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EFFECT OF pH ON GROWTH AND NITROGEN FIXATION OF *AZOTOBACTER* SPP.

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Three species of *Azotobacter* were isolated from prawn-cum-paddy fields of Kerala. These strains were found to have the optimum pH for growth and nitrogen fixation near or slightly above neutrality, *i. e.*, in the pH range of 7.0 to 8.5

INTRODUCTION

Biological nitrogen fixation by free-living bacteria in rice soils has been reported from alluvial, laterite, acid saline and acid sulphate saline soils (Sethunathan *et al.*, 1983). The presence of *Azotobacter* spp. and their rate of multiplication and nitrogen fixation are governed by many factors including soil pH (Jensen, 1961; Roy *et al.*, 1962). Nitrogen fixation by *Azotobacter* spp. as reported remained almost unchanged within the pH range of 6.24 to 8.34 at 10 to 30°C, decreased at more acidic or alkaline pH and complete inhibition was detected above 8.75 and below 5.00. The *Azotobacter* spp. count and nitrogen fixation were reported maximum near or slightly above neutral pH. The soil pH between 7.8 and 8.5 supported maximum population of *Azotobacter* spp. and the count decreased with the increase in pH. However, the efficiency of nitrogen fixation is not affected by the increase of soil pH from 7.0 to 8.7 (Raju *et al.*, 1974; Khullar and Chahal, 1975). Similar conclusions were made by other workers that the optimum pH required for growth and nitrogen fixation was near or slightly above neutrality (Yamagata and Itano, 1923; Rangaswami and Sadasivan, 1965).

MATERIAL AND METHODS

The present investigation was carried out at Narakkal (long. 76° 14' E; lat. 10° 03' N), a fishing hamlet in the Vypeen Island, 15 km north-west of Cochin on the south-west coast of India. Four ponds were selected for regular sampling of which two were perennial prawn culture systems and the other two were seasonal prawn culture systems where prawn and paddy are cultivated during the inter-monsoon (October-May) and monsoon (June - September) periods, respectively.

Water and sediment samples were collected fortnightly for a period of two years. Surface water samples were aseptically collected from the four fixed sites of each pond in sterilised 100-ml capacity polyethylene containers. After thorough mixing, the samples were preserved in 300-ml capacity BOD bottles for bacterial analysis. Sediment samples were collected from the four fixed sites with an impact cover (100 cm x 4 cm diam) made of perspex material from the upper 10-cm layer. About 5 cm depth of surface sediment layer was removed aseptically from each core sample and thoroughly mixed with a sterile spatula in a sterile bottle, followed by vigorous shaking. Sediment samples from each pond were separately preserved in wide-mouthed sterile glass bottles. All the samples were transported to the laboratory in an ice-box. Media for free-living aerobic nitrogen-fixing bacteria and for *Azotobacter* spp. were prepared using double distilled water with 3% NaCl as suggested by Rodina (1972). *Azotobacter* spp. biomass/population was measured in 10^2 to 10^3 dilution in water samples and in 10^3 to 10^4 in sediment samples throughout the investigation period after standardising the population range. The incubation was made for seven days at room temperature (28 ± 2°C), with controls in triplicate. After the incubation period, the presence or absence of bacteria in each tube was estimated from the turbidity of the culture media and the most probable number (MPN) per millilitre of water or per gramme of sediment was calculated from the number of positive tubes in each dilution. The bacteial biomass has been represented in figures as per millilitre of water or per gramme of sediments using monthly mean values.

All the isolates, collected either from the water or from the sediments of the perennial and seasonal ponds, were purified by repeated streaking on nitrogen-free Jensen's agar medium and maintained at room temperature in the same medium throughout the period of investigation. The identification of *Azotobacter* spp. isolates was based on Buchanan and Gibbons (1974). Morphological characters, *viz.*, cell size, colony character, Gram stain, motility and capsule formation, and the biochemical characters, *viz.*, fermentation properties, catalase activity, nitrate reduction, gelatin liquefaction, H_2S production and NH_3 formation, were studied according to Conn *et al.* (1957).

The efficiency of strains to fix atmospheric nitrogen was studied by growing all of them in Jensen's medium of neutral reaction as well as at the pH of the soil from which they were isolated. The pH was adjusted using a buffer of the 0.2 *M* solution of 2-Amino 2-Methyl 1-3 Propanediol (50 ml) with x ml (Table 1) of 0.2 *M* HCl diluted to 200 ml.

Experiments were conducted to study the effect of pH on the growth and nitrogen fixation efficiency of nine (Azv1 to Azv3, Azc1 to Azc3, Azb1 to Azb3) strains of *Azotobacter*. The pH levels tested were 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0. Strains were grown in 100 ml of Jensen's medium (Rodina, 1972) in sterilised Erlenmeyer flasks. The inoculated

Table 1. Quantities (ml) of 0.2 *M* HCl required to produce buffer

Required pH	0.2 *M* HCl (x)	Required pH	0.2 *M* HCl (x)
10.0	2.0	7.0	51.0
9.5	8.5	6.5	58.0
9.0	16.7	6.0	62.0
8.5	34.0	5.5	70.0
8.0	41.0	5.0	78.0
7.5	45.0		

flasks were incubated at 28 ± 2°C for 15 days in dark. The growth of cells was determined by measuring the absorbance at 420 nm wth an Erma colorimeter and the amount of nitrogen fixed in liquid cultures was determined using the modified microkjeldahl method (Rodina, 1972). The experiment was repeated twice.

RESULTS

Nitrogen-fixing bacterial population was observed in the range of 0.11 to 8.1×10^3 cells per millilitre of water and 1.1 to 92.0×10^3 cells per gramme of sediment throughout the period of investigation (Table 2). All the 30 isolates were collected from the water and sediments of the perennial and seasonal ponds. Of the 30 isolates, 13 were identified as *Azotobacter chroococcum*, nine as *A. vinelandii* and eight as *A. beijerinckii*. All the eight isolates of *A. beijerinckii* were Gram-negative circular cells, occurring individually. Their size ranged from 5.4 to 7.4 µm in diameter, and were larger than both *A. chroococcum* and *A. vinelandii*. They were non-motile, non-flagellated, cyst-forming and capsule-producing types, and were capable of producing water-insoluble yellowish pigments. They were unable to utilise starch, mannitol and rhamnose as carbon source.

In *Azotobacter vinelanddii*, a gradual increasing trend was observed with the increasing level of pH in strains Azv1 to Azv3 (up to pH 7.0) which was followed by a sharp increase resulting in maximum growth at pH 7.5 in all the strains of *A. vinelandii*. After the maximum growth, with further increased in pH, a sharp decline was noticed in all the strains of *A. vinelandii*. Nitrogen fixation also increased with the increasing level of pH and the maximum level of pH was recorded at 7.5 in all the strains of *A. vinelandii*. Thereafter, a declining trend was evident (Fig. 1 & 2).

Table 2. Population of nitrogen fixing bacteria isolated from Narakkal

Months	Salinity (‰)	Nitrogen-fixing bacteria		*Azotobacter* spp.	
		Water (cells x 10^3/ml)	Sediment (cells x 10^3/g)	Water (cells x 10^3/ml)	Sediment (cells x 10^3/ml)
October	3.5	0.4	3.6	0.3	3.3
November	6.3	0.3	2.1	0.3	2.0
December	11.4	0.2	1.3	0.1	1.1
January	18.5	6.4	20.0	0.1	17.0
February	20.7	2.0	27.0	2.0	20.0
March	22.6	7.9	78.0	3.3	53.0
April	28.3	3.3	82.0	2.1	79.0
May	34.9	2.7	92.0	1.6	81.0
June	8.5	8.1	81.0	7.9	64.0
July	5.5	0.6	6.4	0.5	5.3
August	4.5	0.9	6.1	0.5	4.6
September	1.5	0.1	4.9	0.1	4.0
October	3.8	0.1	4.0	0.1	3.3
November	6.1	0.8	3.6	0.8	2.1
December	12.5	0.4	3.3	0.3	1.7
January	16.5	7.9	21.0	5.4	33.0
February	21.5	7.8	20.0	4.9	46.0
March	19.6	4.9	61.0	3.5	53.0
April	27.1	2.7	81.0	2.2	79.0
May	35.6	4.6	61.0	4.0	53.0
June	7.9	7.8	49.0	6.4	40.0
July	5.9	0.8	6.4	0.8	5.3
August	4.5	0.2	5.3	0.1	2.7
September	1.5	0.1	2.1	0.1	2.0

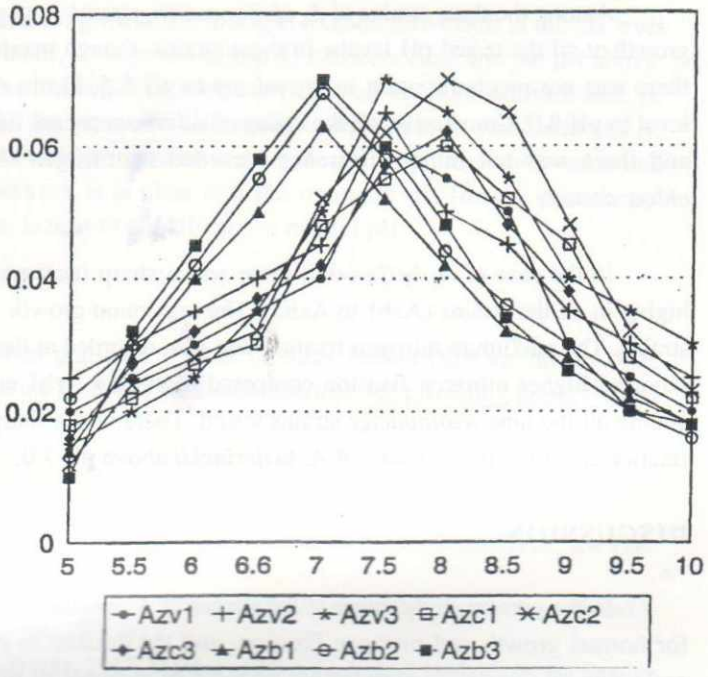

Fig. 1. Growth of *Azotobacter* spp. at different pH levels

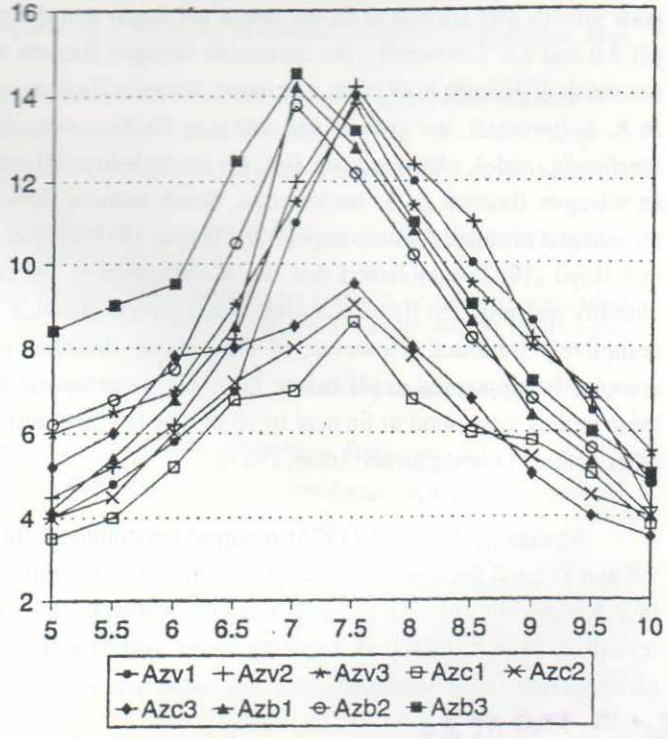

Fig. 2. Nitrogen fixation in *Azotobacter* spp. at different pH levels

Among the three strains of *A. chroococcum*, strains Azc1 and Azc3 showed relatively poor growth at all the tested pH levels. In these strains, though maximum growth occurred at pH 8.0, there was not much variation in growth up to pH 8.5. Strain Azc2 also grew to the maximum level in pH 8.0. Conversely, all the stains of *A. chroococcum* fixed maximum nitrogen at pH 7.5 and there was not much difference recorded in nitrogen fixation in all the strains of *A. chroococcum*.

In the case of *A. beijerinkii*, there was a sharp increase in growth and it was markedly higher in all the strains (Azb1 to Azb3). The maximum growth was recorded at pH 7.0 in all the strains. The maximum nitrogen fixation was also recorded at the same pH. However, strain Azb3 reported higher nitrogen fixation compared to strains Azb1 and Azb2, and it was the highest among all the nine *Azotobacter* strains tested. There was a sharp decline in growth and nitrogen fixation in all the three strains of *A. beijerinckii* above pH 7.0.

DISCUSSION

In the present study, most of the strains of *A. vinelandii* preferred a pH range of 7.0 to 7.5 for normal growth and nitrogen fixation, and the decline in growth and nitrogen fixaton was noticed in all the strains with further increase or decrease of pH. In *A. chroococcum*, relatively poor growth was noticed at all the tested pH levels and the maximum growth occurred between pH 8.0 and 8.5. Conversely, the maximum nitrogen fixation was noticed at pH 7.5 and there was not much difference noticed in maximum nitrogen fixation in all the strains of *A. chroococcum*. In *A. beijerinckii*, the growth and nitrogen fixation were found to be higher compared to *A. vinelandii* and *A. chroococcum*. But, pH levels below 7.0 were detrimental to the growth as well as nitrogen fixation in *A. beijerinckii*. Some authors isolated *A. beijerinckii in-situ* with its growth and nitrogen fixation at pH 5.6 (Tchan, 1953; Jensen, 1955). Koleshko (1961), and Boyd and Boyd (1962) established that the acidification of the medium leads to a sharp decline in viability and nitrogen fixation ability. According to them, at PH 4.5, the growth of the strains is completely inhibited. However, in the present findings, growth and nitrogen fixation were completely suppressed at pH below 7.0 in all the strains of *A. beijerinckii*. The optimum pH for these strains was found to lie near or slightly above neutrality as supported by the work of many other authors (Yamagata and Itano, 1923).

Khullar and Chahal (1975) reported maximum count of *Azotobacter* spp. in the soil pH 7.8 and 8.0, and the counts decreased with the increase in pH. The sediment pH above 9.0 resulted in a decrease in nitrogen fixation in all the strains of *A. chroococcum* and *A. vinelandii*. This reveals a wide variation in capacity to fix atmospheric nitrogen by different isolates of *A. chroococcum* and *A. vinelandii*.

The present study indicates that the growth and nitrogen fixation thrive best at the pH level above 7.0 and below 8.5 in *A. vinelandii*, *A. beijerinckii* and *A. chroococcum*, and the pH above and below this reduces the growth and nitrogen fixation activity in all the nine strains tested. *A. beijerinckii* strains are reported to be the most efficient strains compared to *A. vinelandii* and *A. chroococcum* both in their counts and nitrogen fixation activity. From the present observation and the observations made by other workers, it is clear that the optimum pH for the growth and nitrogen fixation of *Azotobacter* spp. is near or slightly above neutral pH.

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