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ISOLATION AND IDENTIFICATION OF EXOPOLYSACCHARIDE AND IAA PRODUCING BRADYRHIZOBIUM YUANMINGENSE FROM ROOT NODULES OF *ABRUS PRECATORIUS* L.

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ABSTRACT:

It is well known that certain strains of each rhizobial group that are effective plant growth promoters by producing plant growth hormones, in addition to their ability in N_2 fixation. Such strains can be useful not only for their host, but also for other plants. Six *Rhizobium* strains were isolated from root nodules of *Abrus precatorius* collected from different regions of North Gujarat. Four of the *Rizobium* strains produced indole acetic acid (IAA), but maximum amount was produced by only two strains in Yeast Extract Mannitol (YEM) medium supplemented with L-tryptophan. The strains were found to elaborate maximum IAA when fed with 5 mg/ml L-tryptophan. The strains were examined for production of Exopolysaccharide (EPS) and Indole acetic acid (IAA) by utilizing 10 different carbon sources. DNA was isolated from potent producer of EPA and IAA and amplified by using 16S r-DNA primer and then sequenced. By using bioinformatics tool BLAST the nucleotide sequence of isolate was aliened and isolate was found to be *Bradyrhizobium yuanmingense.* The nucleotide sequence was submitted to NCBI gene bank data and the accession number provided by NCBI for this sequence is JN 575481.

Keywords*:* IAA production; EPS production; *Rhizobium* species; *Abrus precatorius; Bradyrhizobium yuanmingense.*

INTRODUCTION:

The legume-rhizobium interaction is the result of specific recognition of the host legume by *Rhizobium*. Various signal molecules that are produced by both Rhizobia and the legume confer the specificity (Phillips, 1991). Exopolysaccharide (EPA) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection (Olivers *et al*., 1984; Raghavendra Joshi and Padder, 2002). It also protects the cell from desiccation and

predation and helps in nitrogen fixation by preventing high oxygen tension (Jarman *et al*., 1978). In addition, *Rhizobium* strains secrete growth hormones like Indole acetic acid (IAA), which shows positive influence on plant growth and also plays an important role in the formation and development of root nodules (Nutman,1977). Hence, the production of EPA and IAA are considered as important traits of plant growth-promoting rhizobacteria.

Plant

Abrus precatorius L. (Fabaceae) is a high climbing, twining woody vine with herbaceous branches. The fruits have more or less the shape of bean pods that contains seeds and are borne in clusters. It grows in tropical climates such as India, Sri Lanka, Thailand, the Philippine Islands, South China, tropical Africa and the West Indies. It also grows in subtropical areas. The most poisonous part of the plant is the seed. The seed contains the toxic poison "abrin" which is close relative to ricin. Ingested seeds can affect the gastrointestinal tract, the liver, spleen, kidney, and the lymphatic system. Infusion of seed extracts can cause eye damage after contact.

Figure: 1 *Abrus precatorius* plant

Traditional uses

The most widespread use of *A. precatorius* L. seeds is in the treatment of eye infections and as a potential contraceptive (Amudar *et al.,* 1991). *A. precatorius* L. seeds are considered as poison which, when administered, inhaled or swallowed, is capable of acting deleteriously on the body (Parikh and Keith, 2007). The present study was aimed at screening the *Rhizobium* strains from *Abrus precatorius* for the plant growth-promoting characteristics such as utilization of different carbon sources, production IAA and EPS.

MATERIALS AND METHODS:

Isolation of bacterial isolates

For the present study different *Abrus precatorius* L. growing wild in the different area of North Gujarat were selected. Fresh roots of *Abrus precatorius* L. collected from field were cleaned with tap water to remove all soil and organic particles. Forceps were used to hold the nodules, and the roots with attached nodules were cut, 2-3mm on each side of the nodules. Intact and undamaged nodules were immersed for 10 second in 95 % ethanol; transferred to a 2.5-3 % (v/v) solution of sodium hypochlorite and soaked for 4-5 min. The segments were then rinsed in five changes of sterile water using sterile forceps for transferring. Forceps sterilized quickly by dipping in alcohol and flaming. Sterile glass Petri dishes used as

containers for the alcohol, sodium hypochlorite, and water. The sterilizing and rinsing fluids changed as required. Mercuric chloride solution (0.1% weight/volume) used for the surface sterilization of nodule. The nodules were crushed in a sterile tube with sterile glass rod and sterile water. The slurry was than diluted and then streaked on the surface of YEMA (Yeast-Extract Mannitol Agar) plates containing congo red. The inoculated Petri plates were incubated at 25-28 ^{o}C for 3 to 10 days, depending on the strain and species until colonies appear. The colonies of rhizobia were mucoid, round and show little or no congo red absorption. The isolate from a single rhizobial colony was then purified and confirmed as *Bradyrhizobium*. Pure cultures were maintained on YEMA medium and used for the further study.

Utilization of different carbon sources

All of the isolated strains were tested for utilization of different carbon sources as sole carbon source by replacing mannitol in YEMA medium with equal amounts of 10 different carbon sources.

Exopolysaccharide (EPS) production

For the estimation of EPS production, *Bradyrhizobium* strains were inoculated into conical flasks containing 100 ml of YEM broth supplemented with 1% of carbon source. The inoculated flasks were incubated at 30 ± 1°C on a gyrorotatory shaker at 200 rpm for 72 h. After incubation, the culture broth was centrifuged 3500×g and the supernatant was mixed with two volumes of chilled acetone (Qualigens, India). The crude polysaccharide developed was collected by centrifugation at 3500×g for 30 min. The EPS was washed with distilled water and acetone alternately, transferred onto a filter paper and weighed after overnight drying at 105 ^{o}C (Damery and Alexander, 1969).

Indole acetic acid (IAA) production

All *Bradyrhizobium* strains were further screened for IAA production by inoculating them into 100 ml conical flasks containing YEM broth supplemented with L-tryptophan (0.1% to 1%) and 1% of different carbon sources. The flasks were incubated at 30 ± 1°C on gyrorotatory shaker at 200 rpm for 72 h. After incubation the medium was centrifuged for 5000×g for 20 min and the cell-free supernatant was used for IAA extraction (Sinha and Basu, 1981). To the 2 ml of supernatant, 2 drops of Orthophosphoric acid and 4 ml of Salkowaski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5M FeCl3) was added and incubated for 30 min under darkness. The amount of IAA produced was determined colorimetrically at 540 nm (Gordon and Weber, 1951).

Characterization and Identification of the isolate.

Morphological analysis

A dried fixed smear was covered with oxalate crystal violet reagent for sixty seconds. The stain was then rapidly washed off with clean water. All the water was then tipped off and the smear was covered with Lugol's Iodine for sixty seconds. The Iodine was then washed off with clean water. The smear was then decolorized rapidly for 10 seconds with acetone alcohol and was washed immediately with clear water. Finally, the smear was covered with Safranine for 30 seconds. The slide was then washed thoroughly in water and blotted dry. The smear was examined under microscope by using immersion oil objective.

Biochemical analysis

The physiological activities of the selected isolate tested through Oxidase, Catalase Motility Indole, Urease (MIU), Methyl red (MR), Acetoin production (Voges-Proskauer), Nitrate reduction, Citrate utilization, Hydrogen Sulfide (H_2S) production, Gelatin liquification and carbohydrate fermentation methods as demonstrated by Collee and Miles (1989).

Genetic analysis

Characterization of isolate using 16S r-DNA sequencing

Culture medium and growth conditions

The pure culture was grown in a 100 ml medium of Yeast Extract Mannitol broth and incubated in an incubator shaker (120 rpm) at 30 °C for 48 hrs. The pellet of culture was obtained by centrifugation of broth.

Genomic DNA extraction from isolate

Genomic DNA was extracted from the isolate using a MEDOX-Bio ^{TM} Ultra pure Genomic DNA Spin Miniprep kit, following the manufacturer's instructions. The cell pellet was re-suspended in 200µl cold TE buffer. 400 µl of digestion solution was added; mixed and 3 µl of proteinase K solution was added and incubated for 5 minutes. Then 260 µl of ethanol was added and thoroughly mixed. The mixture was applied to column placed in 2 ml of collection tube. The mixture was then centrifuged at 8000 rpm for 1 minute. 500 µl of wash solution was added and spin at 8000 rpm for 1 minute. This step was repeated. The flow through and collection tube was discarded. The mini spin column was carefully removed and placed in 1.5 ml micro-centrifuge tube; 30 µl of elution buffer was added and was incubated for 2 minutes at room temperature and then centrifuged at 1000 rpm for 1 minute. The DNA sample was then stored at -20 °C and thawed at room temperature as required.

Determination of DNA concentration, yield and purity

DNA yield was measured by determining the absorption of the elute at 260 nm wavelength. The purity of the DNA was calculated by the ratio of the absorbance at 260 nm and 280 nm,

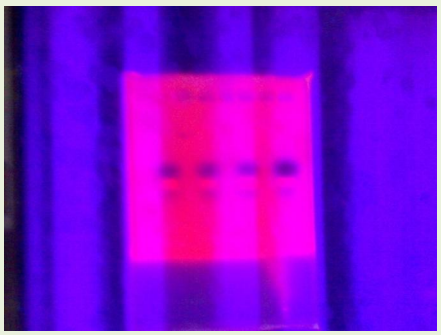
which provided an estimate of the purity with respect to contaminants that absorb UV light, such as protein. Pure DNA has an A260/A280 ratio of 1.8-2.0. DNA concentration was measured using spectrophotometric reading at 260 nm (Shimadzu)

Amplification of 16S r-DNA by Polymerase Chain Reaction (PCR)

In PCR the isolated DNA was amplified by using 16S r-DNA primers (F-5'AGAGTTTGATCCTGGCTCAG 3' and R-5'AAGGAGGTGATTCCAGCC3'), and by using the Taq and GO PCR master mix; which was obtained from MP Bio. The Master mix contained Taq polymerase, Divalent cations Mg^{+2}, dNTPs. The reaction for 50 µl was prepared according to standard protocol provided by MP Biomedicals for amplification. The isolated DNA was added in range of 100pg-100ng. The DNA was amplified by using Thermal cycler (eppendroff) using parameters (viz Denaturation temperature : 94 °C for 20 s, Annealing temperature 55 °C for 40s, Extension temperature 72 °C for 2 min) for 32 cycles. The PCR product was analyzed by gel electrophoresis.

Gel Electrophoresis of PCR product and photography of the gel

Standard electrophoresis protocol was used using a gel electrophoresis. PCR product 5µl was mixed with 2µl of gel loading dye. The reaction was loaded in to the well of a 1% agarose gel prepared in TAE buffer. Etbr was added in gel. The gel was photographed using a Gel Documentation System.



Extraction of PCR Product and DNA sequencing

The PCR product was extracted by a low melting agarose gel method. Sequencing was carried out by Ocimum Biosolutions, Hydrabad. The Forward and Reverse partial 16S r-DNA sequence along with chromatogram of isolate was obtained from Ocimum Biosolution in FASTA format.

Analysis of N_2 fixing activity of isolates

For this, well grown plant along with root nodules was transferred into sterilized glass beaker and nodules were removed from the root system. The nodules were surface sterilized and placed in vials with the help of sterile forceps. Each glass vials have total number of 20 root

nodules. The mouths of the vials were sealed. 10% of the air in the vessel was replaced with equivalent volume of acetylene (C_2H_2) gas and vessels were shaken and incubated for 30 minutes at appropriate temperature. 10 µl of Sample was withdrawn regularly after every 24 hrs for analysis of ethylene produced till 96 hrs of incubation. Ethylene concentration was assayed on a Shimadzu 2010 Gas Chromatograph equipped with a flame ionization detector and a Porapack Q (Internal diameter 2.2 mm, length 6'). The reduction of acetylene to ethylene was depicted in the form of peak (graph). The efficient acetylene reducer was carried forward for characterization and further for mass production.

RESULT & DISCUSSION:

Biochemical characterization and carbohydrate utilization of *Bradyrhizobium yuanmingense* is depicted in Table-1 and 2 respectively. The isolate is gram negative short rod, non spore forming and motile. Isolate also found to be accumulating PHB granules inside the cell. Exopolysaccharide production by using different carbon sources was tested and the isolate was found to be producing highest amount of EPS using Mannitol as carbon source. Amount of exopolysaccharide produced (mg) /gm of carbon source added is shown in Table-3. Isolate produce considerable amount of EPS when provided with galactose, xylose, sucrose and mannitol as carbon source. Less amount of EPS production was found with arabinose and glucose as carbon source. Nitrogen fixation activity of isolate was done indirectly by performing acetylene reduction assay. IAA production by isolate is shown in Table 4. Result shows that production of IAA increases as the amount of tryptophan increases from 1 mg/ml to 5 mg/ml of the medium and again decreases as amount of tryptophan increases beyond 5 mg/ml. The 16S r-DNA partial sequence of isolated organism was aligned by using bioinformatics tool BLAST and it was found that the isolate is showing 99.6% similarity with *Bradyrhizobium yuanmingense* submitted to NCBI Gene bank data. The accession number of the sequence provided by NCBI is **JN 575481**

Table 1:Selected biochemical tests of isolate after 48 hours of incubation at 37±0.5ºC.

Name of the Tests	Response of strain
Gram strain	-
H_2S production	-
Indole production	-
Methyl Red reaction	-
Voges-Proskauer reaction	-
Citrate Utilization	+
Urease activity	-

Catalase	-
Oxidase	+
Gelatin liquefication	+
Starch hydrolysis	+
Lipid hydrolysis	+
Motility	+

Table 2: Selected carbohydrate utilization of isolate after 48 hours of incubation at 37±0.5 ºC.

Carbohydrate test	Response of isolate
D-Glucose	+
Maltose	-
Lactose	-
Mannitol	+
Mannose	+
Sucrose	+
L-arabinose	+
D-fructose	+
D-galactose	+
D-xylose	+

Table-3:Exopolysaccharide production with different Carbon sources

Sugar	EPS production mg/g of Carbon
D-Glucose	325
Maltose	--
Lactose	--
Mannitol	478
Mannose	385
Sucrose	456
L-arabinose	238
D-fructose	397
D-galactose	467
D-xylose	453

Table: 4 IAA production activity

Concentration of Tryptotphan(mg/ml)	IAA produced µg/ml
1	6.45
2	21.11
5	35.67
8	25.3
10	20.64

CONCLUSION:

The isolated organism was identified as *Bradyrhizobium yuanmingense*. This species is a novel type of species isolated from this area first time. The organism has dual capacity of Exopolysaccharide production as well as production of IAA, a very useful plant growth

promoting hormone in addition to Nitrogen fixing capacity. The isolate has shown very promising results in ARA. This shows it's significance in fixation of atmospheric Nitrogen. Along with Nitrogen fixation the isolate produce EPS, which helps the organism to colonize the root system of host plant for effective nodulation.

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