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Determination of Nitrogen Fixing capacity of Bacteria Isolated from the Rhizosphere soil of Crotolaria pallida from the Valley Districts of Manipur, India.

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Abstract: *In the current study an attempt was made for separation of bacteria having nitrogen fixing potential from the rhizosphere of wild leguminous plant Crotolaria pallida. Single spot each from the four valley districts of Manipur viz. Imphal east, Imphal west, Bishnupur and Thoubal was selected for the collection of soil sample. A total of six (6) bacterial isolates were isolated using Burk's nitrogen free medium from the rhizosphere of the plant. Preliminary physiological tests and biochemical tests were being carried out for the isolates for assessment of their nature. Also, "Nitrogenase activities" of the isolates were also determined by performing the "Acetylene Reduction Assay". Five bacterial isolates out of the six showed some nitrogenase activity even though the amounts shown were little.*

Keywords: *Acetylene Reduction Assay, Crotolaria pallida, Nitrogen, Nitrogenase, rhizosphere.*

I. Introduction

Nitrogen is commonly considered one of the foremost restrictive nutrients in plant growth and the major reserve of nitrogen in the biosphere being available in the form of atmospheric nitrogen (molecular nitrogen) which actually cannot be utilised by the plant. The atmospheric nitrogen are made available to the plants when nitrogen fixing bacteria or rhizobia (as they are collectively termed) fixed atmospheric nitrogen and convert them into ammonia (NH_3) in the presence of nitrogenase under different conditions such as, in loose association with other organisms, or in a symbiotic manner with them (Reinhardt *et al., 2008*). The process whereby atmospheric nitrogen is reduced to ammonia in the presence of nitrogenase (found naturally in microorganisms viz. Rhizobium, free living Azotobacter etc) is called Biological nitrogen fixation (BNF). The best example for the symbiotic relationship being the rhizobium-legume-plant symbiosis which is being considered the most proficient type of association between nitrogen fixing microorganisms and plants, and is of major importance for agrricultural practices, such as soybean crops in Brazil (Dobereiner, 1997).

The major conversion of nitrogen from atmospheric N_2 to ammonia (NH_3) can be attributed to the legume plants for which they are considered very significant not only ecologically but also agriculturally. As such the isolation of microorganisms with the ability to fix nitrogen and to further study them has become a major practice of significance in the field of agriculture and science. As a consequence, extending application of biological nitrogen fixation by any means is of huge importance.

The nitrogen-fixing capability of the newly isolated bacterial strains can be utilized, after thorough analysis and studies to more appropriate application, like in agriculture and other plantation works for nitrogen source. Accurate molecular characterization of several unknown nitrogen-fixing organisms can be accomplished in work to be done in future through sequencing of the *nif*H gene, after which the genetic potential of the gene for the nitrogen fixation could also be analyzed (Zehr *et al.*, 1995). It can be employed that *Nif*H genes can be used as markers for the revealing and to study of the genetic multiplicity of diazotrophic organisms in the community of microbial society, for such in the roots of plants like rice (Ueda *et al.*, 1995) or can be acquired from the forest soil (Widmer *et al.*, 1999).

In this study, an effort will be made to isolate several bacterial strains from the wild legume plant *Crotolaria pallida* and investigated to know their skill to fix atmospheric nitrogen by performing Acetylene Reduction Assay.

II. Material And Methods

2.1 Isolation

For isolation of free living diazotrophs, soil samples were collected from area around the vicinity of wild legume plants grown in the valley districts of Manipur. Soil up to the depth of 20cm is collected and kept at 4°C. The soils are then serially diluted and plated on nitrogen free media (Burk's media) for the isolation. Burk's N-free medium comprising: 10g dextrose, 0.41g KH_2PO_4, 0.52g K_2HPO_4, 0.05g Na_2SO_4, 0.2g $CaCl_2$, 0.1g $MgSO_4.7H_2O$, 0.005g $FeSO_4.7H_2O$, 0.0025g $Na_2MoO_4.2H_2O$, and 15g agar for solid medium was used throughout the study (Wilson and Knight, 1952). The medium was adjusted at pH 7.

2.2 Biochemical Tests

Gram staining was performed using regular practice as preliminary physiological test. Biochemical characteristics of the isolates were determined by performing several biochemical tests such as Catalase test, Nitrate reduction test, Citrate test, Urease test, Starch hydrolysis test and Oxidase tests. The tests were performed as per microbiological practical protocols described by Dubey *et al*. (2002) in Practical Microbiology book.

For determining the sensitivity of antibiotics by the bacterial isolates, HI Media Susceptibility discs were used. For the work Six antibiotics were used *viz.* Imipenem(IPM), oxacilin(OX), chloramphenecol(C), novobiocin(NV), ciproflocacin(CIP) and amoxyclav(AMC).

2.3 Acetylene Reduction Assay

Through the method of ARA (Acetylene Reduction Assay) the assessment for determining the activity of nitrogenase enzyme accountable for Nitrogen fixation was elucidated for the bacteria which were isolated. The process of Acetylene reduction assay was executed at IBSD, Imphal by following the method said by Hardy *et al.* (1973). The method of acetylene reduction assay (Hardy *et al*., 1968) is an essential practice for determining the ability of the enzyme nitrogenase, the enzyme that is accountable for the fixation of N_2. The underlying reason for the method is reduction of acetylene into ethylene with the support of enzyme nitrogenase; the process is accessed by using (Thermo scientific CHEMITO CERES 800 plus) gas chromatography at IBSD.

III. Results

From the experiment, a total of six bacterial isolates were assessed for "ARA" activity out of which five bacterial isolates *viz.* CPS.IE-2A, CPS.IE-3, CPS.B-1, CPS.IW-3 and CPS.T- were found to show some ability for fixing Nitrogen (nitrogenase activity). The isolate CPS.B-2 showed negligible amount of reducing Acetylene to Ethylene, thus it could be considered that it may not have the capacity of fixing nitrogen. The ability of nitrogen fixation even though the amount were in small quantity, is visible from the following table and the Acetylene reduction assay curve.

3.1 Biochemical Test

The biochemical characteristics and the antibiotic sensitivity shown by the six bacterial isolates could be considered from the given TABLE 1 and TABLE 2. The zone of inhibition of the bacterial isolates as against the six antibiotics used are given in centimeter (in brackets) on TABLE 2.

Table.1- *Biochemical characteristics of the six bacterial isolates.*

Isolate	Location	Gram stain	Catalase test	Starch hydrolysis	Nitrate Reduction	Urease test	Oxidase test	Citrate test
CPS.B-1	Nambol	−ve	+ve	+ve	+ve	+ve	+ve	+ve
CPS.B-2	Nambol	−ve	+ve	+ve	+ve	−ve	+ve	+ve
CPS.T-1	Khangabok	−ve	+ve	+ve	+ve	−ve	+ve	+ve
CPS.IW-3	Thangmeiband	−ve	+ve	+ve	+ve	−ve	+ve	−ve
CPS.IE-2(A)	Kongpal	−ve	+ve	+ve	+ve	−ve	+ve	−ve
CPS.IE-3	Kongpal	−ve	+ve	+ve	+ve	−ve	+ve	−ve

Table.2- *The zone of inhibition given in centimeters of the six bacterial isolates.*

ISOLATES	NV	OX	CIP	AMC	C	IPM
CPS.B-1	+ve(1.5)	-ve	+ve(3.7)	+ve(0.9)	+ve(1.8)	+ve(3.6)
CPS.B-2	+ve(2.9)	-ve	+ve(2.9)	-ve	+ve(3.0)	+ve(1.8)
CPS.T-1	+ve(1.7)	+ve(2.4)	+ve(4.0)	+ve(4.0)	+ve(4.5)	+ve(5.7)
CPS.IW-3	+ve(2.5)	+ve(2.6)	+ve(3.8)	+ve(3.6)	+ve(3.5)	+ve(4.6)
CPS.IE-2(A)	+ve(2.0)	-ve	+ve(2.4)	+ve(0.7)	+ve(1.9)	+ve(3.4)
CPS.IE-3	+ve(1.0)	-ve	+ve(4.7)	-ve	+ve(2.4)	+ve(4.8)

3.2 Acetylene Reduction Assay

The amount of Acetylene reduced to Ethylene in nano mols by the bacterial isolates are as given in table.3. Out of the total six isolates, five bacterial isolates *viz.* CPS.IE-2A, CPS.IE-3, CPS.B-1, CPS.IW-3 and

CPS.T- showed some ability for fixing Nitrogen (nitrogenase activity) whereas the isolate CPS.B-2 failed to show the ability for fixing nitrogen. The Acetylene reduction curve of the isolates were also given below.

Table.3- *Amount of ethylene produced by the respective bacterial isolates.*

ISOLATES	nM of ethylene
CPS.B-1	1.8
CPS.B-2	0.08
CPS.T-1	0.92
CPS.IW-3	1.66
CPS.IE-2(A)	1.36
CPS.IE-3	1.61

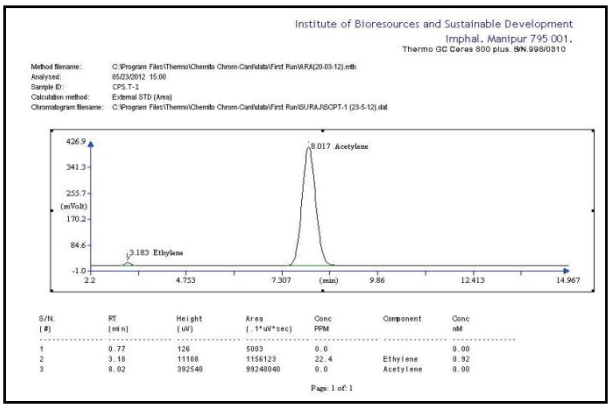

Fig.1: Acetylene reduction assay curve for CPS.T-1.

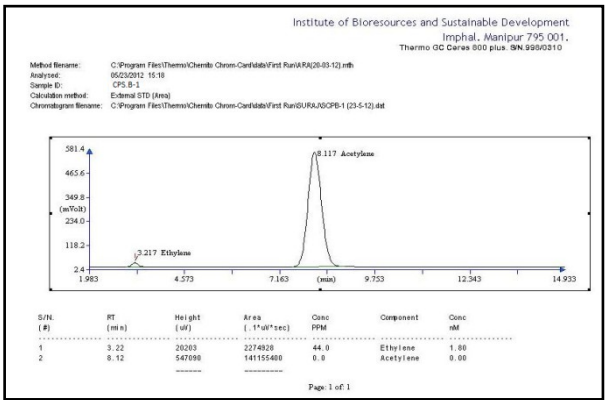

Fig.2: Acetylene reduction assay curve for CPS.B-1.

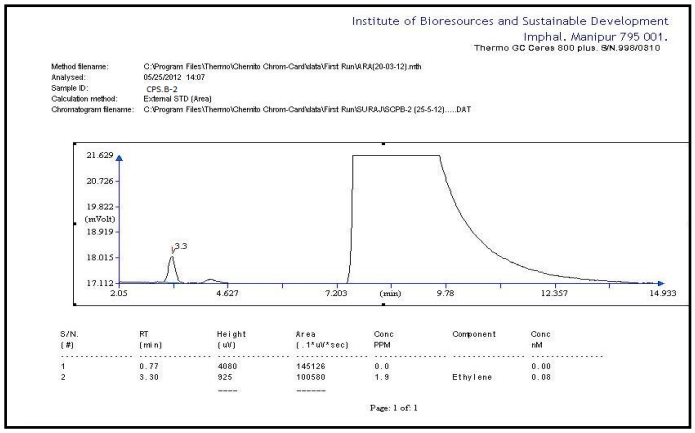

Fig.3: Acetylene reduction assay curve for CPS.B-2.

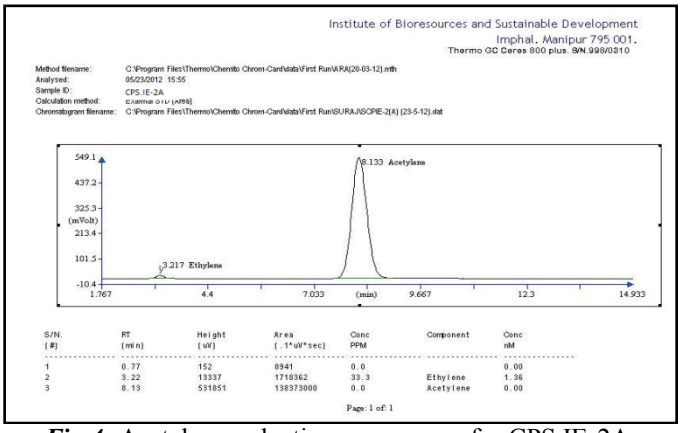

Fig.4: Acetylene reduction assay curve for CPS.IE-2A.

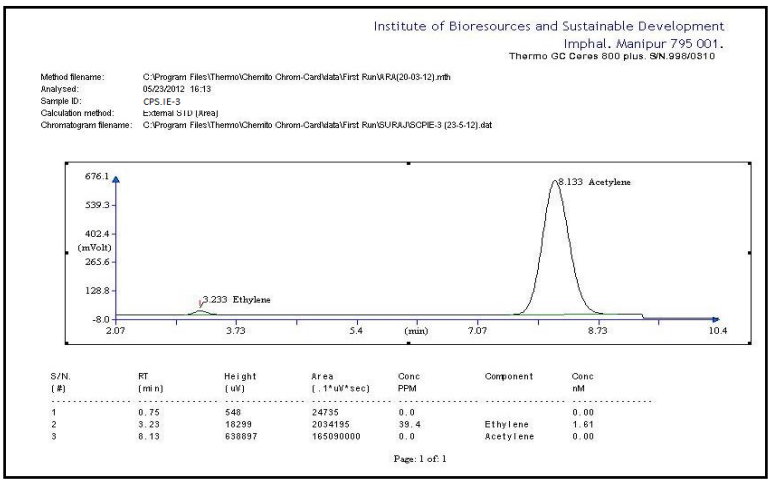

Fig.5: Acetylene reduction assay curve for CPS.IE-3.

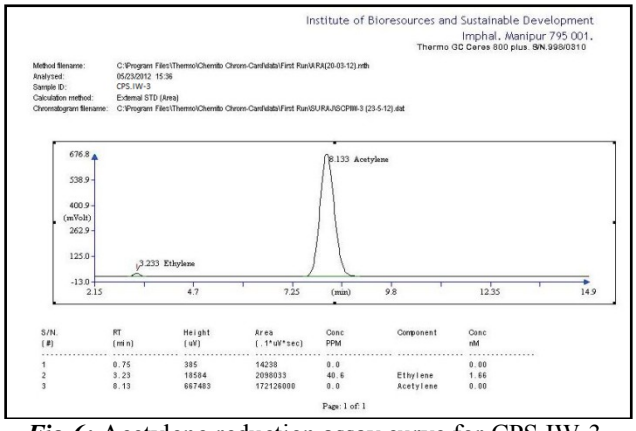

Fig.6: Acetylene reduction assay curve for CPS.IW-3.

IV. Discussion

The curves shown in the above reveals the nitrogen fixing ability of the bacterial isolates *viz.* CPS.IE-2A, CPS.IE-3, CPS.B-1, CPS.IW-3 and CPS.T-1 extracted from the soil rhizosphere of the wild legume plant *Crotolaria pallida*. From the curve it is clear that out of the total six isolates, five isolates do have the capacity of nitrogen fixation, though may be in small amount. The amount shown by the isolate CPS.B-2 is almost negligible, thus it could be concluded that the isolate may be a non nitrogen-fixer.

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