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Deciphering metal toxicity response and exopolysaccharide (EPS) produced by *Rhizobium pusense* KM7

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ABSTRACT
In this study, twenty-six bacterial cultures previously isolated from the soil sample were subsequently screened into five isolates based on their exopolysaccharide (EPS) producing capabilities. Out of the five isolates, a Gram-negative strain, designated as isolate KM7, showed significant EPS production and was identified as Rhizobium pusense KM7 by 16S rRNA sequencing and its 16S rRNA partial nucleotide sequence is submitted to GenBank (NCBI). Furthermore, R. pusense KM7 was characterized morphologically and evaluated for its susceptibility towards different antibiotics. R. pusense KM7 demonstrates an exceptional EPS production of 6.5 g/L on the EPS production medium. KM7 exhibits EPS production, bacterial growth which significantly correlates with the strong biofilm formation. KM7 is evaluated for maximum tolerance concentration towards different heavy metals and depicts tolerance to Arsenic (As) and Lead (Pb), however, it shows maximum tolerance to As since it could thrive at a high level of arsenic (1300 mg/L). Supplementation of As in the EPS fermentation medium results in an increase in EPS yield. Moreover, an increase in the protein content of the obtained EPS is also observed over the period of incubation time. The functional groups of EPS that are crucial in EPS-As binding were discovered using FTIR spectroscopy.
Keywords: *R. pusense, Exopolysaccharide, Heavy metal resistance, As-Tolerance, Biofilm, FTIR*

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INTRODUCTION

Heavy metal pollution has become one of the world's most critical environmental challenges due to the extensive usage of numerous heavy metal compounds in industries and different anthropogenic activities, leading to its continuous emission in the ecosystem. The accumulation of harmful metals in the environment is detrimental to biotic wellness, including human health, due to heavy metal bioaccumulation and biomagnification in living organisms. Heavy metals are not biodegradable and have a longer half-life thus ensuring their persistence in the environment for longer durations. Certain heavy metals, metalloids, and radionuclides pose toxic effects even at the low µM or mM concentration. Arsenic is one of the most hazardous heavy metals found in the environmental bodies and pose serious health hazard. Metal toxicity poses substantial health and environmental risks, necessitating the development of cost-effective and efficient metal-contaminated site remediation strategies [1]. For the remediation or detoxification of heavy metals from the soil, a variety of physical and chemical approaches have been used, which is considered a tough challenge in terms of economic and technical complexity. Application of biological methods is more advantageous, and bioremediation emerges as a potential state-of-the-art approach for the mitigation of pollutants and environmental restoration using bacteria, fungi, algae, or plants [2]. Bacteria are ubiquitous in nature and can even grow in presence of highly toxic metals. Bioaccumulation, biotransformation, biosorption, bioprecipitation, biosolubilization, chelation, speciation, complexation, or extracellular sequestration are the primary mechanisms by which bacteria survive the toxic effects of metals [3]. Microbial biotechnology has developed as a feasible and environmentally beneficial alternative for heavy

metal bioremediation. Bacterial cells and their metabolites possibly collect heavy metal ions in both particulate and soluble forms [4]. Exopolysaccharide (EPS) production is one of the major responses that trigger under heavy metal stress. Extracellular EPS acts as a barrier that protects the bacterial cell and allows its survival under adverse conditions such as the presence of heavy metals by restricting the invasion of metal ions inside the cell. EPS is a high molecular weight biopolymer of microbial origin secreted by the cell and composed of repeating sugar subunits. EPS depicts diverseness in the structure and composition, i.e., homopolysaccharide and heteropolysaccharide [5]. However, the composition of EPS highly depends on the nutrients availability, source of nutrients and culture environment and growth parameter [6]. EPS are made up of organic macromolecules, including proteins, polysaccharides, amphiphilic compounds such as phospholipids, nucleic acids, humic acid, uronic acid as well as non-polymeric low-molecular-weight components [7]. Opt to its strong adsorption potential and ability to thrive in the presence of heavy metals in high concentrations, EPS produced by bacteria can be employed as an efficient biosorbent for heavy metal eradication. Various functional groups in proteins, such as amine, amide, sulfhydryl, and carboxyl groups, as well as hydroxyl and phosphate groups in EPS polysaccharides, have a direct influence on metal complexation on the EPS matrix [8]. . In response to metal toxicity, EPS-producing microbes induce the development of biofilm, which aids in heavy metal detoxification by improving metal tolerance capacity of the microbial cell or by converting harmful metal ions into non-toxic ones [4]. The present study focuses on screening, characterizing, and identifying indigenous bacteria previously isolated from soil samples, with a specific interest in their EPS production and metal tolerance capabilities. Considering the beneficial aspects, the growth condition of the bacterial strain was determined and allowed for the investigation of EPS production in the EPS fermentation medium. Furthermore, the strain's antibiotic susceptibility and biofilm formation are assessed. The strain also exhibited considerable production of exopolysaccharide (EPS) and biofilm. Moreover, the binding behaviour and sorption mechanism between EPS and targeted metal are systematically investigated using Fourier transform infrared spectroscopy (FTIR), which depicts the changes in the functional groups confirming the metal-EPS complex. To the best of our knowledge, an exceptional tolerance up to 1300 ppm is exhibited by the bacterial culture, which will aid us in developing future arsenic removal systems based on bacterial EPS.

MATERIAL AND METHODS

Screening of EPS producing ability of bacteria

25 previously isolated bacterial cultures were obtained from a lab and tested for their ability to produce EPS. The preliminary detection of EPS producing isolates was done on solidified Nutrient agar media (NAM) plates supplemented with 3% (w/v) sucrose. The plates were incubated at 28 ± 4 °C for 48 hours. The appearance of mucoid or slimy colonies on the NAM + 3% sucrose shows an ability to produce EPS. However, MA plates without sucrose supplementation were used for control cultures [9]. The mucoid mucosal bacterial colonies capable of generating EPS were chosen and re-streaked on Luria Bertani (LB) agar plates. Further secondary screening of the isolates was performed on the agar plate amended with dyes.

Congo red agar (CRA) plate and aniline blue agar plate method

The Congo red agar method is a rapid and sensitive method for detecting slime-producing microorganisms. The isolates were cultivated on Brain heart infusion agar (BHIA) amended with 5% (w/v) sucrose and 0.8 g/L congo red dye. The aqueous solution of the dye was prepared in distilled water and autoclaved separately and added in the medium when agar cooled to 45°C. The isolates were incubated at 28 ± 4 °C for 48 hours. Differences in the colony colour, colony diameter and morphology were determined. The results are interpreted based on the colour differentiation of the bacterial colonies. The slime producing strains show blackening of the colonies whereas, the non-slime producer remains pink in colour [10]. Aniline blue agar plate assay is another screening approach using an agar plate with dye. The bacterial isolates were cultivated on the NAM plate supplemented with 0.01% (w/v) trypan blue dye and incubated at 28 ± 4 °C for 48 hours. These dyes increase the mucoidness of the colony [11].

Physiological and biochemical characterization

Gram staining and capsule staining were conducted using commercially available staining kits (K001, HiMedia). The optimal condition for pH and temperature was determined in Nutrient Broth (NB) under aerobic condition. Microorganisms were incubated at several pH (4–11) and temperature (20–45 °C) ranges to assess the pH and temperature ranges for growth. The pH was adjusted by adding 1 M HCl or 1 M NaOH. After 48 hours of incubation, growth was determined by measuring culture turbidity at 600 nm, and growth was considered positive if the optical density (OD 600) was more than 0.300. The biochemical characteristics such as methyl red, Voges–Proskauer, indole production, Catalase and oxidase tests, phenylalanine deaminase and urease, and nitrate reduction were performed. The critical physiological

factors that affect the growth, such as pH and temperature, are determined by allowing bacterial growth in nutrient broth. The Bergey's Manual of Systematic Bacteriology: Volume 2 and 3 was used to identify and distinguish the isolates metabolically. The ability to metabolize various sugars were also determined. The sugar utilization test was performed on Andrade Peptone Water Medium (APWM) (HiMedia - M885) added with readily available sugar discs (HiMedia). A loopful of activated culture were transferred in the medium and incubated for 24 hours. The results are interpreted in the form of a change in colour of the Andrade's reagent [12].

Exopolysaccharide Production

An ideal production medium was selected to study EPS production and bacterial growth kinetics of the bacterial strains KM7. The composition of production media is (in g/L) tryptone 10; NaCl 5 with the adjustment of pH to 7. The production medium was supplemented with 3% (w/v) sucrose as a carbon source. Sucrose was sterilized separately and added to the production medium prior to inoculation. 5%(v/v) of actively growing culture was inoculated in the production medium and allowed to incubate on 150 rpm orbital shaker at 30°C for 144 hrs. The time course of incubation was studied by withdrawing samples aseptically at every 24 h interval up to 144 h and their OD was measured at 600 nm. The optimum incubation time to terminate the production can be determined by growth kinetics. The cultured broth was analysed for changes in pH and viscosity up to 144 h incubation. The viscosity of the EPS produced bacterial broth was measured using Brookfield DVI-Prime viscometer with spindle no. 2. For the EPS recovery, extraction was performed by centrifuging broth at 6000 rpm for 10 min. The supernatant was collected, and 3 volumes of chilled acetone were added and kept overnight at 4°C. The extracted EPS was oven-dried at 80°C until the constant weight was weighted and expressed as EPS (g/L) and then used for further analysis [13].

The gram of EPS produced in 100 mL of the fermentation broth is termed as the %polysaccharide. The residual sugar of the fermentation broth was measured by the dinitrosalicylic acid method using glucose as a standard. EPS yield was determined as a EPS per 100 gram of the sugar consumed. The percent of sugar consumption was calculated as the amount of sugar consumed to the total amount of sugar added multiplied by 100 [14].

Biofilm production

The method proposed by O'Toole, (2010) was used to analyse the biofilm production by bacterial isolates. Biofilm quantification requires activation of bacterial culture on Luria–Bertani (LB) broth. 1:100 (v/v) dilution of the activated inoculum is prepared by successfully transferring in the sterile nutrient broth with 1% sugar. 100 µL of diluted cultures were transferred to the 96-well polystyrene microtiter plate and incubated statically at 28 ± 4 °C for 24 h. The uninoculated nutrient broth was used as a negative control. After incubation, the microtiter plates' content was discarded and gently submerged into the microtiter plate distilled water to remove unattached cells and medium components. 125 µL of a 0.1% aqueous crystal violet (CV) solution are added into wells of microtiter plate wells to stain the adherent biofilm and allowed to incubate at 28 ± 4 °C for 15 min. Rinse the plate vigorously 3-4 times using distilled water and allow to stand overnight in an inverted position to dry out completely. Subsequently, 125 µL 30% (v/v) acetic acid is added to the well and incubated for 15 min at 28 ± 4 °C to solubilize the bounded CV. The quantification of the biofilm was performed by measuring absorbance at 550 nm. 30% (v/v) acetic acid was taken as a blank in the assay. Three replicate wells for each individual analysis were used. The biofilm quantification is categorized into non biofilm former (ODs < ODc), moderate biofilm former (2ODc < ODs < 4ODc), and strong biofilm former (ODs > 4ODc) [15].

Antibiotic susceptibility assay

The bacterial isolate was tested *in vitro* for its sensitivity to different commercially available antibiotics disc, including broad and narrow-spectrum antibiotics using the disc-plate method. The antibiotics used for studies are (a) quinolones and fluoroquinolones– norfloxacin, ciprofloxacin, sparfloxacin; (b) aminoglycosides – gentamycin, netillin and amikacin; (c) cephalosporins – cefuroxime, cefoperazone, ceftazidime, cefactor, cefotaxime, cefadroxil; (d) sulfonamides - co-trimoxazole; (e) penicillins (β-lactam antibiotics) – penicillin, ampicillin, Sulbactam and cloxacillin; (f) Other – chloramphenicol, roxithromycin, clarithromycin, azithromycin. The strength of the antibiotic used was in the range of 5-30 µg. The bacterial inoculum was spread by sterile glass rod into a sterile Mueller-Hinton agar plate. The sterile antibiotic disc (HiMedia IC001) was placed on the surface of inoculated Mueller-Hinton agar plate with the help of a sterile forcep and incubated at 28 ± 4 °C for 24 h. The interpretation of the antibiotic susceptibility was determined by measuring the diameter of the zones showing complete inhibition to the nearest millimeter using calibrated zone scale (HiAntibiotic zone scale). The bacterial isolates were evaluated as resistance (R) or sensitive (S) depends on the obtained zone diameter around antibiotic disc [16].

16S rRNA sequence analysis and identification of bacteria

The potential bacterial strain is identified by the 16S rRNA sequencing method. Using NCBI BLASTn, the obtained 16s rRNA sequence is subjected to check sequence similarity and alignment. The phylogenetic trees were created in MEGA X using the Neighbour-Joining method with a bootstrap value of 1000 simulations. The partial 16S rRNA gene sequence of the strain is submitted to GenBank with accession number.

Assessment of bacterial growth under the stimulation of heavy metals

The tolerance of the bacterial culture KM7 towards different heavy metals was evaluated using turbidity assay. The stock solution of Ba, As, Cu, Cr(IV), Cd, Pb, Co(II), Hg, Cr(VI), Sr, Th, Ce(III) was prepared using respective analytical grade salt. Arsenic trioxide (As_2O_3), Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), Potassium dichromate ($K_2Cr_2O_7$), Cadmium nitrate tetrahydrate ($Cd(NO_3) \cdot 2H_2O$), Lead nitrate ($Pb(NO_3)_2$), Cobalt sulfate heptahydrate ($CoSo_4.7H_2O$), Mercuric chloride ($HgCl_2$), Chromium nitrate nonahydrate ($Cr(NO_3)_3 \cdot 9H_2O$), Strontium nitrate ($Sr(NO_3)_2$), Thorium nitrate ($Th(NO_3)_4$), and Cerium nitrate tetrahydrate ($Ce(NO_3)_2 \cdot 4H_2O$), procured from Hi media. The metal concentration ranging from 10 ppm to 2000 ppm was used to study the tolerance of bacterial isolate. MTC determination was determined by inoculating 0.1 µL bacterial inoculum in 10 ml nutrient broth supplemented with the different metal solution in increasing concentration and incubated at 28 ± 4 °C for 24 h under shaking conditions (200 rpm). The nutrient broth without a metal solution was used as a control. After 24 hours, the bacterial growth in nutrient broth in terms of turbidity was measured spectrophotometrically at 600 nm. The maximum concentration of metal beyond which bacterial culture cannot grow is called maximum tolerance concentration [17].

EPS-Metal binding assessment

The changes in the functional groups present in EPS, metal loaded EPS, were investigated using FT-IR analysis. The pure EPS and metal loaded EPS were prepared separately to compare the interaction of the metal with EPS. For metal loaded EPS, the bacterial culture was allowed to grow in the fermentation medium supplemented with arsenic. Then after the EPS was extracted as per the method described in section 2.6. Both the EPS sample is subjected to FTIR measurement. FT-IR spectrums were recorded by Bruker, ALPHA-ATR system (Bruker Optics GmbH, Ettlingen, Germany), 16 scans in the frequency range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} [18]. The total carbohydrate content of EPS was determined by the phenol sulphuric acid (duboise) method considering glucose as a standard. Similarly, the total protein content was measured by the folin lowry method with bovine serum albumin as standard [19]. All the chemical characterization methods are conducted in triplicates.

RESULTS AND DISCUSSION

Screening of EPS producing ability of bacteria

The phenotypical identification of EPS producer can be performed on the solid media, were the formation of “ropy”, “slimy”, or mucoid colonies confirms the production of EPS [20]. Sugar is the precursor for EPS synthesis and strain depicting the extracellular sucrase activity is highly inducible and substrate-dependent; consequently, a solid medium enriched with sugar such as glucose or sucrose as a carbon source is utilized for the primary screening of EPS producing isolates. All 26 bacterial isolates were subjected to an NAM plate supplemented with 3% sucrose as a sole carbon source for screening of EPS production. The smooth and mucoid colony formation on the sugar supplemented plate serves as an assortment criterion for EPS producers. Comparing colony morphology between induced and non-induced EPS production is an effective technique to evaluate mucoid and slimy colonies. Based on the morphological observation, 5 bacterial isolates show mucoid colony appearance were selected. A similar methodology was applied to screen EPS-producing lactic acid bacteria (LAB). The selected 5 isolates are designated as KM1, KM2, KM3, KM7, and KM9, respectively. The colony characteristic of the selected isolates is presented in Table 1. Similar method was used for the screening of EPS production bacteria were 29 isolates out of 69 show the slimiest and mucoid colony on the De Man, Rogosa and Sharpe (MRS) modified agar medium with 1% sucrose [21]. However, the challenge encountered using this approach is the generation of false-negative results. The secondary screening of the bacterial isolates was performed on the dye amended plates. The dyes such as aniline blue and congo red have been shown to interact with polysaccharides with varying degrees of specificity. This phenomenon can be applied to identify and screen EPS producers [22]. Among all the 5 bacterial isolates KM7 shows maximum mucoidness in all the screening procedures followed by KM3 and KM2. KM7 results in positive EPS production, showing blackening of the colonies after 24 h of incubation on congo red agar plate. The same colony colour was observed after 48 h of incubation, suggesting the bacterial strain KM7 a potential EPS producer as well as biofilm former. On nutrient agar plate containing aniline blue dye, KM7 shows the formation of highly mucoid colonies after 24 h incubation.

Physiological and Biochemical characterization

The cells of the bacterial strain KM7 were found to be gram-negative, short rod, motile and aerobic in nature. The capsule staining confirms the presence of capsular EPS on the outer layer of the cell. Bacteria produce capsular EPS or slime to survive in the stress conditions such as heavy metal toxicity, high/low temperature, salinity, osmotic stress etc. [13]. KM7 can show growth at a temperature range of 20-45°C; however, the optimum growth temperature is 28-32°C. Besides, after 45°C, the strain's development slowed dramatically, and at 55°C, it exhibited no growth. Like temperature, pH is a critical element that significantly impacts microbial growth. The pH dependency revealed that the strain could grow over a pH range of 5 to 11, with the optimal pH growth being between 7 and 8. It shows limited or negligible growth at high acidic and alkaline pH (Fig 1). In the term of substrate hydrolysis, *R. pusense* KM7 is only able to hydrolyze starch, no positive result is obtained in either gelatin or casein. Besides, positive results are obtained in the methyl red test, citrate utilization, catalase, nitrate reductase and oxidase test. The other biochemical test results as such H_2S production, indole and Voges Proskauer were reported to be negative. Out of 12 sugar tested, KM7 utilised carbohydrate and produced acid in dextrose, cellobiose, fructose, sucrose, maltose, mannitol, and trehalose. The delayed positive result is obtained in xylose after 72 hours of incubation. Interestingly, a negative result in term of no carbohydrate utilization is observed in ductitol, melibiose, raffinose, inositol even after 72 hours of incubation. Morphological and biochemical traits of *Rhizobium pusense* KM7 are summarized in Table 2.

Exopolysaccharide production

Natural polymers are in high demand for a variety of applications, which has sparked a surge in interest in EPS production [8]. The kinetics of the growth, EPS production by the *Rhizobium pusense* and amount of residual sugar in the fermentation medium were monitored during the entire fermentation process. The results indicate a significant correlation between bacterial growth, consumption of sugar, exopolysaccharide production, and the presence of residual sugar (fig 2). The maximum EPS production peaked after 96 h of incubation. After 24 h of incubation, a steep increase in the EPS production was recorded till 96 h. However, the EPS yield is decreased by 18% and 23% after 120 and 144 h of incubation, respectively. The fact that the components of EPS, i.e., carbohydrate and protein, may get biodegraded by the cell during times of nutritional scarcity could explain the decline in EPS synthesis over time [23]. Also, glycoside hydrolases might get a release from the cell lysis during either stationary phase or early decline period cause the degradation of EPS [24]. The residual sugar concentration decreases with the increase in bacterial growth and EPS production. More than 96% of the sucrose was consumed over 96 h of incubation and produced 6.5 g/L EPS. Earlier, Sran et al. (2019) reported a similar result were *Rhodobacter johrii* CDRSL 7Cii produce 6 g/L EPS after 72 h in R2A media fortified with 2.5% glucose. Furthermore, the result obtained in terms of incubation time agrees with that of Shukla et al. (2020). Visually apparent parameters like as liquid culture viscosity have also been employed to assess EPS generation. Changes in the pH and viscosity of the broth was recorded from 24 to 144 hrs. Due to vigorous consumption of sugar, the pH of the medium drop from 7 to 6 till 96 h of incubation. After 120 hours the pH stables at 6.5. Viscosity is directly correlated with the EPS concentration in the broth. As the bacterial and EPS production in the broth increases, the viscosity of the broth is also raised. In our study, the maximum viscosity of 2.86 mPa.s was recorded after 96 h of incubation when EPS concentration was maximum. Change in pH and viscosity over fermentation period are demonstrated in Fig. 3.

Biofilm production

Biofilm is the dynamic environment of the bacterial population built on the surface in the defined steps. Extracellular polymeric substances (EPS), also known as “Exopolysaccharide”, plays a prominent role in biofilm development by keeping the bacterial cells in close proximity, thus allowing the formation of an intact biofilm matrix [8]. The biofilm production experiment was executed by inoculating diluted KM7 culture in the nutrient broth supplemented with 1% sugar in the wells of microtitre plates. The OD for the blank obtained after total 48 hours incubation is 0.052. Bacterial strain KM7 shows strong biofilm formation capacity with an average OD of 0.896. EPS is a key component in the production of biofilms since it comprises a variety of functionalities that act as a sorption site for chemical contaminants such as heavy metals from both the terrestrial and aquatic environments.

Antibiotic susceptibility assay

Antibiotic susceptibility assay is carried out to identify the resistance/sensitive profile against various antibiotics. In this study, bacterial strain KM7 were tested for their susceptibility towards 20 known antibiotics. KM7 shows resistance to the various broad-spectrum antibiotics such as Cefoperazone, Roxithromycin, Clarithromycin, Co-Trimoxazole, Cefaclor, Cefadroxil, Ampicillin/Cloxacillin, Penicillin-G. Besides, Cefotaxime, Azithromycin and Ampicillin/Sublactum depicted mid-grade sensitivity having a zone of inhibition diameter 14, 14 and 10 respectively. Similar results are obtained from the *Rhizobium pusense*

isolated from the rhizosphere of chickpea which shows resistance towards ampicillin, nalidixic acid and trimethoprim [27]. The development of antibiotic resistance might be possible due to changes in the outer membrane properties of bacteria such as hydrophobic properties, mutation in porins and other factors [28]. The antibiotic resistance profile of KM7 is shown in Table 3.

Molecular Phylogenetic analysis

The identification is carried out based on the 16s rRNA gene sequence analysis through BLAST searches. The results confirmed that the strain KM7 shows 99% similarity with *Rhizobium pusense* A1143 (JX266311). The phylogenetic tree is constructed of KM7 with the type strain and accepted related species of the Rhizobium genus. Upon the construction of the phylogenetic tree, the strain KM7 was obtained in clade along with *Rhizobium pusense strain* A1143 and *Rhizobium rosettiformans* W3, supported by 100 bootstrap replications (Fig. 4) [25]. The 16S rRNA gene sequence of KM7 was successfully submitted to the GeneBank with accession no. OK035228.

Assessment of heavy metal tolerance

The bacterial culture KM7 shows promising metal-tolerance, and their potential was further explored by integrating various concentrations of heavy metal. The observations were promising since they demonstrated a wide tolerance range to metal toxicity. The development of the isolates was unaffected at the maximal level when the concentration of heavy metals increased progressively. The tolerance of KM7 towards different heavy metals is presented (Fig. 5). *R. pusense* KM7 had higher tolerance ability and showed tolerance to arsenic and lead out of 12 tested metal ions. The maximum tolerance concentration for each metal was as follows: As (1300 mg/L), and Pb (1000 mg/L) (Fig. 6). KM7 shows significant but sluggish growth at arsenic concentration 1300 mg/L. On exceeding the metal concentration of As beyond 1300 mg/L, a steep decrease in bacterial growth is noted. The formation of free radicals and reactive oxygen species (ROS) may be stimulated by the elevated concentration of heavy metal, resulting in the retardation of bacterial growth [29]. The previous research demonstrated that microorganisms evolved to survive in harsh circumstances such as heavy metal toxicity. A similar trend of growth is observed on increasing Pb above 1000 mg/L. Titah et al., (2018) discovered arsenic resistance in *R. radiobacter* and *R. rhizogenes* with MICs of arsenate more than 1500 mg/L and 750 mg/L, respectively. The degree of toxicity of the heavy metal inhibiting the growth of KM7 are in order of Hg > Cd > Cr(VI) > Cu> Th > Co(III) > Sr > Ce. Mercury is extremely detrimental to KM7, and even at a dose of 10 mg/L, the growth rate is exceedingly minimum. In contrast, micronutrients such as Cu, Co, Cr, Cd demonstrated a less inhibitory effect on KM7 especially at a low concentration of 10 mg/L, nevertheless at a high concentration of 100 mg/L for Cu, Co, Cr(VI), Cd and 200 mg/L for Cr(IV) exerted a marked suppressing impact on the bacterial growth. Interestingly, in this study, KM7 can resist radioactive metals such as thorium and strontium up to a concentration of 100 mg/L; however, detrimental effect on the bacterial growth is observed upon increasing the metal concentration to 150 mg/L. Shukla et al., (2020) reported an extraordinary survival of *O. intermedium* AM7 at high thorium concentration of 1000 mg/L. The presence of heavy metals in the medium at high concentration would denature protein, inhibit enzyme activity by attaching the active sites of the enzyme and compete with other necessary cations, thus reducing bacterial activity and growth. Number of literatures are available convincing high degree of tolerance to heavy metals shown by *Rhizobium sp.* Deepika et al. (2016) had investigated the *R. radiobacter* shows multimetal tolerance for As(V), Cu, Pb, Cr, Ni, Cd at 10 mM, 1.5 mM, 0.18 mM, 0.1mM, 0.08 mM and 0.04mM metal concentration, respectively. Ahmad et al. (2013) conducted a study a study were *Rhizobium sp.* RL9 depicts excellent multimetal tolerance. The tolerance profile of RL9 are in the order of Pb (1400 µg/mL) > Zn (1000 µg/mL) > Ni (500 µg/mL) > Cr (400 µg/mL) > Cd and Cu (300 µg/mL). Many bacterial species, in instance, can reduce the toxicity of metal ions by transforming them into a less toxic state by enzymatic reduction [33]. Either chromosomes or plasmid might harbour the genetic factors mediating heavy metal tolerance, However; this resistance may varies among the species. Extracellular efflux through pumps, creation of complexes with other components, redox processes, and intra and extracellular sequestration are among the several probable mechanisms for heavy metal tolerance [34].

Effect of arsenic on EPS Production

In the present study, on evaluating the maximum tolerance concentration of different metals on *R. pusense* KM7, shows the highest tolerance to arsenic metal. Compared to other metals, KM7 offers consistent tolerance to arsenic on increasing the metal concentration. KM7 shows an extraordinary resistance even at 1300 mg/L concentration. Thus, the influence of arsenic on EPS production and composition is evaluated. According to time-course study on EPS production of KM7, maximum EPS production is reported on 4th day of incubation; hence EPS extraction from arsenic amended production medium is performed on the fourth day. When *R. pusense* KM7 is allowed to grow in the arsenic amended production media, EPS production was increased. EPS yield of 7.0 ± 0.14 is obtained on 96 hour of incubation from the production medium

supplemented with 1300 mg/L arsenic, which is higher in contrast to the EPS extracted from the production medium without arsenic. This is due to fact that bacteria tends to produce more EPS under heavy metal stress [19]. Bacteria create extracellular EPS, which acts as a protective barrier to shield bacteria from hazardous heavy metal infiltration, promotes heavy metal sequestration, and prevents heavy metal from accessing cell surfaces [4]. Compared to the production medium without arsenic, the viscosity of the broth was observed to increase in the presence of arsenic. Heavy viscosity of 8 mPa.s was noted, which is 2 folds higher as compare to control medium. However, no change in the pH of the production medium was observed. The major component of the EPS i.e., polysaccharide and protein display different fluctuation tendencies over the incubation period. EPS extracted from the 24 hour incubation shows higher polysaccharide content, which gradually decreases with the incubation time. Under heavy metal stress conditions, the protein content in the EPS gradually increases. Besides, the higher protein content of the EPS responsible for the increase in the viscosity of the medium, rigidity and stability of EPS [35]. The comparison of EPS yield, change in viscosity, total protein and total carbohydrate content of EPS obtained from EPS fermentation media and As amended EPS fermentation media are shown in Table 4. Zeng et al. (2020) reported a similar result, were EPS yield of *Cupriavidus pauculus* 1490 increases under the effect of heavy metal stress. Besides, decrease in the polysaccharide and increase protein content of EPS is reported over the incubation time. The greater amount of protein in EPS and the considerable variations in protein content before and after heavy metal treatment suggested that extracellular proteins played a vital role in metal resistance for *R. pusense* KM7.

FTIR analysis

The structural and compositional makeup of EPS favors the adsorption of metals. The cross-linked structure and presence of amine, sulfhydryl, and carboxyl groups in proteins, phosphodiester (techoic acid), phosphate, hydroxyl groups in polysaccharide impart an overall negative charge to the polymer, which interacts with the positively charged metal ions. Thus, it results in the adsorption of arsenic on the complex cross-linked structure of EPS which is investigated using FTIR (Fig 7). The FTIR spectra of control EPS (without As) and metal loaded EPS (with As) of *R.pusense* KM7 were measured in the range of 400-4000 cm^{-1} to elucidate the involvement of chemical groups of EPS in metal binding. Several peaks belonging to various functional groups were obtained in the metal-loaded and control EPS spectra. In the presence of metals, several shifting in the peaks of functional groups were observed. In the FTIR spectrum, the peak due to stretching of the N-H bond of amino group lies in the region of 3200-3500 cm^{-1}. The interaction of positively charged As with negatively charged functional group of EPS results in the peak shift of N-H bond of amino group and stretching vibration in O-H bond of polysaccharide [36]. In control spectrum, N-H stretching peak is obtained at 3350.61 $cm^{-1,}$ which shifts to 3364.22 in the As amended spectrum. A significant shift of almost 40% is observed in As loaded spectrum, which strongly signifies the strong and active participation of the amino group. Furthermore, in our study, the As-loaded FTIR data are consistent with the As supplemented broth investigation, which shows that the protein content in the obtained EPS increases over the incubation period. Thus, the above peak shifts present the significant interaction of As with N-H bond of amino group. The adsorption peak of carboxyl group of bacterial polysaccharides lies in the spectrum range of 1000-1100 cm^{-1} [36]. A significant shift from 1089.52 cm^{-1} (control) to 1081.72 cm^{-1} is obtained in the presence of As. This shift in the peaks strongly suggests the carboxyl group's involvement in the As adsorption. A minor shift in stretching vibration of C=O in the amide I bond from 1637.57 to 1638.08 is obtained in As loaded EPS. The stretching vibration of C=O from the carboxylic group and deformation vibrations of -OH from the alcohols and phenol group were ascribed to the change in the peak from 1407.20 to 1406.34 in As loaded EPS suggest strong interaction of As with C=O from carboxylic group [37]. A peak at 2320.06 signifies the presence of the sulphydryl group in EPS which depicts its prominent role in As binding [38]. Most significantly, the spectrum of As -loaded EPS showed a characteristic peak of 3731 $cm^{-1,}$ which indicated the stretching of the O-H bond of carboxylic acid, which is attributed to the interaction of carboxylic acid of EPS and arsenic, was missing in the control EPS. Aside from the shift and disappearance of individual group peaks, the strength of some distinctive peaks can also vary dramatically, showing that some functional groups in EPS may be altered under stress conditions [39].

Table 1. Growth characteristics of Five screened bacterial isolates.

Sr No.	Colony characteristics		Isolates			
		KM1	***KM2***	***KM3***	***KM7***	***KM9***
1	Size	Small	Large	Large	Small	Large
2	Shape	Round	Round	Round	Round	Round
3	Margin	Entire	Entire	Entire	Entire	Undulate
4	Elevation	Flat	Raised	Raised	Raised	Flat
5	Opacity	Semi transparent	Opaque	Opaque	Translucent	Opaque
6	Surface	Smooth	Smooth	Smooth	Smooth/ Glistening	Smooth
7	Consistency	Moist	Moist	Butyrous	Dewdropy	Moist
8	Pigmentation	Reddish pink	Off white	White	Nil	Yellow

Table 2. Biochemical characteristics of *Rhizobium pusense* KM7

Sr. No	Characteristics	Activity
Morphology		
1	Gram reaction	Negative
2	Shape	Short rod
3	motility	Motile
Biochemical reaction		
1	Indole test	-
2	Voges Proskauer	-
3	Methyl red test	+
4	Citrate utilization	+
5	Catalase	+
6	Oxidase	+
7	Nitrate reductase	+
8	H_2S production	-
Hydrolysis		
9	Gelatin	-
10	Casein	-
11	Starch	+
Carbohydrate utilization		
12	Dextrose	+
13	Cellobiose	+
14	Melibiose	-
15	Raffinose	-
16	Fructose	+
17	Xylose	*D*
18	Dulcitol	-
19	Sucrose	+
20	Maltose	+
21	Mannitol	+
22	Inositol	-
23	Trehalose	+

+ positive, - negative, *D* delayed result

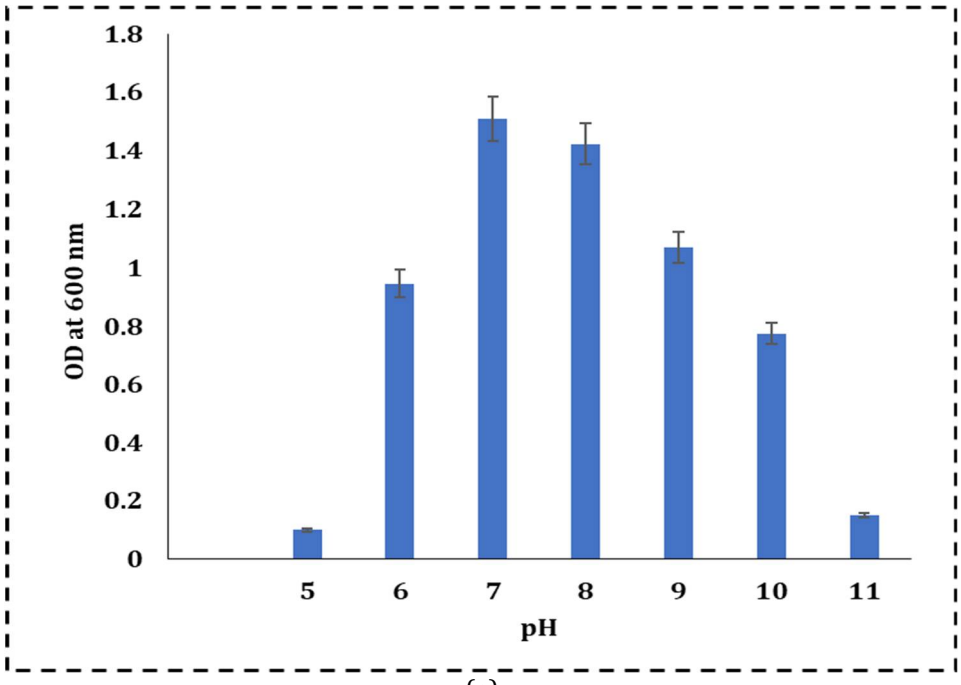
Table 3. Antibiotic susceptibility assay of bacterial strain KM7

Sr. No.	Antibiotics	Concentration (mcg)	Zone diameter (mm)	Results (Resistance/Sensitive)
1	Norfloxacin (NX)	10	28	S
2	Gentamicin (GEN)	10	23	23 mm
3	Chloramphenicol (C)	30	20	S
4	Cefuroxime (CXM)	30	17	S
5	Ciprofloxacin (CIP)	5	27	S
6	Cefoperazone (CPZ)	75	-	R
7	Ceflazidime (CAZ)	30	13	S
8	Roxithromycin (RO)	30	-	R
9	Clarithromycin (CLR)	15	-	R
10	Co-Trimoxazole (COT)	25	-	R
11	Netillin (NET)	30	22	S
12	Cefaclor (CF)	30	-	R
13	Cefotaxime (CTX)	30	14	S
14	Cefadroxil (CFR)	30	-	R
15	Azithromycin (AZM)	15	14	S
16	Ampicillin/Cloxacillin (AX)	10	-	R
17	Penicillin-G (P)	10*	-	R
18	Amikacin (AK)	30	17	S
19	Sparfloxacin (SPX)	5	26	S
20	Ampicillin/Sublactam (A/S)	10/10	10	S

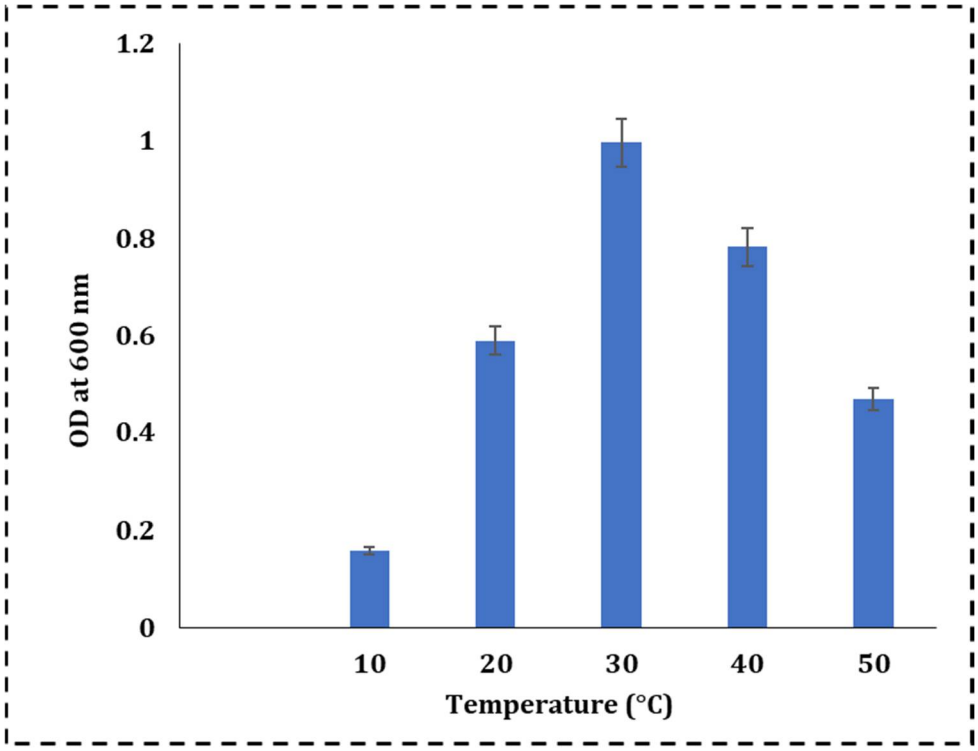
Unit = *; Resistant = R, Sensitive = S

Table 4. The comparison of EPS yield, change in viscosity, total protein and total carbohydrate content of EPS obtained from EPS fermentation media and Ba amended EPS fermentation media

	EPS yield (g/L)	**Viscosity (mPa.s)**	**Total carbohydrate (%w/v)**	**Total protein (%w/v)**
fermentation medium	6.5	2.86	4.17	0.15
As amended fermentation medium	7	8	2.84	0.34



(a)



(b)

Fig 1. Effect of physical parameter (a) pH and (b) Temperature on growth of *R. pusense* KM7.

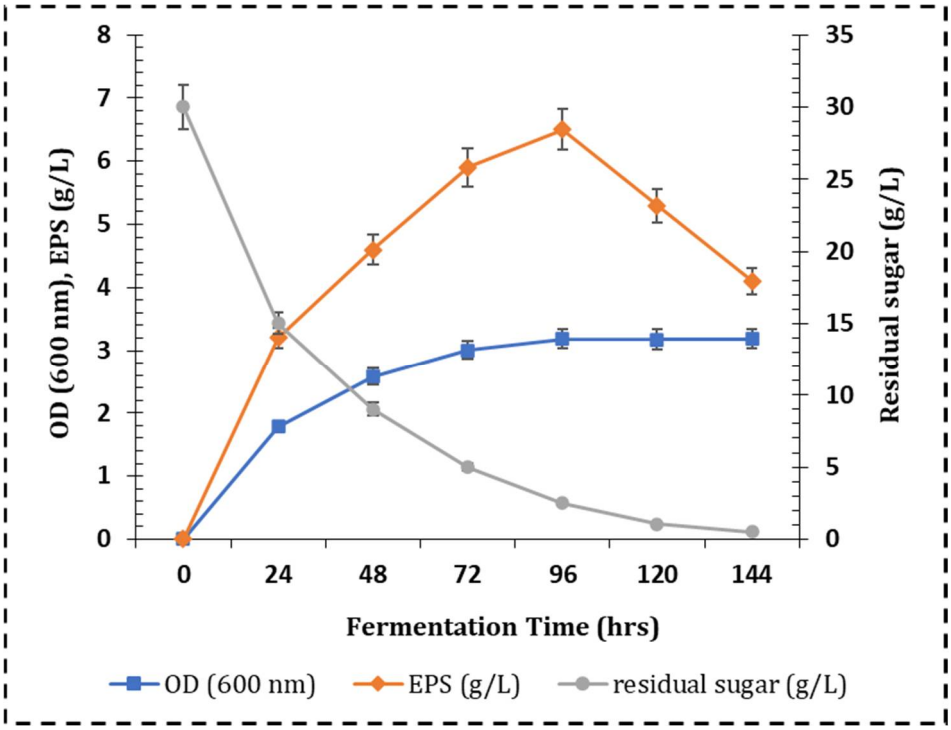


Fig 2. Growth kinetics and EPS production by KM7 in production medium containing 3% sucrose v/s sucrose consumption.

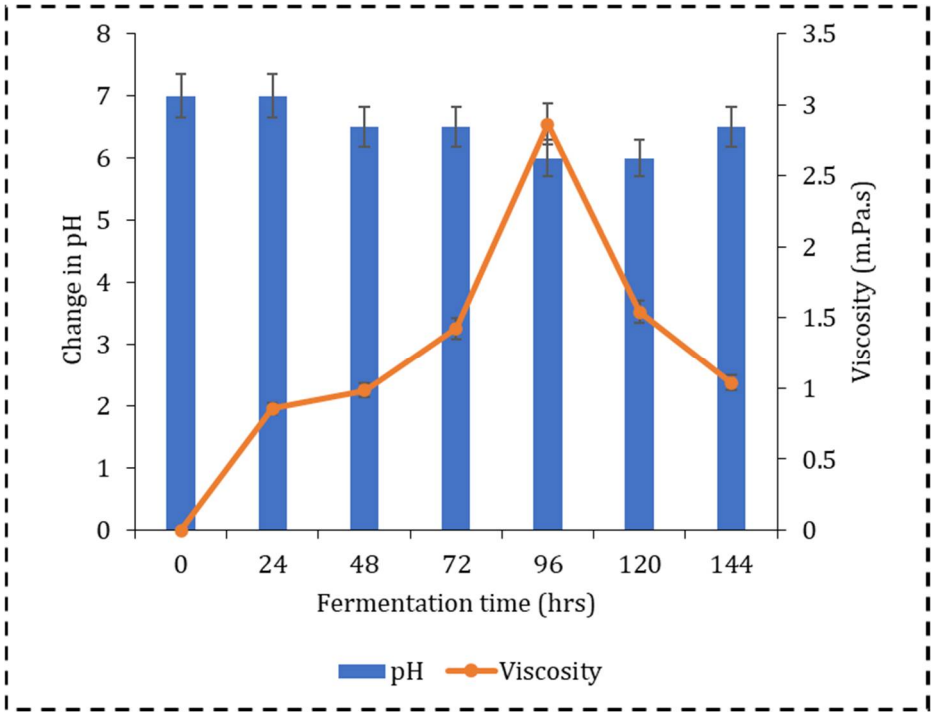


Fig 3. Change in pH and viscosity during EPS production by R. pusense KM7.

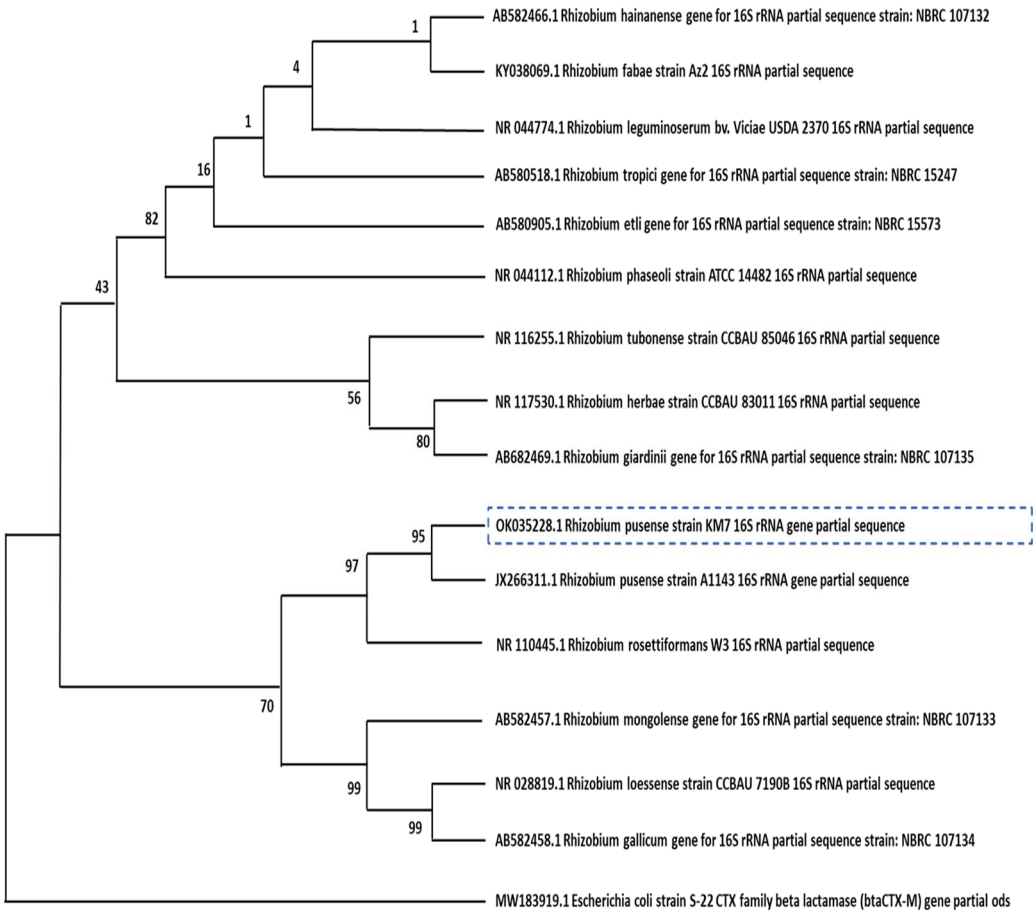


Fig 4. Phylogenetic tree of strain *Rhizobium pusense* KM7 including all fifteen member species of *Rhizobium* genus.

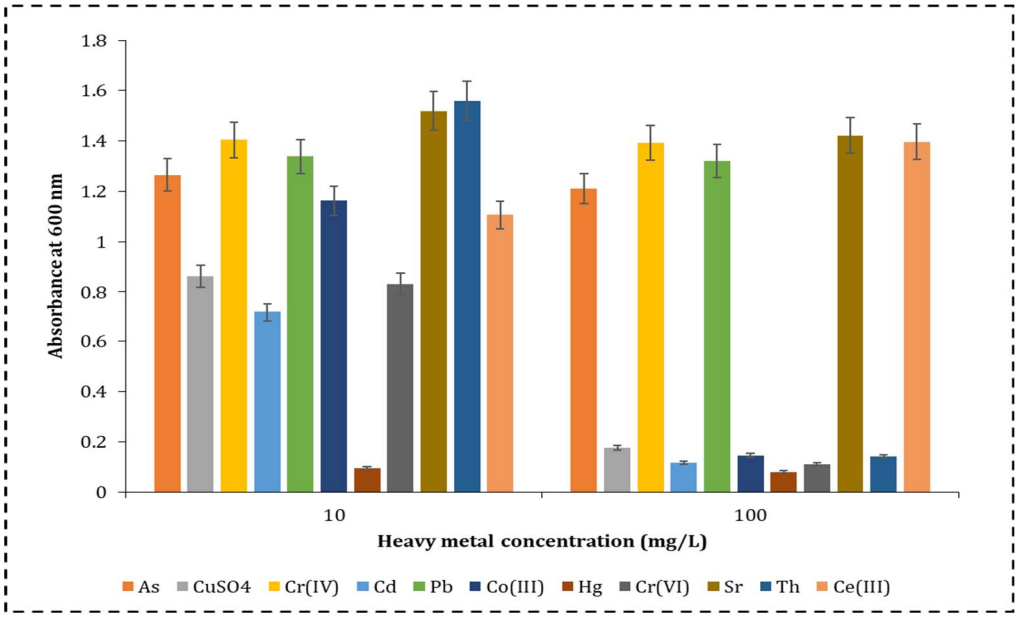


Fig 5. Tolerance index of KM7 against different heavy metal between 10 and 100 mg/L concentration.

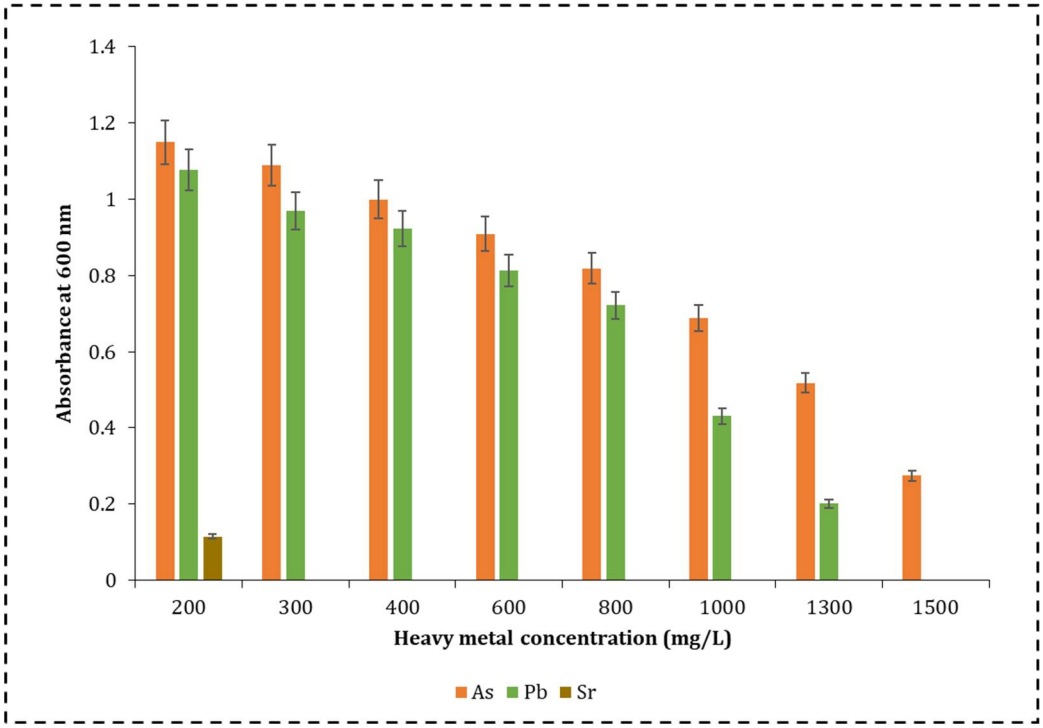


Fig 6. Profile of heavy metal toxicity of KM7 against 200-1500 mg/L concentration.

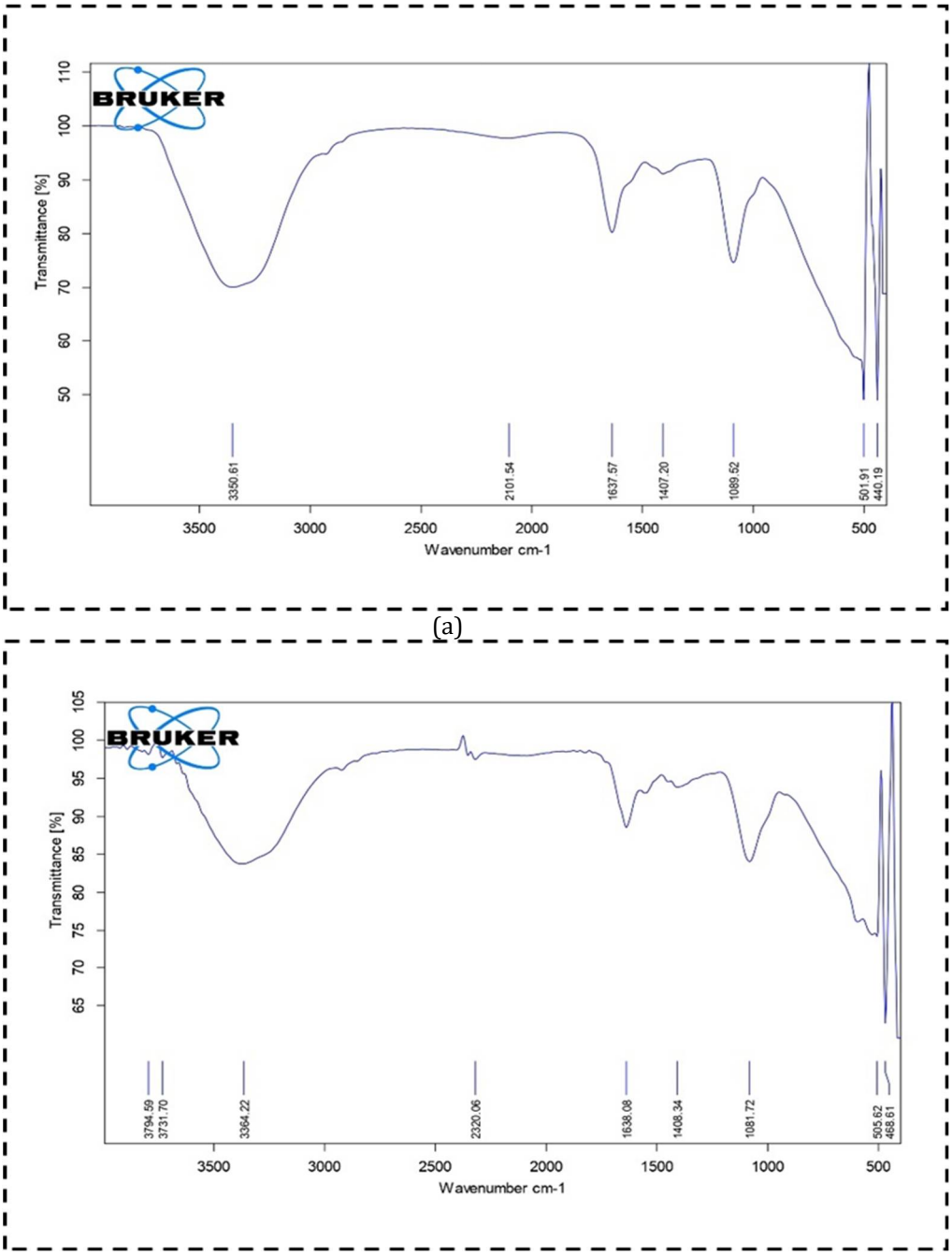


Fig 7. FTIR analysis of *Rhizobium pusense* KM7 EPS (a) control EPS (without metal) (b) As loaded EPS.

CONCLUSION

The indigenous microbiota previously isolated from a soil sample exhibited a diverse range of morphological, biochemical, and metabolic characteristics. A total of 26 bacterial isolates were examined for their exopolysaccharide (EPS) production capabilities. Bacterial culture KM7 demonstrated significant EPS production in plate assays and exhibited biofilm formation potential. Identification of KM7 was achieved through 16S rRNA sequencing, and the nucleotide sequence was submitted to the GenBank database. KM7 was identified as a Gram-negative, short rod-shaped bacterium belonging to the Rhizobium pusense species, designated as Rhizobium pusense KM7. The physicochemical impact of fermentation duration on EPS production was investigated. Over the time course study, the maximum EPS yield was obtained after 96 hours of incubation. Additionally, the heavy metal tolerance ability of bacterial strain KM7 was evaluated, revealing resistance to arsenic (As) and lead (Pb), with luxuriant bacterial growth observed at concentrations of 1300 mg/L As and 1000 mg/L Pb. Notably, EPS yield significantly increased

in As-supplemented media, and a difference in the carbon/nitrogen (C/N) ratio of EPS was reported. Fourier-transform infrared (FTIR) spectroscopy analysis of EPS extracted from As-enriched media revealed the involvement of several major functional groups of EPS in As adsorption. To the best of our knowledge, this is the first study describing the tolerance of As up to 1300 mg/L by any bacterium. The findings of this investigation may be employed to optimize EPS production, thereby facilitating its use in efficient As bioremediation applications.

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