

Original Research Article

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Isolation and Characterization of N₂ Fixing Anaerobic Bacteria from Paddy Ecosystem

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ABSTRACT

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Rice cultivation is taken in flooded soil condition. This creates an anaerobic environment and harbours different group of anaerobes. The continued submergence of soil provides a reducing environment and effects a build-up of anaerobic ecosystem in which two unique processes namely methane emission and reductive transformation of nitrogen occur. But the role played by specific group of anaerobes with respect to nitrogen transformation is still obscure. A study was undertaken to assess the different groups of anaerobes in nitrogen transformation in flooded rice soils. The nitrogen fixing obligate and facultative anaerobes were isolated and characterised from paddy ecosystem. The population of culturable anaerobic bacteria in flooded rice soils was enumerated by the anaerobic roll tube method using Hungates agar medium. Higher AR activity was noticed for the screened obligate and facultative anaerobic bacteria and exhibited maximum activity on 132nd hour (19.81 n moles of C₂H₄ mg⁻¹ protein h⁻¹). In the present investigation, the occurrences of anaerobic nitrogen fixer in the paddy ecosystem and their role in nitrogen fixation have been examined.

Introduction

Rice is one of the most important food crops, as more than a billion people depend on its cultivation for their livelihoods and more than 3.5 billion people depend on rice for more than 20% of their daily calories (Ahn *et al.*, 2012). The most characteristic condition in paddy field is water logging or submergence of the land surface. This brings about anaerobic conditions in the soil due to the very slow diffusion of oxygen through water. After the oxygen reserve in the soil is exhausted and aerobic microorganisms have all died, facultative anaerobes dominate for some times. As anaerobic conditions continue, these microorganisms are gradually

replaced by obligate or strict anaerobes (Kazutake, 2007). The major activities of microorganisms in paddy fields include methanogenesis, methane oxidation and biogeochemical cycling of carbon, nitrogen and sulphur (Reddy *et al.*, 2001; Dubey, 2005).

The biogeochemistry of paddy soils differs from aerated agricultural soils as it is controlled by flooding and the resulting pattern of oxidation and reduction (Neue, 1997). The diffusion of atmospheric oxygen decreases by a factor of 10⁵ due to submergence. Oxygen only penetrates the

first millimetres of the soil where it is rapidly consumed and is therefore a limiting factor in flooded paddy soils (Frenzel *et al.*, 1992). This leads to a coexistence of oxidized and reduced zones forming a compartmentalized system with three major parts: (I) the anoxic bulk soil, (II) the oxic surface soil and (III) the partially oxic rhizosphere with increased substrate concentrations. Diffusive transport of oxygen through the aerenchyma of rice roots leads to the availability of oxygen in the rhizosphere (Gilbert *et al.*, 1998). Between the compartments, well-defined chemical gradients can be measured and form different habitats for microorganisms. Anaerobic microorganisms, such as fermentative bacteria and methanogenic archaea, predominate within the microbial community. Consequently, methane is the final product of anaerobic degradation of organic matter in paddy soils (Liesack *et al.*, 2000). Oxygen at the soil surface and along the roots is a key factor controlling the gradients of other electron acceptors such as nitrate, ferric oxide and sulphate.

In rice cultivation the yield-limiting factor is the availability of nitrogen due to large losses in flooded soils through denitrification or leaching (De Datta and Buresh, 1989, Cassman *et al.*, 1998). Nitrogen limitation of plant productivity in salt marshes has also been demonstrated (Bagwell *et al.*, 2000). Generally, nitrogen fertilizer efficiency in flooded rice fields is poor. Flooded rice crops typically use only 20-40%, whereas upland crops frequently use 40-60% of the applied nitrogen.

Important functions of flooded soil in which the anaerobes involved are nitrate reduction, nitrogen fixation, sulphate reduction and methanogenesis. In aerobic conditions cyanobacteria are significantly responsible for nitrogen fixation. When oxygen is depleted nitrate reduction will pose the Eh at around +

200 mV until it is exhausted and this delay the onset of highly reduced condition (Ponnamperuma, 1972). Under the reduced condition anaerobic nitrogen fixation is carried out mostly by Clostridial community. In the flooded soil many anaerobes are fermentative or stimulated by the fatty acid formed from the fermentation by other bacteria. Though the capacity of nitrogen fixation by Clostridia is known, their potential is yet to be capitalised. The microbial community degrading organic residues in anoxic soil comprises primary fermenters, secondary fermenters, hydrogen-utilizing acetogens, and methanogens (Drake *et al.*, 2009). The intermediary fatty acids and alcohols produced can accumulate up to 25 mM for acetate and 10 mM for propionate and butyrate during the degradation of plant residues (Glissmann and Conrad, 2000; Rui *et al.*, 2009). These fatty acids are then degraded to less than 1 mM, reaching a quasi-steady state. Acetate is a direct substrate for many anaerobes including methanogens. The degradation of other fatty acids like propionate and butyrate, however, is thermodynamically difficult and can be carried out only through the syntrophic interaction between proton reducing and fatty acid-oxidizing syntrophs and H₂-consuming methanogens (Yao *et al.*, 1999).

In terms of N cycling, both biotic and abiotic nitrogen transforming reactions can occur in rice paddy soils, including ammonification, N immobilization, nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), anaerobic ammonium oxidation (anammox), and nitrogen fixation. Although the significance of nitrification and denitrification in rice paddy soils has been well studied, the microbes responsible for these processes were not well understood until recently. In addition, the contributions of recently discovered new processes (Okabe *et al.*, 2010) such as archaeal ammonia

oxidation, fungal denitrification, anaerobic methane oxidation coupled with denitrification, and anaerobic ammonium oxidation (anammox), to N cycling in rice paddy environments have not been well studied.

Biological N₂ fixation is gaining importance in rice ecosystem because of current concern on the environmental and soil health that are caused by the continuous use of nitrogenous fertilizers and the need for improved sustainable rice productivity. Thus, biological fixation of atmospheric N, especially non-symbiotic N₂-fixation in the soil, has been subject of continuing interest in recent decades especially for low input agriculture. Therefore, the objectives of this paper are to assess the contribution of anaerobic nitrogen fixation in flooded rice soil especially *Clostridia*.

Materials and Methods

Collection of soil samples

Soil samples from rhizosphere and non-rhizosphere were collected from the flooded rice field during active tillering stage for enumerating the population of anaerobic microflora. Random samples were collected from the field at the lower horizon (10 cm depth) under anaerobic conditions (Ramasamy *et al.*, 1992).

Enumeration of anaerobic bacteria by roll tube technique

Soil sample (1g) was diluted by tenfold using sterile dilution buffer (100mL) containing sodium carbonate and sodium bi carbonate. After a thorough mixing, diluted sample (1mL) was transferred to another sterile dilution buffer (90mL), which conformed to 10² dilutions. Enumeration and isolation of anaerobic bacteria were carried out with the

anaerobic roll tube method using oxygen-free 95% N₂/5% CO₂ mixed gas as headspace. Each diluted sample was inoculated in triplicate into Hungates medium. The medium was flushed continuously with N₂ using the gassing manifold assembly, simultaneously the sterile test tubes were kept under N₂ atmosphere. The samples were also maintained under N₂ atmosphere. 1ml of the trace element solution and vitamin solution were added to one litre of the media before transferring to the sample. 1ml of the sample was transferred from the desired dilution to the sterile test tube of 25 ml capacity which again was kept under N₂ atmosphere. By using a sterile pipette with silicon tube at one end, 5 ml of the medium was transferred to the test tube with sample which was maintained under N₂ atmosphere. The test tube was immediately stoppered with sterile rubber corks by simultaneously drawing out the gassing jet. The test tube was rolled over the foam, soaked in cold water till the medium uniformly solidified on the sides of the test tube. The test tubes were incubated in an anaerobic jar (Hungate, 1957). The number of colonies that appeared on the agar medium during 12 days of incubation at 30°C was counted to determine the number of culturable anaerobic microbes in the samples. The isolates were purified by repeating the colony isolation by the anaerobic roll tube method, and the strains finally purified were used in this study. The purity of the isolates was confirmed by uniform colony morphology on the roll tube agar and cellular morphology with Gram staining and motility of the cells, as well as observations by phase-contrast microscopy.

Phenotypic characterization of isolates

Catalase activity of cells was tested by the O₂ generation in 3% H₂O₂ where bubble formation shows positive for catalase activity and no bubble formation shows negative for

catalase activity. Utilization of different carbohydrates was tested with different carbon sources *viz.*, glucose, fructose, sucrose, cellulose and cellobiose and incubated anaerobically under N₂ atmosphere. Growth in each medium was monitored by measurement of the optical density at 660 nm with a spectrophotometer at periodical interval. Growth in the medium without supplements was used as control. Spore formation was examined by the observation of cell morphologies staining with malachite green and examined under oil immersion objective.

Analytical methods

Volatile fatty acids and CO₂ production were analysed with a gas chromatograph equipped with a flame ionization detector with N₂ as the carrier gas, by following the method of Holdemann *et al.*, (1977). The column, injector and detector temperatures were 135°, 150° and 150°C respectively. Gas samples were taken from the headspace of culture tubes with a pressure-lock syringe and analysed with a gas chromatograph equipped with a thermal conductivity detector. Denitrification activity was studied by adding 1% potassium nitrate and incubated under N₂ atmosphere and analysed with a gas chromatograph with Helium as the carrier gas. The column, injector and detector temperatures were 80°, 125° and 225° C respectively. Nitrous oxide standard were prepared and the samples were identified and quantified with known standard.

Assay of nitrogenase activity

The acetylene reduction technique (Hardy *et al.*, 1973) was followed for assaying nitrogenase. Gas chromatograph fitted with FID system with poropak N-column served the purpose. The samples were inoculated in serum vials and were flushed with nitrogen

gas to maintain anaerobic condition and incubated at 32° C. After 4 days of growth, a known quantity of (3 ml) pure acetylene gas was injected, withdrawing the same volume of nitrogen and the serum vials were again incubated again at room temperature. Then after the incubation period about 0.5 ml of the gas sample was withdrawn from the serum vials. The samples were injected into a gas chromatograph with FID system with poropak N-column. The column temperature were maintained at 85° C, oven temperature at 70° C and ionisation temperature at 100° C. Nitrogen gas was used as carrier. The area of ethylene peak was recorded for each culture. The acetylene reduction activity was expressed as n moles of ethylene produced h⁻¹ mg⁻¹ protein.

Results and Discussion

The lowland rice field soils favour the growth of anaerobic microflora due to the less concentration of oxygen and have been studied in greater details, for the distribution of various kinds of nitrogen fixing microorganisms. These flooded soils are unique as they experience three conditions *viz.*, aerobic, microaerophilic and anaerobic conditions which favour the activities of the respective group of microorganisms. In India, 90 per cent of the rice fields are grown under submerged conditions and therefore, the present investigation was carried out to isolate the anaerobic nitrogen fixer and to assess the role of anaerobic microflora in nitrogen fixation. The experiments were carried out for isolation of anaerobic nitrogen fixer from flooded rice field having the soil texture of sandy clay loam with a bulk density of 1.27 mg/m³. The porosity and water holding capacity of the soil was 51.32 and 48 per cent and available NPK are 246, 14.3 and 431.8 kg ha⁻¹ respectively. Enumeration of anaerobic N₂ fixing bacteria from paddy ecosystem was carried out for both rhizospheric and non

rhizospheric region by using the Hungates medium. The core sampler (Fig. 1) was used for the sampling purpose from the flooded rice field at the depth of about 10 cm depth. The number of culturable anaerobic N₂ fixing bacteria was at the order of 10² CFU (colony-forming units) for the collected soil samples. Colonies on the roll tube agar inoculated with the soil samples were picked up, and purified strains were obtained (Fig. 2). A total of 11 strains were selected as representatives of the bacterial groups classified by anaerobic growth ability and cellular morphology. Phylogenetic analysis and some phenotypic characterizations, including catalase activity, nitrogenase activity, fermentation products from glucose, were carried out on all 11 strains selected.

Kaku *et al.*, 1997 enumerated the number of culturable anaerobic bacteria in rice plant residue in the rice straw plot by the anaerobic roll tube method using PY4S agar. The number was often more than 100 times higher than those in soil samples of the same paddy field (dry weight basis), and they suggested that rice plant residue served as the main habitat for fermentative microbes in the paddy soil. Similar results were also obtained in this study by the enumeration of anaerobic bacteria in soil samples collected from the flooded rice field.

Anaerobic nitrogen fixer

Microscopic observation of the culture isolated from the rice flooded soil using Hungates medium showed the presence of straight to curved rods with terminal spores (Fig. 3). Motility was observed initially but motility was lost when subcultured. Young cultures were gram positive. Acetate and propionate were detected in the VFA profile (Fig. 4) and CO₂ was detected in head space during growth. No denitrification activity was observed even after seven days of incubation.

The isolate utilized sucrose preferably than the other tested carbon source. Cellulose, glucose, sucrose, cellobiose and fructose were utilized for the growth by the isolate (Table 2). The culture also exhibited nitrogenase activity (Fig. 5). Based on the morphology, growth and nitrogenase activity, it was identified as *Clostridium sp.* Burns (1982) described a *Clostridium pasteurianum* from flooded rice ecosystem and *Clostridium* is considered to be more widely distributed than Azotobacter. Yamagata, (1924) found that *Clostridium* occurred in 95 per cent of paddy fields and their population was high.

Facultative nitrogen fixing isolate

Glistening, raised colonies developed on the roll tubes were sub cultured and observed under microscope. The cells were non motile, straight rods arranged either in single or in pairs and they were gram negative. The VFA profile of the culture exhibited two peaks which were identified as acetate and formate (Fig. 6). CO₂ and H₂ were also detected during the growth of the culture. The isolates utilized different carbon sources *viz.*, cellulose, glucose, sucrose, cellobiose and fructose were utilized for the growth by the isolate and found that sucrose to be preferred carbon source (Table 3). The AR activity was found increasing up to 86hrs and then declined (Fig. 7). Based on the morphology, growth and nitrogenase activity, the isolate was identified as *Klebsiella sp.* The present study was conducted under flooded conditions which encouraged the anaerobic and facultative anaerobic populations. The abundance of *Clostridium* and *Klebsiella* under flooded conditions observed in the present study was the result of conducive 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.

Table.1 Enumeration of anaerobic N₂ fixing bacteria from paddy ecosystem

Stage of the crop	Population of <i>Clostridium</i> (x 10 ² cfu g ⁻¹)	
	Rhizosphere	Non rhizosphere
Seedling	12.3±0.6	8.3±0.5
Tillering	23.7±1.2	12.6±1.0
Panicle initiation	17.6±0.4	10.0±0.3
Flowering	21.6±0.9	15.3±0.6

Table.2 Characterisation of the N₂ fixing anaerobic bacteria isolated from paddy ecosystem

Cell shape	Rod shaped
Colony morphology	Viscoid
Gram reaction	Gram +ve
Sporulation	Terminal spores
Motility	Motile
VFA	Acetic and propionic acid
Gas production	CO ₂ and H ₂
Denitrification	- ve
Catalase activity	-
Nitrogenase	+ve
Utilization of sugar	
Sucrose	+ve
Glucose	+ve
Cellobiose	+ve
Fructose	+ve
Cellulose	+ve

Table.3 Characterisation of the N₂ fixing facultative anaerobic bacteria isolated from paddy ecosystem

Cell shape	Straight rod arranged single or in pair
Colony morphology	Dome shaped glistening colonies
Gram reaction	Gram -ve
Motility	Non motile
VFA	Acetic and formic acid
Gas production	CO ₂ and H ₂
Catalase activity	+
Nitrogenase	+ve
Utilization of sugar	
Sucrose	+ve
Glucose	+ve
Cellobiose	+ve
Fructose	+ve
Cellulose	+ve

Fig.1 Core sampler used for soil sample collection



Fig.2 Total anaerobic bacteria on roll tube with Hungate's medium



Fig.3 Oil immersion objective of the anaerobic culture

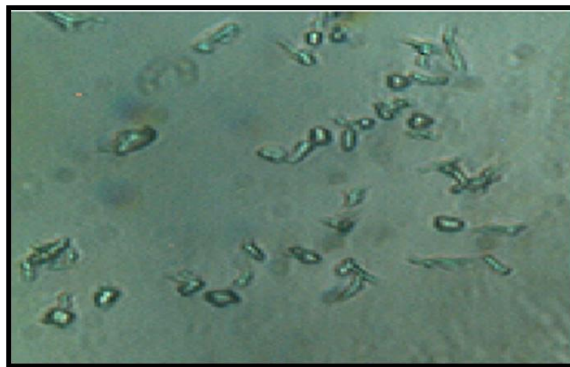


Fig.4 Characterization of anaerobic bacterial isolate TM3 by producing acetic acid

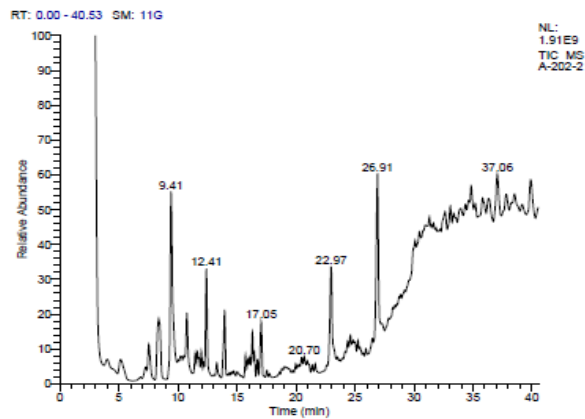


Fig.5 Nitrogenase activity of the anaerobic culture

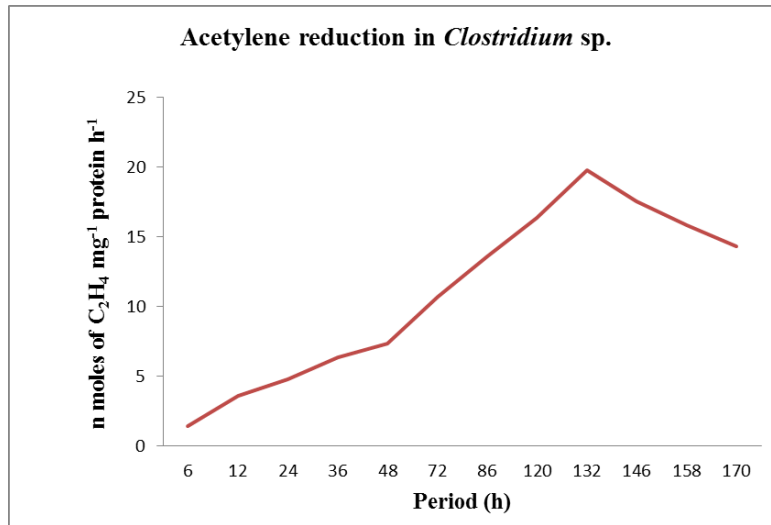


Fig.6 Characterization of anaerobic bacterial isolate Th2 by producing acetic acid

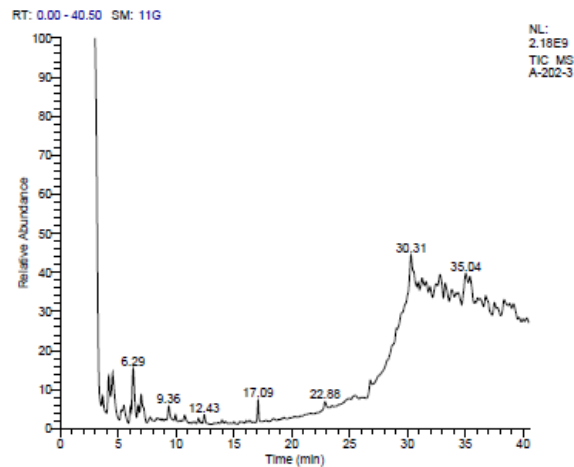
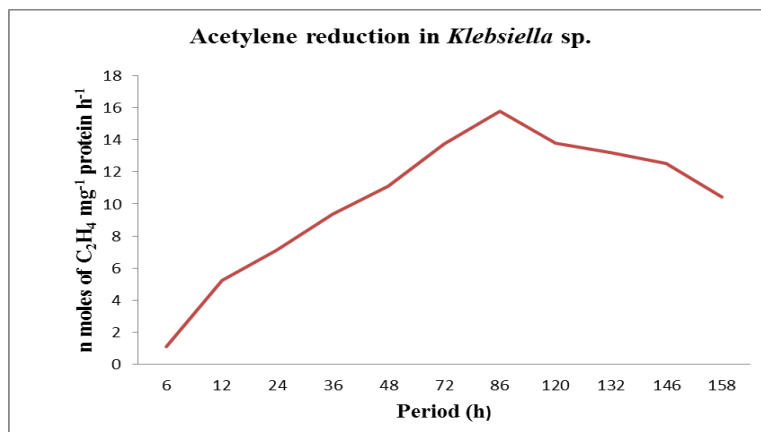


Fig.7 Nitrogenase activity of the Facultative anaerobic culture



In conclusion, the nitrogen-fixing bacterial species within the genus *Clostridium* and *Klebsiella* are traditionally considered representatives of anaerobic, facultative anaerobic, free-living nitrogen-fixers. These anaerobic nitrogen fixers, because of their free-living lifestyle, were not considered active contributors of fixed nitrogen for supporting plant growth and productivity. The isolated nitrogen-fixing clostridia from paddy ecosystem are phylogenetically close to known nitrogen-fixing species, *Clostridium pasteurianum*. The paddy ecosystem was considered as the niche for nitrogen-fixing clostridia and also suggests a more significant role for the obligate anaerobes in supporting plant growth via nitrogen fixation.

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