Molecular characterization of potassium solubilizing bacteria from crop rhizosphere of the North Eastern Region of India

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In this study, isolation and characterization of potassium solubilizing bacteria (KSB) have been carried out which could solubilize a substantial amount of potassium (K) following incubation with mica. Five KSB, isolated from banana and chilli rhizosphere were identified by 16S rDNA sequence analysis. The identified bacteria were *Klebsiella* sp. and *Bacillus cereus.* The isolates had K solubilization potential within the range of 76.3–78.42% compared to control along with significant drop in pH (4.62–4.86). The study reveals the exploitation of *Klebsiella* sp. and *B. cereus* as potential K solubilizing biofertilizers.

Keywords: Crop rhizosphere, molecular characterization, mica, potassium solubilizing bacteria.

POTASSIUM (K) plays a fundamental role in the growth and development of plants. It is absorbed through the soil minerals, organic materials and synthetic fertilizers. K is invariably involved in the plant cellular osmotic pressure adjustment and transportation of compounds in plants. Multifaceted activity of this essential element includes activation of enzymes, utilization of nitrogen (N) and synthesis of protein and sugar as well as boosting the plant photosynthetic activity[1]. Besides providing resistance to plant diseases, K also confers resistance to various abiotic and biotic stresses leading to enhanced production of quality crops[2]. According to the Fertilizer Association of India (FAI) report (2013), the consumption of this important element in the country exceeded 260 lakh tonnes for two consecutive years (2011–2012), wherein the entire amount of potassic fertilizers was imported to meet the demand for agricultural productivity. Despite such huge consumption, the widespread deficiency of K in the rhizosphere of economically important crops has become a constraint for sustainable development in India[3]. On the other hand, mass application of this fertilizer can increase production costs, decrease potassium use efficiency[4] and cause damage to the environment. Thus it is necessary to find a substitute for the chemical K fertilizer. In this regard, exploitation of the potassium reservoir in the soil could be an alternative. The soil is loaded with enough reserves of K, of which only 1–2% can be directly absorbed by the plants for their activities, while bulk of soil K (90–98%) exists in silicate minerals such as K-feldspar and mica[5]. These minerals undergo weathering, thereby slowly releasing the entrapped K from their lattice into the soil. Studies have shown that a variety of soil microbes can cause dissolution of these K-bearing minerals such as K-feldspar, mica and illite. The silicate rock get dissolved by various kinds of organic acids released by soil microbes and this organic acid in turn chelates silicon ion, releasing K ion into the soil[6]. Pertinent literature suggests that there is a wide range of rhizobacteria in the soil, e.g. *Pseudomonas*, *Burkholderia*, *Paenibacillus* sp. and *Acidothiobacillus ferrooxidans*, *Bacillus mucilaginosus*, *Bacillus edaphicus* and *Bacillus circulans*, which can release potassium in accessible form from K-bearing minerals[7]. Polysaccharide and carboxylic acids such as tartaric acid and citric acid are also released by the silicate bacteria *B. mucilaginosus* and *B. edaphicus* to solubilize K compounds[8]. Hence, mitigation of K deficiency in plants could be achieved through microbe-assisted dissolution of K-bearing minerals in the soil[9].

Among 12 mega biodiversity hot spots in the world, India is endowed with two within its boundaries, viz. the North Eastern Region (NER) and the Western Ghats[10]. There is a widespread occurrence of potassium solubilizing bacteria (KSB) reported from different states like Tamil Nadu[11], Karnataka[12], Gujarat[13] and Kerela[14]. The entire NER of India is a biologically rich habitat of different microflora and microfauna. However, there are no reports on KSB from this region. Assam (89°5′–96°1′E and 24°3′–27°58′N) is an important part of the NER, in which muscovite, biotite and K-feldspar are prime K-bearing minerals present in the sand and silt fractions of soil[15]. In view of the vast economic and ecological significance, a study was undertaken to isolate and characterize KSB from crop rhizosphere of NER (Assam) of India.

Soil samples were collected from the rhizosphere of banana and chilli grown in the Jorhat (26°45′N, 92°21′E) and Golaghat (26°31′N, 93°58′E) districts of Assam during March 2015. Soil samples at a depth of 6–10 cm were taken from the rhizosphere after removing the top 5 cm layer. Five samples weighing 20 g each collected from selected sites were mixed to obtain a composite (100 g) sample. As such ten composite samples were obtained from ten different sites. All soil samples were sealed in sterilized ziplock bags and stored at 4°C till further use.

Waste mica, a 2 : 1-type clay mineral of composition $(OH)_4K_2(Si_6Al_2)Al_4O_{20}$, was collected from the dumping sites of mica mines in Koderma district, Jharkhand, India. The mica flakes were ground thoroughly in a mixer-grinder and then passed through a 100 μm sieve. The finely ground waste mica contained 18 ppm of available

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K and had 9.4% total K. Mica was sterilized by dry autoclaving at 20 psi for 120 min, before further use.

Details of chemical constituents of three media which were used in the present study are given below.

Aleksandrov medium[16]: 0.5% glucose, 0.05% magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.0005% iron (III) chloride ($FeCl_3$), 0.01% calcium carbonate ($CaCO_3$), 0.2% calcium phosphate ($CaPO_4$), 0.5% mica powder, 1.5% agar, pH 7.0–7.5. Liquid media[17]: 0.5% sucrose, 0.2% disodium hydrogen phosphate (Na_2HPO_4), 0.05% ammonium sulphate ($(NH_4)_2SO_4$), 0.05% magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.0005% iron (III) chloride ($FeCl_3$), 0.01% calcium carbonate ($CaCO_3$), 0.02% yeast extract, 0.5% mica (0.1% used in original media), pH 7.5.

Nutrient agar media: 0.3% beef extract, 0.5% peptone, 1.5% agar, pH 7.5.

One gram of soil sample from the rhizosphere was serially diluted to 10^{-5}–10^{-6} suspension using 9 ml sterilized water. The soil suspension of 100 µl was then spread over a petri dish containing selective Aleksandrov culture medium with mica as the insoluble source of K for KSB[16]. The petri dishes were placed in an incubator at 30°C for 72 h. Fast-growing colonies with clear zones that grew in Aleksandrov plates were considered as putative KSB. The KSB isolates were purified by repeated streaking on the solid Aleksandrov culture medium. Subsequently the solubilization index of the purified isolate was calculated using Khandeparkar's selection ratio following the growth for seven days in Aleksandrov plates

$$\text{Khandeparkar's selection ratio} = \frac{\text{Diameter of the clear zone } (D, \text{mm})}{\text{Diameter of bacterial growth } (d, \text{mm})}.$$

The solubilization zone was calculated by subtracting the zone of bacterial growth from the zone of clearance ($D - d$; mm). Screened isolates were subjected to Gram staining and preserved in the sterile slant of nutrient agar medium (HiMedia, Mumbai), stored at 4°C till use. As such, nine putative KSB isolates were selected exhibiting higher Khandeparkar's selection ratio (>1.0) of clear zone. These nine selected isolates were examined for colony size and shape, Gram reaction, shape of cells and qualitative polysaccharide production. Broth culture medium as described by Basak and Biswas[17] was prepared and dispensed as 100 ml each into a series of 250 ml conical flasks containing 0.5 g of mica in each flask. The pH was adjusted to 7.5 and autoclaved. Initially the purified KSB isolates were grown in 50 ml Aleksandrov broth (mica replaced by KCl) at 30° ± 1°C for 24 h at 150 rpm. Then, 1 ml culture (>7.0 log cfu ml^{-1}) of each selected isolate was inoculated to the medium in triplicate and incubated in C24 incubator shaker (New Brunswick, Edison, NJ, USA) at 200 rpm and 30° ± 1°C for a period of 40 days, including uninoculated control. At 10 days interval of incubation, 15 ml of the broth cultures was withdrawn and centrifuged at 10,000 rpm for 10 min (C24 Remi refrigerated centrifuge). After centrifugation, the supernatant was filtered through Whatman No. 42 filter paper to remove thick polysaccharide-like exudates. The culture filtrates were used to estimate change in pH and assess soluble K released into the solution. The pH of the culture filtrates was determined using Systronics microprocessor based pH system 361. Available K content was periodically determined at different incubation periods by using the flame photometric method[11].

K-release kinetics: First-order kinetic equation (eq. (1)) was used to determine potentially mineralizable K (K_0) under incubation

$$K_{\min} = K_0(1 - e^{-kt}), \quad (1)$$

where $K_{\min}$ is the cumulative K mineralized at any specific time t (days) and k is the first-order rate constant (day^{-1}). Values of K_0 and k were calculated using regression analysis.

Mineralization rate of K ($dK_{\min}/dt$) was determined by differentiating eq. (1) as follows

$$dK_{\min}/dt = (K_0 k)e^{-kt}. \quad (2)$$

Based on the release kinetics of NH_4OAc-K from waste mica, five isolates were finally selected for phylogenetic analysis. Total genomic DNA from the bacteria was extracted by *N*-cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide (CTAB) method. PCR amplification of the 16S region was done in 20 µl of reaction mixture containing PCR buffer, 1× (HiMedia, procured from Mumbai); $MgCl_2$, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase, 0.05 U; primer, 1 pmol and template DNA, 50 ng. Sterile nuclease-free water was used as negative control. The oligonucleotides used in this reaction were the forward 8–27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse 1521–1540R (5′-AAGGAGGTGATCCAGCCGCA-3′) with a product size of 1500 bp. Amplification was done in a thermal cycler (Eppendorf Mastercycler nexus, Germany) using an initial denaturation at 94°C for 2 min followed by a programme of 30 cycles with each cycle consisting of a denaturation step of 94°C for 50 sec, annealing at 48°C for 30 sec, elongation at 72°C for 1 min 30 sec and a final extension step for 6 min at 72°C. The bands of 1500 bp were obtained from the amplification of the above 16S rRNA gene sequences from the KSB isolates (KSBB3, KSBB8, KSBB9, KSBC5 and KSBC6). The sequencing was carried out on a model 3037 Automated DNA Analyser (Applied Biosystems, Inc.), at the DNA Sequencing Facility (Triyat Genomics, Nagpur). The sequencing results were analysed using NCBI database for homologous sequences of 16S rDNA. Neighbour-joining method was used to build a phylogram.

Table 1. Khandeparkar's selection ratio of potassium solubilizing bacteria exhibiting clear zone

Crop rhizosphere	Isolate	Diameter of zone of clearance (*D*; mm)	Diameter of growth (*d*; mm)	*D*/*d* ratio	Solubilization zone (mm)
Banana	KSBB3	16	5.5	2.90	10.5
	KSBB8	10	9	1.11	1.0
	KSBB9	15	8.9	1.69	6.1
	KSBB5	7	6.5	1.07	0.5
	KSBB6	6	5.71	1.05	0.29
Chilli	KSBC5	20	8.8	2.27	11.2
	KSBC6	11	7.9	1.39	3.1
	KSBC4	18	16.07	1.12	2.0
	KSBC3	17	14.1	1.21	3.0

A total of 37 different types of well-recognized colonies were found to grow on Aleksandrov solid medium from the rhizosphere of banana and chilli, which are widely cultivated in the NER of India. Only 17 colonies were able to exhibit distinct clear zones in the Aleksandrov solid medium. Subsequently, secondary screening was carried out for the isolates to assess their solubilization capacity using Khandeparkar's ratio. Based on zone of clearance and Khandeparkar's selection ratio (>1.0), finally nine isolates were selected for the study. Among them, five isolates belonged to banana rhizosphere (viz. KSBB3, KSBB5, KSBB8, KSBB9, KSBB6) and four (viz. KSBC3, KSBC4, KSBC5, KSBC6) were from chilli rhizosphere (Table 1). A wide range of KSB, namely *B. mucilaginosus*, *B. edaphicus*, *B. circulans*, *Paenibacillus* sp., *A. ferrooxidans*, *Pseudomonas* and *Burkholderia* have been isolated from diverse habitats and successfully demonstrated accessible K release potential[18], which is analogous with the present study.

The solubilization zone of the nine selected isolates was between 0.29 and 11.2 mm in diameter, while their respective solubilization indices ranged from 1.05 to 2.90 (Table 1). Among the isolates, the most apparent K solubilizing zone was shown by KSBC5 (11.2 mm in diameter and corresponding solubilization index of 2.27). KSBB6 had the least solubilization zone of 0.29 mm in diameter compared to the other isolates, with the corresponding solubilization index of 1.05. In case of the K solubilizers obtained from the banana rhizosphere, KSBB3 exhibited the highest solubilization zone (10.5 mm) and solubilization index (2.90). Similarly, KSBC5 obtained from chilli rhizosphere showed the highest solubilization zone (11.2 mm) and solubilization index (2.27). Analogous to the present study, 27 strains of potential K solubilizers from tobacco rhizosphere have been reported[19]. Pertinent literature reveals that the soil rhizosphere serves as host for a variety of potassium solubilizing rhizobacteria (KSR)[6], which augment the plant growth and development activities besides secreting some plant growth promoting substances[20].

Detailed morphological analysis of the isolates has been carried out in this study. The nine selected isolates exhibited distinct morphological character on specific Aleksandrov agar medium with respect to shape, elevation, size, colour and margin of the colony and Gram reaction. The selected isolates were rod-shaped bacteria with circular and raised colonies. Most of the isolates (KSBB3, KSBB8, KSBB9, KSBB5, KSBC5 and KSBC6) had medium-sized colonies, with a few exceptions. KSBC3 had a pinpoint colony, while the colonies of KSBC4 and KSBB6 were the largest. Colonies of KSBB3, KSBB8, KSBB9, KSBB5, KSBC5, KSBC6 and KSBC3 appeared as white on the Aleksandrov agar media, while creamy-white colour colonies were shown by KSBB6 and KSBC4. With respect to colony margin, all the nine isolates had entire smooth margin, except KSBB3 which had lobate margin. Polysaccharide formation, which is one of the important traits of K solubilizing bacteria, was also observed in the study. KSBB3, KSBB8, KSBB9, KSBB5, KSBB6, KSBC3 and KSBC5 were noted as medium to high polysaccharide producers. Colonies of KSBB3, KSBB8 and KSBB6 were purple in colour, while the rest of the isolates were red in their respective Gram reaction tests. Similar study on the successful isolation and characterization of 12 KSR from various crop rhizospheres on the basis of plate assay has been reported[21].

The ability of the nine selected isolates from banana and chilli rhizosphere to solubilize potassium was assessed (Table 2). All the bacteria examined were able to solubilize K from waste mica, which exhibited significant differences among the isolates during the incubation period of 40 days. The isolate KSBC6 from chilli rhizosphere showed maximum cumulative solubilization of K (78.33 μg ml^{-1}) at 40 days of incubation, which was 79.38% more compared to control (16.15 μg ml^{-1}). This was followed by isolates KSBB3 (74.85 μg ml^{-1}), KSBC5 (71.28 μg ml^{-1}) and KSBB8 (69.96 μg ml^{-1}). Zhang and Kong[19] reported the K solubilizing ability (4.4 mg l^{-1}) of *Klebsiella variocola* (HQ2599G1) isolated from tobacco rhizosphere using K-feldspar as the sole source of K after seven days of incubation. The change in pH of the broth culture medium is the confirmatory test for structural disturbances in waste mica by bacterial hydrolysis[22]. In the present study also, during the period of incubation, the K release was associated with significant changes in pH of

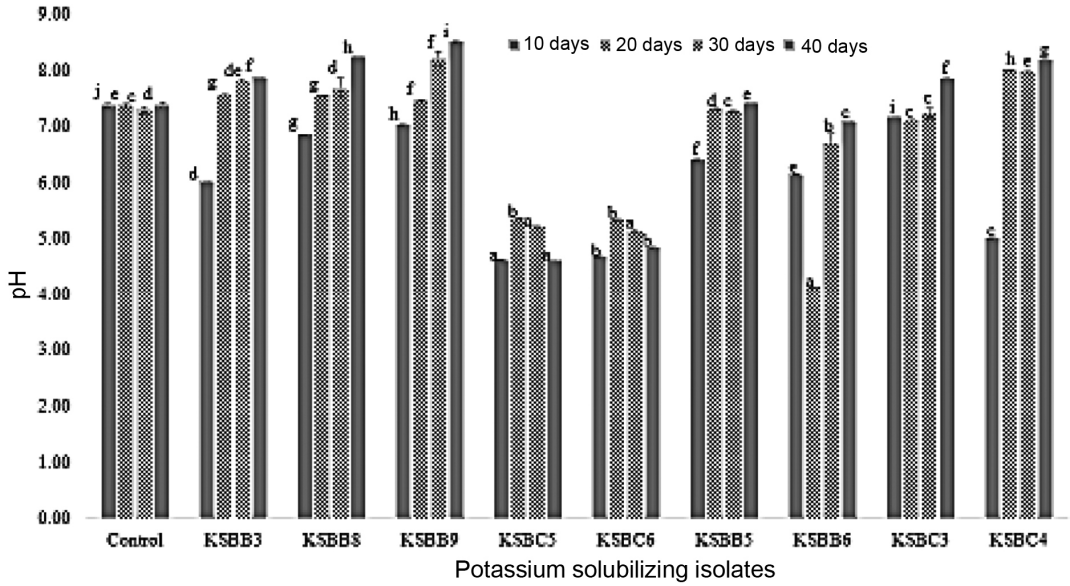

Figure 1. Change in pH of Aleksandrov broth by the isolates during incubation.

Table 2. Potentially mineralizable NH_4OAc-K (K_0), mineralized NH_4OAc-K_{min} and K rate constant (k) in broth culture of selected KSB

Isolate	K_0 (μg ml^{-1})	K_{min} (μg ml^{-1})	Rate constant (k)/day
Control	18.26^{a}	16.15^{a} (± 5.12)	0.054^{ab}
KSBB5	61.40^{e}	55.12^{g} (± 0.11)	0.057^{a}
KSBB8	85.22^{e}	69.96^{ef} (± 0.57)	0.043^{a}
KSBC3	45.06^{cde}	44.06^{e} (± 0.17)	0.096^{ab}
KSBB3	88.32^{e}	74.85+ (± 0.69)	0.047^{ab}
KSBB6	65.77^{de}	61.11^{d} (± 0.69)	0.066^{a}
KSBC5	87.61^{e}	71.28^{f} (± 0.35)	0.042^{ab}
KSBC4	80.99^{e}	68.13^{g} (± 0.23)	0.046^{ab}
KSBB9	74.70^{b}	68.19^{b} (± 0.17)	0.061^{c}
KSBC6	91.11^{cd}	78.33^{e} (± 0.49)	0.049^{s}

Different lower-case letters in each column indicate significant differences between KSB isolates at 5% level of significance according to DMRT.

the broth culture. Compared to the control (pH 7.5), among the nine isolates KSBC5 and KSBC6 displayed significant ($P < 0.05$) drop in pH (4.62 and 4.86 respectively), while the other isolates, viz. KSBB3, KSBB8, KSBB9, KSBB5, KSBB6, KSBC3 and KSBC4 showed pH above 7.0 with concomitant release of soluble K at the end of 40 days of incubation (Figure 1). Reduction in pH could be due to the production of different kinds of organic and inorganic acids by K solubilizers[20]. The decrease in pH is mainly due to the protons associated with the organic acid molecule. This lowering of pH in turn induces cation (Fe, K, Mg, etc.) release capacity of minerals. However, the increase in pH observed for few of the isolates during the incubation period, might be due to accumulation of metabolism products at the later stage, wherein the processes of hydrolysis and reaction between the products of the metabolism and minerals intensified, leading to increase in pH. Binbin and Bin[23] also supported change of pH in media through prolonged K solubilizer under incubation. Moreover, the increase in pH of the broth culture is attributed to the presence of impurities like $CaCO_3$ and $MgCO_3$, which would be released into the medium following decomposition of mica. Those impurities might function as buffer, thereby prevented the broth from being acidified by KSB through production of organic acids[24].

Potassium release kinetics from waste mica under the incubation study was measured from the best-fitted first-order kinetics equation. The potentially mineralizable K (K_0), the mineralized K (K_{min}) as well as the rate constant for K mineralization (k) from waste mica were estimated from the first-order kinetics equation (Table 2). The values of K_0 were significantly ($P < 0.05$) higher for isolates KSBC6, KSBB3, KSBC5, KSBB8 and KSBC4 compared to control and other isolates. The highest K_0 (91.11 μg ml^{-1}) was found for KSBC6, which was 79.95% more than control (18.26 μg ml^{-1}). Rate constant for K mineralization differed among the isolates. It was observed that a significantly higher release rate (k value) was found in case of mica inoculated with KSBC3 compared to rest of the isolates (Table 2). Figure 2 shows the rate of potassium mineralization (dK_{min}/dt) over the incubation period. However, gradual decrease in the value of dK_{min}/dt with the advancement of time was detected for the respective isolates. Significant ($P < 0.05$) effect of inoculation on the initial mineralization rate ($t = 10$ days) was observed in the study; higher values were recorded for the isolate KSBC4 (2.735 mg kg^{-1} day^{-1}). The results of the present study indicate that the initial mineralization rate ($t = 10$ days) has been considered as determinant for assessing and differentiating the efficiency of KSB

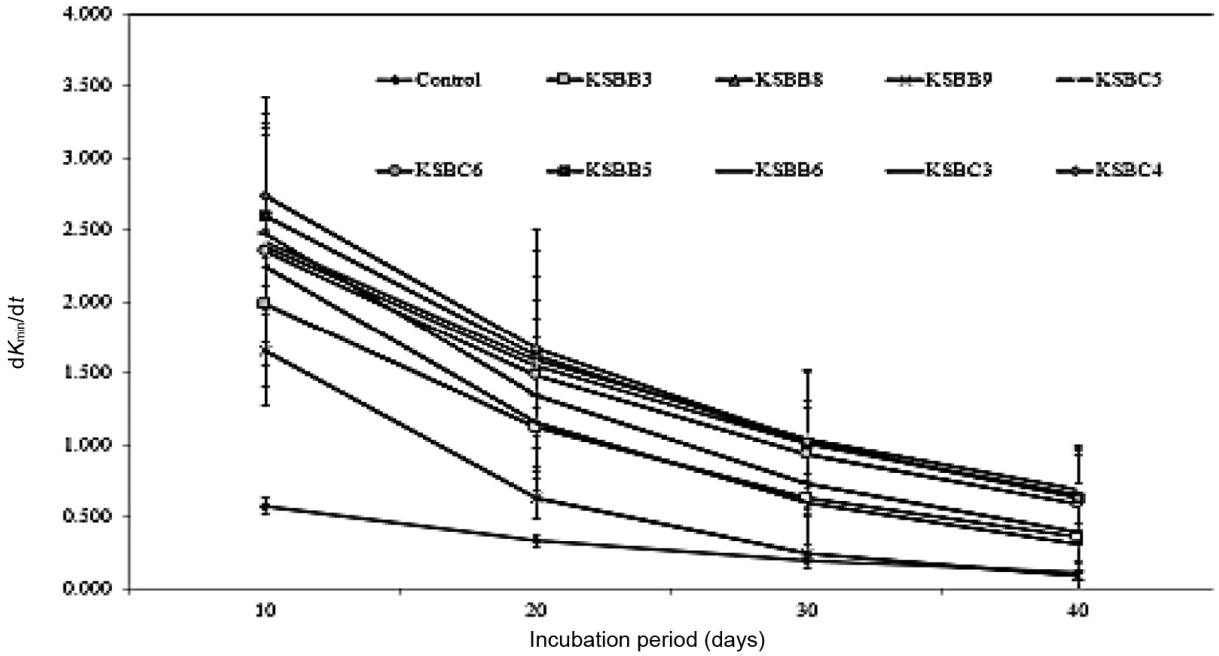

Figure 2. K mineralization rate (dK_{min}/dt) of mica by different potassium solubilizing bacteria isolates.

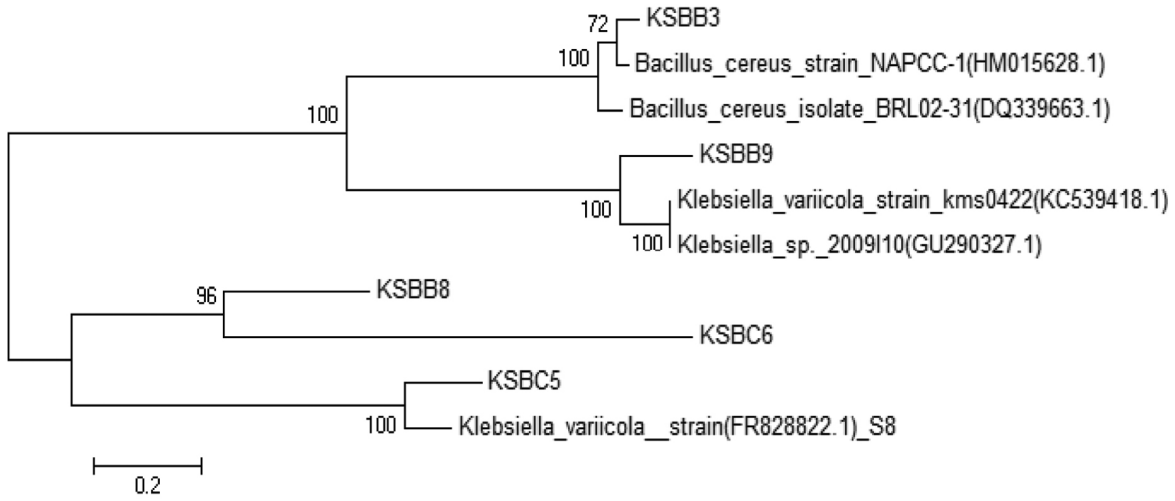

Figure 3. Phylogenetic tree of K-solubilizing bacteria from chilli and banana rhizosphere soil based on 16S rDNA sequences. All tested isolates had their 16S rRNA gene partially sequenced and were grouped according to their genus or species designation.

isolates. Microbial inoculation with waste mica causes dissolution of the mineral and hence it increases the release of K from its otherwise insoluble source. This microbe-mediated dissolution was due to the release of different kinds of organic acids such as oxalic acid, citric acid, tartaric acid and acetic acid[25]. Biswas and Basak[26] also reported higher K release rate from waste mica inoculated with silicate bacteria *B. mucilaginosus* up to 14 days of incubation.

To confirm the identity of the isolates, PCR parameters were optimized for the maximum amplification of 16S rDNA. Amplicons with approximately 1.5 kb length were generated and separated in 1% agarose gel. The 16S rDNA sequences of the five KSB isolates were compared with those of known 16S rDNA sequences using BLAST and GenBank database. The results indicated that KSBB3 showed 100% similarity with its close species strain BRL02-31 (Figure 3). Four isolates (KSBB8, KSBB9, KSBC5 and KSBC6) were found to be phylogenetically closely related to *Klebsiella*, showing 96–100% similarity in their 16S rDNA sequences. KSBB9 was closely related to *Klebsiella variicola* strain kms0422, while KSBC5 was similar to *Klebsiella* sp. 2009I10. A phylogenetic tree was constructed using BLAST analysis in NCBI in order

to confirm the above results. Similar results of dominant *Klebsiella* were also reported in the isolates from tobacco rhizosphere[19]. Another contemporary report on the presence of a diverse range of KSB species belonging to different genera, viz. *Agrobacterium tumefaciens*, *Rhizobium pusense*, *Flavobacterium anhuiense* and *Rhizobium rosettiformans* from the rhizosphere of some *kharif* crops is also available in the literature[21].

In conclusion, promising results of isolation, screening and characterization of five KSB isolates from the soil of NER of India have been obtained in this study. The five K solubilizers identified under *B. cereus* and *Klebsiella* sp. have not been reported earlier in the soil of NER of India. Their K-release capacity through dissolution of waste mica is satisfactory and comparable with other potential reported K solubilizers. Hence it could pave the way for utilizing these isolated KSB as biofertilizers for increasing the productivity of various field crops. Moreover, the subsequent pH drop along with the increase in K concentration in case of two strains of *Klebsiella* sp. clearly shows that acid production is one of the chief mechanisms of K solubilization by KSB. The positive impact of the isolated *Bacillus* sp. and two belonging to *Klebsiella* sp. on pH along with enhanced K release is an another promising result for further exploitation of these bacterial strains in supporting the growth of some acid sensitive crops. The differential capability of the species in releasing the fixed K depicts the diversity among the isolated rhizobacteria. However, detailed studies on the relationship between the findings of this work and plant response as well as uptake of K are required.

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