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Immobilized *Frankia* spores remained viable on dry storage and on restoration to medium regenerated active colonies

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Abstract

Spores of *Frankia* strain ACN^{1AG}, immobilized in calcium alginate beads, germinated to produce colonies that increased in protein content and showed nitrogenase activity. Air dried immobilized spores remained viable for at least 15 days in dry condition, making the storage and transport of *Frankia* strains easy. This also opens the possibility of using beaded spores as inocula.

Introduction

Frankia is a pleomorphic N_2-fixing actinomycete which forms symbiotic association with a number of woody dicotyledonous plants (Normand and Lalonde, 1986). Association of *Frankia* with actinorhizal plants is economically important in forestry and land reclamation (Diem and Dommergues, 1990; Normand et al., 1988). It has been reported that the metabolic activity and efficiency of immobilized cells may change as a result of immobilization due to stabilization of cellular activities (Webb and Mavituna, 1987). Also growth within alginate beads depends on the initial amount of cells encapsulated (Benoist et al., 1992; Diem et al., 1989; Frioni et al., 1994). Encapsulation of *Frankia* in alginate beads may provide growth conditions that permit cultures to retain high infectivity during prolonged storage (Bashan, 1986; Diem et al., 1988). The present study investigated the viability of *Frankia* spores immobilized in alginate beads and the ability of regenerated colonies to reduce N_2. Such a system offers the possibility of transporting the desired strains with relative ease for use as inocula.

Materials and methods

Frankia strain ACN^{1AG}, (LQ 0102001007: Lalonde, 1979) was obtained from the Lyon collection, France and was cultured in defined propionate medium (DPM) without nitrogen (Baker and O'Keefe, 1984). For experimental purpose liquid medium was inoculated with 15 day old ACN^{1AG} grown in batch culture and kept in the dark at 28±1 °C with occasional shaking. After 30 days, filter sterilized ampicillin (2 $\mu g\ mL^{-1}$) was added to the culture medium to induce profuse sporulation (Ganesh, 1993). Spores (11-20 sporangia/colony) were seen with a phase contrast microscope after 7 days of incubation with ampicillin. After 15 days, 5 mL of culture was filtered through a 2.5 μm glass filter (2.5 cm diameter, Sigma, USA) to remove *Frankia* hyphae. The spore size of *Frankia* is about 1 μm (Tjepkema et al., 1980) so the filtrate was then filtered through a 0.9 μm glass filter (2.5 cm diameter, Sigma, USA). Spores retained on this filter were shaken free into 5 mL DPM. The spore density mL^{-1} was estimated using a haemocytometer. One mL of spore suspension was inoculated into 24 mL fresh DPM. The spore density was kept uniform in all cases for further studies. The viability of spores of size <0.9μm was checked by inoculating 25 mL of fresh DPM with the filtrate, incubating at 28±1 °C in the dark with occasional shaking and observing under a phase con-

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trast microscope every alternate day to check spore germination. Growth (increase in protein content, μg mL^{-1}) and nitrogenase activity (acetylene reduction assay, ARA) were also checked.

For protein estimation (Bradford, 1976), 5 mL of liquid culture was centrifuged at 10,000 × *g* for 10 minutes, the pellet resuspended in 5 mL of distilled water and sonicated at 23 kHz in two flushes of 30 seconds each in a Soniprep 250 MSE.

The nitrogenase activity was estimated by ARA using a gas chromatograph (Stewart et al., 1968). Five mL of liquid culture was placed in a 15 mL serum vial and 1 mL air was replaced with 1 mL of pure acetylene. The vials were incubated for 4 h at 28±1 °C with occasional shaking. The ethylene produced in each vial was determined using a Tracor 540 GC with a Porapak "T" column and a flame ionization detector.

For immobilization experiments, two sets of 4% (w/v) sodium alginate solution were prepared in DPM by warming the solution in a water bath. Three mL of the retained spore suspension was added to 47 mL of the sodium alginate solution for one set and 3 mL of final filtrate to the other after the solutions cooled down to room temperature. The mixtures were mixed thoroughly and, using a sterile syringe canula, they were added drop wise into 1% calcium chloride solution in a laminar flow cabinet. Calcium alginate beads (2 mm diameter) formed in the $CaCl_2$ solution were left at 4 °C for 30 minutes for hardening. They were then harvested and washed thoroughly with distilled water and sterile DPM before transferring to DPM. These flasks were kept at 28±1 °C in the dark for further experiments.

Twenty beads from each flask were analyzed for nitrogenase activity and protein content on the first day and then at 5 day intervals for 40 days. After the nitrogenase activity was measured, the same beads were dissolved in 5 mL 0.1 *M* citrate buffer (pH 7.2) at 40 °C in a water bath for 2h, centrifuged at 10,000 × *g* and protein estimated in the pellet by Bradford's method.

Growth was measured in the form of increase in soluble protein content. Observations were recorded at intervals of five days, starting on day ten, when detectable quantities of protein were recorded for all the samples. Since the initial values of the protein, and therefore the biomass, were different for different samples, values were unitized keeping the initial protein content (day ten) as the base in each case, using the

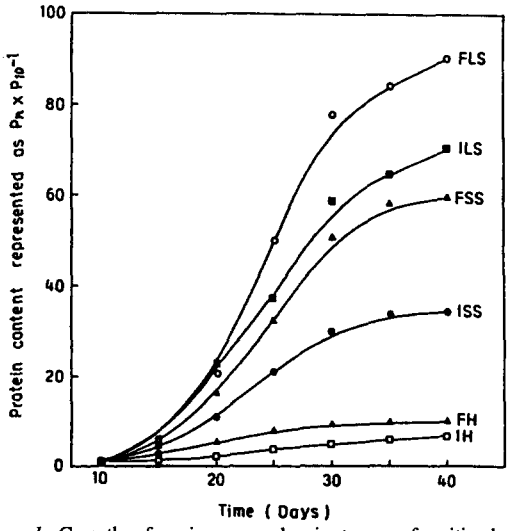

Figure 1. Growth of various samples in terms of unitized values of protein (see Materials and methods for details). (FLS=free larger spores, ILS=immobilized larger spores, FSS=free smaller spores, ISS=immobilized smaller spores, FH=free hyphae and IH=immobilized hyphae).

formula:

$$\text{Unitized protein content} = P_n \times P_{10}^{-1}$$

The percent increase in protein content for different time intervals was estimated as follows:

$$\%\text{ increase in protein content} = (P_n - P_{n-5}) \times P_{n-5}^{-1} \times 100$$

where P is absolute protein content on nth day and n represents 10th, 15th, ... 40th day respectively.

Results

Growth

Figure 1 shows the growth patterns, as measured in terms of soluble protein contents, for different samples. The curves are based on the unitized values as described earlier. Maximum growth per unit soluble protein was recorded for free larger (>9 μm) spores (FLS) followed by immobilized larger spores (ILS), free smaller (<9 μm) spores (FSS), immobilized smaller spares (ISS), free hyphae (FH) and immobilized hyphae (IH) in that order.

Growth for different samples peaked during different time intervals as presented in Figure 2. Free hyphae, free larger spores and immobilized larger

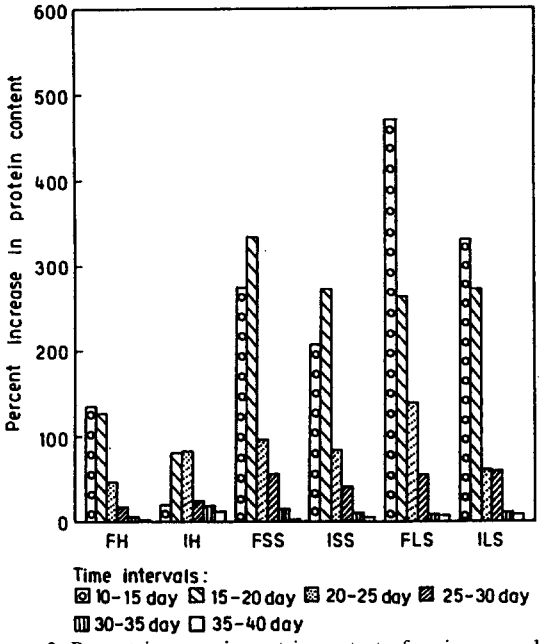

Figure 2. Percent increase in protein content of various samples for different time intervals (see Materials and methods for details). Abbreviations are the same as in Figure 1.

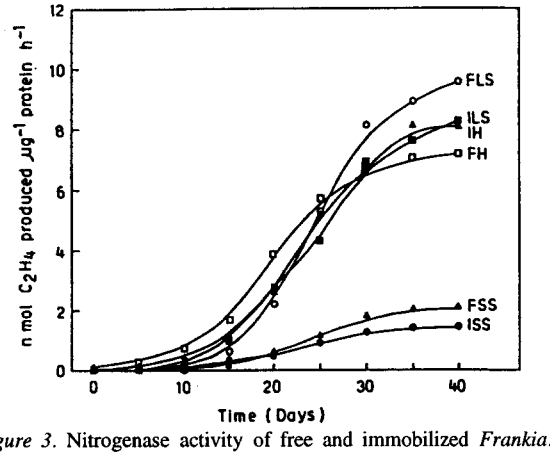

Figure 3. Nitrogenase activity of free and immobilized *Frankia*. Abbreviations as in Figure 1.

spores showed maximum growth during the 10-15 days interval. Growth of free smaller spores and immobilized smaller spores peaked during the 15-20 days time interval. Growth of immobilized hyphae peaked during the 15-25 days dual interval.

Nitrogenase activity

The data on nitrogenase activities of various samples are presented in Figure 3. A comparison of free and immobilized conditions of different samples indicated that the nitrogenase activities in free conditions were higher compared to the corresponding immobilized states during the later phases (day 25 onward), except in the case of hyphae. The nitrogenase activities of free and immobilized hyphae were higher than the corresponding states of larger spores in the initial periods only. By the thirtieth day the nitrogenase activity shown by free larger spores was much higher compared to the corresponding state of hyphae. A notable observation is a positive influence of immobilization on nitrogenase activity of hyphae during the later phases.

Viability of air dried immobilized Frankia *spores*

When alginate beads with *Frankia* spores were air dried for 15 days under sterile conditions, they acquired the size of mustard seeds after drying (Fig.4). These dried beads, when put in sterile DPM, started to swell back to their original size and after 25 days of inoculation in the liquid medium showed emergence of *Frankia* hyphae. These new *Frankia* colonies showed an increase in protein content and in nitrogenase activity indicating growth and viability of these air dried immobilized spores (data not shown).

Discussion

Our results and those of Ganesh (1993) show that addition of ampicillin to the growth medium creates unsuitable growth conditions for *Frankia* which promptly responds by profuse sporulation. The spores when transferred to fresh DPM devoid of ampicillin produced new colonies at a much faster rate than the free hyphae. The size of the *Frankia* spores is about 1 μm (Tjepkema et al., 1980), so the spores retained on the 0.9 μm filter were the ideal size spores which gave rise to viable colonies under suitable growth conditions. The spores that were smaller than 0.9 μm (present in the filtrate), when suspended in the growth medium showed slower growth rate than the retained spores in liquid media. This may be due to the fact that these smaller spores were not mature and were still in the process of developing at the time of spore separation. Therefore, many of them could not give rise to viable *Frankia* colonies within 15 days. However, after 15 days of incubation, significant growth was seen. Their growth in an immobilized condition was found to be even lower (Fig. 1). This may be because these immature spores could not tolerate the restricted growth conditions and limited availability of nutrients in the beads.

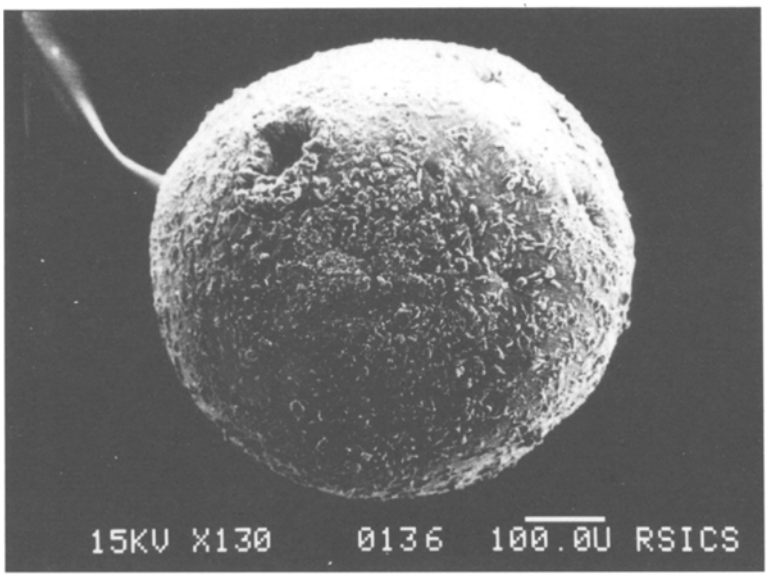

Figure 4. Scanning electron micrograph of 15 day old air dried alginate bead.

As mentioned earlier, the spore density mL^{-1} was uniform in all cases. The initial spore count was 4×10^6 spores mL^{-1}. One mL of this was diluted to 25 mL and since 3 mL of diluted spore suspension was added to 47 mL of alginate solution, (see Materials and methods), the spore count mL^{-1} was reduced to 9600 mL^{-1}. We obtained 100 beads mL^{-1}, therefore the average number of spores per bead was 96.

Metabolic activities of the cells and their efficiencies may undergo changes during immobilization. Further, immobilizing microbial cells leads to increased biocatalytic capacity due to increased densities and stabilized enzymatic activities. This opens up the possibilities of continuous operation (Brouers and Hall, 1986). Therefore, we performed immobilization experiments on *Frankia* cultures to study the changes in their growth pattern and nitrogen fixing capabilities under immobilized conditions. The larger spores (>0.9 μm) under immobilized conditions took about 7 days of incubation before showing any growth. This incubation period was probably necessary to overcome the initial shock of immobilization and also to acclimatize to the restricted availability of nutrients from the medium through the calcium alginate surrounding them. Although the growth of the large spores was less in immobilized condition compared to the free condition (Fig. 1), it was nevertheless much higher compared to free or immobilized hyphae. Therefore, though immobilization may cause a slight reduction in growth rate, the advantages associated with ease of its handling are obvious.

As for nitrogenase activity, much higher levels were attained by the larger spores by the 30th day, although they had lower activity initially. This may be due to the initial presence of the nitrogenase enzyme in hyphae, while it was absent in the spores and it required some time before the genes responsible for nitrogenase production could be expressed in the hyphae generated by spore germination. But once nitrogenase was produced, its activity, as expressed in terms of ARA, increased rapidly in newly produced hyphae.

The fact that *Frankia* spores stayed viable within alginate beads for at least 15 days in an air dried state, makes transportation of *Frankia* strains in the form of alginate beads much easier. They could be used to transport efficient strains that could be used as inoculant for improving nitrogen fixing potential of actinorhizal plants in areas with generally poorer microsymbionts. However, further investigations are required to ascertain the impact of releasing alginate beads in the soil on nodulation frequency.

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