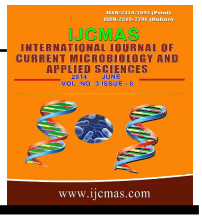
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 6 (2014) pp. 415-422**
http://www.ijcmas.com



Original Research Article

Encapsulation of plant growth promoting inoculant in bacterial alginate beads enriched with humic acid

P.K.Sivakumar, R.Parthasarthi and V.P. Lakshmipriya*

Department of Microbiology, Annamalai University, Annamalai Nagar, Tamil Nadu, India
Corresponding author

Keywords

Encapsulation, humic acid, bacterial alginate, paddy

A B S T R A C T

In this study, encapsulation of a plant growth promoting inoculant *Bacillus megaterium* was attempted with bacterial alginate by enriching the bead microenvironment with humic acid. High viability of the encapsulated bacteria was observed with minimum cell loss upon storage for 5 months. Steady and constant cell release from the bead was observed for 1 week. Successful plant growth promotion of rice by the encapsulated bacteria was also achieved. Feasibility of this improved encapsulation technique is mainly due to the dual benefits of humic acid to microbe and plant and its chemical properties allowing an easy mixing with alginate without interfering in the formation of the alginate gel beads by cross-linking with Ca^{2+} ions. Thus, the encapsulation method described in this study can be effectively used to protect the PGPB inoculum from adverse conditions of the soil for their successful establishment in the rhizosphere.

Introduction

Ability of the microorganisms to produce and release various metabolites affecting plant growth and health is considered one of the most important factors in soil. Plant growth promotion by the PGPR can be either through, phytostimulation, biofertilization, bioremediation, or biocontrol of plant pathogens (Bashan and de-Bashan, 2005; Bloemberg and Lugtenberg, 2001). The ability of phytohormone production, nitrogen fixation, phytopathogen antagonism, cyanogenesis, phosphate solubilization and ACC deaminase activities are the main plant growth promoting related traits beneficial to plant. Soil being a heterogeneous, unpredictable environment, the inoculated bacteria finds it often difficult to establish a niche for survival amongst the competitors and predators.

The immediate response varies considerably depending on bacteria, plant species, soil type, inoculant density, and environmental conditions resulting in a progressive decline in the inoculated bacterial density thereby fails to elicit intended plant response. A major goal of inoculant formulation is to provide more

suitable microenvironment for survival in soil. Formulation of inoculant carrier is an industrial art of converting a promising laboratory proven bacteria to a commercial field product (Bashan, 1998). Immobilization of microbial cells into polymer matrix has proved to be advantageous over direct soil inoculation (Cassidy *et al*., 1996). The main goal of encapsulation of plant growth promoting rhizobacteria is to protect them from harsh soil environment, reduce microbial competition and release them gradually to facilitate colonization of plant roots (Bashan *et al*., 2002; Vassilev *et al*., 2001).

Encapsulation of living cells in polymeric gel is a well established technology in a broad and increasing range of different applications (Park and Chang, 2000). The gel-like matrix allows the cells to remain viable and with its catalytic ability for longer duration. Several studies thus far have used alginate as the encapsulating material as it forms microbeads instantaneously in presence of polyvalent cations by binding the cation to guluronic acid units (Witter, 1996) in one step with sufficient mechanical strength. Moreover, alginate beads are capable of entrapping sufficient number of bacteria (Fenice *et al*., 2000; Zohar-Perez *et al*., 2002). The use of encapsulated cells for environmental applications has several advantages over free cell formulations namely, protection from biotic stresses (Smit *et al*., 1996) and abiotic stresses such as the inhibitory effect of toxic compounds (Cassidy *et al*., 1997), enhanced survival and improved physiological activity (Weir *et al*., 1995), supply of encapsulated nutritional additives (Trevors *et al*., 1993), increased cell densities and preferential cell growth in various internal aerobic and anaerobic zones of encapsulating gel. The encapsulation technologies face the trade-off among several major considerations such as, mechanical stability, controlled release, environmentally responsive, biodegradable, and cost-effectiveness.

Importance of formulation in maintaining the microbial cells or the active ingredient in a metabolically and physiologically competent state in order to obtain the desired benefit when applied to soil is of significant concern. Formulation typically should contain active ingredient in a suitable carrier and additives that will aid in the stabilization and protection of the microbial cells during storage, transport and at the target zone. The development of novel formulations is a challenging task but, regardless of whether the product is newer improved, the product must be stable during storage and transportation, easy to handle and apply, enhance the activity of the organism in the field, be cost-effective and practical.

Bacterial strain, *B. megaterium* (MTCC 3353) for apparent plant growth promoting traits was used in this study. The present study demonstrates an improvement in the encapsulation of plant growth promoting rhizobacteria using bacterial alginate as carrier by supplementation with humic acid to support the bacterial survival. The effect on storage, performance and efficacy of bead encapsulated plant growth promoting inoculum compared to free cell inoculum in promoting the plant growth of rice was also assessed.

Materials and Methods

Bacterial strain, growth and culture conditions

Bacillus megaterium (MTCC 3353) was obtained from Microbial Type Culture Collection, Chandigarh and cultivated in

the recommended culture broth. It was incubated at 30°C for 48 hrs in an orbital shaker (180 rpm). The cells were harvested after centrifugation (4°C, 5,000×g, 10 min) during log phase of growth with a cell density of 4×10^8CFU/ml.

Seed germination bioassay

To evaluate the early plant growth promotion by *B. megaterium*, rice (*Oryza sativa* L.) seed germination bioassay was conducted. The seeds treated with irrigation water served as control. The healthy and uniform sized rice (ADT 36) seeds were surface sterilized with 0.1% mercury chloride for 2- 3 minutes, washed thoroughly with tap water and distilled water to avoid surface contamination. Control treatment received 2 ml of sterile water. The plates were incubated at 28°C.

Bacterial alginate and humic acid

Bacterial alginate was extracted from a mangrove isolate *A.vinelandii* (Lakshmipriya and Sivakumaar, 2013). Alginate solution (2% w/v) was prepared by dissolving it in distilled water with agitation using magnetic stirrer at room temperature and was autoclaved at 121°C for 20 min. Humic acid was extracted from a peat soil sample collected from Indonesia by alkaline extraction (Young *et al*., 2004). Air dried peat soil (200 g) was mixed by shaking with 2,000 ml solution of 0.1MNaOH and 0.1M $Ca(OH)_2$ (1:1) at 270 rpm for 4 h, and centrifuged (12,000×g) for 30 min. The supernatant was acidified with 6 M HCl to pH 1 overnight. The humic acid fraction was filtered on a sintered glass funnel, and dried at 80°C. Humic acid stock solution (10%, w/v) was prepared by dissolving humic acid obtained from soil in deionized water and stored in dark till use.

Preparation of Alginate beads

All the glassware and solutions used in the protocols were sterilized at 121°C for 20 min. Alginate beads were prepared according to the methods outlined in Bashan (1986a) with modifications. Briefly, a mixture of 2.5 ml of 10% humic acid and 750 ml of 30% glycerol were added to 2% bacterial alginate solution to obtain a final volume of 25 ml. Exactly 250 ml of bacterial culture was centrifuged, the cell pellet was washed with saline (0.85% NaCl, w/v) and suspended in 25 ml of alginate-humic acid mixture and mixed thoroughly. This suspension was extruded through a 26-gauze needle drop wise into a pre-cooled sterile 1.5% (w/v) aqueous solution of $CaCl_2$ under mild agitation. The water-soluble bacterial alginate was converted into water-insoluble calcium alginate beads. Thus instantaneously formed beads were allowed to harden for 3–6 h at room temperature. Beads were collected by sieving and were washed several times with sterile water and stored at 4°C in 0.85% (w/v) saline for further investigations. Ten grams of fresh wet beads were frozen at -80°C for 6 h prior to lyophilization at 45°C for 15 h. The lyophilized dry beads were stored in sterile glass bottles for further analysis.

Evaluation of plant growth

The impact of the immobilized microbial inoculant on the germination of Paddy was investigated using trays filled with clay soil. Clay soil was collected from an unpolluted agricultural field.

The soil was transferred in separate trays @ 2 kg/tray, a suitable control tray was also maintained and the trays were irrigated with good quality irrigation

water. The ADT 36 rice seeds 100 nos. already sterilized with 0.1% mercuric chloride were planted in each tray, slightly pressed and allowed to germinate. . The trays were irrigated periodically at every 48 h interval. The percentage of germination was assessed (Rahman *et al*., 2002) at seventh day after sowing (DAS). The number of seeds germinated was counted on 7^{th} day and the germination percentage was calculated and recorded. For assessing the seedling length five seedlings were removed from the tray on the tenth day and washed in water (Yoshida *et al*., 1971).

Results and Discussion

The pH and electrical conductivity (EC) of humic acid was 8.9 and 0.82 mS/cm, respectively. The particle size variation in the humic acid was observed with 60% being of the size 5 mm and remaining 40% of the size range between 0.04 and 0.5 mm. The elemental composition of humic acid was 56%C, 8.3%H, 36.7% of O and 2.6% of N. Fig. 1 shows the external morphology and the cross section of the beads as observed under scanning electron microscope. The uniform distribution of humic acid particles in the beads was also observed. Encapsulation of *B. megaterium* in humic acid enriched alginate yielded beads loaded with significant numbers of bacteria (2×10^8 CFU/g). Lyophilization of the beads resulted in high viability of the cells (1×10^{10} CFU/g) with minimum cell loss. Upon storage of lyophilized beads initial cell densities were retained with minimum cell loss.

The root length of rice was observed in the *in vitro* condition up to thirty days for various treatments as presented in Table Among the different treatments, T_3 (*B.megaterium* – BM + Humic acid) showed a considerable increase in the root length (16.96 cm) and shoot length (24.23 cm) under same experimental conditions after thirty days of transplantation. The least increase in seedling growth was observed in T_1 (control) with 13.17 cm root length and 16.67 cm shoot length. In individual inoculations, T_2 (*B.megaterium* – BM) showed high root and shoot length when compared to other individual inoculations. The effect on germination percentage was studied and the results are presented in Fig. 2. The treatment T_3 recorded the highest germination percentage of 93.33 followed by the other treatments and the least as control. The inoculation with T_3 increased the germination percentage of rice to a higher level compared to control (Fig. 3).

Increased root and shoot elongation was apparent in PGPB treated plants compared to control. Several strains of *B. subtilis* have proven to be efficient in plant growth promotion (Bai *et al.,* 2003; Chanway, 2002). The strain used in the present study also displayed several enzyme activities including IAA production. Several strains of *B. subtilis* strains also have the ability to synthesize phytohormones such as, zeatin, gibberlic acid, and absissic acid in vitro (Tang, 1994). Direct stimulation on plant growth was observed in the present study, wherein, *B. subtilis* treated plants showed increased root growth and shoot growth. The stronger root system leads to an improved uptake of water and nutrients.

It was evident from this study that the growth pattern of the plant In general, basic and the most important requisite of encapsulation are to maintain high cell density with maximum survival even after prolonged storage. In this study no deleterious effects by the supplementation of humic acid on the bacteria was observed.

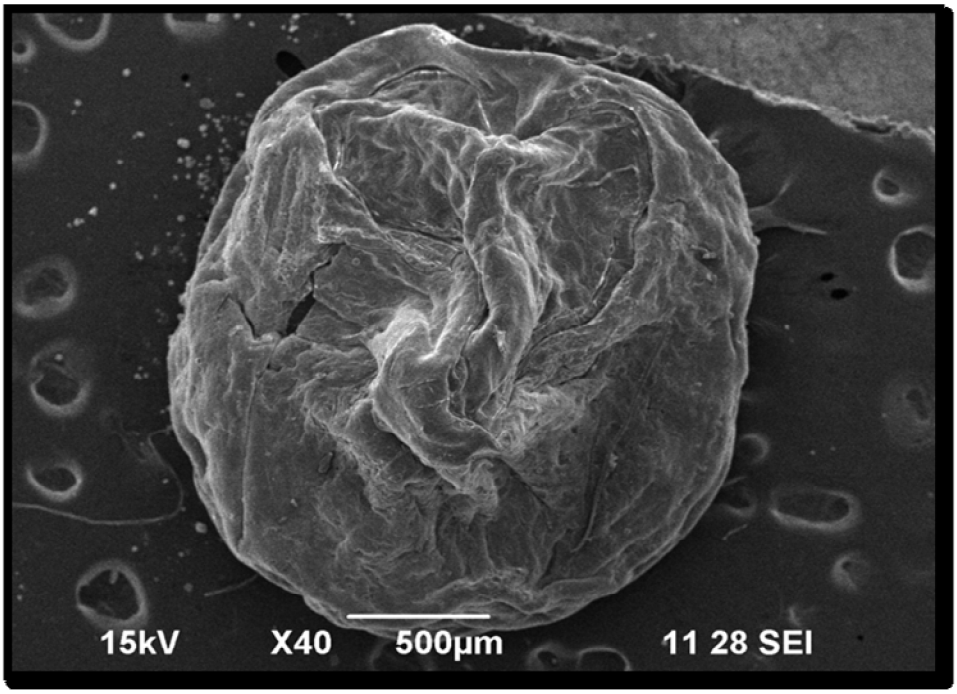


Fig.1 SEM micrograph of bacterial alginate bead without inoculum

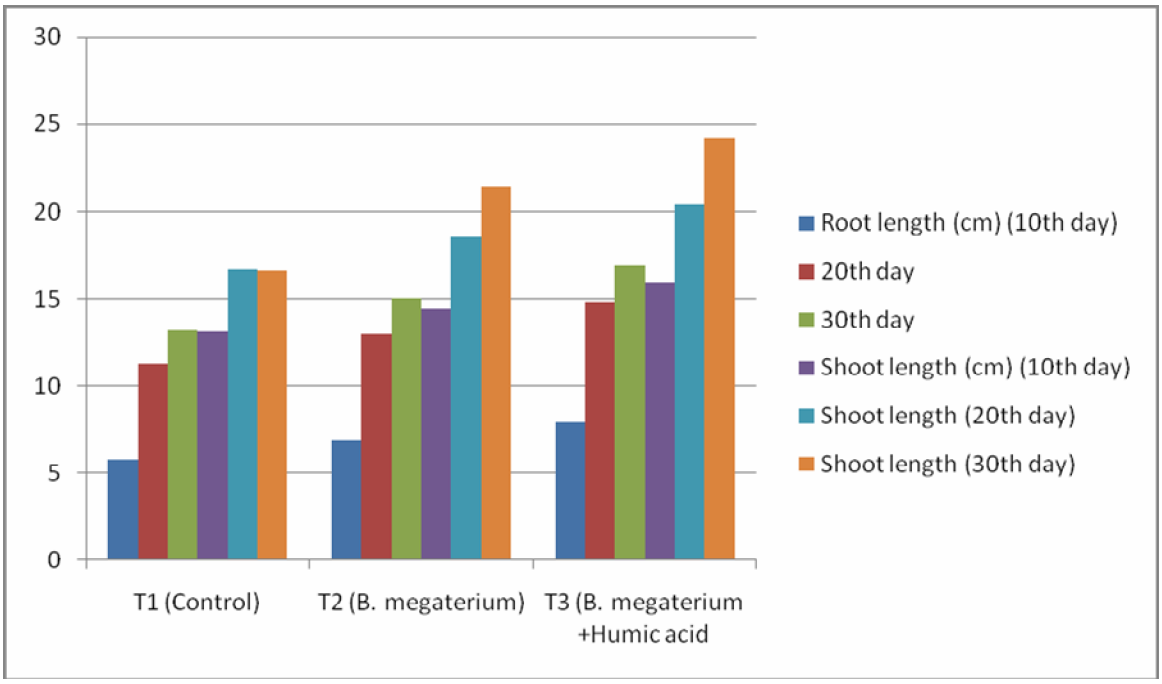


Fig.2 Effect of *Bacillus* sp. cells on root and shoot length of rice ADT-36 *in vitro* condition (Tray culture)

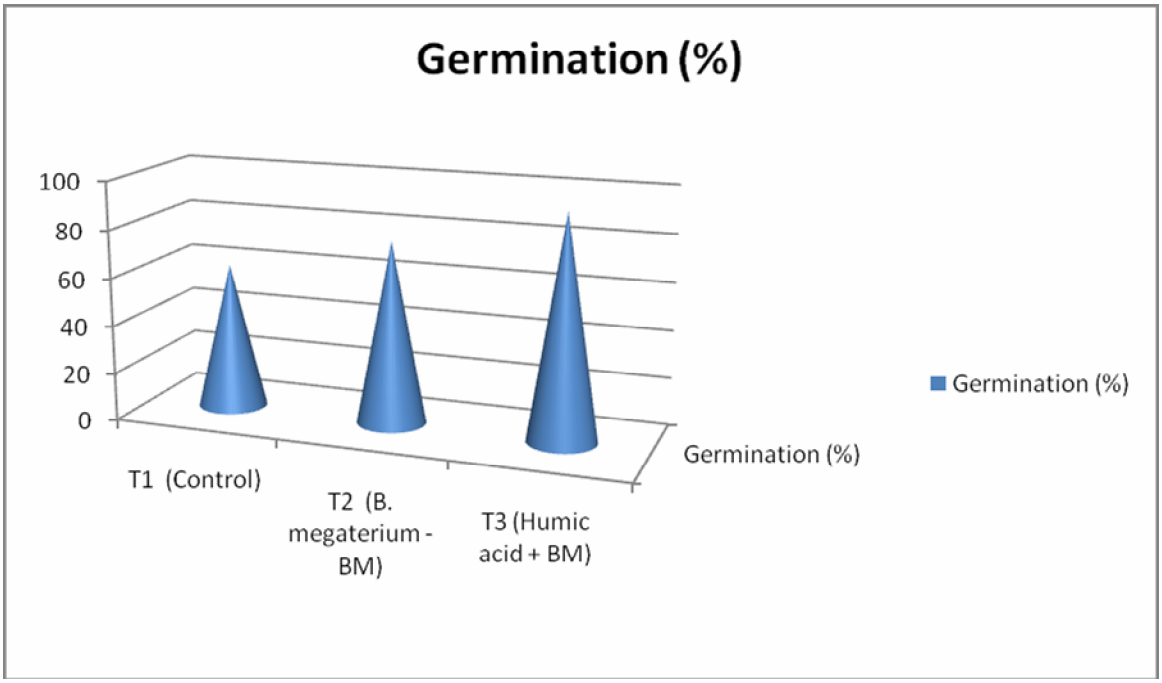


Fig.3 Effect of *Bacillus* on germination of rice ADT-36 (Tray culture)

Even in a few instances it was observed an increased cell numbers in the beads upon storage, which might be due to the porous gel matrix providing the space and the humic acid providing nutrients facilitating the bacterial multiplication within the bead environment. Though in the bead inoculated plants the initial growth was slower, subsequent growth was more vigorous. This may be due to the slow cell release from the beads.

Use of alginate as an immobilizing agent in most applications relies on its ability to form heat stable strong gels, which can develop and set at room temperatures under very mild conditions (Smidsrod and Skjaek-Braek, 1990) and during cross-linking, alginates do not undergo excessive swelling or shrinking, thus maintains its form (Simpson *et al.*, 2003). Further, alginate is the most abundant marine biopolymer, which can be biodegraded in soil (Bashan and Gonzalez, 1999). This alginate gel network supported the bacterial densities in stable numbers even after 5 months of storage. Similar observations on the survival of bacteria even after 14 years in alginate beads were made (Bashan and Gonzalez, 1999; Cassidy *et al.*, 1997). The porous alginate gel matrix as observed in the scanning electron microscope, protects the cells against mechanical stress, facilitates the survival for prolonged storage period as well helps in cell release from the bead. It is also observed that the bacteria tend to occupy the pores present in the gel matrix (Bashan, 1986b).

Additional supplementation of the gel beads with nutrient compounds is presumed to enhance the stability, provides protection and nutrition to the encapsulated cells (Bashan, 1986a). Use of skim milk and clay are among the most applied amendments for gel entrapped soil microbial inoculants that showed better performance in soil (Vassilev *et al.*, 2001).

In this respect, the use of humic acid as a supplementary nutrient for encapsulated PGPB in this study has offered several advantages over the existing enriching compounds. Humic acid in general is the most versatile organic compound. This is mainly due to its natural origin from soil processes, contains chemical structures which can oxidize or reduce elements, photosensitize chemical reactions and enhance or retard the uptake of toxic compounds or micronutrients to plants and microorganisms thereby greatly benefiting plant growth (Bacilio *et al.,* 2003; Nardi *et al.,* 2002).

The feasibility of using humic acid as a nutritional supplement in the present encapsulation technique relies on its polyanionic nature, which facilitate in homogeneous mixing with a similarly charged alginate polymer. In addition, it is expected that humic acid provided the C and N nutrition required for the encapsulated bacteria continuously. A number of recent studies carried out on the application of immobilized, mainly entrapped and encapsulated, cells of microorganisms undoubtedly shows their advantages over traditionally used free cell inoculation in processes such as rock phosphate solubilization and nitrogen fixation (Bashan *et al.,* 2002; Fenice *et al.,* 2000). The potential modification in formulation described here will be useful to agro industry. The versatile nature of humic acid in the soil environment also extends the prospects of this encapsulation technique to the bioremediation of contaminated soil.

Acknowledgement

The authors wish to thank the University Grants Commission (UGC reference No. F 40-122/2011 (SR), dated 04-07-2011), Delhi for its financial support.

References

Bacilio M, Vazquez P, Bashan Y. 2003. Alleviation of noxious effects of cattle ranch composts on wheat seed germination by inoculation with *Azospirillum* spp. Biol Fertil Soils 38:261–266.

Bai Y, Zhou X, Smith DL. 2003. Enhanced soybean plant growth resulting from co-inoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. Crop Sci 43:1774–1781.

Bashan Y. 1986a. Alginate beads as synthetic inoculant carriers for the slow release of bacteria that affect plant growth. Appl Environ Microbiol 51:1089–1098.

Bashan Y. 1986b. Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. Soil Biol Biochem 18:297-301.

Bashan Y. 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol Adv 15:729–770.

Bashan Y, de-Bashan LE. 2005. Bacteria/plant growth-promotion. In: Hillel D, editor. Encyclopedia of soils in the environment. Oxford, UK: Elsevier. Vol. 1, pp 103–115.

Bashan Y, Gonzalez LE. 1999. Long-term survival of the plant growth promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. Appl Microbiol Biotechnol 51:262– 266.

Bashan Y, Hernandez JP, Levya LA, Bacilio M. 2002. Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. Biol Fertil Soils 35:359–368.

Bloemberg GV, Lugtenberg BJJ. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4:343–350.

Cassidy MB, Lee H, Trevors JT. 1996. Environmental applications of immobilized microbial cells: A review. J Ind Microbiol 16:17–101.

Cassidy MB, Lee H, Trevors JT. 1997. Survival and activity of lac-lux marked *Pseudomonas aeruginosa* UG2Lr cells in encapsulated kcarageenan over 4 years at 48C. J Microbiol Meth 30:167–170.

Chanway CP. 2002. Plant growth promotion by Bacillus and relatives. In: Berkeley R, Heyndrickx M, Logan N, De Vos P, editors. B. subtilis for biocontrol in variety of plants. Malden, MA: Blackwell Publishing.pp 219–235.

Fenice M, Selbman L, Federici F, Vassilev N. 2000. Application of encapsulated *Pencillium* variable P16 in solubilization of rock phosphate. Biores Technol 73:157–162.

Lakshmipriya, V.P and P.K. Sivakumaar. 2013.Optimization of certain growth parameters for the production of exopolysaccharides from *Azotobacter* species isolated from mangrove ecosystem. Research J. of Biological Sci., 5(1): 27-33.

Nardi S, Pizzeghello D, Muscol A.Vianello A. 2002. Physiological effects of humic substances in higher plants. Soil Biol Biochem 34:1527– 1537.

Nautiyal CS. 1999. An efficient microbiological growth medium for phosphate solubilizing microorganisms. FEMS Microbiol lett 170: 265–270.

Park JK, Chang HN. 2000. Microencapsulation of microbial cells. Biotechnol Adv 18:303–319.

Rahman, K.S.M., I.M. Banat, T.J. Rhaman, T. Thayumanavan and P. Lakshmanaperumalsamy. 2002. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresource. Technol., 81: 25- 32.

Simpson NE, Grant SC, Blackband SJ, Constantinidis I. 2003. NMR properties of alginate microbeads. Biomaterials 24:4941–4948.

Smidsrod O, Skjaek-Braek G. 1990. Alginate as an immobilization matrix for cells. Trends Biotechnol 8:71–78.

Smit E, Wolters AC, Lee H, Trevors JT, van Elsas JD. 1996. Interaction between a genetically marked *Pseudomonas fluorescens* strain and bacteriophage øR2f in soil: Effects of nutrients, alginate encapsulation, and the wheat rhizosphere. Microb Ecol 31:125–140.

Tang KH. 1994. Environmental soil science. New York: Marcel Dekker Inc. p. 304.

Trevors JT, van Elsas JD, Lee H, Wolters AC. 1993. Survival of alginate encapsulated *Pseudomonas fluorescens* cells in soil. Appl Microbiol Biotechnol 39:637–643.

Vassilev N, Vassileva M, Azcon R, Medina A. 2001. Application of free and Ca-alginate entrapped *Glomus deserticola* and *Yarowia lipolytica* in soil-plant system. J Biotechnol 91:237–242.

Weir SC, Dupuis SP, Providenti MA, Lee H, Trevors JT. 1995. Nutrient enhanced survival of and phenanthrene mineralization by alginate encapsulated and free *Pseudomonas* sp. UG14Lr cells in creosote contaminated soil slurries. Appl Microbiol Biotechnol 43:946–951.

Witter L. 1996. Immobilized microbial cells. In: Baianu IC, Pessen H, Kumosinski TF, editors. Physical chemistry of food processes. New York: Van Nostrand Reinhold. pp 475–486.

Yoshida, S., D.A. Forno and J.H. Cock. 1971. Laboratory manual for physiological studies of rice. *Int. Rice Res. Inst.*, 1**:** 9-12.

Young CC, Su C-H, Li G-H, Wang M-H, Arun AB. 2004. Prospects for nitrogen incorporation into humic acid as evidenced by alkaline extraction method. Curr Sci 87:1704–1709.

Zohar-Perez C, Ritte E, Chernin L, Chet I, Nussinovitch A. 2002. Preservation of chitinolytic Pantoae agglomerans in a viable form by cellular dried alginate-based carriers. Biotechnol Prog 18:1133–1140.