International Journal of Engineering and Advanced Technology (IJEAT)
ISSN: 2249-8958 (Online), Volume-2 Issue-4, April 2013

Pilot Scale Production of AZOTOBACTER BIOFERTILIZER and Its Effect on the Growth Parameters of OCIMUM Sanctum

M Shanmuga Priya, Reyaz Farooq, Divyashree K, A.K Satheesh Babu, M Lakshmi Prabha, Prasad M.P

Abstract *-Azotobacters are predominant in the rhizosphere of plants and they help in phosphate solubilisation. They belong to the family Azotobacteraceae and they are used as broad spectrum biofertilizers. In the present study four samples were collected from the rhizosphere regions of the plants namely Solanum lycopersicum (tomato), Zingiber officinale (ginger), Solanum melongena (Brinjal) and Allium sativum (Garlic). The collected samples were isolated and identified as isolates 1, 2, 3 and 4. Biochemical tests such as motility test, catalase test, oxidase test, starch hydrolysis, litmus milk and phosphate solubilisation were performed. The effect of culture parameters such as Incubation period, pH, Temperature and carbon source at different concentrations were carried out. Mass production of Azotobacter was carried out using batch fermenter. Further pot experiments were conducted to determine the root length, shoot length and number of leaves in Ocimum sanctum on 15th , 30th and 45th day. The results showed an increase in root length, shoot length and number of leaves in biofertilizer treated plant when compared with the control and inorganic fertilizer.*

Keywords**— *Azotobacter*, Biofertlizer, *Ocimum sanctum* and Rhizosphere**

I. INTRODUCTION

Biofertilizers are substances that contain living microorganisms, when they are applied to seeds or plants, they colonize the rizhosphere or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant [1],[2]. In agriculture, the use of artificial fertilizers leads to environmental pollution and causes depletion of important nutrients. Biofertilizers contain symbiotic or non-symbiotic microorganisms that stimulate the growth of plant. Cultivation of plants with biofertilizers can result in higher resistance to diseases, production of phyto hormones and water soluble vitamins [3]-[5].

Rhizobium spp are the first biofertilizers being used commercially as inoculants for various plants [6]. Research in the field of biofertilizers have led to the development of different forms of biofertilizers which includes nitrogen fixing as well as phosphate solubilising microorganisms.

Phosphorus is one of the essential macronutrient, which is required for the maximum yield of agriculturally important crops. However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants [7], [8]. This problem can be resolved by the application of biofertilizers which releases soluble forms of phosphorus, there by facilitates the absorption of phosphorus into soil, which is an important aspect of increasing soil phosphorus availability. Assimilation of phosphate takes place through the enzyme "phosphatase" which is found in various soil microorganisms. Inoculation of the phosphate solubilising bacteria along with seed or soil is found to improve the solubilisation of soil phosphorous resulting in the better crop yield [9]. Phosphorous, facilitates flower formation and also helps in fruit production, quality of fruits, vegetables and grain drops may also be improved [10].

Soil contains a wide range of organic substrates, which can be a source of phosphorus for plant growth. To make this form of Phosphorus available for plants, it must be hydrolyzed to inorganic phosphorus. Mineralization of most organic phosphorous compounds is carried out by means of phosphatase enzyme. Significant amount of phosphatase activity in soil has been reported [11] – [16].

The solubilisation of phosphorus in the rhizosphere is the most common mode of action implicated in plant growth promoting rhizobacteria that increases the nutrient availability to the host plants [17]. Considerable number of bacterial species which are associated with the plant rhizosphere are able to exert a beneficial effect upon plant growth. Many bacterial inoculants such as *Azotobacter*, *Rhizobium*, *Azospirillum* have been used in plants to increase its yield, among them *Azotobacter* is found to be more efficient and they are found in different places such as soil, water, surfaces of roots (rhizosphere) and leaves (phyllosphere). Few species of *Azotobacter* is also found in tropical and Polar Regions but the bacterial population varies in different soil conditions. They are very hardly found in acidic soil but easily found in neutral and alkaline soil conditions [18].

Azotobacter spp are non symbiotic and usually found as gram negative rods with very high respiration rates. It is an obligate aerobe, although it can grow under low oxygen potential. The distribution of *Azotobacter* is very complex and variety of factors determines the presence of this bacterium in the rhizosphere region [19] – [21]. Many evidences which supports the ability of the microorganisms to supply phosphorous to plants as a consequence of phosphate solubilisation has been reported [22] – [24].

Manuscript published on 30 April 2013.
***** Correspondence Author (s)
Dr. M Shanmuga Priya, Department of biotechnology, MVJ college of Engineering, Bangalore, India.
Reyaz Farooq, Department of Biotechnology, MVJ College of Engineering, Bangalore, India.
Divyashree K, Department of Biotechnology, MVJ College of Engineering, Bangalore, India.
Dr. A.K Satheesh Babu, Department of Biotechnology, MVJ college of Engineering, Bangalore, India.
Dr.M. Lakshmi Prabha, Department of Biotechnology, Karunya University Coimbatore, India.
Dr. Prasad M.P, Sangenomics Research Laboratory, Bangalore, India.

Growth of the maize and lettuce was enhanced by the application of phosphate solubilising bacteria was reported by [25].

In the present study four samples of *Azotobacter* were isolated from rhizosphere region of soil and identified as isolates 1, 2, 3 and 4. Biochemical tests such as motility test, catalase test, oxidase test, starch hydrolysis, litmus milk and phosphate solubilisation test were performed. The effect of culture conditions such as incubation periods, pH, temperature and carbon source were carried out. Mass production of *Azotobacter* was carried out using batch fermenter. Further pot experiments were conducted to determine the root length, shoot length and number of leaves in *Ocimum sanctum.*

II. MATERIALS AND METHODS

A. Collection of Soil Samples

Soil samples were collected from different areas in Bangalore, in the month of January 2013. Following are the different samples collected.

1. *Solanum lycopersicum* (tomato) collected from MVJ College of engineering, Channasandra Bangalore.
2. *Zingiber officinale* (ginger) collected from K.R Puram, Bangalore.
3. *Allium sativum* (Garlic) collected from Cox town, Bangalore.
4. *Solanum melongena* (Brinjal) collected from Hoskote, Bangalore.

Soils were collected from the rhizosphere region of the soil. Roots were carefully dug out and the rhizospheric soil samples were taken aseptically into sterile plastic bags and stored at temperature of 4ºc.

B. Isolation of bacteria

Collected samples were serially diluted and inoculated on the Jenson's agar medium in petriplates. Then the bacterial Growth obtained was again sub cultured to obtain pure colonies..

C. Characterization based on colony morphology

Colony morphology of the isolated pure colonies on the streaked plates were examined. Different characteristics such as shape, size, elevation, surface, margin, colour, odour, pigmentation etc were recorded.

D. Gram staining

Gram staining was performed to observe colony morphology.

E. Biochemical Tests

Motility test

Mobility of the bacterial cells were observed using stab agar method.

Catalase test

3% hydrogen peroxide was added to the 48 hr old bacterial colony. The effervescence revealed the positive catalase activity.

Oxidase test

Redox indicator reagent was added directly to the bacterial colony. It was checked for the development of purple colour.

Starch hydrolysis

Starch agar plates (without nitrogen source) were prepared and the plates were aseptically inoculated and kept for incubation. Amylase enzyme produced by the organism degraded the starch and hence the formation of zone was seen and the results were observed.

Litmus milk test

Litmus milk broth was prepared and inoculated. Peptonisation of the milk proteins reveals clearer solution which confirms the positive test.

F. Phosphate solubilisation test

NBRIP (National Botanical Research Institute's Phosphate) media was used in the phosphate solubilisation by *Azotobacter.* Development of the zone surrounding the colony confirms the positive result.

G. Optimization of culture media

Incubation period

The cultures were inoculated and incubated at various incubation periods at the room temperature and the bacterial growth were recorded for each incubation period. *pH*

Media was prepared at varying pH values ranging from 2.0 to 9.0 and its effect on the growth was determined at room temperature.

Temperature

Cultures were grown at different varying temperatures ranging from 20-44ºc and the effect of temperature on the bacterial growth was determined.

Carbon source

Bacterial cultures were inoculated in media containing different concentrations of the D-Glucose ranging from 0.05%-0.4% and its effect on the bacterial growth was studied.

H. Mass production

The isolated strains of *Azotobacter* were grown in slants and transferred to liquid broth in the rotary shaker. Large scale production of *Azotobacter* was carried out in batch fermenter supplement with PVK media. The obtained cultures were harvested using batch culture mode. Later the cultures were mixed with the forest soil and charcoal in the ratio 1:3. The mixture was shade dried, packed in a sterile cover and stored for use as biofertilizer for the desired crop.

I. Pot experiments

The seeds of *Ocimum sacnctum* were planted in three different pots containing various fertilizers and a control was also maintained. The following parameters namely the root length, shoot length and the number of leaves were observed on 15th, 30th and the 45th day after planting. The length of the root was measured from the collar region to the growing tip of the root and expressed in cm. The length of the shoots were measured from the collar region, up to the tip of the shoot and expressed in cm. The total number of leaves in each plant was counted on 15th, 30th and 45th day after planting and expressed as number of leaves per plant for the purpose of statistical analysis. Duncan's multiple range test was preformed for comparing the treatments.

Published By:
Blue Eyes Intelligence Engineering
and Sciences Publication (BEIESP)

III. RESULTS AND DISCUSSION

A. Isolation and identification of the collected samples

The samples were successfully collected from rhizosphere region of *Solanum lycopersicum* (tomato), *Zingiber officinale* (ginger), *Solanum melongena* (Brinjal), *Allium sativum* (Garlic) plants. The collected samples were isolated using Jenson's media. The colonies were round, and creamy in colour. The isolated samples were identified as isolates 1, 2, 3 and 4. All the isolates were gram negative rods. The results of motility, catalase, oxidase, starch hydrolysis and litmus tests were found to be positive for all four isolates.

B. Phosphate solubilization

When the isolates were grown in culture media supplemented with calcium phosphate all the isolates produced halo zone around the colonies indicating the solubilisation of the phosphate. The halo zone is produced due to the solubilzation of insoluble phosphate which is mediated by the production of organic acids in the surrounding medium. Further confirmation of phosphate solubility was determined by phosphomolybdate test. The results indicated that isolate 2 showed greater solubility when compared with other isolates.

C. Optimization

Incubation period

The effect of different incubation periods on the bacterial growth is presented in figure 1. The results showed that the maximum growth of bacteria was observed on the 3rd day.

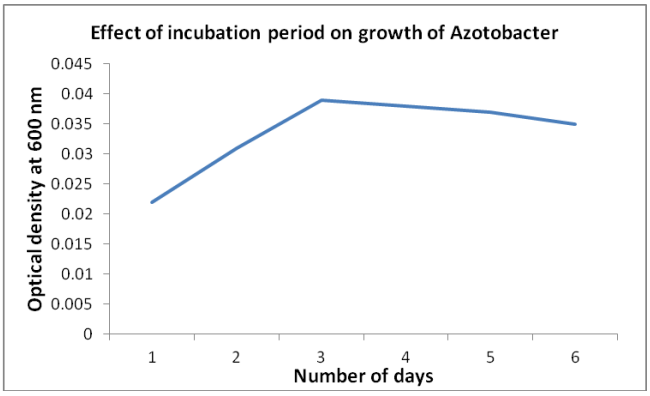

Fig 1

pH

Bacterial culture was grown at various pH and its effect were studied. The results (figure 2) revealed that maximum cell growth was obtained at pH 7.

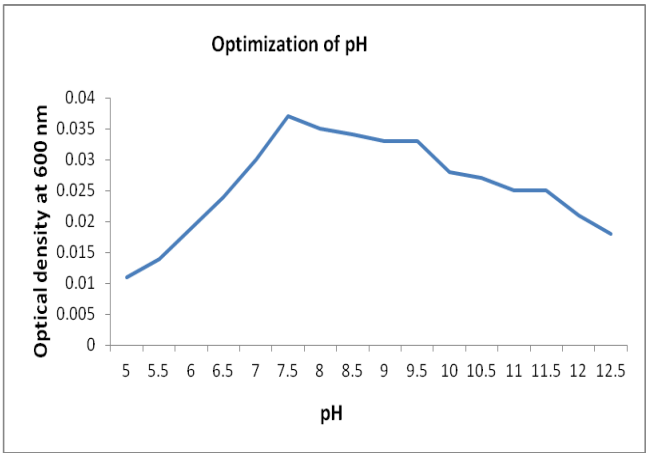

Fig 2

Temperature

Effect of various temperatures on the bacterial cultures were studied at various temperature ranging from 20-44ºC. The results revealed that the maximum bacterial growth was observed at the temperature of 32 ºC (figure 3)

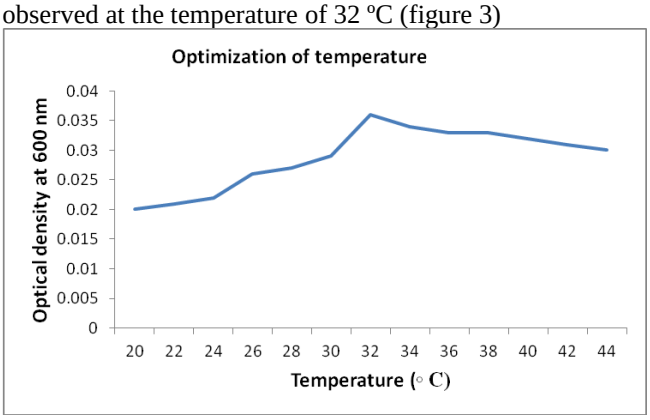

Fig 3

Carbon source

Glucose at a concentration of 3% favoured the maximum bacterial growth of *Azotobacter*. Results are depicted in figure 4.

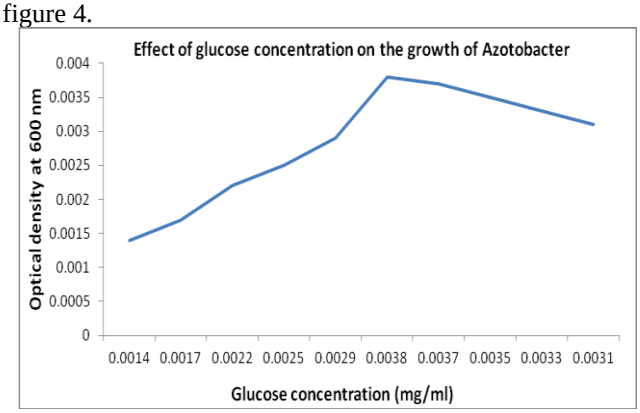

Fig 4

D. Mass production

Mass production of *Azotobacter* was carried out in batch fermenter using PVK media.

Published By:
Blue Eyes Intelligence Engineering
and Sciences Publication (BEIESP)

E. Pot experiments:

In pot experiments, the growth of *Ocimum sanctum at* different time intervals *are* tabulated in Table 1. There is an increase in root length, shoot length and number of leaf count when the seeds were treated with biofertilizer. The *Ocimum sanctum* showed maximum root length 3.99 cm, shoot length 23.29 cm and leaf count 36 in biofertilizer treated plant on 45th day after planting when compared to inorganic manure and control plants. Maximum growth in biofertilizer treated plant was mainly due to the ability of *Azotobacter* to solubilise phosphate and to produce siderophores and hormones (Khan et al. 2009). Statistical data were analyzed by DMRT which resulted in significant differences between the mean of the treated and control plants.

Table 1: Effect of biofertilizer, inorganic fertilizer on growth parameters of *Rosa polyantha.*

Days	Treatment	Root length	Shoot length	No. of leaves
15	Control	1.15	11.34	8
	Inorganic fertilizer	1.75	14.67	13
	Bio fertilizer	1.89	18.78	18
30	Control	2.68	13.64	11
	Inorganic fertilizer	2.89	17.35	19
	Bio fertilizer	2.96	19.77	25
45	Control	2.89	14.99	17
	Inorganic fertilizer	3.33	20.28	27
	Bio fertilizer	3.99	23.29	36

IV. CONCLUSION

From the present study it may be concluded that *Azotobacter* inoculants have significant growth promoting effect on various growth parameters like root length, shoot length and number of leaves in the potted plant. Results suggested that *Azotobacter* can be used as an efficient microbial inoculant for enhancing the growth of plants when compared with inorganic fertilizers.

V. ACKNOWLEDGEMENT

We express our sincere thanks to the Management of MVJ College of Engineering, near ITPB, Channasandra Bangalore- 560067 and SANGENOMICS Biotech research lab Domlur Bangalore, for their constant support and encouragement throughout the period of study

REFERENCES

[1] Revillas JJ, Rodelas B, Pozo C, Martinez-Toledo MV and Gonzalez-Lopez J " Production of B-group Vitamins by two *Azotobacter* strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions," J. Appl. Microbiol, Vol 89, 2000, pp 486-493.

[2] Vessey JK, " Plant growth promoting rhizobacteria as biofertilizers," Plant soil, vol 255, 2003,pp 571-586.

[3] Kumar V, Behl RK and Narula N ," Establishment of phosphate solubilising strains of *Azotobacter chroococcum* in the rhizosphere and their effect on wheat cultivars under greenhouse conditions," Microbiol. Res.vol 156, 2001, pp 87-93.

[4] Kumar GP, Yadav SK, Thawale PR, Singh SK and Juwarkar AA, " Growth of Jatropha curcas on heavy metal contaminated soil amended with industrial wastes and *Azotobacter*-A greenhouse study," Bioresour. Technol. vol 99, 2008, pp 2078-2082.

[5] Behl RK, Sharma H, Kumar V and Narula N," Interactions amongst mycorhiza, *Azotobacter* Chroococcum and root characteristics of wheat varieties" J. Argon. Crop Sci. Vol 189, 2003, pp 151-155.

[6] Kannaiyan S, " Biofertilizers for sustainable crop production In: Kannaiyan. S (ed) Biotechnology and biofertilizers" *Kluwer Academic Publishers*, Derdrecht, The Netherlands, 2002, pp 9-49.

[7] Yadav K.S and Dadarwal K.R," Phosphate solubilisation and mobilization through soil microorganisms" In: Biotechnological Approaches in soil Microorganisms for sustainable Crop production (dadarwal R.K., ed):Scientific Publishers, Jodhpur, India,1997, pp. 293-308.

[8] Dey K.B," Phosphate solubilising organisms in improving fertility status," In: Biofertilizers: Potentialities and Problems (Sen S.P and Patil P., eds). Plant Physiology Forum, Naya Prokash, Calcutta, 1998, pp 237-248.

[9] Yosef, B.B, Rogers, RD,wolfram, JH and Richman, " Isolation of Phosphate Solubilizing Microorganism (PSMs) from Soil," *E.J. Soil Science of America*, 1999, pp 1703-1704.

[10] Bisen P.S and Verma, K, " Handbook of microbiology", *CBS publishers and distrubuters*, New delhi, 1996.

[11] Lynch JM," Microbial matabolites, In: Lynch JM, editor, The Rhizosphere, Chichester, England: John Wiley and sons Ltd, Baffins Lane, Interscience, 1990, pp 177-206.

[12] El-Sawah MMA, Hauka FIA and El-Rafey HH," Study on some enzymes cleaving phosphorus from organic substrates in soil," J Agric Sci, vol 157, 1993, pp 238-43.

[13] Bishop ML, Chang AC and Lee RWK," Enzymatic mineralization of organic phosphorus in a Volcanic soil in chile," Soil Sci, vol 157, 1994,pp 238-43.

[14] Feller C, Frossard E and Brossard M," Phosphatase activity in low activity tropical clay soils, Distributuion in the various particle fractions," Can J Soil Sci, vol 74, 1994, pp 121-9.

[15] Kremer RJ," Determination of soil phosphatase activity using a microplate method," Comun Soil Sci plant Anal, vol 25, 1994, pp 319-25.

[16] Sarapatka B and Kraskova M," Interactions between phosphatise activity and soil characteristics from some locations in the Czech Republic," Rostlinna- Vyroba- UZPI vol 43, 1997, pp 415-9.

[17] Richardson A.E," soil microorganism and phosphorus availability. In: Soil Biota, Management in sustainable farming Systems (Pankhurst C.E., Double B.M., Grupta V.V.S.R., and Grace P.R., eds)": CSIRO Melbourne, Australia,1994, pp 50-62

[18] Jensen H.L, " Non- Symbiotic nitrogen fixation, In: Soil nitrogen-*Academic Press, New York.* 2000, pp 359-371

[19] Dobereiner and F.O Pedrosa," Nitrogen- fixing Bacteria in Non leguminous Crop Plants," 1st ed, Science Tech Publishers, Springer Verlag Inc, Berlin, Germany, 1987, pp 239-245.

[20] Don J Brenner, R.N Krieg and JT Staley," Bergey's Manual of systemic Bacteriology, 1st ed,Michigan State university publishers, 2005, pp 384-402.

[21] J.M Gonzalez-lopes, V Martinez-Toledo and S Reina V Salmeron, J.Technol And Environ Chem, vol 33,1991, pp 69-78.

[22] Richardson AE, " Soil microorganisms and phosphorus availability," In: Pankhurst CE, Double BM, Grupta VVSR, Grace PR, editors, Soil Bista, Management in Sustainable Pharming systems, Melbourne, Australia-CSIRO, 1994, pp 50-62

[23] Krasilnikova M, " On the role of soil bacteria in plant nutrition," *J Gen Appl Microbiol.*, vol 7,1961, pp 128-44

[24] Lifshitz R, Kloepper JW, Kozlowski M, Simonson C, Carbon J, Tipping EM and Zalesca I, " Growth promotion of Canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under genobiotic conditions," *Can J Microbiol.*, 1987,vol 33, 1987, pp 390-5.

[25] Chabot R, Antoun H and Ceseas MP, " Stimulation de la Croissance du mais et de la laitue romaine par desmicrooreganisms dissolvent le phosphate inorganique," *Canadian Journal of Microbiol*, vol 39,1993, pp 941-7