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Isolation of *Azotobacter* and Cost Effective Production of Biofertilizer.

KEYWORDS: Azotobacter, Biofertilizer production, non-symbiotic nitrogen fixing bacteria.

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ABSTRACT *The aim of present study was the production of cost effective biofertilizer using selective and optimized media for Azotobacter to meet increasing nutritional food requirements with biotechnology to increase crop yields to improve socioeconomic condition of rural area to maintain ecological balance for sustainable production in minimum cost with increased percent of proteins, starch, sucrose, vitamins, nitrogen containing products which helps to increase germination rate, gibberlic acid, acetic acid, Vitamin B, Biotin for increase production rate with growth of plants. During study optimized inoculant with coal powder added was more beneficial when compare with laboratories activated charcoal powder for germination rate and growth of plants. Field inoculation of plants with Azotobacter after cultivation of experimental plants it is noted that 20-40 kg nitrogen remain back which benefits for further plantation and reduce use of 25 % chemical fertilizers. Non-legumes can be used to take up excess nitrogen from previous crops and recycle the nitrogen as well as available phosphorus and potassium.*

Introduction:

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or to soil. Biofertilizer from N2 fixing bacteria come in three forms: liquid, solid and lyophilized. For liquid and lyophilized ones, only solution medium is used, but for solid form, carriers such as peat, activated charcoal and chicken dung are needed.The first representative of the genus, Azotobacter chroococcum, was discovered and described in 1901 by the Dutch microbiologist and botanist Martinus Beijerinck. They are found in neutral and alkaline soils [1,2]. Azotobacter is Gram-negative, motile, pleomorphic aerobic bacterium which produces catalase, oval or spherical that form thick-walled cysts and may produce large quantities of capsular slime. Azotobacters are the most intensively investigated heterotrophic group possessing the highest respiratory rates. Members of these genera are mesophilic, which require optimum temperature of about 30°c.There are some microorganism which establish symbiotic relationships with different parts of plants and may develop special structures as the site of nitrogen fixation[3,4].

Non nodule forming diazotrophs for example , Azotobacter, Beijerinckia play an important role in the nitrogen cycle in nature, binding atmospheric nitrogen, which is inaccessible to plants, and releasing it in the form of ammonium ions into the soil. Apart from being a model organism, it respire aerobically which uses the organic matter present in soil to fix nitrogen asymbiotically and receiving energy from redox reactions, using organic compounds as electron donors. Azotobacter can use a variety of carbohydrates, alcohols and salts of organic acids as sources of carbon and can fix at least 10 micrograms of nitrogen per gram of glucose consumed so used by humans for the production of biofertilizers, food additives and some biopolymers. Azotobacter, a free living microbe, acts as plant growth promoting rhizobacteria (PGPR) in the rhizosphere of almost all crops. A group of beneficial plant bacteria, as potentially useful for stimulating plant growth and increasing crop yields has evolved over the past several years to where today researchers are able to repeatedly use them successfully in field experiments [5] Such PGPRs also fix nitrogen for non-legume crops like wheat, rice, sunflower, sugarcane, cauliflower, cotton, maize and sorghum which helps in saving 20-40kg chemical nitrogen i.e.45-90 kg urea per hectare. Yield of several non-legume was increased by PGPRs symbionts through plant growth promoting substances, it helps in root expansion, improve uptake of plant nutrients, protects plants from root diseases and most important improves biomass production of fast growing at wasteland.

Identification of Bacterial culture by Morphological and Biochemical Tests:

The bacterial cultures were classified mainly to their generic level. Morphological characterstics of the isolates viz. shape, size, colour, elevation, surface, margin, gram's nature and motility test. In biochemical characterization IMViC, sugar fermentation, urease, starch hydrolysis. It hydrolyses starch and releases water soluble pigment produced on the nitrogen free medium. Bacterial colonies grown on selective medium after incubation at 300C for 7 days it has been observed large, flat, soft, milky, mucoid and gummy colonies. For further confirmation test for one of the unique features was perfomed which forms an insoluble black brown pigment containing melanin due to its oxidation by tyrosinase. At high salt concentration and humidity it has ability to grow then Azotobacter confirmed at preliminary base (Table 1) and send for identification by using automation system at Indrayani laboratories. This culture used for mass production of Azotobacter.

Culturing of microorganisms

The selective and optimized mediums used for mass culturing of Azotobacter biofertilizers are as follows:

Selective N-free Mannitol broth

Components	Quantity (g L-1)
Mannitol	15.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.2
CaCO3	5.0
Ferric chloride	Trace
MnSO4.4H2O	Trace
pH	7.0
Distilled water	1 L

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Optimized broth were prepared by using additional components calcium chloride, thymidine, Bromothymol blue (0.5 g in 53 ml of 95% ethanol and add 47 ml of distilled water) and Bromocresol purple.

Materials and Methods:
For mass production of Azotobacter bacterial strain 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 300C and continuous agitation for 4-9 days. when cell count reached to 108- 109 cells/ml, the broth used as inoculant. For easy handling, packing, storing and transporting broth is mixed with an inert carrier material which contains sufficient amount of cells. In present study broth is mixed with unsterile soil: Activated charcoal, A. R. (RM 1332): 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

Use of Biofertilizer
There are different methods of applying biofertilizers

- Seed treatment
- Seedling treatment
- Top dressing
- Pouring of slurry

Used 50 ml water with 1% sucrose, boil for 10 min then add 1% gum arabic allow to cool to form slurry which called as sticker solution. In sticker solution add 20gm of biofertilizer mix it properly. Seeds were added to the slurry to form uniform coat of the culture around the seed without damaging seed coat. The inoculated seeds were kept on gunny bags away from sunlight for drying under shade and sown immediately.

Determination of NPK and organic carbon
Soil samples from each treatment were collected and analyzed for NPK content and organic matter under the laboratory condition. Determination of total nitrogen by kjeldahl method[6], Olsen method for phosphorous[7] and Ammonium acetate extractable method for potassium content was done as per in [8,9]. Organic matter and carbon was determined by Ignition method [10].

NPK, organic matter and carbon was calculated by the following formulae.

Available Phosphorus (kg/ha) =

$$R \times \frac{Total\_vol\_of\_extract}{vol\_of\_aliquot\_taken} \times \frac{1}{wt\_of\_soil} \times \frac{2.24 \times 10^6}{10^6}$$

R-from std. curve

$$Available\_K = R \times \frac{Vol\_of\_the\_extract \times 2.24 \times 10^6}{wt\_of\_soli\_taken \times 10^6}$$

Organic matter and carbon determined by

$$\%\ \text{Organic matter} = 100 - (Z + \%\ \text{moisture})\ (\text{Oven dry basis})$$

$$\%\ \text{Organic carbon} = \%\ \text{organic matter x } 0.58^{*}(\text{oven dry basis})$$

*organic matter is assumed to contain 58% organic carbon

Figure 1: Isolated bacterial Colonies of Azotobacter on selective and optimized media

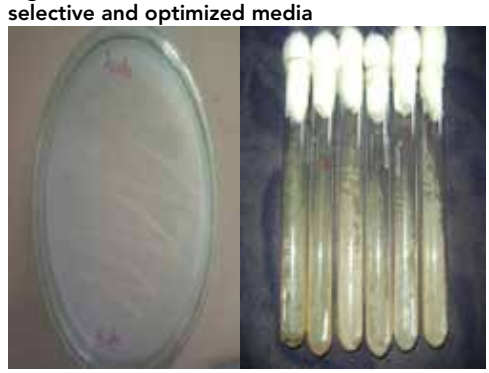

Figure 2: Growth of experimental legume & non-legume crops

2a:Legume crops 2 b:Non-legume crops

Results and Discussion:
Azotobacter forms large, flat, soft, milky, mucoid and gummy colonies (Fig 1). Biofertilizer applied as seed treatment and through pouring of slurry i.e. soil application where it function to mobilize the availability of nutrients especially NPK by their biological activity in particular area and help to enhance soil health by building up microflora to supply different kind of nutrients in the soil. This study clearly indicates that the effect of biofertilizer in wheat was enhanced, when compare with pea plants were inoculated with carrier material compared to uninoculated control. 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. Although, the NPK of soil was measured, it appears that high percent of NPK in pots inoculated with biofertilizer than uninoculated pots. This study not only showing improved growth potentiality with optimized media also considering seed germination found early almost 100% when compared with control. At maturity data regarding plants height, leaf area, root weight, shoots weight and grain yield were recorded using standard procedures. During study N influences vegetative and reproductive phase of plant growth by biofertilizer containing crude coal powder with optimized media were showed enhancement in uptake of NO3, NH4, H2PO4, K, P and improved water status of plants, nitrate reductase activity and antifungal compounds whenever compare with control. For one liter of bacterial culture 5 kg of carrier material is required for biofertilizer preparation.

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Conclusion:

The two experimental plants of mustard and pea have inadequate NPK of soil before inoculation of biofertilizer and improved after inoculation for non-legume plants than legume crops used thus increase yields therefore, use of biofertilizer may be a good option. This study has demonstrated that productivity can potentially be improved through the use of appropriate biofertilizer it resulted in the most of inoculated pots plants colour found to be greenish with proper growth as compare to control. The productivity and quality of mustard than pea can be substantially increased by seed treatment than pouring of slurry to soil so this type of method was showing more potentiality during growth of non-legume crops to get more benefit. In further study media optimization for growth of new improved inoculant may contain Rhizobia and PGP organisms for design of biofertilizer.

Table1. Partial Characteristics of Azotobacter

Charactertics	Observations
Shape	oval or spherical
Texture	Mucoid
Surface	Flat
Gram`s reaction	Negative
Motility	Motile
Indole production	+
Methyl red	+
Voges proskauer	-
Citrate utilization	+
Urease	+
Starch hydrolysis	+
Sugar fermentation	
Glucose	+
Mannitol	+

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