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Azotobacter as Biofertilizer

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- *Azotobacter* belongs to the family *Azotobacteraceae*.
- This family includes various gram negative, aerobic, heterotrophic, catalase positive, free-living diazotrophic bacteria.
- The first species of the genus *Azotobacter*, named *Azotobacter chroococcum*, was isolated from the soil in Holland in 1901 by Beijerinck.

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- Along with *Rhizobium*, *Azotobacter* is the most extensively studied genus among the saprophytes.
- Winogradsky (1932) discovered that *Azotobacter* released ammonia into the soil. This started work on harnessing of *Azotobacter* for the benefit of plant and to improve soil fertility.

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At present six species of *Azotobacter* are known –

1. *A. chroococcum*
2. *A. vinelandii*
3. *A. nigricans*
4. *A. paspali*
5. *A. armenicus*
6. *A. salinestris*

Azotobacter are much more abundant in the rhizosphere of plants than in the surrounding soil and that this abundance depends on the crop species.

A. chroococcum is the most commonly found species in soils.

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Identifying characteristics of the genus *Azotobacter*

1. Large ovoid cells 1.5-2.0 mm or more in diameter.
2. Pleomorphic, ranging from rods to coccoid cells.
3. All the species of this genus form cysts.
4. Motile by peritrichous flagella or non-motile.
5. Aerobic
6. Production of water-soluble and water insoluble pigments.
7. Nitrogen fixers, generally fix nonsymbiotically at least 10 mg of atmospheric nitrogen/g of carbohydrate consumed.
8. Catalase positive.

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Bio-fertilizers

Bio-fertilizers are not fertilizers, which directly give nutrition to crop plants. These are cultures of microorganisms like bacteria, fungi, packed in a carrier material. Thus, the critical input in Biofertilizers is the microorganisms. They help the plants indirectly through better Nitrogen (N) fixation or improving the nutrient availability in the soil.

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Potential of *Azotobacter* in Agriculture

- Gerlach and Vogel (1902) initiated work on artificial inoculation of seeds with *A. chroococcum* and reported an increase of dry matter in buck wheat by 42 per cent.
- Kostychev *et al.* (1926) recommended the use of *Azotobacte*r to improve the growth of agricultural plants and soil properties.
- *A. chroococcum* and *A. vinelandii* have long been used as soil and seed inoculants.
- *Azotobacter* is a broad spectrum biofertilizer and can be used as inoculant for most of agricultural crops.
- With emphasis on development of sustainability in agriculture, *Azotobacter* is an important bioinoculant especially in organic farming.

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- Yield of a number of crops have been improved by inoculation with *Azotobacter* like cereals, millets, vegetables, fruits, and fiber and oil producing commercial crops.
- The yield increases usually range around 10-35%. Higher benefits are accrued at lower fertilizer levels.
- *Azotobacter* when applied as seed inoculant can add 15-20 kg/ha nitrogen to the soil.
- Although benefits obtained due to *Azotobacter* inoculation may not be as visible as that of chemical fertilizers, application of *Azotobacter* bioinoculant should not be viewed from only the angle of nutrient supply to the crops.

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Benefits to the crop

- Increase in percentage of seed germination
- Increased root and shoot length
- Improved nitrogen nutrition
- Reduction in disease incidence
- Increase in grain yield
- Improved post harvest seed quality in terms of germination

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Improvement in crop production due to *Azotobacter* inoculation

S. No.	Crop	Percent increase in grain yield
1.	Cotton	15-23
2.	Wheat	6-17
3.	Maize	15-20
4.	Sorghum	8-35
5.	Potato	6-14
6.	Pea	36-60
7.	Cabbage	33.5
8.	Rice	17.7
9.	Onion	10-17
10.	Chickpea	19-42
11.	Finger millet	37-39
12.	Pearl millet	10-12

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Improvement in fruit production due to *Azotobacter* inoculation

S. No.	Crop	Percent increase in grain yield
1	Mango	30-32
2	Kinnaw	21.1
3	Mandarin	21.1
4	Lemon	25-35

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Mode of action of *Azotobacter*

The beneficial influence of *Azotobacter* on plant growth is attributed to a number of factors. These being –

Direct mechanism of plant growth improvement

- Biological nitrogen fixation under free-living conditions
- Productions of phytohormones like indole 3-acetic acid, gibberrillin-like substances and cytokinins
- Solubilization of insoluble phosphates

Indirect mechanism of plant growth improvement by biocontrol

Antagonism against phytopathogens by

- Production of siderophores
- Production of antifungal compounds
- Induction of defense enzymes

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Plant growth-promoting activities of different strains of *Azotobacter chroococcum* (Apte and Shende, 1981)

Strain	Nitrogen fixation efficiency (mg N/g sucrose consumed)	Excretion of IAA (mg/ml of culture filtrate)	Production of GLS (grades of fluorescence)
A-41	7.9	3.0	+++
B-1	5.3	1.8	++
B-2	6.6	1.1	++
C-1	9.5	1.7	+++
C-2	7.9	2.7	+++
M-2	10.0	2.8	+++
M-4	ND[a]	0.8	+
M-6	1.8	ND	+
P-1	6.2	1.5	++
P-2	6.2	2.5	++
P-4	6.5	3.0	+++
W-2	1.5	1.6	+
W-3	9.2	1.6	+
W-5	7.5	2.5	++

+ yellowish green fluorescence, ++ greenish yellow fluorescence, +++ greenish fluorescence
a Not detectable

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Effect of *Azotobacter* inoculation on seed germination

Crop	% germination over control
Cotton	1.7-33.3
Rice	1.6-16.8
Maize	3-27
Wheat	4.6-24

Biocontrol

- *Azotobacter* is also known to suppress phytopathogens or reduce their deleterious effects thereby improving plant growth. Incidence of fungal, bacterial and viral diseases in the crops is reduced by *Azotobacter* inoculation (Sidorov, 1954; Samitsevich, 1962; Singh, 1977; Meshram, 1984).
- Various mechanisms like production of siderophores, antifungal compounds and defense enzymes have been proposed for antagonistic action of *A. chroococcum* on phytopathogens (Schroth and Hancock, 1982; Verma *et al.*, 2001).
- Flag smut incidence in wheat was significantly reduced due to azotobacterization (Beniwal *et al.*, 1996).
- There was reduction of loose smut of wheat (*Ustilago tritici*), yellow leaf spot of wheat (*Helminthosporium tritici-vulgaris*), powdery mildew (*Erysiphe* sp.) and bacterial blight of bean (*Xanthomonas phaseoli*) by application of *Azotobacter (*Beltyukova, 1953).
- Sidorov (1954) observed a reduction in the attack of *Phytophthora infestans* and *Streptomyces scabies* on potato crop due to *Azotobacter* application.
- Chakrabarti and Yadav (1991) reported that *Azotobacter* treatment resulted in lowering of the disease incidence of downy mildew in opium poppy crop.

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- *Azotobacter* also has a good potential as a biocontrol agent for management of nematodes and insects.
- *Azotobacter* inoculation led to nearly 50% reduction in wheat infected with *Heterodera avenae* (Wollenweber). *A. chroococcum* inhibited the hatching of egg masses of *Meloidogyne incognita* (Kofoid and White) and did not allow the larvae to penetrate into the roots of brinjal to form crown galls (Chahal and Chahal, 1988).
- *A. chroococcum* was observed to inhibit hatching of egg masses of *Spodoptera litura* (Fab.), *Spilarctia obliqua* (Walker) and *Corcyra cephalonica (*Stainton) (Paul *et al.*, 2002). There was also drastic reduction in number of eggs laid/female, per cent pupation and the emergence of adults from pupae.

Inhibitory effects of *Azotobacter chroococcum* strains on growth of various fungi (Pandey and Kumar, 1990)

Fungal strain	Zone of inhibition (mm) produced by strains of *A. chroococcum*			
	A41	C2	W5	M4
Sclerotiorum rolfsii	22	16	14	6
S. sclerotiorum	20	17	8	11
Fusarium moniliforme	18	14	17	5
F. solani	18	12	15	6
F. oxysporum	18	11	15	10
Cephalosporium maydis	2	2	2	2
Alternaria brassicola	17	17	15	6
Colletotrichum falcatum	23	16	14	5
Exserohilum turcicum	19	2	11	2
Chaetomium globosum	11	6	8	10
Penicillium chrysogenum	17	13	11	6
Trichoderma viride	31	15	12	12
Drechslera tetramera	17	12	16	9
Cladosporium herbanum	32	23	18	17

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Maintenance of *Azotobacter* cultures

- For routine maintenance *Azotobacter* should be sub-cultured at monthly or bimonthly with sucrose as carbon source.
- The culture can also be preserved by use of heavy mineral oil (paraffin). Usually *Azotobacter* survives for many months. Routine sub-culturing at 6-monthly interval is adequate.
- The cultures are grown on slants in tubes. Sterile mineral oil is added to these tubes after growth has occurred to completely cover the slope. These can be maintained on the lower most rack of refrigerator.
- Mineral oil is sterilized in an oven for 3 days for 90 min each day at 160^{o}C.

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- *Azotobacter* can also be maintained as glycerol based cultures in a deep freezer.
- Broth cultures are prepared. Glycerol solution (60%) is sterilized by autoclaving and is added to the broth culture to get a final glycerol concentration of 15% v/v.

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Commercial Manufacture of *Azotobacter*

The manufacturing process in short involves

- Selection of suitable strain of the organism for which market demand is identified.
- Mass multiplication.
- Mixing of the culture with carrier material and packing.

under the supervision of trained microbiologist.

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The steps involved are

- Culture selection and maintenance.
- Purchase of desired strains from the identified authentic sources like Agricultural Universities, IARI, some ICAR institutions, Regional biofertilizer labs of MOA, etc. There are international sources of supply also like NifTAL, IRRI etc. These sources maintain pure mother cultures.
- They have to be further sub-cultured and maintained purely for mass production by adopting standard techniques

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Culture augmentation

Culture has to be mass multiplied in two levels

Primary level using **shakers in flasks**

Secondary stage multiplication in **fermenters**

The important factor in this is the preparation of growing medium in which the culture is mass multiplied. There are standard media on which information is available from published sources like Norris and Date, Fred *et al.*, ISI approved etc.

After the media is formulated and sterilized in fermenter, it is inoculated using the shorter cultures multiplied in the flasks at definite ratios usually 5%.

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The bacteria growing medium is called broth and it is continuously aerated by passing sterile air from compressors.

After about 3-4 days fermentation period, the broth will be ready for packing in a carrier material. At various stages the quality is tested by drawing samples.

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Carrier sterilization

- While the broth is getting ready in the fermenter, the carrier material, which is usually the carbon source for the cultures to survive, is sterilized in autoclaves and kept ready for mixing with the broth.
- The carrier is either sterilized in bulk or it is packed and then the packets are sterilized.
- Peat imported from countries like U.S., Australia is reported to be the best source of carrier material. However, as it is costly lignite, charcoal : soil mix are used extensively in India.
- The pH of the carrier material is adjusted to 7 for better results.
- Moisture is generally maintained at 10%.

Mixing and packing

There are 2-3 alternatives depending upon the sophistication and automation of the unit.

Under **non sterile system**, the broth is harvested from the fermenter into sterilized carrier - the mixing is done manually under aseptic condition and packed in polythene bags of desired quantity.

In a **slightly upgraded method**, the broth and sterilized carrier are mixed mechanically in a blender and the material is packed using semiautomatic packing and sealing machine. In a slightly modified method some units are packing by delivering desired quantities of carrier and broth simultaneously from separate pipe conveyance system into the polythene bags.

Under a completely **sterile system** the carrier is taken in autoclavable polypropylene bags and pre sealed - into which the broth from fermenter is directly injected with the help of dispenser. The injection hole is immediately sealed. The packets are kept in incubation room for about a week before transferring to store room.

Sterile system of packing using auto syringe and dispenser is recommended to be the best method and all new units should follow and adopt this system.

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As per BIS specifications, certain tests are required to be conducted, like no. of cells, colony character, reaction etc. Cell number at the time of manufacture should not be less than 10^7 per gram of carrier material for *Azotobacter*. Similarly, the number of cell count and permissible contamination at expiry dates are also specified.

As certification arrangements are not in place at present, legislation for quality monitoring and accredited labs for testing may be needed in future to ensure proper quality and to promote this product.

Indian standard specification for *Azotobacter*

S.N.	Parameters	*Azotobacter* Inoculant IS – 9138-2002
1.	Base	Carrier based
2.	Viable cells	10^7cells/g of carrier within 15 days manufacture.
3.	Cell no at the time of expiry	10^6 cells/g within 15 days before expiry date
4.	Shelf life or expiry period	6 months from the date of manufacture
5.	Permissible contamination	No contamination at 10^5 dilution
6.	PH	6.0-7.5
7.	Moisture %	35-40%
8.	Strain	*A. chroococcum* mentioned
9.	Carrier	Should pass through 100 micron IS sieve
10.	Efficiency test	Minimum amount of N-fixation not less than 10 mg/g of sucrose utilized.

Methods of Application

Application of bio-fertilizer require technical methods, thus it should be shown to farmers.

There are three methods of application

1. **Seed treatment:** Normally, 500 g of bio-fertilizers is required for seeds to be applied in a hectare of field. Bio-fertilizer is mixed with water and adhesive material so that seed coat is not broken, is dried for half an hour before sowing.
2. **Seedling treatment:** Bio-fertilizers are being used for seedling treatment. This is mainly done in transplanted crops. A slurry of *Azotobacter* biofertilizer is prepared. Seedlings are dipped in this slurry for about 15 minutes, allowed to dry and then transplanted.
3. **Soil application :** About 2 kg of bio-fertilizers are mixed with 40-50 kg of decomposed FYM and are broad cast at the time of sowing/prior to sowing.

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Main constraints with bio-fertilizers

Marketing constraints

Short shelf life

Lack of proper storage facilities

Consumer illiteracy

Low awareness amongst consumers

Inadequate guidelines to consumers

Inadequate production/promotion effort

Environmental constraints

Seasonal conditions (high temperature)

Soil pH

Usage of high dose of chemical fertilizers/pesticides/soil amendments.

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At field level the efficiency is limited by several factors

- drought and high summer temperature
- water logging
- unfavourable soil pH
- antagonism from other organisms
- nutrient deficiency
- incompatibility of biofertilizer with fungicide or insecticide coated on the seed
- There is an acute awareness gap among the farmers on the subject.

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How to overcome limitations and constraints

- Broaden the genetic base of mother cultures and go for efficient and effective strains suitable to various agro-environments, or specific for crop and location specific strains.
- Competitive strains capable of surviving and maintaining high populations in soil.
- Improved carrier material with uniform and consistent good quality comparable to imported peat material.
- Avoid contamination in broth mixing and packing stages by using completely closed system of production.

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- Improve packing material to improve shelf life.
- Improve storing conditions, particularly during the distribution period. Avoid exposure to high temperatures and sunlight which destroy the microbial culture. They should be preferably kept in cold storage conditions.
- Employ properly trained microbiologist.
- Maintain proper quality controls and certification procedures.