

# Optimization of growth conditions for *Azotobacter* species and their use as biofertilizer

## Abstract

The present study is concerned with the optimization of growth conditions for *Azotobacter* species. Five isolates were isolated from soil and *Azotobacter* IIB-3 found to be the best; along with strain of *Azotobacter vinelandii* NRRL-14641. The medium M2 gave maximum growth (0.65 and 0.75 mg/ml) of *Azotobacter vinelandii* and *Azotobacter* IIB-3. Optimum temperature, pH and incubation period for growth of *Azotobacter* were 30°C, 8.0 and 48hrs, respectively. The growth of *Azotobacter* was also studied with the supplementation of growth medium with different carbon and nitrogen sources. 2.5% mannitol (as C-source) and 2% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (as N-source) were found best for both *Azotobacter vinelandii* (1.16mg/ml) and *Azotobacter* IIB-3 (1.24mg/ml). 24 hrs old inoculum at a level of 1% was found best for the growth both *Azotobacter vinelandii* and *Azotobacter* IIB-3. The effect of *Azotobacter* biofertilizer was studied on maize plants in pot experiment and it was found that plants inoculated with *Azotobacter* gave better growth as compared to control plants.

Volume 6 Issue 5 - 2018

Hamid Mukhtar, Hina Bashir, Ali Nawaz, IkramulHaq

Institute of Industrial Biotechnology, GC University, PAKISTAN

**Correspondence:** Hamid Mukhtar, Institute of Industrial Biotechnology, GC University, Pakistan, Email hamidwaseer@yahoo.com

**Received:** October 26, 2017 | **Published:** September 12, 2018

## Introduction

Biofertilizer is a large population of a specific or a group of beneficial microorganisms for enhancing the productivity of soil either by fixing the atmospheric nitrogen, by solubilizing soil phosphorus or by stimulating plant growth through synthesis of growth promoting substances.<sup>1</sup> Biofertilizer supply in addition to nitrogen considerable amount of organic matter enriching structure of soil. Inoculants of these microorganisms have proved their technical feasibility, economic viability and social acceptability. Biofertilizers are the most advanced biotechnology invention which is necessary to support developing organic agriculture, sustainable agriculture, green agriculture and non pollution agriculture. The biofertilizer can increase the output and improve the quality of agricultural products.<sup>2</sup> Biofertilizers have definite advantage over chemical fertilizers. Chemical fertilizers supply only nitrogen whereas biofertilizers provide in addition to nitrogen certain growth promoting substances like hormones, vitamins, amino acids etc. to the plants.<sup>3</sup> Crops have to be provided with chemical fertilizers repeatedly to replenish the loss of nitrogen utilized for crop growth. On the other hand biofertilizers supply nitrogen continuously throughout the entire period of crop growth in field under favorable conditions.<sup>4</sup> Biofertilizers are formed by mixing of microorganisms with some carriers like lignite and peat moss in a certain amount. Some of the commonly used biofertilizers are *Azotobacter* biofertilizers, Phospho biofertilizers, Rhizo biofertilizers and *Trichoderma* biofertilizers. A large number of soil microorganisms are used as biofertilizer. Some of these are phosphate solubilizing microbes including *Pseudomonas striata*, *Bacillus megaterium* and *Bacillus subtilis*.<sup>5,6</sup> Some fungi like *Aspergillus* and *Penicillium* are potential solubilizer of bound phosphates.<sup>7</sup> *Azotobacter* is one of the most important microorganisms, which is widely used as biofertilizer (Pereira and Shetty, 1987). *Azotobacter* is Gram negative and polymorphic bacterium i.e. having different sizes and shapes. Their size ranges from 2-3 umin diameter and of various lengths. *Azotobacter* spp. are sensitive to acidic pH high salts and temperature above 35C.<sup>7</sup>

*Azotobacter* spp. shows well growth in a medium containing both

acetate and glucose as carbon source.<sup>8</sup> *Azotobacter* spp. show rapid growth on nitrogen free medium.<sup>9</sup> However under nitrogen fixing conditions *Azotobacter* spp. grow well.<sup>10</sup> Hydrogen ion concentration (pH) is also an important parameter that directly affects the growth of living organisms. *Azotobacter* grows well in Burk s liquid medium at pH range of 7 to 9 but fail to grow below the pH of 6. Moreover other parameters such as temperature, incubation time, aeration rate and inoculum size also have effects on growth of *Azotobacter* species.

The main objective of the present study is to provide a cheaper source of nitrogen to the agriculture industry and to meet the needs of crops as there is spread of inflation day by day due to high prices of petroleum which influences the prices of chemical nitrogenous fertilizers. Also for a sustainable agriculture system, it is imperative to utilize renewable input that can maximize the ecological benefits and minimize the environmental hazards.

## Material and methods

### Microorganisms

Bacterial strains belonging to *Azotobacter* spp. were isolated from different soil samples of local habitat through serial dilution method. 1.0 gram of soil sample was suspended in 100ml of sterilized water in 250ml Erlenmeyer flask and was vigorously shaken. From this stock solution serial dilutions were prepared, and 0.5ml from each dilution was spread on the solidified surface of Burk agar medium plates {(g/L) K<sub>2</sub>HPO<sub>4</sub>, 0.64; KH<sub>2</sub>PO<sub>4</sub>, 0.16; NaCl, 0.20; MgSO<sub>4</sub>, 0.20; CaSO<sub>4</sub>, 0.05; FeSO<sub>4</sub>, 0.003; NaMoO<sub>4</sub>, 0.01; Glucose, 10; Agar, 15}. One strain of *Azotobacter vinelandii* NRRL-14641 was also provided by the culture bank of Institute of Industrial Biotechnology, GC University Lahore. Different morphological and biochemical tests were performed for the identification of isolates, such as Gram staining; capsule staining, motility test, endospore test, protease test and catalase test.

### Inoculum preparation

For the preparation of bacterial inoculum, a loop full of bacteria from slant was transferred to 250ml of Erlenmeyer flask containing 50ml of sterilized nitrogen free medium in laminar air flow chamber.

Flask was then incubated in shaker at 30°C and 200rpm for 24hrs.

### Batch experiments

Batch experiment for the growth of *Azotobacter* was carried out in 250ml shake flasks containing culture medium that was prepared by dissolving 0.2g ammonium sulphate, 0.8g dipotassium hydrogen phosphate, 0.2g magnesium sulphate, 0.1g calcium sulphate, 1ml of Mo solution (0.1mg/ml), 1ml of FeSO<sub>4</sub> solution (1.0mg/ml) and 20g manitol in 1000ml of distilled water. The flasks were cotton plugged and autoclaved at 121°C (15 lb/inch<sup>2</sup> pressure) for 15min. then the medium was cooled down at room temperature and inoculated by adding 1ml of inoculum with the help of micropipette in laminar air flow chamber. The flasks were then incubated at 30°C in a rotary shaker for 48hrs at 200rpm for growth of microorganisms. Fermentation broth was subjected to centrifugation at 6000 rpm for 10 min in pre weighed glass centrifuge tubes. Supernatant was removed and pelleted mass was dried out in oven at 90°C over night. After drying, the centrifuge tubes were again weighed and the weight of biomass was obtained by subtracting the pre weight with after weight.

### Selection of culture medium

Different culture media were screened for the maximum growth of *Azotobacter* spp. the composition of these media is given below.

**M1:** (g/L) glucose, 10.0; di potassium hydrogen phosphate, 0.64; potassium dihydrogen phosphate, 0.16; NaCl, 0.2; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; CaSO<sub>4</sub>·2H<sub>2</sub>O, 0.05; NaMoO<sub>4</sub>·2H<sub>2</sub>O, 0.01; FeSO<sub>4</sub>, 0.003. pH 7.1

**M2:** (g/L) Ammonium Sulphate, 0.2; di potassium hydrogen phosphate, 0.8; MgSO<sub>4</sub>, 0.2; CaSO<sub>4</sub>, 0.1; Mo solution, 1ml (0.1mg/ml); FeSO<sub>4</sub>, 1ml (1.0mg/ml); manitol, 20.0. pH 7.1

**M3:** (g/L) NaCl, 0.4; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4; KH<sub>2</sub>PO<sub>4</sub>, 0.16; K<sub>2</sub>HPO<sub>4</sub>, 0.64; CaCl<sub>2</sub>, 0.084; NaMoO<sub>4</sub>·2H<sub>2</sub>O, 0.002; H<sub>3</sub>BO<sub>3</sub>, 0.003; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.006; CoSO<sub>4</sub>, 0.012; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.0001; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.012; Sucrose, 40. pH 7.2

**M4:** (g/L) K<sub>2</sub>PO<sub>4</sub>, 0.2; MgSO<sub>4</sub>, 0.2; CaCl<sub>2</sub>, 0.2; FeCl<sub>3</sub>, 0.05 ml of 10%; NaMoO<sub>4</sub>, a trace; Manitol, 15.0. pH 7.2

**M5:** (g/L) Beef Extract, 3.0; NaCl, 8.0; Peptone, 5.0. pH 7.1

**M6:** (g/L) glucose, 20.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; K<sub>2</sub>HPO<sub>4</sub>, 0.2; CaCl<sub>2</sub>, 0.05; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.10; NaMoO<sub>4</sub>·2H<sub>2</sub>O, 0.05. pH 7.4.

### Application of biofertilizer

Effect of Bio fertilizer on growth of plants was determined by pot experiment in which seeds of maize inoculated with bacterial growth were sowed in sterilized soil in disinfected pots. Seeds were watered and placed under sunlight. A control was also taken in which seeds were not inoculated with bacterial strains. After the growth of plants, the size of leaves and roots were recorded in all experimental and control pots.

## Results and discussion

Data of Table 1 shows the screening of isolates for maximum growth on synthetic medium in shake flasks. Five different isolates of *Azotobacter* were screened and *Azotobacter* IIB-3 was found best strain showing highest dry cell biomass production i.e. 0.74 mg/ml. so *Azotobacter* IIB-3 was selected for further studies along with *Azotobacter vinelandii* NRRL- 14641. The source of *Azotobacter* IIB-

3 was the soil sample taken from the Ligia garden of GC University Lahore. Other four isolates *Azotobacter* IIB-1, *Azotobacter* IIB-2, A.IIB-4 and IIB-5 showed lesser amount of dry cell biomass i.e. 0.23, 0.38, 0.29 and 0.46 mg/ml, respectively.

**Table 1** Screening of *Azotobacter* spp. for maximum biomass production

S No.	Isolates	Dry cell mass (mg/ml)
1	<i>Azotobacter</i> IIB-1	0.23
2	<i>Azotobacter</i> IIB-2	0.38
3	<i>Azotobacter</i> IIB-3	0.74
4	<i>Azotobacter</i> IIB-4	0.29
5	<i>Azotobacter</i> IIB-5	0.46
6	<i>Azotobacter</i> NRRL-14641	0.63

### Identification of isolates

The isolates were subjected to morphological and biochemical tests (Table 2). It was observed that colonies were white to off white in colour while the growth of colonies were brown to bluish due to production of melanin. The cells were ovoid in shape and their size was about 2-3 μm in diameter. Isolates were observed gram negative, motile and form the cyst. The endospore formation and protease activity was not found but catalase activity was shown by the isolates. According to Bergey's Manual (Kreig *et al.*, 1984) the isolates belonged to the genus *Azotobacter*.

**Table 2** Morphological & Biochemical properties of the selected isolate

Criteria	Characteristics
Shape	Rods
Size	2-3 μm diameter
Colony shape	Raised circular
Colony colour	Off white
Capsule formation	+
Gram reaction	Gram -ve
Motility	+
Endospore formation	-
Cyst formation	+
Florescent pigment	+
Capsular slime production	+
Protease activity	-
Catalase activity	+

### Selection of Culture medium

The growth of *Azotobacter* was investigated using different culture media through batch fermentation (Figure 1). Maximum growth (0.75mg/ml) of *Azotobacter* IIB-3 was observed in medium M2. While maximum growth (0.65 mg/ml) was observed in M2 medium by *Azotobacter vinelandii*. Growth of *Azotobacter* was maximum when medium M2 was used because it contained all the essential nutrients in sufficient amounts required by the bacteria for growth e.g., it contained MgSO<sub>4</sub>, CaSO<sub>4</sub>, NaMoO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> etc. it has been reported that MgSO<sub>4</sub> prolongs the viability of microorganisms

suspended in potassium phosphate buffer (Gunter, 1954). Magnesium ions are essential not only for oxidative function of bacterial cells but also significant in the maintenance of permeability and transport mechanism (Goucner and Kocholaty, 1954).

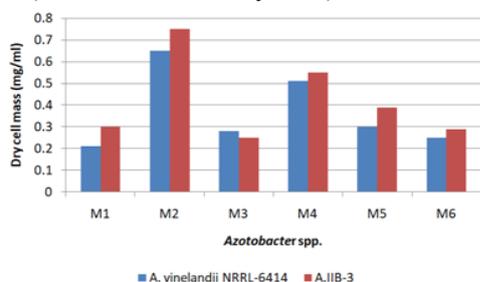


Figure 1 Screening of different media for growth of *Azotobacter* species.

### Effect of pH

The effect of pH on *Azotobacter* spp. were studied by varying the pH of the medium from 5.0 to 9.0 (Figure 2). *Azotobacter* IIB-3 showed maximum growth i.e. 0.77mg/ml at pH 8.0. While *A.vinelandii* showed maximum growth of 0.7 mg/ml at pH 8.0. *Azotobacter* grows on wide range of pH (Kreig and Holt, 1987) but their growth is more on neutral pH (Wachner et al., 2007).

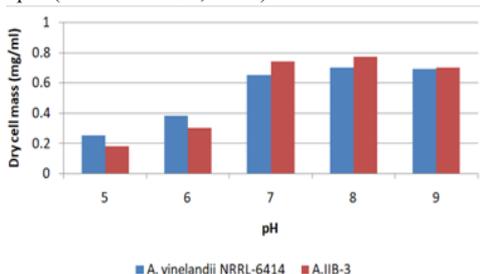


Figure 2 Effect of initial pH of the culture medium on growth of *Azotobacter* species.

### Effect of incubation period

The effect of incubation period on *Azotobacter* spp. were studied by varying the incubation period 24-120 hrs (Figure 3). *Azotobacter* IIB-3 showed maximum growth i.e. 0.91mg/ml after incubation period of 48hrs. While *A.vinelandii* showed maximum growth of 0.85mg/ml in 48hrs incubation period. Incubation period is directly proportional to the growth of bacteria up to a certain extent and after that growth of bacteria start decreasing that can be attributed to the decrease in the supply of nutrients to microorganisms or may be accumulation of some toxic compounds in the broth.

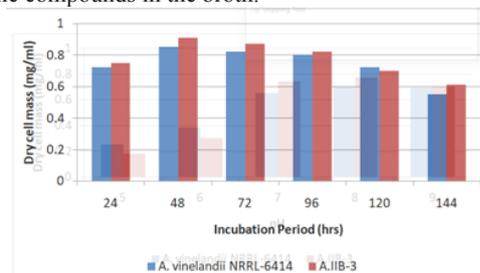


Figure 3 Effect of incubation period on the growth of *Azotobacter* species.

### Effect of incubation temperature

The effect of incubation temperature on *Azotobacter* spp. were studied by varying the incubation temperature 25-40 C hrs (Figure 4). *Azotobacter* IIB-3 showed maximum growth i.e. 0.93mg/ml after incubation at 30C. While *A.vinelandii* showed maximum growth of 0.88 mg/ml at 30C incubation temperature. A little temperature rise increases the growth rate because the velocity of enzyme-catalyzed reaction also increases with it and as the rate of increases; microorganism grows faster (Prescott et al., 1999). Optimum temperature for the growth of *Azotobacter* has been reported as 30C but some species can grow at optimum temperature of 34C (Chen et al., 1984).

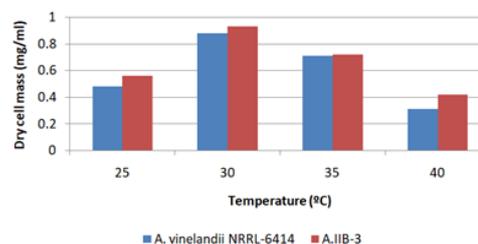


Figure 4 Effect of incubation temperature on the growth of *Azotobacter* species.

### Screening of Carbon Sources

Fermentation medium was supplemented with 2.5% of different carbon sources (Figure 5). *Azotobacter* IIB-3 showed maximum growth (0.95mg/ml) in the presence of manitol as carbon source. While *A.vinelandii* showed maximum growth of 0.92mg/ml in manitol medium. Carbon is essential component of growth medium but an excess of carbon source can cause undesirable metabolic waste products to accumulate into the microorganism while deficiency of carbon source cause lesser growth as well (Prescott et al., 1999). Shawky et al. (2004) has also found manitol as a best carbon source for growth of *A. vinelandii*. Fermentation medium was supplemented with different amounts (0.5–3.0 %) of manitol (Figure 6). *Azotobacter* IIB-3 showed maximum growth (1.12mg/ml) in the medium containing 2.55 manitol while *A.vinelandii* showed maximum growth of 1.10 mg/ml in 2.5% manitol.

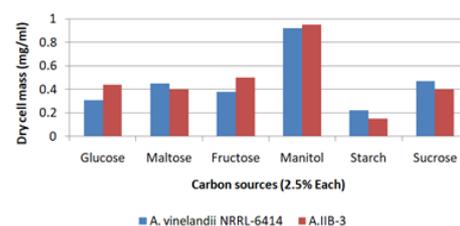


Figure 5 Screening of different carbon sources on the growth of *Azotobacter* species.

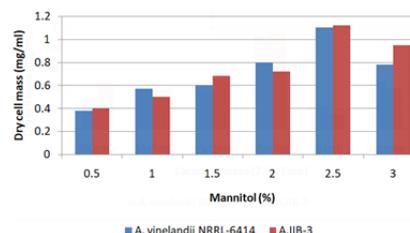
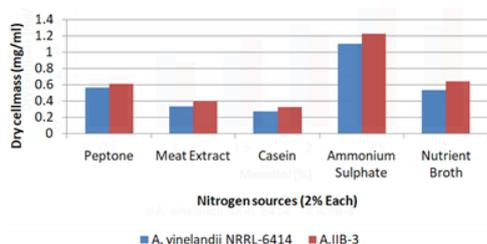


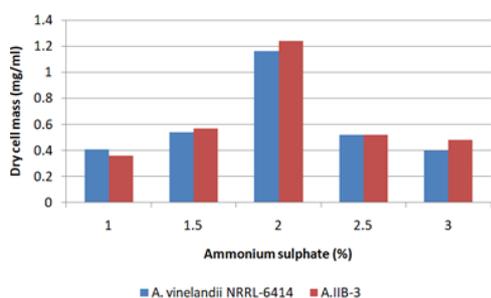
Figure 6 Effect of different concentrations of mannitol in the culture medium on growth of *Azotobacter* species.

### Screening of nitrogen sources

Fermentation medium was supplemented with 2% of different nitrogen sources (Figure 7). *Azotobacter* IIB-3 showed maximum growth (1.12mg/ml) in the presence of ammonium sulphate as nitrogen source. While *A. vinelandii* showed maximum growth of 1.11mg/ml in medium containing ammonium sulphate as nitrogen source. Although *Azotobacter* grows in nitrogen free medium, but it has been reported that nitrogen source was supplied to *Azotobacter* to decrease the lag phase. Gadhari and Stolp (1974) have found ammonium sulphate as best nitrogen source and have reported in the presence of ammonium sulphate, *Azotobacter* growth was increased. Fermentation medium was supplemented with different concentration (1.0–3.0 %) of ammonium sulphate (Figure 8). *Azotobacter* IIB-3 showed maximum growth (1.24mg/ml) in the medium containing 2% ammonium sulphate. While *A. vinelandii* showed maximum growth of 1.16mg/ml in 2.5% ammonium sulphate.



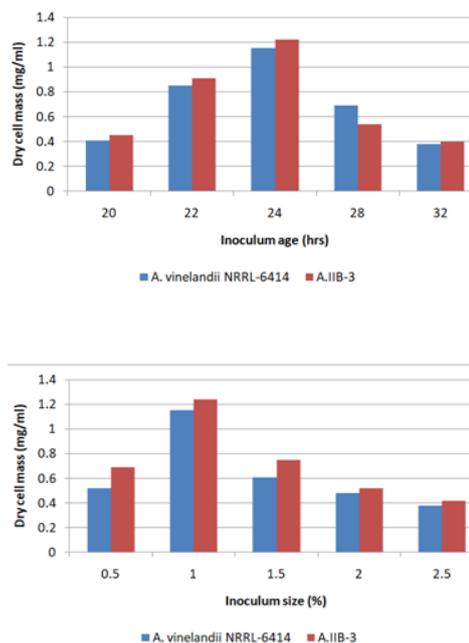
**Figure 7** Screening of different nitrogen sources for enhanced growth of *Azotobacter* species.



**Figure 8** Effect of different concentrations of ammonium sulphat in the culture medium on growth of *Azotobacter* species.

### Effect of inoculum

The effect of inoculum age on the growth of *Azotobacter spp.* was studied by using inoculum of different ages i.e. 20–32hrs (Figure 9). *Azotobacter* IIB-3 showed maximum growth i.e. 1.22mg/ml in 24hrs old inoculum. While *A. vinelandii* showed maximum growth of 1.15mg/ml in the medium containing inoculum of 24hrs old. Effect of inoculum age for the optimization of microorganism growth is also an important parameter. It has been reported 24 hrs old inoculum gave maximum growth of *Azotobacter*. The effect of inoculum size on the growth of *Azotobacter spp.* was studied. For this purpose different sizes of inoculum i.e. 0.5–2.5% were used in the fermentation medium (Figure 10). *Azotobacter* IIB-3 showed maximum growth i.e. 1.24mg/ml in 1% of inoculum size. While *A. vinelandii* showed maximum growth of 1.15mg/ml in the medium containing inoculum size of 1%.



**Figure 9** Effect of age of inoculum on the growth of *Azotobacter* species.

**Figure 10** Effect of size of inoculum on the growth of *Azotobacter* species.

### Application of *Azotobacter* biomass as biofertilizer

Effects of biofertilizers were studied on the growth performance of maize (*Zea mays*) plants (Table 3). Maximum growth (195mm) of leaves and a less extensive (110mm) system of roots was observed in the experimental plants incubated with *Azotobacter vinelandii*. While the seeds inoculated with *Azotobacter* IIB-3 showed leaves size of 197 mm and roots of length 152mm. Results showed that plants inoculated with bacterial strains gave maximum leaf growth which confirms that these contained the maximum nutrients available for their growth and minimum proliferation of roots because sufficient nutrients were present in the soil for uptake of roots due to which their penetration into the soil was not so much high. It has been reported that inoculation of plants with *Azotobacter* increases their growth and yield as well.<sup>10</sup>



**Figure 11** Effect of Biofertilizer on the growth of Maize Seedlings (Leaves size).



**Figure 12** Effect of Biofertilizer on the growth of Maize Seedlings (Roots size).

**Table 3** Effect of biofertilizer application on the growth performance of maize plant

S. No.	Treatments	Leaves size (mm)	Roots size (mm)
1	Control	109	152
2	<i>Azotobacter</i> IIB-3	197	105
3	<i>Azotobacter</i> IIB-5	183	137
4	<i>Azotobacter vinelandii</i> NRRL-6414	195	110

## Conclusion

It was concluded from the above studies that the optimization of cultural and nutrition parameters resulted in enhancement in the growth of *Azotobacter* species during fermentation. Furthermore, the biomass of *Azobacter* species was proved to be good source of biofertilizer.

## Acknowledgements

None.

## Conflict of interest

Author declares that there is no conflict of interest.

## References

- Narayanamma GP, Reddy MSR, Reddy MGR. Studies on improving the productivity of groundnut on problem soil. *Andhra agric J*. 1985;32:6–8.
- Jiskani MM. Biofertilizers better source for protection and production of crops farm inputs 2001.
- Vessey Jk. Plant growth promoting rhizobacteria as bio-fertilizers. *Plant Soil*. 2003;255(2):571–586.
- Sharma AR, Mittra BN. Temperature and dissolved oxygen concentration as parameters of *Azotobacter chroococcum* cultivation for use in biofertilizers. *Biotechnol*. 1990;17(4):367–369.
- Kisten AG, Kerdish IK, Bega ZT, et al. The effects of several factors on the growth of pure mixed cultures of *Azotobacter chroococcum* and *Bacillus subtilis*. *Prikl Biokhim Mikrobiol*. 1996;42(3):315–320.
- Wu SC, ZH Cao, ZG Li, et al. Effects of biofertilizers containing N-fixer, P and K solubilizers and AM Fungi on maize growth: a greenhouse trial. *Geoderma*. 2005;125(1-2):155369166.
- Goenadi DH. Frrtilization efficiency of oil palm through biofertilizer application. Proceeding of International Oil Palm Conference; Nusa Dua, Bali. 1998. p. 370–3776.
- Doebereiner J, Pedrosa FO. Nitrogen fixing bacteria in non leguminous crop plants. *Science Tech*. 1987;24:346–347.
- George SE, Costenbader CJ, Melton T. Diauxic growth in *Azotobacter vinlandii*. *J Bacteriol*. 1985;164(2):866–871.
- Martinez Toledo, Gonzalez-Lopez MV, Rubia J, et al. Isolation and characterization of *Azotobacter chroococcum* from the roots of *Zea mays*. *FEMS Microbiol. Ecol*. 1985;31(4):197–203.
- Allman R, Hann AC, Phillip AP, et al. Growth of *Azobacter vinelandii* with correlation of Coulter cell size, flow cytometric parameters and ultrastructure. *Cytometry*. 1990;11(7):822–831.
- Cerning J, Renard CMG, Thibault JF. Use of Dinitrosalicylic acid Reagent for determination of reducing sugar. *Anal Chem*. 1950;31(3):426–428.
- Topisirovic. Carbon source requirement for exopolysaccharide production by *Lactobacillus casei* and partial structure analysis of polymer. *Appl Environ Microbiol*. 1994;60(11):3914–3919.