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ORIGINAL ARTICLE

Cu-resistant *Kocuria* sp. CRB15: a potential PGPR isolated from the dry tailing of Rakha copper mine

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Abstract Rhizobacteria may enhance biomass production and heavy metal tolerance of plants under stress conditions. The present study was carried out for isolation of metal-resistant bacteria that can be further utilized for phytoremediation process. A potential metal-resistant strain CRB15 was isolated from rhizospheric region of *Saccharum spontaneum* that was found to be resistant against Cu (6.29 mM), Zn (3.25 mM), Pb (1.5 mM), Ni (1.25 mM), and Cd (0.25 mM). SEM analysis was performed for evaluation of morphological changes on bacterial isolate. FTIR analysis observed the change in wavenumbers after the addition of Cu. 16S rDNA sequence analysis showed that CRB15 isolate matched best with genus of *Kocuria* and was named as *Kocuria* sp. CRB15. The isolate *Kocuria* sp. CRB15 was a potential plant growth-promoting rhizobacterium as it had a high IAA (46 µg ml^{-1}), P solubilisation (39.37 µg ml^{-1}), ammonia production (30.46 µmol ml^{-1}), and hydrogen cyanide production capacity. Root–shoot elongation assay conducted on *Brassica nigra* under lab conditions with strain CRB15 demonstrated positive effects of strain CRB15 in root and shoot elongation of Cu-treated seedlings. This study proved the *Kocuria* sp. CRB15 a potential PGPR for bacterial-assisted phytoremediation.

Introduction

Copper (Cu) tailings, produced from mining, smelting, and processing of copper ores, may cause severe destruction to ecosystem including plants, animals, microbes, and human health (Wong 2003). Although, Cu is an essential micronutrient; however, its elevated level may affect the microbially mediated processes. Therefore, it is essential to remove the Cu pollution from the environment. Remediation of polluted sites using the conventional techniques, such as physico-chemical extraction, land filling, soil washing, and excavation, is costly with high energy and chemical consumption (Jeyasingh and Philip 2005). An effective biotechnological approach "Bioremediation" has received increasing attention as an alternative to clean up a polluted environment due to its cost-effective and eco-friendly nature (Wu et al. 2006). To have a crucial role in remediation, microorganisms in heavy metal-contaminated environment must first cope up with the heavy metal stress by means of immobilization or transformation (Ma et al. 2011).

Despite the fact that microbial remediation of Cu has been investigated for many years, there is still considerable interest in the microbe–plant association for heavy metal removal from soil (Taj and Rajkumar 2016; Zaidi et al. 2006). Rhizospheric microbes are considered as important component of phytoremediation technology due to their direct effects to enhanced biomass production and heavy metal tolerance of plants by plant–microbe interaction

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(Glick 2003). Rhizosphere is a complex and dynamic microenvironment for root-microbe association to form unique communities having considerable potential for plant growth promotion (Belimov et al. 2005) and detoxification of hazardous compounds (De Souza et al. 1999). Plant growth-promoting rhizobacteria (PGPR) that solubilize phosphate and synthesize growth-promoting substances (e.g., indole acetic acid, siderophores, ethylene, etc) can be applied in the plant-assisted bioremediation of metal-contaminated soil (Khan et al. 2009).

In the present work, a strain (CRB15) was isolated from rhizospheric region of Cu-tolerant plant, *Saccharum spontaneum* (commonly known as Kans grass) growing on Cu tailing. The metal tolerance and plant growth-promoting abilities of the strain were evaluated.

Materials and methods

Study area and sample collection

The Rakha copper mine selected for this study is situated in the country's prime copper producing belt known as the Singhbhum Copper Belt (SCB) in East Singhbhum, Jharkhand, India. The predominant Cu mineral Chalcopyrite is present with other sulphide minerals, such as pyrite, molybdenite, and marcasite in small quantities. The main gangue minerals include quartz, magnetite, and chlorite (Deb and Sarkar 1973). The climate of the study area is humid tropical. Maximum temperature range to 39 °C and minimum temperature is 10 °C. This area comes under the path of south west (SW) monsoon with average rainfall of about 1400 mm per annum during July to September.

The tailing pond used for sample collection had been abandoned from 2001. Composite sampling of dry tailing from the rhizospheric region of *Saccharum spontaneum* was carried out in January, 2014 and was processed to the laboratory in labelled and air-tight polyethylene bags. The sample was sieved through a <2 mm sieve for removal of plant debris, stones, and soil animals. Part of the sample was kept moist in dark at 4 °C for bacterial isolation. The remaining part of the sample was air-dried at ambient temperature, ground in a porcelain mortar, and sieved through 0.2 mm sieve for chemical analysis.

Analysis of chemical properties

The pH and conductivity (1:2.5; w/v; sample-to-water ratio), organic carbon by rapid dichromate oxidation technique (Walkley and Black 1934), available nitrogen by alkaline permanganate method (Subbiah and Asija 1956), available phosphorus (Bray and Kurtz 1945), and available potassium by ammonium acetate extractant method (Jackson 1973)

were estimated. Total Cu concentration of the sample was determined by acid digestion of 150 mg tailing sample in microwave digestion system (ETHOS One) using a mixture of conc. HNO_3 (68–70%, Merck, EMPARTA), HF (48–51% Merck, EMPARTA), and $HClO_4$ (70% Merck, EMPARTA) in a ratio of 5:1:1 (v/v/v) (Ho et al. 2013) followed by filtration using Whatman no. 42 filter paper and made up to 50 ml with distilled water. Water extractable (WE) (1:8, w/v; sample-to-water ratio) and exchangeable (EX) (1:8, w/v; sample-to-1 M $MgCl_2$ ratio) Cu concentration was also determined. All the samples were analyzed for Cu concentrations by flame atomic absorption spectrophotometer (FAAS, GBC Avanta 200, Australia) using air-acetylene flame. All samples were analyzed in triplicates. The standard solution of Cu was prepared using a stock solution of 1000 mg l^{-1} (Merck) in 1% HNO_3 for calibration.

Total bacterial counts and isolation of copper-resistant bacteria

Erlenmeyer flaks containing 100 ml of sterile physiological salt solution were supplemented with 1.0 g of tailing sample and shaken at 180 rpm for 30 min, followed by tenfold serial dilutions and plating over sucrose-minimal salts low-phosphate (SLP) agar (sucrose 1%; $(NH_4)_2SO_4$ 0.1%; K_2HPO_4 0.05%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; pH 7.0 ± 0.2) for determination of total culturable bacteria. SLP agar amended with 0.8 mM of Cu as $CuSO_4·5H_2O$ was used for determination of copper-resistant bacteria (CRB). After autoclaving, 10 mg fungicidin per litre was supplemented to culture media for inhibition of fungal growth. Plates were incubated at 30 ± 2.0 °C for 48 h. Cu-resistant colonies were purified on the same media by streaking three-to-four times and stored at 4 °C.

Identification of CRB15

The bacterial strain CRB15 was preliminary identified by morphological and biochemical tests using the standard methods mentioned in Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The bacterial strain CRB15 was further identified using 16S rDNA gene sequencing. DNA was isolated from the culture followed by quality evaluation on 0.8% agarose gel, and a single band of high-molecular weight DNA was observed. Isolated DNA was amplified with universal primers 8F (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTAC GACTT-3′) using Veriti® 99 well Thermal Cycler (Model No. 9902). The PCR mixture (25 µl) contained 15.5 µl sterile distilled water, 25 mM $MgCl_2$ (0.5 µl), 10× assay buffer (2 µl), 2.5 mM of dNTPs (0.5 µl), 2 µl each of forward and reverse primers, 2 µl of genomic DNA, and

one unit of Taq DNA polymerase (0.5 µl). The following reaction conditions were followed: initial denaturation step of 94 °C for 3 min, 30 cycles of 30 s for denaturation at 94 °C, 1 cycle of 30 s for annealing at 52 °C, 1 cycle of 1 min for extension at 72 °C, and 1 cycle of 7 min for final extension and stored at 4 °C for further analysis. The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 704F (5′-AGATTTTCCGACGGCAGGTT-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Approximately 1500 bp of PCR product size was observed. All samples were analyzed in triplicate. Both the negative (distilled water + all PCR components) and positive (a DNA template + all PCR components) controls were included in each run.

The 16S rDNA sequence was further subjected to BLAST program and compared against the GenBank database using NCBI Blast program. Gene sequence homology was tested using BLASTn search program (http://www.ncbi.nlm.nih.gov). Gene sequence obtained was aligned by ClustalW using the MEGA 7.0 software, and a neighbour-joining tree was constructed. The gene sequence was submitted to GenBank for assigning of accession number.

Determination of minimum inhibitory concentration (MIC)

The MIC or the lowest concentration of metals at which no growth occurred was determined on the agar plates (He et al. 2010). The isolate, CRB15 was inoculated into SLP agar plates containing different concentrations of $CdSO_4$, $ZnSO_4$, $NiCl_2·6H_2O$, and $Pb(NO_3)_2$ (Merck, EMPARTA). The growing colonies at a given concentration were subsequently transferred to the next higher concentration. The plates were incubated at 30 ± 2.0 °C for 48 h and evaluated for minimum inhibitory concentration. The stock solutions of heavy metals were prepared in double distilled water and sterilized by autoclaving at 121 °C, 15 psi for 15 min.

Effect of Cu on bacterial growth

Effect of Cu on bacterial growth was studied by varying concentration of Cu (0–150 mg l^{-1}) supplemented SLP broth. Culture was grown aerobically in 50 ml medium in 100 ml Erlenmeyer flasks at 30 °C for 24 h. Culture grown in the absence of metal was treated as control. Growth was monitored as a function of biomass by measuring the absorbance at 600 nm against blank in a UV–Visible spectrophotometer (UV-1800, Shimadzu, Japan).

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) analysis

SEM–EDX was carried out to study the surface characteristics of CRB15. During SEM analysis, the cells in the growth medium containing 0–100 mg Cu l^{-1} were prepared and fixed with 0.25% glutaraldehyde at 4 °C overnight, then washed three times with sodium phosphate buffer solution followed by dehydration using a gradient series with ethanol. Samples for SEM analysis were mounted on cover glass and allowed for air drying. The samples were then mounted directly on the specimen metal disc using an adhesive tape. Scanning of samples was carried out at different areas using FE-SEM Supra 55 (Germany). The SEM analysis was carried out under the following analytical conditions: EHT = 10.00 kV, WD = 2.4 mm, Signal A = InLens.

FTIR analysis

FTIR analysis was carried out for determination of functional groups responsible for adhering of Cu(II) to the cell wall. The analysis was determined using dried samples of CRB15 in different copper concentrations and recorded in KBr pellets at room temperature using an FTIR spectrometer (PerkinElmer 2000). The sample/KBr mass ratio was 1:100 and analyzed in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

Plant growth-promoting characteristics of CRB15

Characterization of bacterial indole-3-acetic acid (IAA)

The bacterial strain CRB15 was analyzed for indole-3-acetic acid production in the presence and absence of L-tryptophan and determined by colorimetric analysis (Zaidi et al. 2006). Cells grown in nutrient broth medium in the exponential phase were collected by centrifugation at 10,000 rpm for 15 min at 6 °C, followed by washing with sterile physiological salt solution, and re-centrifugation. An inoculum density of 10^8 colony forming units (cfu) ml^{-1} was prepared by resuspending pelleted cells in sterile physiological salt solution. Bacterial suspensions (1 ml) were cultured for 5 days in flasks containing 20 ml of sucrose-minimal salts (SMS) medium [sucrose 1%; $(NH_4)_2SO_4$ 0.1%; K_2HPO_4 0.2%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; and pH 7 ± 0.2] supplemented with 0–600 µg ml^{-1} of L-tryptophan. After each 24 h of incubation, bacterial culture was centrifuged at 9000g for 15 min. 2 ml of supernatant was collected and mixed with 100 µl of 10 mM orthophosphoric acid and 4 ml of Salkowski reagent (0.5 M FeCl$_3$ in 35% HClO$_4$) and allowed to stand at room temperature for

20 min, after which pink colour developed in the cell suspensions (Gordon and Weber 1951). The absorbance of pink colour was read at 530 nm using UV–Visible spectrophotometer. The IAA concentration in culture was determined using a calibration curve of pure IAA as a standard following the linear regression analysis.

Mineral phosphate solubilizing activity

To analyze the phosphate solubilizing activity, CRB15 was plated onto Pikovskaya's agar medium (Zaidi et al. 2006) with 0.5% of tricalcium phosphate as the inorganic phosphate source. The plates were incubated at 28 ± 2 °C for 72 h, a clear halo around the bacterial colonies indicating the phosphate solubilisation capacity of the bacterial strains. The quantitative estimation of phosphate solubilisation was carried out on Pikovskaya's broth medium, and the change in pH was also observed. The amount of phosphate released in the Pikovaskaya's was quantitatively measured using chlorostannous reduced molybdophosphoric acid blue method (Jackson 1973).

Evaluation of the hydrogen cyanide (HCN) production

The isolate was screened for the production of hydrogen cyanide by adapting the method of Lorck (1948). Briefly, nutrient agar was amended with 4.4 g glycine l^{-1} and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 35 ± 2 °C for 4 days. Development of orange to red colour indicated HCN production.

Production of ammonia

Bacterial isolate CRB15 was tested for ammonia production in peptone water (Cappuccino and Sherman 1992). Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 24–120 h at 30 ± 2 °C. Culture supernatant was mixed with Nessler's reagent (0.5 ml), and volume was adjusted by adding ammonia-free distilled water. Development of yellow-to-brown colour was measured at 450 nm using UV–VIS spectrophotometer. The ammonia concentration in culture was determined using a calibration curve of pure ammonium sulphate as a standard following the linear regression analysis. The analysis was performed in triplicate.

Plant elongation assay on filter paper culture

Based on the plant growth-promoting (PGP) activities of the bacterial strain CRB15, the isolate was used for the plant elongation assay. The plant elongation activity of the bacteria was determined using the modified protocol of Belimov et al. (2001, 2005). Bacteria were grown on nutrient broth medium for 48 h at 30 ± 2 °C and resuspended in sterile distilled water to adjust cell density of 5×10^7 CFU ml^{-1}. 6 mL of bacterial suspensions with 0.5 mM Cu (treatment) as $CuSO_4 \cdot 5H_2O$ was added to glass petri dishes with filter paper. Bacterial suspensions without Cu were considered as control. Blank tests were performed using sterile distilled water or Cu. The seeds of *Brassica nigra* were surface-sterilized with a mixture of ethanol and 30% H_2O_2 (1:1) for 20 min followed by washing with sterile distilled water and aseptically placed on wetted filter paper. Root length and shoot length of seedlings were measured after incubation of closed petri dishes for 7 days at 28 °C in the dark. The assay was repeated three times with four dishes (with ten seeds per dish) for each treatment.

Quality control and quality assurance

Analytical grade, chemicals, and culture media were used for PCR and microbiological analyses, and double distilled deionized Millipore water (Milli-Q system, Millipore) was used for preparation of all reagents and calibration standards. Calibrated glassware was used for experimental work. To avoid other microbial contamination, special care was taken to transfer the samples from the sampling site to laboratory.

Statistical analysis

The experiments were carried out in triplicates. The statistical analyses were conducted to find out mean and standard deviation using MS-Excel 2007 (Microsoft Inc.).

Results and discussion

Chemical properties of tailing

The chemical properties of samples are summarized in Table 1. The pH and electrical conductivity (EC) of the collected samples were 4.14 and 0.29 dS m^{-1}, respectively, with low organic carbon and low available N, P, and K content. The continuous oxidation of pyrite waste was responsible for acidic nature of tailing (Das and Maiti 2008). Along with total concentration of Cu, water extractable and exchangeable forms of Cu were also determined. This is because, the total metal concentration does not describe the bioavailable forms of metal. The concentrations of the total Cu, WE Cu, and EX Cu were 952.83, 10.81, and 14.21 mg kg^{-1}, respectively.

Table 1 Chemical properties and bacterial count of dry tailing

Parameters	Mean $\pm$ SD	Min–max	Median
pH (1:2.5)	4.14 ± 0.05	4.1–4.2	4.13
EC (1:2.5; dS m^{-1})	0.29 ± 0.019	0.281–0.318	0.29
Organic carbon (g kg^{-1})	2.07 ± 0.31	1.8–2.4	2.0
Av. N (mg kg^{-1})	71.97 ± 6.67	64.98–78.27	72.67
Av. P (mg kg^{-1})	1.37 ± 0.23	1.12–1.58	1.42
Av. K (mg kg^{-1})	33.03 ± 3.89	29.57–37.24	32.28
WE Cu (mg kg^{-1})	10.81 ± 1.44	9.25–12.11	11.08
EX Cu (mg kg^{-1})	14.21 ± 5.71	7.84–18.88	15.92
Total Cu (mg kg^{-1})	952.83 ± 160.68	948.7–1115.54	794.25
TBC[a]	138 ± 21.07	116–158	140
CRBC[a]	23 ± 4.35	18–26	25

TBC total bacterial counts, *CRBC* Cu-resistant bacterial counts

[a] Values indicate CFU $\times 10^5$ g^{-1}

Bacterial isolation

The average CFUs of total bacterial count (TBC) and Cu-resistant bacterial count (CRBC) were reported (Table 1). The TBC reported was 138×10^5 CFU g of dry tailing, whereas CRBC was 23×10^5 CFU g of dry tailing. Similar trend between TBC and CRBC was also reported by He et al. (2010). The 15 morphologically different Cu(II) resistant bacterial strains were screened for their Cu(II) tolerance limit. One of the potential strain CRB15 showing a high degree of Cu resistance was selected for further studies.

Identification of CRB15 isolate

The morphological, physiological, and biochemical characteristics (Table 2) and comparative analysis of the sequence with already available sequences in NCBI showed that the strain CRB15 was close to the member of genus *Kocuria*. Phylogenetic tree (Fig. 1) was constructed by neighbour-joining method of MEGA7.0 using 16S rDNA sequences of species belonging to genus *Kocuria* along with 16S rDNA sequence of CRB15. The 16S rDNA gene sequence was submitted to NCBI database under the accession number KX417302.1.

Resistance to heavy metals

The strain CRB15 was observed for very high degree of resistance against selected heavy metals. MIC values varied from 0.25 to 3.25 mM (Table 3). CRB15 showed resistance against 3.25 mM of Zn, 1.5 mM of Pb, 1.25 mM of Ni, and 0.25 mM of Cd, respectively. Among the heavy metals, Zn and Pb were less toxic, whereas Cd and Ni were highly toxic to strain CRB15. The order of resistance of

Table 2 Morphological, biochemical, and physiological characterization of CRB15

Morphology	Traits
Gram reaction	$+$
Shape	Cocci
Colour	Pink and translucent

Biochemical tests	Traits
Catalase	$+$
Indole production	$-$
Methyl red test	$-$
Voges–proskauer test	$-$
Citrate utilization	$-$
Nitrate reduction	$+$
Mannitol	$+$
Motility	$-$
Lactose fermentation	$-$
Sucrose	$-$
Dextrose	$+$
H$_2$S production	$+$
Gas production	$+$
Cellulase activity	$+$
Starch hydrolysis	$-$

Physiological tests	Traits
Growth at 4 °C	$-$
Growth at 41 °C	$+$
Growth at 7% NaCl	$+$

CRB15 was Zn > Pb > Ni > Cd. Growth rates of the strain CRB15 in the presence of heavy metals were consistently slower than that of control. Similar results were reported by Suresh Kumar et al. (2001), Pal et al. (2004) and Raja et al. (2006).

Effect of Cu on bacterial growth

Effect of Cu(II) on *Kocuria* sp. CRB15 was carried out on SLP broth media amended with 0–150 mg/l of Cu(II) as CuSO$_4$·5H$_2$O. The optical density measurements of the cultures incubated for 24 h were in good agreement according to bacterial resistance for Cu(II). In general, the growth rate of the strain CRB15 in the presence of Cu(II) was consistently slower than the control (Fig. 2). The inability to grow at higher concentration was associated with reduction in growth due to the metal binding capacity of CRB15 which resulted by means of interaction between metal cations and functional groups of the cell wall. Similar results were also reported by Friis and Myers-Keith (1986) and Raja et al. (2006).
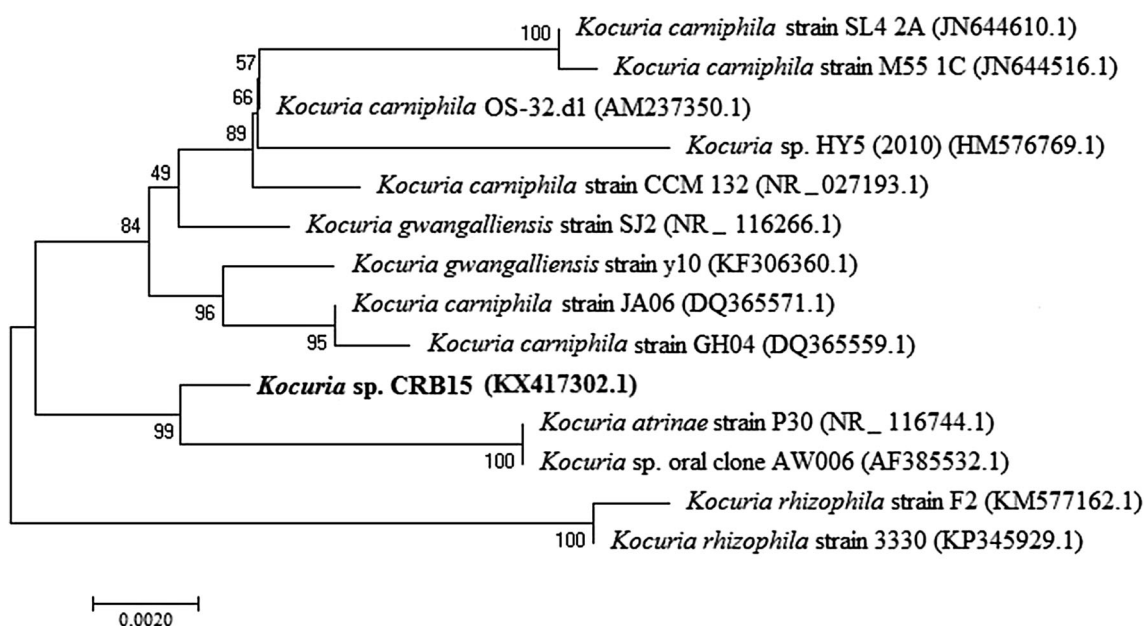
Fig. 1 Phylogenetic neighbour-joining tree based on 16S rDNA gene sequences showing the relationship between *Kocuria* sp. CRB15 and related members of the genus *Kocuria*. Bootstrap numbers indicated the value of 500 replicates. *Bar* represents 0.2% sequence variation. GenBank accession numbers are given in parentheses

Table 3 Minimum inhibitory concentration of heavy metal tolerance of *Kocuria* sp. CRB15

MIC (mM)				
Cu	Zn	Pb	Cd	Ni
6.29	3.25	1.5	0.25	1.25

SEM–EDX analysis

The SEM results revealed that depending on the Cu(II) concentration cell surface morphology changed to some extent due to the extracellular adsorption (Ye et al. 2013). Clear impressions (shown by arrows in Fig. 3c) were easily visualized on the cell surface of bacterial biomass at higher Cu^{2+} concentration, which may lead towards rupturing or deformation of cells (Fig. 3). Furthermore, the EDX spectra showed that some copper peaks could be detected on the cell surface of treated biomass.

FTIR analysis

FTIR study was carried out for identification of functional groups involved in adsorption of Cu^{2+}. The FTIR analysis of living cells was shown in Fig. 4 displaying a number of adsorption peaks indicating the complex nature of the biomass examined. The IR spectrum of metal-free and metal loaded biomass showed difference in the functional groups of –NH, –CH, –CO, –CH$_2$, –PO,
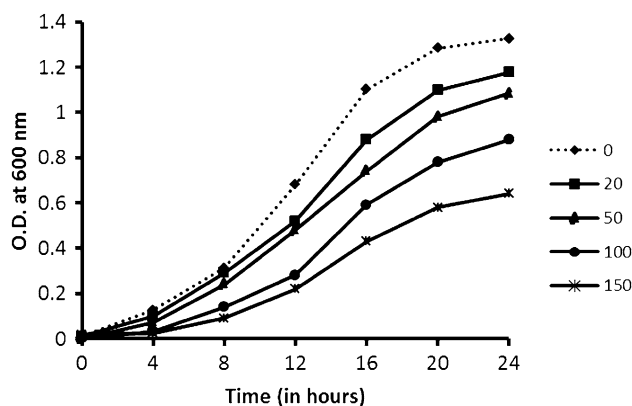

Fig. 2 Growth of CRB15 at 30 ± 2 °C with different concentration of Cu (0–150 mg l^{-1}) in SLP broth

–CN, –H, and –COOH by shifting in their peaks (Table 4). These observations validated the involvement of these functional groups in the adsorption process. Due to the presence of Cu^{2+} in copper-loaded biomass, the peak transmittance and degree of bond stretching became lower than the unloaded biomass. Numerous workers have also reported similar results (Tunali et al. 2006; Giotta et al. 2011). The FTIR spectra of Cu free and Cu loaded biomass revealed a complex and additive influence of chemical structure of bacterial cell surface comprising different functional responsible for regulating the possible cell–cation interactions (Anand et al. 2006).
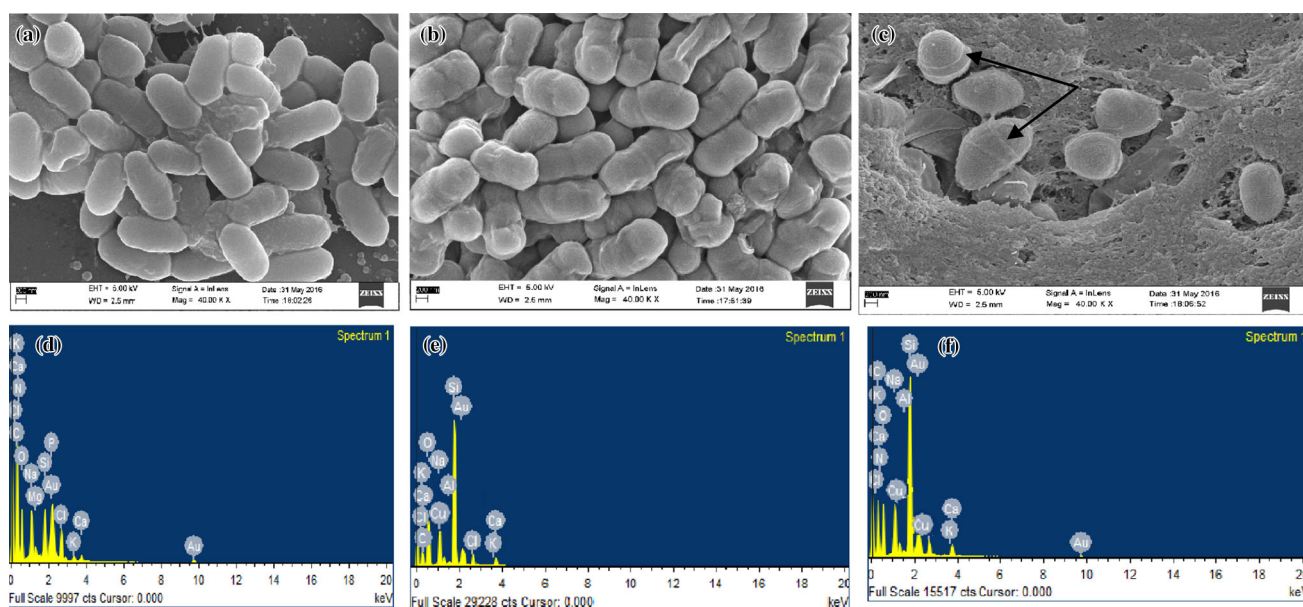
Fig. 3 SEM micrographs of before (**a**) and after copper-loaded (**b** 50 mg L^{-1} and **c** 100 mg L^{-1}) of cell surface and corresponding typical EDX spectra (**d** control, **e** 50 mg L^{-1} copper-loaded, and **f** 100 mg L^{-1} copper-loaded)
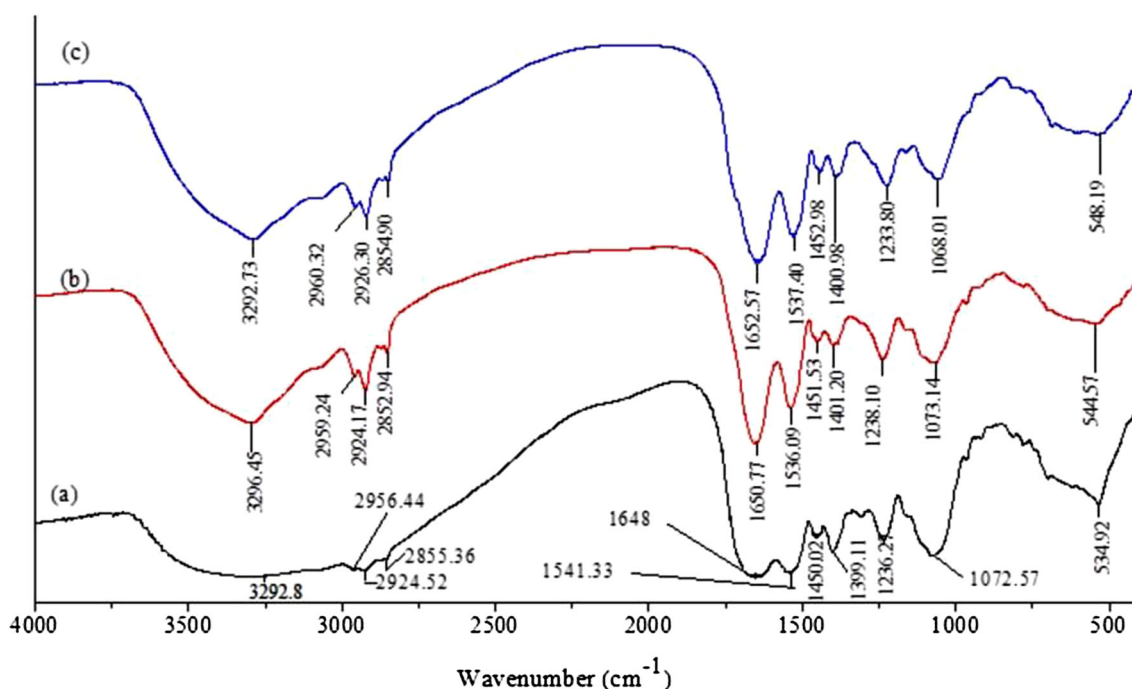
Fig. 4 FTIR spectra of *a* CRB15 (no copper), *b* CRB15 + 50 mg Cu l^{-1} and *c* CRB15 + 100 mg Cu l^{-1}

Plant growth-promoting characteristics of CRB15

Characterization of IAA

The results showed that IAA production was directly proportional to L-tryptophan. Maximum IAA production of 46 µg ml^{-1} was observed after 96 h at 600 µg ml^{-1} L-tryptophan concentrations (Fig. 5). Similar trend of IAA production was also reported by Barazani and Friedman (2000). Increase in IAA was observed until 96 h of incubation, and thereafter, it reached plateau. 5 µg ml^{-1} of IAA was produced in growth medium without L-

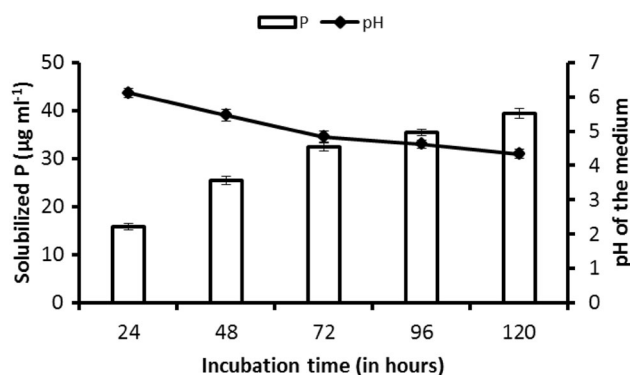**Table 4** Change of IR absorption band in treatments of metals and the possibly corresponding groups

Original main band wave numbers (cm^{-1})	Changed main band wave numbers (cm^{-1})		Functional groups
	50 ppm	100 ppm	
3292.8	3296.45	3292.73	–NH
2956.44	2959.24	2960.32	–CH
2924.52	2924.17	2926.30	–CH
2855.36	2852.94	2854.90	–CH$_2$
1648.0	1650.77	1652.57	–C=O
1541.33	1536.09	1537.40	–COO, –NH, –CH
1450.02	1451.53	1452.98	–CH$_2$
1399.11	1401.20	1400.98	–H
1236.27	1238.10	1233.80	–COOH, –PO
1072.57	1073.14	1068.01	–CO, –CN, –PO
534.92	544.57	548.19	–

Fig. 6 Phosphate solubilisation and drop in pH due to acid production by *Kocuria* sp. CRB15 in various incubation times (24–120 h)

Fig. 5 Correlation between IAA production by *Kocuria* sp. CRB15 after incubation (24–120 h) in different concentrations of L-tryptophan

tryptophan. Similar results were also reported for copper-resistant bacteria *Bacillus* sp. BC21 and *Arthrobacter* sp. MT16 (He et al. 2010). The present study reveals that *Kocuria* sp. CRB15 is a potential producer of IAA than other PGPR, such as *Azotobacter* (20 µg ml^{-1}), *Pseudomonas* (25 µg ml^{-1}), and *Bacillus* (10 µg ml^{-1}) (Ahmad et al. 2008). Some other members of genus *Kocuria*, such as *K. rosea* (Godinho et al. 2010), *Kocuria* sp. PWN-228 (Vicene et al. 2012), and *K. turfanensis* 2M4 (Goswami et al. 2014), have been also reported as potential producer of IAA.

Phosphate solubilizing activities

The major mechanism associated with the solubilization of insoluble phosphate is the organic acid production,

accompanied by acidification of the medium (Puente et al. 2004). The present study represents that a gradual increase in solubilized phosphate concentration was observed from 24 to 120 h. Phosphate concentration varied from 15.78 to 39.37 µg ml^{-1} in the culture filtrates of CRB15. A gradual decrease in pH values from 7.0 to 4.33 was also recorded (Fig. 6). The inverse relationship between pH and soluble phosphorus concentration observed in the present study suggested that acidification of the medium could facilitate the inorganic phosphate solubilization. The present results were also in agreement with other researchers (Illmer and Schinner 1992; Yu et al. 2011), who reported similar negative relationships. It has been suggested that microorganisms which tend to decrease the pH of the medium during growth are efficient P-solubilizers.

HCN production

A colour change was observed in CRB15 indicating its HCN production ability (Fig. 7). Siddiqui et al. (2006) and Akhtar and Siddiqui (2009) reported that HCN is a volatile, secondary metabolite that suppresses the growth of pathogen due to inhibition of metal enzymes, especially cytochrome *C* oxidases in an electron transport system.

Ammonia production

It was observed that CRB 15 isolate has produced ammonia in peptone water. Maximum ammonia production of 30.46 µmol ml^{-1} was reported after 96 h of incubation at 30 ± 2 °C (Fig. 8). Ammonia production by *Kocuria* species were also reported by Goswami et al. (2014).

Root and shoot length promotion

The effects of the Cu-resistant bacterial strain on root and shoot elongation of *B. nigra* in the absence or presence of

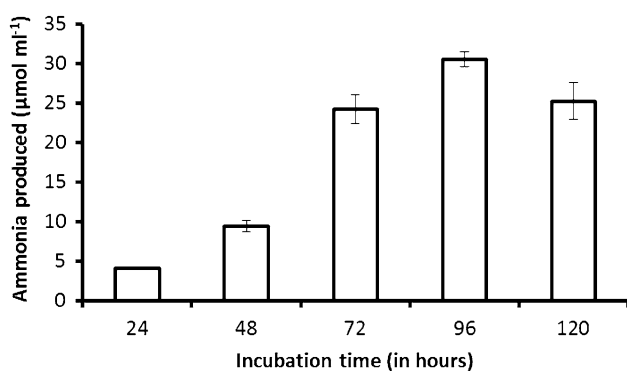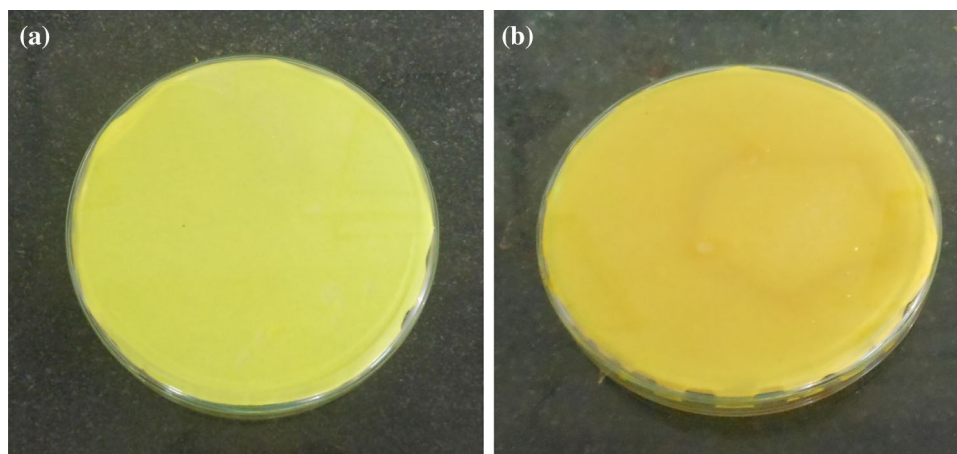Fig. 7 Plates showing HCN production by **a** control-no bacteria and **b** *Kocuria* sp. CRB15

Fig. 8 Ammonia production by *Kocuria* sp. CRB15 in various incubation times (24–120 h)

solubilisation, HCN production, and ammonia production may have the potential for the plant growth promotion and heavy metal resistance, as shown in the root–shoot elongation assay, in which the metal-resistant bacterial strain (CRB15) facilitated *B. nigra* growth even under Cu concentration (Table 5) (Zaidi et al. 2006). Similar results were also reported by He et al. (2010) for *Brassica napus* variety Qinyou-7 seedlings.

Conclusion

In this study, *Kocuria* sp. CRB15 isolated from abandoned Cu tailing was evaluated for its heavy metal resistance and plant growth-promoting activities. These activities may allow the use of isolate for plant growth promotion and bacteria-assisted phytoremediation of Cu, Cd, Ni, Zn, and Pb. SEM–EDX and FTIR analysis evaluated that bacterial cell wall and its functional groups played an important role in metal tolerance. Filter paper assay for root–shoot elongation demonstrated that inoculation with CRB15 lessens the toxic effect of Cu. Future work, such as pot scale study, may be conducted for better understanding and evaluation of effects of *Kocuria* sp. CRB15 on plant growth and phytoremediation efficiency.

Cu are shown in Table 5. The tested strain increased the root length and shoot length of the seedlings in the absence of Cu. Inoculation with strain CRB15 resulted in increased root length of Cu-treated and Cu-untreated seedlings by 20 and 19%, respectively. Similarly, increased shoot length was also observed for Cu-treated and Cu-untreated seedlings by 39 and 37%, respectively. The maximum root length and shoot length promoting effect was observed after inoculation with strain CRB15 only. Bacteria having the characteristics of producing IAA, inorganic phosphate

Table 5 Root–shoot length elongation of *Brassica nigra* seedlings inoculated with copper-resistant bacteria grown in the absence and presence of Cu

Treatment	Root length (in cm)	Bacterial effect (%)	Shoot length (in cm)	Bacterial effect (%)
Distilled water	4.41 (3.94–4.9)	–	6.43 (5.9–7.2)	–
CRB15	5.30 (4.8–5.9)	+20	8.96 (8.6–9.4)	+39
0.5 mM Cu	4.06 (3.8–4.5)	–	5.73 (4.9–6.4)	–
CRB15 + 0.5 mM Cu	4.86 (4.6–5.1)	+19	7.96 (7.6–8.5)	+37

Values in parentheses indicate range

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