Octa Journal of Environmental Research ***Jan.–March, 2014***
International Peer-Reviewed Journal ISSN 2321-3655
Oct. Jour. Env. Res. Vol. 2(1): 32-37
Available online http://www.sciencebeingjournal.com

Research Article

Octa Journal of Environmental Research
(Oct. Jour. Env. Res.) ISSN: 2321-3655
Journal Homepage: http://www.sciencebeingjournal.com

CHARACTERIZATION, BIO-FORMULATION DEVELOPMENT AND SHELF-LIFE STUDIES OF LOCALLY ISOLATED BIO-FERTILIZER STRAINS

Vipin Kumar
Department of Environmental Science & Engineering, Indian School of Mines, Dhanbad - 826 004, Jharkhand, India
Corresponding author's Email:vipinmicro1@gmail.com

Received: 20th Dec. 2013 Revised: 15th March 2014 Accepted: 25th March 2014

Abstract: Nitrogen fixing, phosphate solubilizing and potash mobilizing bacterial strains were isolated from rhizosphere soil of agricultural land, the isolated bacterial strains were further characterized by a series of biochemical reactions and identified as genus *Azotobacter*, *Bacillus* and *Pseudomonas* respectively. A technology for their mass multiplication and their bio-formulation has been developed. Fly-ash was used as carrier materials for bio-formulation development of bio-fertilizer strains. Shelf-life studies of the bio-formulations were carried out during storage period. The selected isolates were found to be potent nitrogen fixer, phosphate solubilizers showing clear halo zone around their colonies and potash mobilizer showing mobilization of potassium on respective medium. A general decline in cfu count was noticed in fly-ash based bio-formulations. All the bio-formulations however, retained more than 10^8 cfu/g viable propagules up to 270 days. The present studies were shown encouraging results in respect to fly-ash as carrier materials for bio-fertilizer strains which are comparable to other commercially available carrier materials.

Key words: Bacterial strains; Bio-fertilizer; Bio-formulation; Fly-ash; Shelf-life.

Postal Address: Dr. Vipin Kumar, Assistant Professor, Department of Environmental Science & Engineering, Indian School of Mines, Dhanbad - 826004, Jharkhand, India, Ph: +91-326-2235643 (O)

INTRODUCTION

The term bio-fertilizer refers to formulations based on beneficial microbes and/or biological product that either fix atmospheric nitrogen or enhance the solubility of soil nutrients and having potential to increase the yield of crops. Being biodegradable, non-toxic and cost effective, bio-fertilizers are emerging as the efficient alternative to agrochemicals used as bio-fertilizers. However in India number of bio-fertilizer production units are engaged in commercial production, the total installed production capacity is still very low compared to their potential demand. The expansion of bio-fertilizer network and its future in the country requires the active participation of research and development agencies as well as public awareness programs. Bio-fertilizer is a product which is largely used now a day in various crops cultivation. There is large demand increases day by day due to replacement of the synthetic fertilizer. Although N_2 is abundant (around 80%) the atmospheric N_2 is not readily available for plant uptake and some bacteria are capable of N_2 fixation from the atmospheric N_2 pool. Many free living N_2 fixing bacteria occur in soil. The amount of N_2 fixed by these organisms is considerable because of the close proximity they have with their host plant. Efficient plant use of field N_2 minimizes volatilization, leaching and denitrification (Nepolean *et al*., 2012). *Azotobacter* is major free living in soils so that it can be cultured and produced in artificial medium. It stimulates the density and length of root hairs, increases the growth through hormonal production, increases biomass, increases survival rate and fixes nitrogen. Phosphate solubilizing Bacteria (PSB) plays a major role in the solubilization and uptake of native and applied soil P (Krishnaveni, 2010). Phosphate is essential for early establishment and better growth of plants and

responsible for biological rescue capability of solubilizing the insoluble inorganic phosphorus of soil. Most of the Indian soils are deficient in P and its requirement is met by the addition of phosphate fertilizers in the form of aluminium or iron phosphate. But these fertilizers are becoming expensive and may have adverse effect on soil. Hence, the phosphate solubilizing bacteria have to be used as they play an important role in the utilization of unavailable native phosphate by bringing about changes insoil producing chelating agents and organic acids. Generally *Bacillus*, *Pseudomonas*, *Flavobacterium* etc. are involved in P solubilization(Gaur, 2006). Thus inoculation of PSM in soil can stimulate plant growth even under the conditions of phosphorus deficiency (Domey *et al*., 1988). Phosphorus is typically insoluble or poorly soluble in soil. Phosphorus is sequestered by adsorption to the soil surface and precipitation by reaction with soil cations, particularly iron, aluminium and calcium (Harris and Lottermoser, 2006). Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms. Assimilation of microorganisms in carrier materials enables easy-handling, long term storage and high effectiveness of biofertilizers. Since the beginning of biofertilizers use at large scale, several carrier materials have been tried like farm yard manure (FYM), compost, peat soil, coal, charcoal, cellulose powder, lignite, talc, bagasse, sedge peat, press mud, teak leaf meal, coconut shell powder etc. Although, fly-ash is basic in nature and it has good mineral content. Use of fly-ash in soil as bio-formulation is useful because it increases pH of soil, converts the nutrients in available form, supplies nutrients to soil and it solves disposal problem of fly-ash (Dwivedi, 2007).

EXPERIMENTAL

Isolation and identification of bacterial strains

Bacterial strains were isolated from rhizosphere soil on respective selective medium bydilution agar plate technique. The selective medium used was Ashby's agar for nitrogen fixing bacteria, Pikovskaya's agar for PSB and modified King's B agar for potash mobilizing bacteria. 10 g of rhizospheric soil was mixed in 100 mL of sterilized distilled water and shaken thoroughly and made a dilution up to 7 fold. 1 mL of suitable dilution of soil sample was inoculated by pour plate method on the agar medium and incubated up to 7 days at 28±2°C. These colonies were picked up and further purified by re-streaking on selective agar plate and were stored at refrigerator temperature for further study. Identification of isolated bacterial strains was performed by morphological and biochemical characteristics comparing with standard references. The microscopic identification was carried out by Gram's staining using compound microscope (OLYMPUS BX 60). Morphological and biochemical tests of the isolates were carried out for their identification as per the procedures outlined in Bergey's Manual of Determinative Bacteriology (Sneath *et al*., 1986).

Multiplication of bacterial isolates

The isolated strains were grown in respective broth medium in culture tube. After checking the culture for purity and proper growth, the culture was transferred from culture tube to small conical flask containing sterilized liquid medium as starter culture. Later the starter culture was transferred to a large conical flask on a rotary shaker at 150 rpm for 5 days at 28±2^{o}C.

Bio-formulation and shelf life studies of bacterial strains

Bio-formulations of selected bio-fertilizer were obtained by mixing broth culture with previously sterilized fly-ash. These were packed in low density polythene pouch and stored at 28±2^{o}C and room temperature. Shelf-life of the formulations was studied by drawing samples at regular interval of 30-days up to nine months from date of mixing and the colony forming unit (cfu) was counted by serial dilution agar plate method.

RESULTS AND DISCUSSION

Isolation and identification of bio-fertilizers strains

The results of morphological and bio-chemical characteristics of isolated strains of bio-fertilizers are summarized in Table 1. The isolated bacterial strains for nitrogen fixing bio-fertilizer was rod shaped,

Gram's negative and identified as *Azotobacter* sp. which is well known as free living N_2 fixing bacteria. Based on the halo zone formation on Pikovkaya's (PKV) agar plate by Bacillus sp. was considered as phosphate solubilizing bacteria. Gyaneshwar *et al*., 1999 also reported that the colonies with clear halo zones are considered to be PSB. Potassium mobilization on modified King's B agar plate was considered as potash mobilizing bacteriaand identified as*Pseudomonas* sp.

Table 1: Morphological and bio-chemical characteristics of isolated strains of bio-fertilizers

Characteristics	*Azotobacter* sp. (N_2 fixer)	*Bacillus* sp. (PSB)	*Pseudomonas* sp (PMB)
Gram reaction	G -ve	G +ve	G -ve
Shape	rods	rods	rods
Colony colour	W/T	Y/O	W/T
Lactose	-	+	+
Dextrose	+	+	+
Sucrose	+	+	+
Mannitol	-	+	+
Indole	-	-	+
Methyl red	-	-	+
Vogues Proskauer	-	+	+
Citrate	+	+	+
H_2S	+	-	-
Oxidase	+	-	+
OF test	+	-	+
Nitrate	+	+	+
Starch hydrolysis	+	+	+
Gelatin hydrolysis	-	+	-

Y/O- Yellow & Opaque; W/T- White & Translucent
PSB-phosphate solubilizing bacteria, PMB-potash mobilizing bacteria

Immobilization of microbial inoculants has been used to improve their effectiveness by supplying nutrients, protection from desiccation and slow cells release (Bashan, 1998; Kim *et al*., 2012). The success of using microbial inoculants introduced into soil requires the survival of adequate numbers of bacteria reaching suitable habitats where they can stay alive (Heijnen and Van Veen, 1991). The principle of immobilization of rhizobacteria is to protect the microorganisms (Schoebitz *et al*., 2012) and to ensure a gradual and prolonged release into the soil (Bashan *et al*., 2002; Wu *et al*., 2011). Most P-solubilizing bacteria and fungi (Achal *et al*., 2007; Aseri*et al*., 2009; Yadav and Tarafdar, 2011) were isolated from the rhizosphere of various plant and are known to be metabolically more active than those isolated from sources other than rhizosphere. Kucey*et al*., 1989 also reported that the *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains as biofertilizers.

Bio-formulation and shelf-life studies

Biofertilizers are usually prepared as carrier based inoculants containing effective microorganisms. These identified strains were used for the preparation of bio-formulations using fly-ash as carrier material and evaluated for their viable cell count during storage period of 270 days in laboratory conditions. A general decline in cfu count was noticed in fly-ash based bio-formulations. All the bio-formulations however, retained more than 10^8 cfu/g viable propagules up to 240 days, though the cfu declined below the optimum level by 270 days. The cfu count stored at 28±2^{o}C was greater than room temperature, in all the formulations (Figure 1, 2 and 3).

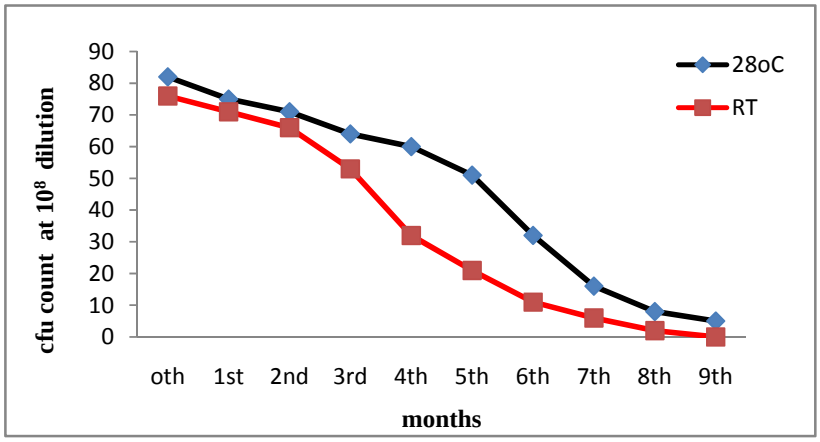

Figure 1. cfu count of *Azotobacter* sp. in fly-ash based formulation

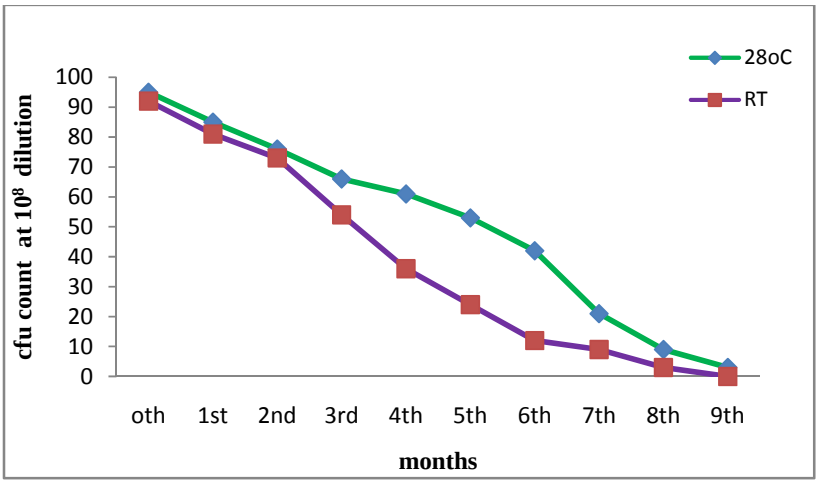

Figure 2. cfu count of PSB in fly-ash based formulation

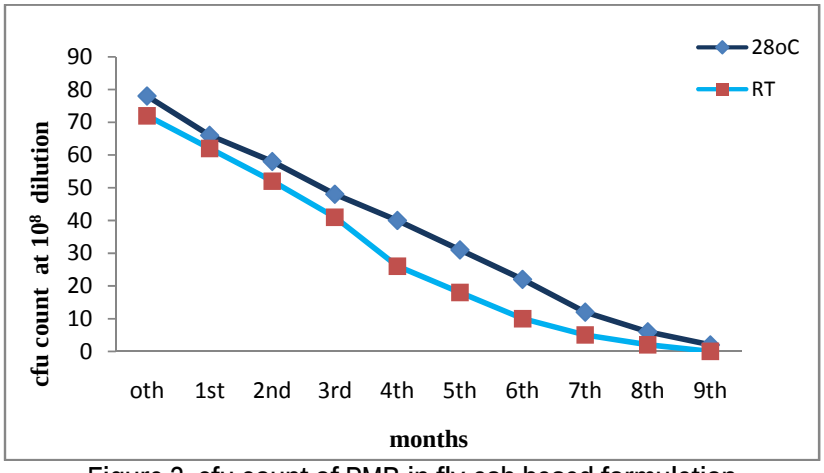

Figure 3. cfu count of PMB in fly-ash based formulation

The numbers of the viable cells were significantly declined in all the three bio-formulations throughout the incubation period. A decline in population on prolonged incubation may be attributed to the depletion of nutrients, moisture and autolysis of cells (Gaind and Gaur, 2003). Statistically, studies suggested that there were significant differences in cfu counts among the formulations at the end of

storage period. All the strains were managed to retain the viable cell count of 10^8cfu g^{-1} in all the formulations at the end of the incubation period of 270 days, which was fulfill the guidelines for biofertilizers of fertilizer control order (FCO). Muniruzzaman and Khan (1992) found that viable counts of two indigenous rhizobial strains (SB-1 and JJS-1) remained more than 10^8 g^{-1} up to 75 days in many carrier materials including charcoal.Many researchers have also suggested that Fly-ash alone and in combination with other materials is a promising carrier for bio-formulation of *Rhizobium* (Kumar and Gupta, 2008) and *Trichoderma viride* and *Trichoderma harzianum* (Kumar *et al*., 2012).

The carrier based inoculants produced in India generally have a short shelf life, poor quality, high contamination and unpredictable field performance. High quality biofertilizers would be expected to have higher population of desired microorganism, sufficient viability, and remain uncontaminated for longer period of storage. Today, advances in inoculant technology are concerned with improving quality, extending useful shelf life and developing new formulations for use under less favorable conditions. Brahmaprakash and Sahu (2012) suggested that liquid inoculants and alginate based granular formulations are two important new inoculant formulations which are an alternative to peat/lignite based ones.In the present study data obtained was suggested that fly-ash can be used as carrier for bio-formulations, similar results were also reported by various researchers, Jayaraj *et al*., 2005 reported that the formulations of *B. subtilis* and *P. fluorescens* are commercially available. Those of *Bacillus* are very stable due to the ability of this bacterium to form spores (Emmert and Handelsman, 1999) that are long-lived, and resist heat and desiccation (Kloepper, 1991).Han and Lee (2005) reported that the phosphate solubilizing bacteria (PSB) *Bacillus megaterium* and potassium solubilizing bacteria (KSB) *Bacillus mucilaginosus* enhanced the nutrient levels in nutrient limited soil planted with eggplant.

CONCLUSION

The selected isolates were found to be potent nitrogen fixer, phosphate solubilizers showing clear halo zone around their colonies and potash mobilizer showing mobilizing potassium on respective medium. The numbers of the viable cells were significantly declined in all the three bio-formulations throughout the incubation period. A decline in population on prolonged incubation may be attributed to the depletion of nutrients, moisture and autolysis of cells. The results of the present studies showed that, fly-ash can be used as carrier materials for biofertilizers strains. The data obtained in respect to fly-ash as carrier materials are comparable to other commercially available carrier materials. Although, fly-ash is basic in nature and it has good mineral content. Use of fly-ash in soil as bio-formulation is useful because it increases pH of soil, converts the nutrients in available form, supplies nutrients to soil and it solves disposal problem of fly-ash. The use of these native strains as bio-fertilizers helps in reducing the use of chemical fertilizers and also effective in reducing the cost of cultivation and maintaining the natural fertility of soil.

Acknowledgements: Author is thankful to Indian School of Mines for providing Laboratory and technical facilities, also thankful to Ministry of human resources and development (MHRD) GoI for financial support.

REFERENCES

Achal, V., Savant, V.V. and Reddy, M. (2007). Phosphate Solubilization by Wide Type Strain and UV-induced Mutants of *Aspergillustubingensis*. Soil Biology and Biochemistry, 39(2):695-699.

Aseri, G.K., Jain, N. and Tarafdar, J.C. (2009). Hydrolysis of Organic Phosphate forms by Phosphatases and Phytase Producing Fungi of Arid and Semi-arid Soils of India. American-Eurasian J. of Agriculture and Environment Science, 5(4):564-570.

Bashan, Y., Hernandez, J.P., Leyva, L.A. andBacilio, M. (2002). Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. BiolFertil Soils, 35:359-68.

Bashan, Y. (1998).Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol Adv, 16:729-770.

Brahmaprakash, G.P., and Sahu, P.K. (2012). Biofertilizers for Sustainability. Journal of the Indian Institute of Science, 92(1): 37-62.

Domey, S., Lippmann, G., Vancura, V., and Kunc, F. (1988). Stimulation of Plant growth by Phosphate Solubilizing bacteria. In Development in soil science, 18ed, 457-461.

Dwivedi, J. and Chauhan, R. (2007). In Proc. of the 44th Annual Convention of Chemists by Indian Chemical Society, Kolkata at MGIAS, Jaipur. pp. D10.

Emmert, E.A.B. and Handelsman, J.(1999).Biocontrol of plant disease: a Gram's positive perspective. FEMS Microbiology Letters, 171:1-9.

Gaind, S. and Gaur, A.C. (2003). Evaluation of fly ash as a carrier for diazotrophs and phosphobacteria. Bioresource Technol, 95:187-190.

Gyaneshwar, P., Pareka, L.J., Archana, G., Podle, P.S., Collins, M.D., Huston, R.A. and Naresh, K.G. (1999). Involvement of a Phosphate Starvation Inducible Glucose Dehydrogenase in Soil Phosphate Solubilization by *Enterobacterasburiae*. FEMS Microbiology Letters, 171(2): 223-229.

Han, H.S. and Lee, K.D. (2005). Phosphate and Potassium Solubilizing Bacteria Effect on Mineral Uptake, Soil Availability and Growth of Eggplant. Research J. of Agriculture and Biological Sciences, 1(2):176-180.

Harris, D.L. and Lottermoser, B.G. (2006). Evaluation of phosphatefertilizers for ameliorating acid mine waste, Appl. Geochem, 21:1216-1225.

Heijnen, C.E. and Van Veen, J.A. (1991).A determination of protective microhabitats for bacteria introduced into soil. FEMS Microbiol Ecology, 85:73-80.

Jayaraj, J., Radhakrishnan, N.V., Kannan, R., Sakthivel, K., Suganya, D., Venkatesan, S. and Velazhahan, R.(2005). Development of new formulations of *Bacillus subtilis* for management of tomato damping-off caused by *Pythiumaphanidermatum*. Biocontrol Science and Technology, 15:55-65.

Kim, I.Y., Pusey, P.L., Zhao, Y., Korban, S.S., Choi, H. and Kim, K.K. (2012).Controlled release of Pantoeaagglomerans E325 for bio-control of fire blight disease of apple. J Control Release, 161:109-115.

Kloepper, J.W. (1991). Plant growth-promoting rhizobacteria as biological control agents of soil borne diseases. In: J. Bay-Peterson (Ed.). The Biological Control of Plant Diseases. Food and Fertilizer Technological Center, Taiwan, 142-152.

Kucey, R.M.N., Janzen, H. and Legget, M.E. (1989). Microbial Mediated Increases in Plant Available Phosphorous. Advances in Agronomy, 42:199-288.

Kumar, V. and Gupta, P. (2008). Efficacy of fly-ash based *Rhizobium* on growth and incidence of powdery mildew in pea.Annals of Plant Protection Sciences, 16(1): 248-249.

Kumar, V., Gupta, P. and Dwivedi, S. (2012). Bio-efficacy of fly-ash based Trichoderma formulations against damping-off and root-rot diseases in tomato (*Lycopersicum esculentum*). Indian Phytopathology, 65(4):404-405.

Muniruzzaman, S. and Khan, S.I. (1992). Suitability of some local agro-industrial wastes as carrier materials for *Rhizobium* sp. infecting *Sesbania bispinosa*. World J. Microbiology and Biotechnology, 8:329-330.

Nautiyal, C.S.(1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol, 170:265-270.

Schoebitz, M., Simonin, H. andPoncelet, D. (2012).Starch filler and osmoprotectants improve the survival of rhizobacteria in dried alginate beads. J Microencapsul, 29:532-538.

Sneath, P.H.A., Mair, N.S., Elisabeth, S.M. and Holt, J.G. (1986). Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, U.S.A.

Wu, Z., Zhao, Y., Kaleem, I. and Li, C. (2011). Preparation of calcium alginate micro-capsuled microbial fertilizer coating *Klebsiella oxytoca* Rs-5 and its performance under salinity stress. Eur J Soil Biol, 47:152-159.

Yadav, B.K. and Tarafdar, J.C. (2011).*Penicilliumpurpurogenum*. Unique P Mobilizers in Arid Agro-Ecosystems. Arid Land Research and Management, 25(1): 87-99.

Gaur, A.C. (2006).Handbook of organic farming and Biofertilizers. Jaipur: Ambica Book Agency,pp. 667.

Nepolean,P., Jayanthi,R., Vidhya, P.R., Balamurugan,A., Kuberan,T., Beulah,T., Premkumar,R. (2012).Role of biofertilizers in increasing tea productivity. Asian Pacific Journal of Tropical Biomedicine, 1443-1445.

Krishnaveni, M.S. (2010).Studies on phosphate solubilizing bacteria (PSB) in rhizosphere and non-rhizosphere soils in different varieties of foxtail millet (*Setariaitalica*), Int J Agricul Food Sci Technol,1(1): 23-39.

CONFLICT OF INTEREST: Nothing