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Characterization of Diazotrophs Associated with Roots of *Leptochloa fusca* (L) Kunth

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ABSTRAK

Sekumpulan sembilan diazotroph diasingkan daripada rumput kallar rizosfora dan dikultur di atas nitrogen bebas sederhana. K_5, K_8, K_9, K_{10}, K_{11}, K_{12}, K_{13}, menunjukkan keaktifan nitrogenase yang tinggi (718 n mol C_2H_4 h^{-1} $riat^{1}$). Sebaliknya, dibandingkan dengan K_7 dan K_{14} ia adalah lebih rendah (< 5 n mol C_2H_4 h^{-1} $riat^{1}$). Keaktifan maksimum nitrogenase didapati dalam kultur muda (24 jam selepas pengeraman) kecuali untuk K_9 dan K_{13} di mana maksimum keaktifan masing-masing ialah selepas 36 dan 48 jam. Semua terikan Gram-negatif, mengeluarkan koloni licin dan pelikel di atas media semi-pepejal. Sel K_5, K_7, K_{10}, K_{11}, K_{12} dan K_{14} adalah pleimortik; K_8 berbentuk rod-rod yang panjang; K_9 kecil dan berbentuk bulat ataupun bujur; manakala K_{14} berbentuk rod-rod yang berlubang. Hanya K_8, K_{13} dan K_{14} didapati dalam bentuk motil. Kesemua pencilan ini berupaya mengecilkan nitrat dan positif untuk oksidase dan katalase. Tiada satupun pencilan-pencilan boleh dinitrifi atau mempunyai keaktifan urase kecuali K_{14} yang positif urease. K_8, K_{13} dan K_{14} difermentasi dan mengeluarkan pigmen merah. Pencilan-pencilan K_5, K_{10} dan K_{12} telah dikaitkan dengan genus Azotobacter *sementara yang lainnya masih tidak dikenalpasti.*

ABSTRACT

A group of nine diazotrophs were isolated from the rhizosphere of kallar grass and cultured on nitrogent-free medium. K_5, K_8, K_9, K_{10}, K_{12}, K_{13} showed high nitrogenase activity (> 18 n mol C_2H_4 h^{-1} $vial^{1}$) whereas in K_7 and K_{14} it was comparatively low (< 5n mol C_2H_4 h^{-1} $vial^{1}$). Maximum nitrogenase activity was found in young cultures (after 24 hours of incubation) except for K_9 and K_{13} where it was maximum after 36 and 48 h, respectively. All strains were Gram-negat, produced smooth colonies and pellicles on semi-solid media. Cells of K_5, K_7, K_{10}, K_{11}, K_{12} and K_{14} were pleiomorphic: K_8 formed long fine rods; K_9 was small, round or oval shaped; while K_{14} formed beaded rods. Only K_8, K_{13} and K_{14} were found to be motile. All isolates were able to reduce nitrate, and were positive for oxidase an catalese. None of them could denitrify or had urease activity except for K_{14} which was urease positive. K_8, K_{13} and K_{14} were fermentative and produced red pigments. The isolates K_5, K_{10} and K_{12} are assigned to the genus Azotobacter *while others remained unidentified.*

Keywords: Diazotrophs, nitrogenase activity, physiological and biochemical characteristics, kallar grass, *Leptochloa fusca*, *Azotobacter*

INTRODUCTION

A number of nitrogen fixing bacteria have been isolated from the roots of different plants (Ahmad, 1979: Cappone and Budin, 1982; Malik and Zafar, 1985; Bilal and Malik, 1987; Reinhold *et al.* 1987; Cavalcante and Dobereiner, 1988; Zafar *et al.* 1988). In Pakistan, attempts have been made to reassess the possible contribution of nitrogen-fixing bacteria on the fertility of saline soil. Attention is being focussed on a salt-tolerant grass, *Leptochloa fusca* (L), which can grow well on low-fertility saline and sodic soils without the addition of nitrogenous fertilizers. Dinitrogen fixation associated with the roots of kallar grass has been reported Malik *et al.* (1980, 1982). Aerobic nitrogen-fixing bacteria have been reported in the rhizophere, rhizoplane and histoplane of *Leptochloa fusco.* Bilal and Malik (1987b) have isolated a nitrogen-fixing zoogloea - forming bacterium from the histoplane of kallar

grass while Niemann *et al.* (1985) and Reinhold *et al.* (1986, 1987) have identified diazotrophs associated with the roots of the same grass as *Azospilirillum*. Facultative anaerobic diazotrophs *Klebsiella* (Malik and Zafar, 1985) and *Enterobacter* (Zafar *et al.* 1988) from the roots of kallar grass have also been reported. The isolation of nine diazotrophs from the rhizophere of *Leptochloa fusca* (L) (Kunth) is reported here.

MATERIALS AND METHODS

After washing with saline (0.85% NaCl) and sterile, distilled water, soil-free roots of kallar grass were excised into 1 cm long pieces and incubated in 5 ml of semi-solid nitrogen free medium (NFM) which contained (g/l in distilled water) $CaCl_2.2H_2O$, 0.02; $MgSO_4.7H_2O$, 2.0; Malic acid, 5.0; KOH, 4.5; NaCl, 0.1; $NaMoO_4.2H_2O$, 0.002; Bromothymol blue, 0.5% in 3 ml of ethyl alcohol; Biotin, 0.1; Yeast extract, 0.002; Agar - for semi-solid, 2.0, for solid, 20.0; K_2HPO_4, 0.5; Na_2Fe (EDTA), 1.64% in 4 ml of water; pH,6.8. Cotton-plugged bottles containing root pieces in NFM medium were incubated at 30°C for 24 - 48 h. Thereafter, fresh vials were inoculated and incubated at 30°C. The cultures were assayed for nitrogenase activity by the acetylene reduction assay (ARA) as per Zafar *et al.* (1988). Cultures from ARA positive vials were streaked on NFM plates and incubated for 48 h. Cultures were then purified by routine streaking methods on nutient agar plate. Pure cultures were transferred to semi-solid NFM medium and incubated at 30°C When growth was observed (between 24- 48 h) the acetylene reduction assay was performed and positive cultures were maintained for further study.

Morphological, cultural and biochemical characters of the isolates were studied using standard bacteriological methods (Gerhardt *et al.* 1981) and QTS-20 and cyto chrome oxidase strips obtained from DESTO (Defence Science and Technology Organisation) Laboratories, Karachi.

RESULTS

ARA performed on freshly-grown cultures (24 h) showed that all the vials gave positive results for nitrogenase activity (> 18 n mol C_2H_4 h^{-1} $vial^{-1}$). These cultures were further streaked to single colonies on plates containing NFM medium. After purifying on nutrient agar plates, twenty single colonies were picked at random and tested for nitrogenase activity. Of these, eleven isolates gave week positive results while nine others (K_5, K_7, K_8, K_9, K_{10}, K_{11}, K_{12}, K_{13} and K_{14}) produced rather strong ARA activities and were selected for subsequent studies. Nitrogenase activity of the selected isolate was determined after 24, 36, 48 and 72 h of incubation. ARA was found to be different for each of these isolates (*Fig.1*) Nitrogenase activity was comparatively low for K_7 and K_{14} and rather strong, with maximum activity after 24 hours of incubation, for K_5, K_8, K_{10}, K_{11} and K_{12}. In K_9 and K_{13}; maximum nitrogenase activity was observed after 36 and 48 hours of incubation, respectively.

All isolates produced round colonies with entire margins (with some variation in size) on NFM and nutrient agar media. The sizes were 1.5 mm for K_5, K_7, K_9, K_{10} and K_{12}; 2.5 mm for K_{11}, K_{13} and K_{14}; and 5.0 mm for K_8 after 24 hours of incubation. All strains formed pellicles in semi solid media. Colony colour variation was observed on different media by different isolates. Colour of all isolates grown on NFM was cream except for K_{14} which was white; on nutrient agar it was yellowwish for K_5, offwhite for K_8 and K_{10}, and cream for K_7, K_9, K_{11}, K_{12}, K_{13} and K_{14}. Colour of colonies cultured on potato extract medium was cream for K_8, K_{11} and K_{14}, and brown to dark brown for K_5, K_7, K_9, K_{12}, and K_{13}. On MacConkey's agar, weak positive results were observed only for K_8, K_{13} and K_{14}. Variation in the cell shape of the different isolates was also recorded. K_5, K_7, K_{10}, K_{11}, K_{12} and K_{13} were irregular or pleiomorphic. K_8 formed long fine rods; K_9 was small, rounded or oval; K_{14} formed beaded rod (*Fig 2*). Only K_8, K_{13} and K_{14} were motile. All strains were Gram-negative.

All isolates, except K_8, showed positive NO_3^- reduction test (Table 1). Denitrification test was negative for all strains except for K_9 which was positive. Urease activity was negative (except for K_{14}) while oxidase and catalase tests were positive for all the isolates. only K_8, K_{13} and K_{14} were able to ferment glucose and manitol (with acid and gas production), and they also produced red pigment. Results or identification strip using QTS-20 reflect great variation in the biochemical characters of the isolates (Table 2).

DISCUSSION

NFM media, which contained nitrogen-free malate compound, were used for the isolation of diazotrophs. Use of N-free semi-solid isolation

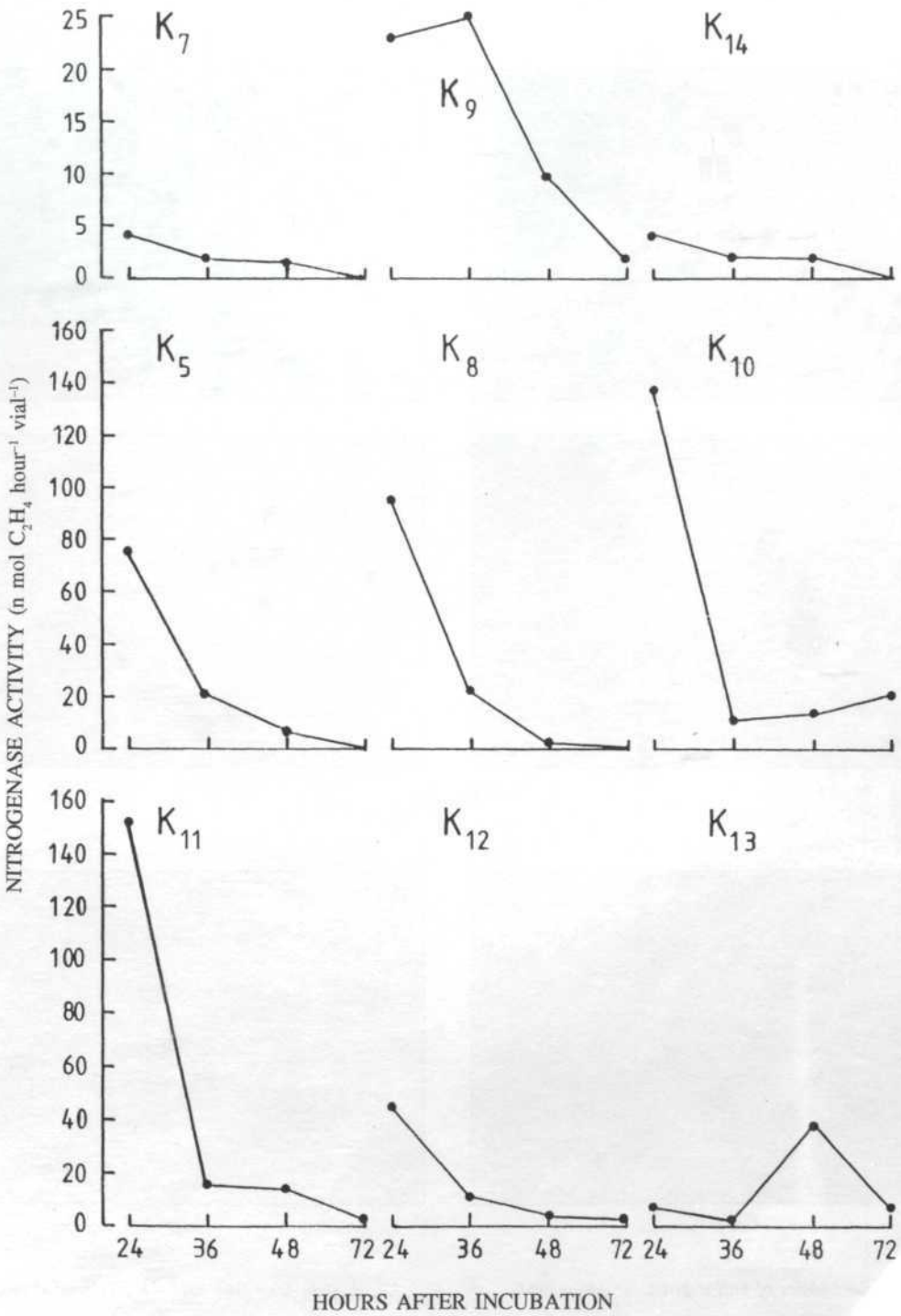

Fig. 1: Nitrogenase activity of the isolates after 24, 36, 48 and 72 hours of incubation

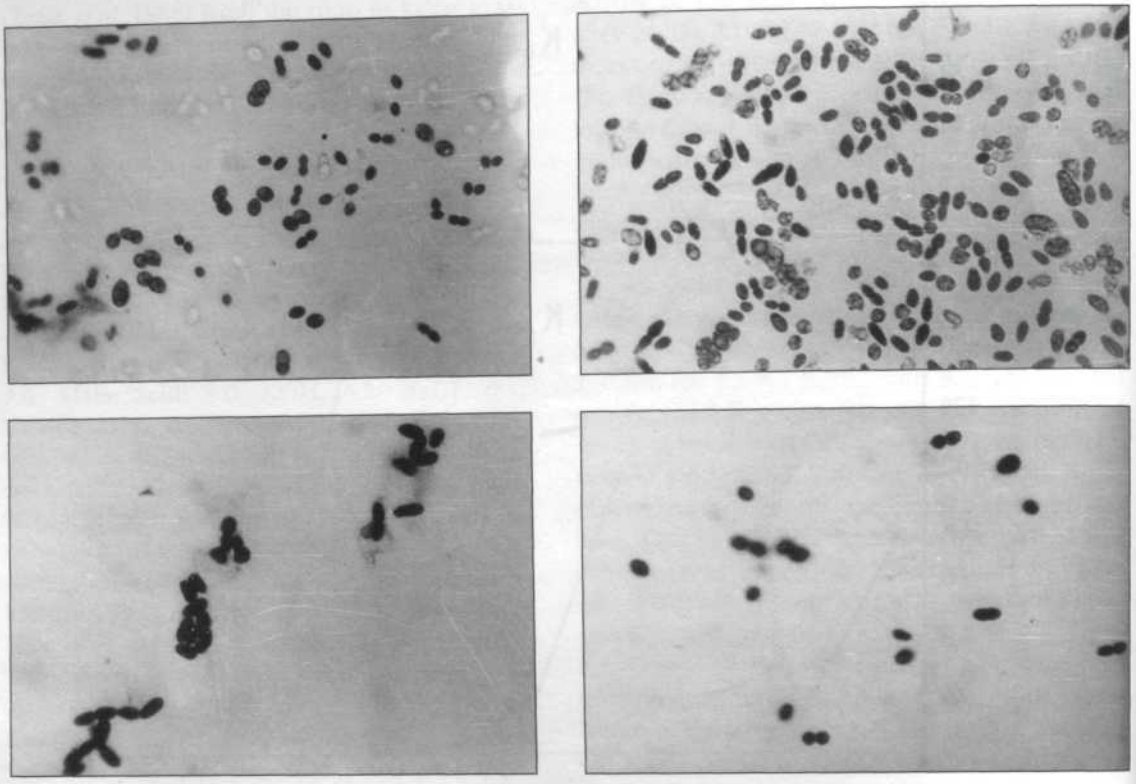

Fig. 2a

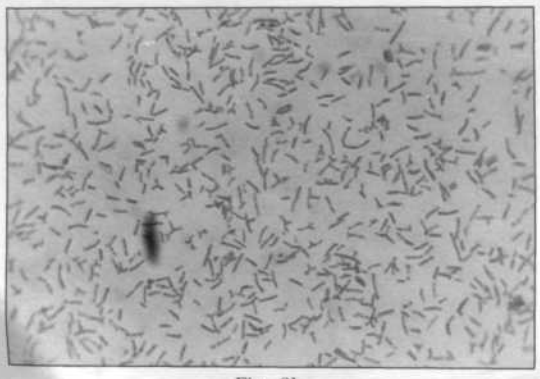

Fig. 2b

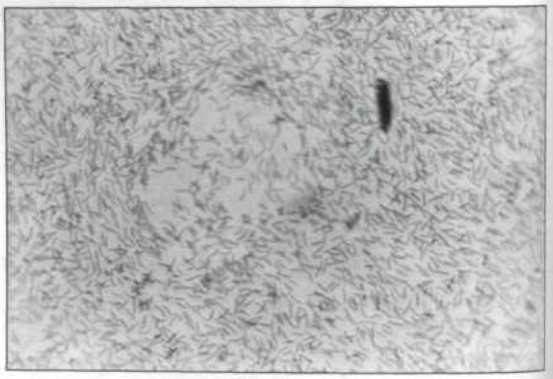

Fig. 2c

Fig. 2: Some isolates of kallar grass, a) pleiomorphic - K_5, K_{10}, K_{12}, K_{13}; b) long fine rods - K_8; c) beaded rods - K14

TABLE 1
Some physiological and biochemical characteristics of the isolates

Isolates	Catalase	Urease	Oxidase	Pigment	NO^-_3 red	Denitrifi cation	Sucrose Utilization	OF	Gram staining
K5	+	–	+w	–	+	–	+	–	–
K7	+	–	+	–	+	–	ND	–	–
K8	+	–	++	red	–	–	–	AG	–
K9	+	–	+W	–	+	+	+	–	–
K10	+	–	+W	–	+	–	+	–	–
K11	+	–	+W	–	+	–	ND	–	–
K12	+	–	+W	–	+	–	+	–	–
K13	+	–	++	red	+	–	–	AG	–
K14	+	+	++	red	+	–	–	AG	–

+, positive; +W, weak positive; –, negative; ND, not determined; AG, acid and gas

TABLE 2
QTS –20 Biochemical characterization of the diazotrophic isolates from the rhizosphere of kallar grass

Isolates Code	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v
K_5	-	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	+
K_7	-	+	+	-	+	+	-	-	-	-	+	-	+	+	+	+	-	-	+	-	+	+
K_8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+
K_9	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	+
K_{10}	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+
K_{11}	-	-	+	+	-	-	-	-	-	-	+	-	+	+	-	+	-	-	+	+	-	+
K_{12}	-	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	-	-	-	+
K_{13}	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	+	-	-
K_{14}	+	-	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	-	+	+	-	-

a, ONPG; b, cilrate; c, malonote; d, lysine; e, arginine; f, ornithine; g, H_2S; h, Urea; i, TDA; j, indole; k, vp; l, gelatine; m, glucose; n, nitrate; o, maltose; p, sucrose; q, mannitol; r, arabinose; s, rhamnose; t, sorbitol; u, inositol; v, oxidase; W+, weak positive.

media provided considerable progress for the isolation of nitrogen fixers (Boddey and Dobereiner, 1984). Species of *Azospirillium* and *Pseudomonas* have been mainly isolated on N-free medium (Barraquio *et al.* 1983; Falk *et al.* 1985; Reinhold *et al.* 1987). K_5, K_8, K_{10} and K_{11} showed reasonable nitrogenase activity (> 70 n mol C_2H_4 h^{-1} $vial^{-1}$) whereas in K_7 and K_{14} nitrogenase activity was rather low (< 5 n mol C_2H_4 h^{-1} $vial^{-1}$). The range of activity was between 4 and 168 n mol C_2H_4 h $vial^{-1}$ and it decreased with the age of the culture, and after 48 hours it was very low except for K_{13} where it was maximum. Tropical grasses show high rates of nitrogenase activity (Ahmad, 1979) and while xeric grasses do show activity, they do so at low rates (Wullstein *et al.* 1979). High nitrogenase activity has been found to be associated with the roots of kallar grass (Malik *et al.* 1982).

Bilal and Malik (1987b) have isolated nitrogen-fixing bacteria from the histoplane of kallar grass, which show very high (500 - 600 n mol C_2H_4 h^{-1} culture-$bottle^{-1}$) nitrogenase activity. Low nitrogenase activity of the isolates described here may be attributed to the low temperature of soil (Thompson *et al.* 1984), since samples for this study were collected in December.

Diazotrophs isolated in this study are Gram-negative, and with the exception of some bacilli (Seldin *et al.* 1984) the majority of N2 fixers are Gram-negative (Oken, 1982; Reinhold *et al.* 1987; Bilal and Malik, 1987b; Zafar *et al.* 1988). On the basics of cytochrome oxidace and fermenting abilities, K_8, K_{13} and K_{14} have been included in Gram-negative, facultative anaerobic rods, while the rest of the isolates were Gram-negative aerobic rods (Krieg and Holt, 1984). K_8, K_{13} and K_{14} were compared with all genera of Enterobacteriaceae but they remained unidentified. Previously, nitrogen-fixing *Klebsiella pneumoniae* have been reported from the rhizosphere of kallar grass (Malik and Zafar, 1985) while aerobic diazotrophic *Zoogloea* (Bilal and Malik, 1987b) and *Azospirilium* (Niemann *et al.* 1985; Reinhold *et al.* 1987) have been isolated from the roots of kallar grass. Zoogloea-forming bacteria were isolated on CCM (Carbon Combined Medium) and *Azospirillum* on NFM. *Zooglae* was pleiomorphic while *Azospirillum* formed vibroid to S-shaped cells. Aerobic rods of K_5, K_7, K_{10}, K_{11} and K_{12} showed pleiomorphic morphology while while K_9 formed small oval rods. Those which had 2 m m dia 3 3 m m length were taken as presumptive *Azotobacter* and *Azomonas*. Considering other biochemical and morphological characters K_5, K_{10} and K_{12} can initially be placed in the genus Azotobacter. These colonies were smooth, glistening, opaque and low convexed. All of them were non-motile, catalase-positive and had ovoid cells which occur singly, in pairs, in irregular clumps or small chains (*Fig. 2a*). Slime formation was also observed. Cysts were also formed in old cultures. All of them could reduce nitrate but none denitrified. All of them can utilize sucrose as the sole carbon source. K_5 and K_{10} utilize rhamnose while K_{12} did not. Table 3 shows the comparison of some biochemical characters of K_5, K_{10} and K_{12}. with six species of *Azotobacter* from Bergy's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Ni % G + C or DNA/RNA or DNA/DNA homology studies were performed with these strains which can escertain the taxonomics position of these isolates. Both *Azotobacter* and *Azimonas* are included in RNA super family II (De Smedt *et al.* 1980) and both of them are able to fix N_2 under atm pO_2.

From the above account it can be surmised that three *Azotobacter* strains (K_5, K_{10}, K_{12}) with comparatively high nitrogenase activity were obtained from the rhizosphere of *Leptochloa fusca*. Two other strains (K_8, K_{11}) also showed high nitrogenase activity but their affinities remain to be established. The extent of nitrogenase activity depends upon the incubation period. Generally, it is highest after 24 hours of incubation but was reduced with the prolonged incubation period.

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TABLE 3
Some biochemical characteristics of K_5, K_{10} and K_{12} along with selected strains of *Azotobacter* from Bergy's Mannual of Systematic Bacteriology (Krieg and Holt, 1984)

	A.chroococcum	*A.Vinelandii*	*A. beijerinchii*	*A.nitrican*	*A. arme niacus*	*A.paspali*	K_5	K_{10}	K_{12}
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	d	+	+	+	+
ND_3 reduction	+	+	+	+	–	–	+	+	+
Denitrification	–	–	–	–	–	–	–	–	–
Rhamnose	–	+	–	–	–	–	+	+	–
Sorbitol	+	+	d	d	+	–	–	+	–
Inositol	–	+	d	–	d	–	–	–	–
Mannitol	+	+	d	d	+	–	–	+	–
Malonate	d	+	+	d	–	–	+	–	+
Sucrose	+	+	+	+	+	+	+	+	+
H_2S production	d	+	d	d	–	+	–	–	–
Pigment	+	–	+	+	–	–	–	–	–
Motility	+	+	–	–	+	+	–	–	–

+. all strains positive; –, all strains negative; d, 11–8 stains are positive

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