

## Biofertilizer: A Novel Approach for Agriculture

Swapnil D. Topre<sup>1\*</sup>, Sreejit S. Panikar<sup>1</sup>, Sarita U. Mahajani<sup>2</sup> and S.B. Patil<sup>3</sup>

<sup>1</sup>Department of Biotechnology, RJSPM's college, Bhosari, Pune - 411 039, Maharashtra, India.

<sup>2</sup>Lokmangal Biotechnology College, Wadala N. Solapur - 413 222, India.

<sup>3</sup>Department of Environmental Science, University of Pune, Ganeshkhind, Pune - 411 007, India.

(Received: 02 November 2010; accepted: 19 December 2010)

Two isolates of agriculturally important nitrogen fixing microbes, *Rhizobium meliloti* and *Azospirillum lipoferum* were isolated from Western Maharashtra soil habitat. The standard isolation technique was carried out in laboratory and were characterized by 16SrDNA method. The growth of microbes was studied at different pH and the optimal pH was found to be 6.5 and 7 for *Rhizobium meliloti* and *Azospirillum lipoferum* respectively. The nodulation activity of *Rhizobium meliloti* was confirmed. Carrier plays an important role in maintaining sufficient shelf life so survival of microorganisms in different carriers at 28°C was deduced and lignite was found to be the most efficient carrier for *Rhizobium meliloti* and *Azospirillum lipoferum*. Further these microorganisms were multiplied for mass production using Lignite as carrier. The biofertilizer was checked for colony count to meet the standard requirement (i.e. 10<sup>7</sup> CFU / gm).

**Key words:** *Rhizobium meliloti*, *Azospirillum lipoferum*, Biofertilizer, Lignite.

Modern agriculture system is completely dependent upon the supply of chemical fertilizers though they are becoming scarcer and more costly. These are major agents for pollution also for water and air. This situation has led to identifying harmless inputs like biofertilizer i.e. microbial inoculum in crop cultivation, which not only increased the nutritional assimilation of plant (total N, P and K), but also improved soil properties, such as organic matter content and total N in soil<sup>1</sup>. Biofertilizers are ready to use live formulation of beneficial microorganisms, which on application to seed, root or soil mobilize the availability of the micro flora and thus soil health.

Habitat plays an important role in shaping the biotic communities. To improve the soil health, it is important to incorporate efficient microflora to the rhizosphere. This can be fulfilled by the use of biofertilizers having beneficial microorganisms which are added into the rhizosphere to enhance the quality of the soil. Microorganisms which can be used as a biofertilizer include bacteria viz. *Rhizobium*, *Azospirillum*, *Azotobacter*, *P-solubilisers* etc., algae, and fungi<sup>2</sup>. Their mode of action differs and can be used alone or in combination. Soil of western Maharashtra region is inert non-lateritic, this triggers the use of fertilizers. Major requirement is fulfilled by using chemical fertilizers; it gives positive result in very short time rather repeated use destroy the soil biota. In nature, there are many beneficial soil microorganisms which help plant to absorb nutrients to improve crop yield<sup>3,4,5</sup>. Their utility can be increased by selecting most efficient strain,

\* To whom all correspondence should be addressed.  
Swapnil D. Topre  
30/4, Golf Residency, Yerwada, Pune - 411006, India.  
Email: swapniltopre@gmail.com

culturing them and applying them to soils directly or through seeds. For easy application, biofertilizers are packed in suitable carrier such as lignite or peat. Carrier also plays an important role in maintaining sufficient shelf life<sup>6</sup>.

**Biological nitrogen fixation (BNF)** refers to the process of micro-organisms fixing atmospheric nitrogen, mostly within subsoil plant nodules, and making it available for assimilation by plants. Nitrogen supply is a key limiting factor in crop production. *Rhizobium* is the most studied and important genera of nitrogen fixing bacteria. It is able to fix atmospheric nitrogen in symbiosis with some types of leguminous plants<sup>7</sup>. *Azospirillum* sp. contribute to increased yields of cereal and forage grasses by improving root development in properly colonized roots, increasing the rate of water and mineral uptake from the soil, and by biological nitrogen fixation<sup>8</sup>. Both of these microbes contribute in making plant self dependent. To the best of our knowledge, less research has been conducted on both of these microbes specifically in western Maharashtra region.

The current research is however focused on isolation and selection of most efficient agriculturally important bacterial strains from western Maharashtra soil habitat, screening and evaluating the nodulation and nitrogen fixing activity, speculating suitable carrier and finally mass production of these biofertilizers.

## MATERIAL AND METHODS

It is certainly cheaper to buy a culture than to isolate from nature, but it is also true that a superior microorganism may be found after an exhaustive search. So soil from the rhizosphere of the specific plants was collected in clean & dry containers. The soil sample was collected from agriculture university field, Pune. For isolation *Rhizobium* spp. root nodule of *Sysbania exaltata* root were selected and for *Azospirillum* spp. soil rhizosphere of sugar cane was taken.

### Isolation technique for *Rhizobium* spp.

Intact root nodules from a healthy *Sysbania exaltata* plant were selected. One of the pink juvenile root nodule was selected and transferred to a drop of sterile water in a petri dish. The nodule in the drop of water was crushed in

between slides causing the release of nitrogen fixing *Rhizobium* bacteria into the drop of sterile water. The smear of the crushed root nodule was streaked onto Yeast Extract Mannitol Agar (YEMA) plate with 1% congo red dye. The culture was then incubated at 20°C to 25°C for 3 days.

### Isolation technique for *Azospirillum* spp.

Juvenile root from a healthy sugar cane plant was taken and kept in saline for 5 minutes. With a forceps, root was immersed to a semisolid Bromothymol blue broth containing 0.8% agar agar in a test tube and incubated at 20°C to 25°C for at least a week. A loopful of culture adjacent to the root in the broth) was transferred to Bromothymol blue media plates. The culture was incubated at 20°C to 25°C for at least a week.

### Genome sequencing and blasting of the 16S rDNA

The confirmation of the microorganisms was given by NCCS, Pune using automated DNA sequence analyzer (ABI 3730 DNA Sequencer).

### Study of nodulation efficiency of the *Rhizobium*

Seeds were germinated for 2 days. Seedlings were transfer to the mixture of broth and course sand in 9:30 ratio. Culture broth was added with dilutions till 10<sup>-10</sup>. After 8 days, plantlets were transferred in soil. Nodulation efficiency was recorded after 3-4 weeks.

### Study of effect of pH on microorganisms

Yeast Extract Mannitol (YEM) media and Bromothymol blue media were prepared. 30 ml of these media were transferred into bumper tubes (10 for each medium). The pH of tubes were adjusted from 2-11 using pH meter. 0.1 ml of respective culture broth was added in each tube and incubated for 7 days. The blank of each set was kept in refrigerator. Growth was determined calorimetrically at 610 nm.

### Selection of suitable carrier for biofertilizer

The lignite powder and peat were autoclaved at 15psi at 121°C for 20 minutes. The culture broth was mixed with both carriers at 30% i.e for 1 kg of carrier 300ml of culture broth. The mixture was spread on a plastic sheet in a closed room for air drying. The viable count of the both biofertilizer was compared for 90 days with 7 days interval.

### Mass production of biofertilizers

The carrier was autoclaved at 15psi at 121°C for 20 minutes. The culture broth was mixed with the carrier at 30% i.e. for 1 kg carrier 300ml of

culture broth. The mixture was spread on a plastic sheet in a closed room for air drying. The biofertilizer was packed in sterile plastic air tight bags & stored.

### RESULTS

1. The microorganisms isolated from agricultural university, Pune were identified as follows –
  - a) *Rhizobium meliloti*
  - b) *Azospirillum lipoferum*
2. The confirmation of *Rhizobium meliloti* & *Azospirillum lipoferum* was given by National Centre for Cell Sciences, Pune using automated DNA sequence analyzer

(ABI 3730 DNA Sequencer).

3. *Rhizobium meliloti* grown luxuriantly at pH 6.5 while *Azospirillum lipoferum* showed optimum growth at pH at 7 as shown in Fig.1.
4. Nodulation activity of *Rhizobium meliloti* was observed till  $10^{-8}$  dilutions as shown in Table 1.
5. Lignite was found to be a better carrier than peat as shown in Fig. 2.
6. Mass production & packing of the biofertilizers was done. The packed bioculture packets were checked for cell count and were found to be above  $10^7$  CFU/g as per the norms of Indian Agricultural Ministry (Table 2).

**Table 1.** Counting nodulation units

Dilution	Nodulation (+) or (-) sets			Total No. of nodulated units
	I	II	III	
$10^{-1}$	+	+	+	4
$10^{-2}$	+	+	+	4
$10^{-3}$	+	+	+	4
$10^{-4}$	+	-	+	3
$10^{-5}$	-	+	+	2
$10^{-6}$	+	+	-	2
$10^{-7}$	-	+	-	1
$10^{-8}$	+	-	-	1
$10^{-9}$	-	-	-	0
$10^{-10}$	-	-	-	0
Total				21

Table 1: (+): Nodulation unit

**Table 2.** Cell count of microbes.

Organisms	Initial count (0 day)/ml	Final count (120 days)/ml
<i>Rhizobium meliloti</i>	$4.24 \times 10^{11}$	$9.4 \times 10^8$
<i>Azospirillum lipoferum</i>	$3.17 \times 10^{11}$	$13.8 \times 10^8$

### DISCUSSION

For a sustainable agriculture system, it is imperative to utilize renewable inputs which can maximize the ecological benefits and minimize the environmental hazards. One possible way of achieving this is to decrease dependence on use

of chemical nitrogen fertilizers by harvesting the atmospheric nitrogen through biological processes. With these considerations, work was done on biofertilizers which indirectly minimize effect of chemical fertilizers by making plant self dependant. Efficiency of biofertilizers was enhanced by improving microbial activity.

Till date many theories based on enhancing plant growth using fungal species have been studied, yet less work is done on bacterial species used for biofertilizers. Biofertilizer component has not received adequate attention in the past. Keeping this in view, Agricultural soil was collected which was rich in nutrients and agriculturally important organisms. Using standard isolation protocol *Rhizobium* spp. and *Azospirillum* spp. were isolated after many subculturing and screening procedures such as colony characteristics and use of selective media. These isolates were confirmed by 16S rDNA technique from NCCS, Pune.

*Rhizobium* grows luxuriantly at pH 6.5 while *Azospirillum* spp. shows optimum pH at 7. So we can conclude that these biofertilizers can work actively in western agricultural area (Maharashtra). Nodulation is another factor, involved in nitrogen fixation in many leguminous plants, has importance in improving product yield. It means that in commercial point of view biofertilizers has great importance, so *Rhizobium* and *Azospirillum* biofertilizers were mass produced and packed using lignite as a carrier due to its advantageous reasons over others. Generally expected expiry given by local manufacturer of biofertilizers is up to 6 months with  $10^7$  CFU/ml,

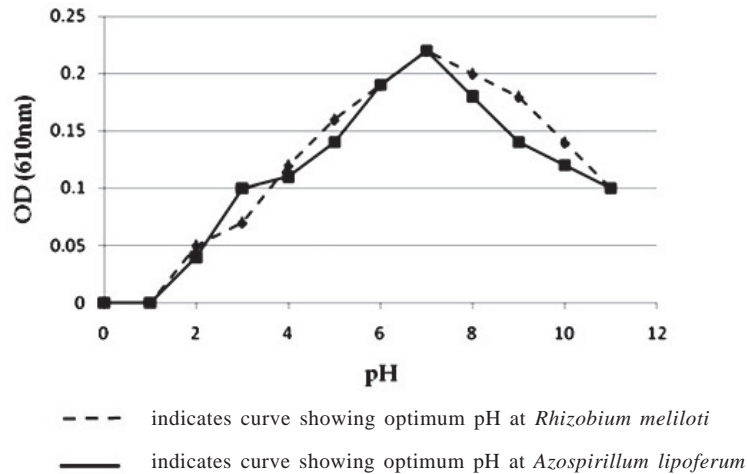


Fig. 1. Growth of microorganism at different pH

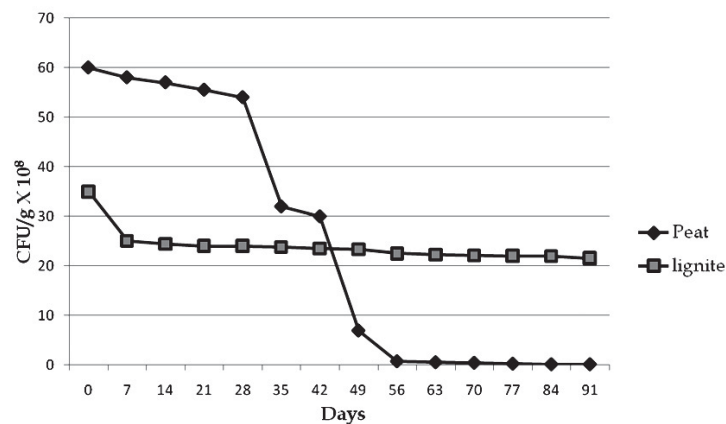


Fig. 2. Survival of microorganisms in peat and lignite

but results obtained showed  $10^8$  CFU with better nitrogen fixing and nodulation ability.

The present study was done to analyze and assess the impacts of biofertilizer used in production systems for fine tuning of biofertilizer technology.

#### ACKNOWLEDGMENTS

We take this opportunity to express our sincere thanks and gratitude Dr. Yogesh Souche, Mr. Sandeep Walunjkar and Mr. Pawar sir for their valuable guidance and help.

Finally we acknowledge with deep appreciation, the indispensable help, encouragement and moral support received from our colleague, lab assistants and dear ones.

#### REFERENCES

1. Wu S.C., Cao Z.H., Li Z.G., Cheung K.C. and Wong M.H. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. *Geoderma*, 2005; **125**(1-2): 155-166.
2. Boraste A., et al. Biofertilizers: A novel tool for agriculture. *International Journal of Microbiology Research*, 2009; **1**(2): 23-31.
3. Rao S., N.S. Biofertilizers in Agriculture. A.A. Blakema, Rotterdam/ New Delhi, 1982; 128-136.
4. Young, C. C., Juang T. C. and Chao C. C.. Effects of Rhizobium and VA mycorrhiza inoculations on nodulation, symbiotic nitrogen fixation, and yield of soybean in subtropical-tropical fields. *Biology and Fertility of Soils* (Germany) 1988; **6**: 165-169
5. Young, C.C. Application and development of N<sub>2</sub>-fixing microorganisms. Proceedings, Symposium on Development and Application of Biofertilizers. *Taiwan Agriculture Research Institute*. 1994; 5-14.
6. Singh, T; Ghosh, T.K.; Tyagi, M.K. and Duhan J.S. Survival of Rhizobia and level of contamination in charcoal and lignite. *Annals of Biology*, 1999; **15**(2): 155-158.
7. Odame, H. Biofertilizer in Kenya: Research, production and extension dilemmas. *Biotechnology and Development Monitor*, 1997; **30**: 20-23.
8. Okon Yaacov, *Azospirillum* as a potential inoculant for agriculture. *Trends in Biotechnology*, 1985; **3**(9): 223-228.