Production of indole-3-acetic acid by immobilized actinomycete (*Kitasatospora* sp.) for soil applications

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Soil isolate *Kitasatospora* sp., an indole-3-acetic acid (IAA) producing strain was encapsulated in calcium alginate bead for soil applications. It was studied for growth pattern culturally, as well as by optical and scanning electron microscopy. The surface of the bead was found to be teaming with mycelial microcolonies showing hyphae formation initially and sporulation by the seventh day of entrapment. The immobilized cells produced higher concentration of IAA over a prolonged period compared to free cells. Similar results were also found when the free and entrapped cells were inoculated in sterile and simulated soil. *Kitasatospora* sp. demonstrated fairly good tolerance to adverse environmental conditions, viz. pH, temperature, salinity and heavy metals, particularly under encapsulation, rendering it suitable for application under variable soil conditions.

Keywords: Encapsulation, indole-3-acetic acid, *Kitasatospora* sp., soil application.

ACTINOMYCETES constitute a significant proportion of the microbial population in most soils, their propagule and spore count being about 10^4–10^6 per gram. The temperate, well-drained soils with neutral to alkaline pH, constituting around 95% of the filamentous actinomycetes as determined by plate spreading are dominated by the genus *Streptomyces*. Rare genera of actinomycetes such as *Kitasatospora* are gaining significance for production of new bioactive compounds and also for identification of unexplored strains using reliable high-throughput techniques[1–4]. Actinomycetes have a long tradition in the research of bioactive compounds. Many species produce a wide variety of secondary metabolites, including anti-helminthic compounds, anti-tumour agents and majority of known antibiotics. Free-living actinomycetes have also been implicated in the enhancement of plant growth by production of plant growth-producing substances such as auxins and gibberellin-like compounds[5–7].

Indole-3-acetic acid (IAA) is the principal form of auxin, which regulates several fundamental cellular processes, including cell division, elongation and differentiation. It also leads to decrease in root length and increase in root hair formation, thus enhancing the capability of the plant to absorb soil nutrients. Besides, there are many developmental processes in which auxin plays a role, including embryo and fruit development, organogenesis, vascular tissue differentiation, root patterning, elongation and tropistic growth, apical hook formation and apical dominance[8–10].

Manulis[6] observed induced synthesis of IAA by six different *Streptomyces* species in the presence of tryptophan and suggested indole-3-acetamide as the main pathway, as *S. violaceus* and *S. exfolitus* catabolized indole-3-acetamide (IAM), indole-3-lactic acid (ILA), indole-3-ethanol (IEt) and indole-3-acetaldehyde (IAAld) into IAA, besides possible presence of other pathways for IAA biosynthesis.

In recent years the method of immobilizing living cells has gained a wide range of applications[11,12]. Encapsulation of microbial cells for soil application provides a range of advantages such as ease of application to the soil, reduced off-site drifting, and protection of cells from environmental stress[13,14]. In addition, they possess high cell-loading capacity, high retention of cell viability, increased rate of production of microbial products and also act as a reservoir, which releases the bacteria at a slow and constant rate[15].

Actinomycetes are known to be sturdy organisms and therefore suitable for soil applications. The spores of most actinomycetes withstand desiccation and show slightly higher resistance to dry or wet heat than vegetative cells. Actinomycetes can colonize dry soil due to their filamentous nature and exist in soil for extended periods as resting arthrospores that germinate in the occasional presence of exogenous substrates[4]. So far, the potential of filamentous actinomycetes in encapsulated state for the production of IAA has neither been fully examined nor used in field conditions to any noticeable extent.

Abiotic soil factors affect the population dynamics of the inoculant, imposing stresses of various nature on the cells. They can also act indirectly, by affecting the activity of the indigenous soil microflora[16]. Typical environmental stresses faced by the organisms in the soil may

include salinity, unfavourable soil pH, extremes in temperature, insufficient or excessive soil moisture, heavy metal toxicity and biocides[17–19].

The purpose of the present study was to evaluate the potential of immobilized *Kitasatospora* sp. as a producer of plant growth-promoting auxin, its ability to grow and remain metabolically active under immobilized conditions, to adapt to various stress and storage conditions, to colonize and survive in the soil.

Materials and methods

Microorganism and growth conditions

Kitasatospora sp., isolated from garden soil previously screened for IAA production was obtained from the Department of Microbiology, Bhavan's College, Mumbai, India. On primary identification using classical methods, the isolate was identified as *Streptomyces* sp. (according to *Bergey's Manual of Systematic Bacteriology*, 1994, vol. 4, 9th edn). However, on molecular identification using 16s rRNA (NCBI, courtesy Nicholas Piramal), the strain was identified as genus *Kitasatospora*. The isolate was grown on Ken Knight agar at room temperature for routine cultivation and maintenance. All growth media selected in the study for actinomycetes were according to the recommendations of Subba Rao[20], and were procured from Hi-Media Laboratory Pvt Ltd, India.

Immobilization of Kitasatospora sp.

Kitasatospora sp. was grown on Ken Knight slants for 48–72 h at 30°C to achieve good sporulation. Uniform spore suspension was prepared in physiological saline supplemented with 0.05% Tween-80 as a surfactant. Spore density was adjusted to approximately 10^6 per ml by measuring absorbance, protein concentration by Folin's method[21] and viable count using Miles and Misra's method[22].

All chemicals and solvents used were of analytical grade and were obtained from Loba Chemicals, Mumbai, India unless mentioned otherwise. For immobilization, 1 ml of spore suspension was aseptically mixed with 9 ml of 3% Na alginate (w/v) in a 25 ml flask and stirred gently for 15 min. With the aid of a 10-ml glass syringe and 18-gauge needle, the cell alginate mixture was added dropwise into gently stirred 100 mM $CaCl_2$ at 30°C. With a total volume of 10 ml of cell alginate mixture, approximately 250 beads were formed, i.e. each bead was formed with 4 µl of original spore suspension and 400 spores were entrapped per bead. The resulting Ca alginate beads (mean diameter 4–5 mm) with the entrapped cells were maintained in $CaCl_2$ at 30°C for an additional 2 h by shaking gently at 50–60 rpm to obtain uniform solid beads, which were then washed twice with distilled water.

The entire procedure was conducted under aseptic conditions.

Growth of Kitasatospora sp. in Ca alginate beads

From the immobilized *Kitasatospora* beads, suspended in 100 ml Waksman broth (nutrient broth + 1% glucose) with 50 mM $CaCl_2$ and incubated on a rotary shaker (180 rpm) at 30°C for 48 h for multiplication, 25 were suspended in 10 ml of physiological saline and the number of cells/propagules within the beads and in the suspending medium was estimated by plate count method on Waksman agar and counts were expressed as number of colony forming units (cfu)/bead. The beads were maintained at 30°C and counts were taken after 0, 2, 4, 7 and 21 days, with change in saline suspension after every estimation. Beads prepared with uninoculated Na alginate were used as control in all steps. As there was confluent mycelial growth of the actinomycetes inside the beads, to measure the cell number, they were first macerated in sterile glass tube with tight-fitting Teflon-coated pestle[23]. Then, 10 ml of 3% tri-sodium citrate was added and shaken gently for 15 min for further dissolution of alginate, followed by dilution and plating on Waksman agar for enumeration. After dissolving 25 beads in 10 ml tri-sodium citrate, protein content of the biomass within the bead was estimated by Folin's method with bovine serum albumin as a standard and the activity of the sample was expressed as microgram per bead.

Scanning electron microscopy

To observe the extent of *Kitasatospora* sp. growth in alginate bead, ten beads were washed in 0.1 M phosphate buffer (pH 7.2) and transferred in 4% gluteraldehyde (Fluka) for 4 h. They were washed in three half-hourly changes of phosphate buffer, which was then completely drained-off and 1% osmium tetra oxide (Sigma) was added and incubated for 3 h. Excess OsO_4 was drained out and phosphate buffer was added to the tubes for 10 min, after which the buffer was drained out and the beads dehydrated slowly through 30, 50, 70, 90 and 100% ethanol (Merck), for 30 min at each concentration. The beads were stored in 100% ethanol till microscopic examination. The entire procedure was performed at low temperature in an ice-bath.

For scanning electron microscopy (SEM), the beads were placed on stubs using double-sided sticker tapes, were sputtered with gold (Polaron SEM-coating unit) and observed under a Philips XL-30 SEM at 10 kV.

Light microscopic examination of beads

Washed beads grown in Waksman broth for 2 and 4 days were placed in 0.5% methylene blue for 15 min and were

embedded in molten paraffin wax kept at 50°C. The paraffin was allowed to solidify and then cut into small cubes, approximately 2 cm^3 sizes. The embedded beads were sectioned into thin slices using a surgical blade. These sections were placed on slides smeared with egg albumin and warmed in an incubator at 60°C for fixation for 15 min. The slides were then observed under light microscope (Leica) at the peripheral, sub-peripheral and central areas for location and growth pattern of the cells.

Production and estimation of IAA

Kitasatospora sp. was immobilized in Ca alginate as described above. Twenty-five beads containing entrapped cells and an equivalent number of free cells (10^5 spores) were inoculated in 50 ml IAA production medium respectively (tryptone 10 g, yeast extract 5 g, NaCl 5 g, tryptophan 0.5 g, 1 l distilled water, pH 7.5) in a 150 ml flask, and were incubated on a rotary shaker (180 rpm) at 30°C. IAA was estimated on days 2, 4, 7, and 21.

IAA extraction: After incubation, the cells/beads were centrifuged at 10,000 rpm for 30 min and the clear supernatant was acidified with 4N HCl to pH 2.8–3.0. Next 0.5 g of activated charcoal was added to the supernatant and shaken for 2 h. Charcoal was separated by centrifugation at 2000 rpm for 10 min. IAA was further extracted with aqueous acetone (16 ml 95% v/v acetone in water). The acetone fraction was air-dried and reconstituted in methanol.

IAA estimation: The procedure of Pilet and Chollet was followed[24], using reagent R1, which contained 12 g FeCl$_3$ per litre in 7.9 M H$_2$SO$_4$. One millilitre of R1 was added to 1 ml of the extract, mixed well and incubated in dark for 30 min at 30°C. Absorbance was determined at 535 nm (Hitachi U 2000 spectrophotometer) against the reagent blank. Concentration of the test was calculated from a standard plot ranging from 0.1 to 10 µ/ml prepared with pure IAA (Sigma).

Effect of stress on free and immobilized Kitasatospora sp.

Kitasatospora spores were immobilized in Ca alginate beads, grown in Waksman broth and washed as described earlier. Ten beads were used for studying each stress. Similarly prepared equivalent number of spores were used as free cells in a volume of 100 µl.

The free and encapsulated cells were subjected to various stresses by suspending them in 5 ml phosphate buffer saline at pH 7.5 (except for pH tolerance), as follows:

Osmotic stress: NaCl at a concentration range 0.1–2 M at intervals of 0.5 M.

Acidity and alkalinity: Buffered saline was prepared at a pH range 4–8.6 using 100 mM acetate buffer for pH 4 and 5; 100 mM phosphate buffer for pH 6–8 and 100 mM tris-maleate for pH 8.6.

Temperature stress: 50°C for 7 h/day for 6 days (stored at 30°C during the remaining period) and 0°C for 1 to 6 months.

Heavy metal toxicity: CuSO$_4$, 0.5–2.5 mM, Cd (NO$_3$), 0.032–0.64 mM and HgCl$_2$, 0.036–0.72 mM.

All stress tests were conducted under sterile conditions and incubation was carried at 30°C for 48 h, unless otherwise specified.

After incubation, the beads were dissolved in minimum amount of tri-sodium citrate (1–2 ml). Then, the cells were washed twice in saline. The sediment was resuspended in 5 ml Waksman broth and survival of the organisms was observed as growth on incubation at 30°C up to one week. Growth could be easily scored visually. However, the quantification was carried out on the basis of relative protein content of the cells after washing them thrice in saline, determined by Folin–Lowry method (with necessary dilutions wherever the growth was greater than the sensitive range of the method).

Growth of Kitasatospora sp. and IAA production in soil

Soil system: Garden soil of clay loam texture pH 6.9, and moisture content 25%, was passed through 4 mm sieve and oven-dried at 105°C overnight. Soil was weighed and distributed in 10 g aliquots in beakers in triplicate for each treatment and sterilized by autoclaving on three consecutive days.

The experiment was designed for two sets of soil – sterile soil and simulated soil.

Preparation of simulated soil: For preparing simulated soil, ten different types of soil microbes (bacteria, including actinomycetes and fungi) were isolated from the garden soil, enumerated, and selected randomly on the basis of their gram nature, morphology and colony characters, such that they could be easily differentiated from the test culture under study. These isolates were introduced in sterile soil approximately in the same numbers as they were encountered in the soil. No attempt was made to identify these isolates. Then 50 µg/g of tryptophan (1/10 of concentration added in the production medium) was added to both sets of soil to enhance IAA production. To include soil phages and other dissolved materials present in the soil, suspension of these cultures was made in soil extract obtained by bacteria-proof filtration of 10% soil solution prepared in sterile water.

Kitasatospora sp. was inoculated in free and encapsulated forms by adding 10^7 spores or 25 beads (with

equivalent number of spores) to each beaker respectively. After adding the inoculants, the moisture content of the soil was maintained at 50% of its water-holding capacity. The whole system was thoroughly mixed, covered with aluminum foil and kept in a humid chamber at 30°C. For every sampling, soil moisture was checked and sterile water was added to compensate the loss[25].

Samples were taken in triplicate from sterile and simulated soils at 0, 30, 60 and 90 days after inoculation for cell count and IAA production by free and immobilized cells. At the same time, the beads from each sample were washed and observed for disintegration by examining under a light microscope (10x) with the following scale: 0, no disintegration; 1, slight visible disintegration on bead edges; 2, one half to three-fourth of the beads degraded; 3, beads fully degraded and not found in the soil. Uninoculated sterile soil was included as control for each sample. This system of observation of beads was adopted from Bashan[14].

Soil samples in the beakers were mixed well and 1 g (in triplicate) from each treatment was evenly crushed and homogenized using tube homogenizer, serially diluted using sterile saline, spread plated on Kenknight and Munaier's agar and incubated at 30°C for 48–72 h. In sterile soil, colonies of only *Kitasatospora* sp. were observed, whereas in simulated soil besides *Kitasatospora* sp. (distinguished as 4–5 mm diameter colonies with greyish spores at 72 h), colonies of other actinomycetes and bacteria were also observed (Figure 1). As fungal growth was not on Kenknight and Munaier's agar; their presence in the system, however, was confirmed by plating diluted soil samples on Rose Bengal chloramphenicol agar.

Estimation of IAA

One gram of soil sample (in triplicate) was suspended in 5 ml distilled water; mixed well in a vortex and centrifuged at 10,000 rpm for 30 min. The clear supernatant was extracted and IAA was estimated as described above.

Experimental design and statistical analysis

Each experiment was set at least in duplicate and repeated three times, where a single flask/tube served as one replicate. Routine controls were prepared similarly where no microorganism was present, in the bead or in soil. Differences due to the experimental factors, encapsulation, aqueous solution for bead preparation, soil preparation, inoculation and sampling depth were considered significant at the 95% confidence level ($P < 0.05$) and are indicated by standard error bars of deviation. Results of all repetitions were combined and analysed by Student's t test, which was used to determine the significance of differences between experiments.

Results and discussion

The genus *Kitasatospora* (formerly *Kitasatosporia*) has had a short but turbulent taxonomic history. The taxon was proposed in 1982, subsequently subsumed within the genus *Streptomyces* in 1992, re-established in 1997 and thereafter recognized in subsequent studies. The genus belongs to the family Streptomycetaceae, together with the genera *Streptacidiphilus* and *Streptomyces*. *Kitasatosporae* are aerobic, Gram-positive, chemo-organotrophic actinomycetes that form an extensively branched substrate mycelium and aerial hyphae that differentiate into long chains of spores and typically produce whole-organism hydrolysates that are rich in galactose and LL- and *meso*-diaminopimelic acid[26]. Due to high similarity with *Streptomyces*, the test culture was initially identified as *Streptomyces* sp. However 16s RNA identification results showed high similarity with *Kitasatospora* sp. In fact, a 100% homology was found with six different species, viz. *K. paranensis*, *K. arboriphila*, *K. terrestris*, *K. gansuensis*, *K. kifunensis*, and *K. nipponensis*. Recent studies have shown that 16s RNA identification is not adequate to differentiate between closely related species of this genus and techniques based on partial RNA polymerase β-subunit gene (*rpoβ*) sequences[2] and microbial diagnostic microarrays (MDMs)[27] have been used for effective differentiation between species and strains of genus *Streptomyces* and *Kitasatospora*.

Growth of Kitasatospora sp. in Ca alginate bead and cell leakage in the suspending media

Traditional methods for assessing the viable cell count of microbial groups have been based on enumerating cfus. As the population of *Kitasatospora* sp. includes spores, mycelia and other resting forms, viable count alone may not give accurate results; therefore, protein estimation was also performed to attain a more dependable growth correlation.

Growth of *Kitasatospora* sp. in microbial beads, as observed by viable count and protein estimation shows an

Figure 1. Growth of *Kitasatospora* spp. in sterile (left) and simulated (right) soil.

increase in count by about two log cycles in 2 days, i.e. from 4.4×10^3/bead to 2.1×10^5/bead with a further increase to 7×10^5/bead in another 48 h (Figure 2). Thereafter, the count was stable up to 7 days, but showed significant reduction to 6.8×10^3/bead in 21 days.

As the objective of the present study was to develop a method for long-term release of microbial flora in the soil, cells released in the suspending medium were also enumerated. The mycelium was visibly discernible (1.5×10^3/ml) in the suspending medium from the second day onwards (Figure 3), which became copious from the fourth day, obviously due to the release of cells from micro colonies from the bead surface. This could also be correlated with increased viable count of 4.4×10^7/ml and high count was maintained at 2.6×10^6/ml even after 3 weeks.

The release of the mycelium in the suspending medium indicated continuous growth of cells in the beads and rupture of Ca alginate membrane. This, however, did not lead to any noticeable reduction in size of the bead,
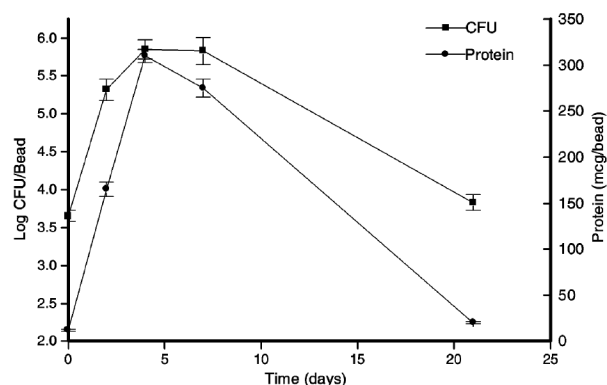

Figure 2. Viable count and protein content of immobilized *Kitasatospora* spp.
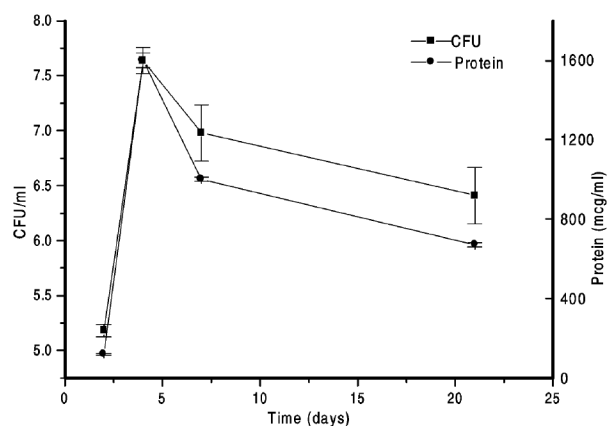

Figure 3. Viable count and protein content of cell leakage from Ca alginate beads.

which was contrary to the report of dissolution of beads after a certain period of time[28].

Besides actinomycetes, we had studied immobilization of *Azotobacter vinelandii*, *Bacillus subtilis*, *Penicillium solitum* and *Gibberella fujikuroi* for soil application (data not shown here). All beads encapsulating filamentous microorganisms appeared tougher in comparison to encapsulated non-filamentous organisms. This is probably due to the mycelial network formed in Ca alginate giving structural strength to the beads, making them perfect candidates for environmental application.

SEM of the bead surface

The immobilized *Kitasatospora* sp. kept in Waksman broth for 48 h showed the formation of numerous bulges all over the bead surface, as seen in the scanning electron micrograph (Figure 4 *a*). At higher magnification (3500x), profuse mycelia growth was observed (Figure 4 *b*). Mahmoud and Rehm[29], in their study on morphological examination of immobilized *Streptomyces aureofaciens* during chlorotetracycline production, also observed the bulge as a hilly surface each made of one micro-colony of mycelia coated with Ca alginate membrane-like structure. Similar results were reported by Bhasan[14] on immobilized *Azospirillum* and *Pseudomonas* for environmental applications.

Light microscopic studies of the bead sections

Examination of thin sections of the beads under a light microscope after 2, 4, and 7 days showed confluent mycelial growth at the periphery of the bead, while the central area was practically devoid of cellular forms. Figure 5 *a* and *b* shows dense hyphal growth after 2 and 4 days respectively, with no conidia formation and Figure 5 *c* shows mycelial branching as well as presence of spores in the bead. Formation of dense layer near the bead surface has been reported for several organisms, indicating that the cells grew at the place where optimal nutrients and oxygen were available for growth[29,30].

One of the criteria for the selection of inoculants for soil application was to withstand environmental adversities; they would either encyst or sporulate to survive unfavourable conditions. Light microscopic photographs at various stages of incubation indicated that when in alginate beads, sporulation was delayed to 7 days compared to normal laboratory conditions, where usually actinomycetes sporulate in 48 h. This was confirmed by SEM observations which showed no signs of sporulation up to 48 h. The 3D effect of scanning brought to life the intensity of growth plus the magnitude of microbial cells that can be delivered into the soil for its intended application by way of these small 4 mm beads.
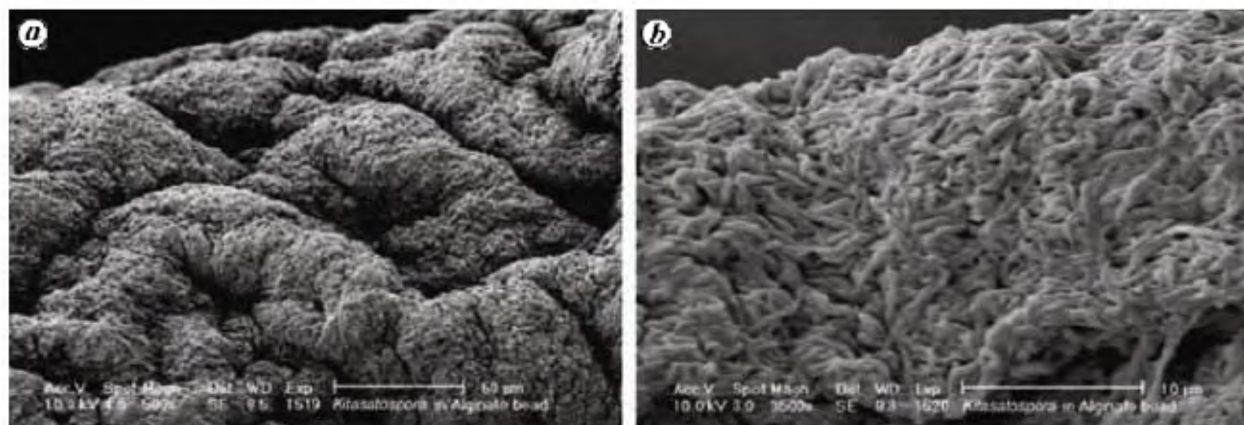
Figure 4. SEM of immobilized *Kitasatospora* spp. showing (*a*) bulges and (*b*) mycelial growth on bead surface.
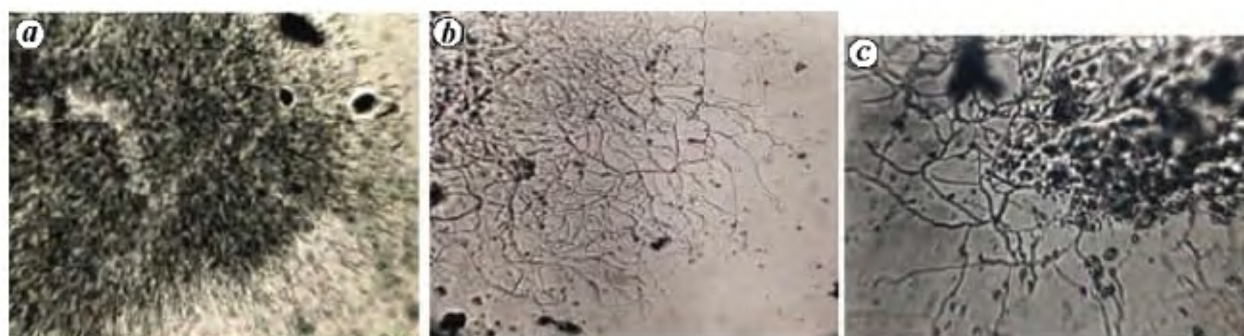

Figure 5 *a–c.* Light microscopic pictures of immobilized *Kitasatospora* spp. *a,* 2 days, 10X; *b,* 4 days, 40X; and *c,* 7 days, 100X.

Production of IAA

The highest rate of IAA production was 1.5 and 2.7 μg/ml, which was observed on the fourth and sixth day under free and immobilized conditions respectively (Figure 6). This was found to coincide with the sporulation phase of the culture under immobilized condition. IAA produced by *Kitasatospora* sp. under immobilized condition was much greater than that by the free cells and higher concentration of IAA was maintained, i.e. 1.8–0.8 μg/ml respectively, up to a study period of 28 days. The increase in productivity seems to have resulted due to increased cell densities at the periphery and stabilized enzymatic activities. Regeneration of the system also opens up the possibility of continuous and prolonged production by the immobilized cells. Borthakur *et al.*[31] and Ogaki *et al.*[32] obtained similar results while working with immobilized *Frankia* sp. and *S. rimosus* for the production of nitrogenase and oxytetracycline respectively.

Tolerance to environmental stress

Kitasatospora sp., when subjected to various environmental stresses were found to withstand them fairly well (Figure 7 *a–d*).
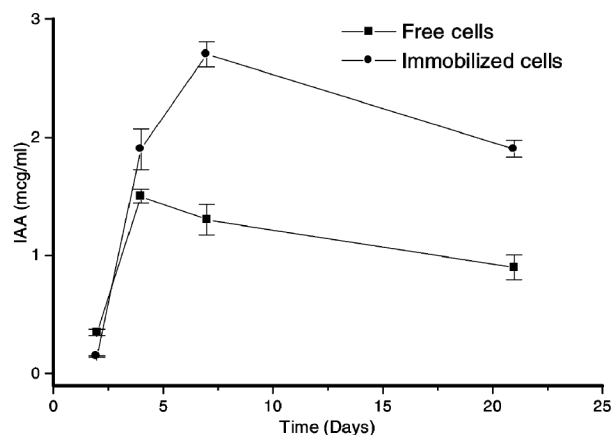

Figure 6. Production of IAA by immobilized *Kitasatospora* spp.

pH: In agreement with the tolerance range reported for other actinomycetes[4], *Kitasatospora* sp. could survive high (9.0) but not low pH (4 and 5) in free state. However, encapsulation offered great protection against pH variation to the cell, particularly at the lower range.
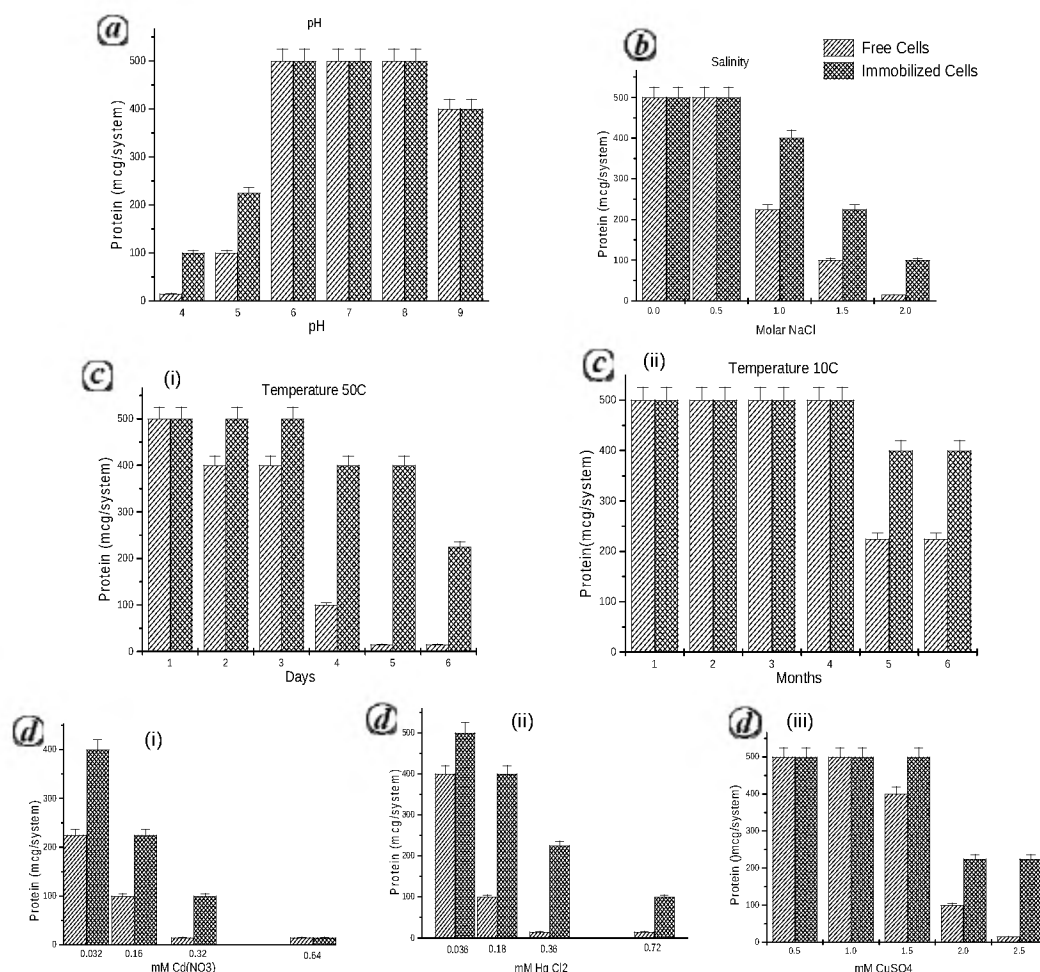
Figure 7. Sensitivity of free and immobilized *Kitasatospora* sp. to pH, salinity, temperature and heavy metals stress.

Salinity: The inoculant could well tolerate high concentration of NaCl, i.e. up to 1.5 and 2.0 M under free and immobilized conditions respectively; thus entrapment enhances adaptation to salinity. Killhamt and Firestone[33] have suggested that actinomycetes are of great importance in saline soils and in environments of high alkalinity since they become an increasingly dominant component of the soil microbiota during prolonged drought.

Temperature: Stress at two extreme levels of temperature was selected for diverse purposes. In field, in most soils where the microbial activity usually occurs, the temperature does not fluctuate more than a few degrees Celsius in any season of the year[34]. However, in many areas in India, the temperature of the upper soil may reach 45–46°C during summer. Between irrigation, therefore the inoculant may be subjected to elevated temperatures and if it can survive the stress, this would be an added attribute for selection of strain. From Figure 7 *c*(i), it is evident that immobilized *Kitasatospora* sp. survived

a daily 7 h high temperature exposure up to 6 days as against 4 days by free cells. Survival of *Kitasatospora* sp. at low temperatures was studied keeping in mind the storage period of the culture between inoculant preparation in the laboratory and actual field applications. It could survive at 10°C up to 6 months, in both free as well as immobilized form.

Heavy metal: In conformity with other stresses, immobilized culture was found to survive the heavy metal stress better than the free cells. It could tolerate $CuSO_4$, $Cd(NO_3)$ and $HgCl_2$ concentrations up to 2.5, 0.32 and 0.72 mM respectively, as against free cells that could also withstand the presence of these heavy metal concentration at 2, 0.16 and 0.18 mM respectively. Thus the sequence of metal toxicity was found to be Cd > Hg > Cu, which varies slightly from that reported by Abbas and Edwards[35], who have studied the effect of metals on a range of *Streptomyces* and have shown the toxicity sequence as Hg > Cd > Cu (Figure 7 *d*).

In agreement with the reports on resistance to environmental stresses[36], the results obtained above proved that this *Kitasatospora* sp. strain was capable of withstanding a wide range of variation in its habitat. This was more pronounced when they were encapsulated in the alginate matrix, as also reported by several others. Young *et al.*[37], in their study on PGPB isolate *Bacillus subtilis*, have similarly proposed the effective use of encapsulated cells in the soil to protect them from adverse conditions. Yong *et al.*[38] studied the survival of Ca alginate-immobilized lactic acid bacteria under different stress conditions, including pH, H_2O_2 and storage period, and found that immobilized cells in alginate bead were more resistant than free cells to adverse environmental conditions. Trevors *et al.*[39] reported a relatively stable population dynamics of encapsulated *P. fluorescens* cells when subjected to wet/dry cycles in soil microcosm, which severely affected the free cell viability and reduced their counts by two log units.

One of the important advantages of immobilization has been in the protection of cells from the external environment perturbation in physical stress such as freeze–thawing, wet–dry cycles, phages and toxic compounds in the effluents. These are of great significance in the environmental applications of immobilized cells. The protective effects have been suggested to be due to absorption of toxic compounds by the matrix, the restricted diffusion of toxic compounds and macromolecules like proteins and phages, or alterations in the membrane composition of immobilized cells[40].

Survival of Kitasatospora sp. in sterile and simulated soil

Growth of a microbial culture and its activity in the natural environment may be different from that under labora-tory conditions. To explore this, soil systems were used where the survival of *Kitasatospora* spp. and its IAA production potential was studied over a period of 3 months.

Similar growth patterns were observed for free and encapsulated *Kitasatospora* spp. in sterile and simulated soil (Figure 8), indicating that soil microflora does not affect its growth. The viable count was maximum up to 30 days and declined gradually over the next two months; however, the counts were still significantly high, to a tune of 10^7 cfu/g of soil.

Omar and Abdul Sater[41] have also reported increase in counts of actinomycetes population used for field application in soil in the ten-week study. Wellington *et al.*[42] have made similar observation in their study with *S. lividans* and *S. violaceolatus*. They also noted that the inoculants survived mostly as spores and the dynamics of survival depended on nutrient status, besides initial inoculum levels and inoculant ratios.

Viability of the cultures in the beads coincided with the visible form of bead deterioration (Figure 9). As expected, the mycelial beads showed only partial degradation up to two months. Disintegration was more accelerated in the second month in simulated than sterile soil, perhaps due to the presence and activity of other microflora. Thus the immobilized inoculants would be released into the soil over a period of two months, which is sufficient for any field crop to release effective produce.

In a similar experiment while studying the degradation of *Pseudomonas* beads in sterile soil, Bashan[14] has explained the process of bead deterioration in soil, which largely depends on soil microflora. The higher the density of the microflora surrounding the bead, the faster is the biodegradation. Biodegradation is carried out from the outside layer inward, thus sequentially exposing different layers of the bead to the surrounding soil until degradation of the bead is complete.
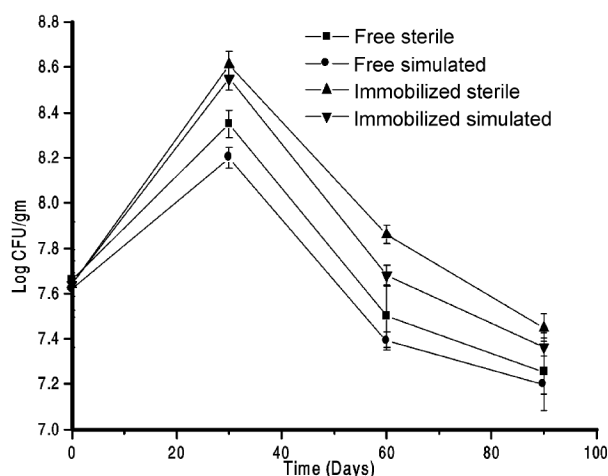

Figure 8. Growth of free and immobilized *Kitasatospora* spp. in oil.
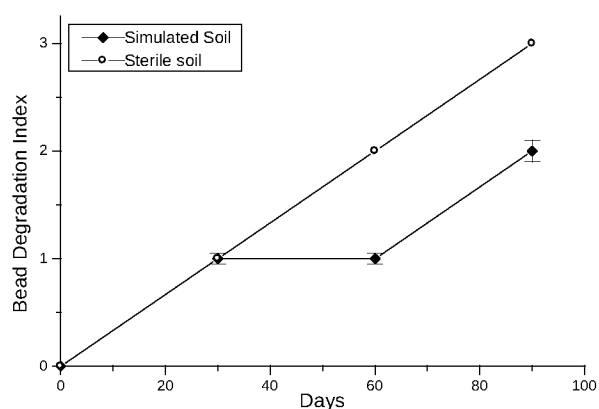

Figure 9. Deterioration of alginate beads in sterile and simulated soil. Degradation index. 0, No disintegration; 1, Slight viable disintegration on bead edges; 2, One half to three-fourth of the bead degraded; and 3, Beads completely degraded and not recovered from the soil.
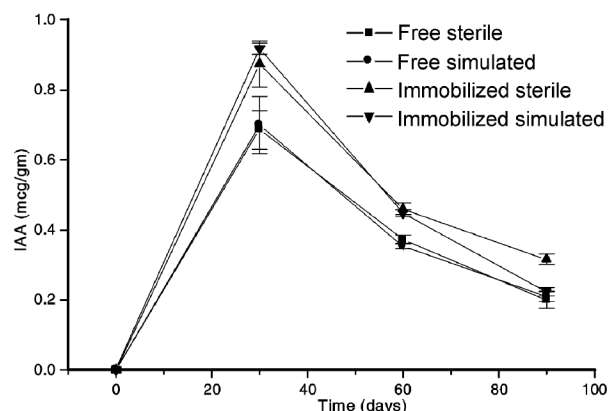
Figure 10. Production of IAA by free and immobilized *Kitasatospora* spp. in soil.

IAA production by Kitasatospora sp. in soil

Peak activity of *Kitasatospora* sp. in terms of IAA production was obtained after one month in soils, which then gradually reduced during subsequent months (Figure 10). The immobilized inoculants compared to free cells demonstrated significantly greater activity (0.87 and 0.92 mcg/g in sterile and 0.68 and 0.7 mcg/g in simulated soil respectively) at the end of one month and correspondingly higher activity levels (0.46 and 0.45 mcg/g in sterile and 0.37 and 0.35 mcg/g in simulated soil respectively) at the end of two months; most likely due to slow and constant release of cells from the alginate beads. Reduction in activity was apparently due to decline in cell population as well as depletion of the easily utilizable soil nutrients, particularly tryptophan which enhances IAA production[9].

Conclusion

Efficient inoculum technology should ensure survival of the inoculant in field under stress conditions. Production of IAA by *Azotobacter* sp. was also studied in broth under similar conditions as *Kitasatospora* sp. (data not shown here). It was found that *Azotobacter* produced greater amounts of the growth hormone. Wheeler *et al.*[43], and Strzelczyk and Burdziej[44], have made similar observations on apparently low production of IAA by actinomycetes compared with non-filamentous bacteria. However, considering the low levels of requirement of IAA by the plants[9], and the advantages of using actinomycetes over non-filamentous bacterial cells such as structural strength of the beads due to mycelial form, better resistance to environmental adversities and survival and multiplication in soil in field conditions for a long period of time, they appear to be ideal candidates for application as biofertilizers. Moreover, non-filamentous bacterial inoculants, viz. *Azospirillum* and *Pseudomonas* have been reported not to survive for long period in soil[14,45]. Therefore, just persis-

tence of plant growth hormone producing *Kitasatospora* spp. in soil in large numbers may be advantageous in the long run. It may serve as inoculum for subsequent crops. With growth and proliferation of roots, nutrients released in the rhizosphere will be accessible, leading to germination of dormant spores, multiplication and biosynthesis of IAA. Further, after production of bioactive compounds from the microbes in the laboratory, they can be processed for field applications. This, however, requires downstream processing, purification, crystallization, etc. that may increase the cost of the product. Instead, PGRP producing viable cells that can withstand environmental stresses can be directly applied in the vicinity (rhizospheres) of plant growth. This will provide a system which will generate the plant growth promoting compounds at a sustained rate over a period of time, right from the germination of seeds to the differentiation of the root system. Thus, the filamentous actinomycete, *Kitasatospora* encapsulated in Ca alginate appears to be a suitable candidate.

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