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Molecular Characterization and Identification of Potassium Solubilizing *Enterobacter hormaechei* (KSB-8) Isolated from Ceramic Industry

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Abstract

Potassium (K) accounts for around 2.5% of the lithosphere. K is present as feldspar (orthoclase and microcline) and mica (biotite and muscovite) among all soil minerals. In agriculture, replenishing potassium remains challenging because of its dependence on fertilizer. Biofertilizers become alternatives to chemical fertilizers to enhance plant nutrition and productivity as they improve soil fertility in an environmental friendly and cost-effective manner. The present study, a novel exploration, aims to identify and select microorganisms from the soils used in the ceramic industry that may solubilize potassium. The potential K solubilizers were determined by morphological, biochemical and molecular characterization through 16S rRNA sequence analysis of the *Enterobacter hormaechei* (KSB -8).

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Introduction

Phylogeny is the study of the evolutionary history of organisms. Cladistic relationships indicate the degree of relatedness between microorganisms, as shown by pathways of ancestry (Cain and Harrison, 1960). Recent advances in genomic analysis have contributed a great deal to understanding the structure and function of microbial communities. Identifying an organism by partial or whole genome sequencing has developed as a consistent and steadfast method. The function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of evolution. This also helps in estimating the rates of species divergence among the bacteria. In bacteria, the small ribosomal subunit contains the 16S rRNA. Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon. The 16S rRNA sequence has hypervariable regions, where sequences have diverged over evolutionary time (Ludwig and Schleifer, 1994).

Some other molecular methods are also available for evaluating phylogenetic relationships, e.g., DNA-DNA and DNA-rRNA hybridization, 5S rRNA and protein sequencing, 16S rRNA oligonucleotide cataloging, enzymological patterning, etc. The identification of organisms by 16S rRNA gene sequencing helps show the minor differences in the closely related members of the species. Reproducible and reliable computational tools are available to analyse the sequences of newly isolated strains. Constructing a phylogenetic tree using BLAST

results can easily deduce the evolutionary relationships. The variability and divergence can be estimated by performing multiple sequence alignments using Clustal W. It has been demonstrated that 16S rRNA gene sequence data on an individual strain with the nearest neighbour exhibiting a similarity score of less than 97% represents a new species.

The 16S rRNA gene sequence can further be used to predict the secondary structure of rRNA. The 16S rRNA plays an essential role during protein synthesis. The helices in the molecule bind to the proteins present in the small subunit of the ribosome, and the loops bind to 5S rRNA present in the larger subunit of the ribosome. We can predict the secondary structure using the Gene Bee package and Vienna RNA package for RNA secondary structure prediction and comparison. Secondary structure in RNA is the list of base pairs that occur in a three-dimensional RNA structure. According to the theory of thermodynamics, the optimal folding of an RNA sequence is that of minimum the total free energy (Lyngso *et al.,* 1999). Thermodynamics tells us that the folding of an RNA sequence in the real world is a probability distribution over all possible structures, where the probability of a specific structure is proportional to an exponential of the free energy of the structure.

Knowledge of the three-dimensional structure of ribosomal proteins and rRNA molecules will help understand the mechanism of interactions between these constituent molecules and, in turn, shed light on the function of ribosomes (Kolaskar *et al.,* 1985).

Materials and Methods

Molecular Characterization and Identification of KSB-8

Bacterial strain selected in this study was the higher potassium solubilizer from the all-isolated potassium solubilizers from ceramic industry soil of Gujarat, India. The medium used for the isolation and screening of the potassium solubilizers was Aleksandrov agar medium constituted 1% glucose, 0.5% Yeast extract, 0.05% $MgSo_4.7H_2O$, 0.0005% $FeCl_3$, 0.01% $CaCO_3$, 0.2% $CaPo_4$ and 0.5% Feldspar, 3 % agar and pH-6.5 (Sugumaran and Janartham, 2007). The selected bacterial strain was identified as *Enterobacter hormaechei* (KSB-8) using standard cultural, morphological and biochemical methodology, but its identity was re-evaluated by 16S rRNA gene sequence analysis.

The 16S rRNA sequencing for bacterial isolate KSB-8 was carried out, DNA was isolated from the pure culture of isolates. Their quality was evaluated on 1.2% Agarose Gel (Sambrook *et al.,* 1989) a fragment of 16S rRNA from the above-isolated DNA.

The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with primers using Micro Sequencer 16S rDNA sequencing kit on Applied Biosystem 3130/3130xl genetic analyser. Using aligner software, a consensus sequence of the 16S rRNA gene was generated from forward and reverse sequence data.

The 16S rRNA sequence obtained was BLAST searched to find out the other closely related species members. [BLASTn (Version 2.2.26) available on the site http://www.ncbi.nlm.nih.gov/Blast (Johnson *et al.,* 2008)]. Sequence data for related species were retrieved, and multiple sequence alignment was performed using the Clustal W program to validate the uniqueness of our sequence (Higgins *et al.,* 1996). Our isolates' hypervariable nucleic acid region was compared with 100 closely related sequences. Using the BLAST package, the phylogenetic tree was constructed via the neighbour-joining algorithm (Saitou and Nei, 1987).

Elucidation of rRNA Secondary Structure for KSB-8

The sequence obtained for KSB-8 was used to predict the secondary structure of RNA (Woese *et al.,* 1980). The secondary structures of rRNA prepared using the multiple sequence alignment result in better results. It checks the conserved region through all the sequences and then gives an appropriate structure. The structures were validated by preparing a secondary structure using 10 closely related sequences of the BLAST result (Lorenz *et al.,* 2011) using the Vienna RNA Website. On comparing the structure of our isolates with the structure generated by using ten closely related sequences, we found the presence of different similar helical regions. These regions must participate in binding proteins in the ribosome and are conserved.

Results and Discussion

Molecular Characterization and Identification of KSB-8

The 16S rRNA genetic marker is present in almost all bacteria with only minute changes, which help differentiate the cultured isolate from its closely related

sequences. In the study, 16S rRNA gene sequence analysis was carried out for KSB-8, the best potassium-solubilizing bacterial isolate. The sequence of KSB-8 is shown in Fig-1. The sequence data of isolate KSB-8 is deposited in the Gene bank Database under the accession number JQ715421.

The obtained sequences of each isolate were compared with deposited sequences in the NCBI database, using BLAST as a search tool. In most cases, the high identity values obtained ensured that KSB- 8 isolate belongs to *Enterobacter spp.* with identity values above 98%. The output result of BLAST performed for KSB-8.

The BLASTn produced for KSB-8 bacterial culture showed significant alignment to 98% similarity with *Enterobacter spp.* The identity score for KSB-8 is 262/267 with E*nterobacter hormaechei* SFK-2 (Fig-2).

Bergey's classification of prokaryotes, well-recognized and widely used for bacterial identification, is based on the phylogeny of prokaryotes from the 16S rRNA gene. If the sequence of 16S rRNA gene of an unknown organism is >95% similar to those in the GenBank (Clarridge, 2004), it is generally considered the same genus. If the 16S rRNA gene sequence is >97% identical to those sequences of any cultures in the Gen Back, it should be considered as the same species but may be a different strain (Embley and Stackebrandt, 1994).

The multiple sequence alignment of 10 nearby strains of the NCBI gene bank was done using Clustal W tool for numerous and pair-wise sequence alignments as well as phylogenetic tree construction aligning of sequences with the nucleic acids of KSB-8 to other nearby strains of NCBI, revealed that the sequence of KSB-8 isolates shares 98% similarity with the *Enterobacter hormaechei.* The phylogenetic tree of KSB-8 with nearby clusters is shown in Fig. 3.

Morales-Gracia *et al.,* (2011) isolated strain UAPS03001 from the rhizosphere of "Rojo–Criollo" maize. Phenotypic tests showed 95 % similarity to *Enterobacter cloacae*, and sequence comparison of 16s rDNA gene showed 96.3 % identity with *Enterobacter* species.

They studied the effect of *Enterobacter* spp. Inoculation in laboratory conditions was reported after twenty days of inoculation, and treated plantlets showed more biomass than non-inoculated ones. In field conditions, kernel biomass was also more incredible than non-inoculated plants.

Patel *et al.,* (2008) reported *Citrobacter* sp. DHRSS, a facultative anaerobe belonging to the *Enterobacteriaceae* family from the rhizosphere of sugarcane, uses stringent buffered conditions with sucrose as the C -source and rock phosphate as the P source to obtain a PSB that can exploit the sucrose and fructose with the intension of P solubilization.

In both cases, it isolates *Citrobacter* sp. DHRSS showed similar growth, acidification, and P solubilization properties. PCR amplification of the rRNA gene and sequence analysis identified this bacterium as *Citrobacter* sp. DHRSS.

Rani *et al.,* (2011) isolated 65 microorganisms from different rhizospheric soils. These isolates were further identified based on 16sRNA sequencing and PCR-RFLP analysis and found to be *Enterobacter* and *Bacillus.*

Isolates showed PGPR activities like IAA production, ACC deaminase abilities, antagonistic activities against fungal pathogens, phosphate solubilization activation, and mineral uptake promotion. The isolates, *Bacillus* and *Enterobacter,* were inoculated with pigeon peas and found to increase in shoot length, root length, dry matter, nodule number, and nodule mass of pigeon peas.

Ogbo *et al.,* (2012) isolated plant growth-promoting *Enterobacter spp.* from the roots of maize, and 16 rDNA identification of isolates revealed to be the closest match at (99.4 %) with *Enterobacter asburiae*. It showed plant growth-promoting activity by producing indole-3-acetic, plant hydrolyzing enzymes, pectinase, cellulose, and ammonia *in vitro*.

Strain KSB-8 was 98% similar to *Enterobacter spp.* Thus, it should be a member of the genus *Enterobacter* but is a new species. In addition, the Minimum Evolution phylogenetic tree also showed that KSB-8 should be a member of the genus *Enterobacter.*

Elucidation of rRNA Secondary Structure of Bacterial Isolate KSB-8

The secondary structure of the rRNA gene sequence can be predicted using various software like Gene Bee, Vienna, RNA structure, and manifold. A secondary structure model for 16S ribosomal RNA proposed for *E. coli* and *B. brevis* was among the early reports based on comparative sequence analysis, chemical modification studies, and nuclease susceptibility data (Woese *et al.,* 1980).

Figure.1 16S rRNA sequence of the Bacterial Isolate KSB-8.

>*Enterobacter hormaechei*

GGATACAAAA AAACTCTCGT GACATGGTGA CGAGAGATAG GGAAAAGGGA
GACACTCTAC CGTCTTCTGG TTGCCCGTTT GGGGGAGAGG AGAAAGCCCG
GGAACGTATG GTTTTTTTTT TCAAAAAAAA GAAAACAAGC TTTTTCTTTT
TTATGGATTC TAGTTGCAAA CTCCAATCCG GACGACTACG CATTTTATGA
GGTCCGCTTG CTCTCGCGAG GTCGCTTCTC TTTGTATGCG CCATTGTAGC
AGGTGGGTAG CCCTACTCGT AAGGGCCATG ATGACTTGAC GTCATCCCCA
CCTTCCTCCA GTTTATCACT GGCAGTCTCC TTTGAGTTCC CGGCCGGACC
GCTGGCAACA AAGGAGAAGG GTTGCGCTCG TTGCGGGACT TAACCCAACA
TTTCACAACA CGAGCTGACG ACAGCCATGC AGCACCTGTC TCAAAGTTCC
CGAAGGCACC AAAGCATCTC TGCTAAGTTC TCTGGATGTC AAGAGTAGGT
AAGGTTCTTC GCGTTGCATC GAAATAAACC ACATGCTCCA CCGCTTGTGC
GGGCCCCCGT CAATTCATTT GAGTTTTAAC CTTGCGGCCG TACTCCCCAG
GCGGTCGACT TAACGCGTTA GCTCCGGAAG CCACGCCTCA CGGGCACAAC
CTCCAAGTCG ACATCGTTTA CGGCGTGAAC TACCAGGGTA TCTCATCCTG
TTTGCTCCCC ACGCTTTCTC ACCTGAGCGT CAGTCTTTGT CCAGGGGGCC
GCCCTCGCCA CCGGTATTCC TCCAGATCTC TACTCAATTC AGCGCTGCAC
TTTCGAATGC GGTTCCCAGG TGGAGCGCGG GGATTTCACA TACGACTTGA
CGGACCGCCG GCGTGCGCTT TACGGCCCAG TAATTCTATG AACGCTGGAG
CCCTCCCTAT TTACCTGCGG CTGCTGGCAC GGAGTTAAAC CGGTGCTTCT
TTCTGCGCGT

Figure.2 Multiple sequence alignment of KSB-8 Isolate (*Enterobacter hormaechei)* with *Enterobacter hormaechei* SFK-2.

Figure.3 Phylogenetic tree depicting the position of KSB-8 (Enterobacter hormaechei) separated from nearby clusters.

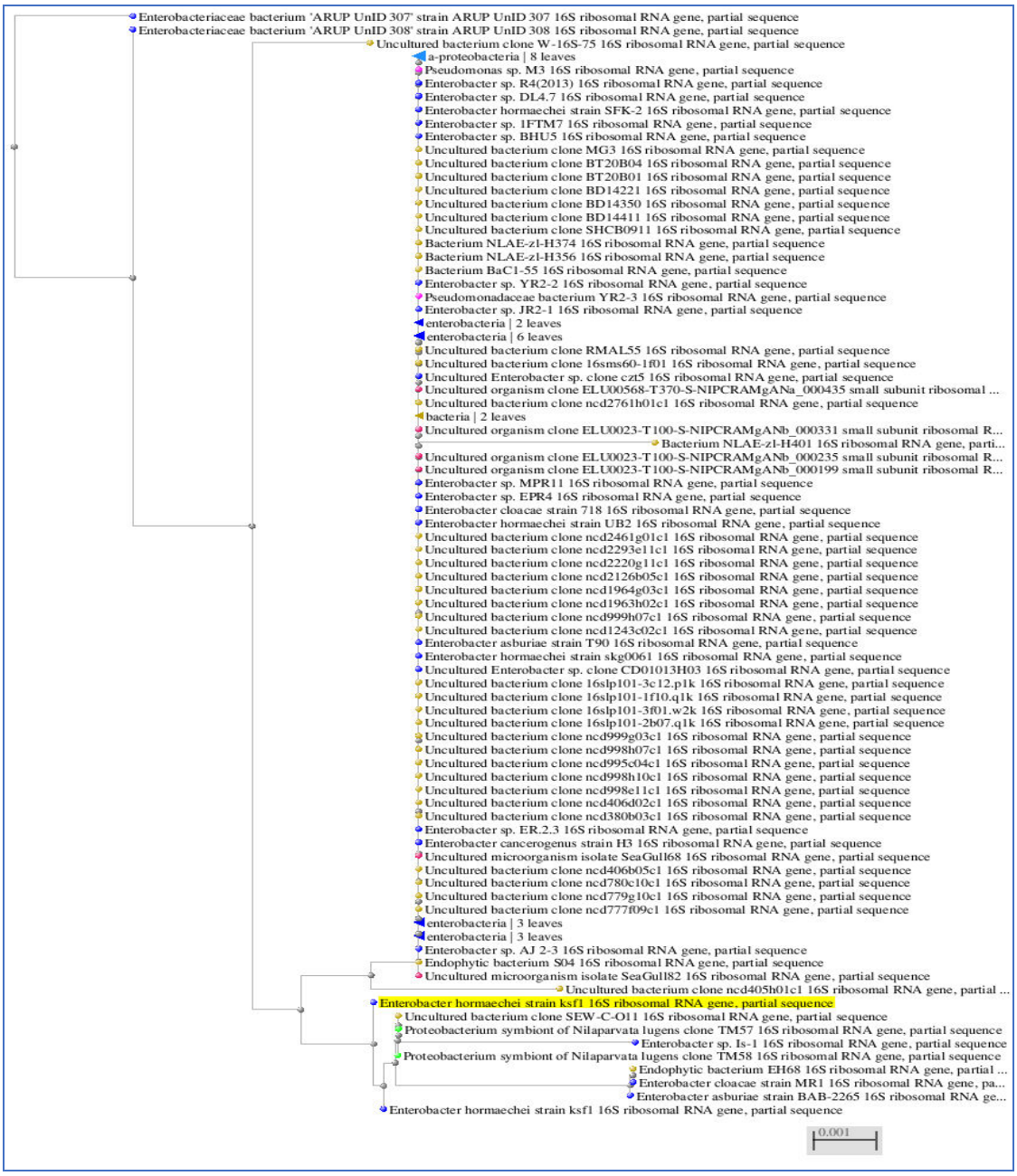


Figure.4 MFE Secondary structure of rRNA for KSB-8 (*Enterobacter hormaechei*).

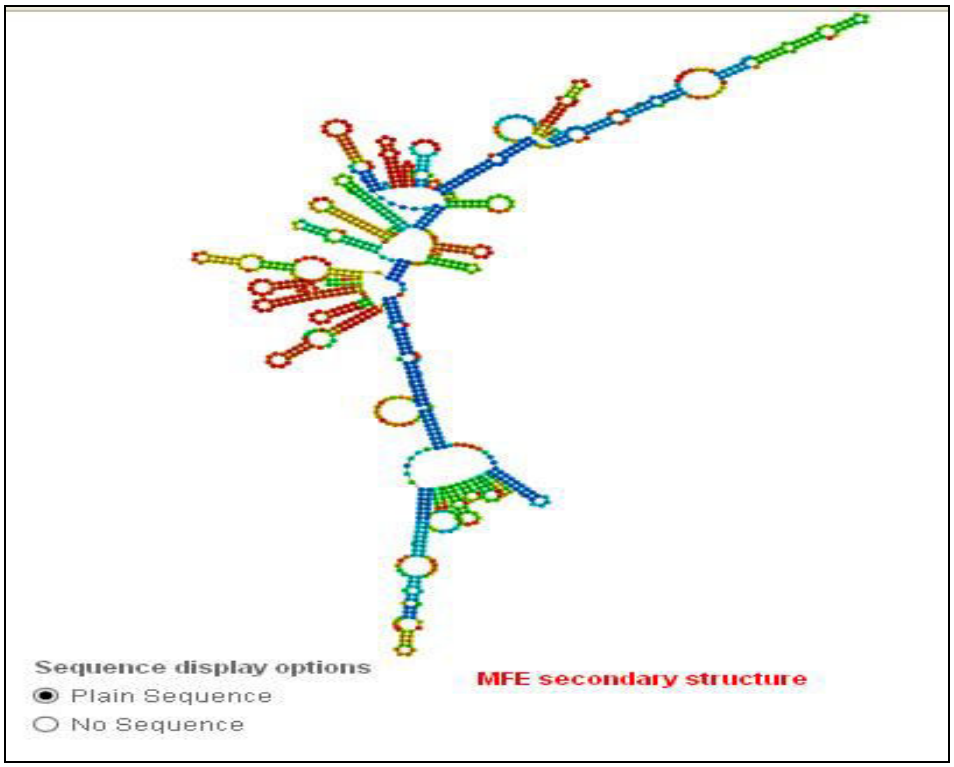
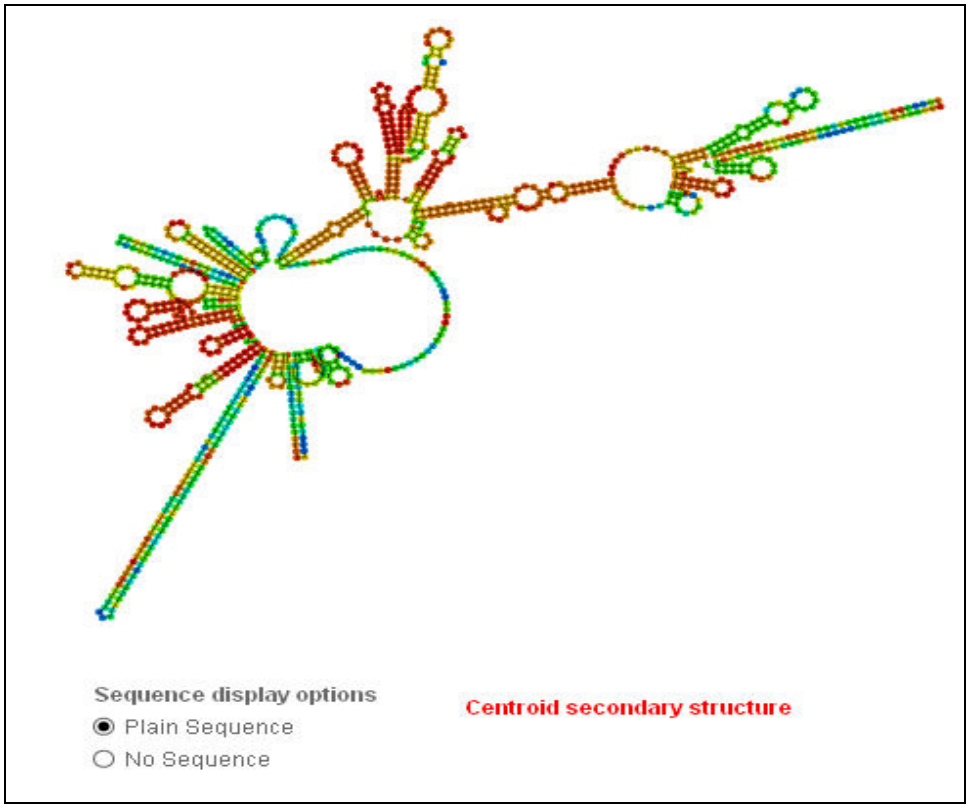


Figure.5 Centroid Secondary structure of rRNA for KSB-8 (*Enterobacter hormaechei*).



A model was proposed (Ignacio, 1971 and Ignacio, 1973) to calculate a folded RNA molecule's stability (in terms of free energy) by adding independent contributions from base pair stacking and loop destabilizing terms from the secondary structure. This model has proven a good approximation of the forces governing RNA structure formation, thus allowing fair predictions of real structures by determining the most stable structures in the model of a given sequence.

The RNA secondary structures are predicted to provide the basic information for phylogenetic analysis (Fig.3). The secondary structural features of KSB-8 regions, as shown in the figure, are analyzed based on the conserved stems and loops, which, in order of preference, were interior loop, hairpin loop and exterior loop in all the isolates.

The secondary structures yielded homologous models that grouped the conserved features. Generally, RNA secondary structure prediction programs rely on free energy minimization using nearest neighbour parameters to predict the overall structural stability in terms of Gibbs free energy at 37^0C. The observed similarities at the secondary structural level are further reflected at the energy level (—ΔG). However, the difference in their topology is due to differences in nucleotide sequence length.

The optimal secondary structure of *Enterobacter hormaechei* (KSB-8) is studied in dot-bracket notation and free energy prediction. The minimum free energy of the secondary structure is -313.40 kcal/mol, and from the thermodynamic ensemble prediction, the free energy is -328.81 kcal/mol, and the ensemble diversity is 259.53. The frequency of the ensemble's minimum free energy (MFE) structure is 0.00 % (Fig-4). When the centroid secondary structure is studied in dot-bracket notation, the minimum free energy is 209.10 kcal/mol (Fig-5). The secondary structure has 15 Hairpin loops, two bulk loops, 4 Junction loops, two stem loops, 23 interior loops, and 1 Pseudoknot that may bind to 23S rRNA in the larger subunit of the ribosome.

All rRNAs have identical functions because all are involved in the production of proteins. The overall three-dimensional rRNA structure that corresponds to this function shows only minor but in highly significant variation. However, within this, nearly constant overall structure, molecular sequences in most molecule regions are continually evolving and changing at the level of their primary structure while maintaining homologous secondary and tertiary structures, which never alters molecular function.

Conclusion

The potential K solubilizers were identified by molecular identification: 16S rRNA analysis of the KSB-8 isolate identified *Enterobacter hormaechei*, KSB-8 bacterial culture shows significant alignment to 98% similarity with *Enterobacter hormaechei.* Thus, bacterial isolate KSB-8 belongs to *Enterobacter spp.,* and the sequences are submitted to the Gene Bank database. The K-releasing bacterial isolated and identified may be used in the amelioration of K-deficient soils and further research can elucidate how these bacteria promoted plant growth and optimize their applications in different agricultural systems and may be used to an alternative mean of K nutrition improvement for use in agriculture.

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