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An Industrial View of *Azospirillum* Inoculants: Formulation and Application Technology

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Abstract

Azospirillum is probably the most studied non-symbiotic N_2-fixer because of its well-known properties of root-growth promotion demonstrated on a very broad range of plant species.

The inoculum formulation and application technology are obviously important parameters conditioning the consistency of field results. They are crucial for the development of commercial *Azospirillum* inoculants, which have been slow to make a significant impact on the market. Optimization of biomass production and downstream processes of formulation are examined for peat-based products and polymer-encapsulated inoculants. The usefulness of a dehydration process related to the problem of survival during storage for a maximum shelf-life is discussed. The advantages and drawbacks of the main application methods, slurry pre-mix with seeds, seed coating, delivery of microgranules and liquid spreading are presented. Finally the industrial, marketing and legal requirements for a successful commercial life of an *Azospirillum* inoculant for crops are introduced.

Keywords: *Azospirillum*, inoculant, formulation, industrial process, application technology

1. Introduction

Bacteria of the genus *Azospirillum* are probably the non-symbiotic plant-growth promoting rhizobacteria (PGPR) that have been the most studied over the last fifteen years.

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They have been isolated from the roots and the rhizosphere of a great diversity of plant species and from a broad diversity of soils in almost all parts of the world, and their genetics, physiology and ecology have been extensively studied (Michiels et al., 1989).

Among several remarkable properties like nitrogen fixation, cyst formation, intracellular poly-β-hydroxy butyrate (PHB) production, their root-growth promotion effect demonstrated on grasses, cereals, legumes and vegetable crops has focused the attention of researchers. Their use as a biofertilizer has recently been reviewed (Bashan and Levanony, 1990).

However, the results of field inoculation still lack of consistency (Kloepper et al., 1989) and the plant-*Azospirillum* associations are generally considered not to be sufficiently understood to ensure a reliable commercial use of *Azospirillum* inoculants on a large scale. Together with the need for more fundamental research on the basic aspects of the association, progress in technological work for inoculant production is expected to be decisive in the matter. The development of appropriate formulations which would ensure survival and protection of the strain and the application technology which would allow easy and precise delivery in the field could be a major step towards this goal.

The paper describes the advantages and drawbacks of the main ways of producing an industrial inoculant of *Azospirillum*.

2. Formulation Technologies

The study of a formulation process is generally undertaken when the choice of an efficient strain has already been made. However, the optimization of formulation is largely independent of the strain since within the *Azospirillum* genus, most of the strains share many properties and it can be assumed that a process developed with a particular *Azospirillum* strain could be transferred with relatively few adaptations to another strain of the same genus, at least of the same species. This assumption is no longer valid in the case of transposition to another genus. Even among Gram negative bacteria, the reactions of bacterial cells to the several stresses exerted during industrial processes may vary tremendously.

Once a strain has been selected for its growth-promotion properties, the problem of formulating it as a practical inoculant arises. A formulation must obviously meet the following major requirements:

- delivery of the right number of viable cells at the right time and place;
- sufficient shelf-life; one to two years at room temperature is often necessary for successful integration in the agricultural distribution system;

- ease of handling, application possible with standard machinery;
- cost-effective production process.

Bacteria can be produced industrially in several different ways. The biomass production, the type of formulation (liquid, powder, granules) and the application methods deserve specific attention.

Biomass production

Most bacteria produced in the pharmaceutical and agro-food industries are obtained by large-scale liquid fermentation. Due to its nutritional requirements, *Azospirillum* can be economically produced in batch cultures.

For batch production in a fermentor, the first step is the optimization of medium and culture conditions. Rather surprisingly, few studies have been devoted to the physiology of *Azospirillum* growth in fermentors for biomass production (Albrecht and Okon, 1980). Most of these physiological studies were in fact related with N_2-fixation activity (Cacciari et al., 1989). For biomass production, growth must not be limited by nitrogen and combined nitrogen salts must be added to the medium. A strict control of sterility is required since the optimal temperature and pH of the culture allow development of all kinds of potential contaminants.

The key parameters to be controlled are:

- *the medium composition*: carbon and energy source, nitrogen source, oligoelements and vitamins (especially with *A. lipoferum* strains). For economical reasons an industrial medium is often much simpler than those use in the lab.
- *the temperature and pH*
- *the oxygen supply*: with a nitrogen source like an ammonium salt, *Azospirillum* is often described as a complete aerobe organism (Krieg and Döbereiner, 1984). A recent study has shown that a *A. lipoferum* strain exhibits a sensitivity to oxygen under these conditions (Paul et al., 1990). High dissolved oxygen tensions (DO) were detrimental while low DO ($< 30\%$ saturation) were favourable. It is likely that most N_2-fixing organisms behave in the same way since such sensitivity was also reported with *Azotobacter* (Senior et al., 1972), *A. brasilense* (Nur et al., 1982) *Arthrobacter* (Cacciari et al., 1985). Therefore programming DO during culture might be necessary in order to maximize yields and productivity and to orient shifts of metabolism if necessary (see next paragraph).

- *the PHB content*: this intracellular reserve material is known for giving improved resistance to various stresses (Tal and Okon, 1985). It has been demonstrated that with *A. lipoferum* grown on glucose, a fine tuning of growth kinetics (carbon source and oxygen consumptions) allows PHB production up to 50%. Its production rate is initiated by a metabolism shift linked to a nutritional limitation (Paul et al., 1990).
- *when cells are harvested*: depending on the downstream process and according to the above-mentioned parameters, the fermentor culture must be stopped at a precise time corresponding to the most adequate physiological state of the cells. This state will give the highest number of viable cells once the product is formulated.

The results of such an optimization for *Azospirillum lipoferum* strain CRT1 are shown in Fig. 1, where the time course of biomass (number of cells and dry weight), glucose consumption and PHB production are plotted. In this culture, 31.5 g/l of glucose were consumed in 26 hr and converted to 15 g/l of biomass (1.6 10^{10} cells/ml; maximum growth rate: 0.50 h^{-1}).

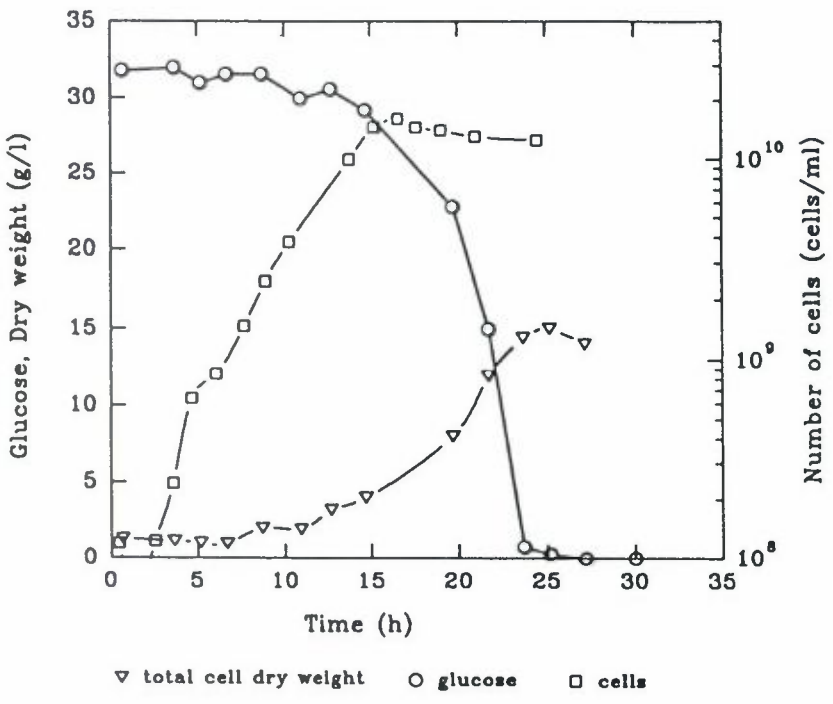

Figure 1. Batch culture of *Azospirillum lipoferum* CRT1 on glucose. Growth kinetics.

The development of optimized fermentation strategies might lead to improved biomass production. Fed-batch cultures where nutrients are added in the medium during the culture can help in reaching the desired physiological state while increasing biomass productivity (unpublished data) and such studies probably deserve more attention.

Formulation process

Most recent work on agricultural inoculants has been devoted to *Rhizobium*. The main features of that work are applicable to *Azospirillum*. However the *Azospirillum* effect of growth-promotion requires quite a high number of cells per plant, 10^5 to 10^6 with wheat (Kapulnik et al., 1985) and 10^7 with corn (Arsac et al., 1990) in the period of 2–3 weeks immediately following sowing. This implies that re-inoculation has to be done every year since *Azospirillum* survives at a much lower level in most of the soils (Bashan and Levanony, 1990). Therefore an *Azospirillum* inoculant must persist long enough to allow the growth-promotion effect to occur but must be biodegradable to avoid any threat to the environment.

Peat-based products

The most classical inoculants are based on carriers mainly peat (or vermiculite), which is generally neutralized, pre-sterilized and inoculated with a culture sample of the desired strain (Somasegaran, 1985). In such formulations the bacteria are metabolically active and sometimes growth continues for a while in the carrier. The level of viable cells is about 10^8–10^9 per gram. The main drawbacks of such formulations are: heat-sterilization of peat may release toxic compounds, a great sensitivity to chemicals – mainly fungicides coated on seeds, a weak tolerance to physical stresses during storage (temperature variations), a sensitivity to contamination and therefore a reduced shelf-life. On the other hand, their production does not necessitate sophisticated technologies and the production cost is relatively low.

Several experimental field trials have been conducted with a peat-based *Azospirillum* inoculant and a commercial product based on vermiculite containing *Azospirillum* strains is on sale in Italy.

Polymer-based products

In the last few years, several experimental *Rhizobium* formulations have been proposed, based on biopolymers like alginate or xanthan gum which have been shown to be good carriers (Jung et al., 1982). These carriers permit an

entrapment of the living cells, which protects the organisms against stresses. In addition, microorganisms are released in the soil only when the polymer is degraded, protection thus being ensured until the degradation occurs. For their conservation before use, such inoculants can be dried (see next paragraph). These polymers are, however, rather expensive and their use requires more technical handling.

Such technology has been proposed for other agricultural inoculants like *Agrobacterium*, *Arthrobacter* and some fungi (Mungnier and Jung, 1985), fungal biocontrol agents (Lewis and Papavizas, 1987) and *Azospirillum* (Bashan, 1986; Fages, 1990).

Dried inoculants

The main problem in the above mentioned technologies is the survival of microorganisms during storage. To overcome this problem, freezing and drying are the most used technologies. For agricultural purposes, dehydration is the most useful.

Dehydrated bacteria are no longer metabolically active and this dormant state ensures much longer shelf-life, better resistance to external stresses, better compatibility with pesticides and near-insensitivity to contamination (Paau, 1988). All these properties come from the lower water availability in the product. The key parameter for following this availability is water activity (a_w). Literature data about the optimal a_w for the storage of dehydrated inoculum are contradictory. Some authors found that the lower the a_w, the more constant the survival rate during storage with *Rhizobium*, *Agrobacterium* and *Arthrobacter* (Mugnier and Jung, 1985). However, with lactobacilli, de Valdez et al. (1985) found that a minimal amount of water must be left to get a satisfactory survival rate. It appears that the conditions reported are different and differences in the process of dehydration and the additives (carriers, protectants, ...) used may explain these results. An exhaustive study on *Azospirillum* dehydration remains to be done. Although a_w controls the survival during storage, the survival just after dehydration depends on several other parameters: the culture medium, the physiological state of the cells when harvested, the process of cell-encapsulation, the use of protecting agents, the process of dehydration (Fages, 1990) and the rate of drying (Mary et al., 1985).

Dehydration of Gram negative bacteria which are not spore forming and especially those of *Azospirillum*, requires excellent know-how to avoid dramatic losses in survival rate during the dehydration process. This dehydration can be carried out by spray-drying, freeze drying or air drying. Kinetics of dehydration is a key factor conditioning final survival. Each process requires

optimization, and the use of protectants and other additives or nutrients is well documented (Mugnier and Jung, 1985; Fages, 1990; Caesar and Burr, 1991).

An example of survival with alginate-encapsulated, dehydrated bacteria is shown in Fig. 2. The storage of *Azospirillum lipoferum* strain CRT1 in our

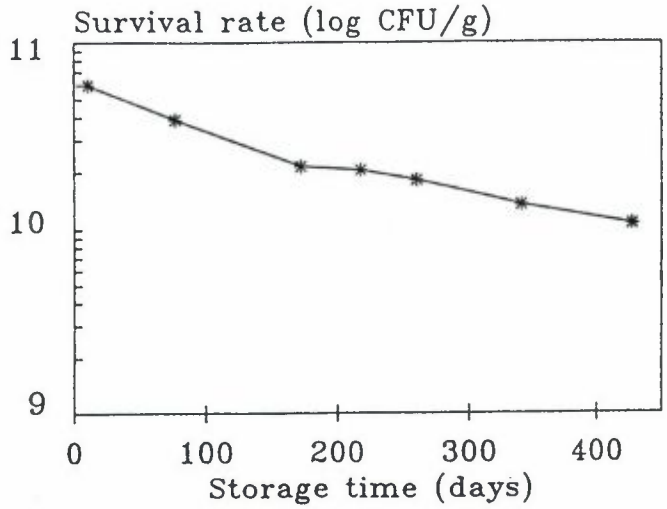

Figure 2. Alginate-encapsulated and dehydrated *Azospirillum lipoferum* CRT1. Time course of survival during storage at room temperature.

laboratory at room temperature, led to viable counts over 10^{10} per gram after one year, which is one logfold higher than classical products.

Liquid products

Liquid products can also be encountered. Numerous experimental inoculation trials with *Azospirillum* were carried out with a freshly prepared liquid suspension of the strain. Developing a stabilized liquid formulation, however, requires optimization. The most-used technique consists of resuspension of bacteria in either mineral or organic oil. Aqueous formulations have also been developed for *Rhizobium* and might be used with *Azospirillum*. The absence of carrier may cause a problem since *Azospirillum* survives poorly in the soil. Therefore, to ensure good colonization of young roots, the inoculation should be delayed for a few days after planting which would lead to additional work for the farmer.

3. Application Methods

The method used to inoculate plants is extremely important. It must match agricultural common practices and must allow the delivery of the right number of cells at the right place with standard equipment.

The choice of application method and the formulation process are linked and have to be made at the beginning of the project.

Two major ways of application can be distinguished: in association with seeds or independently of them.

Application in association with seeds

Pre-mix of inoculum and seeds

This is still the most used with peat-based inoculants (Graham-Weiss et al., 1987). Just prior to sowing, the farmer must mix the seeds and the inoculant, with (or sometimes without) addition of water and sometimes a sticker to improve adhesion. This method has several drawbacks: (1) it requires extra work for the farmer which has to be done in a short period of time; (2) it may decrease the germination level of the seeds if the seeds are hit during the mixing operation; (3) due to their metabolic activity the bacteria are sensitive to sunshine and heat and a fast decrease in viable cells may occur; and (4) it allows moist contact between the chemicals already coated on the seeds and the bacteria. The main advantage is its relatively low cost.

Pre-coating of seeds

This method appears very interesting since most of the drawbacks mentioned for the mixing at planting are avoided. However, it is more costly and more difficult to achieve.

The rhizobacteria must be in a dormant state, i.e. dehydrated to support several months of storage on the seeds. The seeds are not inert, they may produce antagonistic substances to bacteria. Their water activity may not be compatible with that required for good survival of dehydrated bacteria. Close contact with pesticides coated on seeds may be deleterious for the bacterial cells, particularly when rehydration will occur in the soil. Moreover, an efficient and homogeneous adhesion of dehydrated bacteria is required. The use of a polysaccharidic matrix which protects bacteria may avoid most of these disadvantages. The optimal number of *Azospirillum* to get root-growth promotion is about 10^6–10^7 bacteria per seed (Arsac et al., 1990) and this figure must be obtained on every seed after several months of storage.

Application independent of the seeds

Microgranulate application

The use of a microgranulaed inoculum has developed considerably with *Rhizobium* and appears as the best compromise for most agricultural inoculants including *Azospirillum.*

The microgranulate is a chemically inert carrier like marble powder, sand or calcium carbonate, which has previously been mixed, or which must be mixed by the farmer with the inoculum itself. This process is usable with all kinds of inoculum provided compatibility between the carrier and the inoculum has been ensured at all levels: viability, granulometry (300 to 800 μm), density, humidity, pH, etc.

This technique implies a specific granular applicator for the delivery of the product in the furrow. However, in the main agricultural countries, most of the farmers already own this type of equipment for insecticide microgranule delivery. This method allows good control of the number of viable bacteria put in the seed vicinity. With cereals, such an *Azospirillum* inoculant has demonstrated its efficiency in the field with a microgranulate based both on peat (Okon and Hadar, 1987) and on alginate matrix (Berge et al, 1990).

We developed this technology to manufacture an inoculant for maize with *Azospirillum lipoferum* CRT1. With this product, we obtained consistent field trial results on corn for four yeas in 13 locations showing a reproducible improvement of nitrogen nutrition. This product has recently been registered in France under the brand name AZOGREEN^{R}.

Liquid application

The liquid inoculants are less used. However, a new formulation of *Bradyrhizobium* for soybean has recently been authorized for sale in France. This inoculum requires a specific and new system of spreading.

4. Industrial Cost of Production

The cost of production has obviously to be taken into account and must be precisely determined. The more sophisticated the process the more costly it will be. A dried inoculant encapsulated in polysaccharidic matrix will be more expensive than a classical peat-based product. However, quality will be improved and it is the market which will eventually determine which level of sophistication one can afford to reach. The main items which compose this cost are:

- raw materials: components of the culture medium, carriers, additives, etc.;
- utilities: steam, electricity;
- labour;
- fixed costs: depreciation of assets, financial expenses, taxes, maintenance, overheads.

5. Marketing Aspects and Conclusions

Formulation and application technologies condition several parameters of primary importance for ensuring a successful commercial life.

However, several other requirements must be met before commercialization, and one must keep in mind that an efficient strain, an optimized formulation, a cost-effective way of production and a practical delivery system in the field are not sufficient for launching the product on the market.

- The product efficiency and reliability in the large-scale field trials must be statistically demonstrated.
- The registration costs and delays are to be considered. The registration may vary considerably from one country to another even within an economic entity like the E.E.C. For instance, Italy, Germany and France have completely different procedures. French authorities require strict toxicological and field-testing programmes. The French example is worth looking at since many countries are willing to implement registration rules and it is likely that in the coming years the most common feature will be strict reglementation. In other words, a microbial product will have to prove both its efficiency and its innocuity.
- Proprietary rights on industrial processes can be protected by patents or kept as trade secrets.
- A complete market survey has to be made in order to determine customer demands and market size according to the type of formulation and the expected selling price. *Azospirillum* has often been presented as a partial substitute to nitrogen fertilizers since its effect is an improvement in the mineral nutrition of the plant. Therefore, the increasing environmental pressure to diminish the use of chemical fertilizers in most of the agricultural countries may act in favour in this kind of product.

In conclusion, the commercial development of *Azospirillum* inoculants on a significant scale depends on three interrelated major factors:

1. the progress which is expected in the basic understanding of the association with the plant;
2. an optimized formulation and application technology;
3. an evolution in the agrochemical and seed industries' attitudes towards microbial inoculants.

Significant improvements have recently been made in each of these three domains and the near future will indicate if a profitable market niche for *Azospirillum* inoculants does exist.

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