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Azotobacter: A Plant Growth-Promoting Rhizobacteria Used as Biofertilizer

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ABSTRACT

Nitrogen fixation is mainly responsible for improvement of crop yield. In this regard, diazotrophs like *Rhizobium*, *Azotobacter* and *Azospirillum* are important as they enrich nitrogen nutrition in N-deficient soils. Of these, *Azotobacter* promotes plant growth as well as nitrogen fixation. Thus technology has been developed for making use of *Azotobacter* biofertilizer for nitrogen and non-nitrogen fixing plants and popularized by educating about their benefits in agriculture to users for practicing integrated nitrogen management.

Keywords: *Azotobacter*, biofertilizer, growth and yield, nitrogen fixation, organic farming, PGPR

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INTRODUCTION

In agriculture, one of the limiting factors is providing plant nutrients, particularly nitrogen (N) and phosphorous (P), to crops. So the improvement of crop yield by inoculation with diazotrophs like *Azotobacter*, *Rhizobium* and *Azospirillum* has been suggested as an ecofriendly technology (Choudhary and Kennedy 2004). These microorganisms colonize the rhizosphere of plants and remain in close association with roots and influence their growth. Of these, *Azotobacter* is one of the most extensively studied plant growth-promoting microorganisms because its inoculation benefits a wide variety of crops. These are polymorphic, possess peritrichous flagella and produce polysaccharides; they are sensitive to acidic pH, high salts and temperature above 35°C and can grow on a N-free medium thus utilize atmospheric nitrogen (N_2) for cell protein synthesis. Cell proteins are mineralized in soil after death of *Azotobacter* and contribute to N availability to crop plants. Several types of azotobacteria have been found in the soil and in the rhizosphere: *A. chroococcum* (Beijerinck 1901), *A. nigricans* (Krassilnikov 1949), *A. paspali* (Döbereiner 1966), *A. armenicus* (Thompson and Skerman 1981), *A. salinestris* (Page and Shivprasad 1991) and *A. vinelandi* (Lipman 1940). *A. chroococcum* is the species most commonly found in Indian soils. Plant growth promotion by *Azotobacter* may also be attributed to other mechanisms such as ammonia excretion (Narula *et al*. 1981). Besides N_2 fixation, they also produce siderophores and antifungal substances (Suneja *et al*. 1994) and plant growth regulators (PGRs) such as hormones and vitamins (Shende *et al*. 1977; Verma *et al*. 2001). PGRs such as auxin and cytokinin produced by *Azotobacter* sp. have also been reported (Pilet *et al*. 1979; Harmann *et al*. 1983; Horemans *et al*. 1986; Falik and Okon 1989; Nieto and Frankenberger 1989; Taller and Wong 1989; Barbiri and Galli 1993; Patten and Glick 1996; Verma *et al*. 2001; Patten and Glick 2002).

AZOTOBACTER AND NITROGEN FIXATION

Azotobacter belongs to the *Azotobacteriaceae* family. These are Gram-negative, non-symbiotic, aerobic diazotrophs. The young rod-shaped cells vary from 2.0-7.0 to 1.0-2.5 µm and occasionally an adult cell may increase up to 10-12 µm, and be oval, spherical or rod-shaped cells. *Azotobacter* can grow well on simple N-free nutrient medium containing phosphate, magnesium, calcium, molybdenum, iron and carbon sources. Its catabolic versatility in utilizing several aromatic compounds such as protocatechuic acid, 2-4-D (2,4-dichlorophenoxyacetic acid), 2-chlorophenol, 4-chlorophenol, 2,4,6-chlorotriphenol, aniline, lindane, toluene, *p*-hydroxy benzoate, benzoate and benzene is well documented (Hardisson *et al*. 1969; Balajee and Mahadevan 1990; Gahlot and Narula 1996; Moreno *et al*. 1999; Revillas 2000; Thakur 2007). *Azotobacter* contributes significant amounts of fixed N_2 in, on, or near a plant. The energy requirement for the process of N_2 fixation is met by a very high rate of aerobic metabolism which contributes to high oxygen demand for the maintenance of minimal intracellular oxygen tension, a requirement of the oxygen-sensitive nitrogenase to accomplish N_2 fixation (Robson and Postgate 1980).

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Invited Mini-Review

Diazotrophic bacteria in the rhizosphere of plants utilize the products of N_2 fixation for their own growth and release little while they are alive (Imam and Badaway 1978; van Berkum and Bohlool 1980; Apte and Shende 1981; Balajee and Mahadevan 1990; Das *et al*. 1992; Jana and Mishra 1994; Verma *et al*. 2001; Johnson *et al.* 2006; Damir *et al*. 2011; Sayeda *et al*. 2011). When bacteria die, only a small quantity of fixed N_2 is assimilated by the plant. N_2 fixation by heterotrophic bacteria in the rice rhizosphere develops in response to a deficiency in the availability of the combined N_2. When fixed N_2 is not readily available for plant growth, the plant become N_2 deficient where rhizobcteria contribute significantly. The highest rates of root-associated Nitrogenase activity were measured in N-deficient plants (van Berkum and Bohlool 1980; van Berkum and Sloger 1981). In native bacteria the process of N_2 fixation is inhibited by combined N_2 in the environment. The N_2 fixation process in root-associated bacteria can fix N_2 gas in the presence of repressive levels of combined N_2 and export a major portion of the Nitrogenase-produced ammonia or organic N_2 by-product from their cells into the rhizosphere and/or roots. Thus, plants which form associations with desired bacteria have an additional source of combined N_2 available for growth. This microbe possesses three genetically distinct Nitrogenase complexes and the expression of these Nitrogenase varies with vanadium, molybdenum and ammonium in the culture medium (Bishop *et al*. 1980). Nitrogenase-I is expressed only when molybdenum is present in the medium, Nitrogenase-II is expressed only when vanadium is present while Nitrogenase-III is expressed when both molybdenum and vanadium are absent (Chisnelle *et al*. 1988; Kumar *et al*. 1988; Bishop and Joerger 1990; Harvey *et al*. 1990; Falik *et al*. 1991; Joerger *et al*. 1991). Ammonia is responsible for the repression of synthesis of all three N_2ases. Energy requirement for N_2 fixation is obtained from EMP and TCA cycle (Jackson and Dawes 1976). Acetate is utilized via the glyoxalate pathway. Both GS (Glutamine synthase) and GOGAT (Glutamine Oxoglutarate Amino Transferase) accomplish NH_4^+ assimilation though the GS-GOGAT pathway (Kleiner and Kleinschmidt 1976).

Polysaccharide or gum production is one of the characteristic features of *Azotobacter* (Moulder and Brontonegoro 1974). Some of the species produce polysaccharides in more quantities forming a capsule around the cell. These EPS (extracellular polysaccharides) have a composition similar to alginic acid and contain rhamnose, mannose and galactose with trace amounts of glucose (Horan *et al*. 1983; Cote and Krull 1988). The role of EPS has not been clearly established but it has been suggested that they protect against desiccation, mechanical stress, phagocytosis and phage attack, participate in the uptake of metal ions as adhesive agents, ATP sinks or are involved in interactions between plants and bacteria (Fyfe and Govan 1983; Hammad 1998). Pigments are also an important characteristic and are produced by all *Azotobacter* species. *A. chroococcum* produces black, brownish-black water-insoluble melanin like pigment in old cultures (Zinovyeva 1962; James 1970). A yellow-green, fluorescent pigment is excreted by *A. vinelandii* and *A. paspali*. A red-violet or brownish-black pigment is seen in the extracellular product of *A. nigricans* and *A. armeniacus*. These organisms produce a cyst which is a living dormant cell with two coats namely exocytorium and two layers of exine. Cyst is found to be rich in poly-β-hydroxy butyric acid. With the onset of favorable conditions, the cyst gives rise to vegetative cells. Calcium is essential for cyst formation (Page and Sadoff 1975; Gimmestad *et al*. 2009).

USE OF *AZOTOBACTER* AS BIOINOCULANTS/ BIOFERTILIZERS

Azotobacter, a Gram-negative, free living and plant growth-promoting rhizobacteria, was first reported by Kloepper and Schroth (1978). Its use as a biofertilizer was first advocated by Gerlach and Voel (1902) with the purpose of supplementing soil-N with biologically fixed N_2 due to the activity of this microbe. Since then they have been reported to play a multifaceted role in stimulating the growth of plants not only by fixing atmospheric N_2 under free-living conditions but also possess other plant growth-promoting activities like phosphate solubilization, production of PGRs like auxins, gibberellins, cytokinins, vitamins and amino acids (Barea and Brown 1974; Tien *et al*. 1979; Apte and Shende 1981; Barbieri *et al*. 1986; Kerni and Gupta 1986; Das *et al*. 1992; Potdukhe *et al*. 1992; Abbas and Okon 1993; Yadav *et al*. 1996; Paul and Verma 1999; Paul *et al*. 2002; Nagananda 2010; Damir *et al*. 2011). *Azotobacter* has been reported to possess a very high ARA (acetylene reduction assay) and the range of N_2 fixation was observed to be between 2-15 mg N fixed/g of glucose consumed (Apte and Shende 1981). *Azotobacter* sp. has the ability to solubilize phosphates (Shende *et al*. 1975), ranging from solubilization level of phosphates is 8-16% of the substrates used. *Azotobacter* produces IAA (indole-3-acetic acid) when tryptophan is added to the medium (Brakel and Hilger 1965) as tryptophan is the precursor of IAA and is converted to IAA through a primary Trp-aminotransferase reaction. Inoculation of *Azotobacter* improved seed germination rate and enhanced the vegetative growth of the inoculated plants (Apte and Shende 1981). Three gibberellin-like substances and five cytokinins were found in *A. chroococcum* (Brown and Burlingham 1968; Nieto and Frankenberger 1989). *Azotobacter* also improved plant growth indirectly by suppressing phytopathogens or reducing their deleterious effects (Pandey and Kumar 1990) and reduced the incidence of fungal, bacterial and viral diseases of several crops (Meshram 1984; Pandey and Kumar 1990). *A. chroococcum* reduced nematode infection by up to 48% followed by *Pseudomonas* (11%) and *Azospirillum* (4%) (Chahal and Chahal 1988). Competition of iron is one of the well known mechanisms of biocontrol under iron-limiting conditions. The bacterium produces a range of iron chelating compounds or siderophores which have a high affinity to ferric ion. These siderophores bind most of the iron (Fe^{3+}) available in the rhizosphere and thereby making unavailable to pathogens present in soil. Pathogens may not have the ferri-siderophore receptor for uptake of the iron-siderophore complex. So they do not proliferate immediately due to a lack of iron in the soil (Ósulivan and ÓGara 1992; Tindale *et al*. 2000; Duhme *et al*. 1998; Kraepiel *et al*. 2009; Yoneyama *et al*. 2011).

AZOTOBACTER INOCULATION AND CROP YIELD

Improvement in crop production due to *Azotobacter* inoculation has been reported in a number of crops. Artificial inoculation of wheat (*Triticum aestivum*) seeds with *A. chroococum* increased the dry matter 42% more than the control (Gerlach and Vogel 1902). Similarly, a 10-18% increase in yield of bean, corn and potato (*Phaseolus vulgaris*, *Zea mays*, *Solanum tuberosum*) (Sheloumova 1935), 10-23% in wheat (*Triticum aestivum*), 13-19% in oat (*Avena sativa*), and 14-27% in clove (*Eugenia caryophyllata*) more than the control due to *Azotobacter* inoculation has been reported (Krasilnikov 1945; Barea and Brown 1974; Tien *et al*. 1979; Barbieri *et al*. 1986; Kerni and Gupta 1986; Das *et al*. 1992; Abbas and Okon 1993; Paul and Verma 1999; Paul *et al*. 2002; Nagananda 2010; Damir *et al*. 2011).

Azotobacter inoculation also significantly increased the weight of plant, grain yield and N_2 content of plant in wheat (*Triticum aestivum*), maize (*Zea mays*) and cotton (*Gossypium hirsutum*) crops (Apte and Shende 1981). A foliar spray of *Azotobacter* significantly increased the grain and straw yield of rice (*Oryza sativa*) (Kanniyan *et al*. 1980). The use of *Azotobacter* inoculation has great potential in oilseeds and also a diverse array of crops in terms of crop yield. An increase in yield of mustard (*Brassica oleracea*) (Gerlach and Vogel 1902; Schmidt 1960), sunflower (*Helianthus annuus*) (Badve *et al*. 1977; Yadav *et al*. 1996), sugarcane (*Saccharum officinarum*) (Agrawal *et al*. 1987),

Table 1 Summary of the work on use of *Azotobacter* and other growth promoting rhizobacteria as biofertilizer for different crops.

Organism	Strain	Isolated from	Growth condition	Findings	Reference
Azotobacter sp.		Vegetable gardens, grasslands and cultivated fields	All cultures were grown at 0°C on Winogradsky nitrogen-free agar medium and washed off the agar surface for preparation of antisera or antigens for immuno electrophoresis.	All members of the *Azotobacteraceae* have some taxonomic relationship, since they all share some antigens with the reference strains. It is apparent that members of *A. paspali* and *A. vinelandii* are immunologically homogeneous within species, as the immunological distances from the corresponding reference strains ranged from 0 to 0.2 for *A. paspali* and from *0* to 0.08 for *A. vinelandii. A. chroococcum* strains may be considerably less homogeneous, as their immunological distances from the reference strain ranged from 0.5 to 0.3.	Tchan *et al*. 1983
Azotobacter and *Azospirillum*		Obtained from University of Agricultural Science, GKVK, Bangalore and Research Institute, Madurai, Tamil Nadu		Application of *Azotobacter* and *Azospirillum* biofertilizer in irrigated mulberry under graded level of nitrogen was studied. Better response to *Azotobacter* than *Azospirillum* under low nitrogen with 150 kg N/ha was observed. Leaf nitrogen and crude protein were also significantly higher in *Azotobacter*at 150 kg N/ha/year inoculation.	Das *et al*. 1992
Azotobacter	Ale-3	HAU, Hissar		Field trials were conducted in *rabi* seasons of 1987-88 and 1988-89 at research farm, HAU, Hissar. Growth and yield were significantly enhanced with the application of *Azotobacter* with and with out nitrogen. Higher plant height and yield attributes of wheat as compared to other treatments or over control was observed due to *Azotobacter*.	Hooda and Dahiya 1992
Azotobacter and PSB (*Pseudomonas striata*)		Obtained from Microbiology Division, IARI	Jensens and Pikovaskyas tricalcium phosphate broth	Effect of cotton seed (var. 'SRT-1') inoculation with *A. chroococcum* and *Pseudomonas* in combination at graded doses of nitrogen and phosphorous on the uptake of N and P. Plant height, dry matter weight and yield were studied. A combination of fertilizer and *Azotobacter* inoculation saved half on the N and P fertilizers.	Potdukhe *et al*. 1992
A. chroococum	BI2	Soil of West Bengal	N-free Burk's medium containing 1% (w/v) glucose as the carbon source	*A. chroococum* BI2 showed higher nitrogen fixation at pH 7.0 with a low concentration of potassium nitrate (25 mgN/L) and ammonium sulfate (100 mgN/ml) at 28°C. This strain showed tolerance to NaCl (0.12%). A pesticide (Rogor) inhibited growth as well as acetylene reduction at very low concentrations.	Jana and Mishra 1994
A. chroococum	MKU 201; B-8005; BKMB-1030; A-41	Tropical soil	Jensens agar medium	The temperature optima for high survival and efficiency in nitrogen fixation varied among the strains of *A. chroococum*. It ranged between 20-35°C. Performance of temperate strains 8005 and BKMB-1030 was better at low temperature (20°C) and that of tropical strains B and MKU 109 was appreciable even at high temperature (40°C). Strain A-41 exhibited tolerance over a wide range of temperatures; however, extreme temperatures reduced its growth and efficiency of nitrogen fixation.	Rajkumar and Lakshmanan 1995
A. chroococum	RH-30; WH-147; MAC-27; E-12	Department of Plant Breeding CCS HAU, Hisar	Jensen medium containing trace element, sodium glutamate as 'N' source and EDA-HCl (EDA, 0.05-1%) incubated at 30°C for 48-72 h	Ethylene diamine (EDA)-resistant mutants (MAC-27) fixed nitrogen in the presence of high concentration of NH_4^+ and also excreted NH_4^+. E-12, which exhibited low glutamine synthetase (GS) activity, reduced NH_4^+ uptake in the mutant due to GS-induced deficiency in ammonia assimilation. Yield and dry matter in mustard and grain yield of wheat were greater with E-12 inoculation than with parent MAC-27 under greenhouse conditions.	Narula *et al*. 1999
Azotobacter and *Rhizobium*	*Azotobacter* strains: W-5, CBD-15 and C-11; *Rhizobium* strains: BG-256 (chickpea)	Collected from Division of Microbiology, IARI, New Delhi		The effect of inoculation of chickpea seeds with three strains of *A. chroococcum* in combination with *Rhizobium* in a non-sterile soil was studied. Inoculation with either *A. chroococcum* or *Rhizobium* alone increased nodule number, weight and yield of chickpea.	Paul and Verma 1999
A. chroococcum and *Trichoderma viride*	W-5; ITCC 1433; 1662; 2185; 3235; 3255	Obtained from Division of Microbiology, IARI		Strains of *T. viride* were used for solid state fermentation (SSF) of sorghum straw after adjusting the C: N ratio to 35: 1 to study the effect of the fermented residues alone and in combination with *A. chroococcum* W5 as biofertilizer for wheat. Inoculation with W5 alone increased the biomass and yield by 25% over that in control. Fermented residue of *T. viride* ITCC 1433 applied in combination with *A. chroococcum* decreased the yield.	Nain *et al*. 2000

Table 1 (Cont.)

Organism	Strain	Isolated from	Growth condition	Findings	Reference
A. chroococum	Mala-11 and HT54	Collected from Department of Microbiology, CCS, HAU, Hisar	Jensen's N-free medium at 30°C for 18 days	Plant growth regulators (PGRs) like gibberellin, kinetin and indole-3-acetic acid were produced by *Azotobacter*. Out of 20 *Azotobacter* isolates, 4 produced all three PGRs, 14 produced GA_3 and 10 produced kinetin. All the isolates except for Mala-11 and HT-54 produced one of the three PGRs.	Verma *et al*. 2001
Azotobacter sp.		Soil Microbiology Laboratory, G.B. Pant University of Agriculture and Technology, Pant Nagar	Jensen's broth 28 ± 2°C for 7 days	*Azotobacter* culture differed greatly in intrinsic resistance to streptomycin, tetracycline, trimethoprin, nalidixic acid and rifampicin. 14 cultures inhibited growth of *Fusarium oxysporum.* In modified JAM-PDA medium none of the *Azotobacter* strains inhibited the growth of *Microphomina phaseolina* and *Sclerotium rolfsii.* No relationships could be observed between the fungal inhibition and the antibiotic resistance of the diazotrophs.	Agrawal and Singh 2002
B. japonicum and *A. chroococum*				The effect of inoculation of *Bradyrhizobium japonicum* and *A. chroococum* on soybean [*Glycine max* (L) Merill var. Ransom] was studied. Dual inoculation most enhanced plant growth parameters. Inoculation with *Azotobacter* alone was better than uninoculated control.	Bhattarai and Prasad 2003
A. chroococum		Collected from Department of Microbiology, CCS, HAU, Hisar	Grown in Jensen N-free medium for 72 h at 30°C	Sixteen isolates of *A. chroococum* were studied for azide resistance. Azide-sensitive mutants were developed which was widely prevalent among the isolates. Azide resistance showed no significant correlation with rate of respiration, ATP concentration, activity of cytochrome-c-oxidase and nitrogen fixation.	Vasudeva *et al*. 2003
Azotobacter and *Azospirillum*				Total biomass yield was increased under all the soil amendment and inoculation treatments. Highest increase in biomass yield was obtained and influenced by combination with *Azotobacter* and *Azospirillum*.	Pattanayak *et al*. 2004
A. chroococum	BG-13 and BG-33		Jensen's 'N' free medium with sucrose (0.25%) and with 2500 ppm 2,4-D	Four *A. chroococum* strains from soils enriched with 2,4-D were studied for metabolism of the compound. All 4 strains degraded 2,4-D to chlorocatechol even at 2500 ppm in the presence of sucrose as the C source and with out any additional C source in soil. Chlorocatechol formation was observed even at stationary phase of cells indicating co-metabolism of 2,4-D. Nitrogenase activity in these strains remained unaffected up to 50 ppm of 2,4-D. Accumulation of chlorocatechol with less cell density indicates that some strains may not have metabolized the intermediary product.	Gahlot and Narula 2004
Azotobacter		Rice and wheat fields, vegetable gardens, grasslands	Bacteria isolated by serial dilution and plating technique in nitrogen-free medium and general purpose medium (glucose-yeast extract agar)	Total *Azotobacter* population decreased with increasing soil moisture content. Maximum *Azotobacter* population was recorded in March. When the soil moisture content was <15% during May-June declined sharply. Grassland field had highest *Azotobacter* population compared to other fields. Similar trend was also observed in rhizospheric soils collected from vegetable garden and grasslands which were not waterlogged.	Sharma and Bhattacharje e 2004
Azotobacter vinelandii		Strains obtained from the Microbial Genomics section at the Department of Energy Joint Genome Institute (JGI), Virginia, USA	*A. vinelandii* strains were grown at 30°C on modified Burks minimal medium containing 2% sucrose or 2% glucose as the sole carbon source. Ammonium acetate served as the nitrogen source and was added at a final concentration of 13 mM.	Experimental results showed that sucrose catabolic regulon was developed *in A. vinelandii* by using genomic fusions. *IscS*, *IscU*, *HscBA*, and *Fdx* genes are essential in *A. vinelandii* for the functional analysis, whose products are involved in the maturation of [Fe-S] proteins; their depletion causes deficiency in the maturation of aconitase, an enzyme that requires a 4Fe-cluster for its catalytic activity. Depletion of *IscA* results in a null growth phenotype only when cells are cultured under conditions of elevated oxygen.	Johnson *et al*. 2006
Azotobacter vinelandii	ATCC 9046	Rhizospheric soil of *Trigonella* plant	Strain grown in modified Burk's medium and incubated during 24 h at room temperature.	Experimental analysis to scale-up (from shake flasks to fermentor) with a specific growth rate of 0.16 h^{-1} was obtained in a stirred fermentor. Thus, applying the exponential power input (P/V) profile during cultivation, an alginate having 1700 kDa was obtained with respect to the polymer obtained from the cultures conducted in shake flasks	Peña *et al*. 2008
Azotobacter			Bacteria isolated by serially dilution till 10^{-5} and 0.1 ml plated on Ashby's agar medium and incubated at room temperature for 4-7 days.	The root length, shoot length, fresh weight, protein, carbohydrate and chlorophyll content of *Trigonella* plantlets was maximum after 15 days of growth *in vitro*; 100% seed germination was observed when seeds were treated with *Azotobacter* cell at 2.3×10^4 cells ml^{-1}.	Nagananda *et al*. 2010

Table 1 (Cont.)

Organism	Strain	Isolated from	Growth condition	Findings	Reference
Azotobacter vinelandii			N-deficient combined carbon sources medium at 32°C in a rotary shaker for 5 days	In the two dissolved oxygen conditions evaluated, strictly controlled by gas blending at 0.5 and 5% DOT (dissolved oxygen tension), an increase in the agitation rate (from 300 to 700 rpm) caused a significant increase in the OTR_{max} (oxygen transfer rate) from 17 to 100 mmol L^{-1} h^{-1} for 5% DOT and from 6 to 70 mmol L^{-1} h^{-1} for 0.5% DOT). This increase in the OTR_{max} improved alginate production, as well as the specific alginate production rate (SAPR), reaching a maximal alginate concentration of 3.1 g L^{-1}.	Lozano *et al*. 2011
Azotobacter chroococum	AZt			Using ecofriendly biofertilizers instead of chemical ones in fish aquacultures the impact of inoculation of two strains of *Azospirillum brasilense* and *Azotobacter chroococum* was studied. Chemical characteristics of water, specific growth rate, aspartate amino transferase, alanine amino transferase and histopathological changes were analysed in *Oreochromis niloticus* aquaculture. Dissolved oxygen, biochemical oxygen demand, chemical oxygen demand, NPP, NO_3N and O-PO_4 levels were significantly increased by treatment with *Azotobacter* while *Azospirillum* gave lower levels. In other hand single or mixed bacterial treatment increased fish specific growth rate especially treated with *Azotobacter* (34.62% increase in growth). Study induces single inoculation of *Azotobacter* bacteria biofertilizer as a suitable probiotics in aquacultures.	Sayeda *et al.* 2011
Azotobacter chroococcum	DSM 2286	Isolated from *Hordeum vulgare*		Optimization the production of bacterial biomass cultivation of *A. chroococcum* was done by using different media and cultivation techniques (batch, fed batch and repeated batch). Chemically defined and complex media with 20 g/l of sugar were selected as the most appropriate media for batch cultivation in stirred tank bioreactor. Higher fed batch and repeated batch techniques increased the bioprocess efficiency parameters (yield coefficient and productivity). Repeated batch technique appeared to be the most suitable for the bacterial biomass production at industrial scale.	Damir *et al.* 2011

fruit trees (Kerni and Gupta 1986; Pandey *et al*. 1986), pearl millet (Wani *et al*. 1988), sorghum (Jadav *et al*. 1991), jute (Poi and Kabi 1979), cotton (*Gossypium hirsutum*) (Apte and Shende 1981; Paul *et al*. 2002) has been reported. Several vegetable crop like tomato (*Lycopersicon esculentum*), brinjal (*Solanum melongena*), cabbage (*Brassica chinensis*), onion (*Allium cepa*), potato (*Solanum tuberosum*), radish (*Raphanus sativum acanthiformis*), chillies (*Capsicum annuum*) and sweet potato (*Ipomoea batatus*) responded positively to azotobacterisation (Joi and Shinde 1976; Imam and Badaway 1978; Khuller *et al*. 1978; Sethi and Adhikary 2009). Synergestic effect of co-inoculation of *Azotobacter* with *Rhizobium* in pea (*Pisum sativum*) (Paul and Verma 1999), chickpea (*Cicer arietinum*) (Verma *et al*. 2000) and groundnut (*Arachis hypogea*) (Rashid *et al*. 1999) was also observed. Detailed summary of the work carried out on use of *Azotobacter* and other PGPRs as biofertilizers for different crops is provided in **Table 1**.

MASS PRODUCTION PROTOCOLS OF *AZOTOBACTER* BIOFERTILIZER AND FIELD APPLICATION METHODS

Efficient strains of *Azotobacter* can be obtained from established culture collection centres for mass production. Alternatively, region-specific and environmental stress-compatible strains of the bacterium can be isolated and used. For isolation, soil samples adhering to uprooted plants should be collected and cultured through serial dilution and plating techniques using *Azotobacter* isolation media containing (g/l): Sucrose - 20.0, K_2HPO_4 - 1.0, $MgSO_4 \cdot 7H_2O$ - 0.5, Na_2MoO_4 - 0.001, $FeSO_4 \cdot 7H_2O$ - 0.01 and $CaCO_3$ - 2.0, pH - 7.0-7.2 (Yadav and Mowade 2005). The cultures need to be incubated at 28 ± 2°C for 4-5 days to produce bacterial colonies which are white, translucent and circular. After testing the purity followed by this standard protocol for testing purity of bacterial culture by incubating nutrient broth and bromothymol blue, individual strains need to be cultivated in liquid broth (*Azotobacter* isolation media). Basing on higher growth rate and tolerance to different environmental variables strains can be selected for use as biofertilizer. The objective of the paper is not to give details how efficient strains responding to various environmental variables were obtained for use as biofertilizer, hence not given. For this purpose, loops of the respective colonies inoculated in sterile N_2-free medium are grown for 5-7 days. This starter culture is inoculated into a 500-ml flask with a bacterial suspension of 10^5 CFU/ml and grown in rotary shaker at about 120 rpm for 5 days at 30°C. For field experiments, 20-days-old healthy seedlings are used; the roots are dipped in bacterial culture suspension for 20-30 min for proper attachment of microbes and then planted.

CONCLUSION

Azotobacter is a broad spectrum biofertilizer and can be used as inoculant for most agricultural crops. Earlier, its utility as a biofertilizer was not a priority due to its relatively low population in the plant rhizosphere. However, seeding treatment with *Azotobacter* of several crops brought about an increase in yield. Besides, because of its well known N_2 nutritional function, it is now recognized to play a multiple role in helping crop plants to improve their growth potential, yield and maintenance of soil health for sustainable agriculture. Hence there is renewed interest in this rhizobacterium. However, quantitative understanding of the ecological factors that control the performance of biological N_2 fixation systems of the bacterium in crop fields is essential for promotion and successful adoption of the biofertilizer production technology.

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