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Isolation and characterization of nitrogen fixing anaerobes and facultative anaerobes from paddy ecosystem

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Abstract : The importance of nitrogen fixing activity of *Clostridium pasteurianum* and *Klebsiella pneumonia* in flooded soils is known for decades. The anaerobic and facultative anaerobic nitrogen fixing microorganisms were isolated from the flooded paddy ecosystem. Their growth on carbon sources, CO₂ production, denitrification, volatile fatty acid production, nitrogenase activity and protein profile of the cultures were estimated and characterized as *Clostridium* and *Klebsiella*. (**Key words:** anaerobic, facultative anaerobic, nitrogen fixation, *Clostridium*, *Klebsiella*).

Some of the soil anaerobic micro organisms fix atmospheric nitrogen, decompose cellulose, form methane or reduce sulphate and nitrate are of special interest in the flooded rice soil (Skinner, 1986). Nitrogen fixing microorganisms enrich nitrogen in submerged rice soils by atmospheric nitrogen fixation (Grist, 1965). Yoshida and Ancajas (1971) reported that in flooded soil in which rice is growing, some nitrogen apparently occurs in the root rhizosphere. Paul and Newton (1960) reported a number of nitrogen fixing soil organisms such as *Pseudomonas*, facultative bacilli and *Klebsiella* sp. Rice *et al.*, (1967) isolated the nitrogen fixing *Clostridia* from rice soil. *Clostridium pasteurianum* and *Klebsiella pneumonia* are of particular interest because of their ability to fix atmospheric nitrogen. An attempt was made to isolate and characterize the nitrogen

fixing anaerobes and facultative anaerobes from the paddy ecosystem.

Materials and Methods

Isolation of nitrogen fixing anaerobes and facultative anaerobes from paddy ecosystem.

Soil samples at random were collected from the rice during active tillering stage at lower horizon (10 cm depth) under anaerobic conditions (Ramasamy *et al.* 1992) for the isolation of anaerobic nitrogen fixing microflora. Nitrogen fixing anaerobes were isolated using Hungate's (1957) roll tube technique using Hill's medium (Bergerson, 1980). The individual colonies developed in roll tubes were transferred to the vials containing nitrogen free medium and inoculated under nitrogen atmosphere. After the desired growth, the cultures were further purified using roll tube technique.

Table 1. Characterization of the nitrogen fixing organism

Characters studied	Anaerobic	Facultative anaerobe
Cell shape	Rod	Straight rods arranged single or in pair
Colony morphology	Viscoid	Dome shaped glistening colonies, capsulated mucoid colonies
Motility	Motile	Non motile
Gram reaction	Gram +ve	Gram -ve
Sporulation	Terminal spores	Nil
VFA	Acetic and propionic acid	Acetic and formic acid
Gas production	CO ₂ and H ₂	CO ₂ and H ₂
Denitrification	- ve	Not detected
Nitrogenase	+ ve	+ ve
<i>Utilization of Sugar</i>		
Sucrose	+ ve	+ ve
Cellulose	+ ve	+ ve
Cellobiose	+ ve	+ ve
Glucose	+ ve	+ ve
Rhamnose	+ ve	+ ve
Fructose	+ ve	+ ve

Characterization of Anaerobes

i. Cell Morphology

The morphology and motility of the cells were observed under the microscope after Gram staining.

ii. Growth on Different Carbon Sources

The cultures at a log phase with an OD of 0.6 (CFU x 10⁶ ml⁻¹) were inoculated in the vials containing Hill's medium with different carbon sources *viz.* glucose, rhamnose, fructose, sucrose, cellulose and cellobiose and incubated anaerobically under nitrogen atmosphere. The growth rate was measured by observing OD at 600 nm in ECIL GS 5701 Spectrophotometer at periodical intervals.

iii. CO₂ production

Ten ml of the liquid medium was prepared in the infusion bottles and inoculated with the actively growing isolated cultures. The infusion bottles were flushed with N₂ gas to maintain the anaerobic condition and incubated at 28±10°C for 48 hrs. After the incubation period, one ml of the the gas occupying the head space was withdrawn using gas tight syringe and injected into the Chemito Gas Chromatogram with TCD and Chromosorb column for detecting the presence of CO₂ in the infusion bottles (Ramasav *et al.* 1992).

iv. Volatile Fatty acid Production

The volatile fatty acids produced by the isolates were identified and estimated by following the method of Holdeman *et al.* (1977).

v. Acetylene Reduction Activity (ARA)

The acetylene reduction activity of the nitrogen fixers were estimated and described by Bergerson (1980).

vi. Denitrification

Denitrification activity was studied by adding one per cent potassium nitrate to the actively growing isolated cultures and incubated under nitrogen atmosphere. From these vials, 0.5 ml of gas sample was withdrawn after two days of incubation and injected into the GC unit fitted with ECD detector having a Poropak Q column. Helium was used as carrier gas. The column, injector and detector temperature were 80°C, 125°C, and 225°C respectively. Nitrous oxide standards were prepared and run. The samples were identified and quantified with known standards.

vii. Protein profile of the isolate

The cells of isolates, multiplied separately in the bio-engineering fermenter were harvested



Fig.1 Photomicrograph of
N-fixing *Clostridium* sp.



Fig.2 Photomicrograph of
N-fixing *Klebsiella* sp.

at the desired growth stage, washed in saline and suspended at 2000 cycles per second for three min. After sonication, the liberated protein was concentrated using dialysis membrane by placing over dry gel beads. The protein content of the samples was estimated as described by Bradford (1976). The proteins associated with the isolates were fractionated using FPLC (Pharmacia LKB, Sweden). One hundred ml of the sample was injected into the column. The flow rate was maintained at 20 ml per hour and the pressure was maintained at 1.5 m Pa. Similarly standards were also prepared with low and high molecular protein and run. The molecular weight of the separated protein was calculated using the standards (Sara Parwin, Banu and Ramasamy, 1994).

Results and Discussion

Anaerobic Nitrogen Fixer

The importance of nitrogen fixing activity by *Clostridium pasteurianum* and *Klebsiella pneumoniae* in the flooded soil is known (Hoshi *et al.* 1989; Hill, 1976, 1988; Paul and Newton, 1960). In the present investigation, an attempt was made to isolate and characterise the anaerobic and facultative nitrogen fixing organisms.

Microscopic observation of the cultures isolated from the flooded rice soil using Hill's medium showed the presence of straight to curved rods with terminal oval spores. Motility was observed initially but was lost when sub cultured. Young cultures were gram positive (Table.1). Acetate and propionate were detected in the VFA profile and CO₂ was detected in

headspace during growth. No denitrification activity was observed even after seven days of incubation. The isolate utilized sucrose preferably than the tested other carbon sources. Cellulose, sucrose, glucose, maltose and rhamnose were utilized for the growth by this isolate and the results are presented in Fig.3. The cultures also exhibited nitrogenase activity (Table.1). Protein profile of the *Clostridium* sp. showed two major peaks with a molecular weight of 5,80,000 and 6,90,000 Daltons. Based on the morphology, growth and nitrogenase activity it was identified as *Clostridium* sp.(Fig.1) but could not relate to the known species of the genus. Bums (1982) described *C.pasteurianum* from a flooded rice ecosystem and *Clostridium* is more widely distributed than *Azotobacter*. Yamagata (1924) found that *Clostridium* occurred in 95 per cent of Japanese paddy soils and their population was also high.

Facultative Nitrogen Fixing Isolate

Glistening, raised colonies developed on the roll tubes were sub cultured and observed under microscope. The cells were motile, straight rods, arranged either in single or in pairs and they were gram negative. The VFA profile of the cultures exhibited two peaks which were identified as acetate and formate. CO₂ and H₂ were detected during the growth of the culture (Table.1). The cultures utilized different carbon sources *viz.* glucose, sucrose, cellobiose and rhamnose for their growth (fig.4) and sucrose was found to be the preferred carbon source. The acetylene reduction activity of the cultures was found positive. Protein profile of *Klebsiella* sp. showed seven peaks with molecular weight

ranging from 6000 to 60000 Dalton Based on the morphology, growth and nitrogenase activity, the isolate was identified as *Klebsiella* sp. (Fig.2).

The cultures isolated from flooded condition encouraged the role of anaerobic and facultative anaerobic nitrogen fixers in rice ecosystem. Kalininskaya and Lavova (1988) reported the dominant group of nitrogen fixing organism comprised enterobacteria belonging to the species *Enterobacter aerogens*, *Klebsiella pneumonia* and *Citrobacter*. They also isolated clostridial forms which able to fix nitrogen under low pH and temperature. The abundance of *clostridium* and *Klebsiella* under flooded ecosystem observed in the present study was the result of conclusive environment of the rice rhizosphere like low redox potential (-200 mv), availability of organic matter from rice root, rice stubbles, weeds, phytoplankton and hydrolysable carbohydrates.

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