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Research Paper

The use of *lacZ* marker in enumeration of *Azotobacter chroococcum* in carrier based inoculants

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Abstract

A transconjugant of *Azotobacter chroococcum* Mac 27 tagged with *lac Z*(*A. chroococcum* Mac27 L) was found to possess high levels of β-galactosidase activity constitutively.Further, the lac Z marker was found to be stably integrated into the chromosome of the *A. chroococcum* Mac 27 and did not have any adverse effect on growth, nitrogen fixation and excretion of ammonia. A quick method to determine the viable cell number in broth culture and carrier based inoculants has been developed on the basis of β-galactosidase assay. It was found that there was a direct relationship between the number of cell as determined by standard plate count and intensity of colour that developed upon degradation of ONPG due to β-galactosidase activity .The method was found to be sensitive enough to determine 1.7 x 10^6 CFU mL^{-1} in broth culture as well as carrier based *Azotobacter* inoculants. Further, it was observed that when A. *chroococcum* Mac27 L was inoculated on *Brassica campestris*, it could be detected in the presence of other bacteria capable of growing on Burks agar medium containing X-gal on the basis of *lac Z* genetic marker.

Key words: *Lac Z*, transconjugant, β galactosidase, inoculants, *Brassica campestris*.

Introduction

In recent days the importance of *Azotobacter chroococcum* as a biofertilizer to improve crop productivity of non-leguminous crops has been recognized. These inoculants have been found to increase the yield of rice, wheat, pearl millet, sorghum, maize, cotton, oil seeds and several vegetable crops (Pandey and Kumar, 1989; Lakshminarayana *et al.*, 1992; Paul *et al.*, 2011). In India carrier based inoculants are produced by State Agricultural Universities and private entrepreneurs, however, the crop responses are not consistent under different agro-climatic conditions. Quality of the inoculants and their survival and persistence under field conditions are two major problems associated with beneficial effects of inoculants. Many studies have been conducted to monitor and identify the inoculated strains under field conditions using different techniques. However, information on the survival of strains in carrier based culture packets using methods other than standard plate count technique is lacking. Some of the techniques in use to monitor and identify the inoculated strains of bacteria important in agriculture include antibiotic resistance, serological markers, bacteriophage susceptibility, plasmid profile etc. Variation in sensitivity to different antibiotics between species may be a useful taxonomic character for ecological studies and has been used to estimate the survival of fast growing rhizobia from clover and medic growing in non sterile soils (Danso *et al.*, 1973). The disadvantage of antibiotic resistant strains for ecological studies is that selected strains may vary in other important characteristics including ability to fix atmospheric nitrogen. Moreover, loss of antibiotic markers during growth makes this method unsuitable for strain identification. Serological techniques include agglutination, immunogel diffusion, immunofluorescence and ELISA, and have been used to identify *Rhizobium japonicum* grown in culture and from root nodules (Dudman, 1971; Kishinevsky and Bar Joseph, 1978; Olsen and Rice, 1989).However, cross reactive antigens present in other strains give faulty results and limit the

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use of these techniques (Vincent and Humphery, 1970). Susceptibility of a certain bacterial strain to a particular bacteriophage forms the basis for phage typing. Bacteriophage typing has been used for screening an individual isolate from most other strains of diverse origins with sufficient sensitivity. However, the phage typing system has limited applications because bacteriophages for all the strains are not available and some phages have wide host range. The DNA-DNA hybridization and plasmid profiles have been used to fingerprint strains of Gram-negative bacteria (Boyce and Morter, 1986; Cooper *et al.*, 1987; Saano and Lindstorm, 1990). However when the strains contain the same number of plasmids with slight detectable differences in electrophoretic migration pattern, differentiation between strains may be difficult.

Marker genes like *lac* Z, *gus* A, *luc* and *gfp* have been introduced to soil bacteria to identify these bacteria in rhizospheric soil, nodule and within the plant. (De Weger *et al.*, 1991; Bhatia *et al.*, 2002; Kundu *et al.*, 2006; Sunita *et al.*, 2010). These methods are highly specific in identification of introduced bacteria. In the present investigation *lac* Z marked strain of *A. chroococcum* was used to develop a method to enumerate viable cells of *A. chroococcum* in broth culture and charcoal based inoculants on the basis of β-galactosidase activity.

Material and Methods

Bacterial Cultures and growth media

Bacterial cultures(*Azotobacter chroococcum* Mac 27 and *lac Z* transconjugant, *Azotobacter chroococcum* Mac 27 L) used were from the culture collection of Department of Microbiology, CCSHAU, Hisar. These were maintained on Burks medium (Newton *et al.*, 1953) slants. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to precooled sterilized medium.

Growth of *A. chroococcum* Mac27 and Mac 27 L on different carbon sources

Plates of Burk's agar medium were prepared using different organic compounds as sole source of carbon. The carbon sources - xylose, raffinose, fructose, galactose, glucose, sucrose, lactose and ethanol were used at a concentration of 2% while butanol, mannitol, polyethylene glycol, sodium acetate, sodium pyruvate, sodium malate, sodium oxalate, calcium gluconate, calcium lactate, potassium citrate, potassium tartarate and resorcinol were added at the concentration of 1%.

Determination of nitrogenase activity

Slants of Burk's medium with and without NH_4Cl (2 mM,5 mM, 10 mM and 15 mM) and yeast extract (0.05%. 0.1% and 0.2%) were prepared. Four slants of each treatment were inoculated with pure cultures of *A. chroococcum* Mac 27 and *A. chroococcum* Mac 27 L separately. The tubes were incubated for 2 days at 30 °C in a BOD incubator. Reduction of acetylene to ethylene was determined in gas liquid chromatogtaph. Nitrogenase activity is expressed in terms of n moles of C_2H_4 evolved 24 h^{-1} mg^{-1} dry weight of cells.

Ammonia excretion

Ammonia excretion ability of *A. chroococcum* Mac 27 and *A. chroococcum* Mac27 L was studied by growing the cultures in duplicate in 100 mL Burk's broth. The flasks were incubated for 20 days at 30 °C in an incubator under stationary conditions. Aliquots of culture broth (5 mL) were removed at regular intervals of four days, centrifuged at 10,000 rpm for 20 min and ammonia released in the supernatant was determined using the method of Chaney and Marbach (1962).

Stability of *lac* Z marker

A. chroococcum Mac 27, the parent and the transconjugant Mac 27 L were transferred repeatedly on Burk's medium slants with and without kanamycin. The slants were incubated at 30 °C in a BOD incubator. After 50 days, all the slants were restreaked on fresh slants and allowed to grow for 48 h at 30 °C.A loopful of the culture from the freshly streaked slants as well as from the old slants was suspended in 5 mL phosphate buffer(pH 7.0) and β-galactosidase activity was determined by microtitre method using ONPG as the substrate.

Assay of β galactosidase activity

One mL actively growing broth culture of *A. chroococcum* Mac 27 L was taken in Eppendorf tube and centrifuged at 8000 *g* for 10 min. The cell pellet was washed twice with phosphate buffer and resuspended in 500 µL of phosphate buffer (pH 7.0). The cells were permeabilized by vortexing for 10 s with a solution containing 25 µL of toluene. To 100 µL of permeabilized cells, 10 µL ONPG was added. The reaction mixture was incubated at 37 °C in water bath till the development of yellow colour. Reaction was stopped by adding 2 mL of 0.4 M Na_2CO_3 solution. Colour intensity was determined at 420 nm using a spectrophotometer against a blank containing all ingredients except the cell culture. β-galactosidase activity is expressed as Millers unit (Miller, 1972) calculated as per formula given below:

$$\text{Millers unit} = \frac{\text{Absorbance}_{420}}{\text{Absorbance}_{420} \times T \times V} \times 1000$$

where T = time of reaction (min) and V = volume of the cell culture used (mL).

Preparation of charcoal based inoculants

Fifty grams of fine wood charcoal powder was weighed in polypropylene bags and 2 mL water was added

into each packet. The packets were sterilized by autoclaving at 15 psi for 30 min for two consecutive days. To each of these packets, 30 mL freshly grown broth culture of *A.chroococcum* Mac 27 and Mac 27 L were inoculated and incubated at 30 °C.

Enumeration of *A. chroococcum* Mac 27 L by standard plate count (SPC) and by β-galactosidase assay

Five gram of charcoal was added in 45 mL of phosphate buffer and the flask was agitated intermittently on a vortex shaker for about 30 min. One mL sample was withdrawn from this flask and serially diluted upto 10^{-5}. The dilutions ranging from 10^{-3} to 10^{-5} were spread on Burks agar medium containing X-gal and incubated at 30 °C in a BOD incubator for 72 h. For enumeration of *A. chroococcum* Mac 27 L by chromogenic method, five mL samples of charcoal based culture suspended in phosphate buffer were withdrawn and centrifuged at 4500 rpm to settle down the charcoal.β-galactosidase activity was assayed directly in the supernatant by adding ONPG as the substrate after permeabilizing the cells with toluene.

Enumeration of *A. chroococcum* Mac 27 *lac Z* from the rhizoplane of *Brassica campestris.*

Surface sterilized seeds (10 g) of *Brassica campestris* were inoculated with about 10^8 cells of *Azotobacter chroococcum* Mac 27, *A.chroococcum* Mac 27 L and HT-54. Seeds were sown in pots containing 5 kg unsterilized soil. Each treatment was replicated three times. After 30 and 60 days of growth, four plants in three replicates were taken out and cut at the crown after removing the adhering soil. These roots were added to 100 mL sterilized phosphate buffer and vortexed for 15 min. The appropriate serial dilutions were plated on Burks medium plates with and without X-gal. The plates were incubated for 48 h and the number of colonies that appeared on both types of media was counted.

Results

Growth pattern of *A. chroococcum*

The growth of both the strains was compared by using different organic compounds as the sole source of carbon (Table 1). Both *A. chroococcum* Mac 27, the parent as well as *lac* Z marked transconjugant showed good growth on all the substrates tested except xylose, lactose, resorcinol,sodium malate and polyethylene glycol.

Excretion of ammonia by *A. chroococcum* Mac 27 and the transconjugant Mac 27 L

The excretion of ammonia by *A. chroococcum* Mac 27, and a *lac* Z marked transconjugant was determined under stationary conditions. Whereas, there was no ammonia excretion either by the parent or the transconjugant derived from it at the start of the experiment , the excretion of upto 4 μg of ammonia mL^{-1} of broth culture was observed after four days that increased upto twelfth day to 17 μg of ammonia mL^{-1} in both the cultures (Table 2). The quantity of ammonia excreted decreased after sixteenth day and no ammonia excretion was observed after 24 days. It was observed that maximum amount of ammonia was excreted during the stationary phase of growth.

Table 1 - Growth of *A. chroococcum* on various organic compounds.

Sr. No.	Carbon source (organic)	*A. chroococcum* Mac 27	*A. chroococcum* Mac 27 L
1	Control	-	-
2	Xylose	-	-
3	Raffinose	+	+
4	Fructose	+	+
5	Galactose	+	+
6	Glucose	+	+
7	Sucrose	+	+
8	Lactose	-	-
9	Ethanol	+	+
10	Butanol	+	+
11	Mannitol	+	+
12	Polyethylene glycol	-	-
13	Sodium acetate	+	+
14	Sodium pyruvate	+	+
15	Sodium malate	-	-
16	Calcium oxalate	+	+
17	Calcium gluconate	+	+
18	Calcium lactate	+	+
19	Potassium citrate	-	-
20	Potassium tartarate	-	-
21	Resorcinol	-	-

Nitrogenase activity

The nitrogenase activity expressed as n moles C_2H_4 evolved h^{-1} mg^{-1} dry weight of cell was found to be 281.3

Table 2 - Ammonia excretion* by *Azotobacter chroococcum* Mac 27 and Mac 27 L at 30 °C under stationary conditions.

Time of Sampling (days)	*A. chroococcum* Mac 27	*A. chroococcum* Mac 27 L
0	0.00	0.00
4	4.05	3.75
8	6.26	5.82
12	17.00	16.60
16	5.62	5.36
20	2.55	2.31

*Ammonia produced is expressed in μg mL^{-1}.

units and 300 units in the parent and transconjugant respectively in the absence of combined nitrogen. When the Burks medium was supplemented with organic nitrogen added in the form of yeast extract from 0.05% to 0.2%, the nitrogenase activity decreased in both the strains. When Burks medium was supplemented with ammoniacal nitrogen varying from 2 mM to 15 mM, nitrogenase activity upto 115.3 and 134 units was observed in *A. chroococcum* Mac 27 and transconjugant respectively. Nitrogenase activity was completely suppressed in the presence of 5 mmM or more of ammoniacal nitrogen (Table 3).

Determination of stability of lac marker in *A. chroococcum* Mac 27 L

To check, if the lac Z marker present in *A. chroococcum* Mac 27 L was stable or lost during growth in the presence or absence of kanamycin, the culture was transferred every alternate day for 50 days and β-galactosidase activity was determined in all the twenty four transfers by chromogenic method using ONPG as the substrate (Table 4). Though the β-galactosidase activity varies in different transfers but it was present even after twenty four transfers.

Enumeration of *A. chroococcum* Mac 27 L in pure culture and in charcoal based culture packets

A. chroococcum Mac 27 L strain was grown in Burks medium in the presence of kanamycin for 72 h. The cells were centrifuged and washed using phosphate buffer. Cell suspensions of different concentrations varying from 0.02 to 0.5 were prepared by dilution in the phosphate buffer under aseptic conditions. Aliquots of 200 μL of cell suspension were withdrawn from each dilution in triplicate and colour was developed using ONPG as the substrate after permeabilizing the cells with toluene. The intensity of the colour after 15 min was determined by using a spectrophotometer. Viable number of cells was also determined in all the six dilution by plating appropriate dilutions of each suspension on Burk's medium plates containing kanamycin in duplicate. The viable number of cells was determined after 72 h of growth. Correlation between the intensity of colour and viable number of cells is represented in Table 5.

There is almost a linear relationship between the intensity of colour due to β-galactosidase activity and number of viable cells. The method is sensitive and cell number upto 1.7 x 10^6 can be determined by calorimetric method and cells upto 3.9 x 10^6 and more can be estimated by visual observations. The number of viable cells in charcoal based inoculants determined by using this chromogenic technique was found to be similar to those determined by standard plate count (SPC) technique (Table 6).

Table 3 - Effect of combined nitrogen on the nitrogenase activity of *A. chroococcum* Mac 27 and Mac 27 L.

Treatment	Strain	
	Mac 27	Mac27 L
	Nitrogenase activity*	
Burks Medium(BM)	281.3	300
BM+Yeast extract		
0.05%	180.6	188.5
0.1%	166.4	169.0
0.2%	145.5	136.0
BM+ NH_4Cl		
2 mM	115.3	134.0
5 mM	6.5	19.5
10 mM	0.0	0.0
15 mM	0.0	0.0

*nm C_2H_4 evolved 24 h^{-1} mg^{-1} dry wt. of the cell.
Average value of 4 replications.

Table 4 - Effect of repeated transfer of *A. chroococcum* Mac 27 L on β-galactosidase activity.

Transfer No.	Incubation time (days)	β-galactosidase Activity(M.U.)	
		Burk's Medium	Burk's Medium + Kanamycin
1	49	22300	15120
2	47	23300	13060
3	45	16200	19600
4	43	14700	19100
5	41	12710	17320
6	39	15100	18060
7	37	13500	15500
8	35	18100	15500
9	33	10300	9500
10	31	17400	13300
11	29	16300	10200
12	27	15400	7700
13	25	14200	8700
14	23	86400	9200
15	21	13600	14900
16	19	93500	15400
17	17	13900	14700
18	15	5140	12640
19	13	9330	8200
20	11	16100	4220
21	9	10400	3010
22	7	16370	7380
23	5	14710	4190
24	3	15100	4090

Effect of the other bacteria on the β-galactosidase activity of Mac 27 L

β-galactosidase activity of *A. chroococcum* Mac 27 L was determined in the presence and absence of some of the common soil bacteria in different proportions.The β-galactosidase activity was found to be 11000-12000 MU in the presence or absence of other bacteria (Table 7).

Table 5 - Enumeration of *A. chroococcum* Mac 27 L in pure culture.

Absorbance (600 nm)	Absorbance (420 nm)	Viable count	M.U	Colour intensity (visual)
0.015	0.018	1.7×10^6 (6.23)	4460	-
0.036	0.047	3.9×10^6 (6.59)	4850	+
0.133	0.0192	1.5×10^7 (7.17)	5360	+
0.267	0.403	3×10^7 (7.47)	5600	+
0.444	0.695	4.8×10^7 (7.68)	5810	+
0.558	0.893	6.8×10^7 (7.83)	5940	+

*Values in the parentheses represents the log number of viable cells.

Table 6 - Enumeration of *A. chroococcum* Mac 27 L in charcoal based culture packets.

Time of sampling (days)	No. of cells (SPC)	No. of cells (calculated)
0	1.2×10^7	1.3×10^7
7	10^7	1.1×10^7
14	3×10^6	2.4×10^6
21	10^6	9×10^5
28	4×10^5	7×10^5
42	2×10^4	N.D.
56	10^3	N.D.

Enumeration of *A. chroococcum* Mac 27 L from the rhizoplane of *Brassica campestris*

The transconjugant of *A.chroococcum* Mac 27 containing the *lac Z* fusion was used to monitor its survival in the rhizoplane of *Brassica campestris*. The transconjugant was detected by direct plating on Burks medium containing X-gal. Colonies that appeared blue on these plates were formed by lacZ tagged transconjugant. It was observed that out of 1.2×10^6 cfu/plant root, 2.5×10^5 cfu/plant root were that of *A.chroococcum* Mac 27 L after 30 days of sowing in plants treated with *A.chroococcum* Mac 27 L (Table 8). However no blue coloured colony was observed in other treatments.

Discussion

There are around 169 bio-fertilizer production units in India with the capacity to produce 67 thousand tons of

Table 8 - Enumeration of different strains of *Azotobacter* from the rhizoplane of *Brassica campestris*.

Time of sampling (days)	Treatment *A. chroococcum*	cfu/plant/root	
		Total	*Lac Z* marked
30	Mac 27 L	1.2×10^6	2.5×10^5
	Mac 27	1.55×10^6	-
	HT-54	4.2×10^6	-
	Control	10^7	
60	Mac 27 L	1.2×10^5	2.0×10^4
	Mac 27	1.3×10^5	-
	HT-54	2.8×10^5	-

Table 7 - β-Galactosidase activity of *A. chroococcum* Mac 27 L as affected by other bacteria.

Culture	Cell concentration (600 nm)		Colour intensity	
	Mixed Culture	*Lac Z* marked	Absorbance (420 nm)	M.U.
Mac 27	1.25	-	0.011	-
Mac 27 L	1.20	1.20	1.0	11700
Mac 27 +Mac 27 L(1:1)	1.22	0.61	0.49	11300
Mac 27 +Mac 27 L(4:1)	1.28	0.256	0.215	11800
Bacillus	1.482	-	0.016	-
Mac 27 L+ *Bacillus* (1:1)	1.33	0.665	0.56	11900
Mac 27 L+ *Bacillus* (1:4)	1.46	0.292	0.24	11600
Pseudomonas	0.95	-	0.017	-
Mac 27 L+ *Pseudomonas* (1:1)	1.1	0.55	0.47	12000
Mac 27 L+ *Pseudomonas* (1:4)	1.0	0.2	0.17	12000
Mac 27 L+ *Pseudomonas* + *Bacillus* (1:1:1)	0.96	0.32	0.27	11900
Mac 27 L+ *Pseudomonas* + *Bacillus* (1:2:2)	0.9	0.18	0.15	11900
Mac 27 L + *Bacillus* (1:9)	1.026	0.103	0.072	11400

biofertilizers, current annual production is over 20 thousand tons.However, because of lack of quick and reliable methods to determine the quality of these bioferlizers, some of the biofertilizers being produced by these agencies may be of poor quality. Thus there is a need to develop a reliable, rapid and reproducible method for enumeration of viable number of cells in these inoculants which is one of the major indicator of quality of inoculants.Drahos *et al.* (1986) developed a marker system based on expression of *E. coli* lac operon genes into *Pseudomonas fluorescens* enabling them to cleave the chromogenic substrate 5-bromo 4-chloro-3-indolyl-β-D galactopyranoside (X-gal) to a coloured compound. A *lac* Z marked transconjugant of *A. chroococcum* Mac 27 developed earlier in for ecological studies (Garg, unpublished) was used in the present investigation. Although insertion of the transposon does not alter the eological behavior of the host but in some transconjugants the marker have been found to be inserted into some essential genes (Sessitsch *et al.*, 1997). To determine if *lac* Z insertion into *A. chroococcum* Mac 27 has resulted in loss of any other metabolic property of the parent, the characteristics like growth pattern, utilization of different carbon sources, nitrogenase activity and excretion of ammonia of *lac* Z marked *A. chroococcum* were studied and it was observed that the insertion of the *lac* Z containing transposon has not damaged any of the essential property of the *A.chroococcum* Mac 27, the parent and the present strain can be reliably used as biofertilizer for various non leguminous crops. Hofte *et al.* (1990) constructed a *lac* Z marked strain of *Pseudomonas aeruginosa* and a detailed comparison was made between the wild type strain 7NSK2 and the *lac Z* marked strain MPB1. The results showed that none of the genes essential for growth, siderophore production, survival, plant growth stimulation or root colonization had been damaged in the MPB1 strain.

Further, this marker can be used effectively for monitoring of *A. chroococcum* Mac 27 L in the environment since *lac* Z insertion is found to be stable as confirmed by repeated and successive transfers on Burks medium slants with and without kanamycin.Similar observations have also been reported by Pal *et al.* (2000) in case of *lac* Z marked *Pseudomonas glumae*.In the present study, it has been found that β-galactosidase activity of *lac* Z marked strain of *A. chroococcum* Mac 27 is directly proportional to the cell concentration. After establishing a direct relationship between cell concentration and the intensity of the colour developed by the cleavage of chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) due to β-galactosidase activity, it was presumed that this property may be useful in enumeration of *lac* Z tagged strain of *A. chroococcum* either in pure culture or carrier based inoculants. When the number of viable cells in charcoal based inoculants was determined by using this chromogenic technique, the number of cells in suspension was found to be similar to those determined by standard plate count. These results confirmed our hypothesis that number of cells can be determined by measuring the intensity of the colour that appear due to β-galactosidase activity in the *lac*Z tagged *A. chroococcum* Mac 27. Further, *lac Z* marked *A. chroococcum* was used to monitor its survival in the rhizoplane of *Brassica campestris*. However there are a variety of contaminants present in the environment which may interfere in the expression of β galactosidase activity of the transconjugant. That the contaminants do not affect the β-galactosidase activity of *A. chroococcum* Mac 27 L has been confirmed by determining the β-galactosidase activity of *A. chroococcum* Mac 27 L in the presence and absence of some of the common soil bacteria in different proportions.There was no effect on the expression of β-galactosidase activity of the transconjugant by the presence of *Bacillus* spp. in concentration as high as 10 times that of *A.chroococcum* Mac 27 L. The *lac Z* marked transconjugant has been found to be reliable for the enumeration of number of viable cells in culture packets, this has great practical application for quality control of microbial inoculants as well as proving a strain for ecological studies.

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