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Virtual Screening And Molecular Dynamics Simulations of Efficient Potassium Solubilizing Bacteria Isolated From Rhizosphere Soils of Rice At Veeranam Command Area in Cuddalore DistricT

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ABSTRACT

A macronutrient that is essential for plant growth and development is potassium. Potassium (K) is one the important components for plant growth, metabolism, and development of crops. Potassium deficiency in soils has major consequences for all kinds of life. The potassium reserves in soil are ample but most of it being insoluble is unavailable for plant uptake. Soil microbes play a central role in nutrient cycling and influence the availability of soil mineral nutrients. For this investigation, 15 potassium solubilizing bacterial isolates which are isolated and characterized from rhizosphere soil samples of Rice gathered from 15 different locations at Veeranam command area in Cuddalore district were screened and molecularly identified. The 15 isolated and characterized potassium solubilizing bacteria in Rice rhizosphere (KSBR) were screened for their ability to produce plant growth promoting substances such as IAA and GA_3, siderophore production, polysaccharide production, and organic acids production. From the results of screening, the isolate KSBR-12 was considered to be the most efficient and had the maximum production of IAA (10.96 µg/25 ml), GA_3 (3.06 µg/25 ml), and siderophore production (280.17 µg/L). In addition, the isolate KSBR-12 showed higher polysaccharide production and produced more organic acids. Hence, the most efficient isolate, KSBR-12 was identified as *Frateuria aurantia* by molecular methods.

Keywords: Screening of isolates, 16S rRNA analysis, *Frateuria aurantia*

I. INTRODUCTION

Potassium (K) is one the important components for plant growth, metabolism, and development of crops. Potassium is considered by plant physiologists to be second to nitrogen in improving plant growth and yield (Meena *et al*., 2014). Soil consists of four different forms of potassium such as unavailable K (mineral K), available K (soluble K), non-exchangeable K (fixed or trapped K), and exchangeable K (ionic K) (Olaniyan *et al*., 2022). The mineral potassium makes up more than 90–98% of all soil potassium. Potassium is found in silicate minerals such as muscovite, orthoclase, biotite, feldspar, illite, mica, vermiculite, smectite, etc. in mineral soils. The fixed K in the soil is a stock source of potassium, while the exchangeable form of K (ionic K) is readily taken up by the plant's root system and substituted for potassium on the exchange sites (Nain *et al*., 2023). Potassium uptake by plants varies with different plants, and it is most needed at the early growth stage of the plant more than nitrogen and phosphorus (Sattar *et al*., 2019). Its uptake is mainly affected by soil moisture, temperature, and tillage system (Mouhamad *et al*., 2016). The K sources in soil are ample but most of it being insoluble is unavailable for plant uptake. Lack of potassium in the soil leads to poor development of roots and shoots, fewer branches, slow growth, and smaller seeds with poor yield (Verma *et al*., 2016).

Microorganisms can play a vital role in the solubilization, transport, and deposition of metals and minerals in this environment. Among these microorganisms, there is a group of soil bacteria known as plant growth promoting rhizobacteria (Bharathiraja and Tholkappian, 2011). These PGPRs promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus, and vital minerals) or modulating plant hormone levels and indirectly by reducing the inhibitory effects of numerous pathogens in the forms of biocontrol agents (Ranjitha and Bharathiraja, 2021).

In India, the farmers regularly use more amount of chemical fertilizers for crop production this way Indian soils receive pollutants which leads to pollution and ultimately causes health hazards. In order to avoid

environmental pollution, especially soil pollution most scientists are recommending the use of biofertilizers to replace the use of inorganic fertilizers (Bharathiraja, 2022).

Soil microbes have played a crucial role in maintaining potassium balance in soil. The use of plant growth promoting rhizobacteria (PGPR) along with potassium solubilizing microorganisms as biofertilizers, has become advised as a sustainable solution to enhance plant nutrients and production (Badr *et al*., 2006). Potassium solubilizing microorganisms (KSMs) present in the soil help to convert complex potassium in soil into simple forms and make them available to plants. Potassium solubilizing microorganisms like *Acidothiobacillus ferrooxidans*, *Aspergillus terreus*, *Bacillus circulans*, *B. edaphicus*, *B. mucilaginosus*, *Burkholderia*, *Pseudomonas,* and *Paenibacillus* solubilize fixed form of K in soil and making them available to plants via the processes such as organic acid production such as citric acid, acetic acid, malic acid, formic acid, oxalic acid, etc., acidolysis, polysaccharides secretion (Kour *et al*., 2020), biofilm formation on mineral surfaces (Etesami *et al*., 2017), complex formation and exchange reactions (Rai *et al*., 2020) hence, improve the growth and yield of crops (Meena *et al*., 2016). In addition, different types of amino acids, vitamins, and plant growth promoting substances such as gibberellic acid (GA_3) and indole-3-acetic acid (IAA) are also released by K-solubilizing bacteria, which help plants attain better growth (Singh *et al*., 2015).

KSMs possess the potential to improve the potassium availability in soils and hence can play an important role in potassium nutrient management under the condition of K-limited soils. The potassium solubilizing bacteria (KSB) are frequently found in much higher concentrations in the rhizosphere than in the non-rhizospheric soil. Most of them belonged to the *Bacillus*, *Pseudomonas*, *Acidithiobacillus*, and *Burkholderia* genus families.

In the previous study, 15 potassium solubilizing bacterial isolates were isolated from the rhizosphere soil samples of rice collected from 15 different locations at the Veeranam command area in Cuddalore district, Tamil Nadu. The isolates were characterized based on morphological and biochemical analysis and further screened for their efficiency to solubilize potassium from an insoluble potassium source (Mica) both qualitatively and quantitatively. Based on the morphological and biochemical characterization, the chosen isolates have been identified up to the genus level.

Thus, the present study aimed to screen the isolates for the production of plant growth promoting substances such as IAA and GA_3, siderophore production, polysaccharide production, and organic acids production. From the results of screening, the efficient isolate was molecularly characterized by 16S rRNA gene sequencing which helps to identify the isolate up to species level.

II. MATERIALS AND METHODS

The present study was carried out to screen and identify the efficient potassium solubilizing bacteria up to the species level from the rhizosphere soils of rice. The screened efficient isolate was subjected to a genotypic study. Laboratory studies were done to find out the efficient potassium solubilizing bacteria from the rhizosphere soils of rice.

2.1. Screening of potassium solubilizing bacterial isolates

2.1.1 Production of plant growth promoting substances by the potassium solubilizing bacterial isolates

2.1.1.1 Estimation of IAA and GA_3 production by potassium solubilizing bacteria

All fifteen isolates were subjected to qualitative analysis for IAA (Bric *et al*., 1991) and GA_3 production (Brown and Burlingham, 1968).

The Luria agar supplemented with 0.06 percent sodium dodecyl sulphate and one percent glycerol was prepared and plated. The surface area of the agar medium was divided into squares of 2 cm x 2 cm by marking on the bottom of each plate. The overnight culture of each isolate grown on Luria agar was spotted with a sterile toothpick in each square. The spotted plates were overlaid immediately with a sterile disc of Whatman No. 1 filter paper. Plates were incubated until the colonies reached the size of 0.5 to 2.0 mm in diameter. After an appropriate incubation period, the filter paper discs were removed from the plates and treated with Salkowski's reagent (2 % of 0.5 M $FeCl_3$ in 35 % perchloric acid) by soaking in a Petri dish containing the reagent. The reaction was allowed to proceed until adequate color was developed. The bacteria producing IAA were identified by the formation of a characteristic red halo zone around the colony on filter paper. The paper discs after treatment with Salkowski's reagent were viewed under UV light. The spots giving typical green fluorescence were taken as positive for GA_3 production. The isolates showing IAA and GA_3 production were further examined for the amount of IAA and GA_3 production as detailed below.

2.1.1.2 Quantitative estimation of IAA and GA_3 production

The overnight cultures of the isolates which showed the production of IAA and GA_3 in qualitative estimation were inoculated to 50 ml of sterilized Aleksandrov's solution and incubated at 37°C for seven days

in the dark. After the incubation period, the cultures were centrifuged at 6000 rpm for 20 minutes. The supernatant was collected in a conical flask and used for the estimation of IAA and GA_3.

2.1.1.3 Estimation of IAA (Loper and Scroth, 1986)

Quantitative analysis of IAA was performed using the technique of Loper and Scroth (1986) at different concentrations of tryptophan (150 mg ml^{-1}). Potassium solubilizing bacteria cultures were grown for 48 hrs on their AMs at 28 ± 2°C. Fully grown cultures were centrifuged at 6000 rpm for 20 minutes. The supernatant obtained (2 ml) was then mixed with two drops of ortho-phosphoric acid and 4 ml of the Salkowski reagent (50 ml of 35 % Perchloric acid, 1 ml of 0.5 M $FeCl_3$ solution). The optical density of the pink color was taken at 530 nm with the help of a spectrophotometer. The quantity of IAA in the culture filtrate was estimated from the standard curve prepared with known concentrations of IAA and expressed as µg/25 ml of the medium.

2.1.1.4 Estimation of GA_3 (Paleg, 1965)

To assess the gibberellic acid production, freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated at 37 ± 2°C for 24-72 h, after the Nessler's reagent (0.5 ml) was added in each tube. The development of color from brown to yellow indicated a positive response for gibberellic acid production (Cappuccino and Sherman, 1992). An individual colony of the test strain was streaked on NA plates supplemented with 4.4 g glycine L^{-1}, to screen for cyanide production. Thereafter, a piece of filter paper (Whatman filter paper No. 1) drenched with 0.5 % picric acid (yellow) and 2.0 % sodium carbonate was placed in the lid of each Petri dish. The Petri dishes were sealed with parafilm and kept at 28°C for 48-96 h. Gibberellic acid production was measured according to Paleg (1965). The amount of GA_3 present in the extract was calculated from the standard curve and expressed as µg/25 ml of the medium. The standard curves of IAA and GA_3 were prepared by using graded concentrations of IAA and GA_3.

2.1.2 Siderophore production by potassium solubilizing bacterial isolates (Schwyan and Neilands, 1987)

For the assay of siderophore production, all the glasswares were first soaked in 2N HCl solution for 24 h to avoid contamination of iron from the glasswares. The Chrome azurol S (CAS) solution was prepared by dissolving 60.5 mg dehydrated Chrome azurol S in 50 ml double distilled water and further mixing with 10 ml of iron solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). This was then slowly added with 40 ml aqueous solution containing 72.9 mg acetyl trimethyl ammonium bromide with continuous stirring and the final solution was autoclaved. The King's B agar was prepared using PIPES buffer (30.2 g) and Difco agar (18.0 g) and the pH was adjusted to 6.8 by the addition of 0.1N NaOH before autoclaving. After cooling, the CAS solution (100 ml) was added along with the wall of a flask with gentle agitation to mix without the formation of foam. The CAS agar thus prepared was poured into the plates. After solidification, the plates were kept in the refrigerator (4°C) for 24 h. The overnight cultures of *Frateuria* sp. (10 µl each) were spotted on these CAS agar plates and incubated at 28°C for 48 hours. The formation of orange-colored zone around the colony was taken as positive for siderophore production. The diameter of the orange-colored zone was recorded visually and scored as a narrow clear zone (+) and wide clear zone (++) by the strains.

2.1.3 Polysaccharide production by potassium solubilizing bacterial isolates

All the efficient potassium solubilizing bacteria were tested for polysaccharide production by spotting 10 µl of overnight culture on glucose minimal agar medium (Sambrook *et al*., 1989). The plates were incubated at 28 ± 2°C for 24 to 48 h. The amount of polysaccharide produced on glucose minimal agar medium was observed visually and scored as weak polysaccharide production (+), moderate polysaccharide production (++), and high polysaccharide production (+++) by the strains.

2.1.4 Organic acid production by potassium solubilizing bacterial isolates

One ml of 24 hours old culture of each isolate was inoculated to 25 ml of Aleksandrov broth and incubated at 28 ± 2°C for 10 days. The inoculated culture broth was centrifuged at 10,000 rpm for 10 minutes. The supernatant so obtained was concentrated to nearly 1/10th of the original volume in a water bath at 60°C. The concentrated material was then used for the estimation of organic acids by paper chromatography in comparison with standard organic acids (Gaur, 1990). Pure organic acids were prepared at 20 g/ml stock. About 10 µl of these standard acids and 15 µl of culture supernatants were spotted on Whatman No.1 chromatographic filter paper and then dried with a hair dryer.

A descending chromatography was run using a solvent mixture of n-butanol acetic acid and water in a 12:3:5 ratio in a chromatographic chamber pre-saturated with solvent for six hours. The chromatogram was run for 6 hours and air-dried for three days. The air-dried paper was sprayed with 0.04 percent bromocresol green (40 g BCG in 1000 ml methanol pH 7.0). The paper was air-dried at room temperature. The RF values (chromatography) and the intensity of yellow spots of organic acids developed on a blue background were measured and compared with the RF values (chromatography) of the standard organic acids for identification.

2.2 Genotypic identification study (16S rRNA analysis)

The bacterial isolate was identified by a 16S rRNA gene sequence study processed at Medauxin, Genomics R & D Services company in Bangalore. The genomic DNA was extracted as per the protocol (Manufacturer guide). Its quality was evaluated on 1.0 % agarose gel, and a single band of high-molecular weight DNA was observed. The fragment of 16S rRNA gene was amplified by 16S rRNA-F and 16S rRNA-R primers. When resolved on agarose gel, a single discrete PCR amplicon band of 1500 bp was observed. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16S rRNA-F and 16S rRNA-R primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic analyzer. Using aligner software, the consensus sequence of the 16S rRNA gene was generated from forward and reverse sequence data. The 16S rRNA gene sequence was then used to carry out BLAST with the 'nr' database of the NCBI Genbank. The first ten sequences were selected based on the maximum identity score and aligned using the multiple alignment software program Clustal W. Distance matrix and the phylogenetic tree was constructed using MEGAXI.

III. RESULTS AND DISCUSSION

3.1 Screening of isolates for IAA and GA_3 producing potential

Among the fifteen potassium solubilizing bacterial isolates, the isolate KSBR-12 produced the maximum amount of 10.96 µg IAA $25ml^{-1}$ (Table 1). The minimum amount of IAA was produced by KSBR-5 (7.16 µg IAA 25 ml^{-1}). The maximum amount of GA_3 (3.06 µg 25 ml^{-1}) was produced by KSBR-12. The minimum amount of GA_3 was produced by KSBR-5 (1.13 µg 25 ml^{-1}).

Similarly, Nagaraju *et al*., (2017) isolated 7 potassium solubilizing bacterial isolates and screened them for their efficiency to produce IAA. Also, similar findings were made by Singh *et al*., (2018), Yaghoubi Khanghahi *et al*., (2018) and Raji and Thangavelu (2021).

3.2 Screening of isolates for siderophore production

All fifteen potassium solubilizing bacterial isolates were able to produce orange halo zone around the colony (Table 2). Among them, the isolates KSBR-2, KSBR-4, KSBR-7, KSBR-9, KSBR-10 and KSBR-12 produced wider clear zone. Whereas, the isolates KSBR-1, KSBR-3, KSBR-5, KSBR-6, KSBR-8, KSBR-11, KSBR-13, KSBR-14 and KSBR-15 produced narrow clear zone. Among the fifteen potassium solubilizing bacterial isolates, the isolate KSBR-12 recorded the maximum production of siderophores (280.17 µg L^{-1}) followed by other isolates.

According to Chinachanta and Shutsrirung (2021), out of nine selected potassium solubilizing bacterial isolates, five isolates were able to produce orange halo zone around the colony and exhibited higher siderophore production. Similar observations were reported by Nagaraju *et al*., (2017) and Raji and Thangavelu (2021).

3.3 Screening of isolates for polysaccharide production

All fifteen potassium solubilizing bacterial isolates were able to produce polysaccharides and were categorized into three groups such as high, moderate, and low polysaccharide production (Table 3). Among the fifteen isolates, the isolates KSBR-12 and KSBR-4 recorded higher amounts of polysaccharide production whereas, the isolates KSBR-2, KSBR-6, KSBR-8, KSBR-11, and KSBR-15 recorded moderate amounts of polysaccharide production and the remaining isolates such as KSBR-1, KSBR-3¸ KSBR-5, KSBR-7, KSBR-9, KSBR-10, KSBR-13, and KSBR-14 produced low polysaccharide.

In the same way, Sheng and He (2006) showed the production of polysaccharides by the cultures of *Bacillus edaphicus*. Similar observations on the production of polysaccharides by KSB have been made by several workers (Liu *et al*., 2006; Buragohain *et al*., 2018).

3.4 Screening of isolates for organic acids production

All the isolates were found to produce one or the other organic acid tested (Table 4). Among all the organic acids produced by the potassium solubilizing bacterial isolates, citric acid was found to be the most common organic acid produced by all the fifteen isolates and all these isolates were not able to produce lactic acid and showed negative response. The other organic acids produced by the isolates include malic acid (eight strains), succinic acid (six strains), gluconic acid (ten strains), oxalic acid (eight strains), and tartaric acid (eight strains). Among the isolates, the isolate KSBR-12 showed positive responses for the production of more organic acids.

According to Kammar *et al*., (2016), all 28 potassium solubilizing bacterial isolates were found to produce one or the other organic acid tested for and it was found that the oxalic acid and citric acid were the most common organic acids produced by all the isolates as well as by the reference strain (*Frateuria aurantia*). The production of organic acids like oxalic acid, tartaric acid, citric acid, and malic acid by potassium solubilizing bacteria has been reported earlier by various workers (Sheng and He, 2006; Liu *et al*., 2006).

Molecular analysis

From the results of screening, out of 15 bacterial isolates, the most efficient strain KSBR-12 was molecularly identified up to the species level by using 16S rRNA gene sequencing. For molecular analysis, the efficient strain KSBR-12 was sent to Medauxin, Genomics R & D Services company, Bangalore. The isolate KSBR-12 was identified as *Frateuria aurantia* (OR681910) by 16S rRNA gene sequencing.

>KSBR-12

AGCACCGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTTAATCGGAATTACTG
GGCGTAAAGCGTGCGTAGGCCGGTTTGTTAAGTCTGTTGTGAATCCCCGGGCTCAACCTGGGAATGGC
AATGGATACTGGCAAGCTAGAGTGTGATAGAGGATGGTGGAATTCCCGGTGTAGCGGTGAAATGCGTA
GAGATCGGGAGGAACATCAGTGGCGAAGGCGGCCATCTGGATCAACACTGACGCTGAGGCACGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGT
CTCAACTCGGAGATCAGTGTCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGAC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGC
GAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGTAGAGATATGGGAGTGCCTTCGGGAATCAG
AACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTACAATGGT
CGGTACAGAGGGTTGCAATACCGCGAGGTGGAGCCAATCCCAGAAAGCCGATCCCAGTCCGGATCGA
AGTCTGCAACTCGACTTCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCTATGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACAGACCGCCC

Figure 1: PCR amplification profile

Agarose (1.5%) gel electrophoresis of PCR using 27F and 1492R primers bacterial sample.

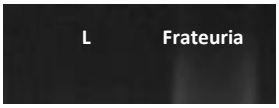

Figure 2: Phylogenetic tree of *Frateuria aurantia* by 16S rRNA gene sequence

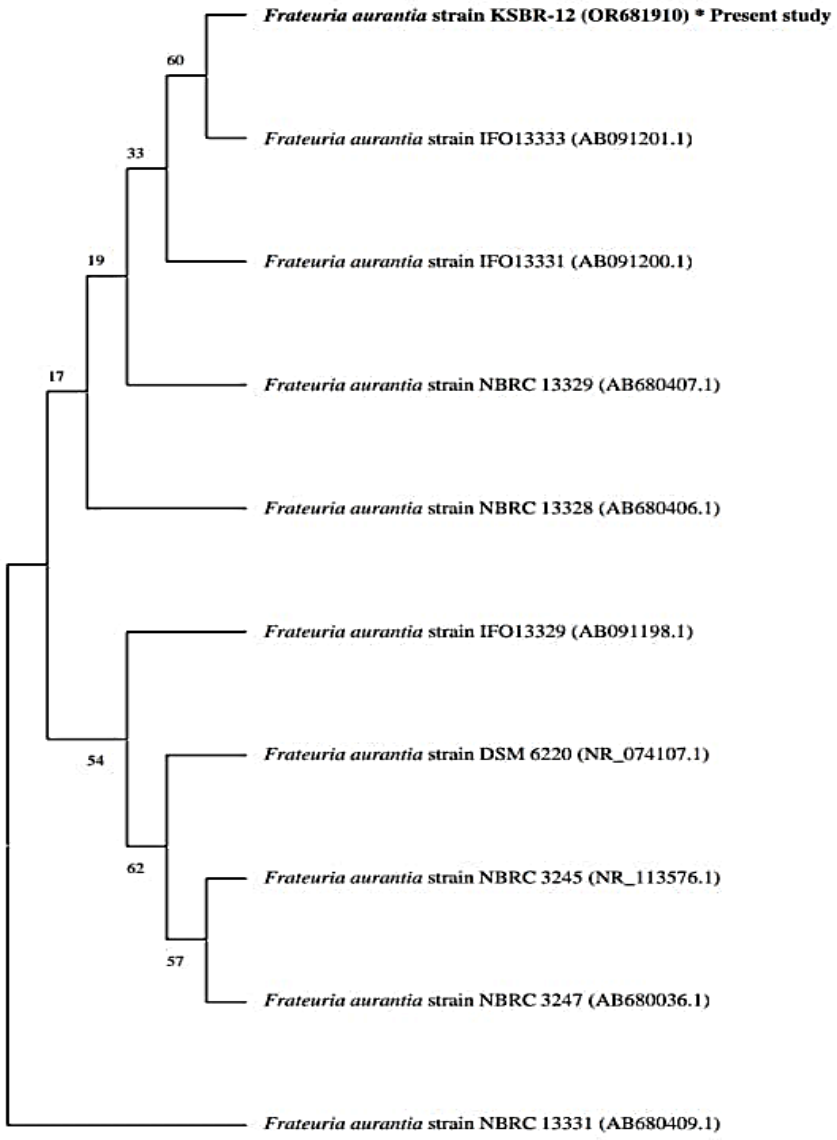

Table 1: Screening of potassium solubilizing

bacterial isolates for indole-3-acetic acid (IAA) and gibberellic acid (GA_3) producing potential

Isolates	IAA (µg 25 ml^{-1} of broth)	GA_3 (µg 25 ml^{-1} of broth)
KSBR-1	7.43	1.40
KSBR-2	8.13	1.94
KSBR-3	8.35	2.01
KSBR-4	9.14	2.81
KSBR-5	7.16	1.13
KSBR-6	7.68	1.69
KSBR-7	8.06	1.73
KSBR-8	7.34	1.25
KSBR-9	8.59	1.97
KSBR-10	7.83	1.54
KSBR-11	7.99	1.82
KSBR-12	10.96	3.06
KSBR-13	7.28	1.17
KSBR-14	7.73	1.39
KSBR-15	8.54	2.13

Table 2: Screening of potassium solubilizing bacterial isolates for siderophore production

Isolates	Orange halo zone	Siderophore content (µg L^{-1})
KSBR-1	+	68.37
KSBR-2	++	162.50
KSBR-3	+	169.26
KSBR-4	++	212.54
KSBR-5	+	97.36
KSBR-6	+	110.83
KSBR-7	++	174.62
KSBR-8	+	86.35
KSBR-9	++	188.50
KSBR-10	++	72.49
KSBR-11	+	196.35
KSBR-12	++	280.17
KSBR-13	+	65.93
KSBR-14	+	83.33
KSBR-15	+	202.41

(+) Narrow clear zone; (++) Wide clear zone

Table 3: Screening of potassium solubilizing bacterial isolates for polysaccharide production

Isolates	Polysaccharide production
KSBR-1	+
KSBR-2	++
KSBR-3	+
KSBR-4	+++
KSBR-5	+
KSBR-6	++
KSBR-7	+
KSBR-8	++

KSBR-9	+
KSBR-10	+
KSBR-11	++
KSBR-12	+++
KSBR-13	+
KSBR-14	+
KSBR-15	++

(+++) High; (++) Moderate; (+) Low

Table 4: Screening of potassium solubilizing bacterial isolates for organic acids production

Strain	Malic acid	Gluconic acid	Tartaric acid	Succinic acid	Oxalic acid	Citric acid	Lactic acid
KSBR-1	-	+	+	-	-	+	-
KSBR-2	+	-	-	+	+	+	-
KSBR-3	+	+	-	+	-	+	-
KSBR-4	+	+	+	-	+	+	-
KSBR-5	-	-	+	-	+	+	-
KSBR-6	-	+	+	-	+	+	-
KSBR-7	+	-	-	+	+	+	-
KSBR-8	-	+	+	-	-	+	-
KSBR-9	+	-	+	+	-	+	-
KSBR-10	+	+	-	-	-	+	-
KSBR-11	-	+	+	-	+	+	-
KSBR-12	+	+	-	+	+	+	-
KSBR-13	-	-	+	-	+	+	-
KSBR-14	-	+	-	-	-	+	-
KSBR-15	+	+	-	+	-	+	-

(+) Showed positive response; (-) Showed negative response

IV. CONCLUSION

The potassium solubilizing bacteria isolated from the rhizosphere soil samples of rice were molecularly characterized and screened for IAA and GA_3 producing potential, siderophore production, polysaccharide production, and production of organic acids. From the results, out of all 15 isolates, KSBR-12 was found to be the most efficient isolate. The isolate KSBR-12 was then molecularly identified as *Frateuria aurantia* OR681910 by 16S rRNA gene sequencing. The isolate KSBR-12 (*Frateuria aurantia* OR681910) can also solubilize inorganic sources of potassium. So, this isolate will be applied alone or in a consortium mode which aid in enhancing the potassium content in various crops such as cereals, vegetables, flower crops, and medicinal crops while simultaneously enhancing the growth and yield.

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