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# A study on synthesis of amylase, cellulase L-asparaginase enzymes and potassium solubulizing efficiency of rhizobacteria from the *Piper betle* fields of Guntur District, Andhra Pradesh, India

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**Key words:** Betel vine, Rhizosphere, Rhizoacteria, *Bacillus endophyticus* ASN-5, Strain

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**Abstract**

The present study was aimed to screen the potassium solubilizing rhizobacteria from Betel vine plant (*Piper betle*) of Guntur district (Nutakki, Revendrapadu), Andhra Pradesh, India. In present study nutrient agar and Aleksandrov medium (A) were used for the isolation of rhizobacteria. A total of 15 rhizobial strains designated as ASN-1 to ASN-15 were isolated. All the strains were tested for their efficiency in synthesizing amylase, cellulase L-asparaginase enzymes. Apart from these all the strains are also tested for their Potassium solubulizing efficacy. Highly potent strain was based on its morphological, biochemical and molecular characteristics (16s r RNA sequencing). Of all the rhizobial strains, ASN-5 showed high potassium solubilization efficiency 15 mm at 25 µg/ml on Aleksandrov medium after 72 h of incubation at 28±2 $^0$C. Beside potassium solubilization strain ASN-5 is able to produce amylase, cellulase and L-asparaginase enzymes. The potential strain was characterized as *Bacillus endophyticus* ASN-5 (MW537708).

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## Introduction

Potassium (K) is the third major essential macronutrient after Nitrogen (N), Phosphorus (P) for plant growth, development, metabolism (Protein synthesis, Photosynthesis) and Resistance to diseases (Sardans *et al.*, 2012). It was considered as a major constituent within all living cells. With insufficient potassium levels, the plants will grow slowly, produce small seeds, have poorly developed roots and have lower yields. Though the Potassium was an abundant element in soil only 1-2% of this is available to plants as natural or synthetic fertilizers and the rest being bound with other minerals and therefore unavailable to plants. In soil, K is present in several forms such as exchangeable K, non-exchangeable K, mineral K and soluble K. The soluble potassium concentrations in the soil are usually very low and above 90% of potassium in the soil exist in the form of silicate minerals and insoluble rocks (Archana *et al.*, 2012; Bahadur *et al.*, 2014).

Rhizosphere microflora especially bacteria have been found to dissolve potassium from insoluble potassium.The popular rhizosphere bacteria includes *Pseudomonas, Burkholderia, Acidothiobacillus, Bacillus* and *Paenibacillus* etc., has been reported to release potassium in accessible form from potassium-bearing minerals in soils (Dixon and Weed, 1989; Jain *et al.*, 2022). The potassium solubilizing bacteria (KSB) werefound to dissolve potassium and other nutrients (aluminium and silicon) from insoluble K-bearing minerals by excreting organic acids which directly dissolved rock K.

Moreover, because of imbalanced fertilizer application, K-deficiency is becoming major barrier in crop productionand need to have an alternative source of K for plant uptake and to maintain K levels in soils for sustainable crop production (Meena *et al.*, 2014). Since, Soil microbes have been reported to play a key role in the natural K cycle and therefore, potassium solubilizing microbes present in the soil could make potassium was available for plants uptake (Khan *et al.*, 2007). Currently, the application of K solubilizing bacteria as biofertilizer or bio control agents for agriculture improvement can reduce the use of agrochemicals, enhance soil fertility and support eco-friendly crop production (Han *et al.*, 2006; Matias *et al.*, 2019).

In view of beneficial role of K in agriculture sector, the present research was focused on isolation and screening of efficient potassium solubilizing bacterial strains from rhizosphere soil of *Piper betle* at Guntur district (Nutakki and Revendrapadu villages), Andhra Pradesh, India.

## Materials and methods

*Collection of rhizosphere soil samples*

*Piper betle* (Betel vine) fields from Nutakki and Revendrapadu villages (Guntur district), Andhra Pradesh, India were selected for collection of rhizosphere soil samples. The soil samples from rhizosphere Betel vine fields were taken in randomly at a depth of 10-15 cm and brought to the laboratory. All the collected samples were shade dried at room temperature, processed by removing stones and sand particles. Samples were then grinded in mortar and pestle then used for further studies (Rekha and Sreeramulu, 2015).

*Isolation of bacteria from rhizosphere soil*

The soil sample (10 g) was dissolved in de-ionized water (100 ml) and shaken for 15 minutes. The serial dilutions were made up to $10^{-6}$ and 0.1 ml of each diluted suspension was spread over Nutrient agar medium (NAM). Nystatin (25 µg/ml$^{-1}$) was supplemented to the NAM as antifungal agent to control the fungal contamination. Plates were incubated at 28±2 $^0$C for 3-4 days (Prajapati and Modi, 2012). After incubation, appeared bacterial colonies were selected and sub-cultured on NAM slants. All the bacterial isolates were stored at 4 $^0$C in refrigerator for further studies.

*Screening of rhizobacteria for K-solubilization*

The isolated bacteria were subjected to screen potassium solubilization on Aleksandrov medium (A) and pH 7.0 was maintained (Alexander, 1985). All the isolated bacterial strains were spotted over

Aleksandrov medium and incubated at 28±2 ⁰C for 3 days. After incubation, the bacterial colonies showing clear zone or formation of solubilization zone were considered to be KSB (Potassium solubilizing bacteria). Solubilization index (SI) was measured by Khandeparkar's selection ratio (Ratio=Diameter of zone of clearance/Diameter of colony growth) (Rajawat *et al.*, 2016).

*Quantitative Estimation of K-solubilization efficiency*

The isolated K-solubilising strain was quantitatively estimated on Aleksandrov broth to solubilize $K_2HPO_4$ (0.50%) was determined by using Sodium cobaltinitrite solution (5 ml). The Aleksandrov Broth supplemented with 0.50% $K_2HPO_4$ was prepared, the strain ASN-5 was inoculated and incubated at 28±2 ⁰C for 8 days and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted, precipitate collected and treated with ethanol. 10 ml of conc. HCl was added to the precipitate and incubated at 30±2 ⁰C for 15-20 minutes and absorbance was measured at 600 nm using the spectrophotometer (Keshavarz *et al.*, 2013).

Characterization of the rhizobacterial strain ASN-5

*Morphological and biochemical identification*

Characterization of isolated potential potassium solubilizing bacteria ASN-5 was studied through inoculated on NAM and followed by incubation at 28±2⁰C for 2 days. The strain ASN-5 was examined for their colony morphology through microscopic observation by Gram's reaction. Different biochemical tests such as Voges-proskauer (VP) test, catalse test, citrate utilization, Indole production and Methyl-red (MR) test, were performed to identify the bacteria. Starch hydrolysis, $H_2S$ production and Gelatin liquefaction were also evaluated (Holt *et al.*, 1994; Capuccino and Sherman, 2001; Bhat and Vyas, 2014).

*Molecular characterization*

Genomic DNA (gDNA) extraction and PCR amplification Pure genomic DNA was extracted from pure bacterial culture using the GenElute Bacterial Genomic DNA extraction kit according to the manufacturer's instructions. The extracted DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Waltham, MA, USA) and used as a template for the amplification of the 16S rRNA gene region. Nearly full-length 16S rRNA gene sequences was PCR-amplified using a universal bacterial primer pair 27F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3'). The PCR was carried out in a l 10x PCR buffer, 0.75 µl $MgCl_2$, 25 µl reaction and consisted of 2.5, 0.5 µl dNTPs, 0.5 µl of each primer, 0.2 µl platinum Taq (Invitrogen), 1 µl of template DNA and 19.05 µl of water. Amplification was performed with initial heating at 95 °C for 30 sec followed by 30 cycles of denaturation at 95 °C for 50 sec, annealing at 54 °C for 50 sec and extension at 72 °C for 1 min and a final extension period at 72 °C for 5 min using MJ Research PTC-225 Peltier Thermal Cycler. Amplicons were confirmed by visualization on 1% ethidium bromide stained agarose gels under gel documentation chamber and PCR products were sequenced (Tamura *et al.*, 2013).

*Phylogenetic analysis*

Sequences were compared using BLASTN analysis and sequence alignment was performed using the CLUSTAL program (http://www.clustal.org) against the similar strains. A phylogenetic tree of the aligned sequences was constructed using MEGA 6 software. Evolutionary distances were computed using the Maximum parsimony method. To obtain statistical support values for the branches, bootstrapping was conducted with 1000 replicates. All sites, including gaps in the sequence alignment, were excluded pair-wise in the phylogenetic analysis. Using the resultant neighbor-joining tree, each isolate was assigned to the Bacillus taxonomic group. The16S rRNA gene sequences of the strain ASN-5 were submitted in NCBI (National Center for Biotechnology Information) (Felsenstein, 1985).

*Multiple enzyme screening of the strain ASN-5*

*Screening of amylase enzyme*

Amylase production of the strain ASN-5 was determined by employing Starch casein agar medium (SCA), amended with soluble starch (1%) and sterilized at 121 ⁰C for 15min. The strain ASN-5 was

streakedover SCA medium and incubated for 3-4 days at 28±2 ⁰C. Thereafter, plates were flooded with 1% Gram's iodine and amylase production was confirmed by formation of a clear zone around the colony (Pranay *et al.*, 2019).

*Screening of cellulase enzyme*

Carboxyl methyl cellulose (CMC) agar medium was used for screening for cellulolytic ability of the strain ASN-5. The strain ASN-5 was screened for cellulase through observation of a clear zone around the culture on CMC agar. The culture was streaked on CMC plates and incubated for 3-4 days at 28±2 ⁰C. After incubation, the plates were flooded with Gram's iodine and cellulose enzyme production was visualized in plates by appearance of a zone of clearance (Ariffin *et al.*, 2006).

*Screening of L-asparaginase enzyme*

Rapid plate method was used to screen the strain ASN-5 for anticarcinogenic L-asparaginase enzyme (Gulati *et al.*, 1997). The L-asparaginase activity was carried out by using asparagine dextrose (ADS) agar, at pH 7.0 and phenol red (0.009%) was supplemented as pH indicator. The ADS agar was sterilized in an autoclave for 30 min at 121⁰C/15 lbs pressure. The culture ASN-5 was inoculated and plates were incubated for 3-4 days at 28±2⁰C. After incubation, the appearance of a pink zone around the culture colony indicated the positive of L-asparaginase production (Wakil and Adelegan, 2015).

*Statistical analysis*

Readings were taken as the mean ± standard deviation of the mean of three replicates calculated using Microsoft Excel XP 2007.

**Results and discussion**

*Isolation and screening of rhizobacteria for k-solubilization*

A total of fifteen rhizobacterial strains were isolated from the agriculture fields of *Piper betle* from Guntur district (Nutakki, Revendrapadu), Andhra Pradesh, India. All the isolates were sequentially designated as ASN-1 to ASN-15. The isolated bacterial strains were screened for their enzyme production and potassium solubilizing capacity by using Aleksandrov medium supplemented with 0.50% of $K_2HPO_4$. A loopful of each bacterial strain was spot inoculated on Aleksandrov medium plates and growth was observed after 24-48 h (Table.1). Among the 20 strains screened, the isolate ASN-5 exhibited predominant zone of potassium solubilization (Fig. 1).

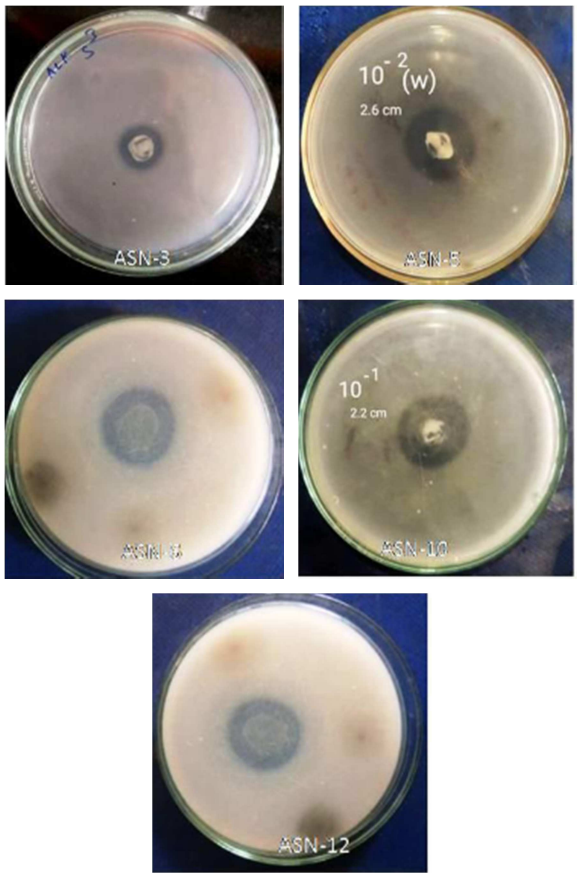


**Fig.1.** Clear zone of Potassium solubilization of the strains on Aleksandrov medium

*Estimation of potassium solubilization efficiency*

The potassium solubilizing capacity of the strain ASN-5in Aleksandrov Broth indicated that the strain exhibited K-solubilizing nature (Table 1). ASN-5 strain was produced 80.32 µg/mL$^{-1}$ soluble phosphate in the PK broth. Similarly, the bacterial strains WPS73 and NNY43 showed largest largest potassium solubilisation zone (48.00 mg/l; 41.0 mg/l) (Matthew and Shivakumar, 2021). Whereas the bacteria *Cellulosimicrobium funkei* solubilized potassium better with glucose as carbon source in Aleksandrov medium (7.04 mg/l) (Parmar and Sindhu, 2013).

**Table 1.** Potassium (K) solubilization efficiency of the isolated strains

| Strain | Diameter of zone (D) of clearance (mm) | Diameter of growth (d) of colony (mm) | D/d ratio |
|---|---|---|---|
| ASN-1 | 1 | 1 | 1 |
| ASN-2 | 7 | 6 | 1.16 |
| ASN-3 | 4 | 8 | 0.5 |
| ASN-4 | 6 | 4 | 1.5 |
| ASN-5 | 26 | 8 | 3.25 |
| ASN-6 | 6 | 14 | 0.4 |
| ASN-7 | 2 | 4 | 0.5 |
| ASN-8 | 12 | 9 | 1.3 |
| ASN-9 | 7 | 6 | 1.16 |
| ASN-10 | 22 | 11 | 2 |
| ASN-11 | 3 | 3 | 1 |
| ASN-12 | 8 | 16 | 0.5 |
| ASN-13 | 2 | 4 | 0.5 |
| ASN-14 | 6 | 8 | 0.75 |
| ASN-15 | 14 | 9 | 1.5 |

Khandeparkar's ratio: D/d. D = Diameter of zone of clearance, d = Diameter of growth of isolate

**Table 2.** Morphological, biochemical and physiological characteristics of ASN-5

| Characteristics | ANS-5 |
|---|---|
| Gram's reaction | + |
| Cell morphology | Rod shaped |
| Temperature for growth | 30±2$^0$C |
| pH for growth | 7.0 |
| Catalase activity | + |
| Oxidase activity | + |
| Gelatin liquefaction | - |
| Citrate utilization test | - |
| Methyl red reaction | + |
| Indole production test | + |
| Voges-proskauer reaction | - |
| $H_2S$ production | + |
| Starch hydrolysis | + |

(+) indicates positive; (-) indicates negative

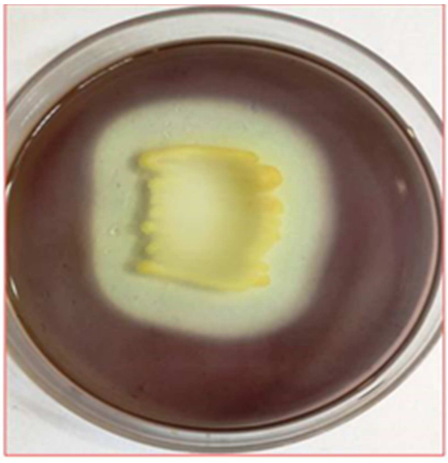

**Fig. 2.** Starch hydrolysis of the strain ASN-5

*Biochemical and physiological characteristics of the strain ASN-5*

Several tests were conducted for identifying strain ASN-5 (Table 2). The strain exhibited Gram positive, non-motile with rod shaped cells. The strain ASN-5 exhibited a positive response to oxidase activity, catalase activity, indole production and methyl red reactions but negativeto citrate utilization, Gelatin liquefaction and Voges-Proskauer reactions. The strain ASN-5 possess positive reaction to starch hydrolysis (Fig. 2) and $H_2S$ production.

*Molecular characterization of the strain ASN-5*

Based on the morphological, biochemical and molecular characteristics, the strain has been included under the genus *Bacillus* and deposited at NCBI genbank with an accession number MW537708. The partial sequence was aligned and compared with all the 16S rRNA gene sequence available in the GenBank databaseby using the multisequence advanced BLAST comparison tool that is available in the website of NCBI. The phylogenetic analysis of the 16S rRNA gene sequence was aligned using the CLUSTAL W program from the MEGA 6 Version**.** Phylogenetic tree was constructed using MEGA software Version 6 using maximum parsimony method (Fig. 3).

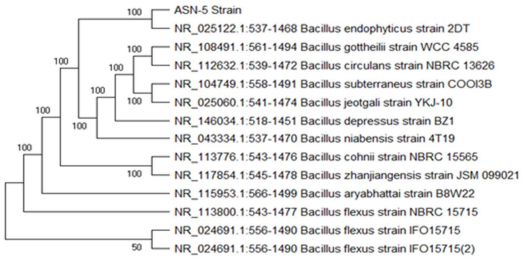


**Fig. 3.** Starch hydrolysis of the strain ASN-5

*In-vitro multiple enzyme screening*

Initial screening of the strain ASN-5 for amylase production was detected through observation of zones of hydrolysis on starch casein agar plates. 1% Gram's iodine was flooded into the culture plates and visualized a clear zone around the colony. Which represents the positive activity for amylase production (Fig. 4A)?

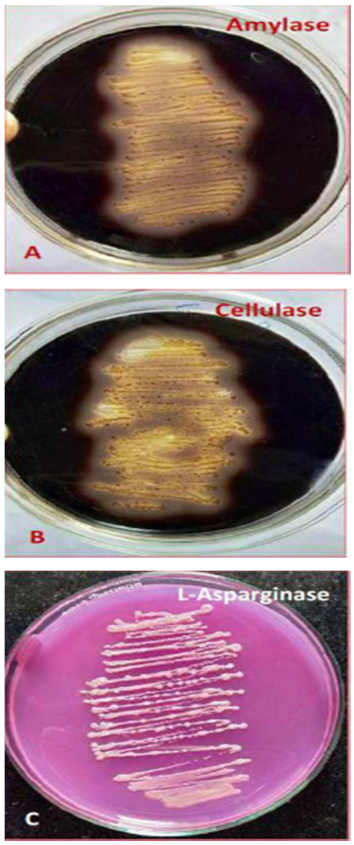


**Fig. 4.** Enzyme production of ASN-5

After inoculation of the strain ASN-5 on CMC agar and incubation period, plates were flooded with Gram's iodine (1%) solution for 3-5min the cellulolytic ability was visualized as positive reaction by the appearance of a zone of clearance (Fig. 4B).

L-asperginase production of *Bacillus* ASN-5 was evidenced by formation of a pink zone around the colonies which indicates the hydrolysis of L-asparginase. Since ADS agar medium appeared as yellow (acidic pH) and at alkaline pH turns pink, thus formation of a pink zone around the culture colonies was determine as L-asparginase production (Fig. 4C).

## Conclusion

In the current study, *Bacillus endophyticus* ASN-5 isolated from *Betel vine* rhizosphere showed encouraging potassium solubilization activity in Aleksandrov medium and produced different industrially important enzymes (amylase, cellulose and L-asperginase). Further study on optimization process of parameters of the strain ASN-5 for potassium solubilization is in progress.

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## Conflicts of interest

Author declares that there are no conflicts of interest.

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