Exploring potential applications of a novel extracellular polymeric substance synthesizing bacterium (*Bacillus licheniformis*) isolated from gut contents of earthworm (*Metaphire posthuma*) in environmental remediation

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Abstract The aim was to isolate, characterize, and explore potentials of gut bacteria from the earthworm (*Metaphire posthuma*) and imply these bacteria for remediation of Cu(II) and Zn(II). An extracellular polymeric substance (EPS) producing gut bacteria (*Bacillus licheniformis* strain KX657843) was isolated and identified based on 16S rRNA sequencing and phylogenetic analysis. The strain showed maximum tolerance of 8 and 6 mM for Cu(II) and Zn(II) respectively. It removed 34.5% of Cu(II) and 54.4% of Zn(II) at 25 mg L^{-1} after 72 and 96 h incubation respectively. The bacteria possessed a great potential to produce indole acetic acid (38.49 μg mL^{-1}) at 5 mg mL^{-1} L-tryptophan following 12 days incubation. The sterilized seeds of mung beans (*Vigna radiata*) displayed greater germination and growth under bacterium enriched condition. We observed that the bacterial strain phosphate solubilization ability

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with a maximum of 204.2 mg L^{-1} in absence of Cu(II) and Zn(II). Endowed with biosurfactant property the bacterium exhibited 24% emulsification index. The bacterium offered significant potential of plant growth promotion, Cu(II) and Zn(II) removal, and as such this study is the first report on EPS producing *B. licheniformis* KX657843 from earthworm which can be applied as powerful tool in remediation programs of Cu(II) and Zn(II) contaminated sites.

Keywords Gut bacteria · Indole acetic acid · *Vigna radiata* · Biosurfactant · Heavy metal removal · Phosphate solubilization

Introduction

Increasing anthropogenic activities, industrialization, and urbanization have resulted in a large scale environmental pollution and land degradation (Antoci et al. 2017; Boente et al. 2017; Fan et al. 2017). The global scientific community is always searching for environmental technologies which are 'economically competitive', 'environmentally sustainable and biodegradable' and 'socially acceptable' (Virapongse et al. 2016; Antoniadis et al. 2017). Implicating organisms to protect the environment can combine all these benefits together. Bioremediation is such a nature based solution exploiting biological agents to eliminate toxic substances from the environment (Balseiro-Romero et al. 2017; Alvarez et al. 2017). The modification of the physical environment by organisms is a critical interaction in most ecosystems. Of the innumerable life forms that inhabit the soil, earthworms form one of the major soil macrofauna to maintain dynamic equilibrium and regulate soil fertility and ecosystem health (Kale and Karmegam 2010; Martinkosky et al. 2017). The gut of the earthworm constitutes a mobile anoxic microzone to which the microorganisms of aerated soils are subjected. The in situ factors of the earthworm gut, including anoxia and high concentrations of organic substrates greatly stimulate a subset of ingested heterotrophic soil bacteria capable of anaerobiosis (Drake and Horn 2007; Wu et al. 2015). The occurrence of high amounts of mucus, plant derived saccharides, organic acids, denitrification-derived nitrous oxide (N_2O), molecular hydrogen (H_2) and anoxia indicates that the earthworm gut acts as a bioreactor and provides a high diversity of metabolic processes and associated microbial taxa of which a huge number of active bacterial taxa is still unexplored (Byzov et al. 2009; Ravindran et al. 2015). Microorganisms have several potential uses in the environment, for purposes as diverse as agriculture, pollution control, mining, oil recovery, biopolymer production etc. Recently, the potential of improving microorganisms for selected environmental management applications has received greater attention and speculation (Abhilash et al. 2016; Dzionek et al. 2016).

The gut inhabiting microbes are potentially more dynamic than the microbes in the soil of earthworm microcosm. Microbial communities that are mostly dominated in the soil are selectively activated in the earthworm gut and the enzymatic activities that develop in the earthworm gut actually originate from the ingested, activated microbes, especially those of nitrogen fixing, phosphate and nitrogen mineralization (Liu et al. 2017). Earthworm casts are also enriched with several micro- and macro-nutrients and have been shown to have enhanced microbial and enzymatic activities. It is already established that earthworms hold greater diversity of microbes in their gut, which are responsible for various activities, including mineralization and chelation of several ions in the soil (Adnan and Joshi 2013). Fresh casts of endogeic earthworms are considered as hotspots of microbial activity that exhibit greater carbon mineralization than the bulk soil (Abail et al. 2017).

Microorganisms are well adapted to their soil microhabitat where they live together in consortia, interacting with other biota, including earthworms.

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For some microorganisms, earthworms' gut represent mobile micro-habitats in which dormant microorganisms can find available food, mobility and shelter to develop (Lavelle et al. 1997). In recent times many environmental media have been subjected to unprecedented stress due to recalcitrant chemical contaminants like heavy metals, which can cause an array of ecotoxicological insults and injury in diverse life forms in the environment ranging from microbes to man. For example as a trace metal Cu(II) which is naturally found in soil at concentrations ranging from 13 to 24 mg Kg^{-1} in excess amounts can cause brain and kidney damage, liver cirrhosis and chronic anaemia in the human body (Cornu et al. 2017; Biswas et al. 2018). Zn(II) generally considered as a micronutrient at lower concentrations, can lead to neurosensory and neuropsychiatric disorders when present at higher concentrations (Pani et al. 2017). Hence, it is essential for removal of excess amount of Cu(II) and Zn(II) from contaminated sites and the use of biological agents particularly microbes in metal removal is both cost-effective and environmentally safe. By their natural right microorganisms have drawn current attention of the global scientists as emerging candidates for heavy metal removal where the microbial metabolic means like biodegradation, biotransformation and biocatalysis can be exploited for their remediation (Gupta and Diwan 2017). Several strains of bacteria are being utilized in plant growth promotion, biosurfactant production and toxic elements (TEs) remediation (Ashraf et al. 2017).

Although certain earthworms have been successfully tried and tested for several environmental management applications in general and bioremediation in particular, little is known on gut resident bacteria—contaminated soil and/or bacteria-phytoremediating plant interactions and their potential role in phytoremediation of metal contaminated soils. We hypothesize that specific gut resident bacteria of the earthworms could serve as the 'black box' of bioremediation for unveiling the linking loop between earthworm and the remediation processes and identifying the real role players. To bridge the existing knowledge gap the present study was carried out with an aim of assessing the potential of the gut isolate in environmental remediation, comprising of the following objectives: (1) to isolate and characterize bacteria from endogeic earthworm *Metaphire posthuma* thriving in its gut, (2) to evaluate heavy metal resistance of the isolate and its removal efficiency for Cu(II), and Zn(II), (3) to assess ability of the isolate for phosphate solubilizing capacity, indole acetic acid (IAA) production and its effect on seed germination, and (4) to test the biosurfactant potential of the gut isolate.

Materials and methods

Source of the earthworm sample

The used earthworm (*Metaphire posthuma)* is a medium size endogeic geophagous earthworm, ~ 10 cm in length, and 5 mm in diameter on average at the adult stage. Earthworm samples were collected in sterile bags from the garden soil of Kalyani University campus (Lat 22.9862°N, Long 88.4464°E) and used for further examination and isolation of bacteria.

Isolation and characterization of microorganism from earthworm gut

The earthworm samples were surface sterilized with 70% ethanol and was washed in sterile water. The gut content was released by aseptically squeezing intact worms from the anterior to the posterior end. Gut contents thus obtained were used for isolating microbes by the pour plate technique using denitrifying agar media. From different colonies obtained on denitrifying agar media, a characteristic slimy, mucoid colony was isolated, and cultured for further characterization and detailed study.

The composition of the denitrifying agar media utilized for isolating the microorganism includes potassium nitrate—1 g, L-asparagine monohydrate—1 g, sodium citrate—8.5 g, potassium dihydrogen phosphate—1 g, magnesium sulphate—1 g, calcium chloride—0.2 g, ferric chloride—0.0001 g, agar—15 g, distilled water—1000 mL. The pH of the medium was adjusted to 7.5 before sterilization. Biochemical characterization of the culture was done using a series of tests such as indole production, methyl red, Voges Proskauer, citrate utilization, carbohydrate fermentation, starch hydrolysis, catalase test, nitrate reduction, and gelatin liquefaction tests (Benson 2015).

The bacterial isolate (*B. licheniformis* strain KX657843) as reported in the previous study by

Biswas et al. (2017a) was incubated into Luria–Bertani (LB) medium which contains casein enzymic hydrolysate—10 g, yeast extract—5 g, sodium chloride—10 g, and distilled water—1000 mL. The pH of the medium is in the range 7.5 ± 0.2; the pH of the medium was adjusted to obtain different pHs (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0) in order to get the optimum pH for bacterial growth. Also, salinity tolerance (halotolerance) was tested by incubating the LB medium supplemented with 0, 2, 4, 6, 8, 10, 12, 14, and 16% (w/v) NaCl. Optimal growth in the LB medium was evaluated by measuring the increase in OD at 600 nm with a spectrophotometer. A 24 h culture was used for the entire study.

16S rRNA gene sequencing was performed to identify the strain. To sequence the 16S rRNA gene, genomic DNA of the bacterial strain was extracted and amplification of 16S rRNA gene was performed by polymerase chain reaction (PCR). For PCR bacterial genomic DNA was used as the template and bacterial universal primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). Twenty μL PCR mixtures contained 60 ng templates, 2 μL of 10× Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.6 μL of dNTP mix (10 mM each), 0.2 μL of 5 $U\mu L^{-1}$ Taq DNA polymerase and 3.75 pmol primers (each). The PCR was performed in Applied Biosystems Thermo Cycler with the temperature profile as followed, initial hot starting at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 90 s, and final extension at 72 °C for 7 min.

Electrophoresis of the PCR product was done in 1% agarose and stained with ethidium bromide; 1.5 kb band was purified by HiPurA Quick Gel Purification Kit (HiMedia Laboratories, India). Then the purified 16S rRNA gene was transformed into JM109 competent *Escherichia coli* cells using pGEM-T Easy Vector System I (Promega Corporation, USA). Plasmid DNA was isolated from the transformed cell by QIAprep Spin Miniprep Kit (Qiagen, Germany) and was sent for sequencing in Eurofins Genomics, Bengaluru, India. Using BLAST (Basic local alignment search tool, BLAST at NCBI) 16S rRNA gene sequence was compared to the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Based on the maximum identity score, the first ten sequences were selected and aligned using the multiple alignment software program, Clustal W. The phylogenetic tree was constructed based on neighbor-joining method and Jukes–Cantor distance correction using MEGA7 software (Choudhary and Sar 2011) (Fig. 1). The 16S rRNA gene sequence was submitted to the GenBank.

Examining the resistance of *Bacillus licheniformis* to Cu(II) and Zn(II)

The resistance of the isolated microorganism (*B. licheniformis)* to Cu(II), and Zn(II) were tested subjecting it to gradually increasing concentrations, starting from 0.5 mM up to 8 mM and 6 mM for Cu(II) and Zn(II) respectively, with an interval of 0.5 mM. The upper limits were the maximum metal concentrations that were tolerated by the bacterial isolate. Stock solutions (500 mM) of the studied elements as $CuSO_4{\cdot}5H_2O$ and $ZnSO_4{\cdot}7H_2O$ were filter sterilized. Selected concentrations were added to sterilize nutrient agar (NA) medium containing peptic digest of animal tissue—5 g, beef extract—3 g, sodium chloride—5 g, agar—15 g and distilled water—1000 mL and pH in the range 6.8 ± 0.2 from the stock solutions. Plates were then spot inoculated and incubated at 32 °C for 2 days. The maximum tolerable concentration (MTC) of heavy metals was designated as the highest concentration of heavy metals that allowed growth of the organism after 2 days (Srivastava et al. 2012; Jebelli et al. 2017).

Determination of Cu(II) and Zn(II) removal efficiency

Determination of removal efficiency of Cu(II) and Zn(II) was carried out using three metal concentrations (25, 50 and 100 mg L^{-1}). Nutrient broth media containing the desired concentration of metals were inoculated with 2% of the bacterial isolate and kept in the shaker incubator at 32 °C at 180 rpm. The pH of the medium was kept at 7.0 ± 0.2. Media containing the same concentrations of metal solution without the bacterial culture was kept as control. The experimental set up was maintained in triplicates. An aliquot of 10 mL from the culture free control media as well as the metal and bacteria inoculated medium was withdrawn after 24 h interval, centrifuged at 9500 rpm for 10 min and the supernatant was digested with acid mixture (HCL:HNO_3) in the ratio 3:1. Then the amount of residual metal concentrations remaining

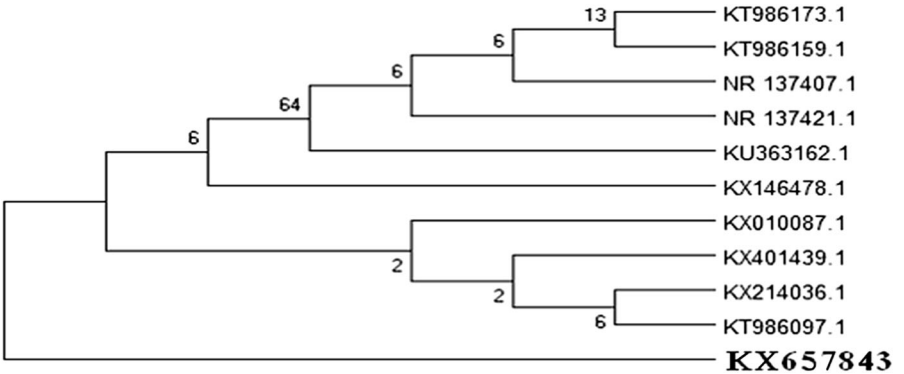

Fig. 1 Molecular phylogenetic analysis by maximum likelihood method

in the digested supernatant both in the culture treated and culture free control media was determined using AAS (Perkin-Elmer). The metal removal efficiency was calculated using the following formula (Xiao-xi et al. 2009; Syed and Chinthala 2015) (Eq. 1):

$$\text{Removal efficiency } (\%) = \frac{\text{Initial metal concentration} - \text{Final metal concentration}}{\text{Initial metal concentration}} \times 100. \quad (1)$$

Estimation of phosphate solubilizing efficiency

The isolated bacterium was grown on Pikovskaya's agar (PKA) medium (Kim et al. 1997) consisting of following constituents: glucose—10 g, tri-calcium phosphate (TCP)—5 g, yeast extract—0.5 g, ammonium sulphate—0.5 g, potassium chloride—0.2 g, sodium chloride—0.2 g, magnesium sulphate—0.1 g, ferrous sulphate-trace, manganese sulphate-trace, agar—15 g, distilled water—1000 mL. The pH was adjusted to 7.0 ± 0.2 before sterilization and incubated at 32 °C for 48 h to determine its phosphate solubilizing ability. The qualitative analysis of phosphate solubilization potential of selected bacterial isolate was measured in vitro by determining available soluble phosphate in the Pikovskaya's broth (PKM) supplemented with 0.5% TCP. The broth medium was inoculated in triplicates with the isolated bacterium. The flasks were incubated at 32 °C for 5 days on rotary shaker at 180 rpm. An aliquot of 3 mL was withdrawn periodically from each culture flask at 24 h interval. The samples were centrifuged at 9500 rpm for 10 min. Phosphomolybdate method was used for determination of available soluble phosphate in culture supernatant (Olsen and Sommers 1982; Horta and Torrent 2007). The pH of the broth medium was also measured with a digital pH meter (Hach HQ4d multi) after regular intervals (Paul and Sinha 2016).

To determine the ability of phosphate solubilization even in the presence of heavy metals, Pikovskaya's broth medium were supplemented with 50 mg L^{-1} Cu(II) and Zn(II). The flasks were incubated at 32 °C for 5 days on rotary shaker at 180 rpm. An aliquot of 3 mL was withdrawn periodically from each culture flask at 24 h interval. The samples were then centrifuged at 9500 rpm for 10 min. Phosphomolybdate method was used for determination of available soluble phosphate in culture supernatant (Olsen and Sommers 1982; Horta and Torrent 2007). The pH of the broth medium was also measured with a digital pH meter (Hach HQ4d multi) after regular intervals.

Quantification of indole acetic acid (IAA) production

To determine the amounts of IAA produced by the *B. licheniformis* strain, a colorimetric technique was performed with Van Urk Salkowski reagent using the Salkowski's method (Wahyudi et al. 2011). L-Tryptophan ($C_{11}H_{12}N_2O_2$) is a precursor for IAA, to check the effect of tryptophan on IAA production the isolates were grown in Luria–Bertani (LB) medium supplemented with 2 and 5 mg mL^{-1} L-tryptophan and incubated at 32 °C for 12 days. The results were obtained after 6 days and 12 days of incubation. The broth was centrifuged at 9500 rpm for 10 min after incubation. Then 2 mL of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) was added to 1 mL of the supernatant and incubated in the dark for 30 min. Development of pink colour indicates IAA

production while the intensity of the colour reflects the IAA concentration which is measured spectrometrically at 533 nm optical density (OD). The quantification of IAA was carried out using a standard curve with known concentrations of pure IAA.

Seed germination test

Seeds of mung beans (*Vigna radiata*) were surface sterilized with 2% sodium hypochlorite solution for 1 min and washed 5 times in single distilled water and dried. The 48 h culture of the isolated *B. licheniformis* strain grown on LB medium was used to obtain cell pellet by centrifugation at 9500 rpm for 5 min and the pellet obtained was washed twice with single distilled water. The bacterial pellets were suspended in 1 mL single distilled water, vortexed and used for seed treatment. The sterilized seeds were treated with the bacterial suspension for 10 min and then seeds were dried and placed on sterilized Petri dishes containing two layers of moistened filter paper and incubated at room temperature. Fifteen seeds were placed on the Petri dishes. The experiment was performed in triplicate. Seeds treated with water instead of bacterial suspensions were established as control. The number of bacterial cells per seed, determined via serial dilutions (Biswas et al. 2017b), was approximately 10^8 CFU $seed^{-1}$. The germination rate was recorded and the root length, shoot length, fresh weight, dry weight was measured after seventh day. The germination rate and vigor index were calculated according to the following equations (Islam et al. 2016) (Eqs. 2 and 3):

$$\text{Germination rate } (\%) = \frac{\text{No of germinated}}{\text{Total no of seeds}} \times 100, \tag{2}$$

$$\text{Vigour index} = \% \text{ Germination} \times \text{Total plant length}. \tag{3}$$

Characterization for biosurfactant production of extracted EPS

In oil spreading method oil film spread on water surface is displaced by a biosurfactant producing microorganism creating a clear zone on the oil. Here, used frying oil was added on the water surface and then to it 100 µL of cell free extract was added.

Emulsion capacity is evaluated by an emulsification index (E_{24}). This was done by adding 2 mL of cell free broth containing EPS in a test tube and 2 mL of kerosene. Then this mixture was vortexed at high speed for 2 min and allowed to stand for 24 h then E_{24} is calculated by the following formula (El-Sheshtawy et al. 2015) (Eq. 4).

$$\text{Emulsification index } (\%) = \frac{\text{Height of emulsion formed}}{\text{Total height of emulsion}} \times 100. \tag{4}$$

Statistical analysis

All data obtained were subjected to statistical analysis. Means were compared using one way analysis of variance (ANOVA) test followed by LSD test. The degrees of correlation among related factors and EPS production were determined by regression analysis. The level of statistical significance was accepted at $P < 0.05$.

Results

Identification of the gut isolate

The phylogenetic tree based on the full 16S rRNA sequences of strain isolated and related species of *Bacillus* is shown in Fig. 1. The full 16S rRNA sequence analysis revealed that the gut isolate exhibited close similarity to *Bacillus licheniformis* strain LiF4 (KX010087.1), *Bacillus licheniformis* strain LY-13 (KU363162.1), *Bacillus licheniformis* strain XY1 (KX401439.1), *Bacillus paralicheniformis* strain KJ-16 (NR_137421.1), *Bacillus glycinifermentans* strain GO-13 (NR_137407.1), *Bacillus licheniformis* strain 33CL (KX214036.1), *Bacillus licheniformis* strain Xmb047 (KT986173.1), *Bacillus licheniformis* strain Xmb031 (KT986159.1), *Bacillus licheniformis* strain Lmb032 (KT986097.1), and *Bacillus licheniformis* strain 08BF01CA (KX146478.1). The 16S rRNA gene sequence of strain isolated was submitted to Gene Bank under accession number KX657843 (Biswas et al. 2017a).

Characteristics of the isolated microorganism

The isolated strain produced highly mucoid slimy colonies denoting the production of extracellular polymeric substances. The microorganism was characterized by microbiological techniques and biochemical characterization as determined by different biochemical tests performed (Table 1).

The isolated strain was found to be Gram positive, rod shaped bacteria. The strain was facultatively anaerobic. The pH range for growth was 3.0–12.0 with optimal pH being 6.0. The isolate grew in a range of 0–16% NaCl with optimum growth at 6%. The organism showed a positive response for methyl red, citrate utilization, starch hydrolysis, catalase and nitrate reduction tests. Acid production from glucose, sucrose, mannitol, and lactose was observed. The tests were negative for production of H_2S, urease, and gelatin utilization.

Resistance and removal efficiency of the gut isolate to Cu(II) and Zn(II)

The isolated strain of *B. licheniformis* could tolerate varying concentrations of Cu(II), and Zn(II). The maximum tolerable concentration (MTC) for the gut isolate was 8 mM for Cu(II) and 6 mM for Zn(II).

The gut isolate was capable of removing the tested metals up to 34.54% for Cu(II) and 54.42% for Zn(II) after 72 and 96 h of incubation respectively, when the culture media contained the original individual metal concentration of 25 mg L^{-1}. The data revealed that the metal removal (%) was found to be a function of incubation period up to 72 h irrespective of the metal ions and their basal concentration in the culture media except at 25 mg L^{-1} Zn(II) where the removal rate continued to increase up to 96 h. For Cu(II), with lapse of time after 24 and 72 h there resulted a corresponding increase in metal removal from 12.5 to 34.54% for the original metal concentration of 25 mg L^{-1}, 5.5 to 23.4% for 50 mg $L^{-1,}$ and 4.3 to 13% for 100 mg L^{-1} concentrations. Similarly, for Zn(II) after 24 and 72 h the increases in metal removal were registered from 18 to 36.42% for the original metal concentration of 50 mg L^{-1}, and 14 to 25.2% at 100 mg L^{-1}. The maximum removal (54.42%) of Zn(II) was obtained at 96 h incubation which far exceeded the removal rate measure at 24 h of incubation (21.75%) (Fig. 2).

The trend of metal removal (Fig. 2) further showed that over the entire incubation period of 120 h the removal percentages tended to increase gradually with time, reached their respective peak at either 72 or 96 h followed by a plateau (steady state) or a slight declining phase. In case of Cu(II) at concentrations

Table 1 Phenotypical characterization of the enteric microbial isolate from the used earthworm; here '+' sign indicates a positive response while '−' sign indicates negative response

Characteristics	Interface	
Shape	Long rod	
Gram character	+	
Indole production	−	
Methyl red	+	
Voges Proskauer	−	
Citrate utilization	+	
Starch hydrolysis	+	
Gelatin liquefaction	−	
Catalase	+	
Carbohydrate fermentation	Acid production	Gas production
Glucose	+++	−
Sucrose	+++	−
Lactose	+++	−
Triple sugar iron test	++	−
Oxygen requirement	Acid butt and slant; no gas; no H_2S	
Nitrate reduction	Facultative anaerobe	
Urease	+	
Lactose	−	

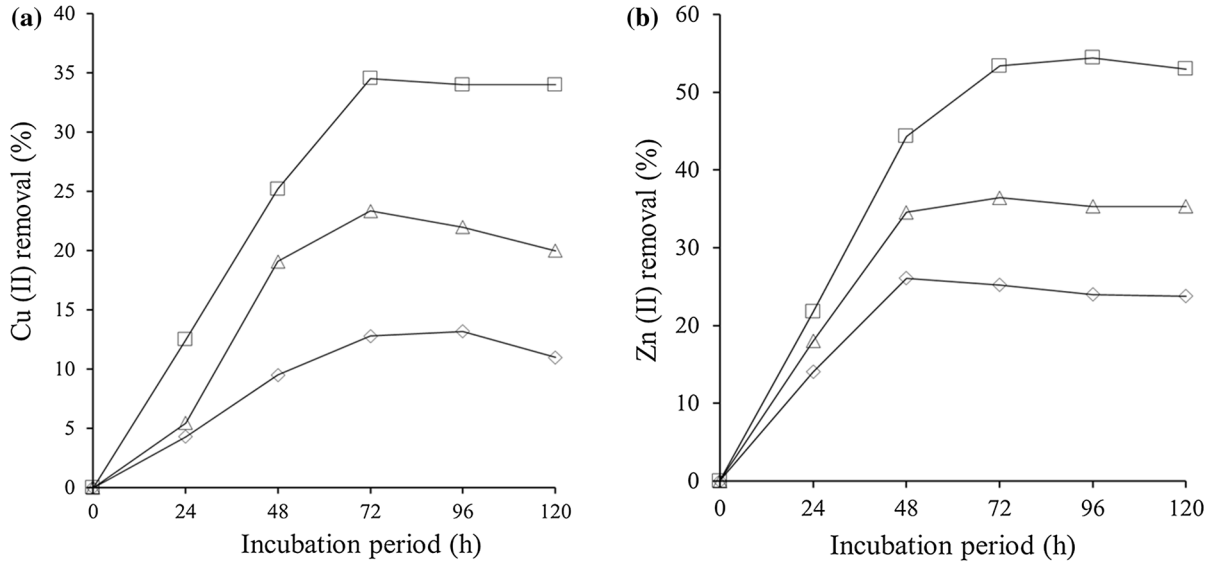

Fig. 2 Removal of Cu(II) and Zn(II) by *Bacillus licheniformis* KX657843. Open square 25 mg L^{-1}, open triangle 50 mg L^{-1}, open diamond 100 mg L^{-1}

of 50 and 100 mg L^{-1} there witnessed a slight decline in metal removal to the tune of 20 and 11% respective. For Zn(II) at 25 mg L^{-1} metal concentration there was a slight decline in metal removal percentage after 96 h incubation, the metal removal percentage being 53%. However, increase in metal concentrations from 25 to 100 mg L^{-1} did not result in increase in removal for both the metals.

Phosphate solubilization efficiency

The bacterial isolate was found to be a phosphate solubilizer. The phosphate solubilizing efficiency of the isolated *B. licheniformis* strain indicated that the strain solubilized inorganic phosphate in the PKM medium. The concentration of solubilized phosphate (mg L^{-1}) increased gradually from 35.1 mg L^{-1} at 24 h to 204.2 mg L^{-1} at 96 h and stabilized thereafter. The treatment variability of phosphate solubilization was significant ($P < 0.05$) with the following order: PKM > PKM + Zn(II) > PKM + Cu(II). Maximum phosphate was solubilized at 96 h incubation in all medium with and without metal solutions. Figure 3b shows that the isolate produced 204.2 mg L^{-1} soluble phosphate after 96 h incubation period. No phosphate solubilization was witnessed in absence of bacterium as reflected in the controls. In presence of Cu(II) and Zn(II) the isolate also showed gradual increase in solubilized phosphate from 33.5 mg L^{-1} at 24 h to 148.4 mg L^{-1} at 96 h for Cu(II) and 33.4 mg L^{-1} at 24 h to 180.5 mg L^{-1} at 96 h for Zn(II).

In liquid medium, the solubilization of tri-calcium phosphate was accompanied by a significant decline in pH of culture supernatant from an initial pH of 7.0 ± 0.2 to 5.0 ± 0.5. The maximum drop in pH value was related to elevated levels of phosphate solubilization. The isolated bacteria declined the pH to 4.6 after 96 h in absence of any metal solution. The pH value dropped down to 5.1, and 4.9 in presence of Cu(II), and Zn(II), respectively after a period of 96 h (Fig. 3a).

Potential of IAA production

The *Bacillus licheniformis* strain produced more IAA with increasing concentration of L-tryptophan and also with increasing incubation time (Fig. 4). The organism produced 38.49 μg mL^{-1} of IAA at 5 mg mL^{-1} L-tryptophan concentration after 12 days of incubation.

Induction of seed germination

The treatment of bacteria on *V. radiata* seeds had significant effect on the germination rate, vigor index,

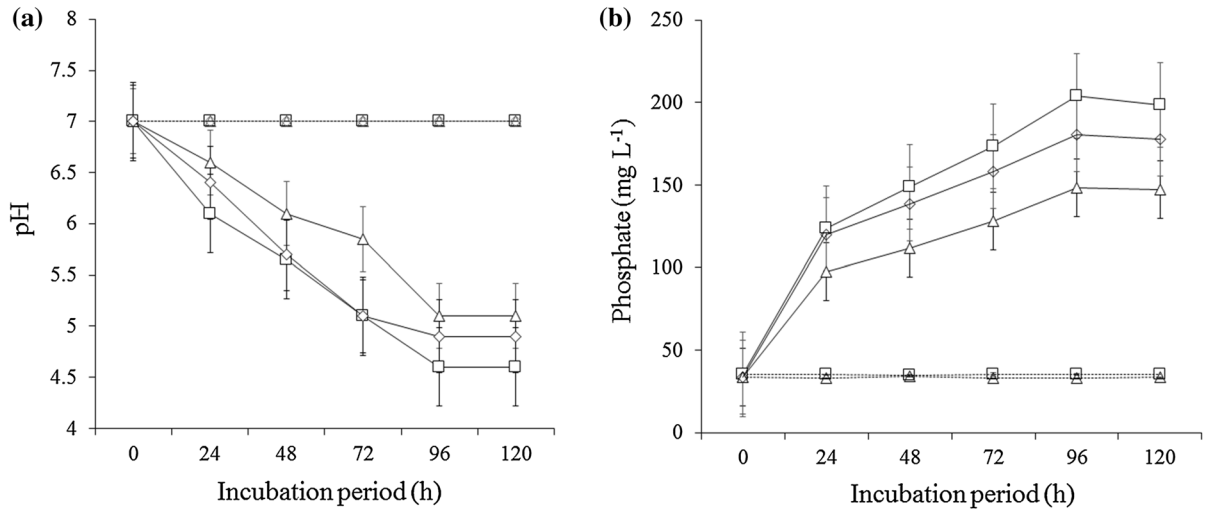

Fig. 3 Phosphate solubilization by the isolated *Bacillus licheniformis* KX657843. **a** pH change in presence and absence of Cu(II) and Zn(II). **b** Soluble phosphate in culture medium in presence and absence of Cu(II) and Zn(II) heavy metals. Open square PKM, open triangle PKM + Cu(II), open diamond PKM + Zn(II), dashed lines control

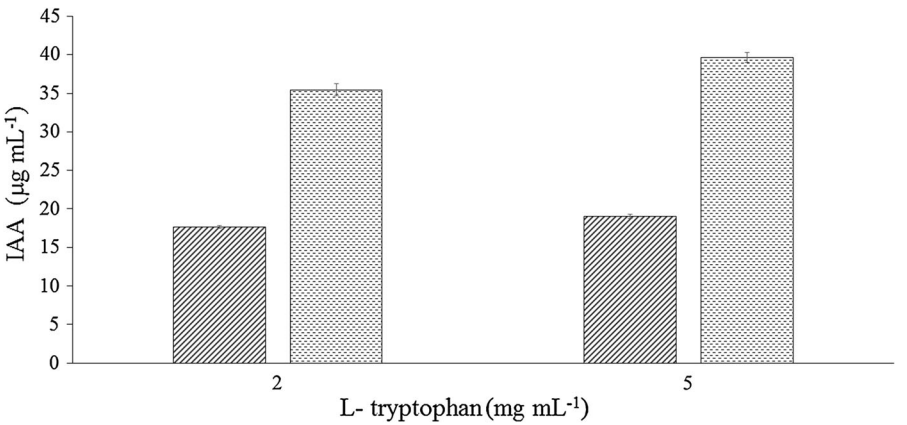

Fig. 4 Indole acetic acid production after 6 days incubation (square filled with cross lines) and after 12 days incubation (square filled with dashed lines) by *Bacillus licheniformis* KX657843

Table 2 Effect of *Bacillus licheniformis* strain KX657843 on *Vigna radiata* seed germination

Seed germination parameters	Unit	Control	Treated seeds
Germination rate	%	85 ± 5	100
Vigor index	%	635.2 ± 13.3	1183.0 ± 3
Average root length	cm	4.8 ± 0.15	7.8 ± 0.44
Average shoot length	cm	2.3 ± 0.42	3.9 ± 0.11
Fresh weight	g	4.7 ± 0.16	5.1 ± 0.042
Dry weight	g	0.52 ± 0.01	2.0 ± 0.43

The data presented are means of replicates ± standard deviations

root length, shoot length, fresh weight and dry weight as compared to those of the control. Table 2 clearly shows the effect of the isolated bacterium on seed germination.

Potential of biosurfactant production

The oil-spreading technique showed that the EPS solution when added to oil in plates containing distilled water the oil film was displaced producing a clear zone and the diameter of the clearing zone of displacement was found to be 5 cm. The emulsification capacity of the EPS was calculated as 24%.

Discussion

The activities of earthworms profoundly affect the physical, chemical, and biological properties of soils (Liu et al. 2017). The earthworm gut itself acts as a bioreactor contributing to a wide spectrum of metabolic processes mediated by the associated microflora. It provides a dynamic environment wherein the microbial community and its activities can be adapted to the ambient nutrient levels, pressure, salinity, pH, and levels of oxygen and nitrogen. All of these environmental conditions can influence the production of extracellular polymeric substances (EPS) or biofilm formation by microorganisms. Microbial communities that are mostly dominated in the soil are selectively activated in the earthworm gut and can cause beneficial environmental effects (Gómez-Brandón et al. 2011).

The present study focused on the isolation of a novel EPS producing *B. licheniformis* strain from the gut of the earthworm *Metaphire posthuma* and exploring its potential as a heavy metal resistant, plant growth promoter, and biosurfactant producer. Figure 1 represents that the isolated strain showed a close relation with a number of *B. licheniformis* strains obtained from NCBI Gene bank, the isolated strain was given an accession number KX657843 and was found to have a unique property of producing EPS only in the presence of L-asparagine monohydrate. The biochemical characterization of the isolated strain shown in Table 1 reveals the isolated strain to be facultatively anaerobic, nitrate reducing, Gram positive bacteria (Biswas et al. 2017a).

Some toxic elements are stable and persistent environmental contaminant since they cannot be degraded or destroyed. Moreover, the non-biodegradable nature of heavy metals ensures its prolonged presence in the environment. Presently, prokaryotic as well as eukaryotic microbial biomasses (living or dead cells), like bacteria, fungi, yeast and few microalgae are emerging candidates of bioremediation specially by biosorption, which can uptake and reduce toxic metal ion concentration from contaminated sources in eco-friendly manner (Gupta and Diwan 2017). With this backdrop the metal resistance properties of the isolated strain were examined so that its bioremediating ability can be determined and it was observed that the strain could tolerate up to 8 mM Cu(II) and 6 mM Zn(II).

The use of microorganisms in removing heavy metals from metal contaminated sites is profound. The abilities of *Bacillus* strains in removing toxic elements are remarkable. There are reports on the potential of *B. licheniformis* strain in removing metals from contaminated sites (Basha and Rajaganesh 2014; Gupta et al. 2014). It is reported that *B. licheniformis* exhibits growth and tolerance in the presence of higher concentration of chromium (225 mg L^{-1}) (Kavitha et al. 2011).

Microorganisms are metal-immobilizing agents able to sequester metals directly in (or on) their biomass by biosorption, bioaccumulation and biomineralization (Cornu et al. 2017). Metal ion sequestration by microbial biosorption usually works passively in a metabolism independent manner, where live as well as dead biomass can uptake metal ions through mechanisms like ion exchange, complexation, precipitation, chelation etc.

In the present study the examination of the metal removal efficacy of gut isolate revealed that with the lapse of time the percentage of metal removal increased up to a certain time period after which there was no further increase in metal removal but increase in metal concentrations resulted in decrease in the metal removal efficiency (Fig. 2a, b). Decline in metal removal percentage after a certain time period [72 h at 50 and 100 mg L^{-1} for Cu(II) and 96 h at 25 mg L^{-1} for Zn(II)] may be attributed to either of the facts that there was no further increase in bioaccumulation of metals after attaining the saturation level or there might have caused a decline in the number of live cells able to accumulate metal ions in the metal removal system. Previous studies have shown that the level of metal removal and bacterial growth depends on the initial concentration of metal ions (Malik 2003). The decrease in metal removal percentage with increasing metal concentration could be due to the lack of sufficient surface area present on bacterial surface in

order to accommodate more metal available in solution. Moreover, live cells can utilize an array of possible mechanisms to transform metal ions but beyond a particular concentration, maintaining their viability becomes challenging (Gupta and Diwan 2017). As a result, increasing metal concentrations in the present study resulted in decrease in metal uptake.

The study also reveals that irrespective of higher MTC for Cu(II) than Zn(II), the removal efficiency of Zn(II) was greater than that of Cu(II). In heavy metal polluted environment bacteria accumulate Zn(II) by a fast but unspecific uptake mechanism (Nies 1999) since Zn(II) is an essential trace element, biologically redox non reactive in nature. Moreover, Zn(II) has comparatively more metabolic demand and is less toxic to bacterial cells. Cu(II) is generally utilized by bacterial cells in small quantities in biosynthesis of metabolic enzymes like, cytochrome c oxidase and bacterial exposure to high concentrations of Cu(II) due to its wide application in mining, industry processes, and agricultural practices (Singh et al. 2010) lead to the evolution of several types of mechanisms to defend against the high copper concentration and copper induced biotoxicity (Spain and Alm 2003).

Thus, fast and unspecific mechanisms of Zn(II) uptake mechanisms could be the reason for the greater uptake of Zn(II) than that of Cu(II) in the present study. High accumulation of heavy metals in the environment forces the bacteria to adopt diverse metabolic mechanisms under metal stress conditions (Gillan et al. 2014). Such microorganisms may bear the legacy of continuous exposure to metal challenged circumstances, and possess the adaptation capacity and inherent resistance aptitude to the specific heavy metals; the degree of uptake, utilization, resistance and removal vary depending on the historical past of metal exposure (Munoz et al. 2006; Biswas et al. 2017c). In the process they develop various acclimatization, adaptation and resistance strategies at molecular cellular, population and community levels to cope with metal stress and toxicity (Jarosławiecka and Piotrowska-Seget 2014).

Studying the ability for phosphate solubilization is an important aspect for plant growth promotion. Solubilization of mineral phosphate is naturally exhibited by different microorganisms. Maximum phosphate solubilization by the isolated gut isolate was after 96 h of incubation. The present study also reveals that phosphate solubilization is directly related with decrease in pH (Fig. 3a, b). The decrease in pH can be attributed to microbe-mediated solubilization of phosphate and to the production of organic acids and CO_2 by microbial metabolism (Garg et al. 2006). Maximum phosphate solubilization along with maximum drop in pH at 96 h has also been reported in case of *Pseudomonas aeruginosa* as well as fungus *Trichoderma* sp. (Kapri and Tewari 2010; Paul and Sinha 2016). This indicates that the mechanism of phosphate solubilization is related to acidification of the culture medium. Various organic acids like gluconic acid, 2-ketogluconic acid, lactic acid, isovaleric acid, isobutyric acid, acetic acid, oxalic acid, citric acid etc., produced by phosphate solubilizing bacteria enhances solubilization of insoluble phosphates (Ma et al. 2009). The organic acids produced by the phosphate solubilizers decrease the pH to bring phosphate into solution (Pradhan and Shukla 2005). Further the quality of the acid and simultaneous production of different organic acids contribute to the greater potential for solubilization of insoluble inorganic phosphates rather than that of the total amount of acids produced by phosphate solubilizing organisms (Scervino et al. 2010; Marra et al. 2012).

Phosphate solubilization and pH drop was also observed in the presence of the heavy metals (Fig. 3a, b). In this study maximum solubilization and pH drop was similar to that observed in case of media without metal addition. The study reveals media without metal amendments solubilize better than media supplemented with metals. The toxicity of metals towards the gut isolate and also buffering effect of culture media due to metal addition could have been the reasons for decrease in phosphate solubilization (Onyia et al. 2014). Decrease in phosphate solubilization in presence of Ni stress by *Bacillus megaterium* strain has also been observed (Rajkumar et al. 2013; Oniya et al. 2014).

The gut isolate has the potential to produce IAA, one of the most physiologically active auxins (Fig. 4). The production of IAA by the gut isolate in presence of L-tryptophan indicates that the strain utilized L-tryptophan as a precursor for IAA production. Therefore, the isolate showed an increase in IAA production with increasing L-tryptophan and also with incubation time (Fig. 4). IAA is a common product of L-tryptophan metabolism produced by several microorganisms. Studies on production of IAA by *B. licheniformis* strain have been reported (Kayasth et al. 2013; Nabti

et al. 2013). The property of synthesizing IAA is considered as an effective tool for screening beneficial microorganisms suggesting that IAA producing bacteria have profound effects on plant growth. Overall, bacterial IAA increases root surface area and length, and thereby provides the plant with greater access to soil nutrients. In addition, bacterial IAA loosens plant cell walls and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Glick 2012).

Treatment of *V. radiata* seeds with the earthworm gut isolate improved seedling germination and growth and also enhanced seed germination and vigour index as compared to that of control (Table 2). Such enhanced seed germination in presence of IAA producing bacteria has been observed. One of the most commonly reported mechanisms is the production of phytohormones such as IAA (Patten and Glick 2002). There are reports of enhanced growth, production of *V. radiata* seeds by IAA producing and phosphate solubilizing bacteria (Shahab et al. 2009).

Several microorganisms have the potential of producing surface active agents known as biosurfactants. Low toxicity, high biodegradability and ecological acceptability are the main characteristics of such surface active materials. An indicator of biosurfactant production is measuring the emulsification index (E_{24}). The EPS extracted from the gut isolate possesses an emulsification index of 24%. El-Sheshtawy et al. (2015) showed the ability of *B. licheniformis* in producing biosurfactant (El-Sheshtawy et al. 2015). Studies on microorganisms producing surface-active compounds when growing on crude petroleum-oil hydrocarbons have been performed (Chhatre et al. 1996; Jazeh et al. 2012). The present research work establishes that *B. licheniformis* strain KX657843 isolated from the gut of earthworm *Metaphire posthuma* can resist and remove high concentration of heavy metals and also can promote plant growth through production of phytohormone. Thus the isolate can be a promising candidate for application in bioremediation of toxic elements in contaminated environments. Inoculation of plants with this gut isolate can promote phytoremediation of metal in contaminated soil through bioaugmentation.

The inherent ability of plant growth promoting heavy metal resistant bacterium can help host plants adapt to unfavourable metal-stress conditions and enhance the efficiency of phytoremediation by promoting plant growth, alleviating metal stress, reducing metal phytotoxicity, altering metal bioavailability in soil and metal translocation in plant. Overall the bacterial endophytes promote phytoremediation process in metal polluted soils by two distinct means, i.e. enhancement of plant metal tolerance and promotion of plant growth, additionally it has also well enough potential for biosurfactant production which offers its promise as environmental remediation tool.

Acknowledgements The study was financially supported by Department of Science and Technology (DST), Government of India in the form of providing INSPIRE Fellowship (Code No.: 1F131101) to Anurupa Banerjee for carrying out research work under the supervision of Dr. Jayanta Kumar Biswas. The authors also duly acknowledge the infrastructural support provided by Department of Ecological Studies, University of Kalyani as well as the facility available under the DST-PURSE programme.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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