Journal of Agricultural Biotechnology and Sustainable Development Vol. 3(10), pp. 205-208, December 2011
Available online at http://www.academicjournals.org/JABSD
DOI: 10.5897/JABSD10.042

Full Length Research Paper

Biofertilizer: A novel approach for agriculture

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Accepted January 18, 2011

The nodulation activity in some legume plants was estimated using two nitrogen fixing microorganisms that produced and introduced in different growth conditions. Two isolates of agriculturally important nitrogen fixing microorganisms, *Rhizobium meliloti* and *Azospirillium lipoferum* were isolated from Western Maharashtra soil habitat. The standard isolation technique was carried out in laboratory and was characterized by 16S rDNA method. The growth of mentioned microorganisms was studied at different pH, the optimal pH was found to be 6.5 and 7 for *R. meliloti* and *A. lipoferum* respectively. The nodulation activity of *R. meliloti* was confirmed. Carrier plays an important role in maintaining sufficient shelf life, so, survival of mentioned microorganisms in different carriers at 28°C was deduced. However, lignite was found to be the most efficient carrier for *R. meliloti* and *A. lipoferum* to introduce as biofertilizer. The mentioned biofertilizer was checked for colony count to meet the standard requirement (that is, 10^7 cells/gm). Furthermore, the mentioned microorganisms were multiplied for mass production using lignite as carrier.

Key words: *Rhizobium meliloti*, *Azospirillium lipoferum*, biofertilizer, lignite.

INTRODUCTION

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 of water and air. This situation has led to identifying harmless inputs like biofertilizer, that is, microbial inoculums 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 (Wu et al., 2005). Biofertilizers are ready to use as live formulation of beneficial microorganisms, when it amended to seed, root or soil, it mobilizes the availability and utility of the microorganisms 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 microorganisms to the rhizosphere. This can be fulfilled by the use of biofertilizers having beneficial microorganisms which are added into the rhizosphere to enhance the soil fertility. Microorganisms which can be used as a biofertilizer include bacteria viz. *Rhizobium*, *Azospirillium*, *Azotobacter*, P-solubilisers etc., algae, and fungi (Boraste, 2009). 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 than repeated use which destroys the soil biota. In nature, there are many beneficial soil microorganisms which help plant to absorb nutrients to improve crop yield (Rao, 1982; Young, 1994; Young et. al., 1988). Their utility can be increased by selecting most efficient strain, 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 (Singh et. al., 1999). Biological nitrogen fixation (BNF) refers to the process of

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microorganisms 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 (Odame, 1997). *Azospirillum* spp. 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 (Okon, 1985). 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 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.

MATERIALS 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 and dry containers. The soil sample was collected from Agriculture University, Pune, India. For isolation of *Rhizobium spp.* and *Azospirillium spp.*, the root nodules of *Sysbania exaltata* root and soil rhizosphere of sugar cane were used respectively.

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 two glass 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 to 25°C for 3 days (Boraste, 2009).

Isolation technique for *Azospirillium* spp.

Juvenile root from a healthy sugar cane plant was taken and kept in saline for 5 min. With a forceps, root was immersed to a semisolid Bromothymol blue medium broth containing 0.8% agar in a test tube and incubated at 20 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 to 25°C for at least a week.

Genome sequencing and blasting of the 16s rRNA

The confirmation of the microorganisms was given by National Centre for Cell Science, 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 transfered to the mixture of broth and course sand in 9:30 ratio. Culture broth was added with dilutions till 10^{-10}. After 8 days, plantlets were transferred into soil. Nodulation efficiency was recorded after 3 to 4 weeks (Boraste, 2009).

Study of pH effect on mentioned 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 was adjusted from 2 to 11 in gradient manner using Interlab's digital pH meter. 0.1 ml of respective last mentioned culture broth was added into each tube and then incubated for 7 days. The blank of each set was kept in refrigerator. The microorganisms growth was determined calorimetrically at 610 nm.

Selection of suitable carrier for making the biofertilizer

The lignite powder and peat obtained from Bio agro Ferticons, Mundwa, Pune, India were autoclaved at 15 psi at 121°C for 20 min. The last mentioned culture broth was mixed with both carriers at 30%, that is, for 1 kg of carrier, 300 ml of culture broth. The mixture was spread on a plastic sheet in a closed room for air drying. The viable count of both biofertilizer was compared for 90 days with 7 days interval.

Mass production of biofertilizers

The carrier was autoclaved at 15 psi at 121°C for 20 min. The culture broth was mixed with the carrier at 30%, that is, for 1 kg carrier, 300 ml 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 and stored.

OBSERVATIONS AND RESULTS

The microorganisms isolated from Agricultural University, Pune were identified as follows: (a) *R. meliloti*; (b) *A. lipoferum.* The confirmation of *R. meliloti* and *A. lipoferum* was given by National Centre for Cell Sciences, Pune using automated DNA sequence analyzer (ABI 3730 DNA Sequencer). *R. meliloti* was grown luxuriantly at pH 6.5 while *A. lipoferum* showed optimum growth at pH 7 as shown in Figure 1. Nodulation activity of *R. meliloti* was observed till 10^{-8} dilutions as shown in Table 1. Lignite was found to be a better carrier than peat as shown in Figure 2. Mass production and 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).

DISCUSSION

For a sustainable agriculture system, it is imperative to utilize renewable inputs which can maximize the

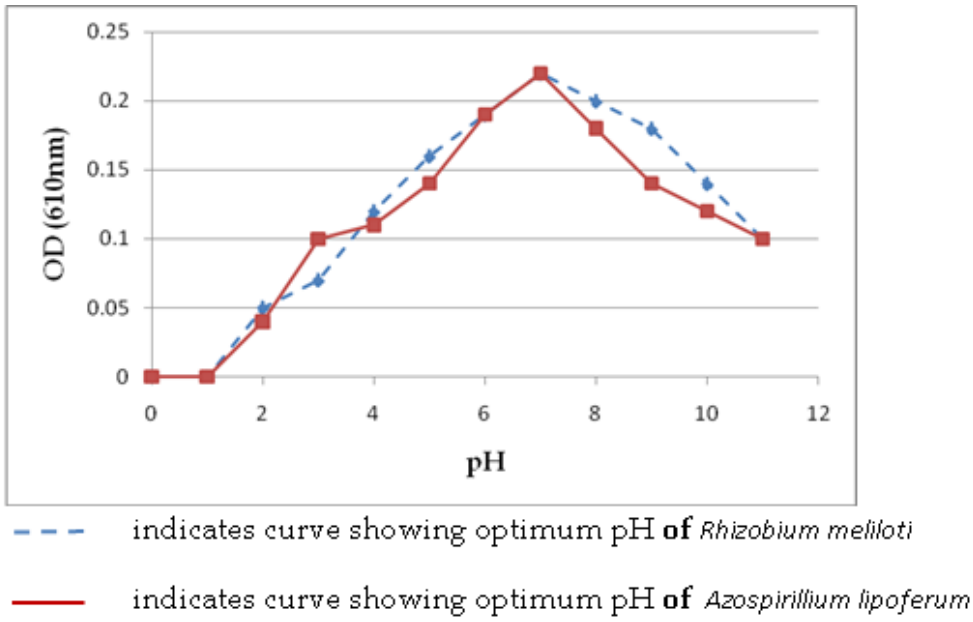

Figure 1. Growth of microorganism at different pH.

Table 1. Nodulation activity of *Rhizobium meliloti*.

Dilution	Nodulation (+) or (-) sets			Total number 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

(+): Nodulation unit.

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 the use 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 in western Maharashtra region. Biofertilizer component has not received adequate attention in the past. Keeping this in view, experimental soil was collected from agricultural area which was rich in nutrients and agriculturally important microorganisms. Using standard isolation protocol *Rhizobium* spp. and *Azospirillium* spp. were isolated after many sub culturing and screening procedures such as colony characteristics and use of selective media. These isolates were confirmed by 16S rDNA technique from NCCS, Pune, India.

Rhizobium grows luxuriantly at pH 6.5 while *Azospirillium* spp. shows optimum growth at pH 7. So we

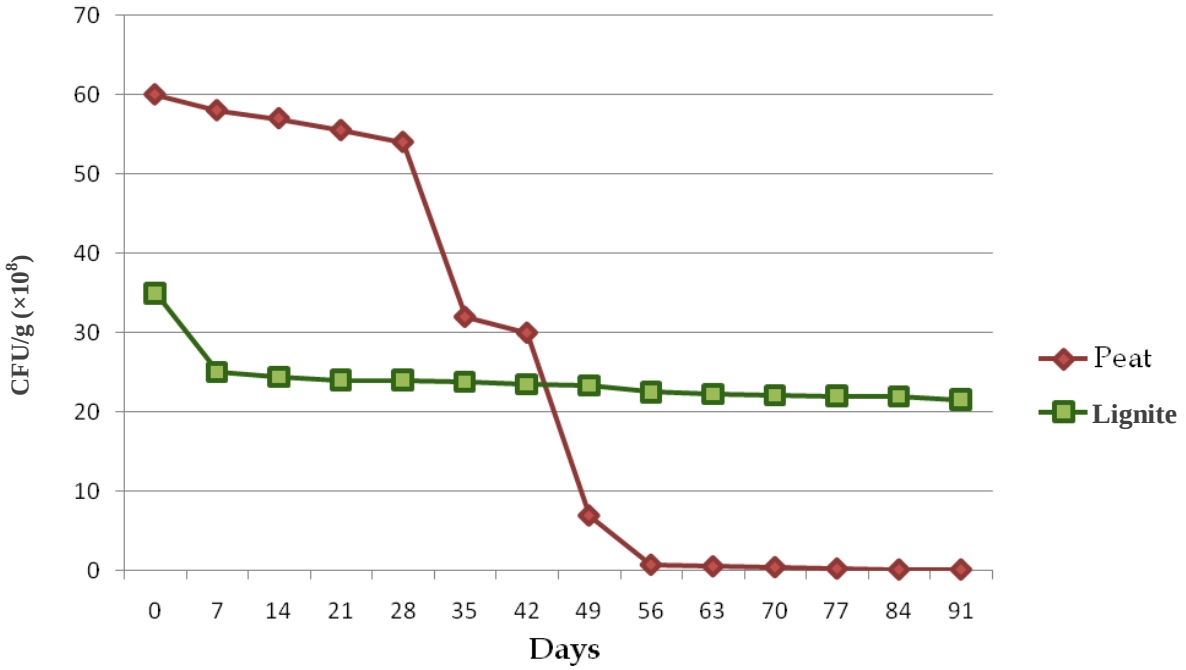

Figure 2. Survival of organisms in peat and lignite.

Table 2. Cell count of microbes.

Organism	Initial CFU/ml (0 days)	Final CFU/ml (120 days)
Rhizobium meliloti	4.24×10^{11}	9.4×10^{8}
Azospirillium lipoferum	3.17×10^{11}	13.8×10^{8}

can conclude that these biofertilizers can work actively in Western Maharashtra Agricultural Area. Nodulation as 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 have great importance, so *Rhizobium* and *Azospirillium* 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, but results obtained showed 10^8 cells with better nitrogen fixing and nodulation ability. Further work was done to analyze and assess the impacts of biofertilizer used in production systems for fine tuning of biofertilizer technology.

ACKNOWLEDGEMENTS

We use this opportunity to express our sincere thanks and gratitude to 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, laboratory assistants and dear ones.

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