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Isolation and mass production of *Azotobacter* species potential useful in agricultural application

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Abstract
Great many papers on this genus have dealt with its significance in plant nutrition and its contribution to soil fertility. Because of this and for better clarity, this paper has been divided into three sections: *Azotobacteria* and their natural habitat (the soil and plant); production of growth substances and their effects on the plant; and possibility of using *Azotobacteria* in agriculture. As soil bacteria, *Azotobacteria* cannot be studied without their natural environment. The main focus of study was the soil as the natural habitat of these bacteria. A bacterium of the *Azotobacter*ia genus synthesizes IAA and these growth materials are the primary substances controlling the enhanced growth. These hormonal substances, which originate from the rhizosphere or root surface, affect the growth of the closely associated higher plants. In order to guarantee the high effectiveness of inoculants and microbiological fertilizers it is necessary to find the compatible partners, i.e. a particular plant genotype and a particular *Azotobacteria* strain that will form a good association.

Keywords: *Azotobacter* sps, microbiological fertilizer, plant, soil

Introduction

Symbiosis is close and often long-term interaction between two different biological species. Microorganisms can be physically associated with other organisms and plants in a variety of ways. There also are many cases in which microorganisms live on both the inside and the outside of another organism, a phenomenon called ecto/endosymbiosis. These interactions can be positive or negative manner. Non-symbiosis or free living is defined as living independently of other organisms [8].

A Bio fertilizer is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Bio-fertilizers add nutrients through the natural processes of nitrogen fixation, solubilising phosphorus and stimulating plant growth through the synthesis of growth-promoting substances. The microorganisms in bio-fertilizers restore the soil's natural nutrient cycle and build soil organic matter [27].

Bio-fertilizers such as *Rhizobium*, *Azotobacter, Azospirilium* and Blue Green Algae (BGA) have been in use a long time. *Azotobacter* species are free-living, nitrogen-fixing bacteria; usually gram negative, motile, oval or spherical bacteria that form thick-walled cysts and may produce large quantities of capsular slime, in contrast to *Rhizobium* species, they normally fix molecular nitrogen from the atmosphere without symbiotic relations with plants, although some *Azotobacter* species are associated with plants [12].

Azotobacter produces pigments. For example, *Azotobacter chroococcum* forms a dark-brown water-soluble pigment melanin. It has a full range of enzymes needed to perform the nitrogen fixation: ferredoxin, hydrogenase, and an important enzyme nitrogenase [9].

Indole-3-acetic acid (IAA) is the most naturally-occurring plant growth hormone of the auxin class [6]. Bacteria of the genus *Azotobacter* synthesize auxins i.e IAA, cytokinins, and Glutamic Acid like substances and these growth materials are the primary substance controlling the enhanced growth of plants [3].

It is presumed that Plant Growth Promoting Rhizobacter producing plant growth regulators play a critical role in plant growth promotion. Effects of plant growth regulators including IAA on the plant will be concentration dependent. To assess this hypothesis, local isolates of *Azotobacter* sp. were screened for their intrinsic ability to produce IAA in the presence of varying amounts of L-tryptophan and their effect on root elongation of germinating seeds of test plants.

Methodology

Collection and processing of soil sampling

Soil sample were collected from earth surface at a depth of 10-15cm below the surface, collected in to sterile vials, sieved through a 4-mm-mesh sieve and stored field moisture content at 4°c.

Isolation and identification of *Azotobacter*

On gram of soil was serially diluted up to 10^{-8} dilution, 0.1 ml of each dilution was Ashby's agar using spread plate technique. The plates were incubated at room temperature for 4-7 days. Selected colonies from nutrient agar were streaked in selective and differential media were subjected to macroscopy, microscopy and biochemical tests for identification.

Screening of isolates based on IAA production

All the test strain of *Azotobacter* sp. were screening for IAA production. Test bacterial culture was inoculated in the respective medium (Jenson's/Nutrient broth) with tryptophan (1, 2 and 5mg/ml) or without tryptophan incubated at 28 ± 2°C for 15 days for *Azotobcter.* Cultures were centrifuged at 3000 rpm for 30 minutes. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of solawaski's reagent (50ml, 35% perchloric acid; 1ml 0.5% $FeCl_3$) development of the pink color indicates. IAA production Optical Density was read at 530 nm. The level of IAA production was estimated by a standard IAA graph [14].

Mass production of *Azotobacter*

For mass production of *Azotobacter* strain is isolated from various regions and grown on slants for preservation as per need culture from slant were transferred to liquid broth of selective as well as optimized medium in the rotary shaker for 4 days to prepare starter culture. Later on the starter cultures is transferred to the fermenter in batch culture mode with proper maintenance of 300°C and continuous agitation for 4-9 days. The mass broth is mixed with unsterile soil: Activated charcoal: CaCo3 in a ratio of 1:2:1where as other set prepared by using unsterile soil: crude coal powder: CaCo3 in same ratio over the carrier in such a way that 40% moisture is maintained. After proper mixing carrier containing inoculant was left for 7days and above formulated microbial inoculants used as biofertilizer.

Cell harvesting

The broth culture were centrifuged 5000 rpm for 30 minutes at 4°c, the pellets were resuspended physiological saline and stored at 4°C.

Field application

Soil was collected from the agricultural field, sterilization at 121°c for 15 minutes at 15 lbs pressure. The sterile soil was cooled to room temperature and dispensed in to a sterile pot and watered. The seeds coated with different concentration of (10-40%) co-inoculums of *Azotobacter* sp. (with and without charcoal) were sowed in the pots and were maintained in green house. After 15 days phenotypical traits and physiological estimations were carried out as per standard protocols [15].

Quality analysis of *Azotobacter* activity

The biochemical Studies of *Azotobacter* soil were carried out by using standard methods. The reagents and chemicals used in the biochemical studies were Analar Grade (AG). The optical density readings of the bio chemical estimations like the protein, carbohydrate and chlorophyll content were taken on UV Spectrophotometer.

Statistical analysis

All the values were expressed as mean ± SD (standard Deviation). Statistical analysis was carried out by using Origin software package (version 6.0). Statistical significance of differences between the control and experimental groups was assessed by One-way ANOVA. The value of probability less than 5% ($P < 0.05$) was considered statistically significant [16].

Result and discussion

The microbe-plant interaction in the rhizosphere can be beneficial, neutral, variable, or deleterious for plant growth. *Azotobacter* sp. was exerts beneficial effects on plant development are termed plant growth-promoting rhizobacteria. The term rhizobacteria is used for bacteria that aggressively colonize the rhizosphere**.**

Isolation of *Azotobacter sp.*

The present investigation the soil samples were in differently collected from the rhzosphere of corn and common lawn grasses. Moreover, as the use of fertilizers in soils tend to favor the development of micro-organism unable to fix nitrogen. The *Azotobacter* in soil is also greatly influenced by the PH value. In fact, their populations reach the highest levels in soils with PH above 6.5, where as they rarely occur collected showed PH values within the range 6.5-9.0 and were there for used for *Azotobacter* isolation.

Among the 10 nitrogen fixers, only 3 types of strains (strain-1, strain-2 and strain-3) were selected for the identification on the basis of their colonies appearances on Nutrient Agar media. The appearances of the colonies of strains (strain-1, strain-2 and strain-3) on the nutrient agar (NA) plate were circular, flat, raised and convex in elevation; small and pinpoint in size; dark brown, yellow, green, off white in color, respectively.

All the selected strains were identified as gram-negative rods and mostly true motile. All the strains were Oxidase positive, reduced nitrite to nitrate, strain-1 & 2 was found to be H_2S positive. The results obtained from the biochemical and carbohydrate fermentation tests are stated in Tables 2. According to Bargey's Manual of Systemic Bacteriology, the biochemical and carbohydrate fermentation tests indicated that the characters represented by the strain-1 is similar to *A. chroococcum,* strain-2 is as *A. vinelandii* and strain-3 is as *A. macrocytogenes*.

Direct isolation of *Azotobacter* like colonies on selective Brown medium from 16 out of 35 soil samples utilised. All members of genus *Azotobacter* produced slimy, glistening, smooth, whitish, weakly convex, 2–10-mm in diameter colonies. However, slight differences in size and sliminess of colonies were observed among different species. *A. vinelandii* formed circular, 2–3 mm in diameter, weakly slimy colonies, while only strain DSM87 produced a green fluorescent undiffusible pigment. *A. armeniacus* formed irregular, large, very slimy colonies and did not reveal any pigment production. *A. chroococcum* produced regular, very small colonies which turn brown with age. *A. paspali* formed rough, matt colonies with irregular edges (Aquilanti *et al.,* 2004) [2].

Azotobacter forms large, flat, soft, milky, mucoid and gummy Colonies (Gandora *et al.,* 1998) [11]. The soil samples were successfully collected from rhizosphere region of *Solanum lycopersicum* (tomato), *Zingiber officinale* (ginger), *Solanum melongena* (Brinjal), *Allium sativum* (Garlic) plants. The collected samples were isolated using Jenson's media. The colonies were round, and creamy in colour. The isolated samples were identified as isolates 1, 2, 3 and 4. All the isolates were gram negative rods. The results of motility, catalase, oxidase, starch hydrolysis and litmus tests were found to be positive for all four isolates [20].

The morphologial and bochemical characters of phosphobacter were found to be gram negative with rod shaped and non motile characteristics. The organism showed positive results for indole production, methyl red and catalase test. The organism thus identified were cultivated in mass usinng the specific medias and used as biofertilizer [26]. The microorganisms remained in the soil for a longer duration and enrich the soil. Dependence on chemical fertilizer for further agricultural growth would further loss in soil fertility, water contamination and unsustainable burden in the fiscal system (Promoting Biofertilizer in INDIA by Nilabja Ghosh).

Screening of isolate based on IAA production

The bacterial isolates were screened for their ability to produce plant growth regulator IAA. The range of IAA production in *Azotobacter chroococcum* was 2.68-10.80

mg/ml, *Azotobacter vinelandii* was 1.02 – 7.65 mg/ml and *Azotobacter macrocytogenes* was 0.87 – 5.61 mg/ml without tryptophan. The range of IAA production in *Azotobacter chroococcum* was 4.50-14.06 mg/ml, *Azotobacter vinelandii* was 2.22 – 9.72 mg/ml and *Azotobacter macrocytogenes* was 1.09 – 7.54 mg/ml with tryptophan.

The amount of IAA produced from some isolates, which range from 2.31 to 9.43 mol ml^{-1}. It is required to utilize potential application of the IAA high-producing bacteria. Seven siderophore-producing bacteria found in this study including the reference strain *A. vinelandii* Mac 259 are good candidates to be used for plant growth promotion, especially in neutral to alkaline soil. Four P-solubilizing bacteria have good prospects to improve plant growth, especially in soil with large amount of precipitated phosphate [7].

A total of 21 bacterial isolates (*Azotobacter sp.,* 10 and fluorescent *Pseudomonas sp.,* 11) were isolated from different rhizospheric soils in the vicinity of Aligarh city and characterized as per standard methods. These isolates were further tested for the production of indole acetic acid (IAA) in a medium with 0, 1, 2 and 5 mg/ml of tryptophan. A low amount (2.68-10.80 mg/ml) of IAA production was recorded by *Azotobacter* strains without tryptophan addition (Figure 1). Seven *Azotobacter* isolates showed high level (7.3 to 32.8 mg/ml) production of IAA at 5 mg/ml of tryptophan while at 1 and 2 mg/ml the production was in the range of 1.47 to 11.88 and 5.99 to 24.8 mg/ml, respectively. Production of IAA in fluorescent *Pseudomonas* isolates increased with an increase in tryptophan concentration from 1 to 5 mg/ml in the majority of isolates. In the presence of 5mg/ ml of tryptophan, 5 isolates of *Pseudomonas* produced high levels (41.0 to 53.2 mg/ml) of IAA while 6 other isolates produced IAA in the range of 23.4 to 36.2 mg/ml [10].

There are numerous soil microflora involved in the synthesis of auxins in pure culture and soil [4]. Some microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. The effects of auxins on plant seedlings are concentration dependent, i.e. low concentration may stimulate growth while high concentrations may be inhibitory. Different plant seedlings respond differently to variable auxin concentrations and type of microorganisms [18].

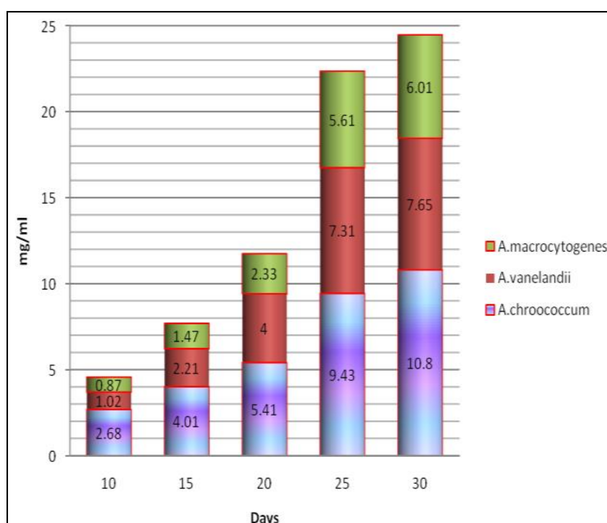

Fig 1: Estimation of IAA in culture filtrates of *Azotobacter* sp. without tryptophan

Mass production

When the cell count reached to 108- 109 cells/ml, the broth used as inoculants. For easy handling, packing, storing and transporting broth is mixed with an inert carrier material which contains sufficient amount of cells. Mass production, from 10 litres of *Azotobacter* strain bacteria media charcoal powder mixed 23 kgs of bio-fertilizer with 108-9 cells g -1 carrier material prepared for 5-7 days reported well growth on the broth culture. After 7 days doing next process of centrifuge is done centrifuged strain was stored at physiological saline.

Using a classical media optimization approach, *A. vinelandii* was cultivated under different concentrations of glucose, yeast extract, ammonium sulfate and phosphate salts. Cultivations using the optimized medium in a16-L stirred tank bioreactor without pH control yielded the maximum increase of approximately 275% in cell mass when compared to shake flask cultures using the unoptimized medium. Further improvement in cell mass production is currently carried out in our laboratories using different fed-batch cultivation strategies [24].

The consistent efficacy of *Azotobacter chroococcum* inoculants at 108-9 cells/g charcoal carrier material used as nitrofert bio-fertilizer application in mulberry garden crop. S1635 mulberry variety in Paired Row System of plantation with (150+90) x 60 cm spacing under irrigated condition with two treatments. Average leaf yield of 7.35 & 7.34 and total biomass of 12.95 & 13.0 tons ha-1 obtained in T1 and T2 respectively and quality of leaves on economic characters found without significant difference between the treatments revealed the consistent efficiency of *Azotobacter chroococcum* in fixing atmospheric nitrogen in the soil of mulberry garden to reduce nitrogenous chemical fertilizer and expenditure without affecting the quality linked leaf productivity and mass culture of the bacteria for preparation of Nitrofert bio-fertilizer [17].

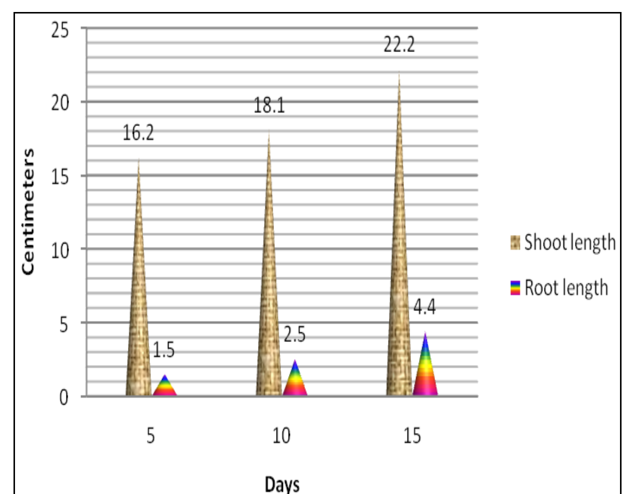

Fig 2: Effect of *Azotobacter* sp. in seed germination of *Vigna mungo*

Field application

In pot experiments, the growth of *vigna mungo, vigna unguiculata* at different time intervals are tabulated in Table 5 & 6. There is an increase in root length, shoot length and number of leaf count when the seeds were treated with biofertilizer. The both plants showed range of root length 1.5 to 4.4 of cm of *vigna mungo* and 2.1 to 5.6cm of *vigna unguiculata*, shoot length range from 16.2 to 22.2cm of

vigna mungo (Figure 2) and 16 to 20.6 cm of *vigna unguiculata* biofertilizer treated plant on 15th day after planting when compared to inorganic manure and control plants. Maximum growth in biofertilizer treated plant was mainly due to the ability of *Azotobacter* to solubilise phosphate and to produce siderophores and hormones. Statistical data were analyzed by DMRT which resulted in significant differences between the mean of the treated and control plants [13].

All parameter were significantly showed favorability more for seed treatment with biofertilizer than pouring of slurry in soil over control. Plant height was one of the parameters used to assess performance for crops showed that treatments involving *Azotobacter* with crude coal powder were more pronounced growth throughout the growing period than activated charcoal where nutrient contents studied showed good nutritional value with crude powder and enhance N-fixation thus disease free high growth of plants was resulted and economically it is better to use biofertilizer of *Azotobacter* alone for non-leguminous plants by using mass inoculums of optimized as well as selective media. However Mustard and pea plants grown in control pots were showing less growth in figure 3 [23].

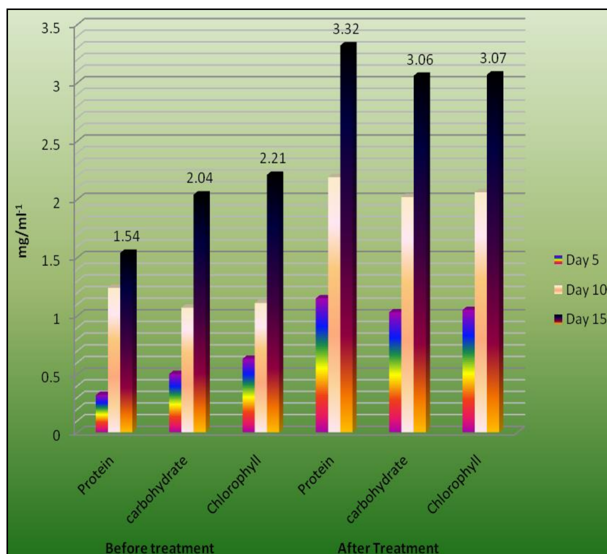

Fig 3: Effect of *Azotobacter* inoculums on plants before & after treatments

It is a natural method without any problems like salinity, alkalinity, soil erosion etc., In vast areas of low input agriculture as in crops like sugarcane these products will be of much use to give sustainability to production. Bio fertilizer soaks up and holds these dissolved nutrients substances so that the roots have more time to absorb them. Bio fertilizer also adds small amount of zinc, copper, boron and other vital nutrients to the soil and protects water quality [1].

Estimation study

The biochemical contents such as, chlorophyll, carbohydrate and protein content were determined to find out the variation in these single and dual inoculations of plant and control. Results showed that *Azotobacter* treated plants had the highest chlorophyll, carbohydrate and protein contents. The protein content of plants was analysed that range from 1.15 to 3.32, Carbohydrate content range from 1.03 to 3.06 and Chlorophyll content range from 1.05 to 3.07 after treatment. Similar results were reported by the biofertilizer significantly improved chlorophyll concentration in chilli and in black gram [21]. This is because, N is the chief constituent of protein, essential for the formation of protoplasm, which leads to cell enlargement, cell division and ultimately resulting in increased plant growth, *Azospirillum* augment the plant growth is mainly due to the biosynthesis of growth promoting substances like vitamin B12 and auxin. Growth and yield were significantly higher when the biofertilizers were inoculated with combined treatment (*Azotobacter* and *Azospirillum*) compared to individual inoculation and control. This could be due to the collective effect of biofertilizers. Plants inoculated with bio-VAM and *Azospirillum* fixed more nitrogen and produced more grain yield than singly inoculated plants in pearl millet [25]. Similar growth increase was recorded in black pepper earlier also with combined inoculation of biofertilizers (*Azospirillum, Azotobacter* and Phosphobacteria) [5].

Sharma and Johri (1988) showed bio-fertilizers including growth prompting bacteria improved the sunflower yield and qualitative parameters in compared with control (non-inoculation) treatment and as a result caused to increasing protein content. Studying interaction of bacteria treatment showed that double-inoculation caused to nitrogen content and yielded highest protein content using double-inoculation. Since *Rhizobium* with Phosphobacteria is nitrogen fixing bacteria and nitrogen is basic matter to forming protein treatment [21].

Conclusion

A bio-fertilizer is technically living; it can symbiotically associate with plant roots. Involved microorganisms could readily and safely convert complex organic material in simple compounds, so that plants are easily taken up. Microorganism function is in long duration, causing improvement of the soil fertility. It maintains the natural habitat of the soil. *Azotobacter chroococcum, Azotobacter vanelandii* and *Azotobacter macrocytogenes* inoculation was found to improve plant growth and biomass significantly. The *Azotobacter* sp. has high ability to increase Root and shoot as well as IAA production acquisition for plants.

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