an atomic number of 82 has been utilized by humans for centuries, leading to its widespread presence in various environments. Lead has historically been utilized in a variety of applications, including paints, pipes, batteries, and gasoline additives. Lead exposure occurs through ingestion, inhalation, and ingestion, disrupting various biological processes and leading to an imbalance in living organisms [16]. Lead, on the other hand, is a serious concern due to the significant environmental pollution it has created. One of the most serious issues with lead is its toxicity to living organisms. Even in low concentrations, lead can be harmful to many living organisms, including humans.

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Regulatory steps, cleanup plans, and advocating for alternative materials in many businesses are all part of the effort to reduce lead pollution. Despite all these efforts, lead pollution remains a significant environmental problem [4]. On a global scale, lead ranks 5th in industrial metal production, following iron (Fe), copper (Cu), aluminium (Al), and zinc (Zn). In 2015, worldwide refined lead production amounted to 10.04 million tons, showing an 8.2% decline from previous years. Subsequently, there was a nearly 3.7% reduction in global refined lead production in 2019 and 2020, equivalent to almost 11.7 million metric tons less compared to 2015 [17]. In terms of reserves, India holds an estimated 2.48 million tons of lead metal, with a total metal content of 13 million tons [8]. Numerous studies on lead exposure have been conducted on several mammalian species, with the results indicating that lead-exposed animals' kidneys showed higher levels of lipid peroxidation. Free radicals quickly interact with lipid electrons in cell membranes, resulting in lipid peroxidation, an oxidative breakdown of lipids that damages cells. Because of their high concentration of long-chain polyunsaturated fatty acids, the kidneys are especially vulnerable to reactive oxygen species (ROS) damage [10]. The WHO's 2022 update on the Public Health Impact of Chemicals: Knowns and Unknowns revealed that over 50% of the 2 million deaths reported in 2019 due to exposure to known chemicals were associated with lead exposure (International Journal of Environmental Health Research 7).

Remediation Approach

Microbial remediation is a potentially successful method for reducing lead pollution in a range of environmental media, including sediments, water, and soil, by utilizing the metabolic processes of microbes. Bacteria and archaea are bacteria capable of transforming, immobilizing, or eliminating lead from the environment via complex metabolic processes. Biosorption, in which microbial cells accumulate lead ions on their surfaces, is a well-known microbial mechanism in lead bioremediation. Some bacteria sp, such as *Pseudomonas*, *Bacillus*, and *Desulfovibrio*, exhibit a high affinity for lead ions due to the presence of functional groups on their cell wall. Lead's potential toxicity is reduced when it binds to these microbial surfaces, lowering the amount of soluble lead in the environment. Microbes also participate in the precipitation process, which makes lead immobile. When sulfate is reduced in the presence of lead ions, sulfate-reducing bacteria such as *Desulfovibrio* and *Clostridium* cause the precipitation of lead sulfide (PbS). The resulting insoluble PbS is less mobile and bioavailable, lowering the risk of lead exposure. Some microorganisms can enzymatically transform soluble lead ions into less toxic ones. Bacteria such as *Shewanella* and *Geobacter* can mediate the reduction of soluble lead ions to elemental lead or lead sulfide to change the chemical state of lead in the environment [18] [13]. It is known that these microbes can create and use a variety of detoxification strategies,including bioaccumulation, biotransformation, biosorption, and biomineralization. Because it happens spontaneously and is affordable, it is a widely acknowledged method of remediation [7]. In metal-stressed environments, bacteria exhibit diverse mechanisms to endure metal ion uptake, ensuring their survival. These mechanisms encompass the accumulation of metal ions in less toxic states, precipitation of metal ion efflux outside the cell, biosorption to cell walls, entrapment in the extracellular capsule, and chemisorption of metal ions inside the cell [14]. Thus, the present research focused mainly on the isolation of bacterial strain that not only, sequesters but also reduces heavy metals to nonbioavailable forms.

2. Methods

All chemicals used for experimental purposes were procured from Hi-Media. Dehydrated media were utilized according to the manufacturer's instructions for the biochemical characterization of the isolates. Borosilicate glassware, including test tubes, beakers, and Erlenmeyer flasks, was utilized throughout the experiments.

Sample collection

Industrial effluent samples were collected from the different regions of Himachal (Baddi) (30.93957^0 N, 76.81218^0 E), Haryana (30.13082^0 N, 77.27570^0 E), and Punjab (30.31989^0 N, 76.41113^0 E) (30.89471^0 N, 75.86287^0E). Samples were collected in sterile cans and brought immediately to the laboratory for further processing.

Physiochemical parameters of effluent samples

The effluent samples were subjected to nitric–perchloric acid digestion, following standard protocols described earlier [21]. The samples were digested at temperatures

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ranging from 70-100^{0}C until yellow HNO_3 fumes and white $HClO_4$ fumes appeared. For the dissolution of the digested samples, distilled water was used, followed by a filtration process to remove impurities, and the final volume was increased to 25 ml [6]. The processed samples were transferred to polythene bottles and subjected to metal analysis via ICP-MS.

Enrichment and isolation of lead tolerant bacterial strains

Heavy metal-contaminated effluent samples were collected from different regions. The lead-tolerant strains were isolated by the method described by [12]. For enrichment and isolation of potent bacterial strains, the samples were inoculated in 2 distinct media: Tryptone Soya Broth (TSB), Composed of (g/l): Casein peptone-17.0 g, Soymeal peptone-3.0 g, Dextrose-2.5 g, Sodium chloride-5.0 g, Di-potassium hydrogen phosphate-2.5 g and Bushnell Hass Broth (BHB), Composed of (g/l): Magnesium sulfate -0.2 g, Calcium chloride - 0.02 g, Mono-potassium phosphate -1.0 g, Di-ammonium hydrogen phosphate - 1.0 g, Potassium nitrate- 1.0 g, Ferric chloride - 0.05 g supplemented with 0.2% glucose in 100ml broth after sterilization at 121^{o}C in autoclave.

Then, lead was added to the media at a concentration of (1 mg/l a.i.). For the enrichment process, small aliquots of metal-tolerant bacterial strains were transferred from overnight flasks to fresh media with increasing concentrations of metals. Pure cultures were maintained and preserved in glycerol at 4^{0}C until further use.

Tolerance studies

All ten isolates (AS-1 to AS-10) were screened to check their tolerance towards varying concentrations of lead chloride (i.e.1, 2, 5, 10, 25 and 50 mg/l). Isolates were further processed by taking half strength of TSB and BHB media and further incubated under aerobic conditions at 37°C. The turbidity inside the medium act as an indicator of growth, which further correlated with the tolerance to different heavy metals.

Growth Kinetics

To perform growth profiling studies, $PbCl_2$ salt was employed to expose isolates to 50 mg/l of lead chloride dissolved in 100 ml of fresh medium. A (2%) of inoculum from overnight broth culture was then transferred to TSB and BHB fresh media supplemented with lead salt. The cultures were incubated on an orbital shaker at 120 rpm and 37 °C, alongside positive (+) and negative (-) control. Growth was monitored spectrophotometrically by measuring the OD, at 600 nm, at 24 h intervals up to 96 h.

Biotransformation Approach

Mass balance studies: Specifically, AS-3 strain was chosen due to its intensified tolerance to lead metal. Growth profiling was conducted following the methodology outlined [12]. Initially, actively growing AS-3 cultures were inoculated into Tryptone Soya Broth, enriched with varying concentrations of lead, and then incubated at 37°C for 96 hours. Samples were collected for growth and mass balance profiling studies at the peak of the experiment (i.e., 96 h). It involved the collection of cell-free supernatant and biomass, after centrifugation at 8000 rpm at 4°C for 10 minutes. These fractions were subjected to acid digestion, following the protocol outlined by the United States Environmental Protection Agency (USEPA) in 1996, with subsequent dilution. For the digestion process, biomass and supernatant samples containing sequestered lead was separately treated with a mixture of concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$) in a 3:1 ratio [21]. Digestion continued until the appearance of white HNO_3 fumes, indicating complete digestion. After digestion, the samples were subjected to analysis via ICP-MS.

ICP-MS

The digested samples were analysed using inductively coupled plasma-mass spectrometry (ICP-MS) at Choksi Lab Ltd. in Panchkula, India, to quantify the metal content in various fragments. The equipment was operated under inert conditions where the flow of argon gas was maintained throughout the plasma, auxiliary and nebulizer. The samples were subjected to atomization with the help of plasma formation at 6000^o C, which was further detected by mass spectrometry.

Biochemical and molecular characterization

Bacterial isolates were identified through various biochemical tests which were performed in accordance with standard methodology from Bergey ‘s Manual [5]. The molecular characterization and identification of AS-3 was performed via 16S rRNA gene sequencing followed by phylogenetic analysis with available databases at the National Centre for Biotechnology

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Information (NCBI) [9]. 16SrRNA sequences are used to study the phylogeny and taxonomy of bacteria. It is widely used technique because almost in all bacteria 16SrRNA sequences are present. The most attractive feature of 16SrRNA is that it provides the information of genes and species name of the identical isolates. Phylogenetic analysis was performed using MEGA 11 for maximum likelihood.

Statistical exploration

Growth profiling and mass balance studies were conducted in triplicate, with all the results presented as mean values with standard error in the respective figures.

3. Results and Discussion

Screening based on tolerance

Out of 10 isolates only 5 isolates (AS-1, AS-3, AS-5, AS-7 and AS-9) were reported to tolerate 50 mg/l of lead (Fig.1). Selected 5 isolates were subjected to growth profiling studies in the presence of lead metal followed by biotransformation and sequestration studies.

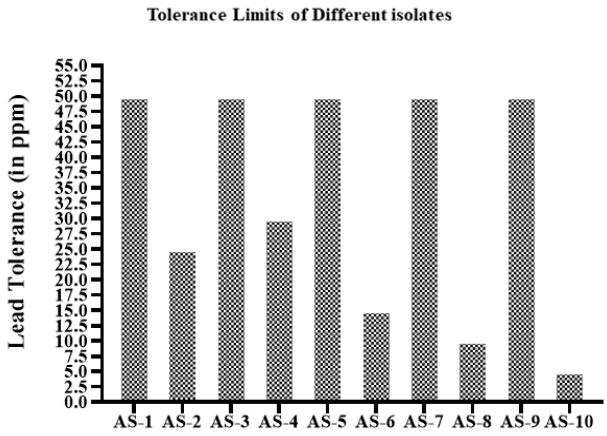


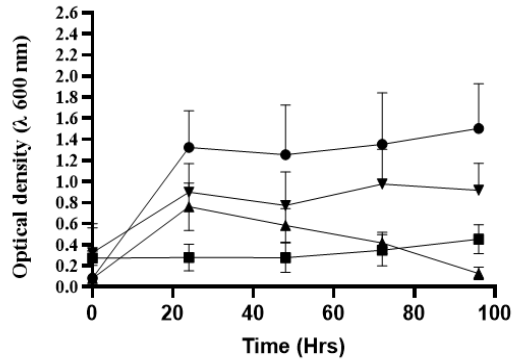
Figure 1. Lead tolerance of different isolates

Growth kinetics and metal sequestration

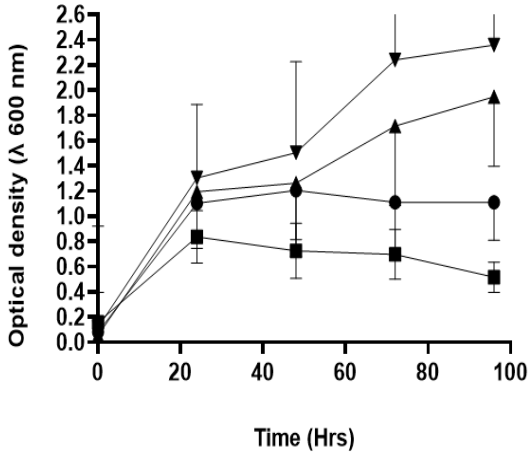
Growth kinetic experiments were performed using selected heavy metal-tolerant isolates in lead-supplemented media with spectrophotometric monitoring conducted over a 96 hrs period to observe changes in growth patterns (Fig. 2). The isolates exhibited remarkable growth patterns in media containing Pb^{2+}.

For strain AS-1 a significant expansion of the lag phase was noted in TSB media, which served as the positive control and was supplemented with lead, compared to a marginal effect observed in same strain in lead supplemented BHB media (Fig. 2a). After 72 h strain AS-3 displayed an unusually prolonged lag phase in lead supplemented BHB compared to other isolates (Fig. 2b).

In TSB media supplemented with lead strain AS-5, AS-7 and AS-9 showed shorter lag phases compared to strains AS-1 and AS-3. The growth patterns observed in control TSB and lead- supplemented TSB were consistent with the results obtained from previous isolates. However, in BHB media supplemented with lead there was a notable increase in growth.



(a)



(b)

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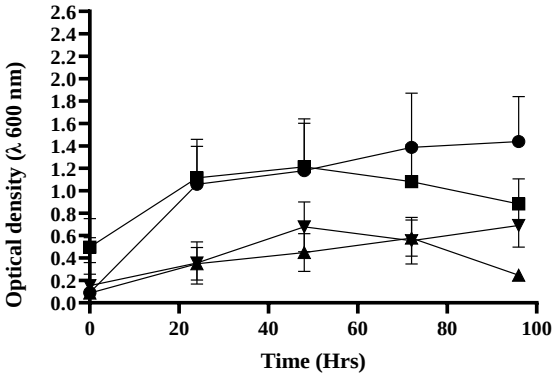

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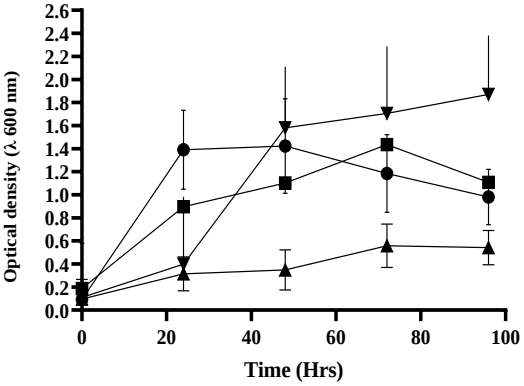

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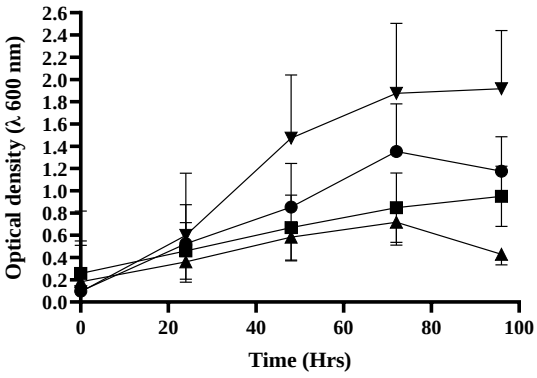
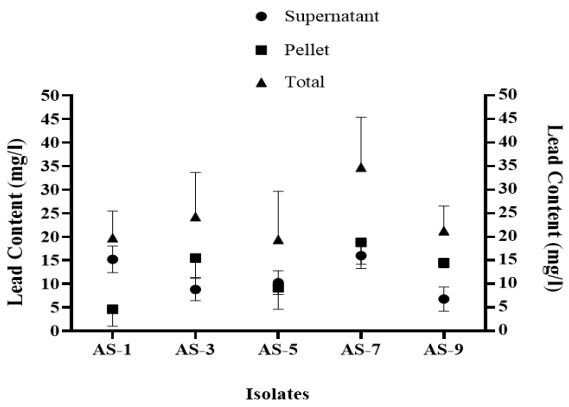


(e)

Figure 2. Comparative growth profile of different isolates with lead (50mg/l) and control in TSB and BHB. (a)AS-1; (b) AS-3; (c) AS-5; (d) AS-7; (e) AS-9.

Metal sequestration/Mass balance studies

In addition to growth kinetics, metal sequestration studies revealed significant lead sequestration by strain AS-3, AS-1 and AS- 9, as indicated by the concentration of lead in cell pellet compared to the metal concentration in the supernatant of TSB media (Fig. 3a). Specifically, in TSB 68% of the lead was sequestered by strain AS-9 followed by AS-3 and AS-7, while only marginal sequestration was observed in AS-5 with a moderate accumulation in AS-1. Conversely, in BHB media, all strains efficiently sequestered lead, except for AS-1, where 48% of the metal was found in the biomass. Mass balance studies showed nearly 100% biosorption in strain AS-3, followed by AS-9, compared to the other isolates (Fig. 3b). Various pathways have been proposed for lead tolerance and resistance in microorganisms, including efflux mechanisms where transformed metal ions are expelled from cells using energy-dependent systems (Chatterjee et al, 2012). However, in this study, sequestration results indicate that a significant portion of transformed metal remains inside the cells (AS-1, AS-3 and AS-9 in BHB, and AS-3 and AS-7 AS-9 in TSB). This retention may be due to the formation of organic compounds within the cells. Uptake studies of AS-1 was consistent with its growth kinetics, showing relatively lower growth in TSB media. Complexation of lead ions with organic nutrients may also contribute to lower metal sequestration. This effect varies among isolates, with metal sequestration levels correlating to isolate growth. In BHB media maximum metal sequestration occurs, evidenced by increasing growth over the time, likely because mineral salt media prevent metal ion complexation, leaving them available for culture.



(a)

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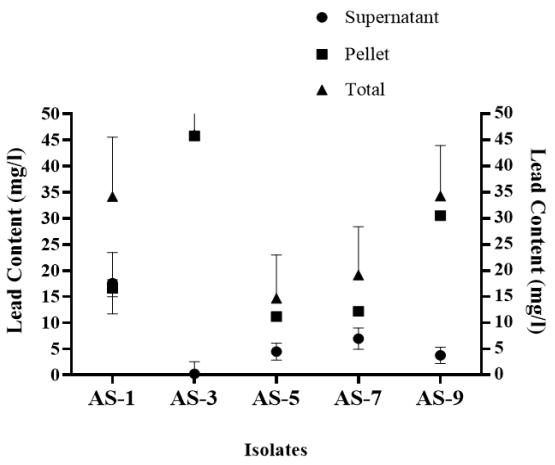

(b)

Figure 3. Sequestration of lead after 96 h. (a): in Tryptone soya broth; (b) in Bushnell Hass broth

Molecular Characterization and identification

AS-3 was characterized morphologically, physiologically, and biochemically which is represented in Table 1. Strain was found to be a non-spore former, gram-negative, rod-shaped, motile and does not produce any fluorescent pigments. Physiologically, this strain prefers neutral pH (pH 7) but it has that ability to grow at pH as high as 9, *Stutzerimonas* grows optimally and offered a wide range of temperature from 20–44 ℃ and a high salt concentration (5% w/v). Growth profiling studies and maintenance of culture was carried out under aerobic conditions, it tests positive for both catalase and oxidase along with positive nitrate reduction. Analysis of the 16S rRNA sequence of AS-3 showed 99% homology with that of *Stutzerimonas* sp. The evolutionary history was confirmed using the maximum likelihood method. Sequences were aligned using ClustalW[24], and phylogenetic analysis was performed using MEGA-11[19]; subsequently, a phylogenetic tree was generated using the maximum likelihood (Fig. 4)

Table I: AS-3 Strain characterization

Morphological	
Cell Shape	Rods
Cell Size	1–3 µm long and 0.5–0.8 µm wide
Gram Stain	Gram-ve
Motile	monotrichous
Capsule	-ve
Colony Morphology	Dark brown
Pigmentation	No pigment

Physiological	
Growth at 4^0C	+ve
Growth at 42^0C	+ve
Growth with 1% NaCl	+ve
Growth with 5% NaCl	+ve
Growth with 6% NaCl	+ve
Growth at pH-2	-ve
Growth at pH-5	+ve
Growth at pH-8	+ve
Growth at pH-11	-ve

Biochemical Characteristics	
Indole Utilization	-ve
Methyl Red	-ve
Voges Proskeur	-ve
Citrate Utilization	+ve
Nitrate Reduction	+ve
H_2S Production	-ve
Urease	-ve
Starch Hydrolysis	-ve
Casein Hydrolysis	-ve
Gelatin Liquefaction	-ve
Oxidase	+ve
Catalase	+ve

Carbohydrate Fermentation Test	
Sucrose	+ve
Glucose	+ve
Lactose	-ve

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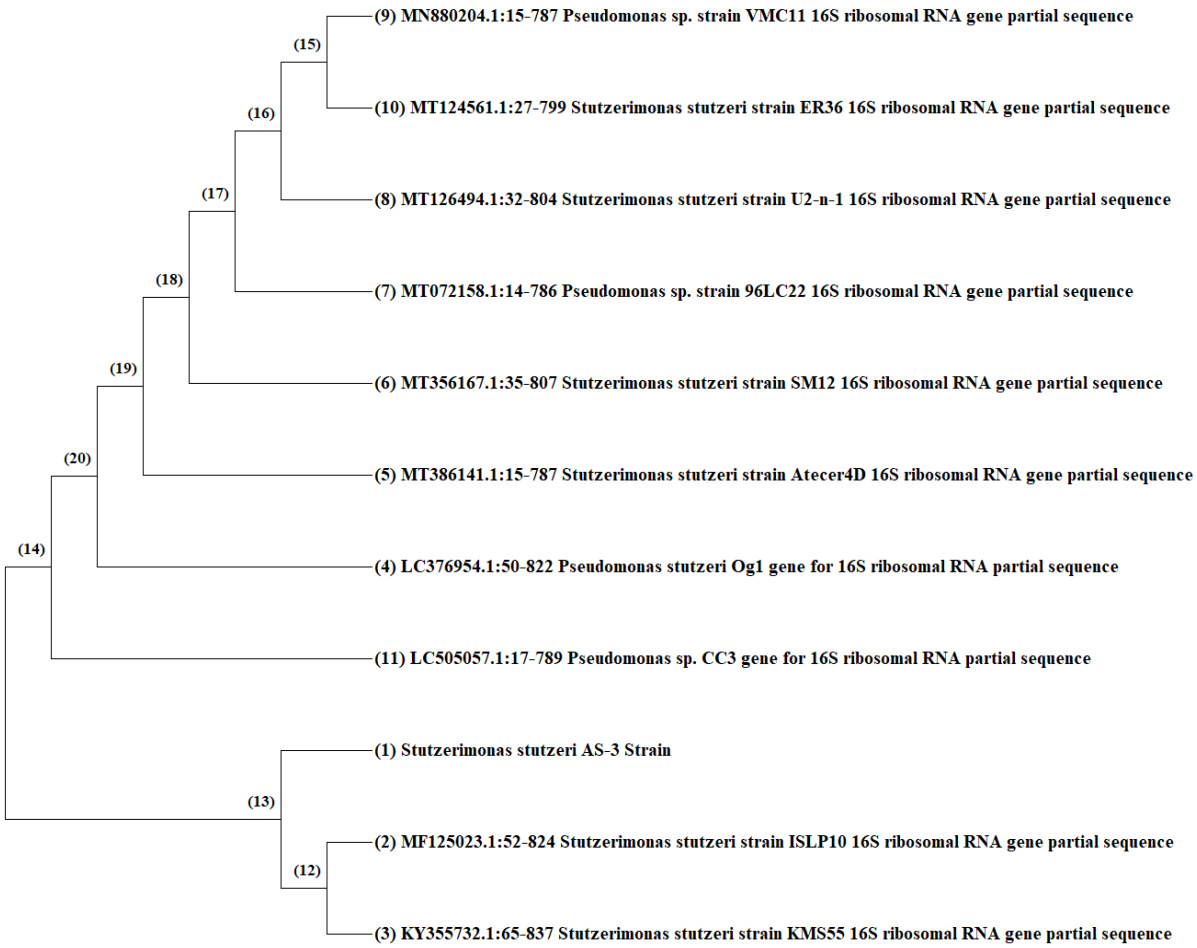

Figure 4. Molecular characterization and phylogenetic relatedness of selected bacterial strain

Discussion

In this present study our research focussed to have the exploitation of indigenous organisms for the sequestration of lead from industrial effluents, which further helps in reducing its discharge into surrounding water bodies which otherwise will enter biological systems leading to bioaccumulation and biomagnification, ultimately affecting all life forms. In this research we have isolated 5 bacterial strains from industrial effluent and are currently studying the mechanism(s) of lead transformation by these isolates. The isolates in this study may be exploited further in managing the heavy metal contamination with the help of biomass associated sequestration of lead resulting in immobilization of heavy metal. Studies on bioremediation of lead by facultative bacteria have been conducted on a wide variety of species including *Pseudomonas aerugonisa* from residual water sample [21] *Rhodotorula mucilaginosa* [20] and *Bacillus thuringiensis* [15]. However, in the present study, 99% sequestration was observed in mineral salt medium where complexation is not possible. The isolate AS-3 identified as an *Stutzerimonas sp*. Inherited feature of *Stutzerimonas* through exopolysaccharide production and biosorption promotes the heavy metal uptake and its binding may facilitate efficient immobilization for in-situ metal sequestration under lab conditions. The isolate AS-3 identified as *Pseudomonas sp*. Inherited feature of *Pseudomonas* through exopolysaccharide production and biosorption promotes the heavy metal uptake and its binding may facilitate efficient immobilization for in-situ

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metal sequestration under lab conditions. The ability of AS-3 to sequester lead within the biomass further highlights this characteristic of *pseudomonas* sp. Growth kinetics data of various isolates and their affinity for lead salts showed distinct patterns, with AS-3 exhibiting better growth and sequestration compared to other isolates. Approximately 99% sequestration of Pb^{2+} ions was observed in mineral salt medium by AS-3 with lead accumulation in the cell pellet, on or within the biomass. The efficient detoxification and transformation mechanisms employed by these organisms to combat higher concentrations of lead metal/cation supports the result obtained. In case of TSB medium, the complexation of lead ions by organic ingredients prevented Pb uptake, resulting in comparatively better growth rates. Although the speciation of intracellular lead was not quantified in this study, the uptake and sequestration of lead were evident. Over 96 hrs, the profile of total lead in various fractions such as cell-free supernatant and biomass, indicated substantial lead uptake.

Conclusion

Biochemical, physiological, molecular, and phylogenetic characterization of AS-3 strain confirmed it as *Stutzerimonas Stutzeri* which is gram-negative rod-shaped in nature that has been isolated from the industrial effluent which showed a promising potential to bio transform and sequester Pb-metal up to 50 mg/l maintained at lab conditions in terms of availability of nutrient. Here, this research work presents one of the few findings among other Pb-accumulating bacteria from contaminated environment. This strain may be exploited further for its efficiency towards the bio-transformation of heavy metals in bound form within the biomass and for alleviating lead polluted environment.

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Competing Interests

There is not conflict of interest with work from any authors to disclose.

Author Contributions

Abhijit Kumar: Conceptualization, methodology, experimentation, data collection and draft writing; Saurabh Gupta: Data analysis, curation, editing and representation, supervision; draft editing, curation; Gunjan Mukherjee: primary draft preparation and data collection.

Ethical Approval

Not applicable.

Consent to Participate

No consent is required

Consent to Publish

Present study is not a case study; hence no consent to publish is required.

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