

EVALUATION OF ENDOPHYTIC ESTABLISHMENT OF MICROBIAL BIOCONTROL AGENT ON BLACK GRAM AND GREEN GRAM

Submitted in partial fulfillment of the requirements for the award for the award of Bachelor of Science degree in Biotechnology

BY

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BONAFIDE CERTIFICATED

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ABSTRACT

Plants nutrients are essential for the production of crop and healthy food for the world's ever increasing population . Soil management strategies today are mainly dependent on inorganic chemical – based fertilizer, which cause a serious threat to human health and the environment . Biofertilizer has been indentified as an alternative for increasing soil fertility and crop production in sustainable farming . The exploitation of beneficial microbes as bio- fertilizers has become of paramount importance in agricultural sector due to their potential role in food safety and sustainable crop production .

Biofertilizer can be an important component of integrated nutrients management . Microorganisms that are commonly used as bio- fertilizer components include nitrogen fixers (N- fixer) , potassium and phosphours solubilizer , growth promoting rhizobacteria (PGPRs) , endo and ecto mycorrhizal fungi cyanobacteria and other useful microscopic organisms. The use of bio- fertilizer leads to improved nutrients and water uptake, plant growth and plant tolerance to abiotic and biotic factors. These potential biological fertilizers would play a key role in productivity and sustainability of soil and also in protecting the environment as eco- friendly and cost effective inputs for the farmers .

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CHAPTER 1

1.INTRODUCTION

1.1 AGRICULTURE IN INDIA

Agriculture is the production of food and goods through farming and forestry . Agriculture was the key development that led to the rise of human civilization, with the husbandry of domesticated animals and plants creating food surpluses that enabled the development of more densely populated and stratified societies. The study of agriculture is known as agricultural science. Agricultural practices are irrigation, crop rotation ,fertilizers and pesticide

1.1.1 IMPORTANCE

*Agriculture is the major source of income for about three- fourths of india' population who live in villages.

*Agriculture provides not only food but also raw materials for manufacturing industries like textiles , sugar , vegetable oil ,jute and tobacco.

*Agriculture is not only an important occupation of the people, but also way of life ,culture and custom. Most of the indian customs and festivals are observed in consonance with agriculture seasons , activities and products.

*The importance of agriculture can be depicted from a fact that it is the most important sector in national economic development .

1.1.2 USE OF BENEFICIAL ORGANISMS IN AGRICULTURE

Agriculture production begins with the process of photosynthesis by green plant , which requires solar energy , water , and carbon dioxide. These materials are freely available . Therefore, it can be defined that agriculture is to produce something from nothing . But present agriculture has an extremely low efficiency . This is due to the low utilization efficiency of solar energy by plants. The potential utilization rate of solar energy by plants has been estimated theoretically to be between 10 and 20% . However, the actual utilization rate is less than 1% . Even the utilization rate of C4 plants , such as sugar cane which have a high photosynthetic efficiency , barely exceeds 6 or 7% during the maximum growth period .

Past studies have shown that photosynthetic efficiency of the chloroplasts of host crop cannot be enhanced further. This means biomass production capacity has reached a maximum. Therefore, the best opportunity for increasing biomass production is to utilize the visible light and the infrared radiation. This together accounts for about 80% of the total energy. And also the ways of recycling organic energy contained in plant and animal residues have to be explored through direct utilization of organic molecules by plants.

In the presence of organic matter, photosynthetic bacteria and algae can utilize wavelengths ranging from 700 to 1200nm. Green plants do not use these wavelengths. Fermenting microorganism can also break down organic matter, thereby releasing complex compounds such as amino acids for plant use. This increases the efficiency of organic matter for crop production. Thus a key factor for increasing crop production is the availability of organic matter, which has been developed by utilizing solar energy and the presence of efficient microbes to decompose these materials. This increases the utilization efficiency of solar energy.

One of the biggest agricultural problems is the lack of nutrients and top soil due to chemicals, row-crop production and intensive tillage. Effective microorganisms (EM) culture solves this problem by reinstating millions of microorganisms to the soil turning into eco-gold environment. This sustainable soil called zymogenic soil creates the most conducive growing conditions. A soil rich in minerals and regenerative microorganisms provides optimum production levels, reduces disease and produces top quality crops without chemicals.

Agriculture has a vital role in the economy of many developing as well as developed countries. In India, over twenty-two percent of the rural households depend on agriculture as the principal means of livelihood (Sahayraj, 2004).

Agriculture is India's big economy. Although the share of agriculture in the total national income has been gradually decreasing on account of development of the secondary and tertiary sectors, its contribution continues to be significant. In 1950, the share of agriculture was 57% but it is only 26% now. The more developed a country is, the lesser is the contribution of agriculture. Today almost 60% of the population depends directly or indirectly on agriculture. The greater independence of working population on agriculture indicates the underdevelopment of non-agriculture activities in the country. Agriculture provides raw materials to our leading industries such as cotton textiles and sugar industries. Not only this, the workers in industries depend on agriculture for their food.

Agriculture also provides the market for a variety of goods. A number of the agricultural commodities like tea, coffee, spices and tobacco constitute our main items of exports. These amount to almost 15% of our total exports. Hence agriculture provides foreign exchange which helps us to buy

machines from abroad . It also maintains a balance of payments and makes our country self-sufficient. Tertiary sector provides helpful services to the industries and agriculture like banking ,warehousing ect .Internal trade is mostly done in agriculture produce . For example , various means of transport get bulk of their business by the movement of agricultural goods. Our agriculture has brought fame to the country . India enjoys first position in the world as far as the production of tea and groundnuts are concerned .

Agriculture plays a important role in the internal trade . It is because of the fact that 90% of our population spends 60% of their income on the purchase of the items like food , tea , milk etc .(vivek chowdhury ,2010)

Paroda (1993) report that the total annual crop loss due to insect pests alone exceeded Rs . 6000 crores during 1973 and it was further increased to about 25, 000 crorse in 2001-2002 (Ignacimuthu, 2002). Among the various biotic and a-biotic constraints in agriculture production , the insect pests , disease causing pathogens , weeds and nematodes as biotic stress play a vital role . The biotic stress was estimated to cause an annual loss of about 33- 35 percent of the potential food production worldwide . Furthermore, the pest related damges result in an estimated Rs. 50,000 crore annually in agricultural production in the field as well as storage in india (Ignacimuthu, 2002) . The rapid increase in area under intensive cultivation of crop , lack of natural enemy of pests , pest's resurgence ,climatic condition and the spread of pests from one place to another are largely responsible for the serious pest deprivation in any agro – ecosystem (jayaraj, 2002) . The key challenge today is to adopt strategies that will address the concern of maintaining and enchancing the integrity of natural resources and improved productivity .

1.1.3 CROP YIELD

Agricultural productivity is measured as the ratio of agricultural outputs to agricultural inputs . while individual products are usually measured by weight, their varying densities make measuring overall agricultural output difficult. Therefore , output is usually measured as the market value of final output , which excludes intermediate products such as corn feed used in the meat industry. This output value may be compared to many different type of inputs such as labour and land (yield).These are called partial measures of productivity. Agricultural productivity . Agricultural productivity may also be measured by what is termed total factor productivity (TEP) . This method of calculating agricultural productivity compares an index of agricultural inputs to an index of outputs. This measure of agriculture productivity was established to remedy the shortcomings of the partial measures of productivity notably that it is often hard to identify the factor cause them to change . Changes in TEP are usually attributed to technological improvements .

1.1.3.1 Importance of agricultural productivity

The productivity of a region's farms is important for many reasons. Aside from providing more food, increasing the productivity of farms affects the region's prospects

for growth and competitiveness on the agricultural market, income distribution and savings, and labour migration. An increase in a region's agricultural productivity implies a more efficient distribution of scarce resources. As farmers adopt new techniques and differences in productivity arise, the more productive farmers benefit from an increase in their welfare while farmers who are not productive enough will exit the market to seek success elsewhere (Mundlak, Yair, 2007). As a region's farms become more productive, its comparative advantage in agricultural products increases, which means that it can produce these products at a lower opportunity cost than can other regions. Therefore, the region becomes more competitive on the world market, which means that it can attract more consumers since they are able to buy more of the products offered for the same amount of money.

Increases in agricultural productivity lead also to agricultural growth and can help to alleviate poverty in poor and developing countries, where agriculture often employs the greatest portion of the population. As farms become more productive, the wages earned by those who work in agriculture increase. At the same time, food prices decrease and food supplies become more stable. Labourers therefore have more money to spend on food as well as other products. This also leads to agricultural growth. People see that there is a greater opportunity earn their living by farming and are attracted to agriculture either as owners of farms themselves or as labourers.

However, it is not only the people employed in agriculture who benefit from increases in agricultural productivity. Those employed in other sectors also enjoy lower food prices and a more stable food sup. At the same time, they may see their wages rise as well. Agricultural productivity is becoming increasingly important as the world population continues to grow. India, one of the world's most populous countries, has taken steps in the past decades to increase its land productivity. Forty years ago, North India produced only wheat, but with the advent of the earlier maturing high-yielding wheats and rices, the wheat could be harvested in time to plant rice. This wheat/rice combination is now widely used throughout the Punjab, Haryana, and parts of Uttar Pradesh. The wheat yield of three tons and rice yield of two tons combine for five tons of grain per hectare, helping to feed India's 1.1 billion people (R. Brown, Lester, 2006) .

1.2 FACTORS CONTRIBUTION TO DECLINE OF AGRICULTURE

Slow Down in Agricultural and Rural Non-Farm Growth: Both the poorest as well as the more prosperous 'Green Revolution' states of Punjab, Haryana, Andhra Pradesh and Uttar Pradesh have recently witnessed a slow-down in agricultural growth and it ultimately lead for farmer's suicide. Some of the factors hampering the revival of growth are:

1.2.1 Poor Composition of Public Expenditures

Public spending on agricultural subsidies is crowding out productivity-enhancing investments such as agricultural research and extension, as well as investments in rural infrastructure, and the health and education of the rural people. In 1999/2000, agricultural subsidies amounted to 3 percent of GDP and were over 7 times the public investments in the sector.

1.2.2 Over-regulation of Domestic Agricultural Trade

While economic and trade reforms in the 1990s helped to improve the incentive framework, over-regulation of domestic trade has increased costs, price risks and uncertainty, undermining the sector's competitiveness.

1.2.3 Government Interventions in Labor, Land, and Credit Markets

More rapid growth of the rural non-farm sector is constrained by government interventions in factor markets -- labor, land, and credit -- and in output markets, such as the small-scale reservation of enterprises.

1.2.4 Inadequate Infrastructure and Services in Rural Areas

Infrastructure is also a significant factor in the process of development but country like our rural Bharat has not possess the infrastructure such as roads, electricity, fertilizer and pesticides availability which caused the vulnerable damage to the growth of agriculture.

1.3 WEAK FRAMEWORK FOR SUSTAINABLE WATER MANAGEMENT AND IRRIGATION

1.3.1 Inequitable Allocation of Water

Many states lack the incentives, policy, regulatory, and institutional framework for the efficient, sustainable, and equitable allocation of water.

1.3.2 Deteriorating Irrigation Infrastructure

Public spending in irrigation is spread over many uncompleted projects. In addition, existing infrastructure has rapidly deteriorated as operations and maintenance is given lower priority (G. C. Aggarwal, 1995).

1.3.3 Stringent Land Regulations Discourage Rural Investments

While land distribution has become less skewed, land policy and regulations to increase security of tenure (including restrictions or bans on renting land or converting it to other uses) have had the unintended effect of reducing access by the landless and discouraging rural investments.

1.3.4 Computerization of Land Records has Brought to Light Institution

State government initiatives to computerize land records have reduced transaction costs and increased transparency, but also brought to light institutional weaknesses.

1.3.5 Rural Poor have Little Access to Credit

While India has a wide network of rural finance institutions, many of the rural poor remain excluded, due to inefficiencies in the formal finance institutions, the weak regulatory framework, high transaction costs, and risks associated with lending to agriculture.

1.3.6 Weak Natural Resources Management

One quarter of India's population depends on forests for at least part of their livelihoods.

1.3.7 A Purely Conservation Approach to Forests is Ineffective

Experience in India shows that a purely conservation approach to natural resources management does not work effectively and does little to reduce poverty.

1.3.8 Weak Resource Rights for Forest Communities

The forest sector is also faced with weak resource rights and economic incentives for communities, an inefficient legal framework and participatory management, and poor access to markets.

1.3.9 Weak Delivery of Basic Services in Rural Areas

Low bureaucratic accountability and inefficient use of public funds: Despite large expenditures in rural development, a highly centralized bureaucracy with low accountability and inefficient use of public funds limit their impact on poverty. However, the transfer of authority, funds, and functionaries to these local bodies is progressing slowly, in part due to political vested interests. The poor are not empowered to contribute to shaping public programs or to hold local governments accountable (Ramaiah Bheenaveni, 2007).

1.4 BIOFERTILIZER

The name itself is self explanatory. The fertilizers are used to improve the fertility of the land using biological wastes, hence the term biofertilizers, and biological wastes do not contain any chemicals which are detrimental to the living soil. They are extremely beneficial in enriching the soil with those micro-organisms, which produce organic nutrients for the soil and help combat diseases. The farm produce does not contain traces of hazardous and poisonous materials. Thus those products are accepted across the world as Organic ones. Hence for organic farming the use of biofertilizers is mandatory.

Biofertilizers, more commonly known as microbial inoculants, are artificially multiplied cultures of certain soil organisms that can improve soil fertility and crop productivity.

'Biofertilizer' is a substance which contains living microorganisms which, when applied to seed, 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(Vessey, J.k. 2003). Biofertilizers add nutrients through the natural processes of Nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth promoting substances. Biofertilizers can be expected to reduce the use of chemical fertilizers and pesticides. The microorganisms in biofertilizers restore the soil's natural nutrient cycle and build soil organic matter. Through the use of biofertilizers, healthy plants can be grown while enhancing the sustainability and the health of soil. Since they play several roles, a preferred scientific term for such beneficial bacteria is plant-growth promoting rhizobacteria (PGPR). Therefore, they are extremely advantageous in enriching the soil fertility and fulfilling the plant nutrient requirements by supplying the organic nutrients through microorganism and their byproduct. Hence, biofertilizers do not contain any chemicals which are harmful to the living soil. Biofertilizers are Eco-friendly organic agro-input and more cost effective than chemical fertilizers. Biofertilizers like Rhizobium, Azotobacter, Azospirillum and blue green algae(BGA) are in use since long time ago. Rhizobium inoculant is used for leguminous crops. Azotobacter can be used with crops like wheat, maize, mustard, cotton, potato and other vegetable crops. Azospirillum inoculants are recommended mainly for sorghum, millets, maize, sugarcane and wheat. Blue green algae belonging to genera Nostoc, Anabaena, Tolypothrix and Aulosira fix atmospheric nitrogen and are used as inoculants for paddy crop grown both under upland and low land conditions. Anabaena in association with water fern Azolla contributes nitrogen up to 60 kg/ha/season and also enriches soils with organic matter. Other types of bacteria, so-called phosphate solubilizing bacteria like Pantoea agglomerans strain P5, and Pseudomonas putida strain P13 are able to solubilize the insoluble phosphate from organic and inorganic phosphate source. In fact, due to immobilization of phosphate by mineral ions such as Fe, Al and Ca or organic acids, the rate of available phosphate (Pi) in soil is well below plant needs. In addition, chemical Pi fertilizer are also immobilized in the soil immediately so that less than 20 percent of added fertilizer is absorbed by plants. Therefore, reduction in Pi resources, on one hand, and environmental pollutions resulted from both production and applications of chemical Pi fertilizer, on the other

hand, have already demanded the use of new generation of phosphate fertilizers globally known as phosphate solubilizing bacteria or phosphate biofertilizers.

Although the 3 beneficial effects of legumes in improving soil fertility was known since ancient times and their role in biological nitrogen fixation was discovered more than a century ago, commercial exploitation of such biological processes is of recent interest and practice. The commercial history of biofertilizers began with the launch of 'Nitragin' by Nobbe and Hiltner, a laboratory culture of Rhizobia in 1895, followed by the discovery of *Azotobacter* and then the blue green algae and a host of other micro-organisms. *Azospirillum* and Vesicular Arbuscular Micorrhizae (VAM) are fairly recent discoveries. In India the first study on legume *Rhizobium* symbiosis was conducted by N.V.Joshi and the first commercial production started as early as 1956. However the Ministry of Agriculture under the Ninth Plan initiated the real effort to popularize and promote the input with the setting up of the National Project on Development and Use of Biofertilizers (NPDB).

1.4.1 How Biofertilizers are Applied to Crops?

1. Seed treatment

200 g of nitrogenous biofertilizer and 200 g of Phosphotika are suspended in 300-400 ml of water and mixed thoroughly. Ten kg seeds are treated with this paste and dried in shade. The treated seeds have to be sown as soon as possible.

2. Seedling root dip

For rice crop, a bed is made in the field and filled with water. Recommended biofertilizers are mixed in this water and the roots of seedlings are dipped for 8-10 hrs.

3. Soil treatment

4 kg each of the recommended biofertilizers are mixed in 200 kg of compost and kept overnight. This mixture is incorporated in the soil at the time of sowing or planting.

1.4.2 How Could One Get Good Response to Biofertilizer Application?

- Biofertilizer product must contain good effective strain in appropriate population and should be free from contaminating microorganisms.
- Select right combination of Biofertilizers and use before expiry date.
- Use suggested method of application and apply at appropriate time as per the information provided on the label.
- For seed treatment adequate adhesive should be used for better results.
- For problematic soils use corrective methods like lime or gypsum pelleting of seeds or correction of soil pH by use of lime.
- Ensure the supply of phosphorus and other nutrients.

1.4.3 What would be Probable Reasons for not Getting Response from the Application of Biofertilizers?

1. On account of quality of product
 - Use of ineffective strain.
 - Insufficient population of microorganisms.
 - High level of contaminants.
2. On account of inadequate storage facilities
 - May have been exposed to high temperature.
 - May have been stored in hostile conditions.
3. On account of usage
 - Not used by recommended method in appropriate doses.
 - Poor quality adhesive.
 - Used with strong doses of plant protection chemicals.

4. On account of soil and environment
 - High soil temperature or low soil moisture.
 - Acidity or alkalinity in soil.
 - Poor availability of phosphorous and molybdenum.
 - Presence of high native population or presence of bacteriophages.

1.4.4 What Precautions One Should take for using Biofertilizers?

- Biofertilizer packets need to be stored in cool and dry place away from direct sunlight and heat.
- Right combinations of biofertilizers have to be used.
- As *Rhizobium* is crop specific, one should use for the specified crop only.
- Other chemicals should not be mixed with the biofertilizers.
- While purchasing one should ensure that each packet is provided with necessary information like name of the product, name of the crop for which intended, name and address of the manufacturer, date of manufacture, date of expiry, batch number and instructions for use.
- The packet has to be used before its expiry, only for the specified crop and by the recommended method of application.
- Biofertilizers are live product and require care in the storage
- Both nitrogenous and phosphatic biofertilizers are to be used to get the best results.
- It is important to use biofertilizers along with chemical fertilizers and organic manures.
- Biofertilizers are not replacement of fertilizers but can supplement plant nutrient requirements

1.4.5 Importance of use of Biofertilizers

Biofertilizers are microorganisms that help plants to grow by increasing the quantity of nutrients. Since these fertilizers contain living microorganisms, it increases or promotes the supply of important nutrients crucial for the overall productivity of the soil. An increasing number of farmers and agriculturists are turning to the use of biofertilizers as these are gentler on the soil as against chemical fertilizers.

It is easier to fully appreciate the importance of biofertilizers when we know how harmful chemical fertilizers can get for the soil and the crop. Chemical fertilizers are meant to boost the growth of plants and increase the fertility of the soil; however they cause significant damage to the environment. These chemical based fertilizers also make use of nitrogenous fertilizers or chemicals, are expensive and not as conveniently available. The value of biofertilizers has further increased in an increasingly eco-conscious world. Since these fertilizers are eco-friendly they can be used generously to promote healthy crops. The quality of the soil is also improved thanks to these environmentally friendly fertilizers.

1.4.6 Benefits of using Biofertilizers

As it is living thing, it can symbiotically associate with plant root. Involved microorganisms could readily and safely convert complex organic material in simple compound, so that plant easily taken up. Microorganism function is in long duration causing improvement of the soil fertility. It maintains the natural habitat of the soil. It increases crop yield by 20-30%. Replace chemical nitrogen and phosphorus by 25% in addition to stimulating of the plant growth. Finally it can provide protection against drought and some soil borne diseases.

1.4.7 Advantages of Biofertilizers

Cost effective relative to chemical fertilizer and reduces the costs towards fertilizers use, especially regarding nitrogen and phosphorus. It is environmentally friendly fertilizer that not only prevents damaging the natural source but helps to some extent clean the nature from precipitated chemical fertilizer. And can provide better nourishment to plants.

1.4.8 Commonly Explored Biofertilizers in India are Mentioned below along with some Salient Features.

Rhizobium (RHZ): These inoculants are known for their ability to fix atmospheric nitrogen in Symbiotic association with plants forming nodules in roots (stem nodules in sesabaniamrostrata). RHZ are however limited by their specificity and only certain legumes are benefited from this Symbiosis.

Azotobacter (AZT): This has been found beneficial to a wide array of crops covering cereals, millets, vegetables, cotton and sugarcane. It is free living and non-symbiotic nitrogen fixing organism that also produces certain substances good for the growth of plants and antibodies that suppress many root pathogens.

Azospirillum (AZS): This is also a nitrogen-fixing micro organism beneficial for non-leguminous plants. Like AZT, the benefits transcend nitrogen enrichment through production of growth promoting substances.

Blue green Algae (BGA) and Azolla: BGA are photosynthetic nitrogen fixers and are free living. They are found in abundance in India. They too add growth-promoting substances including vitamin B12 improve the soil's aeration and water holding capacity and add to bio mass when decomposed after life cycle. *Azolla* is an aquatic fern found in small and shallow water bodies and in rice fields. It has symbiotic relation with BGA and can help rice or other crops through dual cropping or green manuring of soil.

Phosphate solubilizing (PSB)/mobilizing biofertilizer: Phosphorus, both native in soil and applied in inorganic fertilizers becomes mostly unavailable to crops because of its low levels of mobility and solubility and its tendency to become fixed in soil. The PSB are life forms that can help in improving phosphate uptake of plants in different ways. The PSB also has the potential to make utilization of India's abundant deposits of rock phosphates possible, much of which is not enriched.

Trichoderma: It is a non- pathogenic and eco-friendly product. The product is antagonistic hyper parasitic against different pathogens in the field and economically well established biocontrol agent

Composter: (Decomposing Culture): Composter breaks down any organic matter such as dead plants farm yard waste, cattle waste etc. thereby increasing the soil productivity.

Tricho-Card: Trichogramma is an efficient destroyer of eggs of many leaf and flower eaters, stems, fruit, shoot borers etc. It can be used in a variety of crops as well as in horticultural and ornamental plants, such as sugarcane, cotton, brinjal, tomato, corn, jawar vegetables, citrus, paddy apple etc.

Vermi Compost: It is 100% pure eco-friendly organic fertilizer. This organic fertilizer has nitrogen phosphorus, potassium, organic carbon, sulphur, hormones, vitamins, enzymes and antibiotics which helps to improve the quality and quantity of yield. It is observed that due to continuous misuse of chemical fertiliser soil loses its fertility and gets salty day by day. To overcome such problems natural farming is the only remedy and Vermicompost is the best solution.

Biocompost: It is eco-friendly organic fertilizer which is prepared from the sugar industry waste material which is decomposed and enriched with various plants and human friendly bacteria and fungi. Biocompost consists of nitrogen, phosphate solubilizing bacteria and various useful fungi like decomposing fungi, trichoderma viride which protects the plants from various soil borne diseases and also help to increase soil fertility which results to a good quality product to the farmers.

1.5 RHIZOBIUM

Rhizobia (Frank, 1889) are soil bacteria that fix nitrogen (diazotrophy) after becoming established inside root nodules of legumes (Fabaceae). Rhizobia require a plant host; they cannot independently fix nitrogen. Morphologically, they are generally gram negative, motile, non-sporulating rods **Scientific Classification**

<i>Rhizobium</i>	
Kingdom:	<u>Bacteria</u>
Phylum:	<u>Proteobacteria</u>

Class:	<u>Alphaproteobacteria</u>
Order:	<u>Rhizobiales</u>
Family:	<u>Rhizobiaceae</u>
Genus:	<u>Rhizobium</u>

1.5.1 History

The first species of rhizobia, *R. leguminosarum*, was identified in 1889, and all further species were initially placed in the Rhizobium genus. However, more advanced methods of analysis have revised this classification, and now there are many in other genera. Most research has been done on crop and forage legumes such as clover, alfalfa, beans, and soy; recently, more work is occurring on North American legumes.

The word rhizobia comes from the Ancient Greek $\rho\acute{\iota}\zeta\alpha$, "rhíza," meaning "root," and $\beta\acute{\iota}\omicron\varsigma$, "bios," meaning "life." The word *Rhizobium* is still sometimes used as the singular form of rhizobia.

1.5.2 Taxonomy

Rhizobia are a paraphyletic group which fall into two classes of the proteobacteria—the alpha- and beta-proteobacteria. As shown below, most belong to the order Rhizobiales but several rhizobia occur in distinct bacterial orders of the *Proteobacteria*.

(i) <u>α-proteobacteria</u> <u>Rhizobiales</u> <u>Bradyrhizobiaceae</u>	<u>Rhizobiaceae</u>	(ii) <u>β-proteobacteria</u> <u>Burkholderiales</u> <u>Burkholderiaceae</u>
<u>BradyRhizobium</u> <u>B. canariense</u> <u>B. elkanii</u> <u>B. japonicum</u> <u>B. liaoningense</u> <u>B. yuanmingense</u> <u>Brucellaceae</u>	<u>Rhizobium</u> <u>R. cellulosilyticum</u> <u>R. daejeonense</u> <u>R. etli</u> <u>R. galegae</u> <u>R. gallicum</u> <u>R. giardinii</u>	<u>Burkholderia</u> <u>B. caribensis</u> <u>B. dolosa</u> <u>B. mimosarum</u> <u>B. phymatum</u> <u>B. tuberum</u> <u>Cupriavidus</u>

<u>Ochrobactrum</u> <u>O. cytisi</u> <u>O. lupini</u> <u>Hyphomicrobiaceae</u> <u>AzoRhizobium</u> <u>A. caulinodans</u> <u>A. doebereineriae</u> <u>Devosia</u> <u>D. neptuniae</u> <u>Methylobacteriaceae</u> <u>Methylobacterium</u> <u>M. nodulans</u> <u>Phyllobacteriaceae</u> <u>MesoRhizobium</u> <u>M. albiziae</u> <u>M. amorphae</u> <u>M. chacoense</u> <u>M. ciceri</u> <u>M. huakuui</u> <u>M. loti</u> <u>M. mediterraneum</u> <u>M. plurifarum</u> <u>M. septentrionale</u> <u>M. temperatum</u> <u>M. tianshanense</u> <u>Phyllobacterium</u> <u>P. ifriqiense</u> <u>P. leguminum</u> <u>P. trifolii</u>	<u>R. hainanense</u> <u>R. huautlense</u> <u>R. indigoferae</u> <u>R. leguminosarum</u> <u>R. loessense</u> <u>R. lupini</u> <u>R. lusitanum</u> <u>R. mongolense</u> <u>R. miluonense</u> <u>R. sullae</u> <u>R. tropici</u> <u>R. undicola</u> <u>R. yanglingense</u> <u>SinoRhizobium (Ensifer)</u> <u>S. abri</u> <u>S. adhaerens</u> <u>S. americanum</u> <u>S. arboris</u> <u>S. fredii</u> <u>S. indiaense</u> <u>S. kostiense</u> <u>S. kummerowiae</u> <u>S. medicae</u> <u>S. meliloti</u> <u>S. mexicanus</u> <u>S. morelense</u> <u>S. sahelii</u> <u>S. terangaie</u> <u>S. xinjiangense</u>	<u>C. taiwanensis</u> <u>Oxalobacteraceae</u> <u>Herbaspirillum</u> <u>H. lusitanum</u>
(i) <u>α-proteobacteria</u> <u>Rhizobiales</u> <u>Bradyrhizobiaceae</u>	<u>Rhizobiaceae</u>	(ii) <u>β-proteobacteria</u> <u>Burkholderiales</u> <u>Burkholderiaceae</u>
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<u>Devosia</u>	<u>R. mongolense</u>	
<u>D. neptuniae</u>	<u>R. miluonense</u>	
<u>Methylobacteriaceae</u>	<u>R. sullae</u>	
<u>Methylobacterium</u>	<u>R. tropici</u>	
<u>M. nodulans</u>	<u>R. undicola</u>	
<u>Phyllobacteriaceae</u>	<u>R. yanglingense</u>	
<u>MesoRhizobium</u>	<u>SinoRhizobium (Ensifer)</u>	
<u>M. albiziae</u>	<u>S. abri</u>	
<u>M. amorphae</u>	<u>S. adhaerens</u>	
<u>M. chacoense</u>	<u>S. americanum</u>	
<u>M. ciceri</u>	<u>S. arboris</u>	
<u>M. huakuui</u>	<u>S. fredii</u>	
<u>M. loti</u>	<u>S. indiaense</u>	
<u>M. mediterraneum</u>	<u>S. kostiense</u>	
<u>M. plurifarum</u>	<u>S. kummerowiae</u>	
<u>M. septentrionale</u>	<u>S. medicae</u>	
<u>M. temperatum</u>	<u>S. meliloti</u>	
<u>M. tianshanense</u>	<u>S. mexicanus</u>	
<u>Phyllobacterium</u>	<u>S. morelense</u>	
<u>P. ifriqiense</u>	<u>S. saheli</u>	
<u>P. leguminum</u>	<u>S. teranga</u>	
<u>P. trifolii</u>	<u>S. xinjiangense</u>	

These groups include a variety of non-symbiotic bacteria. For instance, the plant pathogen Agrobacterium is a closer relative of Rhizobium than the BradyRhizobium that nodulate soybean (and may not really be a separate genus). The genes responsible for the symbiosis with plants, however, may be more closely related than the organisms themselves, acquired by horizontal transfer (via bacterial conjugation) rather than vertical gene transfer (from a common ancestor).

1.5.3 Importance in agriculture

Although much of the nitrogen is removed when protein-rich grain or hay is harvested, significant amounts can remain in the soil for future crops. This is especially important when nitrogen fertilizer is not used, as in organic rotation schemes or some less-industrialized countries. Nitrogen is the most commonly deficient nutrient in many soils around the world and it is the most commonly supplied plant nutrient. Supply of nitrogen through fertilizers has severe environmental concerns.

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1.5.4 Symbiosis

Rhizobia are unique because they live in a symbiotic relationship with legumes. Common crop and forage legumes are peas, beans, clover, and soy.

1.5.5 Infection and signal exchange

The symbiotic relationship implies a signal exchange between both partners that leads to mutual recognition and development of symbiotic structures. Rhizobia live in the soil where they are able to sense flavonoids secreted by the root of their host legume plant. Flavonoids trigger the secretion of Nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses such as ion fluxes. The best known infection mechanism is called intracellular infection, in this case the rhizobia enter through a deformed root hair in a similar way to endocytosis, forming an intracellular tube called the infection thread. A second mechanism is called "crack entry", in this case no root hair deformation is observed and the bacteria penetrate between cells, through cracks produced by lateral root emergence. Later on bacteria become intracellular and an infection thread is formed like in intracellular infections. The infection triggers cell division in the cortex of the root where a new organ, the nodule appears as a result of successive processes.

1.5.6 Nodule formation and functioning

Infection threads grow to the nodule, infect its central tissue and release the rhizobia in these cells where they differentiate morphologically into bacteroids and fix nitrogen from the atmosphere into a plant usable form, ammonium (NH_4^+), utilizing the enzyme nitrogenase. In return the plant supplies the bacteria with carbohydrates, proteins, and sufficient oxygen so as not to interfere with the fixation process. Leghaemoglobins, plant proteins similar to human hemoglobins help to provide oxygen for respiration while keeping the free oxygen concentration low enough not to inhibit nitrogenase activity. Recently, it was discovered that a *Bradyrhizobium* strain forms nodules in *Aeschynomene* without producing Nod factors, suggesting the existence of alternative communication signals other than Nod factors (Eric Giraud, *et al.*, and L. Vallenet, D. *et al.*, 2007).

The legume–*Rhizobium* symbiosis is a classic example of mutualism rhizobia supply ammonia or amino acids to the plant and in return receive organic acids (principally as the dicarboxylic acids malate and succinate) as a carbon and energy source but its evolutionary persistence is actually somewhat surprising. Because several unrelated strains infect each individual plant, any one strain could redirect resources from nitrogen fixation to its own reproduction without killing the host plant upon which they all depend. But this form of cheating should be equally tempting for all strains, a classic tragedy of the commons. There are two competing hypotheses for the mechanism that maintains legume *Rhizobium* symbiosis (though both may occur in nature). The sanctions hypothesis suggests that plants police cheating rhizobia. Sanctions could take the form of reduced nodule growth, early nodule death, decreased carbon supply to nodules, or reduced oxygen supply to nodules that fix less nitrogen (R. F. Denison, 2000). The partner choice hypothesis proposes that the plant uses pre-nodulation signals from the rhizobia to decide whether to allow nodulation and chooses only non-cheating rhizobia. There is evidence for sanctions in soybean plants, which reduce *Rhizobium* reproduction (perhaps by limiting oxygen supply) in nodules that fix less nitrogen (E.T. Kiers, *et al.*, 2003). Similarly, wild lupine plants allocate less resources to nodules containing less-beneficial rhizobia, limiting rhizobial reproduction inside. This is consistent with the definition of sanctions just given, although called "partner choice" by the authors (Simms, *et al.*, 2006).

However, other studies have found no evidence of plant sanctions and instead support the partner choice hypothesis (K. D. Heath, and P. Tiffin, 2009 ; D. E. Marco, *et al.*, 2009).

1.6 AZOTOBACTER

Azotobacter is a genus of usually motile, oval or spherical bacteria that form thick-walled cysts and may produce large quantities of capsular slime. They are aerobic, free-living soil microbes which 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 is used by humans for the production of biofertilizers, food additives and some biopolymers. The first representative of the genus, *Azotobacter chroococcum*, was discovered and described in 1901 by the Dutch microbiologist and botanist Martinus Beijerinck. *Azotobacter* are Gram-negative bacteria. They are found in neutral and alkaline soils(V. Gandora, *et al.*, 1998 and S. Martyniuk, *et al.*, 2003) in water and in association with some plants(N. Tejera, *et al.*, 2005 and R. Kumar, *et al.*, 2007).

Scientific Classification

Domain:	<u>Bacteria</u>
Kingdom:	<u>Bacteria</u>
Phylum:	<u>Proteobacteria</u>
Class:	<u>Gammaproteobacteria</u>
Order:	<u>Pseudomonadales</u>
Family:	<u>Pseudomonadaceae/Azotobacteraceae</u>
Genus:	<i>Azotobacter</i>

Some important species are:*Azotobacter agilis*, *Azotobacter armeniacus*, *Azotobacter sp.AR*, *Azotobacter beijerinckii*, *Azotobacter chroococcum*, *Azotobacter sp. DCU26*, *Azotobacter sp. FA8*, *Azotobacter nigricans*, *Azotobacter paspali*, *Azotobacter salinestris*, *Azotobacter tropicalis*, and *Azotobacter vinelandii*.

1.6.1 Taxonomy

Martinus Beijerinck (1851–1931), discoverer of the genus *Azotobacter*. The *Azotobacter* genus was discovered in 1901 by the Dutch microbiologist and botanist Martinus Beijerinck, who was one of the founders of environmental microbiology. He selected and described the species *Azotobacter chroococcum* – the first aerobic, free-living nitrogen fixer (M. W. Beijerinck, 1901).

1.6.2 Biological Characteristics

1.6.2.1 Morphology

Cells of the genus *Azotobacter* are relatively large for bacteria (1–2 micrometres in diameter). They are usually oval, but may take various forms from rods to spheres. In microscopic preparations, the cells can be dispersed or form irregular clusters or occasionally chains of varying lengths. In fresh cultures, cells are mobile due to the numerous flagella (A. Baillie *et al.*, 1962). Later, the cells lose their mobility, become almost spherical and produce a thick layer of mucus, forming the cell capsule. The shape of the cell is affected by the amino acid glycine which is present in the nutrient medium peptone (G. R. Vela, *et al.*, 1972).

Under microscope, the cells show inclusions, some of which are colored. In the early 1900s, the colored inclusions were regarded as "reproductive grains", or gonidia – a kind of embryo cells (D. H. Jones, 1920). However, it was later demonstrated that the granules do not participate in the cell division (I. M. Lewis, 1941). The colored grains are composed of volutin whereas the colorless inclusions are drops of fat, which act as energy reserves (I. M. Lewis, 1937).

1.6.2.2 Cysts

Cysts of the genus *Azotobacter* are more resistant to adverse environmental factors than the vegetative cells, in particular, they are twice more resistant to UV light. They are also resistant to drying, ultrasound and gamma and solar irradiation, but not to heating (M. D. Socolofsky, and O. Wyss, 1962). The formation of cysts is induced by changes in the concentration of nutrients in the medium and addition of some organic substances such as ethanol, n-butanol or β-hydroxybutyrate.

Cysts are rarely formed in liquid media (J. S. Layne and E. J. Johnson, 1964). The formation of cysts is induced by chemical factors and is accompanied by metabolic shifts, changes in catabolism, respiration and biosynthesis of macromolecules (H. L. Sadoff, 1975) it is also affected by aldehyde dehydrogenase (Gama-Castro S, 2001) and the response regulator AlgR (C. Núñez, *et al.*, 1999). The cysts of *Azotobacter* are spherical and consist of the so-called *central body* – a reduced copy of vegetative cells with several vacuoles and the *two-layer shell*. The inner part of the shell is called *intine* and has a fibrous structure (L. M. Pope, and O. Wyss, 1970). The outer part has a hexagonal crystalline structure and is called *exine* (W. J. Page and H. L. Sadoff, 1975). Exine is partially hydrolyzed by trypsin and is resistant to lysozyme, in contrast to the central body (H. L. Sadoff, 1969). The central body can be isolated in a viable state by some chelation agents (L. T. Parker, and M. D. Socolofsky, 1968). The main constituents of the outer shell are alkylresorcinols composed of long aliphatic chains and aromatic rings. Alkylresorcinols are also found in other bacteria, animals and plants (N. Funa, H. Ozawa; A. Hirata, S. Horinouchi, and B. Loperfido, *et al.*, 2006).

1.6.3 Physiological properties

Azotobacter respire aerobically, 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. Nitrogen fixation requires molybdenum ions, but they can be partially replaced by vanadium ions. The source of nitrogen can be nitrates, ammonium ions or amino acids. The optimal pH for the growth and nitrogen fixation is 7.0–7.5, but growth is sustained in the pH range from 4.8 to 8.5 (M. George, 2005). *Azotobacter* can also grow mixotrophically, in a nitrogen-free medium containing mannose; this growth mode is hydrogen dependent. Hydrogen is available in the soil, thus this growth mode may occur in nature (T.Y. Wong, and R. J. Maier, 1985).

While growing, *Azotobacter* produce flat, slimy, paste-like colonies with a diameter of 5–10 mm, which may form films in liquid nutrient media. The colonies can have dark brown, green and other colors, or may be colorless, depending on the species. The growth is favored at a temperature of 20–30 °C (E.Z. Tepper, *et al.*, 1979).

1.6.3.1 Pigments

Azotobacter produce pigments. For example, *Azotobacter chroococcum* forms a dark-brown water-soluble pigment melanin. This process occurs at high levels of metabolism during the fixation of nitrogen and, is thought to protect the nitrogenase system from oxygen. Other *Azotobacter* species produce pigments from yellow-green to purple colors, including a green pigment which fluoresces with a yellow-green light and a pigment with blue-white fluorescence (D. B. Johnstone, 1955).

1.6.3.2 Distribution

Azotobacter are ubiquitous in neutral and weakly basic soils, but not acidic soils. They are also found in the Arctic and Antarctic soils, despite the cold climate, short growing season and relatively low pH values of these soils. In dry soils, *Azotobacter* can survive in the form of cysts for up to 24 years (J. Moreno, 1986).

Representatives of the genus *Azotobacter* are also found in aquatic habitats, including freshwater (H. J. Dicker, and D. W. Smith, 1980) and brackish marshes. Several members are associated with plants and are found in the rhizosphere, having certain relationships with the plant. Some strains are also found in the cocoons of the earthworm *Eisenia fetida*. (D.B. Johnstone, 1967)

1.6.3.3 Nitrogen fixation

Nitrogenase – a key enzyme of nitrogen fixation. *Azotobacter* are free-living nitrogen-fixing bacteria, 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 (Bürmann H., et al., 2003). Nitrogen fixation is inhibited in the presence of available nitrogen sources, such as ammonium ions and nitrates (H. Bürmann, et al., 2003).

Azotobacter have a full range of enzymes needed to perform the nitrogen fixation (J. M. Young and D. C. Park, 2007): ferredoxin, hydrogenase and an important enzyme nitrogenase.

The process of nitrogen fixation requires an influx of energy in the form of adenosine triphosphate (ATP). Nitrogen fixation is highly sensitive to the presence of oxygen, and therefore *Azotobacter* developed a special defensive mechanism against oxygen, namely a significant intensification of metabolism that reduces the concentration of oxygen in the cells (M. C. Durrant, *et al.*, 2006). There is also a special protein *shethna*, which protects nitrogenase and is involved in protecting the cells from oxygen (R. J. Maier, *et al.*, 2000). Mutants not producing this protein, are killed by oxygen during nitrogen fixation in the absence of a nitrogen source in the medium (J. B. Howard, and D. C. Rees, 2006). Homocitrate ions play a certain role in the processes of nitrogen fixation by *Azotobacter* (M. C. Durrant, *et al.*, 2006).

1.6.3.4 Nitrogenase

Nitrogenase is the most important enzyme involved in nitrogen fixation. *Azotobacter* species have several types of nitrogenase. The basic one is molybdenum-iron nitrogenase (C. Rüttimann Johnson, *et al.*, 2003). An alternative type contains vanadium; it is independent of molybdenum ions (R. L. Robson, *et al.*, 1986) and is more active than the Mo-Fe nitrogenase at low temperatures. So it can fix nitrogen at temperatures as low as 5 °C and its low-temperature activity is 10 times higher than that of Mo-Fe nitrogenase. An important role in maturation of Mo-Fe nitrogenase plays the so-called P-cluster. Synthesis of nitrogenase is controlled by the *nif* genes. Nitrogen fixation is regulated by the enhancer protein NifA and the "sensor" flavoprotein NifL which modulates the activation of gene transcription of nitrogen fixation by redox-dependent switching. This regulatory mechanism, relying on two proteins forming complexes with each other, is uncommon for other systems.

1.6.4 Importance

Nitrogen fixation plays an important role in the nitrogen cycle. *Azotobacter* also synthesizes some biologically active substances, including some phytohormones such as auxins, thereby stimulating plant growth. They also facilitate the mobility of heavy metals in the soil and thus enhance bioremediation of soil from heavy metals, such as cadmium, mercury and lead. Some kinds of *Azotobacter* can also biodegrade chlorine-containing aromatic compounds, such as 2,4,6-trichlorophenol. The latter was previously used as an insecticide, fungicide and herbicide but later found to have mutagenic and carcinogenic effects.

1.6.5 Applications

Owing to its ability to fix molecular nitrogen and therefore increase the soil fertility and stimulate plant growth, *Azotobacter* are widely used in agriculture, particularly in nitrogen biofertilizers such as *Azotobacterin*. They are also used in production of alginic acid (E400), which is applied in medicine as an antacid, in the food industry as an additive to ice cream, puddings and creams, and in the biosorption of metals (G. Emtiazia, *et al.*, 2004).

1.6.6 Characteristics of *Azotobacter*

Azotobacter is Gram negative bacteria, polymorphic i.e. they are of different sizes and shapes. Their size ranges from 2-10x1-2.5 μ m, young cells possess peritrichous flagella and are used as locomotive organs. Old population of bacteria includes encapsulated forms and have enhanced resistance to heat, desiccation and adverse conditions. The cyst germinates under favourable conditions to give vegetative cells. They also produce polysaccharides. *Azotobacter* spp. are sensitive to acidic pH, high salts, and temperature above 35°C. There are four important species of *Azotobacter* viz. *A. Chroococcum*, *A. agilis*, *A. paspali* and *A. vinelandii* of which *A. chroococcum* is most commonly found in our soils.

1.6.8 *Azotobacter* in Soil

Soils are inhabited by a very large number of microbial species. The co-existence of the relative populations of each one of the species is determined by ecological factors prevailing in the soil. These various species survive in soil while maintaining a balance of population between various microbial species within certain limits. A normal population of *Azotobacter* could be about 10 thousand to 1 lakh/g of soil and it is mostly influenced by other micro-organisms present in soil. There are some micro-organisms which stimulate the *Azotobacter* population in soil thereby increasing the nitrogen fixation by *Azotobacter*. On the other hand there are some micro-organisms which adversely affect the *Azotobacter* population and hence nitrogen fixation process is hampered. For example *Cephalosporium* is most commonly found organisms in soil which restricts the growth of *Azotobacter*.

1.6.9 Functions of *Azotobacter*:

Azotobacter naturally fixes atmospheric nitrogen in the rhizosphere. There are different strains of *Azotobacter* each has varied chemical, biological and other characters. However, some strains have higher nitrogen fixing ability than others. *Azotobacter* uses carbon for its metabolism from simple or compound substances of carbonaceous in nature. Besides carbon, *Azotobacter* also requires calcium for nitrogen fixation. Similarly, a medium used for growth of *Azotobacter* is required to have presence of organic nitrogen, micro-nutrients and salt in order to enhance the nitrogen fixing ability of *Azotobacter*.

Azotobacter also produces fixation of Thiomin, Riboflavin, Nicotin, indol acitic acid and gibberalin. When *Azotobacter* is applied to seeds, seed germination is improved to a considerable extent, so also it controls plant diseases due to above substances produced by *Azotobacter*.

1.6.10 Growth of *Azotobacter*

Usually *Azotobacter* is grown on a solid medium free of nitrogen. After some times (6 months) old growth of *Azotobacter* is transferred to a fresh solid medium to renew the growth. This procedure is repeated periodically so that the culture can be maintained in good condition.

1.6.11 Production

- i. **Mother culture:** A pure growth of any organism on a small scale is called as a mother culture. Mother culture is always prepared in a conical flask of 500 or 1000 ml. Capacity and then this mother culture is used for further production.

For this purpose, one litre conical flasks are taken to which 500 ml of broth of nitrogen free medium is added and these flasks are then plugged with non-absorbent cotton, sterilized in an auto slave for 15-20 minutes at 75 lbs pressure for 15 minutes. Flasks are then inoculated with mother culture with the help of inoculating needle aseptically. The flasks are transferred to shaker and shaking is done for 72-90 hours so as to get optimum growth of bacteria in broth. Bacteria are multiplied by binary method i.e. cell division. After about 90 days, the number of per milliliters comes to about 100 crores. Total growth of bacteria in this broth means starter culture or mother culture, which should carefully be done, since further purity of biofertilizer or quality of biofertilizer depends upon how mother culture is prepared.

- ii. **Production on a large scale:** *Azotobacter* is multiplied on a large scale by two ways viz. Fermenter and Shaker. The fermenter is most automatic and accurate method of multiplication of any micro-organism. In this method, the medium is taken in a fermenter and then sterilized. After this pH of the medium is adjusted and 1% mother culture is added. In order to get an optimum growth of the *Azotobacter* required temperature and oxygen supply is adjusted so that concentrated broth is made. This concentrated broth of the culture is then mixed with a carrier previously sterilized and bio-fertilizers is prepared. Depending upon the demand and supply suitable fermenter is selected.

In the 2nd method i.e. shake method, a suitable medium is prepared transferred to conical flask of suitable capacity. These flasks are then sterilized in an autoclave at 15 lbs pressure for 15 minutes. Each flask is inoculated with 10 ml mother culture and they are transferred to shaker for multiplication where they are kept for 72-90 hours. This broth is mixed with a suitable carrier previously sterilized. Thus biofertilizer is prepared, filled in plastic bags and stored in cool place.

1.6.12 Features of *Azotobacter*

1. *Azotobacter* contributes moderate benefits
2. *Azotobacter* is heaviest breathing organism and requires a large amount of organic carbon for its growth.
3. It is poor competitor for nutrients in soil and hence its growth promoting substances, fungistatic substances.
4. It can benefit crops by Nitrogen fixation, growth promoting substances, fungi static substances.
5. *Azotobacter* is less effective in soils with poor organic matter content.
6. It improves seed germination and plant growth
7. It thrives even in alkaline soils.

1.6.13 Benefits

- It improves seed germination and plant growth
- *Azotobacter* are tolerant to high salts.
- It can benefit crops by Nitrogen fixation, growth promoting substances, fungistatic substances.
- *Azotobacter* is heaviest breathing organism and requires a large amount of organic carbon for its growth.
- It is poor competitor for nutrients in soil and hence its growth promoting substances, fungistatic substances.
- It thrives even in alkaline soils.
- *Azotobacter* is less effective in soils with poor organic matter content.

1.6.14 Additional Benefits

Azotobacter also produces some substances which check the plant pathogens such as *Alternaria*, *Fusarium* and *Helminthosporium*. Hence *Azotobacter* also acts as a biological control agent.

1.7 AZOSPIRILLUM

Azospirillum (Tarrand, et al., 1979) is a plant growth-promoting rhizobacterium associated with roots of monocots, including important crops, such as wheat, corn, and rice. Both in greenhouse and in field trials, *Azospirillum* was shown to exert beneficial effects on plant growth and crop yields, under various soil and climatic conditions

Scientific Classification

<i>Domain:</i>	Bacteria
<i>Phylum:</i>	<u>Proteobacteria</u>
<i>Class:</i>	<u>Alphaproteobacteria</u>
<i>Order:</i>	<u>Rhodospirillales</u>

<i>Family:</i>	<u><i>Rhodospirillaceae</i></u>
<i>Genus:</i>	<i>Azospirillum</i>

The actual benefit from biological nitrogen fixation has been questioned, and plant growth promotion by *Azospirillum* seems to be due mainly to production of phytohormones (Y. Bashan, *et al.*, 2004 and S. A. Dobbelaere, 2001). The most abundant phytohormone produced is indole-3 acetic acid, allowing an increase in the number of lateral roots and root hairs; this results in a higher absorption of water and minerals from the soil (9). Bacterial populations, especially in soil or in the rhizosphere, have to endure fluctuating environmental conditions. Bacteria have evolved different strategies to adapt to these environments. Phase variation is one adaptive process by which bacteria undergo frequent, usually reversible phenotypic changes resulting from genetic or epigenetic alterations at specific genetic loci (I.R. Henderson, *et al.*, 1999). This process is used by several bacterial species to generate intrapopulation diversity that increases bacterial fitness and is important in niche adaptation or to escape host defenses (L. Salau, *et al.*, 2000; M. W. Van der Woude, and A. J. Baumler, 2004).

Bacteria of the genus *Azospirillum* (K-subclass of proteobacteria) are known for many years as plant growth promoting rhizobacteria (PGPR) (Y. Okon, 1994) and Y. Okon, and J. Vanderleyden, 1997). They were isolated from the rhizosphere of many grasses and cereals all over the world, in tropical as well as in temperate climates. Both in greenhouse and in field trials, *Azospirillum* was shown to exert beneficial effects on plant growth and crop yields (R.M. Boddey, *et al.*, 1986). Ammonium, nitrate, nitrite, amino acids and molecular nitrogen can serve as N-sources. In unfavorable conditions, such as desiccation and nutrient limitation, azospirilla can convert into enlarged cyst-like forms. This morphological change is accompanied by the development of an outer coat of polysaccharides and by the accumulation of abundant poly-L-hydroxybutyrate granules, which can serve as C- and energy source under conditions of stress and starvation (S. Tal, and Y. Okon, 1985)

Azospirillum represents the best characterized genus of plant growth-promoting rhizobacteria. Other free-living diazotrophs repeatedly detected in association with plant roots, include *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Azoarcus spp.* and *Azotobacter*. Four aspects of the *Azospirillum* plant root interaction are highlighted: natural

habitat, plant root interaction, nitrogen fixation and biosynthesis of plant growth hormones. Each of these aspects is dealt with in a comparative way. *Azospirilla* are predominantly surfacecolonizing bacteria, whereas *A. diazotrophicus*, *H. seropedicae* and *Azoarcus* sp. are endophytic diazotrophs. The attachment of *Azospirillum* cells to plant roots occurs in two steps. The polar flagellum, of which the flagellin was shown to be a glycoprotein, mediates the adsorption step. An as yet unidentified surface polysaccharide is believed to be essential in the subsequent anchoring phase.

Bacteria belonging to the genus *Azospirillum* are highly motile. *A. brasilense*, *A. lipoferum* and *A. irakense* display a mixed pattern of flagellation (P.G. Hall, and N.R. Krieg, 1984). One polar flagellum is synthesized during growth in liquid medium and is primarily used for swimming. Additional lateral flagella are induced during growth on solidified media and are responsible for swarming of the bacteria over solid surfaces. *A. halopraeferens* and *A. amazonense* only display the polar flagellum. Motility offers the bacterium the advantage of moving towards favorable nutrient conditions. *Azospirilla* exhibit positive chemotaxis towards organic acids, sugars, amino acids (Zhulin, *et al.*, 1993) and aromatic compounds as

1.8.1 Usage

1.8.1.1 Agricultural and horticultural use

1.8.1.1.1 Natural biocontrol and elicitor

In agriculture, chitosan is used primarily as a natural seed treatment and plant growth enhancer, and as a ecologically friendly biopesticide substance that boosts the innate ability of plants to defend themselves against fungal infections (Linden, J., *et al.*, 2000). The natural biocontrol active ingredients, chitin/chitosan, are found in the shells of crustaceans, such as lobsters, crabs, and shrimp, and many other organisms, including insects and fungi. It is one of the most abundant biodegradable materials in the world. Degraded molecules of chitin/chitosan exist in soil and water. Chitosan applications for plants and crops are regulated by the EPA, and the USDA National Organic Program regulates its use on organic certified farms and crops. EPA approved biodegradable chitosan products are allowed for use outdoors and indoors on plants and crops grown commercially and by consumers. The natural biocontrol ability of chitosan should not be confused with the effects of fertilizers or pesticides upon plants or the

environment. Chitosan active biopesticides represent a new tier of cost effective biological control of crops for agriculture and horticulture(F. A. Goosen, 1996). The biocontrol mode of action of chitosan elicits natural innate defense responses within plant to resist insects, pathogens, and soil-borne diseases when applied to foliage or the soil. Chitosan increases photosynthesis, promotes and enhances plant growth, stimulates nutrient uptake, increases germination and sprouting, and boosts plant vigor. When used as seed treatment or seed coating on cotton, corn, seed potatoes, soybeans, sugar beets, tomatoes, wheat and many other seeds, it elicits an innate immunity response in developing roots which destroys parasitic cyst nematodes without harming beneficial nematodes and organisms(R. Smiley, *et al.*, 2002). Agricultural applications of chitosan can reduce environmental stress due to drought and soil deficiencies, strengthen seed vitality, improve stand quality, increase yields, and reduce fruit decay of vegetables, fruits and citrus crops (see photo right). Horticultural applications of chitosan increases blooms and extends the life of cut flowers and Christmas trees. The US Forest Service has conducted research on chitosan to control pathogens in pine trees and chitosan's ability to increase pine tree resin pitch outflow by 40% to resist pine beetle infestation(O'Toole, 2009).

Chitosan has a rich history of being researched for applications in agriculture and horticulture dating back to the 1980s. By 1989, Bentech Labs patented chitosan salt solutions applied to crops for improved freeze protection or to crop seed for seed priming. Shortly thereafter, Bentech's chitosan salt received the first ever biopesticide label from the EPA. Numerous other chitosan patents for plants soon followed. Chitosan applications to protect plants have been used in space as well. NASA results revealed chitosan induces increased growth (biomass) and pathogen resistance due to elevated levels of beta 1-3 glucanase enzymes within plant cells. NASA confirmed chitosan elicits the same effect in plants on earth(Linden, J.,2008). Over 20 years of R&D by DuPont/ConAgra Ventures (DCV) and AgriHouse Inc have gone into developing non-toxic low molecular weight chitosan polymer solutions safe enough for broad spectrum agricultural and horticultural use. In 2008, AgriHouse Inc, Denver (Berthoud), Colorado, was granted EPA natural broad spectrum elicitor status for YEA! Yield Enhancing Agent, a liquid solution containing an ultra low molecular active ingredient of 0.25% chitosan. YEA! is a next generation natural chitosan elicitor solution for agriculture and horticultural uses, and was granted an amended label for foliar and irrigation applications by the EPA in June,

2009. A milliliter of YEA! contains over 14.4×10^{13} bioactive low molecular weight chitosan molecules, and it is 600 times more effective than common Chitosan. Given its low potential for toxicity and its abundance in the natural environment, chitosan does not harm people, pets, wildlife, or the environment when used according to label directions. Agricultural chitosan facts are located on USDA and EPA web sites. The US Forest Service tested chitosan as an eco-friendly biopesticide, to pre-arm pine trees to defend themselves against mountain pine beetle.

1.8.1.1.2 Water Filtration

Chitosan can also be used in water processing engineering as a part of a filtration process. Chitosan causes the fine sediment particles to bind together, and is subsequently removed with the sediment during sand filtration. Chitosan also removes phosphorus, heavy minerals, and oils from the water. Chitosan is an important additive in the filtration process. Sand filtration apparently can remove up to 50% of the turbidity alone, while the chitosan with sand filtration removes up to 99% turbidity (Alan Woodmansey, 2002). Chitosan has been used to precipitate caseins from bovine milk and cheese making.

Chitosan is also useful in other filtration situations, where one may need to remove suspended particles from a liquid. Chitosan, in combination with bentonite, gelatin, silica gel, isinglass, or other fining agents is used to clarify wine, mead, and beer. Added late in the brewing process, chitosan improves flocculation, and removes yeast cells, fruit particles, and other detritus that cause hazy wine. Chitosan combined with colloidal silica is becoming a popular fining agent for white wines, because chitosan does not require acidic tannins (found primarily in red wines) with which to flocculate (Terry, Rayner, 2008).

1.8.1.1.3 Potential industrial use

Scientists have recently developed a polyurethane coating that heals its own scratches when exposed to sunlight, offering the promise of scratch-free cars and other products. The self-healing coating uses chitosan incorporated into traditional polymer materials, such as those used in coatings on cars to protect paint. When a scratch damages the chemical structure, the chitosan

responds to ultraviolet light by forming chemical chains that begin bonding with other materials in the substance, eventually smoothing the scratch. The process can take less than an hour.

Marek W. Urban, a scientist working on this project, said the polymer can only repair itself in the same spot once, and would not work after repeated scratches.^[30] Whether this technology can be applied to industrial materials, however, depends on a number of factors (long-term persistence of "healability", stiffness and heat resistance of coating, knowledge of the exact mechanism of healing, etc.) not present in initial studies; further investigation into these factors can potentially take decades to rectify.

1.8.1.1.4 Biomedical uses

Chitosan's properties allow it to rapidly clot blood, and has recently gained approval in the United States and Europe for use in bandages and other hemostatic agents. Chitosan hemostatic products have been shown in testing by the U.S. Marine Corps to quickly stop bleeding, and result in 100% survival of otherwise lethal arterial wounds in swine and to reduce blood loss. Chitosan hemostatic products reduce blood loss in comparison to gauze dressings and increase patient survival (Pusateri, 2003). Chitosan hemostatic products have been sold to the U.S. Army and are currently used by the UK military. Both the US and UK have already used the bandages on the battlefields of Iraq and Afghanistan. Chitosan is hypoallergenic and has natural antibacterial properties, which further support its use in field bandages (Kevin McCue, 2003).

Chitosan's properties also allow it to be used in trans-dermal drug delivery. One of these properties is that it is mucoadhesive in nature. Another property that is important to chitosan's ability to aid in drug delivery is that it is fairly reactive so it can be produced in many different forms. The most important property of chitosan with regards to drug delivery is its positive charge under acidic conditions. This positive charge comes from protonation of its free amino groups. Lack of a positive charge means chitosan is insoluble in neutral and basic environments. However, in acidic environments, protonation of the amino groups leads to an increase in solubility. The implications of this are very important to biomedical applications. This is a molecule that will maintain its structure in a neutral environment but will solubilize and

degrade in an acidic environment. This means that chitosan can be used to transport a drug to an acidic environment, where the chitosan packaging will then degrade, releasing the drug to the desired environment. One example of this drug delivery has been seen in the transport of insulin(Sunil A. Agnihotri, 2004).

1.8.1.1.5 Claimed health benefits

Chitosan is frequently sold in tablet form at health stores as a "fat binder": It is supposed to have the capability to interact with lipids (fat) from the digestive system and limit their absorption in the body. Therefore, chitosan can be an effective complement to help lose weight during diet period or to stabilise one's weight. In the 2007 Cochrane meta-analysis ^[36] which evaluated all available clinical trials performed with chitosan on the subject of weight loss, it was concluded that body weight and all parameters related to cholesterol changed in favor of chitosan compared to placebo. The mean difference in body weight was -1.7 kg (range: -2.1 to -1.3) in favor of chitosan. This change in body weight was statistically significant. There was no difference between chitosan and placebo concerning side effects. The various qualities (in terms of duration, sample size, doses, subject characteristics, type of diet, chitosan quality and characteristics, etc.) of the clinical trials performed to evaluate the effect of chitosan on body weight might account for some of the disparities observed in clinical trial results (Schiller RN,2001)and the subsequent critics regarding the real efficacy of chitosan. In an experimental model of the stomach and duodenum tract, chitosan has shown to interact with oil, which inhibited duodenal absorption and enhanced lipid excretion (Rodríguez MS, 2005). However, the mechanism of interaction between chitosan and fat is not very well understood and has not been really proved clinically yet. This is certainly the reason why the FDA has issued in 2004 Warning Letters to two companies who made inappropriate claims according to the regulator. Although detractors claim that using chitosan may have the deleterious effect of rendering ineffective certain minerals found in foodstuffs, several animal studies contradicted this statement by showing no or little effect. In mice, dietary ingestion of chitosan did not depress the level of iron, zinc or copper(L. Zeng, 2008). Moreover, there is no proof of any adverse events, in particular regarding nutrient absorption, in humans.

1.8.1.1.6 Medical Research

Chitosan is currently the focus of much medical research, as it is a polyglucosamine (the second-most-common dietary fiber, after cellulose) (D. Knorr, 1991). Studies have shown chitosan has the following properties:

- As a soluble dietary fiber, it increases gastrointestinal lumen viscosity and slows down the emptying of the stomach.
- It alters bile acid composition, increasing the excretion of sterols and reducing the digestibility of ileal fats. It is unclear how chitosan does this, but the currently favored hypotheses involve the increase of intestinal viscosity or bile acid-binding capacity.
- Chitosan is relatively insoluble in water, but can be dissolved by dilute acids, which would make it a highly-viscous dietary fiber. Such fibers might inhibit the uptake of dietary lipids by increasing the thickness of the boundary layer of the intestinal lumen, which has been observed in animal experiments (I. Ikeda, 1993).

Having very few acetyl groups, chitosan contains cationic groups. This may cause chitosan to have bile acid-binding capacity, which causes mixed micelles to be entrapped or disintegrated in the duodenum and ileum. This would interrupt bile acid circulation, causing reduced lipid absorption and increased sterol excretion, which has also been observed in animal experiments (I. Ikeda, 1993).

The green revolution brought impressive gains in food production but with insufficient concern for sustainability. In India the availability and affordability of fossil fuel based chemical fertilizers at the farm level have been ensured only through imports and subsidies. Dependence on chemical fertilizers for future agricultural growth would mean further loss in soil quality, possibilities of water contamination and unsustainable burden on the fiscal system. The Government of India has been trying to promote an improved practice involving use of bio- fertilizers along with fertilizers. These inputs have multiple beneficial impacts on the soil and can be relatively cheap and convenient for use. Consistent with current outlook, the government aims not only to encourage their use in agriculture but also to promote private

initiative and commercial viability of production. There has been no accelerated growth in distribution with time, inadequate spatial diffusion and despite entry of small private units into the industry there is no clear indication of the success of privatization.

1.9 FORMULATION

A **formulation** is a mixture that is prepared according to a specific procedure (called a “formula”). Formulations are made for particular applications and normally are more effective than their individual components when these are used singly. Formulation is used to improve self life of biofertilizer and crop yield and allow the organism to persist in the environment for long time. Formulations are commercially produced for drugs, cosmetics, coatings, cleaning agents, foods, paints, pesticides and many other products.

1.9.1 Components

Components (also called ingredients), when mixed together according to a formula, create a formulation.

Some components impart specific properties to the formulation when it is put into use. For example, certain components (polymers) are used in paint formulations to achieve deforming or levelling properties. Some components of a formulation may not be active in a particular application.

A formulation may be created for any of the following purposes:

- to achieve effects that cannot be obtained from its components when these are used singly
- to achieve a higher degree of effectiveness
- to facilitate any potential synergistic action of their components
- to improve handling properties and often safety for the user

1.9.2 Pesticide Formulation

The biological activity of a pesticide, be it chemical or biological in nature, is determined by its active ingredient (AI - also called the *active substance*). Pesticide products very rarely consist of pure technical material. The AI is usually formulated with other materials and this is the product as sold, but it may be further diluted in use. Formulation improves the properties of a chemical for handling, storage, application and may substantially influence effectiveness and safety.

Formulation terminology follows a 2-letter convention: (*e.g.* GR: granules) listed by CropLife International (formerly GIFAP then GCPF) in the *Catalogue of Pesticide Formulation Types*. Some manufacturers do not follow these industry standards, which can cause confusion for users.

1.9.3 Water-Miscible Formulations

By far the most frequently used products are formulations for mixing with water then applying as sprays. Water miscible, older formulations include:

- EC Emulsifiable concentrate
- WP Wettable powder
- SL Soluble (liquid) concentrate
- SP Soluble powder

Newer, non-powdery formulations with reduced or no use of hazardous solvents and improved stability include:

- SC Suspension concentrate
- CS Capsule suspensions
- WG Water dispersible granules

1.9.4 Other Pesticide Formulations

Other common formulations include granules (GR) and dusts (DP), although for improved safety the latter have been replaced by microgranules (MG *e.g.* for rice farmers in Japan). Specialist formulations are available for ultra-low volume spraying, fogging, fumigation, *etc.* Very occasionally, some pesticides (*e.g.* malathion) may be sold as technical material (TC - which is mostly AI, but also contains small quantities of, usually non-active, by-products of the manufacturing process).

A particularly efficient form of pesticide dose transfer is seed treatment and specific formulations have been developed for this purpose. A number of pesticide bait formulations are available for rodent pest control, *etc.*

Formulation is a crucial aspect for producing inoculants containing an effective bacterial strain and can determine the success or failure of a biological agent (Bashan, Y. 1998). Formulation typically consists of establishing the active ingredient (i.e., microorganism) in a suitable carrier together with additives that aid in the stabilization and protection of the microbial cells during storage and transport, and at the target site. Whether a product is new or improved, it is imperative that the formulation be stable during production, distribution, storage, and transportation. The formulation should also be easy to handle and apply so that it is delivered to the target in the most appropriate manner and form, protects the agent from harmful environmental factors, and maintain or enhance activity of the organism in the field (Jones, K. A., and Burges, H. D. 1998). Another important consideration is the cost-effectiveness of the formulation. Therefore, several critical factors including user preference have to be considered before delivery of the final product.

1.9.5 Current Inoculant Formulations

Commercial inoculant formulations are available as powder, granule, and liquid. Generally, peat has been the preferred carrier in powder form (Burton, J. C. 1982). The rhizobial cells in the inoculant are metabolically active and continue to grow and multiply as long as favorable nutrient and environmental conditions are maintained. Superior seed adhesion is

achieved with a finer particle and therefore it generally is recommended the particle size should allow at least 50% of the peat particles to pass a 0.075-mm sieve (Strijdom, B. W., and Deschodt, C. C. 1976).

1.9.6 Future Prospects

Formulation is a challenging and often success-limiting step in the successful commercialization of new microbial inoculants. Although formulation research is progressing slowly, several developments including liquid and granular formulations have contributed to the ease of use at the farm combined with the economic benefits of increased crop yield levels.

1.10 NANOPARTICLES - A GREEN REVOLUTION IN FUTURE

The potential uses and benefits of nanotechnology are enormous. These include agricultural productivity enhancement involving nanoporous zeolites for slow release and efficient dosage of water and fertilizer, nanocapsules for herbicide delivery and vector and pest management and nanosensors for pest detection. Nanoparticles help to produce new pesticides, insecticides and insect repellants.

There have been a number of studies exploring the use of nanoparticles in the controlled release of bioactive substances in wood and the use of micro and nanoparticles as a tool for plant science. Recently the use of silica nanoparticles has been effective in the controlled release of substances into protoplasts within plant cells. Work in Spain has focused on developing tools for controlled and targeted release of substances in plant organs which are susceptible to pathogens. Further work from this group reported more recently has shown a deepening in the understanding of the mechanisms of interaction between plant cells and nanoparticle.

1.11 BACILLUS MEGATERIUM

Isolation of *Bacillus megaterium*, majorly an aerobic spore forming bacterium that is found in widely diverse habitats. The research paper gives a brief account about the bacteria, bacterial sources and its uses for environment and human welfare in brief. The methodology undertaken for the cultivation and isolation of the bacteria by the HiCrome Bacillus Agar medium preparation method is well-reasoned. The physiology and commercial products of *B. megaterium* like amylase, glucose dehydrogenase, Penicillin amidase, vitamin B12, oxetanocin and other antimicrobial agents and bioremediation are provided with thoughtful clarification. The interpretation part contains the addendum of the bacteria and its application in the market and its potential future.

Bacillus megaterium (*Bacillus megaterium*) was first identified over 100 years ago and is a curio in the present, due to its physiology and unusually useful enzymes. It has the capacity of sporulation and a simple cell differentiation cycle, which helps in understanding gene regulation during temporal and morphological developments. *Bacillus megaterium* has large size vegetative cell and spore. Though considered a soil organism, it may also be found in diverse environment like rice paddies, dried food, seawater, sediments, fish, and normal flora and even in bee honey. Since *B. megaterium* is able to grow on a wide variety of carbon sources, they are found in many ecological niches such as waste from meat industry or petrochemical effluents.

Bacillus megaterium has economic importance because of production of commercially important enzymes like penicillin amidase, steroid dehydrolase, amylase, and products like hydroxyl citric acid (HCA), during its growth cycle. It is the major aerobic producer of vitamin B, and is one of the organisms involved in fish spoilage. An extensive review emphasizing its commercial applications have recently been published (Vary, 1992); major research areas and commercial applications are summarized in the illustration that appears on the cover of this issue of Microbiology [140(5)]. During the 1980s, genetic techniques of transduction, plasmid transformation, protoplast fusion and transposition became developed enough in *B. megaterium* to apply them to the study of many of its metabolic and developmental functions. Moreover, it is increasingly used as a host to produce foreign genes since it has been found to express, secrete and process foreign proteins without degradation.

Gram Staining:

The Gram staining is done in initial stages of the study to confirm the type of bacteria in the inoculums (Gram+ve or Gram-ve) and to characterize the initial features of the organism present in the inoculums. The samples are heat fixed on glass which was treated with Crystal violet then treated with a decolorizer 70% Ethanol after suitable incubation with dye after the treatment with an alcohol it is treated with Saffron dye and is washed after suitable incubation. *Bacillus megaterium* is isolated by enrichment culture techniques. Soil suspension at different dilutions were drawn and poured onto the surface of HiCrome Bacillus Agar medium being a selective medium for isolating *Bacillus megaterium*. Organisms like *B. megaterium* yield yellow coloured colonies. The cultures isolated were characterized through a number of microbiological and biochemical tests. The colonies identified as catalase-positive, Gram-positive, endospore-forming rods. *Bacillus megaterium* has many physiology and commercial uses. They secrete enzymes like α -amylase, P-amylase, penicillin amidase, neutral protease, P-glucanase, megacins, glucanotransferase and chitosanase. They also synthesize unusual enzymes such as an epoxide

hydrolase, an isomerase for maleylpyruvate, a α -glucanase used as a probe for structural analysis of yeast cell walls, and the enzyme alfostatin, which inhibits alkaline phosphatase. There are many other commercial products that have been mentioned above. *Bacillus megaterium* is a very useful organism due to all the above properties mentioned and its isolation is relatively simple and can be done on an industrial level.

CHAPTER -2

REVIEW OF LITERATURE

Jean-Charles Cote, in 2001 reported that, Persistence of insecticidal activity of novel bioencapsulated formulations of *Bacillus thuringiensis* var. *kurstaki* against *Choristoneura rosaceana*. DiPel™, a registered *Bacillus thuringiensis* var. *kurstaki* (Btk)-base6 formulation, and experimental bio-encapsulated Btk formulations were sprayed in an apple orchard. Their persistence was assessed in the laboratory against obliquebanded leaffroller (*Choristoneura rosaceana*) larvae for three consecutive years. In 1995, DiPel and the bio-encapsulated formulation caused significantly higher larval mortality than the control at least 1 day, and up to 27 days after treatments, respectively. In 1996, although 6.4 mm of rain fell during the day of treatments, DiPel and the bio-encapsulated formulation caused respectively 21 and 33% larval mortality immediately after treatments. DiPel caused significantly higher mortality than the control for up to 3 days while the bio-encapsulated formulation caused significantly higher mortality for up to 14 days. In 1997, two Btk strains (HD-1 and BMP123) were sprayed at different concentrations in a.i. (3 and 8%) and doses (30 and 240 g 15 L⁻¹ applied). Higher concentrations of a.i. in the Btk formulations and higher doses sprayed increased the persistence of the insecticidal activity. Among the meteorological factors that were measured (i.e. precipitations, temperature and solar radiations), precipitations reduced the persistence of the insecticidal activity. Solar radiations may have modulated the persistence of the formulations.

Daniel Uribe in 2003 described the Role of Microbial Biofertilizers in the Development of a Sustainable Agriculture in the Tropics. The new social and economic world order has created

new challenges for the development of agriculture in the tropics. Functional microbial biofertilizers have been used in some tropical countries for more than half a century in both small and large farms. Biological nitrogen fixation, plant growth promotion, phosphorus solubilization, and translocation to host plants are the major benefits of biofertilizer use, observed or claimed by researchers and product developers. However, a major constraint for the further development of the microbial biofertilizer industry is the demonstration of consistent field effects of the marketed products. This study discusses the main issues related to the functional characterization and promotion of microbial biofertilizer in tropical countries, and the potential of these biofertilizers as tools for small- and large-scale sustainable crop production.

Ilungo J. Xavier, *et al.*, in 2004 studied the Development of rhizobial inoculant formulations. A key constraint to successfully commercializing beneficial microorganisms is overcoming difficulties in formulating a viable, cost-effective, and user-friendly final product. The live nature of the active ingredient (i.e., the microbial agent) underscores the importance of formulation in maintaining the microbial cells in a metabolically and physiologically competent state in order to obtain the desired benefit when applied. The development of new microbial formulations is a challenging task and requires greater effort in terms of funding and research towards making significant advances in this field. Worldwide, legumes are grown on approximately 250 million hectares and fix about 90 Tg (or 9×10^{13} g) of dinitrogen (N_2) per year (12). *Rhizobium* spp. and *Bradyrhizobium* spp. are major contributors to overall N_2 fixation through the legume-*Rhizobium* symbiosis and have been used for over a hundred years as beneficial microorganisms. For example, the improvement of crop production through mixing of soil containing naturally-occurring rhizobia with seeds became a recommended practice in the USA by the end of the 19th century (24). Since then considerable improvement in this inoculant technology has been made, resulting in the successful commercialization of rhizobial inoculants by several companies around the world.

Rice, W. A., *et al.*, in 2004 studied the Rhizobial inoculant formulations and soil pH influence field pea nodulation and nitrogen fixation. Crop production systems that include field pea (*Pisum sativum* L.) in rotation are important for sustainable agriculture on acid soils in northwestern Canada. Greenhouse experiments were conducted to compare the ability of liquid inoculant

applied to the seed, powdered peat inoculant applied to the seed, and granular inoculant applied in a band with the seed to establish effective nodulation on field pea grown at soil pH(H₂O) 4.4, 5.4 and 6.6. Plants were grown to the flat pod stage, and then total plant biomass dry weight, dry weight of nodules, number of nodules, plant nitrogen content, and proportion of plant nitrogen derived from the atmosphere (%Ndfa) were measured. Granular and powdered peat inoculants produced greater nodule numbers and weight, plant nitrogen content, %Ndfa and total biomass than liquid inoculant in at least two of the three experiments. Only granular inoculant was effective in establishing nodules at soil pH 4.4, but granular and powdered peat inoculants were effective at pH 5.4, and all three formulations were effective at pH 6.6. The results showed that granular inoculant has potential for effective nodulation of field pea grown on acid soil.

In 2005 Nelson Duran *et al.*, evaluated the mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains. Extracellular production of metal nanoparticles by several strains of the fungus *Fusarium oxysporum* was carried out. It was found that aqueous silver ions when exposed to several *Fusarium oxysporum* strains are reduced in solution; thereby leading to the formation of silver hydrosol, the silver nanoparticles were in the range of 20-50 nm in diameter. The reduction of the metal ions occurred by a nitrate dependent reductase and a shuttle quinone extracellular process. The potentialities of this nanotechnological design based in fungal biosynthesis of nanoparticles for technical applications are important, including their high potential as antibacterial material.

Yan Wu *et al.*, (2005) proposed the Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate. The ammonium glycyrrhizinate-loaded chitosan nanoparticles were prepared by ionic gelation of chitosan with tripolyphosphate anions (TPP). The particle size and zeta potential of nanoparticles were determined, respectively, by dynamic light scattering (DLS) and a zeta potential analyzer. The effects, including chitosan molecular weight, chitosan concentration, ammonium glycyrrhizinate concentration and polyethylene glycol (PEG) on the physicochemical properties of the nanoparticles were studied. These nanoparticles have ammonium glycyrrhizinate loading efficiency. The encapsulation efficiency decreased with the increase of ammonium glycyrrhizinate concentration and chitosan concentration. The introduction of PEG can decrease significantly the positive charge of particle surface. These studies showed that chitosan can complex TPP to form stable cationic nanoparticles for subsequent ammonium glycyrrhizinate loading.

David Fairhurst, and Andrew Loxley *et al.*, (2005) predicted the Micro- and Nanoencapsulation Of Water- and Oil-soluble Actives for Cosmetic and Pharmaceutical Applications. Skin, hair and mucosal surfaces are useful targets for the delivery of active compounds, botanicals and, importantly, drugs. Encapsulation provides an invaluable tool to the cosmetic and/or pharmaceutical formulator, providing great flexibility in the choice of delivery mechanisms and excipients that can be used. Dispersions of solid lipid nanoparticles (SLNs) were prepared using biodegradable materials generally regarded as safe by a melt-emulsify-chill (MEC) method. Among the materials encapsulated in SLNs were hydrophobic active pharmaceutical ingredients, antiviral and fungicidal compounds, organic UV absorbers and fluorescent dyes. The loading, adhesion and intracellular localization of the SLNs were studied as a function of surface charge. The MEC process can easily, and cheaply, produce a range of solid lipid nanoparticles (SLNs) - “smart colloids” - that offer flexibility in formulation, increased efficacy and decreased formulation complexity. The method is general and can be applied to many water- or oil- soluble cosmetic and pharmaceutical actives. Multiple actives can be co-encapsulated. The process is cost-effective, reproducible, robust and scalable. The bulk physical characteristics and surface chemical properties can be varied to allow systems to be custom designed to fit specific applications; use can be made of this in the targeting of encapsulated actives, botanicals and therapeutics to skin and hair.

Francesco Lai *et al.*, (2006) reported the Artemisia arborescens L Essential Oil–Loaded Solid Lipid Nanoparticles for Potential Agricultural Application: Preparation and Characterization. The aim of this study was to formulate a new delivery system for ecological pesticides by the incorporation of Artemisia arborescens L essential oil into solid lipid nanoparticles (SLN). Two different SLN formulations were prepared following the high-pressure homogenization technique using Compritol 888 ATO as lipid and Poloxamer 188 or Miranol Ultra C32 as surfactants. The SLN formulation particle size was determined using Photon correlation spectroscopy (PCS) and laser diffraction analysis (LD). The change of particle charge was studied by zeta potential (ZP) measurements, while the melting and re-crystallization behavior was studied using differential scanning calorimetry (DSC). In vitro release studies of the essential oil were performed at 35°C. Data showed a high physical stability for both formulations at various storage temperatures during 2 months of investigation. In particular, average diameter

of *Artemisia arborescens* L essential oil-loaded SLN did not vary during storage and increased slightly after spraying the SLN dispersions. In vitro release experiments showed that SLN were able to reduce the rapid evaporation of essential oil if compared with the reference emulsions. Therefore, obtained results showed that the studied SLN formulations are suitable carriers in agriculture.

Lilian van Vlerken and Mansoor Amiji (2006) reported the multi – functional polymeric nanoparticles for tumour targeted drug delivery. The use of nanoparticles as delivery vehicles for anti cancer therapeutics has great potential to revolutionise the future of cancer therapy. As tumour architecture causes nanoparticles to preferentially accumulate at the tumour site, their use as drug delivery vectors results in the localization of a great amount of the drug load at the tumour site, thus improving cancer therapy and reducing the harmful non – specific side effects of chemotherapeutics. In addition formulation of these nanoparticles with imaging contrast agents provides a very effective system for cancer diagnostics. In this report the exhaustive possibilities available to polymeric nanoparticle chemistry, research has quickly been directed to multi – functional nanoparticles , combining tumour targeting, tumour therapy and tumour imaging in an all - in - one system providing a multi – modal approach in the battle against cancer.

In 2007, Burak Erdinc evaluated the Micro/Nanoencapsulation of Proteins within Alginate/Chitosan by spray drying. Currently, therapeutic proteins and peptides are delivered subcutaneously, as they are readily denatured in the acidic, protease rich environment of the stomach or gastrointestinal track and low bioavailability results from poor intestinal absorption through the para-cellular route. Encapsulation of therapeutic peptides and proteins into polymeric micro- and nano-particle systems has been proposed as a possible strategy to overcome limitations to oral protein administration. Furthermore, it was shown that nanoparticles having diameters less than 51m are able to be taken up by the M cells of Peyer's patches found in intestinal mucosa. However, the current methodologies to produce particles within desired range involves organic solvents and several steps. In this study, spray drying was investigated as a microencapsulation alternative, as it offers the potential for single step operation, producing dry particles, with the potential for extending the micro particle size into the nano-range. The particles were produced by spray drying of alginate/protein solutions. The effect of spray drying

operational parameters on particle properties such as recovery, residual activity and particle size was studied. Particle recovery depended on the inlet temperature of the drying air, whereas the particle size was affected by the feed rate and the alginate concentration of the feed solution. Increase in alginate-protein ratio increased protein stability during the process and shelf live experiments. Presence of 0.2 g trehalose/g particle increased the residual activity up to 90%. The resulting spherical micro and nanoparticles had smooth surfaces. Stable glycol-chitosan-ca-alginate particles were produced with single step operation. The resulting particles had mean diameter around 3.5 μ m and released 35% of the initial protein content to the simulated stomach environment within 2 hours. The protein distribution within the particle was studied by confocal laser scanning microscope with florescent labeled protein. The image showed protein deposition toward the surface of the particles. Total drying time and Peclet number was calculated for the particles and found to be 8.5 ms and 240, which indicates that particle formation was governed mainly by convection, which resulted in a hollow central region and protein distribution toward the particle surface. This study shows that stable alginate particles containing proteins can be produced in a single step by spray drying, where the particles had a mean size lower than the critical diameter necessary to be orally absorbed by M cell's of the Peyer's patches in the gastrointestinal tract and thus can be considered as a promising technology for oral peptide and protein delivery.

Amir Dustgania *et al.*, (2008) reported the Preparation of Chitosan Nanoparticles Loaded by Dexamethasone Sodium Phosphate. Biodegradable nanoparticulate carriers, have important potential applications for administration of therapeutic molecules. Chitosan based nanoparticles have attracted a lot of attention upon their biological properties such as biodegradability, biocompatibility and bioadhesivity. The aim of the present investigation was to describe the synthesis and characterization of novel biodegradable nanoparticles based on chitosan for encapsulation of dexamethasone sodium phosphate. To achieve this objective, ionic gelation method were used. Drug containing nanoparticles were prepared with different amounts of drug. The mean size and size distribution of nanoparticles were measured by dynamic laser light scattering. The mean particle size, varied in the range of 250-350 nm. Values of loading capacity and loading efficiency varied between 33.7%-72.2% and 44.5%-76.0% for prepared nanoparticles.

Dharmendra Singh *et al.*, in 2009 discussed the development and characterization of chitosan nanoparticles loaded with amoxycilli. Size analysis indicates a uniform particle size. The encapsulation efficiency decreased with increased of amoxicillin and chitosan concentration. The drug entrapment efficiency of nanoparticles having same ratio of polymer and drug was about 88%. The physical stability of nanopaticles was good as studied over a period This work presents result of the development and characterization of nanoparticles for entrapping Amoxicillin, an anti miicrobial agent. The present study was to focus on the development of amoxicillin loaded chitosan TPP nanoparticles. Chitosan concentration and drug polymer ratio in the nanoparticles influence the physicochemical characteristic such as zetapotential, polydispersity index, and average nanosize diameter or percentage encapsulation efficiency of amoxicillin. An ionization method was used to entrap amoxicillin in a chitosan-matrix. The particle of three weeks. The study of the release of drug from nanoparticles exhibited a prolonged release profile (79%) as studied over 28 hr. the drug release was constant showed that formulation was long term treatment. These studies showed that chitosan can complex TPP to form stable nanoparticles for amoxicillin loading, which can be useful for microbial therapy.

Murray Isman *et al.*, (2010) evaluated the Commercial opportunities for pesticides based on plant essential oils in agriculture, industry and consumer product. In spite of intensive research on plant natural products and insect-plant chemical interactions over the past three decades, only two new types of botanical insecticides have been commercialized with any success in the past 15 years, those based on neem seed extracts (azadirachtin), and those based on plant essential oils. Certain plant essential oils, obtained through steam distillation and rich in monoand sesquiterpenes and related phenols, are widely used in the flavouring and fragrance industries and in aromatherapy. Some aromatic plants have traditionally been used for stored product protection, but the potential for development of pesticides from plant essential oils for use in a wide range of pest management applications has only recently been realized. Many plant essential oils and their major terpenoid constituents are neurotoxic to insects and mites and behaviourally active at sublethal concentrations. Most plant essential oils are complex mixtures. In our laboratory we have demonstrated that individual constituents of oils rarely account for a major share of the respective oil's toxicity. Further, the results suggest synergy among constituents, including among those that appear non-toxic in isolation. Repellent effects may be

particularly useful in applications against public health and domestic pests, but may be useful in specific agricultural applications as well. In all of these applications, there is a premium on human and animal safety that takes priority over absolute efficacy. In agriculture, the main market niche for essential oil-based pesticides is in organic food production, at least in developed countries, where there are fewer competing pest management products. There is also scope for mixing these oils with conventional insecticides and for enhancing their efficacy with natural synergists.

Atanu Bhattacharyya *et al.*, (2010) investigated the Nano-particles - A recent approach to insect pest control. Nanotechnology, a promising field of research opens up in the present decade a wide array of opportunities in the present decade and is expected to give major impulses to technical innovations in a variety of industrial sectors in the future. The potential uses and benefits of nanotechnology are enormous. These include agricultural productivity enhancement involving nanoporous zeolites for slow release and efficient dosage of water and fertilizer, nanocapsules for herbicide delivery and vector and pest management and nanosensors for pest detection. The atom by atom arrangement allows the manipulation of nanoparticles thus

Influencing their size, shape and orientation for reaction with the targeted tissues. It is now known that many insects possess ferromagnetic materials in the head, thorax and abdomen, which act as geomagnetic sensors. In this paper, our discussion is focused on nanoparticles in insects and their potential for use in insect pest management.

Vipin Kumar and Gurdeep Singh(2010) evaluated Fly-ash as possible carrier *Azotobacter* and *Azospirillum* formulations. *Azotobacter* and *Azospirillum* strains were isolated from healthy wheat rhizospore soil and bio-formulated in fly ash(300meshes). Fly ash based *Azotobacter* and *Azospirillum* formulation alone and in combination with chemical fertilizer was evaluated for bio-efficacy on wheat. Population of *Azotobacter* and *Azospirillum* was also evaluated in treated soil. The results showed that, seed treatment with *Azotobacter* and *Azospirillum* and soil treated with chemical fertilizer alone and in combination significantly enhanced the seed germination, plant height, plant bio-mass and crop yield compared to control. Chemical fertilizer treated wheat plant observed more effective bio- efficacy than bio-fertilizers treated with with

wheat but reduced (destroyed) the microbial population in soil, whereas *Azotobacter* and *Azospirillum* treated soil observed significantly enhanced the microbial population with slightly lesser plant grown as compared to chemical fertilizer. They concluded that utilization of fly ash as carrier in bio-fertilizer formulation emerged as safe and effective alternative and use of fly ash as carrier in these formulation is an effective way of utilization of problematic fly ash waste in a useful manner.

E. Corradini *et al.*, in 2010 evaluated A preliminary study of the incorporation of NPK fertilizer into chitosan nanoparticle. The use of slow release fertilizer has become a new trend to save fertilizer consumption and to minimize environmental pollution. Due to its polymeric cationic, biodegradable, bioabsorbable, and bactericidal characteristics, chitosan (CS) nanoparticle is an interesting material for use in controlled release systems. However, there are no attempts to explore the potential of chitosan nanoparticles as controlled release for NPK fertilizers. In this work chitosan nanoparticles were obtained by polymerizing methacrylic acid for the incorporation of NPK fertilizers. The interaction and stability of Chitosan nanoparticle suspensions containing nitrogen (N), phosphorus (P) and potassium (K) were evaluated by FTIR spectroscopy, particle size analysis and zeta-potential. The FTIR results indicated the existence of electrostatic interactions between Chitosan nanoparticles and the elements N, P and K. The stability of the CS-PMAA colloidal suspension was higher with the addition of nitrogen and potassium than with the addition of phosphorus, due to the higher anion charge from the calcium phosphate than the anion charges from the potassium chloride and urea. The mean diameter increase of the CS-PMAA nanoparticles in suspension with the addition of different compounds indicated that the elements are being aggregated on the surface of the chitosan nanoparticles.

Partha Saha *et al.*, in 2010 studied the Formulation and Evaluation of Chitosan-Based Ampicillin Trihydrate Nanoparticles. The purpose of their study was to develop ampicillin trihydrate-loaded chitosan nanoparticles by modified ionic gelation method and evaluate their antimicrobial activity. Ampicillin trihydrate-loaded chitosan nanoparticles were prepared by ionic gelation method with the aid of sonication. Parameters such as the zeta potential, polydispersity, particle size, entrapment efficiency and in vitro drug release of the nanoparticles were assessed for optimization. The antibacterial properties of the nanoparticle formulation were evaluated and compared with that of a commercial formulation (reference). Scanning electron microscopy

revealed that the nanoparticles were in the nanosize range but irregular in shape. Concentrations of 0.35 %w/v of chitosan and 0.40 %w/v sodium tripolyphosphate (TPP) and a sonication time of 20 min constituted the optimum conditions for the preparation of the nanoparticles. In vitro release data showed an initial burst followed by slow sustained drug release. The nanoparticles demonstrated superior antimicrobial activity to plain nanoparticles and the reference, due probably to the synergistic effect of chitosan and ampicillin trihydrate. Modified ionic gelation method can be utilized for the development of Chitosan nanoparticles of ampicillin trihydrate. Polymer and crosslinking agent concentrations and sonication time are rate-limiting factors for the development of the optimized formulation. The chitosan nanoparticles developed would be capable of sustained delivery of ampicillin trihydrate.

Abdelbasset El Hadrami *et al.*, 2010 reported the study of Chitosan in Plant Protection Chitin and chitosan are naturally-occurring compounds that have potential in agriculture with regard to controlling plant diseases. These molecules were shown to display toxicity and inhibit fungal growth and development. They were reported to be active against viruses, bacteria and other pests. Fragments from chitin and chitosan are known to have eliciting activities leading to a variety of defense responses in host plants in response to microbial infections, including the accumulation of phytoalexins, pathogenrelated (PR) proteins and proteinase inhibitors, lignin synthesis, and callose formation. Based on these and other proprieties that help strengthen host plant defenses, interest has been growing in using them in agricultural systems to reduce the negative impact of diseases on yield and quality of crops. This review recapitulates the properties and uses of chitin, chitosan, and their derivatives, and will focus on their applications and mechanisms of action during plant-pathogen interactions.

CHAPTER - 3

Aim and Scope of present investigation

Bacterial bio inoculant agents mainly phosphate solubilizing agents are the well known biofertilizers often the effective growth of many agricultural important crop . It is necessary to the factor influencing survival of bacterial agents . In this present study the effect of salt concentration factor study will be helpfull to celline bacterial biofertilizer or effective plant growth promoting agent.

CHAPTER - 4

MATERIALS AND METHODS

4.1 COLLECTION OF SEEDS

Healthy seeds Black gram and Green gram were collect from the supermarket and brought laboratory



Fig 4.1 Black gram and Green gram Seeds

4.2 COLLECTION OF BIOFERTILIZER

Biofertilizer was in the study *Bacillus megatherium* which was collected from the Stanes symbion-[P] of Thiruporur , Tamil Nadu, India



Fig 4.2 Biofertilizer Of Bacillus Magatherium

4.3 SEED TREATMENT

Respect seeds were washed thoroughly the sterilize distilled and immersed in filed *Bacillus Megatherium* suspension for 1 hour and ambient temperature . Treat seed were allow shadow dry after shade dried the inoculated seeds were transparent moisture tissue paper that prepared towel , Incubator in dark observation was record every day to record seedling emergent from this data germination index was calculated control was also maintain distilled water .



Fig 4.3 Black gram and Green gram

4.4 INFERNECES SALINITY ON *BACILLUS MEGATHERIUM* COLLECTED SEEDS

In this study common sodium carbonate was used to tested to salinity influence or (salt tralert)

The seeds were embred biofertilizer submission 1% of sodium carbonate seeds were incubator 1 hour at ambient condition after incubation the seed kept on tissue paper

4.5 EVOLUTION OF STRESS

Salt induced on the collection the respective test was evaluation determination of stress enzyme like lipid peroxide(LP) , Gremination Total chlorophyll contain and protein proliferation by SDS PAGES electrophoresis

4.6 DECRIPITION OF THE BIOFERTILIZER :

4.6.1 SEED INOCULATION

Healthy seeds of tested plant was immersed in 0.1 , 0.25 , 0.5 , and 1% concentration of NaCl in a tap water filtered by addition of biofertilizer (filter concentration) . After 1 hour of incubation , the treated seeds were shade dried at room temperature. After drying the treated seeds were kept on tissue paper moisture with distilled water. Incubation at 28*c in the work germination index was shedied . Results indicate that the bacteria exhibited high salt concentration and the salt didn't affect germination index and colonization . Biochemical parameters of tested seedings also reveals no dirtect effect. The presents study would suggest the possible utilization of Bacillus Megatherium

CHAPTER- 5

RESULTS & DISCUSSION

1. Estimation of photosynthetic pigments (mg/g.f. wt)

Chlorophyll a and b contents were extracted from respective dose of leaves and estimated according to the method of Arnon (1949).

Two hundred mg of fresh young leaves were ground with 10ml of 80 per cent acetone Pestle mortar and spun at 2500 rpm for 10 min at 20⁰ c in centrifuge. The homogenate was re-extracted with 80 per cent acetone until the green color was disappears in the residue and the extract was pooled and made up to 20 ml with 80 per cent acetone. Two ml of extract was transferred into a cuvette and the absorbance/ optical density (OD) was read at 663, 645 in a spectrometer (Elico Made) against 80 per cent acetone as a blank, to determination chlorophyll a and band carotenoids content.

Chlorophyll content was calculated using the formula of Arnon (1949).

$$\text{Chlorophyll 'a' (mg/g f wt)} = (1.27) \times (A.663) - (0.269) \times (A.645)$$

$$\text{Chlorophyll 'b' (mg/g f wt)} = (2.29) \times (A.645) - (0.468) \times (A.663)$$

$$\text{Total chlorophyll (mg/g f wt)} = (2.02) \times (A.645) + (0.802) \times (A.663)$$

The spectrum graph for chlorophyll Pigment observed from 200nm to 800nm with blank 80% acetone.

2. Anti-oxidant assay

A. Catalase assay

CAT activity was assayed by the method of Goth (1991) with slight modifications. The enzyme extract (0.5mL) was added to the reaction mixture, containing 1mL of 0.01mol/L phosphate buffers (pH 7.0), 0.5mL of 0.2 mol/L H₂O₂ or 30% H₂O₂, 0.4mL H₂O, and incubated for 10mins. The enzymatic reaction was stopped with 1ml of 32.4mmol/l ammonium molybdate and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm against blank 3.

$$\text{Sample CAT activity (kU/l)} = \frac{A_{(\text{Blank 1})} - A_{(\text{Sample})}}{A_{(\text{Blank 2})} - A_{(\text{Blank 3})}} \times 271$$

Where:

Blank 1 contained 1.0 ml substrate, 1.0 ml molybdate and 0.2 ml sample; blank 2 contained 1.0 ml substrate, 1.0 ml molybdate and 0.2 ml buffer; blank 3 contained 1.0 ml buffer, 1.0 ml molybdate and 0.2 ml buffer.

A. Estimation of reduced glutathione (GSH)

Estimation of reduced glutathione was done according to method of Jollow et al. With slight modification plant sample homogenate (500μL of 0.1M potassium phosphate buffer (pH7.4)) was precipitated by the inclusion of 4% sulfosalicylic acid (500μL). After keeping for 1 h at 4°C, the samples were centrifuged for 20min at 1200g. The supernatant (33μL) was gathered and added to aliquots consisting of 900μL of 0.1M potassium phosphate buffer (pH7.4) and 66μL of 100mM dithiobis (2-nitrobenzoic acid) (DTNB). GSH reacts with DTNB and produces a yellow colored complex. Absorbance was taken at 412 nm within 10min 100μl of extract was treated as above and the absorbance was taken at 412 nm. Blank tubes having all the reagents except glutathione solution and the extract were also included.

B. Glutathione S-transferase assay GST

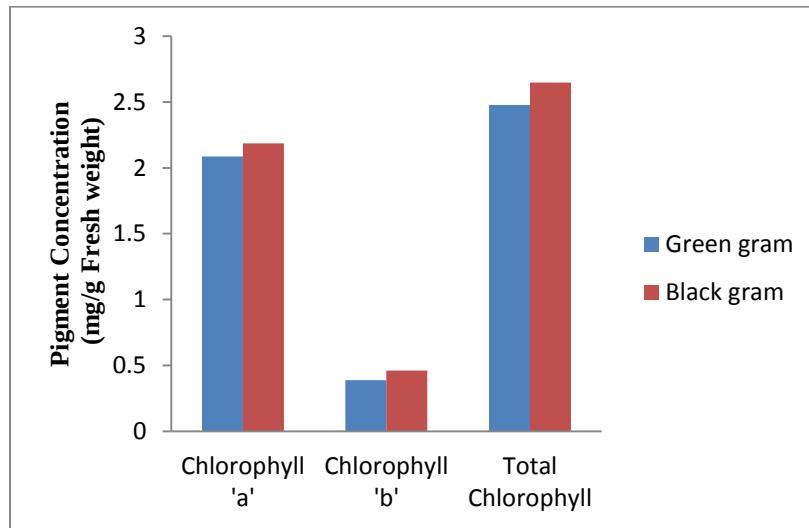
30 mg of each sample was weighed and dissolved in 3ml of DMSO solution and mixed well. This extract was further used. A 2ml of phosphate buffer (pH7.4) and 2ml of plant extract were added to blank and sample wells, respectively. Reaction was initiated by adding 1ml of

CDNB (1mM/ml) to both the wells and mixed well. The absorbance was read at 340nm. The extinction coefficient for CDNB is $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340nm. Protein was estimated using Bradford's assay.

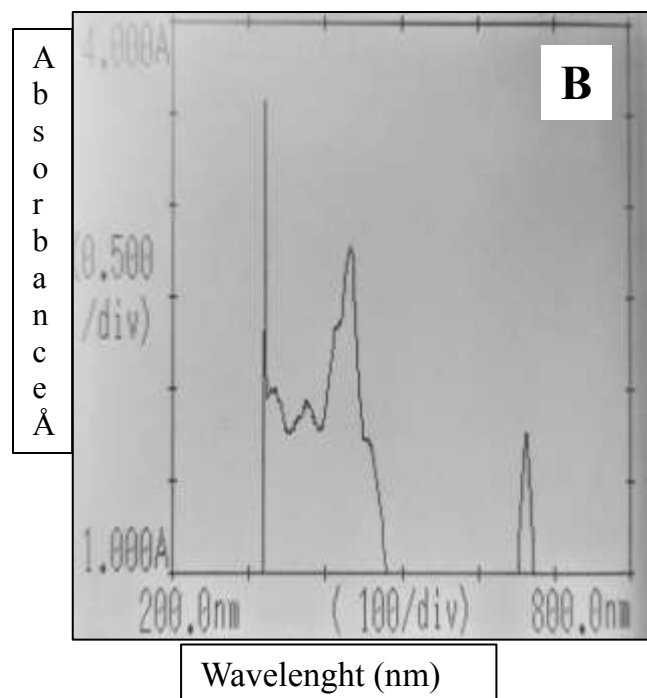
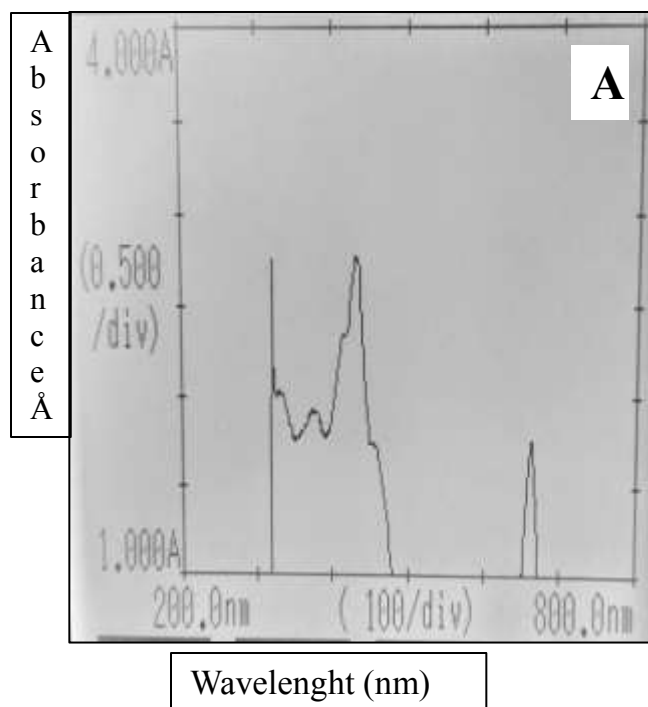
A. Quantification of Lipid Peroxidation :

Thiobarbituric acid-reactive substances (TBARS) assay is used to detect lipid peroxidation. In this assay, malondialdehyde forms a complex with thiobarbituric acid, which can be quantified spectrophotometrically. The plant sample was homogenized with ice cold KCl Buffer then the homogenized sample was collected in eppendrof. Then added 0.2ml of 8.1% SDS, 1.5ml of 20% acetic acid mixed and incubated for 10min, then added 1.5ml of 0.8% TBARS and 0.7ml of distilled water to the solution. This reaction mixture was incubated for 1 h at 95°C and then cooled to 25°C and centrifugation at 5000g for 15min to remove debris. Absorbance was recorded at 532nm. The untreated sample was taken as a control. The standard was performed with MDA (Malondialdehyde). The sample values compared with standard and the concentration expressed as μM .

1. Chlorophyll pigment Assay



The photosynthetic pigment such as chlorophyll a, b and total chlorophyll (Chl-a, b and Tol-chl) content were decreased in Green gram compared to Black gram.

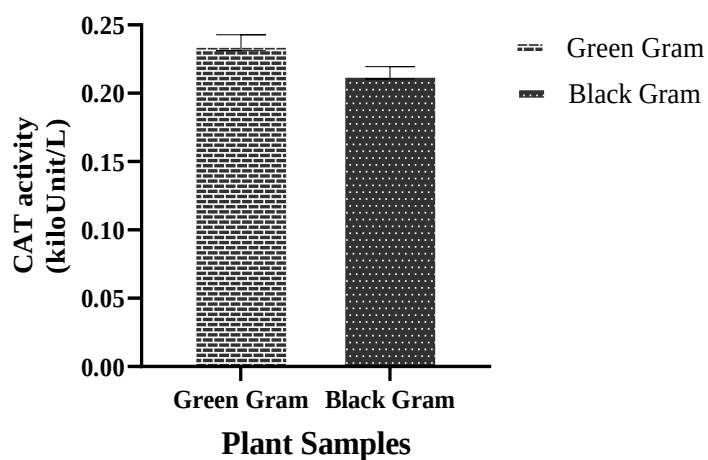


A) Green gram, B) Black gram

The first absorption peak was observed at 320nm for Green gram and at 376nm for Black gram, whereas the highest absorption peak was observed at 432nm for Green gram and 432nm for Black gram.

2. Anti-oxidant assay

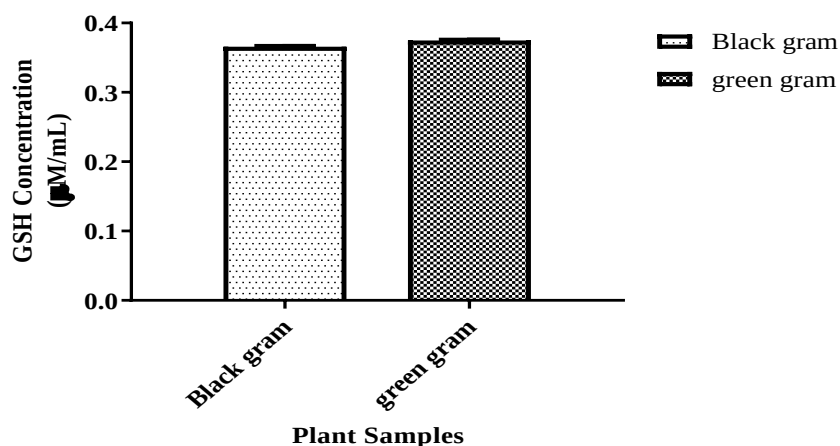
A. Catalase activity



The total content of Catalase activity was performed amid the treated and untreated plant sample values are expressed as Mean ($\pm$ SD) analyzed. The graphical representation

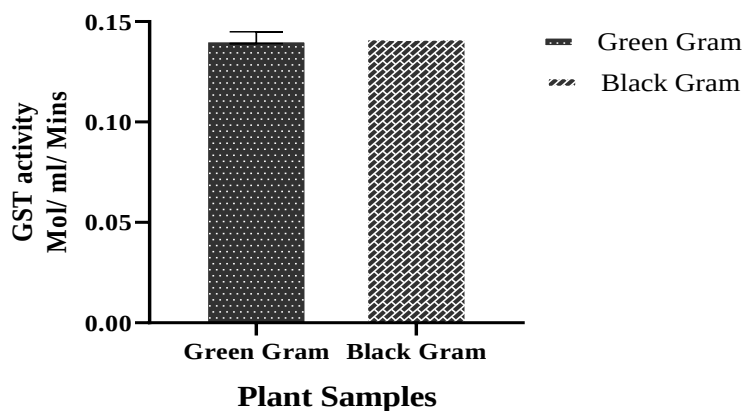
shows the different Catalase activity in Black gram and Green gram, these clearly indicate that there were increased Catalase activities in the Green gram compared to Black gram.

A. Estimation of reduced glutathione (GSH)



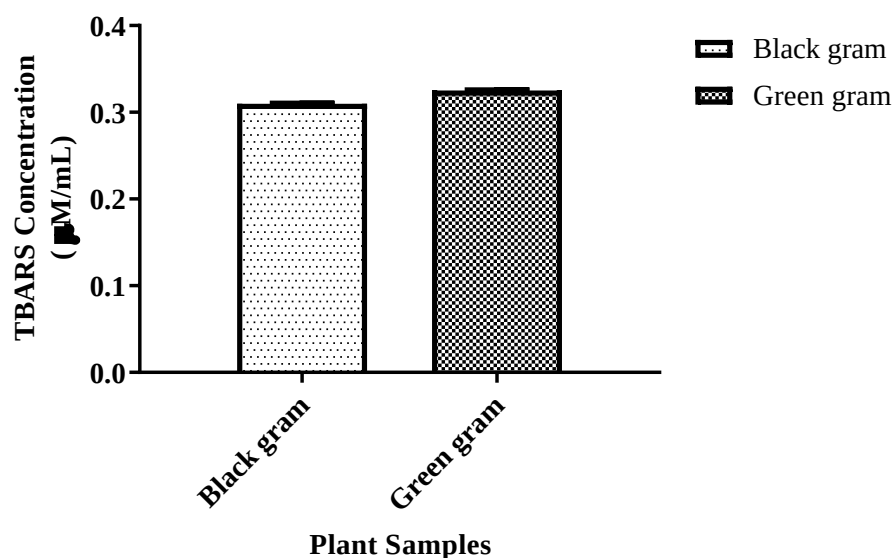
Total content of Glutathione (GSH) among the plant samples values are expressed as mean ($\pm$ SD). The graphical representation shows the different in GSH content between Black gram and Green gram; these clearly indicate that there were increased GSH contents in the Green gram compared to Black gram.

A. Glutathione S-transferase assay GST



The total content of Glutathione S-transferases activity was performed amid the treated and untreated plant sample values are expressed as Mean ($\pm$ SD) analyzed. The graphical representation shows the nearly equal GST activity in Black gram and Green gram.

A. Quantification of Lipid Peroxidation



Total content of Thiobarbituric acid reactive substances (TBARS) among the plant samples values are expressed as mean ($\pm$ SD). The graphical representation shows the different TBARS content in Black gram and Green gram; these clearly indicate that there was increased TBARS content in the Green gram compared to Black gram.

CHAPTER - 6

SUMMARY AND CONCLUSION

Biofertilizers based on bacteria are the promising plant growth promoters of various crops.

Bacillus Megatherium among the one of the major bacterial agent known useful for its unique phosphate solubilizing efficiency. In this study, salt stress incubated *Bacillus megatherium* and its influence on molecular mechanism of *Bacillus megatherium* were shown any undesirable effect on the total chlorophyll content and anti oxidative enzymes. No sign of toxic effect clearly indicate the salt tolerance efficiency.

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