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Enumeration of Nonsymbiotic Nitrogen-Fixing Bacteria Using Enzyme-Linked Immunosorbent Assay (ELISA)*

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Abstract. Serological relationships amongst different genera and also different species of nonsymbiotic N_2-fixing bacteria were studied using Enzyme-linked Immunosorbent Assay (ELISA). *Azospiritlum lipoferum* strains ICM 1001 and 4ABL were serologically distinct from strains of *A. brasilense* & *A. amazonense*; however, all of 10 *A. lipoferum* strains showed weak cross reactions, suggesting that these strains shared some common antigens with the *A. lipoferum* strains. Antisera of *A. brasilense* strains (SP 7a, SL 33, and SM 6M) did not cross react with 12 strains of *A. lipoferum*, 4 strains of *A. amazonense*, nor with 17 strains of *A. brasilense* tested. An exception was the B 25 strain, which showed the same reaction as that of homologous antigen against SP 7a antiserum. Antisera of azospirilla did not cross react with other genera of N_2-fixing bacteria tested using ELISA. Antiserum of *Azotobacter chroococcum* (ICM 2001) was genus and species specific. *Enterobacter cloacae* antiserum was genus specific but strains of *E. cloacae* and *E. aerogenes* shared some common antigens. The use of the ELISA to enumerate azospirilla in peat inoculants and broth culture and *A. chroococcum* in broth culture was investigated. A minimum of 10^3 and 10^4 cells of *Azotobacter* and *Azospirillum*, respectively, are required for a detectable ELISA reaction. Heat killed cells interfere with the ELISA reaction, limiting the use of this technique when the number of bacterial cells is low and all the cells in a sample are killed.

Key words. Azospirilla, *Azotobacter*, ELISA, Enumeration, N_2-fixing bacteria (nonsymbiotic)

Introduction

The distribution of N_2-fixing bacteria associated with nonlegumes is widespread in nature. Several groups of N_2-fixing bacteria have been found associated with cereals and grasses (1–5). Among the several types of N_2-fixing bacteria, the genera *Azospirillum* and *Azotobacter* have been widely studied. The genus *Azospirillum* includes two species, viz., *A. lipoferum* and *A. brasilense* (6). Two more species, *A. amazonense* and *A. halopraeferans* have been proposed (7–8). In *A. brasilense* two groups have been defined ; Nir^+ (denitrifier) and Nir^- (non-denitrifier). These species and groups have been characterized serologically (9–12). Using fluorescent antibody (FA) technique it was shown that *A. brasilense* and *A. lipoferum* are serologically distinct. Nonetheless, in immunodiffusion tests both the species showed cross reaction (11-12). *A. lipoferum* represented a more homogenous group in FA tests, in contrast, *A. brasilense* consisted of at least 3 subgroups (11).

Enumeration of these N_2 fixing bacteria is necessary in quality assessment of peat inoculants and in ecological studies also. Enumeration of azospirilla is generally done by

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dilution-plate count (DPC) or the most probable number (MPN) method based on acetylene reduction activity. Both methods are laborious and require at least 3-4 days for completion.

The enzyme-linked immunosorbent assay (ELISA) is more sensitive than the immunodiffusion or FA techniques. The direct or double antibody sandwich form of ELISA has been widely used for the detection of viruses in plant tissues (13–15) and *Rhizobium* in nodules (16–18) and also for enumerating rhizobia from peat inoculants and soil samples (19).

The objectives of this work are to study the possibility of using ELISA for a) studying the serological relationships amongst different groups of N_2-fixing bacteria and b) to enumerate azospirilla in broth and peat inoculants and *A. chroococcum* in broth culture using ELISA. The later is useful for assessing the quality of the inoculants produced for agricultural use.

Materials and methods

Chemicals and reagents : Unless otherwise mentioned, all chemicals and reagents used were from Sigma Chemicals, St. Louis, MO, USA.

Source of cultures : *Azospirillum lipoferum* (ICM 1001) and *Azotobacter chroococcum* (ICM 2001) were isolated from the rhizosphere of sorghum cv CSH 1 and *Cenchrus ciliaris* plants respectively (20). *A. brasilense* strains used were SL 33 and SM 6M (from Dr. F. V. MacHardy, University of Alberta, Edmonton, Canada), SP 7a (from Dr. J. Dobereiner, EMBRAPA, PAISNLCS-CNPA, Brazil), and *A. lipoferum* strains 4 ABL (from Dr. J. Balandreau, CNRS, Nancy, France). *Enterobacter cloacae* (ATCC 13047) was obtained from the American Type Culture Collection.

Antisera production and preparation of peat inoculants : All the cultures used for raising antisera were grown as thick lawns on nutrient agar slopes prepared in 350 ml bottles, using 100 ml medium and incubated at 33°C for 72 h. Bacterial cells were harvested in 5–10 ml of 0·85% sterilized physiological saline and washed three times, using the same solution. The washed cells were suspended in 0·85% saline to give a final viable cell number of 10^9 cells ml^{-1} for all cultures except for *E. cloacae* ($1·6 \times 10^{10}$ cells ml^{-1}).

Antisera for azospirilla, *A. chroococcum* and *E. cloacae* were prepared by injecting live cells into eight weeks old New Zealand white rabbits (schedule given in Table 1). Seven days after giving the last injection, test bleeding was done by puncturing the marginal ear vein. The antisera were collected and the antibodies titre in the serum was estimated using tube precipitation method. In atl the cases the titre was usually about 1 : 1600. The antisera collected from the nonimmunized rabbits did not show presence of any antibodies by precipitation method and also no positive reaction by ELISA was observed against the strains used for antisera production.

Peat inoculants of *A. lipoferum* (ICM 1001) and *A. brasilense* (SL 33) were prepared as described earlier (20).

Purification of antibodies from antiserum : Gamma globulins (antibodies) were collected by sodium sulphate precipitation (21). The serum was diluted in an equal volume distilled water and an equal volume of 36% aqueous sodium sulphate added. The precipitated protein was collected by centrifuging for 15 min at 6000 g, washed twice with 18% aqueous

Table 1. Immunization schedule for *Azospirilla, A. chroococcum*, and *E. cloacae*

Days	Quantity of antigen injected (ml)[a]	Route of injection[b]
Azospirilla and *A. chroococcum*		
1	0·5	IV
	1·0[c]	IM
2	1·0	IV
3	1·5	IV
7	1·5	IV
8	2·0	IV
9	2·0	IV
	1.0	IM
10	2·0	IV
25	2·0	IV
Enterobacter cloacae		
1	1·0[d]	IM
5	1·0	IM
12	1·5	IM
19	2·0	IM
23	2 0	IV

[a]Live cells counts in the antigen suspension for all the cultures were 10^9 cells ml^{-1} except for *E. cloacae* ($1{\cdot}6 \times 10^{10}$ cells ml^{-1})

[b]Intravenous (IV) injections were given in the marginal ear vein. Intramuscular (IM) injections were given in left and right thigh muscles.

[c]Equal amounts of antigen and Freund's complete adjuvant were injected.

[d]Heat killed cells were injected only at first injection time, subsequently live cells were used for injection.

sodium sulphate, resuspended in phosphate buffered saline (PBS) and dialysed at 4°C against three changes of PBS for 3 h each. The antibody preparation was diluted with PBS to give a final protein concentration of 1 mg ml^{-1} as estimated spectrophotometrically, using an extinction coefficient of 1·40.

Antibody-enzyme conjugation : The purified γ-globulins were conjugated with the enzyme, alkaline phosphatase, as described by Kishinevsky and Bar-Joseph (16). For all the experiments γ-globulins (conc. 1 mg ml^{-1}) were diluted to 1 : 500, 1 : 1000, 1 : 2000, for all the bacterial cultures except for *A. chroococcum* (ICM 2001) where globulins were diluted up to 1 : 3000 in conjugate buffer (PBS plus 0·05% Tween 20 plus 2% PVP and 0·2% ovalbumin). In all the cross reaction studies and enumeration experiments γ-globulins (1mg ml^{-1}) were diluted up to 1 : 1000 except that γ-globulins from *A. brasilense* (SL 33) and *A. chroococcum* (ICM 2001) were diluted to 1 : 500 and 1 : 2000, respectively.

Preparation and dilution of antigen : For studying the cross reactions, cultures except *rhizobium* strains were streaked on nutrient agar plates and the plates were incubated for 72 h at 33°C. The strains of *Rhizobium* were grown on yeast extract mannitol congo red agar and plates were incubated at 33°C for 72 h for fast-growing strains and 5 days for slow-growing strains. The growth was scraped from the agar surface and suspended in extraction buffer (Phosphate buffered saline, 0·02 mol/l phosphate, 0·15 mol/l NaCl, 0·003 mol/l KCl, pH 7·4, plus 0·05% Tween 20 and 2% polyvinylpyrrolidone, PVP-40T) to obtain 10^8 cells ml (PBS) (19).

One set each of peat inoculants of *A. lipoferum* (ICM 1001) and *A. brasilense* (SL 33) was incubated at 4°C and 40°C. Each set was sampled at 2 and 9 weeks after incubation and cell numbers were determined using the ELISA method and the dilution-plate count (DPC) method. For enumerating cells of *A. lipoferum* in broth, 100 ml broth was inoculated with one ml of inoculum (10^8 cells ml^{-1}). Two replicated flasks were incubated at each test temperature i.e., 32°, 45°, and 55°C under stationary conditions. The broth samples were aseptically collected from each flask at 0, 12·5 and 90 h interval, diluted and the cell numbers were estimated using the two methods. The cell numbers of *A. chroococcum* (ICM 2001) and *A. brasilense* (SL 33) in broth culture were estimated at 35°C incubation. Serial dilutions of peat inoculum or broth culture were prepared as follows : Peat inoculum (1 g) or culture broth (1 ml) was mixed with 9 ml of antigen extraction buffer. Serial dilutions were made in antigen extraction buffer.

Effect of autoclaving and heat killing of antigen on ELISA reaction : Serial dilutions of peat inoculum or broth culture were prepared in triplicate and one set was autoclaved for 15 min at 1kg cm^{-2}. Then second set was heated for one hour in a waterbath at 65°C, and the third set was used without any treatment.

Detection and estimation of antigen : The direct ELISA was used wherein alkaline phosphatase enzyme was used to conjugate with γ-globulin and P-nitrophenyl phosphate was used as a substrate. The procedure for direct (double-antibody sandwich) ELISA was followed as described by Kishinevsky and Bar-Joseph (16) by following the steps given below :

(1) Polystyrene microtitre plates (Dynatech Laboratories, INC : Alexandria VA 22314) were coated with 200 µl of specific diluted antisera $well^{-1}$ (1 : 1000 concentration) raised against test strains and the plates were incubated overnight at 4°C.

(2) Aliquots (200 µl) of serial dilution of antigen in extraction buffer were added to each well and incubated at 32°C for 3 h.

(3) To each well 200 µl of diluted γ-globulin-alkaline phosphatase enzyme conjugate (concentrations mentioned earlier) was added and the plates were incubated at 32°C for 3 h. After completing each of the above steps of ELISA, the plates were washed three times with PBS Tween (PBS plus 0·05% Tween).

(4) Substrate buffer 200 µl (80 mg p-Nitrophenol disodium phosphate in 100 m^{-1}) was added to each well and plates were incubated for 30 min at room temperature. Reaction was stopped with 50 µl of 3 m NaoH and the O. D. of P-nitrophenol produced in individual wells was read at 410 nm using a Dynatech MR 590 reader.

Results and Discussion

Cross reaction studies : The results of the cross reaction studies of different bacteria tested against the specific antisera of *A. lipoferum* (ICM 1001, 4 ABL), three strains of *A. brasilense* (Sp 7a, SL 33, and SM 6M), *A. chroococcum* (ICM 2001) and *E. cloacae* (ATCC 13047) are presented in Table 2. The number of strains used belonging to particular species are mentioned in Table 2, however only the results for the strains showing reactions different from the rest of the strains are given in the table. All the 11 strains of *A. lipoferum* group cross reacted weakly against antiserum of *A. lipoferum* (ICM 1001). Similar results were also observed with 4 ABL strain. These results suggested that strains of *A. lipoferum*

Table 2. Cross reaction studies with different groups of bacteria against antisera of *A. lipoferum*, *A. brasilense*, *A. chroococcum* and *E. cloacae* strains[a]

Test organism	Cross reactions with antiserum of						
	A. lipoferum (ICM 1001 4ABL)		*A. brasilense* (SP 7a	SL 33	SM6M)	*A. chroococcum* (ICM 2001)	*E. cloacae* (ATCC 13047)
A. lipoferum (12)[b]							
ICM 1001	2·0[c]	0·22	—[d]	—	—	—	—
4 ABL	0·22	1·72	—	—	—	—	—
Other strains	0·15-0·25	0·15-0·25	—	—	—	—	—
A. brasilense (17)							
SP 7a	—	—	2	—	—	—	—
SL 33	—	—	—	2	—	—	—
SM6M	—	—	—	—	2	—	—
B25	—	—	2	—	—	—	—
Other strains	—	—	—	—	—	—	—
A. amazonense (4)	—	—	—	—	—	—	—
A. chroococcum (2)							
ICM 2001	—	—	—	—	—	2·0	—
ATCC 480	—	—	—	—	—	—	—
A. vinelandii (2) (AVOP, SMG 85)	—	—	—	—	—	—	—
A. paspali A101 (1)	—	—	—	—	—	0·21	—
E. cloacae (4)							
ATCC 13047	—	—	—	—	—	—	1·11
1975	—	—	—	—	—	—	0·90
D14	—	—	—	—	—	—	—
7 ATZ	—	—	—	—	—	—	0·35
E. aerogenes (2)							
ATCC 13048	—	—	—	—	—	—	0·35
1976	—	—	—	—	—	—	—

[a]The cross reaction experiments were repeated 2–5 times. The concentration of antigens used for cross reaction studies were 10^9 cells ml^{-1}.

[b]The strains of different nitrogen-fixing bacteria used in cross reaction studies were :

A. lipoferum strains 114, 117, SP 59, SP3A, M83, M87, S82 and S93 were obtained from Dr. J. Dobereiner, Brazil, 4ATZ and 4ABL were obtained from Dr. J. Blandreau, France, and Br 10 was obtained from Dr. R. J. Rennie, Canada.

A. Brasilense strains (SP 7a, 34, 107, 109, PH1, SP 245 were obtained from Dr. J. Dobereiner, SL 1, SL 33, SL 77, SM 6M were obtained from Dr. F. V. MacHardy, Canada, AB 1 & AB 2 were obtained from Dr. N. S. Subba Rao, India, B 25 was obtained from Dr. S. A. Dhala, India and 12 S was obtained from Dr. B. S. Kundu, India.

A. amazonense strains (AM 14, AM 76, AM 91, and AM 132) were obtained from Dr. J. Dobereiner. In addition to above mentioned cultures following bacterial cultures were also used in cross reaction studies. All these cultures mentioned below showed no cross reaction against any of the test antisera. *Derxia gummosa*, *P. aeurginosa* (ATCC 15442, 2242), *P. paucimobilis* (5 AJ, SY 1), *P. pseudoalcaligenes* (ATCC 17440 and ATCC 17443), *P. stutzeri* (ATCC 17832), *K. pneumoniae* (K 11 and M 5A1), *Rhizobium* spp. (IHP 69, IHP 100, IHP 195, IC 76, IC 165, IC 6006, NC 92, TAL 176, F 75, and 51A 70). [c] O. D. values, [d] no cross reaction observed.

cross reacted serologically in ELISA tests. Similar findings using immunodiffusion (11-12) and FA techniques have been reported (11). No cross reactions were observed with 72 h old other bacterial cultures tested including 17 strains of *A. brasilense* and 4 strains of *A. amazonense.* Species specificity in the genus *Azospirillum* has been reported earlier using the FA technique (10-12). However, with cultures older than 48 h such species specificity was not found (11). Using immunodiffusion it may not be possible to differentiate the species of azospirilla since common precipitin bands are found with *A. lipoferum* and *A. brasilense* (11–12). However, using ELISA, which is a more sensitive assay, it is possible to differentiate the species and also the strains of *A. lipoferum* based on the quantitative measurement of the intensity of the reaction. These results showed that the strains of *A. lipoferum* vary serologically from each other even though, they share some common antigens. Using the FA technique, results suggested that strains of *A. lipoferum* form a homologous group (11). However, our studies using ELISA measuring quantitative reactions, suggest that they do not form a homologous group but that they only share some common antigens. Direct ELISA is highly specific whereas immunodiffusion technique has broader specificity. This is also one of the reasons why ELISA studies reported here are able to detect differences amongst *A. lipoferum* strains.

Seventeen strains of *A. brasilense* isolated from a wide range of geographical regions were tested against antisera of three additional strains (SP 7a, SL 33 and SM 6 M) for cross reaction studies. In general, there were no cross reactions amongst the strains of *A. brasilense* except that strain B 25 from India showed similar reaction against SP 7a antiserum as that of homologous strain. On the other hand *A. brasilense* strains isolated in Brazil did not cross react with the antiserum of SP 7a. The strain B 25 did not show any cross reaction against the antisera of SL 33 or SM 6 M strains. None of the strains belonging to *A. lipoferum, A amazonense* and other groups of bacteria tested showed any cross reaction against antisera of the three strains of *A. brasilense* indicating that *A. brasilense* strains are distinctly different from other species of *Azospirillum* and other groups of bacteria. The three strains of *A. brasilense* tested were serologically distinct from each other. Using FA technique the strains of *A. brasilense* were found to be serologically different (11), and the strains were grouped into 3 subgroups (11). However, there were 3 strains which could not be grouped into any of the 3 subgroups of *A. brasilense.* The findings of the present investigations suggest that all the 3 strains that we tested are serologically distinct and no relationships exist amongst the strains tested based on their nir^+ or nir^- characters as observed by earlier workers using FA technique (11). ELISA, which is more sensitive technique than FA or immunodiffusion, may therefore be employed for enumeration of a given strain from multi-strain samples and also may be employed for studying the persistence of inoculated strains in the field, after further, standardization of the method.

Azospirillum amazonense is a third species proposed in the genus *Azospirillum* (8). Our studies showed that none of the four strains of *A. amazonense* tested showed any cross reaction against the antisera of *A. lipoferum* or *A. brasilense* strains tested. Further, studies using antiserum produced against the type species of *A. amazonense* would indicate the serogroups amongst the strains belonging to *A. amazonense.*

The results with the antiserum against *A. chroococcum* (ICM 2001) showed that the antiserum was genus and species specific. The strain ICM 2001 was serologically distinct

from the strain of *A. chroococcum* (ATCC 480) tested in these studies. However, strains specificity in *A. chroococcum* needs to be checked in detail using more strains of the group.

Enterobacter cloacae was included in the present study as it is generally found in the rhizosphere of sorghum and pearl millet and showed considerable nitrogenase activity when grown as mixed culture with other bacterial cultures that did not show N_2ase *in-vitro* in pure culture (22). *Enterobacter cloacae* (ATCC 13047) was serologically distinct from the other genera of bacteria tested. Of the three strains used in this investigation one strain (1975) showed similar reaction as that of the homologous strain and two strains showed weak cross reactions. These results suggest that the strains of *E. cloacae* share some antigens and that few strains cross react serologically. Two strains of *E. aerogenes* were tested and one strain showed weak cross reaction against the antiserum of *E. cloacae* suggesting that some strains of *E. aerogenes* also shared some antigens with *E. cloacae*. However, this observation needs to be confirmed duing additional strains of *E. aerogenes*.

Enumeration of azospirilla and azotobacters using ELISA : In ELISA, the intensity of the colour reaction could be measured spectrophotometrically permiting quantification of the

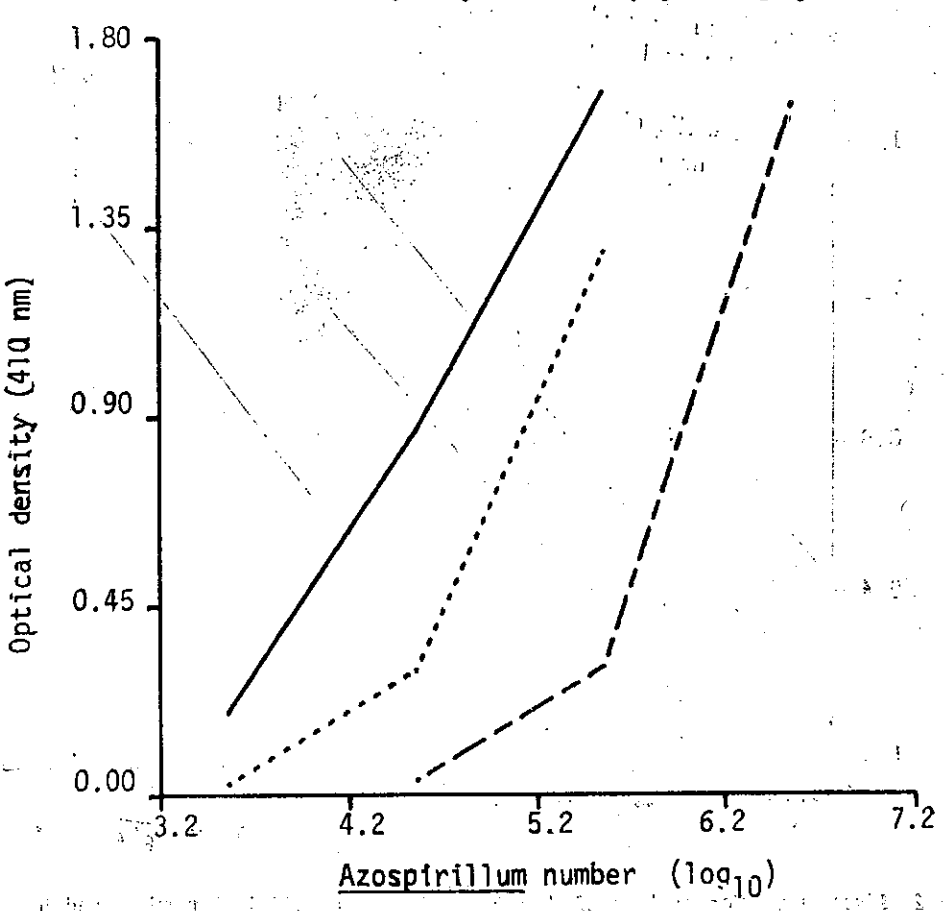

Fig. 1. Relationship between *Azospirillum lipoferum* (log_{10}) and intensity of alkaline phosphatase reaction estimated as O. D. using driect ELISA. γ-globulin enzyme conjugate dilutions used were 1 : 500(——), 1 : 1000 (.....) and 1 : 2000 (-----).

antigen (19). We have used ELISA for the enumeration of azospirilla and azotobacters from broth and peat inoculants. Figure 1 shows the relationship between the numbers of *A. lipoferum* (ICM 1001) in a broth culture and the corresponding O. D. values obtained from ELISA. There is no linear relationship across the range of azospirilla number (10^4—10^8 cells) using different γ-globulin enzyme conjugate dilutions (Fig. 1). Similar results were observed with *A. brasilense* (SL 33) and *A. chroococcum* (ICM 2001) also. As explained by Nambiar and Anjaiah (19) in case of *Rhizobium*, this is probably because the intensity of the colour developed is dependent on the amount of reagents (antiserum, antibody, antiserum-enzyme conjugate) bound and equilibrium reached during each incubation step, rather than directly proportional to the concentration of the antigen. These results show that linear relationship can be obtained with a particular concentration of γ-globulin enzyme concentration up to a certain range of bacterial cell numbers (Figures 2–4).

Figures 2, 3 and 4 show regression analyses for different concentration of γ-globulin

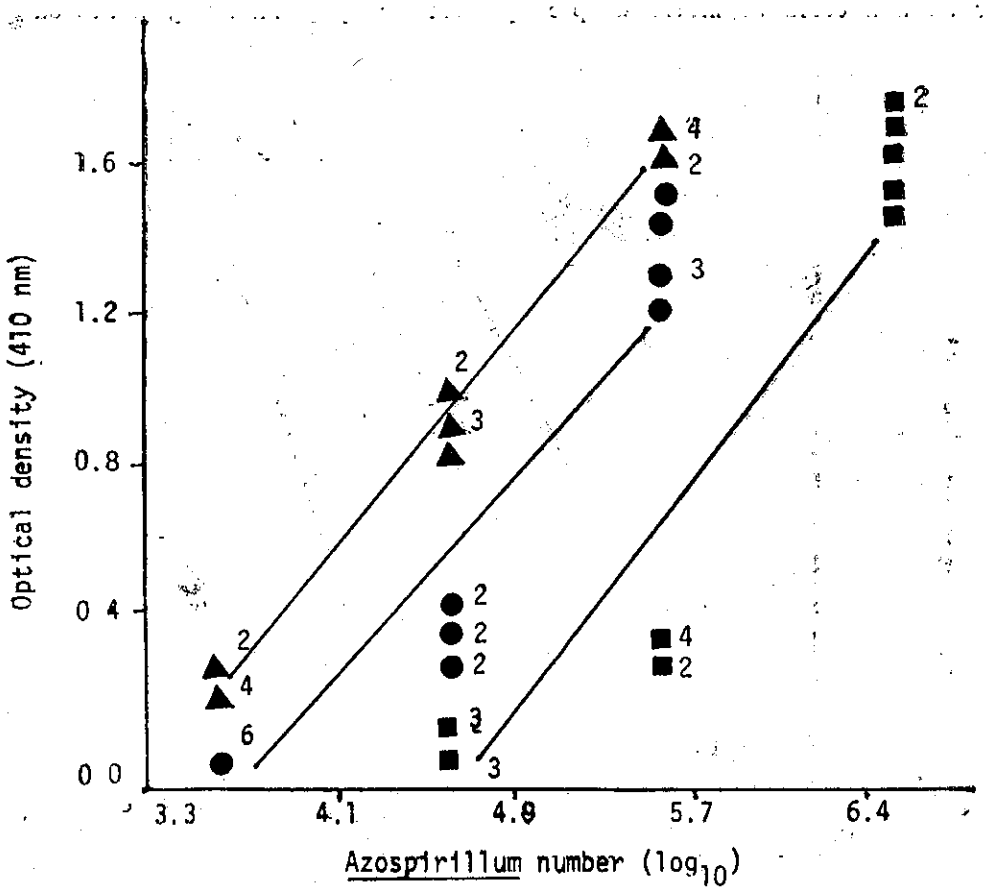

Fig. 2. Linear regression analyses of *Azospirillum lipoferum* (ICM 1001) numbers and intensity of alkaline phosphatase reaction estimated as OD by ELISA. The assays were done at 3 γ-globulin enzyme conjugate dilutions. 1. ▲.....1 : 500, y= −2·4156+0·7342 x (r^2=0·99); 2. ●....1 : 1000, y=−2·451+0·6617 x (r^2=0·87); 3. ■..... 1 : 2000, y= −3·793+0·8025 (r^2=0·85).

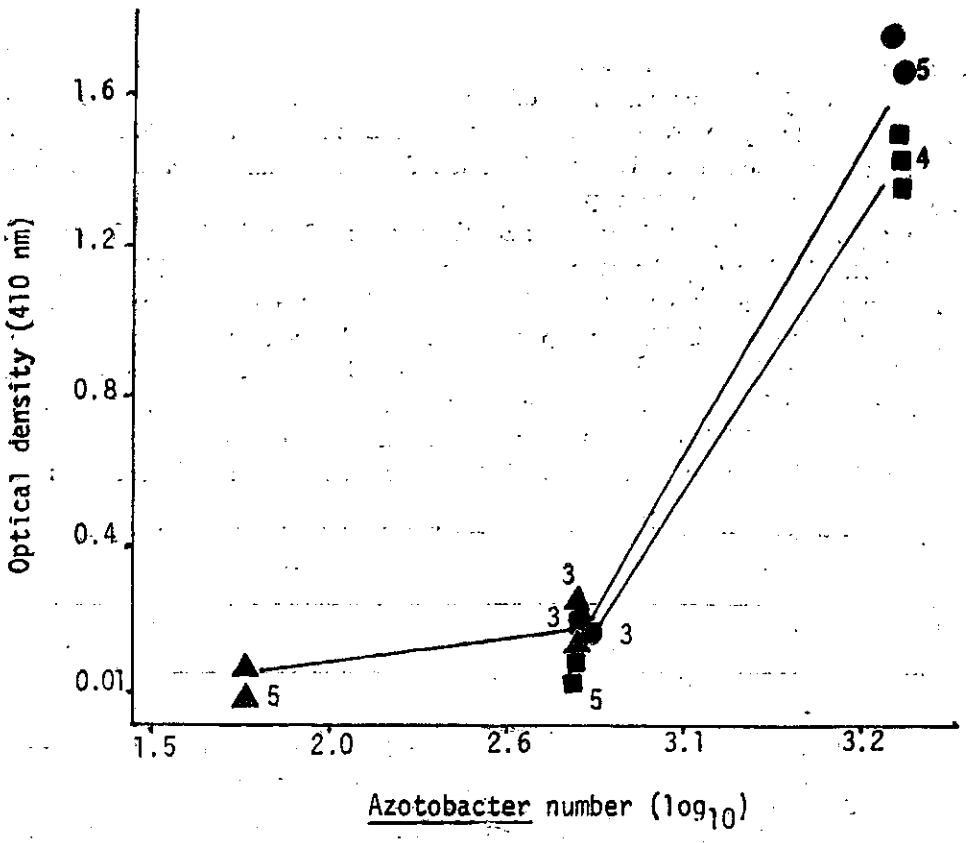

Fig. 3. Line regression analyses of *Azotobacter chroococcum* (ICM 2001) numbers and intensity of alkaline phosphatase reaction estimated as OD by ELISA. The assays were done at 3y-globulin enzyme conjugate dilutions 1. ▲.....1 : 1000, y = −0·2718+0·1767 x (r^2=0·86); 2. ●...1 : 2000, y = −4·2537+1·5717 x (r^2=0·99); 3. ■.....1 :3 000, y = −3·6497+1·3500 x (r^2=0·99).

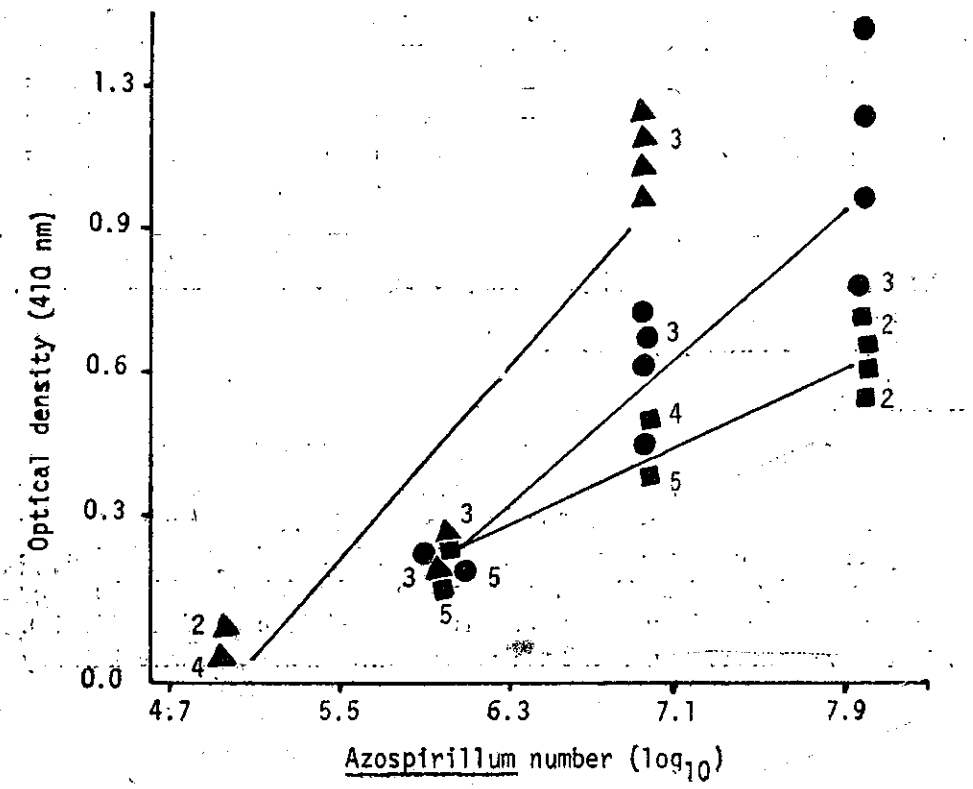

Fig. 4. Linear regression analyses of *Azospirillum brasilense* (SL 33) numbers andintensity of alkaline phosphatase reaction estimated as O. D. by ELISA. The assays were done at 3y-globul in enzyme conjugate dilutions. 1. ▲.....1:500, y = −2·7 73+0·5408 x (r^2 =0·86), 2. ● ...1: 1000, y=-2:180 + 0·4008x (r^2 0·84); 3. ■-1 : 2000, y = −1·083+0·2175x (r^2=0·93).

enzyme concentrations and number of live bacterial cells of *A. lipoferum*, *A. brasilense* and *A. chroococcum* respectively. These results indicate that by using a higher concentration of the γ-globulin enzyme conjugate lower numbers of bacterial cells could be detected (Figures 2 and 4). Similar relationships were observed between O. D. measurements and number of live bacterial cells in peat inoculants. Using ELISA therefore the enumeration of bacterial cells in broth culture and peat inoculant is possible.

Quality assessment of broth and peat inoculants : *Broth culture* : Data in Table 3 showed similar counts of *A. lipoferum* in broth cultures incubated at 32°C by ELISA and DPC techniques. However, the samples incubated at 45°C and 55°C showed a higher cell number at all three sampling times with ELISA as compared to the DPC technique. There was no multiplication of bacteria in the broth incubated at 45°C and the number declined considerably. For sets incubated at 55°C, all cells were killed within 12·5 h. However, ELISA results showed quite high bacterial numbers in 12·5 h and 90 h samplings. Results in Table 3 show that the live cell numbers of *A. chroococcum* and *A. brasilense* in broth

Table 3. Enumeration of azospirilla and azotobacters cells in broth incubated at different temperatures using ELISA[a] and dilution-plate count techniques

Incubation temperature (°C)	Method	Age (h)					
		0	12·5	21	42	90	96
A. lipoferum							
92	DPC[b]	$2·7\times10^6$	$3·0\times10^7$	—[c]	—	$1·3\times10^7$	—
	E	$1·4\times10^6$	$6·0\times10^7$	—	—	$1·1\times10^7$	—
45	DPC	$2·7\times10^6$	$3·0\times10^6$	—	—	$1·2\times10^4$	—
	E	$1·4\times10^6$	$4·0\times10^7$	—	—	$4·7\times10^6$	—
55	DPC	$2·7\times10^6$	d	—	—	d	
	E	$1·4\times10^6$	$5·7\times10^6$	—	—	$3·5\times10^6$	—
A. brasilense							
35	DPC	—	—	$3·5\times10^7$	$2·1\times10^7$	—	$5·1\times10^7$
	E	—	—	$3·2\times10^7$	$3·2\times10^7$	—	$3·7\times10^7$
A. chroococcum							
35	DPC	—	—	$1·6\times10^6$	$1·6\times10^7$	—	$2·6\times10^7$
	E	—	—	$5·1\times10^6$	$2·8\times10^7$	—	$5·1\times10^7$

[a]O. D. values were converted to cell numbers by using regression equations for test strains. For *A. brasilense*, *A. lipoferum* and *A. chroococcum*, γ-globulin enzyme conjugate concentrations used were 1:500, 1 : 1000, and 1 : 2000 respectively.

[b]DPC=Dilution–plate count technique, E=ELISA

[c]Not determined.

[d]No live cells detected.

cultures incubated at 35°C under stationary condition as enumerated by using ELISA or DPC were the same at all 3 sampling intervals. These results suggest that the cells killed instantly interfere to a great extent in the enumeration of cell numbers by ELISA. However, if a few cells die naturally and live cells are present in the broth then the dead cells don't cause interference in enumeration by ELISA. This may be due to the breakdown of dead cells by the extracellular enzymes secreted by living cells as explained in case of rhizobia (19). Similar observations have been made with the broth cultures where in the cells were killed by

autoclaving or by heating the broth at 65°C for 1 h. The bacterial cells killed instantly gave higher O. D. values than the O.D. values obtained with live cells. As ELISA gives results in less time than the DPC technique, this technique is useful for enumerating bacteria from broth cultures. However, if there is a possibility that cultures have been exposed to conditions causing instant killing of all the cells, then it is mandatory to streak the broth on plates to test the viability of the cells.

Peat inoculants : The results of *A. lipoferum* and *A. brasilense* cell numbers in peat inoculants are given in Table 4. The cell numbers in peat estimated either by ELISA or by the DPC

Table 4. Comparison of dilution plate count and ELISA[a] methods for counting *A. brasilense* and *A lipoferum* from peat inoculants[b]

Organism	Method	Incubation Time (weeks)			
		2		9	
		Incubation temperature (°C)			
		4	40	4	40
A. brasilense	DPC[c]	$7{\cdot}4\times10^7$	9×10^7	2×10^6	1×10^6
	E	8×10^7	9×10^7	$4{\cdot}3\times10^6$	5×10^6
A. lipoferum	DPC	$6{\cdot}8\times10^7$	$6{\cdot}8\times10^7$	$1{\cdot}3\times10^7$	9×10^6
	E	$6{\cdot}5\times10^7$	$6{\cdot}5\times10^7$	$1{\cdot}5\times10^7$	$4{\cdot}6\times10^6$

[a]O. D. values were converted to cell numbers by using regression equations. For *A. brasilense* and *A. lipoferum* γ-globulin-enzyme conjugate concentrations used were 1 : 500 and 1 : 1000 respectively.
[b]Average of two replicated packets. From each packet two replicated samples were used for preparing dilutions.
[c]DPC=Dilution–plate count method, E=ELISA

were similar. The cell numbers of *A. lipoferum* in the peat inoculants incubated at 40°C declined to a greater extent as compared to the number in the inoculants incubated at 4°C. The decline in the cell counts was equally evident using both the methods. However, if the cells in the peat inoculants were killed instantly by heating the peat inoculants at 65°C for 1 h or by autoclaving, the ELISA gave high O. D. values whereas no live cells were detected using DPC count technique. These results confirm the findings that instantly killed cells interfere in enumeration studies using ELISA. ELISA could be however, successfully used to monitor the number of viable cells in the peat inoculants for monitoring the quality.

ELISA technique can be employed to test the quality of broth culture before inoculating in the peat as well as the quality of the inoculants prepared at the production center. However, it will be difficult to test the quality of the inoculants at distributing ends using ELISA unless other methods are used in conjunction with those to test the inoculants, after exposure to high temperatures. Our studies indicate that ELISA can differentiate the strains, species and genera of nonsymbiotic N_2-fixing bacteria more effectively. Being a quantitative technique, it can be used for the enumeration of bacterial cells in broth culture or peat inoculants in less time than that needed for enumeration by MPN or the DPC technique. ELISA could also be a tool in ecological studies of nonsymbiotic N_2-fixing

bacteria and in studying the competitive ability and persistence of inoculated strains in the field.

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