Potential application of selected metal resistant phosphate solubilizing bacteria isolated from the gut of earthworm (*Metaphire posthuma*) in plant growth promotion

Jayanta Kumar Biswas[a,b,*], Anurupa Banerjee[a], Mahendra Rai[c], Ravi Naidu[d,e], Bhabananda Biswas[e,f], Meththika Vithanage[g], Madhab Chandra Dash[h], Santosh Kumar Sarkar[i], Erik Meers[j]

[a] *Enviromicrobiology, Ecotoxicology and Ecotechnology Research Unit, Department of Ecological Studies, University of Kalyani, Kalyani, Nadia, 741235, West Bengal, India*
[b] *International Centre for Ecological Engineering, University of Kalyani, Kalyani 741235, West Bengal, India*
[c] *Department of Biotechnology, SGB Amravati University, Amravati 444602, Maharashtra, India*
[d] *Global Centre for Environmental Remediation (GCER), The University of Newcastle, Callaghan, NSW 2308, Australia*
[e] *Cooperative Research Centre for Contamination Assessment and Remediation of the Environment, ACT Building, The University of Newcastle, Callaghan 2308, NSW, Australia*
[f] *Future Industries Institute, University of South Australia, Mawson Lakes, SA 5095, Australia*
[g] *Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka*
[h] *Sambalpur University, Jyoti Vihar, Sambalpur, Odisha 768019, India*
[i] *Department of Marine Science, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700 019, West Bengal, India*
[j] *Laboratory of Analytical Chemistry and Applied Ecochemistry, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium*

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ABSTRACT

The present study focuses on the isolation of three phosphate solubilizing bacteria (PSB), PSB1, PSB2 and PSB3 from the gut of earthworm *Metaphire posthuma*. The three stains were identified as *Bacillus megaterium* (MF 589715), *Staphylococcus haemolyticus* (MF 589716) and *Bacillus licheniformis* (MF 589720) through 16 S rRNA gene sequencing and biochemical characterization. The strains showed resistance to the metals Cu and Zn at significant concentrations and could solubilize phosphate even in the presence of metals. Maximum phosphate was solubilized by strain PSB3 with a production of 222 ± 2.0 mg L^{-1} soluble phosphate followed by PSB1 (213.7 ± 1.3 mg L^{-1}) and PSB2 (193.5 ± 1.5 mg L^{-1}) at 96 h of incubation. The strains were able to produce indole acetic acid (IAA) in presence of L-tryptophan and possessed ammonium ion production potential in the order PSB3 > PSB1 > PSB2 ($P < 0.05$). The sterilized seeds of mung beans (*Vigna radiata*) displayed greater germination rate and higher growth under bacterium-enriched conditions. The effect on seed germination traits by the isolated strains followed the order of PSB3 > PSB1 > PSB2 ($P < 0.05$). Our results suggest that the three isolated PSB strains from earthworm gut possess intrinsic abilities of growth promotion, metal resistance and solubilization of phosphate which could be exploited for plant growth promotion and bioremediation even under metal-stress conditions.

1. Introduction

Earthworm is an important zoological group of soil macrofauna that dominates in the contribution of soil biomass of invertebrates (Liu et al., 2017). The fundamental role of earthworms in the decomposition of organic matter and nutrient cycling and its impact on agriculture is well established (Andriuzzi et al., 2016; A. Singh et al., 2016; P. Singh et al., 2016; Thomason et al., 2017). The feeding behavior of earthworm, which is the ingestion of soil and litter, can ultimately host other potential soil biological components "microorganisms" in their guts (Eisenhauer et al., 2012; Ali et al., 2015). These gut-dwelling microbes play an important role in biogeochemical processes of soil elements through gut-passage processes, such as producing cast (Sruthy et al., 2013; Aria and Dominguez, 1998; Clause et al., 2015). Thus, the earthworms together with the microbiota are responsible for decomposition and turnover of substances in nature and thereby regulate the

biogeochemistry of terrestrial soils (Byzov et al., 2015; Arai et al., 2017). Soil enzymes along with microbial biomass are important biochemical parameters that define biological activities in soils (A. Singh et al., 2016; P. Singh et al., 2016). Although there are some reports on earthworm gut epithelium serving as a source of luminal enzyme activities (Sanchez-Hernandez et al., 2009), majority of the enzymatic activities in the earthworm gut are actually originated from the ingested, activated microbes, especially those of nitrogen fixing (Hussain et al., 2016), nitrifying and denitrifying (Horn et al., 2005; Drake and Horn, 2006), and phosphate solubilizing categories (Hussain et al., 2016).

Phosphorus (P) plays an important role in plant maturation and is required for photosynthesis, root establishment, energy transfer, good flowering, fruit quality etc. (Bhat et al., 2017). Of the various chemical forms of P, plants take up only negatively charged primary and secondary orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) as nutrient, but most of P in nature exists in various organic and inorganic forms. The insoluble and inaccessible forms of P are hydrolyzed to soluble and available forms through the process of solubilization of inorganic P and mineralization of organic P (Koch et al., 2018; Khan et al., 2014). The insoluble forms of P such as tricalcium phosphate ($Ca_3PO_4)_2$, aluminium phosphate ($AlPO_4$), or iron phosphate (Fe_3PO_4), may be converted to soluble P by phosphate-solubilizing microorganisms and enzymes (e.g. phosphatase, phosphotriesterases) that are present in soils (Sharma et al., 2013). Such release of P can be performed by earthworm gut phosphatases and phosphate-solubilizing microbes present in the gut (Bhat et al., 2017). Some studies suggest that the phosphatase activity in the casts was significantly higher than the soil; the major contribution of this enzyme was the earthworm gut microorganisms than the epithelium of the gut itself (Vinotha et al., 2000).

Also such P solubilizing microbes exhibit multifunctional activities that benefits plants by synthesizing siderophores, indole acetic acid (IAA), and gibberellic acid etc. (Khan et al., 2013). However, in soils which are contaminated with high levels of metals like copper (Cu) and/or zinc (Zn), microbial functions and earthworm actions may be adversely affected resulting in decreased P release (Doelman and Haanstra, 1989; Wang et al., 2007). In recent years, metal resistant microbes have been employed because they display a high potential to alter the metal mobility and bioavailability (Park et al., 2010; Mohamed and Almaroai, 2017). However, multifunctional microorganisms that are capable of P solubilization and are resistant to high level of metals (e.g. Cu, Zn) should be available in the soil and soil-inhabiting organisms have rarely been explored. We hypothesize that if the specific gut resident bacteria of the earthworms are found to be endowed with unique attributes like phosphate solubilization and metal resistance they could elucidate earthworm's role in enhancing the soil fertility and metal remediation. This could establish such gut inhabiting bacteria as ‘connecting link’ and ‘behind the screen’ role players in those ecological services. Hence, the present study focuses on the isolation of PSB from the gut of endogeic earthworm *Metaphire posthuma*, and investigates their properties as a plant growth promoter and also their resistance towards metals.

2. Materials and methods

2.1. Source of the earthworm sample

Endogeic geophagous earthworms (*Metaphire posthuma*) used in this study were adult with a medium body size (Length: ~10 cm, Diameter: ~5 mm).Earthworms were collected from the garden soil of Kalyani University campus (22.9862°N, 88.4464°E) and stored in sterile bags and used for further examination and isolation of bacteria from its gut.

2.2. Isolation of PSB from earthworm gut

The earthworms were surface sterilized with 70% ethanol and washed with sterile water. The gut contents were released from the anterior to the posterior end by aseptically squeezing intact worms. Gut contents were then stored at 4 °C unless used immediately for the isolation of target bacteria. The isolation of potential PSB was executed following the pour plate technique using Pikovskaya's agar medium (dextrose 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 1 L; the pH was adjusted to 7.0 ± 0.2 before sterilization). After 48 h of incubation at 28 ± 2 °C, three bacterial colonies showing discrete hallo zones were isolated and sub-cultured for further characterization.

2.3. Identification and characterization of PSB

Several biochemical tests of the isolated PSB were carried out for the identification of strains (Benson, 2015).

The 16S rRNA gene sequencing was performed to identify the strains (Biswas et al., 2017). 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). The PCR reaction mixture (20 µL) 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 μL^{-1} Taq DNA polymerase and 3.75 pmol primers (each). The PCR performed (Applied Biosystems Thermo Cycler) with an initial heat step for 5 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and extension step 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 stained with ethidium bromide and; 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 *E. 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 at 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/).

2.4. Effect of pH and NaCl on PSB strains isolated

The bacterial isolates were incubated into Luria Bertani (LB) medium consisting of casein enzymic hydrolysate 10 g; yeast extract 5 g; sodium chloride 10 g and distilled water 1 L in order to determine the optimum pH and salinity tolerance (halotolerance) for those isolates. 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) using drops of 0.05 M HCl or NaOH. In the other case, LB medium was supplemented with various concentrations of NaCl (0, 2, 4, 6, 8, 10, 12, 14, and 16) %, (w/v) in order to assess the salinity tolerance of the strains. The experiments were performed in triplicates. Optimal growth in the LB medium was evaluated by measuring the increase in optical density (OD) at 600 nm with a spectrophotometer (UV-1800 UV–Vis Shimadzu).

2.5. Examining the resistance of PSB strains to metals

The resistance of the isolated PSB strains to Cu and Zn was tested. Stock solutions (metal concentration 500 mM) of the elements in the forms of $CuSO_4{\cdot}5H_2O$ and $ZnSO_4{\cdot}7H_2O$ were sterilized using 0.45 µm pre-sterile syringe filter (Millipore filter paper). Further, increasing concentrations (0.5 mM to 8 mM) of the metal salts were added from the stock solution to sterile nutrient agar (NA) medium (composition: peptic digest of animal tissue 5 g; beef extract 3 g; sodium chloride 5 g;

agar 15 g and distilled water 1 L with pH adjusted to 6.8 ± 0.2 before sterilization). Plates were then spot inoculated and incubated at 32 °C for 2 days. The maximum tolerable concentration (MTC) of Cu and Zn was designated as the highest concentration of metals that allowed growth of the organism after 2 days (Srivastava et al., 2012; Jebelli et al., 2017).

2.6. Estimation of phosphate solubilizing efficiency

The phosphate solubilization potential of selected bacterial isolates (Chen et al., 2006) was measured in vitro by determining available soluble phosphate in the Pikovskaya's broth (PKM) supplemented with 0.5% TCP (equivalent to 5000 mg L^{-1}). In brief, the broth medium was inoculated in triplicates with the isolated PSB strains and similar set-up without culture (uninoculated) was used as control. The flasks were incubated at 28 ± 2 °C for 5 days on a 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 the culture supernatant (Watanabe and Olsen, 1965). 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 metals (Cu and Zn) Pikovskaya's broth medium was supplemented with 50 mg L^{-1} of Cu and Zn using $CuSO_4{\cdot}5H_2O$ and $ZnSO_4{\cdot}7H_2O$, and similar individual set-ups without culture (uninoculated) and Cu and Zn metal at 50 mg L^{-1}concentrations served as control. This being the minimum tolerable concentration for PSB1 and PSB2 subjected to metal (Cu and Zn) stress that particular concentration has been chosen and used to assess how phosphate solubilization potentials of all the three strains perform under such condition. The incubation and the determination of soluble phosphate were performed following the procedure described above. Here also the experiments were carried out in triplicates.

2.7. Applicability test of PSB for plant growth promotion

2.7.1. Characterization for indole acetic acid (IAA) production

To determine the amounts of IAA produced by the PSB strains, a colorimetric technique was performed using the Van Urk Salkowski reagent (Wahyudi et al., 2011). The isolates were grown in triplicates in LB medium supplemented with 0, 2 and 5 mg mL^{-1} of L-tryptophan and incubated at 32 ± 2 °C for 12 days. The results were obtained after 6 days and 12 days. The broth was centrifuged at 9500 rpm for 10 min after incubation and then to 1 mL of the supernatant 2 mL of Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$ solution) was added and the mixture was incubated in the dark for 30 min. Development of pink color indicated IAA production which was recorded spectrophotometrically at 533 nm and measured against the standard curve with known concentrations of pure IAA (Sigma-Aldrich) purchased.

2.7.2. Determination of ammonium ion concentration in solution

To test the ammonium ion production activity, the bacterial isolates were inoculated to peptone water (medium: peptone 10 g; sodium chloride 5 g and distilled water 1 L and pH was adjusted to 7.0 ± 0.2 before sterilization) and incubated with constant shaking at 140 rpm for 7 days at 30 ± 2 °C. Uninoculated medium served as control. 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. The ammonium ion produced was colorimetrically determined by phenol-hypochlorite method (APHA, 2005). The experiment used triplicate sets.

2.7.3. Seed germination and early plant growth potential

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 followed by air-drying. The cell pellets of potential PSB were obtained from the pre-grown culture broth by centrifugation at 9500 rpm for 5 min, washed twice with sterile distilled water and the pellets were resuspended in 1 mL sterile distilled water, vortexed for 20 s and used for seed treatment. About twenty sterilized seeds were treated with 5 mL bacterial suspensions for 10 min, and then seeds were air-dried and placed on sterilized Petri dishes (9 cm) containing two layers of moistened filter paper and incubated at room temperature. Fifteen seeds were placed on the Petri dishes. Triplicates of this treatment were maintained and seeds treated with sterile water (no bacterial suspensions) were also established. The number of bacterial cells per seed, determined via serial dilutions, was approximately 10^8 CFU $seed^{-1}$. The seeds were incubated in a light incubator. The germination rate was recorded and the root length, shoot length, fresh weight and dry weight were also measured after seventh day. The germination rate and vigor index were calculated according to the following equations (Islam et al., 2016):

$$\text{Germination rate (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100 \quad (1)$$

$$\text{Vigor index} = \%\text{germination} \times \text{total plant length} \quad (2)$$

2.8. Statistical analysis

All data obtained were statistically analyzed using SPSS 20.0 (IMB, USA). Means were compared using a one-way analysis of variance (ANOVA) test followed by the post-hoc analysis with LSD test. The degrees of correlation among related factors were determined by regression analysis. The level of statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Identification

To characterize the three isolated strains of PSB named strains designated as PSB1, PSB2 and PSB3 several biochemical tests were performed; the strains showed a varied response to different biochemical tests but the tests were negative for H_2S gas production and other gas productions in all the cases (Table 1).

The16S rRNA gene sequencing revealed the proximity of 99% of PSB1 with *Bacillus megaterium* strain Amic 4 (KX228234.1), *B. megaterium* strain MP8 (KX298860.1), *B. megaterium* strain SA1 (KX197921.1). PSB2 showed 100% proximity with *Staphylococcus haemolyticus* strain S4 (KX611317.1), *S. haemolyticus* strain PAH-3

Table 1
Phenotypic characterization of enteric microbial isolate PSB1, 2, 3 from earthworm (*Metaphire posthuma*). The '+' sign indicates positive response while the '−' sign indicates the negativity.

Characteristics	PSB1	PSB2	PSB3
Shape	Rod	Coccus	Rod
Gram character	+	+	+
Indole production	−	−	−
Methyl red	−	+	−
Voges Proskauer	−	−	+
Triple sugar iron test	Acid slant; no gas; no H_2S	Acid slant; no gas; no H_2S	Acid slant and butt; no gas; no H_2S
Starch hydrolysis	+	−	+
Gelatin liquefaction	−	−	+
Catalase	+	+	+
Citrate utilization	+	−	+
Oxygen requirement	Aerobe	Facultative anaerobe	Aerotolerant anaerobe

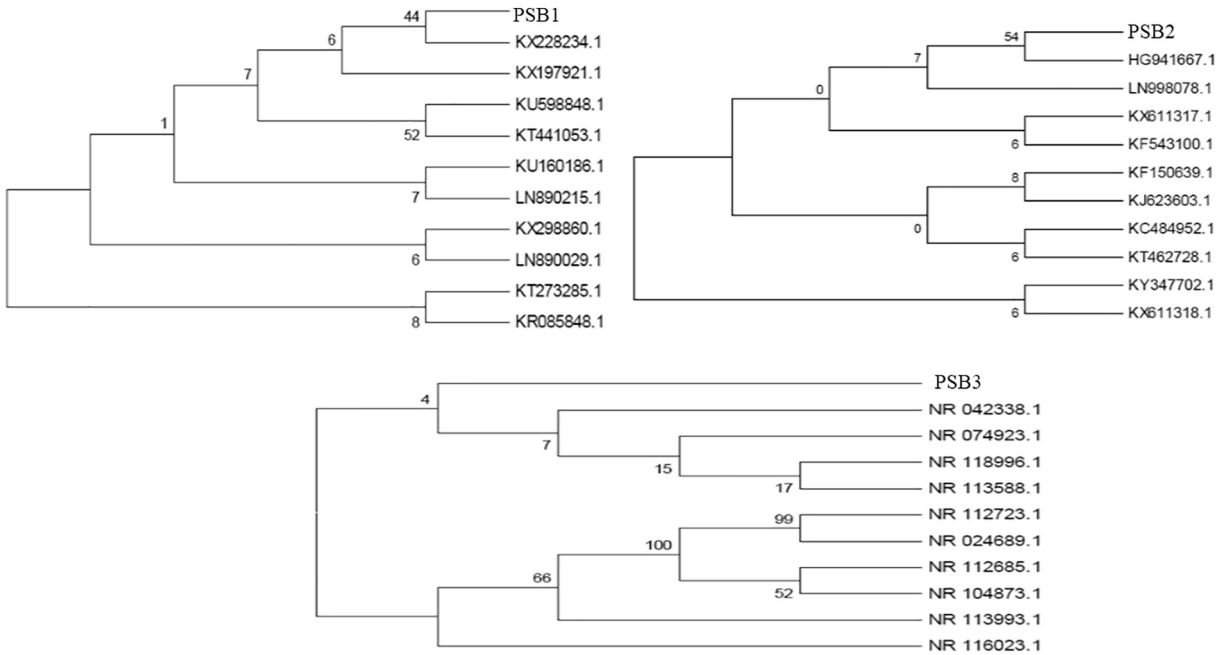

Fig. 1. Molecular identification of *Bacillus megaterium* (MF 589715) (PSB1), *Staphylococcus haemolyticus* (MF 589716) (PSB2) and *Bacillus licheniformis* (MF 589720) (PSB3) strains.

(KF543100.1), *S. haemolyticus* strain SH6 (KF150639.1) and PSB3 showed 99% similarity with *B. licheniformis* strain DSM 13 (NR_118996.1), *B. licheniformis* strain NBRC 12200 (NR_113588.1), *B. licheniformis* strain BCRC 11702 (NR_116023.1) (Fig. 1).

The 16S rRNA gene sequence of strains isolated was submitted to Gene Bank under accession numbers MF 589715 for PSB1, MF 589716 for PSB2 and MF 589720 for PSB3.

3.2. pH and NaCl tolerance

For all the three strains, the optimum pH for attaining the maximum growth was 7. However, the organisms could also grow in a range of pH from 5 to 10. Among the strains, PSB1 could tolerate slightly higher alkalinity (pH = 8–10) than that for other strains (LSD test; $P < 0.05$). The potential PSB strains tolerated upto 8% of NaCl in the culture media. For all the strains the maximum growth was observed at 2% NaCl concentration (Fig. 2). However, PSB3 could tolerate comparatively less salinity. Complete growth inhibition of all strains occurred at 10% of NaCl ($P < 0.05$).

3.3. Resistance of PSB strains to Cu and Zn

The isolated strains could tolerate varying concentrations of Cu and Zn. The maximum tolerable concentration (MTC) of PSB1 was 2 mM for Cu and Zn. On the other hand, PSB2 and PSB3 could tolerate Cu significantly higher (MTC = 6 mM for both); however, in the case of Zn, these two strains responded differently as MTC was 2 for PSB2 and 5 for PSB3.

3.4. Estimation of phosphate solubilizing efficiency

The phosphate solubilizing efficiency of isolated strains in Pikovskaya's broth indicated that the strains efficiently solubilized inorganic phosphate in the medium containing tri-calcium phosphate. In the broth of all the three strains, the concentrations of solubilized phosphate increased gradually from 24 h to 96 h and almost stabilized after that. The phosphate was solubilized at its maximum at 96 h incubation in all treatments with and without metal solutions resulting in the order of PSB3 > PSB1 > PSB2 (LSD test; $P < 0.05$; Fig. 3a). For instance, PSB3 produced 222 ± 2.0 mg L^{-1} soluble phosphate followed by PSB1 (213.7 ± 1.3 mg L^{-1}) and PSB2 (193.5 ± 1.5 mgL^{-1}) at 96 h incubation.

Solubilization of phosphate was also observed in the presence of Cu and Zn but the solubilization efficiency of the strains was less than that of the medium without Cu and Zn. In the presence of Cu, the solubilization followed the order PSB2 > PSB3 > PSB1 (LSD test; $P < 0.05$). PSB2 produced 162.6 ± 2.4 mg L^{-1} soluble phosphate followed by 153.2 ± 2.8 mg L^{-1} and 135.9 ± 0.01 mg L^{-1} soluble phosphate by PSB3 and PSB1 respectively at 96 h incubation. In the presence of Zn, PSB3 produced maximum soluble phosphate of 156.8 ± 1.2 mg L^{-1} followed by PSB1 and PSB2 producing 138 ± 0.9 mg L^{-1} and 136.8 ± 1.1 mg L^{-1} soluble phosphate respectively at 96 h incubation.

In liquid medium, the solubilization of tri-calcium phosphate by the isolated strains was accompanied by a significant decline in pH of the culture supernatant in case of both metal and nonmetal medium from an initial pH of 7.0 ± 0.2. Maximum pH drop of 3.7 ± 0.1 was observed for PSB3 followed by 4.3 ± 0.25 and 5.2 ± 0.01 for PSB1 and PSB2 respectively in the absence of Cu and Zn solutions at 96 h incubation. In the presence of Cu, pH declined from 7.0 to 5.5 ± 0.05, 5.6 ± 0.1 and 5.7 ± 0.1 for PSB2, PSB1 and PSB3, respectively at 96 h incubation. In the presence of Zn, pH declined from 7.0 to 4.8 ± 0.1 for PSB3, from 7.0 to 5.4 ± 0.1 for PSB1 and from 7.0 to 5.4 ± 0.05 for PSB2, at 96 h incubation (Fig. 3b).

3.5. Characterization for IAA production

The IAA production for all the three strains was directly proportional to the L-tryptophan concentration under different incubation period (R^2 = 0.995 and 0.984), and their incubation period (R^2 = 0.922 and 0.996) at different concentrations (Table 2) while no IAA was produced in the absence of L-tryptophan. In all the cases the production of IAA was better for 5 mg mL^{-1} L-tryptophan after 12 days incubation rather than at 2 mg mL^{-1} and 6 days incubation. PSB2 produced less amount of IAA (26.65 ± 1.4 µg mL^{-1} IAA at 5 mg mL^{-1} L-tryptophan after 12 days of incubation) as compared to PSB1 and PSB3; PSB3 produced maximum IAA among the three isolates, with 41.43 ± 0.6 µg mL^{-1} IAA at 5 mg mL^{-1} L-tryptophan concentration after 12 days of incubation. PSB1 produced 38.56 ± 0.6 µg mL^{-1} IAA

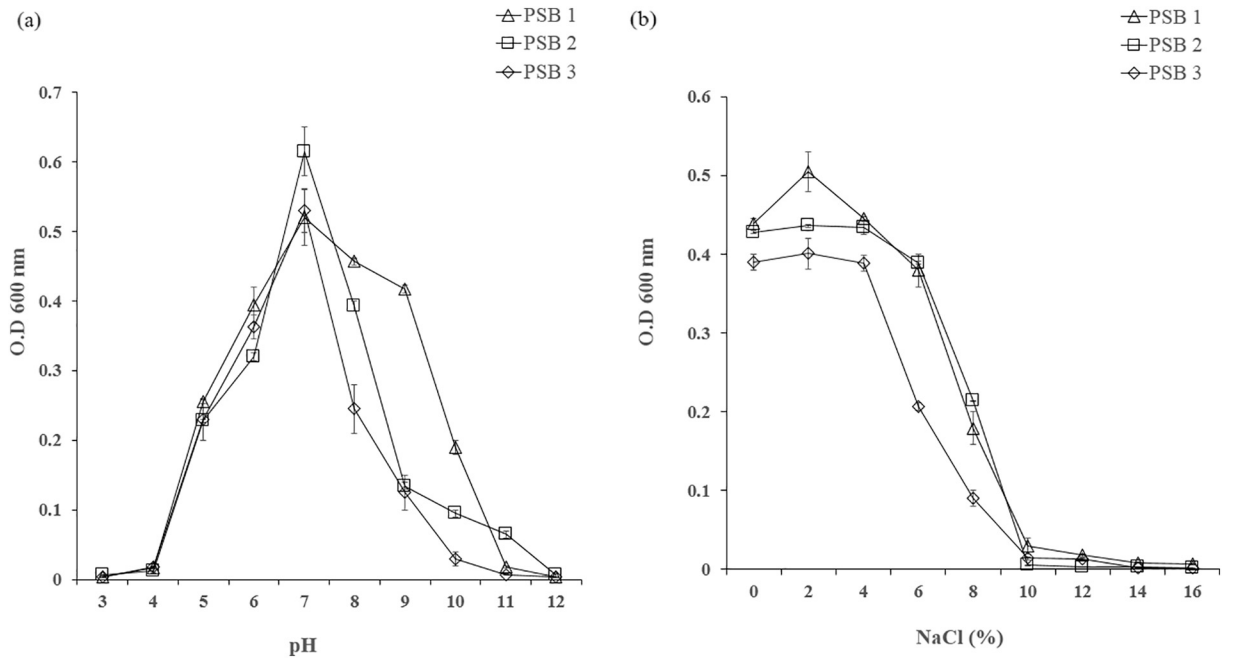

Fig. 2. Effect of pH (a) and NaCl (b) on *Bacillus megaterium* (PSB1), *Staphylococcus haemolyticus* (PSB2), *Bacillus licheniformis* (PSB3) strains.

at 5 mg mL^{-1} L-tryptophan concentration after 12 days of incubation.

3.6. Ammonium ion production

The production of ammonium ion increased with an increase in incubation period. Ammonium ion production potential was in the order of PSB3 > PSB1 > PSB2 (LSD test; $P < 0.05$). PSB3 produced 253 mg L^{-1}, PSB1 produced 238 mg L^{-1} and PSB2 produced 176 mg L^{-1} ammonium ion after 7 days of incubation. The production of ammonium ion reached its saturation point after 4 days of incubation (Fig. 4).

3.7. Seed germination

The treatment of bacterial strains on *Vigna radiata* seeds had a significant effect ($P < 0.05$) on the germination rate, vigor index, root length, shoot length, fresh weight and dry weight as compared to those of the control. However, these effects of potential PSB varied depending on the isolates. In all the germination and growth indices, there appeared significant differences among the impact of isolated strains on seed germination in the order: PSB3 > PSB1 > PSB2 ($P < 0.05$; Table 3).

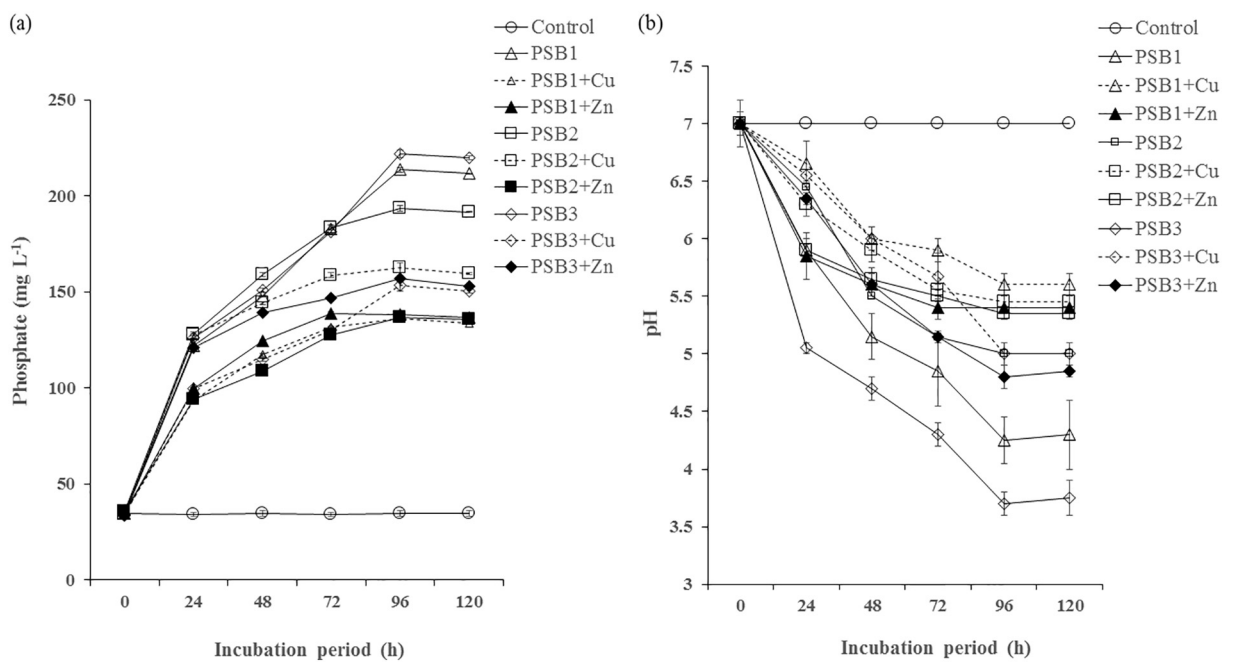

Fig. 3. Phosphate solubilization by *Bacillus megaterium* (PSB1), *Staphylococcus haemolyticus* (PSB2), *Bacillus licheniformis* (PSB3) strains (a) phosphate solubilization in presence and absence of Cu and Zn (b) decline in pH after solubilization in presence and absence of Cu and Zn.

Table 2
IAA production by bacterial isolates PSB1, 2 and 3 in presence of L-tryptophan. The numerical values represent means of replicates ± standard deviations. The different letters in superscript (a, b, c) indicate the significant difference at 95% level of confidence, based on LSD test in ANOVA.

Organism	L-Tryptophan (mg mL^{-1})	IAA production (μg mL^{-1})	
		6 days	12 days
PSB1	2	14.61 ± 1.5^{b}	28.89 ± 1.0^{b}
	5	18.86 ± 1.4^{a}	38.56 ± 0.6^{b}
PSB2	2	8.97 ± 1.0^{c}	22.93 ± 2^{c}
	5	12.71 ± 1.3^{b}	26.65 ± 1.4^{c}
PSB3	2	16.5 ± 1.5^{a}	31.87 ± 2^{a}
	5	19.37 ± 1.4^{a}	41.43 ± 0.6^{a}

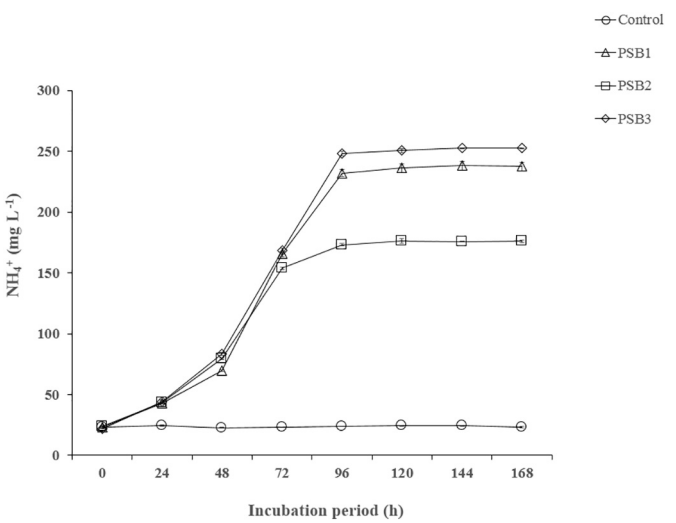

Fig. 4. Ammonia production potentials of *Bacillus megaterium* (PSB1), *Staphylococcus haemolyticus* (PSB2), *Bacillus licheniformis* (PSB3) strains.

4. Discussion

Earthworms are considered as natural bioreactors where microorganisms proliferate and provide favorable conditions for mineralization (Rorat et al., 2017). Earthworm passes comminuted organic matter through their gut, thereby increasing the surface area for the gut associated microbes and their digestive enzymes to act upon the matter which finally leaves the gut in partially digested form as 'casts' (Lazcano et al., 2008). The earthworm casts are known to be enriched in base exchangeable phosphorus, potassium, and total exchangeable calcium that enhance soil fertility. The release of P is performed by phosphatases and phosphate-solubilizing microbes which have been reported to have their enteric origin in the earthworms (Bhat et al., 2017). The present study has successfully isolated three PSB strains namely, *Bacillus megaterium* (MF 589715), *Staphylococcus haemolyticus* (MF 589716) and *Bacillus licheniformis* (MF 589720) from the gut of endogeic earthworm *Metaphire posthuma*, and assessed their potential in plant growth promotion under metal-free and metal-contaminated conditions. The presence of related bacteria, such as *Bacillus* sp. and *Staphylococcus* sp. was also reported in the gut of earthworms (Pathma and Sakthivel, 2012). Although the optimum growth conditions for the three strains were not different (pH = 7.0, salinity ~2% NaCl), the *Bacillus* strains (PSB3 and PSB1) could solubilize higher amount of phosphate than *Staphylococcus* strain (PSB2). The phosphate solubilizing efficiency by *Bacillus* sp. has also been reported elsewhere (Mohamed and Almaroai, 2017). With regard to the incubation time required for maximum P solubilization (Fig. 3a), the result in the present work were in conformity with other reports where maximum phosphate solubilization efficiency of isolated *Bacillus* sp. was at 96 h incubation (Banerjee et al., 2010). This might be due to the cellular growth of the strains that reached its exponential phase. It was also observed that phosphate solubilization was directly related to the drop in pH as reported in *Pseudomonas aeruginosa* as well as fungus *Trichoderma* sp. (Kapri and Tewari, 2010; Paul and Sinha, 2016). In the above study, the acidification of the culture supernatants clearly indicates that the involvement of low molar mass organic acids secreted by microorganisms is the principal cause of phosphate solubilization (Khan et al., 2013). 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 phosphate solubilization potential of the studied bacteria in presence of Cu and Zn is further supported by the semi-quantitative index as "tolerable metal concentration" that the bacterial isolates could resist the direct exposure of Cu and Zn (Section 3.3.). There are reports on the decrease in phosphate solubilization in presence of metals (Paul and Sinha, 2016). The toxicity of metals towards the gut isolates under metal stress condition might be responsible for the decrease in phosphate solubilization (Onyia et al., 2014). Decrease in phosphate solubilization was not due to the precipitation of phosphate with metal ions, since there was no change in the uninoculated control PKM medium supplemented with metals Cu and Zn. However, the metal resistant capacity (2–6 mM of Cu and Zn) of the isolated strains observed in this study could certainly be beneficial to soil productivity in soil environment contaminated with Cu and Zn. As obtained in this case, *Bacillus* sp. offered potential in metal remediation and plant growth promotion, which was supported by other metal-resisting *Bacillus* spp. in soils (Zaidi et al., 2006; Rajkumar et al., 2008). Recently, the use of metal resistant bacteria in soils has received greater attention since bacteria affect mobility of toxic elements and their uptake by plants through various reactions such as metal biosorption, oxidation/reduction and metal-ligand complexation etc. (Mohamed and Almaroai, 2017).

There are also various mechanisms proposed for the phosphate induced immobilization of metals, which include direct metal adsorption by P compounds, phosphate anion-induced metal adsorption, direct precipitation of metals with P adsorption by P compounds, direct precipitation of metals with P in solution as metal phosphates precipitation through the liming action of rock phosphate, and rhizosphere

Table 3
Effect of enteric bacterial isolates PSB1, 2 and 3 on *Vigna radiata* seed germination. The numerical values represent means of replicates ± standard deviations. Means of replicates (n = 3) ± standard deviations. The different letters in superscript (a, b, c) indicate the significant difference at 95% level of confidence, based on LSD test in ANOVA.

Seed germination parameters	Unit	Control	PSB1	PSB2	PSB3
Germination rate	%	85 ± 5^{c}	100 ± 2^{a}	95 ± 5^{b}	100 ± 3^{a}
Vigor index	%	635.2 ± 13.3^{d}	1188.5 ± 48^{b}	1100 ± 36^{c}	1298 ± 23^{a}
Average root length	cm	4.8 ± 0.15^{c}	7.9 ± 0.39^{b}	7.9 ± 0.3^{b}	8.4 ± 0.2^{a}
Average shoot length	cm	2.3 ± 0.42^{d}	4.2 ± 0.05^{b}	3.7 ± 0.05^{c}	4.6 ± 0.025^{a}
Fresh weight	g	4.7 ± 0.16^{d}	9.9 ± 0.18^{b}	9.4 ± 0.43^{c}	11.51 ± 0.18^{a}
Dry weight	g	0.52 ± 0.01^{c}	1.11 ± 0.43^{a}	1.02 ± 0.023^{b}	1.14 ± 0.016^{b}

modification through acidification and mycorrhizal association (Park et al., 2010).

In addition to the metal-tolerance capacity, the isolates also showed plant growth-promoting functions (Table 3). For instance, the effect of PSB on seed germination of *Vigna radiata* was, in accordance to the other indices, higher than without any isolates. One of the most commonly reported mechanisms of improved seed germination is the production of phytohormones such as IAA (Patten and Glick, 2002). Enhanced seed germination in the presence of IAA producing bacteria has also been observed (Islam et al., 2016). The IAA production test in the present study, confirmed that the production of IAA was tryptophan dependent (Table 2). L-Tryptophan being the precursor for IAA production has been used here to examine the effects of L-tryptophan on IAA production by the isolated strains. The isolated strains showed tryptophan dependent IAA production. IAA producing microorganisms can increase root growth and root length of plants by stimulating plant cell elongation or effecting cell division, which constitutes a greater root surface area that enables the plant to get more nutrients from the soil (Davies, 2010; Glick, 2012).The ability of *Bacillus* sp. and *Staphylococcus* sp. in IAA production has also been reported in the literature (Kayastha et al., 2013; Rajkumar et al., 2013; Orhana, 2016; Alibrandi et al., 2017). The increase in the amount of IAA in the presence of tryptophan in the medium is in agreement with other reports where keratinolytic *Stenotrophomonas maltophilia* and *Bacillus subtilis* showed a similar response (Tamreihao et al., 2017). Kadyan et al. (2013) also isolated IAA-producing bacterial strains from rhizospheric soil and reported that those isolates were *Jeotgalibacillus* sp., *T. saccharophilus*, *T. goriensis*, *B. megaterium*, *B. simplex* and *B. aryabhattai*. Nitrogen and nitrogen-containing compounds play an essential role for the growth of plants (Orhana, 2016). Ammonia is taken up by plants as nitrogen source. In general, soluble phosphates and ammonia directly support plant growth as they act as macro-nutrients (Bhattacharyya and Jha, 2012). Here also all the three strains (PSB1, PSB2, and PSB3) isolated from the gut of the earthworm were able to produce ammonia with increasing incubation period (4–7 days) (Fig. 4). Similar to the performance for the other tests (e.g. phosphate-solubilization, metal tolerance, and IAA production) conducted in the present study, *Bacillus* strains (PSB3 and PSB1) were more potent in ammonia production than *Staphylococcus* (PSB2). The production of ammonia by these bacteria could also be an important trait within the properties of plant growth promotion (Ahmad et al., 2008; A. Singh et al., 2016; P. Singh et al., 2016). Reports also suggested that several stains of *Oceanobacillus* sp., *Halomonas* sp., *Exiguobacterium* sp., *Zhihengliuella* sp. and *Bacillus* sp. isolated from coastal soil could be ammonia producing bacteria (Siddikee et al., 2010). In this study, the 16S rRNA-based identification revealed that two *Bacillus* sp. and one *Staphylococcus* sp. had a high potential for ammonium and ammonia production that supported the growth of a model plant (*Vigna radiata*); the potentials of these two groups of bacteria have also been reported by Orhana (2016).

5. Conclusions

In the present study, we isolated potent PSB from the gut of an endogeic earthworm *Metaphire posthuma*. The isolated *Bacillus megaterium* (MF 589715), *Staphylococcus haemolyticus* (MF 589716) and *Bacillus licheniformis* (MF 589720) strains showed promising results towards seed germination and plant growth promoting potentials like IAA production, phosphate solubilization, and ammonium ion production. The ability of the strains to resist metals Cu and Zn and also their ability to solubilize phosphate in the presence of these metals show their potential to be used as a plant growth promoter under environmental stress conditions. Among the identified strains *Bacillus* sp. (PSB3 and PSB1) showed significantly higher performance than the *Staphylococcus* sp. This bench scale findings would contribute significant insights for the use of integration of earthworm and associated bacteria as the powerful biofertilizer in the sustainable crop production. Future studies can also be focused on the beneficial effects of these strains under long-term field conditions and with the adverse and changing environmental conditions, such in the presence of multiple emerging contaminants in the soils.

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References

Ahmad, F., Ahmad, I., Khan, M.S., 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res. 163, 173–181.

Ali, U., Sajid, N., Khalid, A., Riaz, L., Rabbani, M., Syed, J.H., Malik, R.N., 2015. A review on vermicomposting of organic wastes. Environ. Prog. Sustain. Energy 34, 1050–1062.

Alibrandi, P., Cardinale, M., Rahman, M.M., Strati, F., Ciná, P., de Viana, M.L., Giamminola, E.M., Gallo, G., Schnell, S., de Filippo, C., Ciaccio, M., Puglia, A.M., 2017. The seed endosphere of *Anadenanthera colubrina* is inhabited by a complex microbiota, including *Methylobacterium* spp. and *Staphylococcus* spp. with potential plant-growth promoting activities. Plant Soil. http://dx.doi.org/10.1007/s11104-017-3182-4.

Andriuzzi, W.S., Schimidt, O., Brussaard, L., Faber, J.H., Bolger, T., 2016. Earthworm functional traits and interspecific interactions affect plant nitrogen acquisition and primary production. Appl. Soil Ecol. 104, 148–156.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC.

Arai, M., Miura, T., Tsuzura, H., Minamiya, Y., Kaneko, N., 2017. Two-year responses of earthworm abundance, soil aggregates, and soil carbon to no-tillage and fertilization. Geoderma. http://dx.doi.org/10.1016/j.geoderma.2017.10.021.

Aria, M., Dominguez, J., 1998. Changes in nutrient pools, microbial biomass and microbial activities in soil after transit through gut of three endogeic earthworm species of genus *Prostandilus* Qui and Bouche. J. Soils Sediments 14, 1335–1340.

Banerjee, S., Palit, R., Sengupta, C., Standing, D., 2010. Stress induced phosphate solubilization by *Arthrobacter* sp. and *Bacillus* sp. isolated from tomato rhizosphere. Aust. J. Crop. Sci. 4, 378–383.

Benson, H.J., 2015. Microbiological Application Laboratory Manual in General Microbiology, thirteenth ed. McGraw Hill Education, New York.

Bhat, S.A., Singh, J., Vig, A.P., 2017. Earthworms as organic waste managers and biofertilizer producers. Waste Biomass Valoriz. http://dx.doi.org/10.1007/s12649-017-9899-8.

Bhattacharyya, P., Jha, D., 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28, 1327–1350.

Biswas, J.K., Banerjee, A., Majumder, S., Bolan, N., Seshadri, B., Dash, M.C., 2017. New extracellular polymeric substance producing enteric bacterium from earthworm, *Metaphire posthuma*: modulation through culture conditions. Proc. Zool. Soc. http://dx.doi.org/10.1007/s12595-017-0250-y.

Byzov, B.A., Tikhonov, V.V., Nechitailo, T.Y., Demin, V.V., 2015. Taxonomic composition and physiological and biochemical properties of bacteria in the digestive tracts of earthworms. Eur. J. Soil Sci. 48, 268–275.

Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A., Young, C.C., 2006. Phosphate solubilizing bacteria from subtrophical soil and their Tricalcium phosphate solubilizing abilities. Appl. Soil Ecol. 34, 33–41.

Clause, J., Barot, S., Forey, E., 2015. Effects of cast properties and passage through the earthworm gut on seed germination and seedling growth. Appl. Soil Ecol. 96, 108–113.

Davies, P.J., 2010. The plant hormones: their nature, occurrence and functions. In: Davies, P.J. (Ed.), Plant Hormones. Springer, Dordrecht, pp. 1–15.

Doelman, P., Haanstra, L., 1989. Short- and long-term effects of heavy metals on phosphatase activity in soils: an ecological dose-response model approach. Biol. Fertil. Soils 8, 235–241.

Drake, H.L., Horn, M.A., 2006. Earthworms as a transient heaven for terrestrial denitrifying microbes: a review. Eng. Life Sci. 6, 261–265.

Eisenhauer, N., Cesarz, S., Koller, R., Worm, K., Reich, P.B., 2012. Global change below ground: impacts of elevated CO_2, nitrogen, and summer drought on soil food webs and biodiversity. Glob. Chang. Biol. 18, 435–447.

Glick, B.R., 2012. Plant growth-promoting bacteria: mechanisms and applications. Scientifica 15. http://dx.doi.org/10.6064/2012/963401.

Horn, M.A., Ihssen, J., Matthies, C., Schramm, A., Acker, G., Drake, H.L., 2005. *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. Int. J. Syst. Evol. Microbiol. 55, 1255–1265.

Hussain, N., Singh, A., Saha, S., Kumar, M.V.S., Bhattacharya, P., Bhattacharya, S.S., 2016. Excellent N-fixing and P-solubilizing traits in earthworm gut isolated bacteria: a vermicompost based assessment with vegetable market waste and rice straw feed mixtures. Bioresour. Technol. 222, 165–174.

Islam, S., Akanda, M.A., Prova, A., Islam, M.T., Hossain, M.M., 2016. Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. Front. Microbiol. 6, 1360.

Jebelli, M.A., Maleki, A., Amoozegar, M.A., Kalantar, E., Shahmoradi, B., Gharibi, F., 2017. Isolation and identification of indigenous prokaryotic bacteria from arsenic-contaminated water resources and their impact on arsenic transformation. Ecotoxicol. Environ. Saf. 140, 170–176.

Kadyan, S., Panghal, M., Kumar, S., Singh, K., Yadav, J.P., 2013. Assessment of functional and genetic diversity of aerobic endospore forming Bacilli from rhizospheric soil of *Phyllanthus amarus* L. World J. Microbiol. Biotechnol. 29, 1597–1610.

Kapri, A., Tewari, L., 2010. Phosphate solubilization potential and phosphatase activity of rhizospheric *Trichoderma* spp. Braz. J. Microbiol. 41, 787–795.

Kayastha, M., Kumar, V., Gera, R., 2013. Exploring the potential of PGPR strain *Bacillus licheniformis* to be developed as multifunctional biofertilizer. Cent. Eur. J. Exp. Biol. 2, 12–17.

Khan, M.S., Ahmed, E., Zaidi, A., Oves, M., 2013. Bacteria in agro biology: crop productivity. In: Maheshwari, D., Saraf, M., Aeron, A. (Eds.), Bacteria in Agrobiology: Crop Productivity. Springer, Berlin, Heidelberg, pp. 237–265.

Khan, M.S., Zaidi, A., Ahmed, E., 2014. Mechanism of phosphate solubilization and physiological functions of phosphate-solubilizing microorganisms. In: Khan, M.S., Zaidi, A., Mussarat, J. (Eds.), Phosphate Solubilizing Microorganisms. Springer International Publishing, Switzerland, pp. 31–62.

Koch, M., Kruse, J., Eichler-Löbermann, E., Zimmer, D., Willbold, S., Leinweber, P., Siebers, N., 2018. Phosphorus stocks and speciation in soil profiles of a long-term fertilizer experiment: evidence from sequential fractionation, P K-edge XANES, and ^{31}P NMR spectroscopy. Geoderma 316, 115–126.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. Chichester, John Wiley & Sons, United Kingdom, pp. 115–175.

Lazcano, C., Gomez-Brandon, M., Dominguez, J., 2008. Comparison of the effectiveness of composting and vermicomposting for the biological stabilization of cattle manure. Chemosphere 72, 1013–1019.

Liu, D., Lian, B., Wu, C., Guo, P., 2017. A comparative study of gut microbiota profiles of earthworms fed in three different substrates. Symbiosis. http://dx.doi.org/10.1007/s13199-017-0491-6.

Ma, Y., Rajkumar, M., Freitas, H., 2009. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. J. Environ. Manag. 90, 831–837.

Mohamed, H.M., Almaroai, Y., 2017. Effect of phosphate solubilizing bacteria on the uptake of heavy metals by corn plants in a long-term sewage wastewater treated soil. Int. J. Environ. Sci. Develop. 8, 366–371.

Onyia, C.E., Anyanwu, C.U., Onuigbo, A.C., Mbegbu, E., 2014. Impact of heavy metals and initial pH variations on the phosphate solubilizing fungi (PSF) isolated from Nsukka pepper and garden-egg plants rhizosphere. J. Pharm. Biol. Sci. 9, 2278–3008.

Orhana, F., 2016. Environmental microbiology alleviation of salt stress by halotolerant and halophilic plant growth-promoting bacteria in wheat (*Triticum aestivum*). Braz. J. Microbiol. 47, 621–627.

Park, J., Bolan, N., Megharaj, M., Naidu, R., 2010. Isolation of phosphate-solubilizing bacteria and characterization oftheir effects on lead immobilization. Pedologist 53, 67–75.

Pathma, Jr, Sakthivel, N., 2012. Microbial diversity of vermicompost bacteria that exhibit useful agricultural traits and waste management potential. Springerplus 1. http://dx.doi.org/10.1186/2193-1801-1-26.

Patten, C.L., Glick, B.R., 2002. Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. Appl. Environ. Microbiol. 68, 3795–3801.

Paul, D., Sinha, S.N., 2016. Isolation and characterization of phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12 with antibacterial potential from river ganga India. Ann. Agrar. Sci. 15, 130–136.

Rajkumar, M., Ma, Y., Freitas, H., 2008. Characterization of metal-resistant plant-growth promoting Bacillus weihenstephanensis isolated from serpentine soil in Portugal. J. Basic Microbiol. 48, 500–508.

Rajkumar, M., Ma, Y., Freitas, H., 2013. Improvement of Ni phytostabilization by inoculation of Ni resistant *Bacillus megaterium* SR28C. J. Environ. Manag. 128, 973–980.

Rorat, A., Wloka, D., Grobelak, A., Grosser, A., Sosnecka, A., Milczarek, M., Jelonek, P., Vandenbulcke, F., Kacprzak, M., 2017. Vermiremediation of polycyclic aromatic hydrocarbons and heavy metals in sewage sludge composting process. J. Environ. Manag. 187, 347–353.

Sanchez-Hernandez, J.C., Mazzia, C., Capowiez, Y., Rault, M., 2009. Carboxylesterase activity in earthworm gut contents: potential (eco)toxicological implications. Comp. Biochem. Physiol. C 150 (4), 503–511.

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A., 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springerplus 2. http://dx.doi.org/10.1186/2193-1801-2-587.

Siddikee, M.A., Chauhan, P.S., Anandham, R., Han, G.H., Tongmin, S.A., 2010. Isolation, characterization, and use for plant growth promotion under salt stress, of ACC deaminase-producing halotolerant bacteria derived from coastal soil. J. Microbiol. Biotechnol. 20, 1577–1584.

Singh, A., Tiwari, R., Sharma, A., Adak, A., Singh, S., Prasanna, R., Saxena, A.K., Nain, L., Singh, R.V., 2016. Taxonomic and functional diversity of the culturable microbiomes of epigeic earthworms and their prospects in agriculture. J. Basic Microbiol. 56, 1009–1020.

Singh, P., Mitra, S., Majumdar, D., Bhattacharyya, P., Prakash, A., Borah, P., Paul, A., Rangan, L., 2016. Nutrient and enzyme mobilization in earthworm casts: a comparative study with addition of selective amendments in undisturbed and agricultural soils of a mountain ecosystem. Int. Biodeterior. Biodegrad. 119, 437–447.

Srivastava, S., Verma, P.C., Singh, A., Mishra, M., Singh, N., Sharma, N., Singh, N., 2012. Isolation and characterization of *Staphylococcus* sp. strain NBRIEAG-8 from arsenic contaminated site of West Bengal. Appl. Microbiol. Biotechnol. 95, 1275–1291.

Sruthy, P.B., Anjana, J.C., Rathinamala, J., Jayashree, S., 2013. Screening of earthworm (*Eudrilus eugeniae*) gut as a transient microbial habitat. Adv. Zool. Bot. 1, 53–56.

Tamreihao, K., Devi, L.J., Khunjamayum, R., Mukherjee, S., Ashem, R.S., Ningthoujam, D.S., 2017. Biofertilizing potential of feather hydrolysate produced by indigenous keratinolytic *Amycolatopsis* sp. MBRL 40 for rice cultivation under field conditions. Biocatal. Agric. Biotechnol. http://dx.doi.org/10.1016/j.bcab.2017.04.010.

Thomason, J.E., Savin, M.C., Brye, K.R., Gbur, E.E., 2017. Native earthworm population dominance after seven years of tillage, burning, and residue level management in a wheat-soybean, double-crop system. Appl. Soil Ecol. 120, 211–218.

Vinotha, S.P., Parthasarathi, K., Ranganathan, L.S., 2000. Enhanced phosphatase activity in earthworm casts is more of microbial origin. Curr. Sci. 79, 1152–1162.

Wahyudi, A.T., Astuti, R.P., Widyawati, A., Meryandini, A., Nawangsih, A.A., 2011. Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. J. Microbiol. Antimicrob. 3, 34–40.

Wang, Y., Shi, J., Wang, H., Lin, Q., Chen, X., Chen, Y., 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. Ecotoxicol. Environ. Saf. 67, 75–81.

Watanabe, F.S., Olsen, S.R., 1965. Test of an ascorbic acid method for determining phosphorous in water and $NaHCO_3$ extracts from soil. Soil Sci. Soc. Am. J. 29, 677–678.

Zaidi, S., Usmani, S., Singh, B.R., Musarrat, J., 2006. Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere 64, 991–997.