MASS PRODUCTION OF FREE-LIVING AND SYMBIOTIC NITROGEN-FIXING BIOFERTILIZERS AND COMPARATIVE EFFICACY STUDY OF DIFFERENT CARRIERS

A

Dissertation Submitted to the **Department of Microbiology**,

Central Campus of Technology,

Tribhuvan University, Dharan, Nepal,

In Partial Fulfillment of the Requirements for the Award of Degree of Master of Science in Microbiology

(**Agriculture**)

Submitted by

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T.U. Registration Number 5-2-8-49-2012

2022

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ACKNOWLEDGEMENT

I owe my deepest gratitude and heartfelt appreciation to my respected supervisor Asst. Professor **Mr. Hemanta Khanal** for his continuous support, encouragement and expert guidance throughout my research work. Without his valuable help, it would not have been possible to complete this dissertation work successfully. I want to express my sincere gratitude to the **Ministry of Industry, Tourism, Forest and Environment province 1**for providing me grant support to complete my academic thesis.

I am much obliged to my Campus Chief **Dr**. **Dil Kumar Limbu**, **Mr. Om Prakash Panta** Head of Department of Microbiology, Central Campus of Technology for providing me with the required facilities and instructions for the dissertation work.

Similarly, I would like to thank all the teachers, especially Asst. Professor **Shiv Nandan Sah and Dinesh Shrestha**for his good works and support and laboratory staff **Mr. Ain Bahadur Karki**, **Prajwal Bhandari**, librarian **Mr. Om Khatiwada** and library staff for their great cooperation and help.

Finally, I would express my gratitude to my friends, especially **Mr. Sunil Regmi, Ms. Deepa Rai, Susmita Phattepuri, Priskila Tolangi, and Mr. Pradeep Kafle** for their help and support. I would like to convey my regards to my entire family members for motivating and supporting me during the thesis work.

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ABSTRACT

There is a high demand for food worldwide which has caused the increase in the use of chemical fertilizer to fulfill the global need for food. The use of chemical fertilizer has a negative impact on the environment which has exponentially raised the global interest in microbial fertilizer. This study focuses on the easy and cost-effective use of carrier material that can be used in the production of microbial fertilizer. For these symbiotic bacteria (*Rhizobium*) and non-symbiotic bacteria (*Azotobacter*) were used as microorganisms and charcoal, rice husk, and farmyard manure as carrier material were used. *Rhizobium* species and *Azotobacter* species were isolated from pea plant root nodule and soil sample respectively. These isolated organisms were blended with carrier material and kept in two different temperatures i.e. at room temperature and refrigerator to know the survivability of microorganisms in normal conditions and preserved condition.

A decline in moisture and pH on prolonged incubation was observed in both organisms. A high survivable rate of *Rhizobium* species is seen in rice husk and *Azotobacter* species is seen in farmyard manure stored in both temperatures. Whereas least survivability was seen in charcoal for both organisms stored in both temperature. Moisture and pH are normally responsible for more 50% of change in CFU in all carrier material except for rice husk blended with *Rhizobium* and farm yard manure blended with *Azotobacter* stored in room temperature. CFU in all the carrier material with respect to storage and carrier material have significant difference except for *Azotobacter* stored in room temperature. The rhizobia and *Azotobacter* strains population significantly decline over time regardless of the carrier material and storage temperature. A decline in population on prolonged incubation may be attributed to the depletion of nutrients, moisture, and cell death. Rice husk, however, demonstrated extraordinary potential, particularly in respect of shelf life.

KEYWORDS: *Rhizobium*, *Azotobacter*, carrier, biofertilizer

LIST OF ABBREVATIONS

NA	Nutrient agar
YEMA	Yeast Extract Mannitol agar
FYM	Farmyard manure
N	Nitrogen
BNF	Biological Nitrogen fixation
PGPB	Plant growth promoting biofertilizers
PGPR	Plant growth promoting rhizobacteria
HCN	Hydrogen, carbon and nitrogen
IPNM	Integrated plant nutrient management
CFU	Colony forming unit
µl	Microliter
Mm	Millimeter
^{0}C	Degree centigrade

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CHAPTER-I

INTRODUCTION AND OBJECTIVE

1.1 Background of the study

Nepal`s financial system in large part relies upon its agriculture sector. In 2018 agricultural sectors furnished 7.35 billion US back to the country`s financial system by supplying employment to 65.56% of people. Priority strategies, both technical and legislative, had been installed region to gain sustainable agricultural productivity. Several studies research regarding the utility of contemporary-day agriculture technology had been performed to deal with the trouble of low crop production, soil fertility, and nutrient imbalance (Reddy *et al.* 2020).

After the Green Revolution, extensive use of chemical fertilizer and pesticides is seen in agriculture. Fertilizers are natural or manmade chemicals that, when applied on the plant or to soil or by fertigation (applying by irrigation water), can supplement natural soil nutrients and augment crop growth and soil fertility (Edgerton 2009). Chemical fertilizer makes the environment hazardous when used discriminately. To avoid the negative effects biofertilizers are used as the alternative source of fertilizer as it increases crop yields and is environment friendly (Kaljeet, Keyeo and Amir 2011).

According to FAO Biofertilizer: a broad term used for a product containing living or dormant micro-organisms inclusive of bacteria, fungi, actinomycetes and algae, alone or in combination, which on utility assist in fixing atmospheric nitrogen or solubilize/mobilize soil nutrients (Timmusk *et al.* 2017).

Various microorganisms like *Azotobacter*, *Rhizobium*, and Cyanobacteria are used as biofertilizers for imparting vital vitamins inclusive of nitrogen and phosphorus. (Hari and perumal) defined biofertilizer as the selected strain of useful soil microorganism cultured within side the laboratory and packed in an appropriate carrier (Ju *et al.* 2018). These microorganisms also are vital to preserve nutrient

flow from one system to every other and to decrease ecological imbalance (Novinscak *et al.* 2008).

Plant vitamins are factors crucial for the growth and development of flora, commonly now no longer which includes carbon, hydrogen, and oxygen. Plant vitamins encompass the number one vitamins nitrogen, phosphorus, potassium, and others which include sulfur, calcium, magnesium, boron, chlorine, copper, iron, manganese, molybdenum, zinc, and others (Fukami, Cerezini and Hungria 2018).

Nitrogen is crucial for protein manufacturing in flora and is the important thing number one plant nutrient accountable for the growth of green parts in flora. Nitrogen constitutes approximately 78% of the atmospheric gases and greater than 99% of it is far found in non-reactive N_2 form. The reactive N (ammonia, ammonium, nitrogen oxides) is anticipated to be much less than 1% in the atmosphere. Rhizobia related to legumes and a few different organisms, such as *Azotobacter* spp can restorative non-reactive N_2 and make it available to developing flora (Kumar 2014).

Rhizobium is a (0.5-1.0) long and (1.2-3.0) widthmicron Gram-negative bacillus. Non-spore forming, motility by peritrichous flagella, and+respiratory metabolism with oxygen as terminal electron acceptors. Optimal temperature of *Rhizobium* is $(25\text{-}30)^0$C and pH is (6-7) (Gomare, Mese and Shetkar 2013).

Azotobacter is a free-living, Gram-negative, aerobic, nitrogen-fixing bacterium, so it is used as a biofertilizer instead of a chemical fertilizer. It grows in the temperature and pH range of $(28\text{-}30)^0$C and (7.0-7.5) respectively. It uses sugar, alcohol, and organic acid salts for growth. Generally, it fixes non–symbiotically about 10mg of atmospheric nitrogen/gram of carbohydrates (usually glucose) consumed. It is non-spore forming but can form a cyst in adverse conditions, and in older cultures grown with sugar as the carbon source (Mahato and Kafle 2018).

Microbial inoculant has a completely short shelf existence so a good carrier material is wanted to grow its effectiveness. Biofertilizers are commonly

organized on the idea of carrier substances used. Incorporation of microorganisms in provider substances permits smooth handling, long-time storage, and excessive effectiveness of biofertilizers (Ali *et al.* 2005).

Various sorts of substances are used as a carrier for seed and soil inoculation. Some of the carrier substances are filter mud, lignite, coal, wheat straw, rice husk, coconut shell , and aggregate of those substances (Chaot and Alexander 1984). Peat is the maximum, often used substance for seed inoculation. Peat is extensively used for legume inoculants, even though it isn't always quite simply to be had or is pricey in comparison to different substances. Mineral soil is an especially appealing carrier material because it is to be had in all farming soil and is inexpensive. Some strains of *Rhizobium meliloti* can live in soil for 30-forty years (Chaot and Alexander 1984). Some of the carrier substances used conventionally used had beenhumic acid, compost, peat, and vermicompost. Some different carrier substances often used are peat, vermicompost, and humic acid which might be excessive in natural content material and beautify the general increase of plant (Siddiq *et al.* 2018).

Rice husk: The hard covering of rice grain is called rice husk. The use of rice husk as a carrier material has been demonstrated. Rice husk was discarded as it was considered waste. Therefore utilization of rice husk was started by the development of rice husk into carrier material. It was mixed with local kaolin for the improvement of water activity in the carrier material. kaolin also protects the plant from insect pathogen as it irritates the insect that is attached to the plant (Kaljeet, Keyeo and Amir 2011).

Charcoal: Charcoal is mostly pure carbon, made by cooking wood with low oxygen. The process can take days and burns off volatile compounds such as water, methane, hydrogen, and tar, and leaves about 25% of black lumps and powder of the original weight. The quality of charcoal is defined by various chemical characteristics, although properties are interrelated, they are measured and appraised separately. Most of the specifications that control charcoal quality have originated in the steel or chemical industry (Open and Publisher 2014).

Farmyard manure: Degradation of organic matter under the controlled condition of temperature, pressure and oxygen is termed as compost. It is a good source of nutrients for the plant as it provides an easily degradable and stable source of carbon. Easily degradable provides carbon to the plant as stable carbon help to hold the nutrient and does not allow them to leach down (Siddiq *et al.* 2018).

Biofertilizer formulation determines its potential success in the market, normally being the key obstacle to their commercialization (Fages 1992). The formulation involves different steps that result in single or numerous microbial strains incorporated in the suitable carrier material, which provides a safe environment that protect them from adverse storage conditions, guaranteeing their survival and establishment after their inoculation (Herrmann and Lesueur 2013).

The type of carrier, storage temperatures pH and moisture are the main factors that determine the shelf life of biofertilizers (Kremer and Peterson 1983). Biofertilizers with an adequate shelf life of at least one to two years at room temperature are desirable for effective integration into farming systems (Catroux, Hartmann and Revellin 2001). Storage temperature can affect biofertilizer activities pre or post-microbial application (Malusá, Sas-Paszt, and sCiesielska 2012), while the physiological processes might decline rapidly if storage is not properly done (Kaljeet, Keyeo and Amir 2011). Optimal lasting storage conditions for rhizobia survival have assumed that temperature and moisture conditions are significant for rhizobia growth (Bohlool 1983). Strains used in liquid formulation normally grow at 37°C and are tolerant to temperatures of up to 45 °C for two years or more. Solid-based shelf life is hardly up to 3 months, since soaring in temperatures beyond 35°C causes a rapid decline in organism's population(van Schreven 1970).

In the decades that followed, there were several reports on the benefits of new PGPB and advances in the inoculant industry, but modest interest from research and industry was observed. It is understandable that the number of studies on new inoculant development (Gundi *et al.* 2018; Santos, Nogueira and Hungria 2019), new strain identification and new inoculation methods (Zvinavashe *et al.* 2019) is

increasing. Protein Based biomaterial that can encapsulate and protect rhizobia inoculated into seeds even after sowing, enhancing the effectiveness of inoculation has been developed. According to the Web of Science database, between 2015 and 2019, 68 of his papers (not revised) with the key words "inoculant" or "biofertilizer" followed by "production" or "development" were published. Therefore, inoculant involving both microbes and technology are expected to be announced in the coming years (Çakmakçı 2019). Before initiating a large-scale inoculation program with rhizobia, it is essential to evaluate the need for inoculation and perform a cost-benefit analysis (Stephens and Rask 2000).

Though biofertilizer is environmentally friendly related to chemical pesticides, their applications are limited because of cost efficacy. So keeping the view the recent scenario of mass production of biofertilizer and application, the present study will be conducted to study the efficacy of different carrier materials on selected microorganisms and to select the most suitable carrier material that can enhance the effectiveness and shelf life of selected microorganism. This study also contributes to the effective utilization of bioorganic waste material for the production of suitable carrier material.

1.2 Objective

1.2.1 General objective

Mass production of free-living (*Azotobacter* species) and symbiotic (*Rhizobium* species) nitrogen-fixing biofertilizers and comparative efficacy study on different carriers.

1.2.2 Specific objectives

- To isolate *Rhizobium species* and *Azotobacter species* from pea plant and soil sample.
- To prepare different carrier materials for microbial inoculants.
- To study the viability of *Rhizobium species* and *Azotobacter species* on different carriers with different time and temperature relationships.

CHAPTER-II

LITERATURE REVIEW

2.1 Fertilizer

Fertilizers are materials that deliver plant vitamins or amend soil fertility (IFA, 1992) and are carried out to grow crop yield and/or quality, in addition, to preserve soil ability for destiny crop production. According to not unusual place dictionaries, fertilizers can encompass each manures and plant residues, in addition to evidently going on critical factors which have been mined (e.g. P and K) or, within side the case of N, constant from the surroundings and included into synthetic fertilizers. However, agronomist's use this time period differently, and on this guide, the word "fertilizer" refers to synthetic nutrient re assets except in any other case especially noted (Alley and Vanlauwe 2009).

Increased use of fertilizers and high-productivity systems has also caused environmental problems such as soil, surface water and groundwater degradation, air pollution, loss of biodiversity and suppression of ecosystem functioning (Lam *et al.* 1995; Paper 1999; Vance 2001). The most restrictive nutrients for plant growth are N and P (Schachtman *et al.* 1998). Soil can contain large amounts of both nutrients, but most are not readily available to plants. There is an urgent need for sustainable agricultural practices on a global scale. Developed countries need to reduce energy and environmental costs. Developing countries need efficient and sustainable practices to be able to cost-effectively produce enough food for their growing populations. Through more efficient use of nutrients or availability of nutrients to overcome the ecological problems caused by plant nutrient loss and increase crop yields in the absence of resources to obtain expensive fertilizers. Microorganisms can provide efficient use of nutrients (Santos, Nogueira and Hungria 2019).

The cost of chemical fertilizers will be very high that the marginal farmer will not be able to use in coming future. The commercial nitrogen fixation now no longer

simplest depletes our finite reserves of fossil fuels, however additionally generates big portions of essential greenhouse gas, nitrous oxide, that's three hundred instances greater poisonous than carbon dioxide (Bashyal 2013).

Environmental infection can be the main risk to the survival of residing organisms. The misuse of chemical fertilizers and insecticides can make contributions to the deterioration of the environment. It is critical to apply renewable resources (biofertilizer) to maximize crop yields and decrease the environmental dangers related to chemical residues (Kaosol 2009).

2.2 Biofertilizer

We agree with the definition of biofertilizer proposed by Prof. Dr. ZulkifliHj. Shamsuddin, University Putra Malaysia, in Inaugural Lecture of seventeenth June 2005. “Biofertilizer is a substance which includes residing microorganisms which, while carried out to seed, plant surfaces, or soil, colonizes the rhizosphere or the indoors of the plant and promotes increase in supply or availability of number one nutrient to the host plant (Vessey 2003). Plant growth-promoting rhizobacteria are thus used as biofertilizers (Fnca *et al.* 2006).

This definition separates biofertilizer from natural fertilizer. The latter carries natural compounds which directly, or through their decay, grow soil fertility. Likewise the time period biofertilizer ought to know no longer be used interchangeably with the terms, inexperienced manure, manure, intercrop, or natural-supplemented chemical fertilizer. Not all plant growth promoting rhizobacteria (PGPR) may be taken into consideration as biofertilizers.

The term biofertilizers or which may be greater as it should be called `microbial inoculants' may be typically described as an preparation containing live or latent cells of efficient strain of nitrogen-fixing, phosphate solubilizing or cellulolytic microorganisms used for the utility of seed, soil or composting regions with the goal of growing the numbers of such microorganisms and boost up sure microbial procedure to augment the quantity of the provision of vitamins in a shape which

may be without problems assimilated through the plant. In a big sense, the time period can be used to encompass all-natural resources (manure) for plant growth which might be rendered in an to be had the shape for plant absorption through microorganisms or plant institutions or interactions (Alley and Vanlauwe 2009). Biofertilizers containing microorganisms like bacteria, fungi, and algae were advised as possible answers for big-scale agricultural practices which now no longer best are natural, eco-friendly, and economic however additionally preserve soil shape in addition to biodiversity of agricultural land (Singh 2019).

2.2.1 Integrated plant nutrient management

Eighteen elements are essential for higher plants: carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), sulphur (S), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo), chlorine (Cl), nickel (Ni) , and cobalt (Co). All elements are not essential for all plants. Carbon, H, and O are obtained from the atmosphere and water and are not considered mineral elements. The remaining essential elements can be divided into primary macronutrients (N, P, K), secondary macronutrients (S, Mg, Ca), and micronutrients (Fe, Mn, Zn, Cu, B, Mo, Cl, Ni, Co) based on average concentrations in plants.

Primary and secondary macronutrients are found in plants at levels of 0.2 to 5.0% or greater, while plant concentrations of micronutrients range from 0.1 to 100 μg/g (Alley and Vanlauwe 2009). Plant growth-promoting microorganisms (PGPM) are heterogeneous in nature comprising bacteria, fungi, and actinomycetes that survive in and around the root rhizosphere. PGPM enhances plant growth and yield either directly or indirectly (Hariprasad *et al.* 2009). Direct plant growth promotion involves the solubilization or mobilization of important nutrients (phosphorous, potash, zinc, sulfur, , and iron) or fixing atmospheric nitrogen for the uptake of plants (Arshadl 1998).

2.2.2 Nitrogen and its uses

Our attitude towards mineral nitrogen (N) fertilizers is ambivalent. N fertilizers have on one hand increased our supply of food, feed, and other bio-based raw materials tremendously and also improved the use efficiency of land and labor, but have on the other hand a negative impact on the quality of the environment and contributed to the depletion of fossil fuel reserves. This awareness has resulted in strong pleas to spend much more attention to the recycling of N containing downstream "wastes". It is, however, naive to assume that even perfect recycling suffices to offer the same number of people the same diet without inputs of "new" N, as inevitable losses of N make compensations indispensable. "New" N can be derived from either biological N fixation ("legumes") or industrially fixed N ("fertilizer").

Although N is abundant in the air, it is the most restrictive nutrient for plant growth because it is not available for plant uptake. Some bacteria can immobilize N_2 from N pools in the atmosphere (Catroux, Hartmann and Revellin 2001). Board (2004) suggested that inoculation is likely to be beneficial to plant productivity at a population density of fewer than 100 rhizobia per gram of soil. At such low population densities, vaccination has proven cost-effective, regardless of the N_2 fixation efficiency of native rhizobia (Stephens and Rask 2000).

Between 2000 and 2010, about 120 million tons of fertilizer nitrogen was used in global food production. Other sources, namely animal manure and waste, nitrogen fixation and deposition (including rain, irrigation water and animal grazing), represent 57, 60 and 70 million tons respectively. About the same amount of Fertilizer-N was removed by crops (122 million tons). N losses from leaching and runoff, ammonia volatilization and denitrification were 37, 95 and 25 million tons, respectively, resulting in a positive balance (soil accumulation) of 28 million tons per year (Kumar 2014).

2.3 Microorganism as biofertilizer

The presence of those microorganisms makes the soil a dwelling and lively system. These microorganisms play a large position with inside the lifestyles cycle of flora and animals through some of the procedures consisting of decomposition, solubilization, nutrient fixation and supply of flora. Biofertilizer means use of living organisms as fertilizers, both to restoration atmospheric nitrogen and to solubilize mineral vitamins like phosphorus. The microbial inoculants have attained unique importance in present-day agriculture (Board 2004). The method is liable for solving atmospheric nitrogen through microbes into ammonium so that it can be used for flora is called biological nitrogen fixation (BNF) (Desbrosses and Stougaard 2011). Keeping in view the growing cost of chemical fertilizers and the negative buying ability of Indian farmers numerous microorganisms which may be utilized in agriculture (Board 2004).

Table 1: Nitrogen fixing capacity of microorganism.

Microorganism	***Nutrient fixed (kg/ha/year)***
Actinorrhizae (frankia spp.)	150 kg N_2/ha
Algae	25 kg N_2/ha
Azolla	900 kg N_2/ha
Azospirillum	10-20 kg N_2/ha
Rhizobium	50-300 kg N_2/ha
Azotobacter	0.026-20 kg N_2/ha
Mycorrihizae	Solubilize food phosphorus (60%)
Phosphate solubilizing bacteria and fungi	Solubilize about 50-60% of the fixed phosphorus in the soil

2.3.1 Biological nitrogen fixation

The N fertilizer industry is using solar energy that has been captured in the past ("fossil energy"), whereas legumes use the current solar energy. As a result of that, legumes consume land because one cannot use solar energy to fix N (more precisely: allocate photosynthates to the N-fixing bacteria living in symbiosis with legumes) without affecting the amount of solar energy that is left over for the production of food, feed, fiber, and biofuel. In addition to land, legumes also demand other inputs of which the availability can be limited such as water. As far as the consumption of land is concerned, it must be noted that land consumption per unit N that is effectively available for crops that are deemed to benefit from these green manures, is even higher than indicated by the N fixation per hectare, as the N fertilizer replacement value of ploughed-in legumes will be less than 100%, just as that of any other organic source of N. If one assumes that one hectare of legume land fixes 100 - 300 kg N per year (Herridge, Peoples and Boddey 2008)and that three-quarters of this amount can effectively convert into mineral N equivalents for subsequent crops (Schroder 1997.Pdf), the current global application rate of mineral fertilizer N (22 kg N per ha) would equate with an additional arable land claim of almost 30% - 100%.

Schroder & Sorensen presented a simple model N directed at calculating the demand for land in the function of choices with respect to the sources of N (mineral fertilizer N, urban residues, legumes), dietary aspirations, and attainable yield levels. Their model simulations indicate that an intensive strategy (ambitious yield levels, mineral fertilizer N) needs half the amount of land of an extensive strategy (modest yield levels, biologically fixed N in addition to the recycling of urban residues). This outcome mirrors the combined effects of lower food yields per hectare resulting from extensification and the dilutions of these yields per hectare with the additional hectares needed for growing legumes. The loss of N per hectare of the intensive system is higher but the global N loss (i.e. the product of the loss per hectare and the number of hectares needed) of both strategies are quite similar. However, an affluent diet produced according to the intensive strategy needs as much land as a moderate diet produced according to

the extensive strategy, whilst losing around twice as much N per hectare. It shows that N losses are just as much determined by decisions on what we eat ourselves than by decisions on how we feed our crops (Schröder 2014)

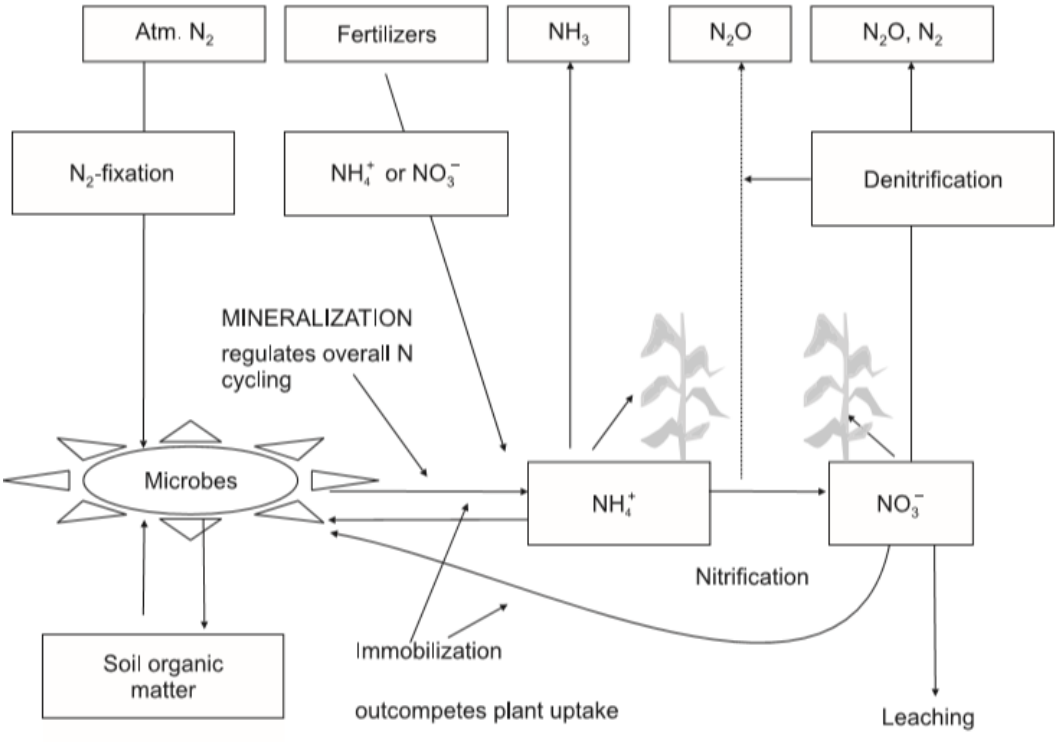


Figure1: Biological nitrogen fixation.

2.4 *Azotobacter* and *Rhizobium*

2.4.1 *Rhizobium*

Rhizobium is suitable to be used as the microbial inoculant as the close propinquity between rhizobia and host plant allows resourceful use of fixed nitrogen (Kaljeet, Keyeo and Amir 2011). These bacteria fix nitrogen after they are established inside root nodules of legumes so they are called symbiotic nitrogen-fixing bacteria. Different types of rhizobia belong to the family rhizobiales, a probably monophyletic group of proteobacteria. These are unique

soil bacteria that have the potential to fix atmospheric nitrogen by infecting the root of legumes. These bacteria form an intimate symbiotic relationship with the legumes by responding chemotactically to flavonoids released as a signal by the plant. These plant compounds help to induce the expression of the nodulation gene in rhizobia. And in return produce lipochitooligosaccharides signal that starts the mitotic cell division in the cell of plants roots. This symbiosis is an example of a mutation. It turns out that the legumes of plants guide the evolution of rhizobia towards greater mutation by reducing the oxygen supply to nodules that fix less nitrogen by reducing the cheaters in the next generation (Baset Mia and Shamsuddin 2010).

2.4.2 N_2 Fixation in a Legume-*Rhizobium* Symbiosis

The N_2 fixation in legume-*Rhizobium* symbiosis is carried by the enzyme dinitrogenase (EC 1.18.2.1). It is a multimeric protein complex made up of two proteins of different sizes; molybdoferredoxin (Mo-Fe) protein and azoferredoxin (Fe) protein. Nitrogenase catalyzes the reduction of atmospheric N_2 to NH_3. The Mo-nitrogenase requires high energy (16 mol ATP) for reducing each mole of N_2. Moreover, the enzyme is extremely oxygen-sensitive, whereas the symbiotic rhizobacteria are strictly aerobic. The photosynthetic derivatives provided by the legume host plant in the form of abundant carbohydrate and citric acid cycle intermediates accomplish the energy requirement, and root nodules provide the anoxygenic environment required for N_2 fixation. The synthesis, processing, and assembly of the nitrogenase complex are carried by the nif genes. The number of nif genes varies with the physiology of the colonizing bacterium. Based on the previous studies, 15 nif genes have been reported in *A. caulinodans* and *B. japonicum*(Kaneko *et al.* 2002), and 8 and 9 nif genes have been found in *R. leguminosarumbv. Viciae*(Kumar Deshwal and Chaubey 2014) and *S. meliloti*(Dupont *et al.* 2012), respectively. The nifA, nifB, nifDK, and nifEN are the core nif genes (Masson-boivin *et al.* 2009).

In rhizobia, the NifJ and NifF electron transfer proteins are missing and replaced by the fixABCX gene products. Similarly, the nifS and nifU genes are substituted with the icsS and iscA (housekeeping paralogs), respectively. NifY is replaced by the NifX (absent in *R. leguminosarumbv. viciae*) but is not involved in stabilizing apo-NifDK. The nifW and nifZ genes are also absent in rhizobia. Therefore, the nitrogenase assembly machinery in *S. meliloti* and *R. leguminosarumbv. viciae* is trimmed.

It was previously suggested that nif gene numbers might vary according to the physiology of rhizobacteria(Rubio and Ludden). Alternatively, unidentified proteins might replace the missing nif products. Rhizobialnif genes are regulated by the NifA protein. The synthesis and tran_scriptional activity of NifA in rhizobia are restricted by oxygen due to the extended cysteine-rich domain, but the transcriptional regulation varies in different rhizobia. The nifA expression of A. caulinodans, B. japonicum, and S. meliloti is carried out by FixLJ contrasting with *R. leguminosarumbv. viciae* that lacks the fixLJ(Gray *et al.* 1996). In this two-component regulatory system, FixL is the O_2-binding heme-based sensor. Although rhizobia exhibit plasticity for the nif gene composition and regulation, N_2 fixation occurs under apparent physiological condition (Rubio and Ludden).

2.4.3 *Azotobacter*

Among biofertilizers, *Azotobacter* traces play a key position in the nitrogen cycle in nature that binds atmospheric nitrogen inaccessible to flowers and liberates it in the shape of ammonium ions to be had to flowers in the soil solving a median 20kgN/ha consistent with year. It is capable of developing at a pH variety of 4.8–8.5 and fixes N at the surest pH of 7.0–7.5 (Dilworth, Eadyt and Eldridge 1988). *Azotobacter* can repair at the least 10μg of nitrogen consistent with a gram of glucose consumed. Inoculation impact of free-residing *Azotobacter* species are in large part related to nitrogen fixation (Bulletins) 1981), formation of diverse physiologically lively increase hormones like gibberellin, auxin and cytokinin(Kathiresan and Masilamani 2006), ammonia, nutrients and increased

materials accountable for seed germination (Moreno, Gonzalez-lopez and Vela 1986; Kumar, Narula and Merbach 2015), safety in opposition to root pathogens (Sindhu, Rakshiya and Sahu 2009; Kumar, Narula and Merbach 2015), stimulation of useful rhizospheric microorganisms and enhancement of plant yield (Wu *et al.* 2009).

However, the precise mode of motion with the aid of using which *Azotobacter* complements the increase in the plant isn't always absolutely understood. The abundance of *Azotobacter* in soil relies upon many elements including soil physicochemical (e.g. natural matter, pH, temperature, soil moisture) and microbiological properties. However, the abundance varies as consistent with the intensity of the soil profile (Uma Maheswari and Kalaiyarasi 2015).

2.4.4 Non-symbiotic nitrogen fixation

The N-fixing enzyme, nitrogenase, convert atmospheric nitrogen (N_2) to ammonia (NH_3), which is then aminated into glutamine or other amino acid. The enzyme is very sensitive to oxygen. This reduction reaction is endothermic, with an energy cost of about 35 kJ mol^{-1} for every N fixed, while the actual cost might be about 15–30 g of carbohydrates for every gram of ammonia-N (George and Marschner 1995; Gutschick 2014). Nitrogenase activity can be measured by an acetylene reduction assay, which is based on the observation that the enzyme also converts acetylene to ethylene. The majority of non-symbiotic N fixation studies used the acetylene reduction method with some modifications (Silvester *et al.* 1988). Biological N fixation is, to some extent, a self-regulated process and is induced or inhibited by changes in the levels of inorganic N (Roper and Gupta 2016).

Nitrogen fixation involves enzymes that are highly sensitive to oxygen, particularly Fe-protein components. Generally, it is stimulated by reduced oxygen levels and low redox potentials (Bulletins) 1981). Soil moisture levels are linked to N fixation through its influence on oxygen supply and the transportation of mineral nutrients. Most N-fixing microorganisms are mesophilic in temperature, and N fixation is often more affected by temperature than general growth and

photosynthesis. Most N-fixing ecosystems have a soil pH optimum close to 7, but generally show a broad range around this value. It has been demonstrated frequently that various mineral nutrients such as P, Mo, Fe, Co, Mg, P, W and Ca are essential for N fixation. Non-symbiotic N-fixing organisms are directly dependent on light quality and intensity for nitrogenase activity. Heterotrophic N-fixing organisms can generally utilize several carbohydrates, alcohols, organic acids or aromatic compounds as energy sources. As N fixation requires electrons and energy, the efficiency of the process depends on the pathway through which the organisms metabolize the available substrates (Bulletins) 1981). Figure 2 shows the function of plant growth promoting bacteria (Santos, Nogueira and Hungria 2019)

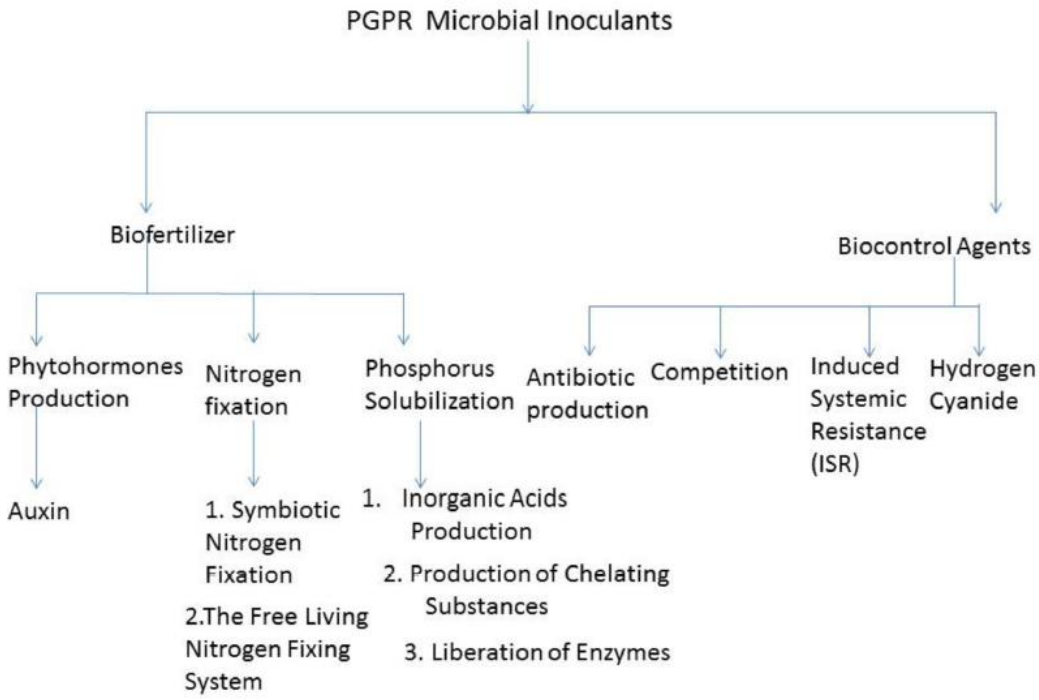


Figure 2: Flow chart showing the function of plant growth-promoting bacteria

2.5 Carrier materials

Biofertilizers are usually manufactured as carrier-based inoculants containing effective microorganisms. Incorporation of microorganisms into carrier materials facilitates handling, enables long-term storage, and provides high effectiveness of organic fertilizers. Among the different types of biofertilizers, bacterial inoculants

represent a major group, including rhizobia, nitrogen-fixing rhizobia, plant growth-promoting rhizobia, phosphate-soluble bacteria, etc. (Fnca *et al.* 2006).

2.5.1 Types of carrier material

- **Farmyard manure**: farmyard manure refers to the decomposed mixture of dung and urine of farm animals along with litter and leftover material from roughages or fodder fed to the cattle. On average well-decomposed farmyard manure contains 0.5 percent N, 0.2 percent $P_2O_5$and .0.5 percent K_2O. The present method of preparing farmyard manure by the farmers is defective. Urine, which is wasted, contains one percent nitrogen and 1.35 percent potassium. Nitrogen present in urine is mostly in the form of urea which is subjected to volatilization losses. Even during storage, nutrients are lost due to leaching and volatilization. However, it is practically impossible to avoid losses altogether, but can be reduced by following improved methods of preparation of farmyard manure. Trenches of size 6 m to 7.5 m length, 1.5 m to 2.0 m width and 1.0 m deep are dug (Javaid 2011).
- **Rice husk**: Rice husk constitutes around 20% of the paddy grain. According to Food and Agriculture Organization of the United Nations (FAO) data, the majority of current world production rates 410 million tons of rice (Javaid 2011), is geographically located in developing countries. Even though numerous research findings about the utilization of this waste as fuel (rice husk) have been published, these wastes can be seen in heaps of various sizes around processing facilities, where they not only constitute a nuisance and health risks but may also occupy and put large areas of land out of use heaps. Heaps of rice husk behind mill at Abakaliki, Nigeria Being very abundant and practically of no economic value in many developing countries, rice husk already meet two important requirements of carrier materials (Taylor, Mansaray and Ghaly 2007).
- **Charcoal**: Charcoal is a lightweight black carbon residue produced by strongly heating wood in minimal oxygen to remove all water and volatile

constituents. In the traditional version of this pyrolysis process, called charcoal burning, often by forming a charcoal kiln, the heat is supplied by burning part of the starting material itself, with a limited supply of oxygen. The material can also be heated in a closed retort. Modern "charcoal" briquettes used for outdoor cooking may contain many other additives, e.g. coal (K. S. Gomare, M. Mese and Y. Shetkar 2011).

This process happens naturally when combustion is incomplete and is sometimes used in radiocarbon dating. It also happens inadvertently while burning wood, as in a fireplace or wood stove. The visible flame in these is due to the combustion of the volatile gases exuded as the wood turns into charcoal. The soot and smoke commonly given off by wood fire results from incomplete combustion of that volatiles. Charcoal burns at a higher temperature than wood, with hardly a visible flame, and releases almost nothing except heat and carbon dioxide (Gaind and Gaur 1990).

Regarding the quality of charcoal, better chemical properties of charcoal are reached with higher levels of fixed carbon and lower levels of ash and volatiles. It is associated with high levels of lignin and low levels of holocelluloses and extractives in wood (Gaind and Gaur 1990).

Various types of material are used as a carrier for seed or soil inoculation. For the preparation of seed inoculant, the carrier material is milled to a fine powder with a particle size of 10 -40 μm. According to the (Hoben 2016), the properties of good carrier material for seed inoculation are (1) non-toxic to inoculant bacterial strain, (2) good moisture absorption capacity, (3) easy to process and free of lump-forming materials, (4) easy to sterilize by autoclaving or gamma-irradiation, (5) available in adequate amounts, (6) inexpensive, (7) good adhesion to seeds, and (8) good pH buffering capacity. Needless to say, (9) non-toxic to plant, is another important property (Fnca *et al.* 2006).

The survival ability of resident microbes depends upon: (1) variations in cell physiology, such as membrane composition; (2) survival mechanisms, such as the capacity to form spores or cysts; and (3) environmental variables, such as

temperature and the nature of the substrate used. Post-irradiation conditioning of the surviving microorganisms to environmental conditions has also been reported to affect survival numbers (Ben Rebah *et al.* 2007).

2.6 Biofertilizer formation

The production of biofertilizers consists of very important steps for success, including several things such as microbial growth profile, species and optimal condition, prescription of inoculum, application method, storage method, etc. We need to consider product. Selection of active microorganisms, separation and selection of target microorganisms, selection of growth and support substances, phenotypic and large-scale testing are six important steps in biofertilizer production (Ju *et al.* 2018).

Therhizobial inoculum material can be manufactured and used in a variety of ways. Inoculum materials can be prepared as powder, liquid, and granular formulations. Granular formulations are useful because they allow for placement and control of coverage (Stephens and Rask 2000). Another important feature of the N inocurum is the choice of support (peat, pearlite, mineral earth, charcoal, etc.). The presence of a carrier requires its sterility to maximize the survival of the inoculum and subsequent infection rates (Stephens and Rask 2000; Catroux, Hartmann and Revellin 2001).

2.7 Industrialization of biofertilizer in around the world

The connection between research and industry allows not only the inoculum production for field trials but also testing of the industrial scale inoculum production for direct marketing (Stephens and Rask 2000).

Different types of biofertilizers provide optimal nutrients for crops, minimize environmental damage and improve soil biodiversity. An overall increase in demand for fertilizers to produce more food on limited arable land, plus depletion of raw materials/fossil fuels (energy crisis), rising cost of chemical fertilizers and declining soil fertility, their consumption is expected to increase in the future on environmental hazards and growing threats to sustainable agriculture. The

biofertilizer market share is projected to reach USD 1.66 billion by 2022, further strengthening the annual growth rate of 13.2% from 2015 to 2022 (Timmusk *et al.* 2017). History of industrialization in biofertilizers is shown in fig 3 (Banjara 2019).

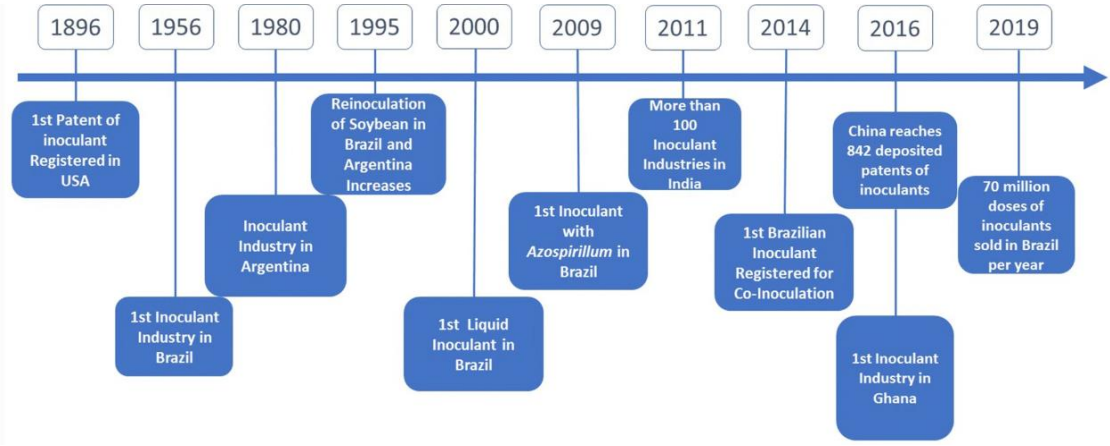


Figure 3: History of industrialization in biofertilizers.

2.8 Current aspect of biofertilizer (perspective for the future)

China currently leads the number of inoculant-related patents registered, with over 800, and India already has over 100 inoculant industries. These numbers are expected to increase in other countries as well (Gadal *et al.* 2019)

Challenges to developing new inoculant arise from growing concerns about climate change. Rising temperatures and dry periods expected over the next few years will have a major impact on agriculture. According to García-Fraile et al. (2012) in tropical regions, maize and rice yields can decline by 5–10% and 2–5%, respectively, for each degree increase in temperature. Climate change will reduce the available planting area. Therefore, it is imperative to look for more effective microbial inoculants under stressful conditions. On the other hand, microbial inoculant can also help mitigate the effects of other related abiotic pressures such as climate change and salinity (Cerezini et al. 2016; Fukami, Cerezini and Hungria 2018). The use of microbial inoculants is expected to increase dramatically in the coming years due to increased availability of quality products

and government commitments to more sustainable agricultural systems (Santos, Nogueira and Hungria 2019).

2.9 Benefits of the use of biofertilizer

They are also known to produce various plant-growth-promoting hormones like indole acetic acid, gibberellic acid, cytokinins and ethylene (Arshadl 1998). PGPM also indirectly reduces the deleterious effect of phytopathogens. The modes of action of PGPM though not completely explored, the possible reasons could be: 1. Production of plant growth regulators, 2. Symbiotic and asymbiotic N_2 fixation (Dent and Cocking 2017), 3. Antagonistic activity to phytopathogens by the production of siderophore, antibiotics (Shanahan *et al.* 1992) and HCN (Gray *et al.* 1996), 4. Solubilization of mineral phosphates and other nutrients (Han, Aidi and Ani 2007), 5.Substrate competition, 6. Chitinase production, 7.Cellulase, pectinase, protease & starch hydrolysis and 8.Sclerotial and mycoparasitization. In addition to these traits, an effective PGPM should be a rhizospheric competent, able to cope with the biotic and abiotic stresses and colonize in the rhizosphere(Cattelan, Hartel and Fuhrmann 1996).

Biofertilizers are often linked with disease control and plant health in agriculture. Biofertilizers can act through various methods in plant health improvement. Antibiotics production is the main mechanism of plant Growth promoting bacteria (PGPB) to counteract the damaging effect of phytopathogens. *Pseudomonads* produce a variety of compounds such as 2, 4-diacetylphloroglucinol (DAPG), amphisim, hydrogen cynide, phenazine, oomycin A, tropolone, pyoluterin, tensin, pyrrolnitrin, and cyclic lipoprotein. Streptomyces, bacillus, and strophomonas produce kanosamine, oligomycin A, Xanthobaccin, Zwitttermicin etc. These antibiotics have been identified to have antibacterial, antiviral, antifungal, antihelminthic, antimicrobial, cytotoxic, phytotoxic, antioxidant and antitumor properties (K.C. Kirankumar and Rudresh 2017).

CHAPTER-III

MATERIALS AND METHODS

3.1 Materials

The materials required for this work are listed in Appendix I.

3.2 Methods

3.2.1 Place of study

The study was conducted in the microbiology and molecular biology laboratory of Central Campus of Technology, Dharan.

3.2.2 Study period

The study was performed from 2076 magh to 2077 chaitra.

3.2.3 Sample size and types

This research is qualitative. 28 pea (*pisummsativum*) plants for *Rhizobium* species and 20 soil samples for *Azotobacter* species were collected.

3.2.4 Sample collection and transportation

Pea plants were collected from the agricultural land of Ramdhuni municipality of Sunsari district. Pea plants were uprooted with soil kept in a normal plastic bag and transported to the lab. Soil samples were collected in a sterile plastic bottle from Dharan Sunsari and transported to the lab.

3.3 Isolation of microorganism

3.3.1 *Rhizobium*

Pea plants were uprooted carefully to get nodules that are attached in the roots. These were brought to the laboratory within same day. Healthy pea nodules were detached from the root and further isolation of root nodulating rhizobia was carried out. The detached root nodules were washed in tap water to remove the adhering soil particles from the nodule surface. Nodules were dipped in 0.1% mercuric chloride ($HgCl_2$) solution for 30 sec and later washed successively ten

times with sterilized distilled water to remove the traces of $HgCl_2$. Surface sterilized nodules were transferred in the test tube containing 5 ml sterilized distilled water. These nodules were crushed with the help of a sterilized glass rod to obtain a milky suspension of bacteriods. These were streaked on YEMA containing congo red. *Rhizobium* colonies were remained white, translucent, elevated and mucilaginous, after 72 h, whereas contaminations turned red. The colony was picked up and transferred to the YEMA slant for further characterization (Ngakou *et al.* 2010).

3.3.2 *Azotobacter*

Isolation, identification and characterization of *Azotobacter* were done according to Bergey's manual of Bacteriology. For the isolation of *Azotobacter* spp. 1g of soil sample was subjected to serial dilution up to 10^{-6} dilution in sterile water. Nitrogen-free media (Ashby's and Jensen's media) were prepared, autoclaved at 121ºC for 15 minutes for sterilization and then poured in sterile plates. 0.1ml suspension from dilution 10^{-3}, 10^{-5} and 10^{-6} were spread on Ashby's and Jensen's media plates uniformly with the help of a sterile dolly rod. Then, the inoculated plates were incubated at 32ºC for 5 days. After incubation, the plates were observed and colonial characteristics like color, shape, margin, opacity, elevation, consistency etc. were noted. Creamy and dark brown colored colonies from Ashby's media and water droplet type of colonies from Jensen's media were selected and sub-cultured on Nutrient Agar (NA) (Roychowdury Debojyoti. Manibrata Paul & Sudip Kumar Banerjee 2017).

3.4 Identification of microorganism

3.4.1 *Rhizobium* spp.

Biochemical tests such as growth on glucose peptone agar(Okon, Eshel and Henis 1972), growth in presence of 8% KNO_3, hydrolysis of urea, growth on 1, 2% NaCl(Sadowsky, Keyser and Bohlool 1983), Gram stanning, gelatinase activity, H_2S (Lead *et al.* 1934), acid production in YEM broth (Jordan and Ogren 1984), catalase activity (P.H. Graham and C.A. Parkes (Springer) 1964), acid reaction in litmus milk (Jordan and Ogren 1984), precipitation in calcium glycerol phosphate

(Hofer 1939), starch hydrolysis, utilization of different carbon source was done. The organism was identified by using standard microbiological techniques as described in Bergey's Manual of Systematic Bacteriology-1986.

3.4.2 *Azotobacter*

After obtaining the pure culture, the organism was identified by using standard microbiological techniques as described in Bergey's Manual of Systematic Bacteriology-1986. For the identification of *Azotobacter* from pure culture, colonies from NA plates were taken and Gram stained. The colonies having rod-shaped bacteria were again sub-cultured in NA. Then further identification was done by performing biochemical tests like motility test, catalase test, starch hydrolysis test, citrate utilization test, urease test, indole test and MR-VP test. Carbohydrate fermentation (glucose, sucrose and fructose) tests were performed for further confirmation of *Azotobacter*.

To perform the biochemical test; biochemical media were prepared in test tubes, autoclaved and slants or broth were made. Colonies to be tested were stabbed or inoculated with the help of a sterile inoculating needle/loop and incubated at 32ºC for 24 hours. After incubation, the result was noted by observing the change in color (without or after adding reagents).

3.5 Obtaining pure culture

After incubation, the single colonies from each NA plate with different colony morphology were taken and streaked on NA plates by quadrant streaking method and again incubated at 30^0C for 48 hours. The single colonies then obtained were used as a pure culture of colonies for further use.

3.6 Preparation of carrier material

The inert solid carriers used in the formulations are rice husk, farm yard manure and charcoal. The carrier materials were collected from different sources and also their physicochemical properties were tested and the details are mentioned in the

table. Carriers were sun-dried for 7 days. They were grinded to form small powered. Carrier was sieved and autoclaved at 121^0 C at 15 lbps for 20min.

3.6.1Determination of physicochemical properties of carrier material

Moisture content:

100 g of soil sample was taken in a pre-weighted petridish and placed in an oven at 70℃ for 24 hours. Then the petridish with dried soil samples were weighted. The soil moisture content was calculated by using the formula,

$$\text{Moisture content (\%)} = \frac{weight\ lost}{dry\ wight\ of\ sample} \times 100$$

pH:

20 gm of soil sample was taken in a beaker. 100 ml of distilled water was added to it and stand for 30 minutes. Then the pH of the solution was noted using calibrated electronic pH meter.

3.7 Inoculum build-up

A loopful of *Azotobacter* spp and *Rhizobium* spp was transferred into 250ml Erlenmeyer flask containing 100ml of Ashby's broth and YEM broth separately and incubated at 28^oC and 30^0C on 120 rpm rotary shaker for 72 hours respectively. After incubation, 10ml of the inoculum was transferred to 1000ml of respective broth and kept in a shaking incubator for mass multiplication (Open and Publisher 2014).

3.8 Packaging

750 milliliters of broth culture was mixed thoroughly with 1000 g of each sterile carrier, adjusted the moisture content to 75 % water holding capacity, hand packaging in sterile polyethylene bags under sterile condition and sealed in zip lock plastic (Phiromtan, Mala and Srinives 2013).

3.9 Storage

Packed inoculum polyethyelene bags were stored at room temperature and in freeze.*Azotobactor* species was stored for 7weeks and *Rhizobium* species was stored for 11 weeks.

3.10 Total plate count

1 gram of inoculants was weighed from packed plastic bag for each carrier material in aseptic environment. It was then transferred to test tube with sterile 10ml water and serial dilution was performed. Total plate count was carried out by the pour plate technique. Plate Count Agar (PCA) media was used. After performing serial dilution, 1 ml aliquot from each dilution was added to sterile petri plates in which molten and cooled (40-45ºC) PCA was poured. The plates were gently rotated for uniform distribution throughout the medium. The plates were allowed to solidify and then incubated at 32ºC for 24-48 hours (Aneja KR., 2003). The plates were screened for the presence of discreet colonies after 24 hours and the actual number of bacteria was estimated as colony forming unit per ml (CFU/ml).

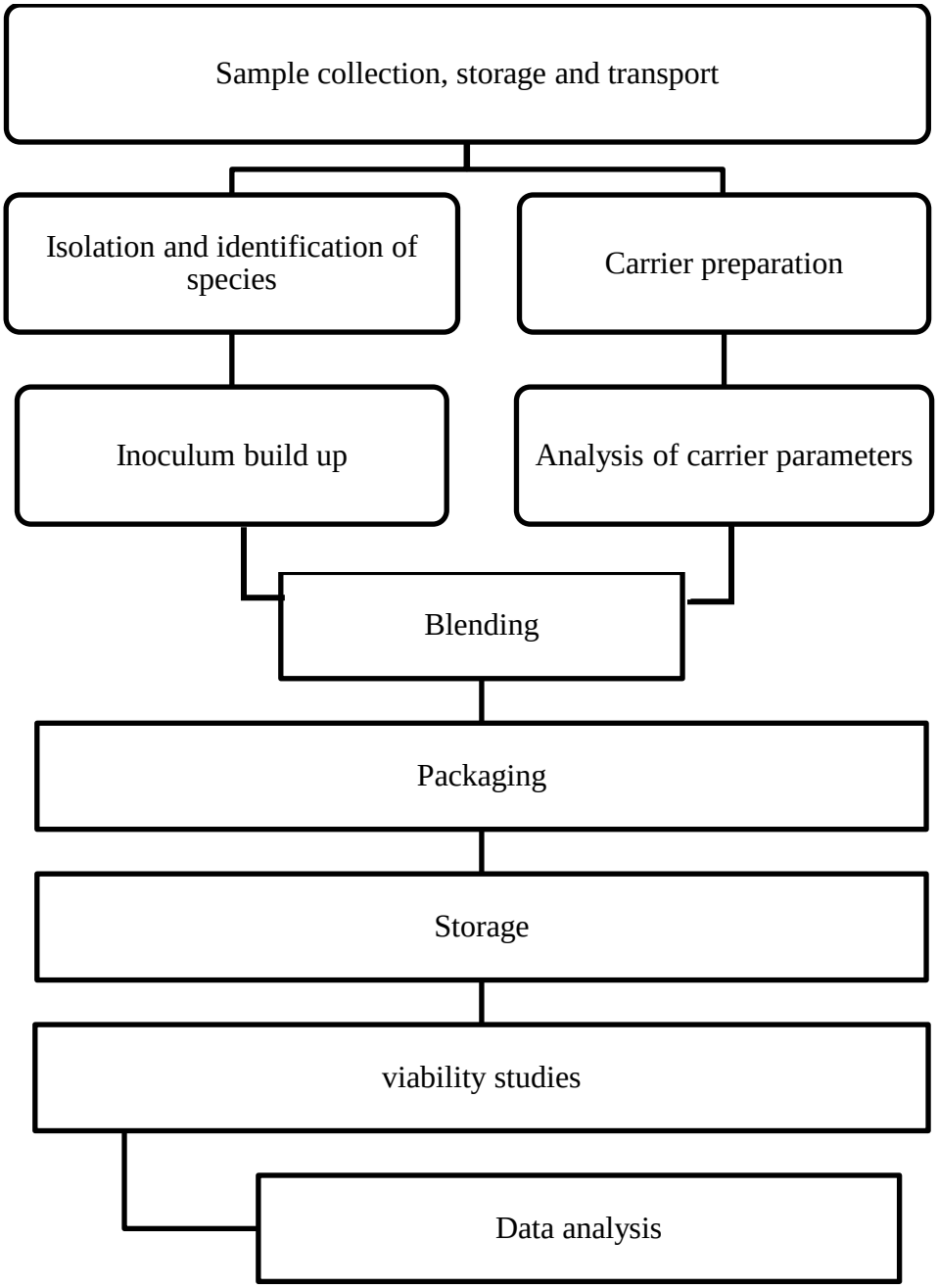
$$\text{CFU/gm} = \frac{number\ of\ colony \times dilution\ factor}{volume\ of\ sample}$$

3.11 Survival rate of bacteria

Survival rate was calculated using Microsoft excel.

$$\text{Survival rate} = \frac{cfu\ at\ the\ end\ of incubaton\ week \times 100}{cfu\ in\ week\ 0}$$

3.12 Research design



CHAPTER-IV

RESULTS

4.1 Distribution of *Rhizobium* spp in pea plant and *Azotobacter* spp in soil sample

Root nodule of pea plant, soil and three carrier materials were taken as sample in this study. Isolation of *Rhizobium* species was done from pea plant root and *Azotobacter* was isolated from soil sample. These microorganisms were than mixed with carrier material whose physical properties were checked. Prepared inoculants were kept in two different temperature and pH, moisture and shelf life of microorganism was analyzed for comparative study of used carrier material.

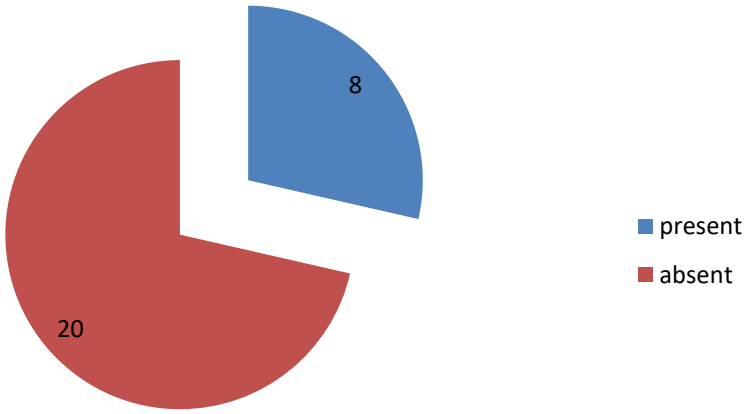


Figure 4: Presence of Rhizobium spp in the total sample.

Among 28 pea plants collected from the agricultural land of Ramdhuni. *Rhizobium* spp were present in only 8 root nodules and Rhizobium spp was absent in 20 root nodules.

Among the 20 soil samples, *Azotobacter* spp was isolated from 16 soil samples. *Azotobacter* spp was absent in 4 soil samples.

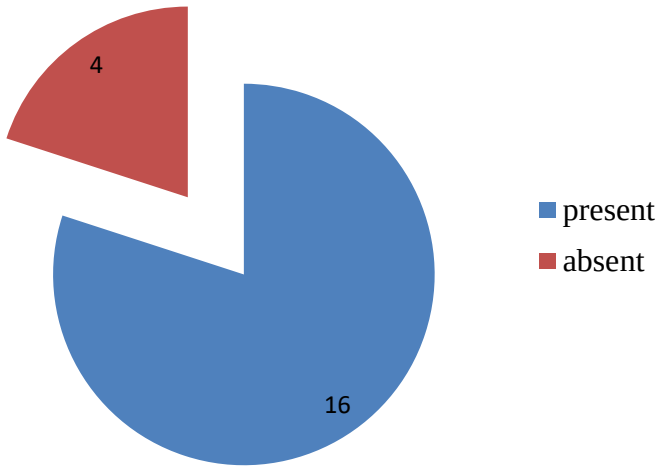


Figure 5: Presence of *Azotobacter spp* in the total soil sample.

4.2Isolation and identification

4.2.1*Rhizobium*

Several colonies were observed in YEMA containing congo red among them white, translucent colonies were sub cultured in NA plates until pure colonies were obtained. Biochemical test was performed for suspected colonies and bacteria were selected based on the following biochemical result.

Table 2: Biochemical test of *Rhizobium* species

Absorb congo red in YEMA	**Growth in GPA**	**Gram reaction**	**Motility test**	**Growth in 2% Nacl**	**H_2s production**	**Catalase**	**Starch hydrolysis**
Negative	Negative	Negative	Positive	Negative	Negative	Positive	Negative

4.2.2*Azotobacter*

Colonies were observed in Ashyb's media among them brown, viscous, medium sized circular colonies with oval rod in shape were sub cultured in NA plates until pure colonies were obtained. Biochemical test was performed for suspected colonies and bacteria were selected based on the following biochemical result.

Table 3: Biochemical test of *Azotobacter* species.

Gram's reaction	**Motility**	**Catalase**	**Starch hydrolysis**	**MR**	**VP**	**Indole**	**Citrate**
Negative	Positive	Positive	Positive	Positive	Negative	Negative	Negative

Table 4: Utilization of carbon source by bacteria

Carbohydrate	*Rhizobium spp*	*Azotobacterspp*
Fructose	Positive	NA
Mannitol	Positive	Positive
Sucrose	Positive	NA
Maltose	Positive	Positive
Glucose	Positive	Positive

Rhizobium spp used five carbohydrates as sugar source and *Azotobacter* spp used only three carbohydrates that are mannitol, maltose and glucose. Fructose and sucrose was not used by *Azotobacter* species.

Table 5: Physical and chemical properties of the carrier

Carrier	pH	Water holding capacity(water observed in ml) in 10gm carrier.	Particle size	Color	Source
Rice husk	8.2	9.5	≤0.63mm	Light brown	Rice mill Jhumka
Charcoal	7.5	16	≤0.63mm	Black	Dharan
Farm yard manure	6.3	5.25	≤0.63mm	Dark brown	Jhumka

Physical properties were measured rice husk showed basic but charcoal and farmyard manure showed acidic in pH. Water holding capacity was high for charcoal and medium for rice husk and farmyard manure. They were passed from o.63mm sieve.

4.3 Effects of temperature on moisture content, pH and biomass during incubation with rice husk, charcoal and farmyard manure in Azotobactor species.

Moisture is decreased slightly from 52% to 42% and 26.5% in refrigerator and room temperature respectively. Room temperature moisture is less than refrigerator and there is high difference in first week in both temperatures.

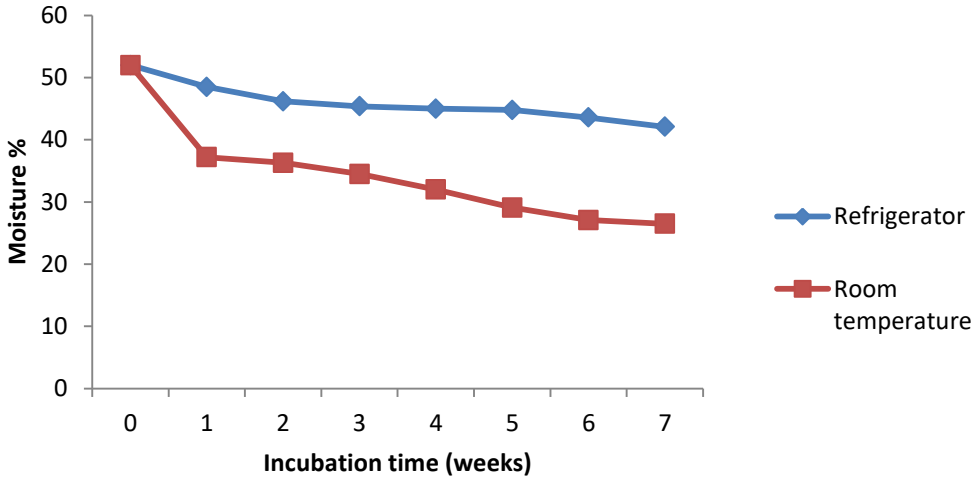


Figure 6: Moisture content of *Azotobacter* species blended in rice husk at different temperature.

pH in both temperature is decreased as the incubation time is increased. It is decreased from 8 to 6 in both temperatures making blending material acidic. Room temperature pH is less than refrigerator at the end of 7th week.

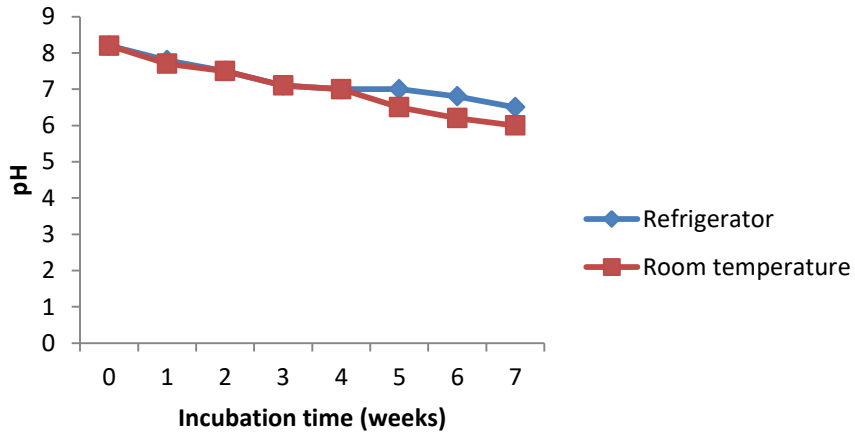


Figure 7: pH of *Azotobacter* species blended with rice husk at different temperature.

The population of *Azotobactor* is increased in first week of incubation but then decreased gradually decreased with increase in incubation period at both temperatures. At room temperature population is increased by double then initial phase and in refrigerator it is increased from 8^7 to 10^7. At the 7^{th} week room temperature population is less then refrigerator temperature.

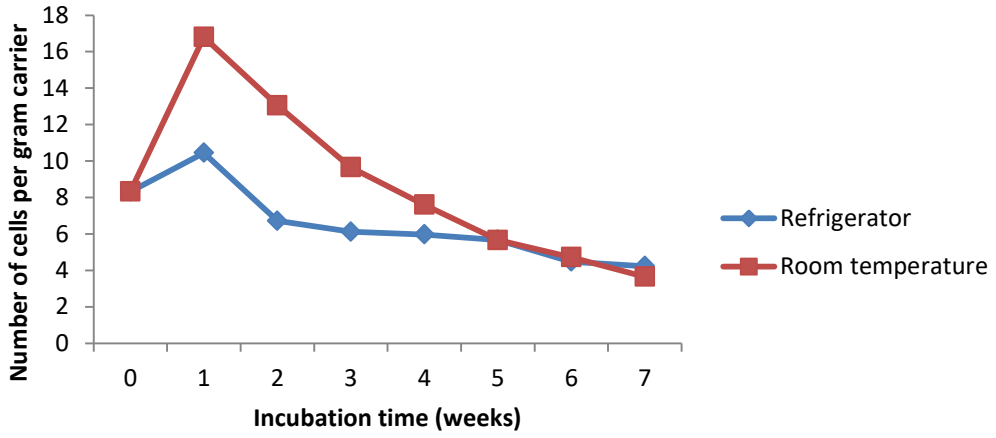


Figure 8: Biomass of *Azotobacter* species blended with rice husk at different temperature.

Moisture decreased with increase in incubation time in both temperatures. Inoculants stored in room temperature lost more moisture reaching nearly 30% in 7^{th} weeks whereas refrigerator moisture was more than 40%.

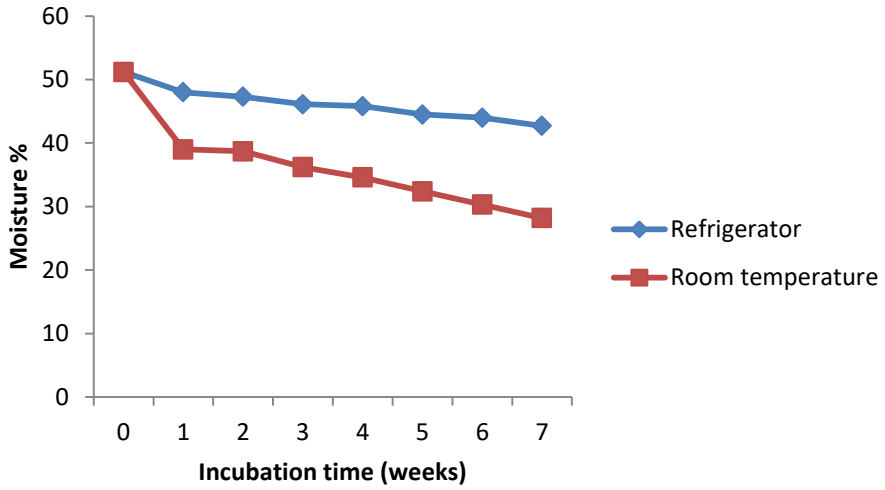


Figure 9: Moisture content of *Azotobacter* species blended in charcoal at different temperature.

pH of charcoal is same in both the temperature in most of the week observation. Charcoal inoculants is turned acidic as the pH decreased with the increased in incubation time.

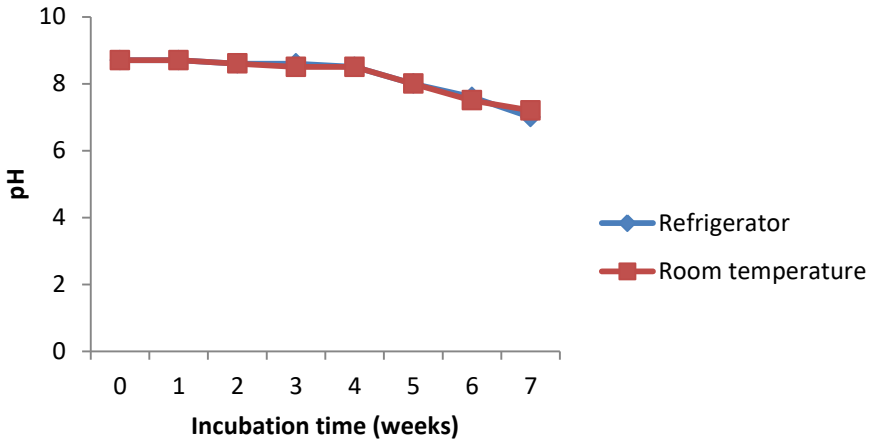


Figure 10: pH of *Azotobacter* species blended with charcoal at different temperature.

Population of inoculants stored in both temperature increased in 1st week and gradually decreased. Room temperature inoculants population increased more than refrigerator inoculant in first week. At 7th week population of inoculants stored in room temperature was less than refrigerator.

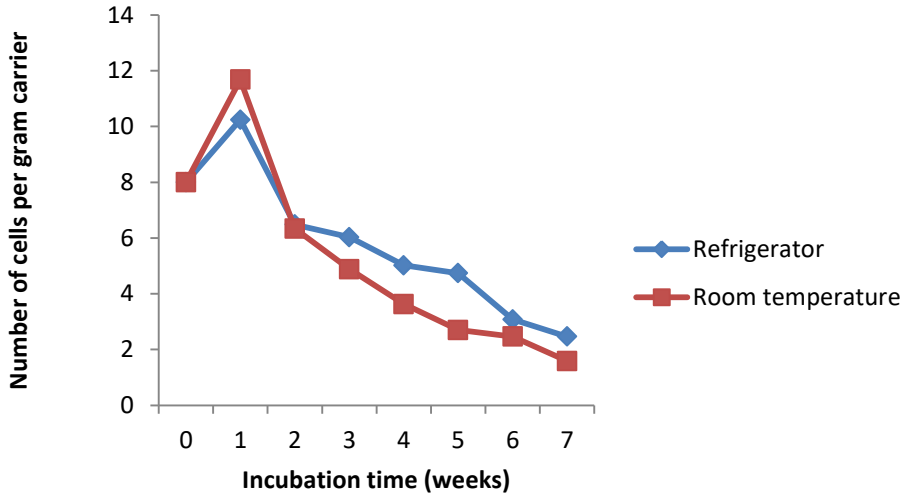


Figure 11: Biomass of *Azotobacter* species blended with charcoal at different temperature.

Moisture is decreased from 1st week of incubation. Inoculant stored in room temperature has lost more moisture than stored in refrigerator. At 7th week moisture was nearly 30% and more than 40% in room temperature and refrigerator respectively.

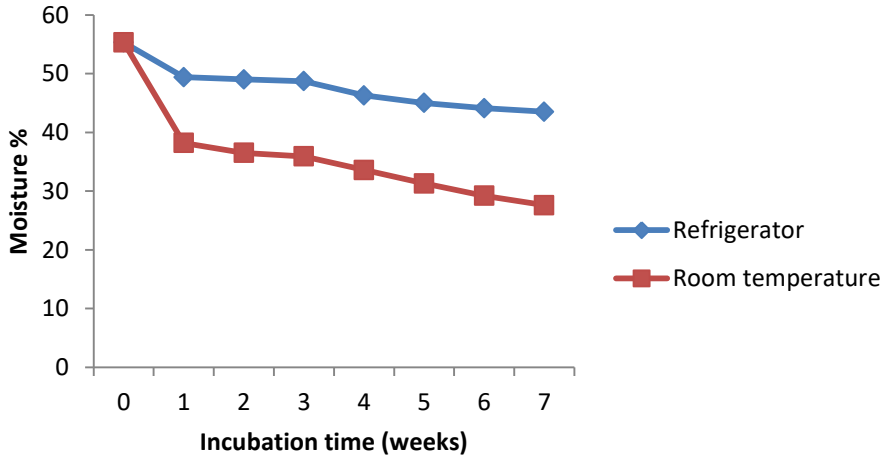


Figure 12: Moisture content of *Rhizobium* species blended in farmyard manure at different temperature.

pH decreased from 1st week in inoculants stored in both temperature but in 5th week pH of inoculants increased and then decreased. Inoculants stored in room temperature was acidic than that of refrigerator.

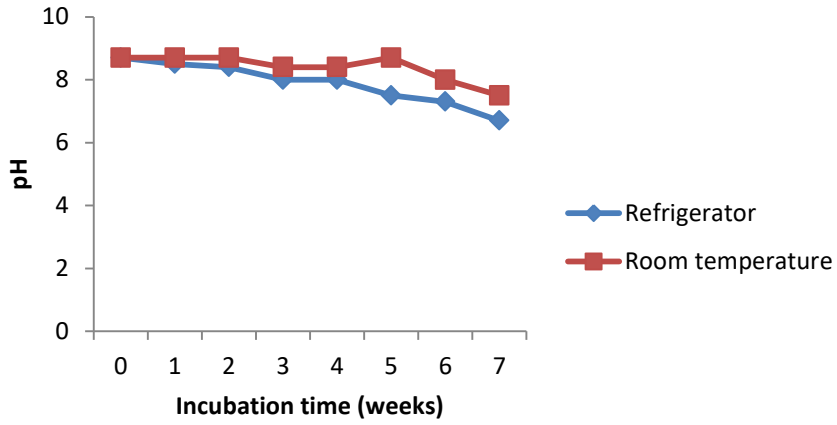


Figure 13: pH of *Azotobacter* species blended with farmyard manure at different temperature.

Population of *Azotobacter* species blended with farmyard manure increased in first week and gradually decreased. Inoculants stored in refrigerator showed more growth than stored in room temperature in first week but at 7^{th} week the population was nearly equal.

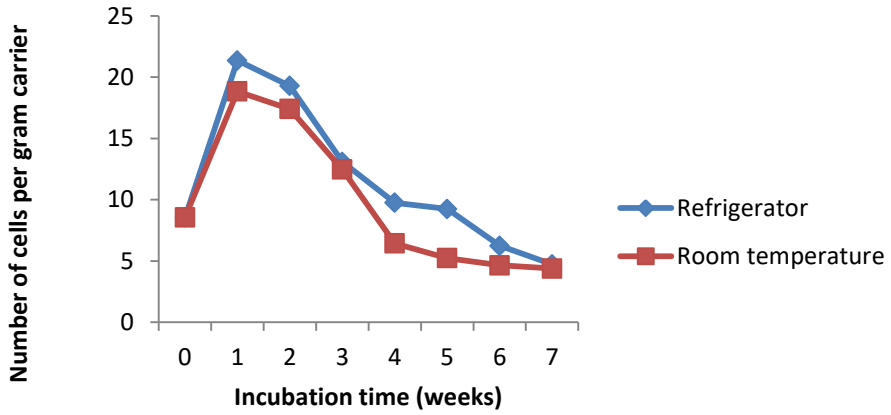


Figure 14: Biomass of *Azotobacter* species blended with farmyard manure at different temperature.

4.4 Effects of temperature on moisture content, pH and biomass during incubation with rice husk, charcoal and farmyard manure in Rhizobium species.

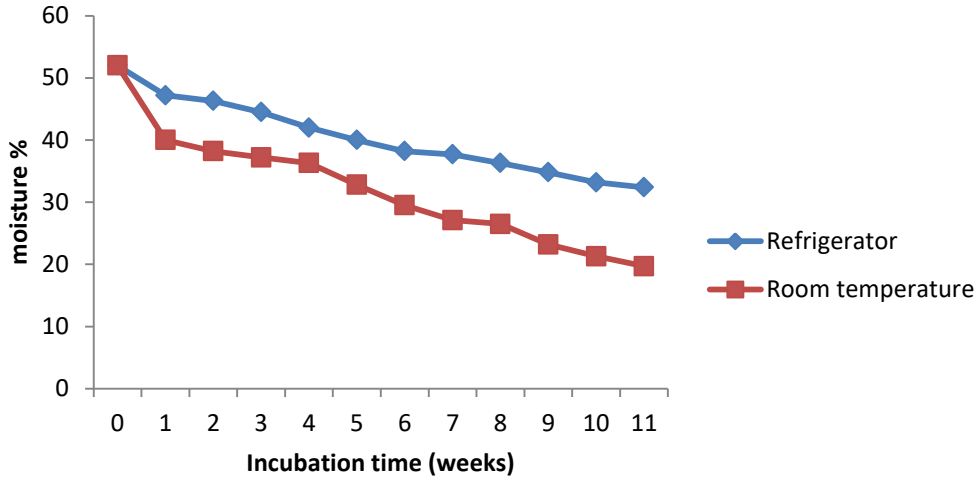


Figure 15: Moisture content of *Rhizobium* species blended in rice husk at different temperature.

Moisture decreased as incubation time is increased in both temperature. Room temperature moisture is less than 20% at 11th week but refrigerators moisture is more than 30% at same time.

pH decreased with increase in incubation days at both temperature. Refrigerator pH is more acidic than room temperature but there is no big difference.

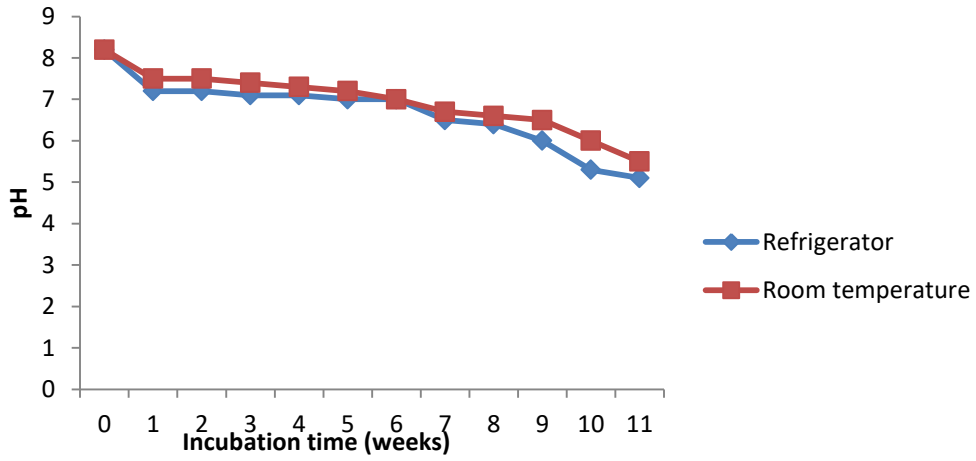


Figure 16: pH of *Rhizobium* species blended with rice husk at different temperature.

Population of *Rhizobium* species increased in 1st week and gradually decreased at both temperatures. Population in room temperature is more than refrigerator. In 1st week population increased more than by double in room temperature.

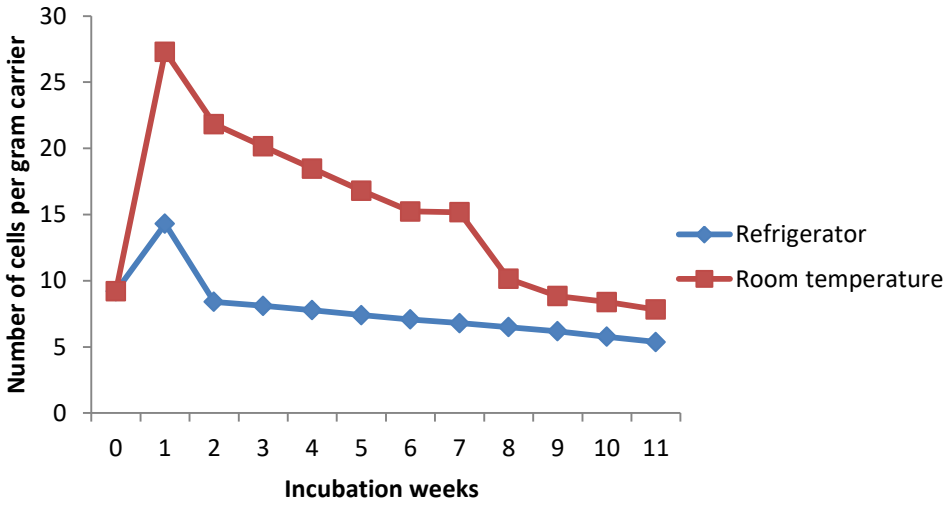


Figure 17: Biomass of *Rhizobium* species blended with rice husk at different temperature.

Moisture decreased from 1st week of incubation and at 11th week reached nearly 20% and 35% in room temperature and refrigerator respectively. Moisture is consistently less in room temperature innoculant then refrigerator.

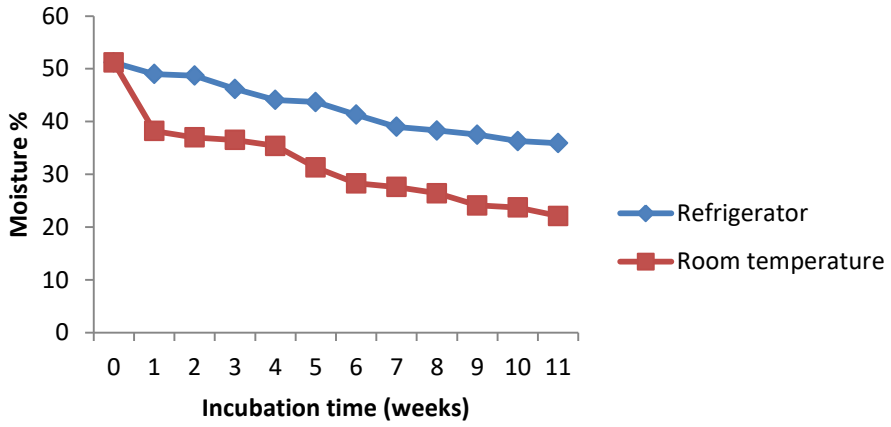


Figure 18: Moisture content of *Rhizobium* species blended in charcoal at different temperature.

pH is nearly equal in *Rhizobium* species when blended with charcoal at both temperatures. pH decreased with increase in incubation period making it more acidic.

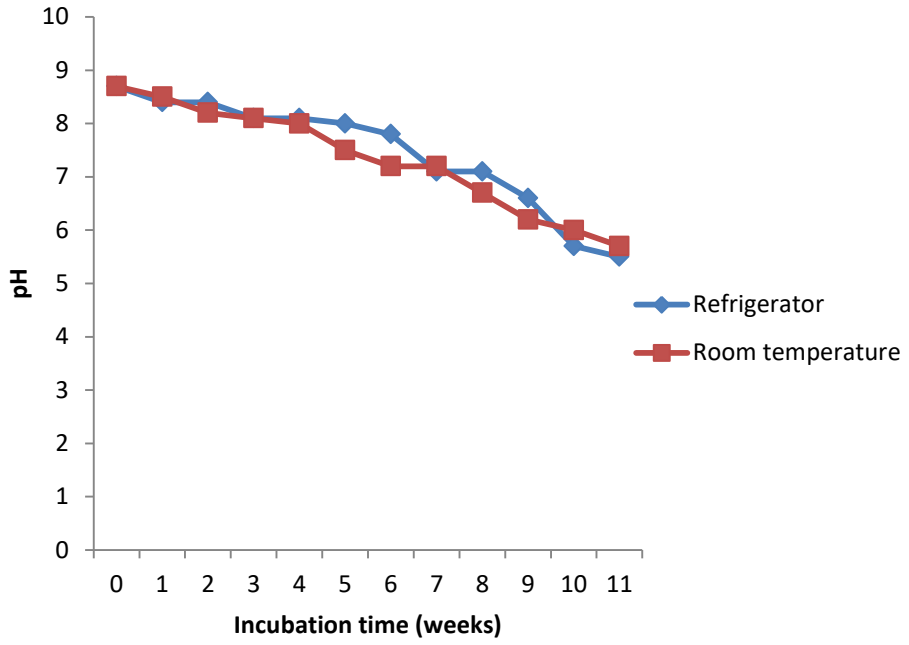


Figure 19: pH of *Rhizobium* species blended with charcoal at different temperature.

Population of *Rhizobium* species in charcoal decreased with longer incubation period. During 1st week biomass increased and increment is high in room temperature and low in refrigerator. At the end of 11th week biomass in room temperature is more than refrigerator.

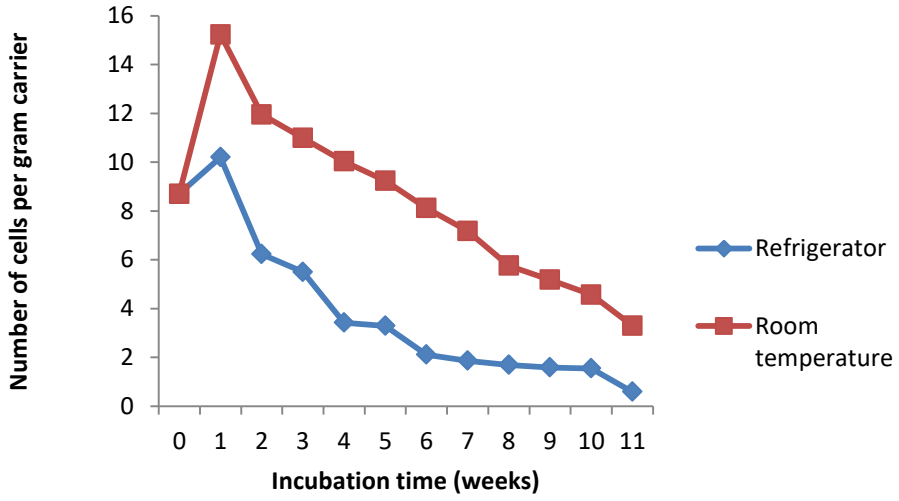


Figure 20: Biomass of *Rhizobium* species blended with charcoal at different temperature.

Moisture in farmyard manure decreased as the incubation time is increased. Moisture of room temperature is less than that of refrigerator. At the end of 11th week moisture at room temperature is 20% and refrigerator is nearly 40%.

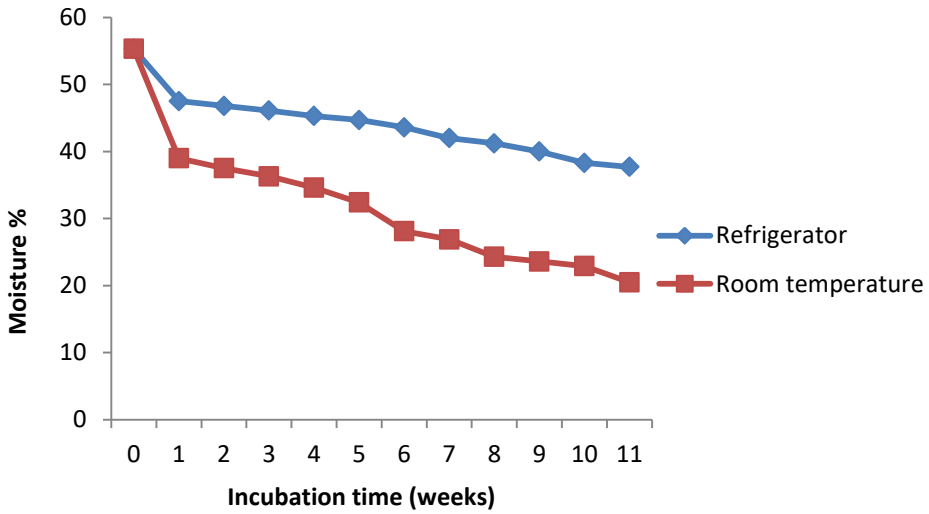


Figure 21: Moisture content of *Rhizobium* species blended in rice husk at different temperature.

There is slight decrease in pH *Rhizobium* blended with farmyard manure. At the end of 11th week pH in refrigerator is less then room temperature with 5 and 6 respectively.

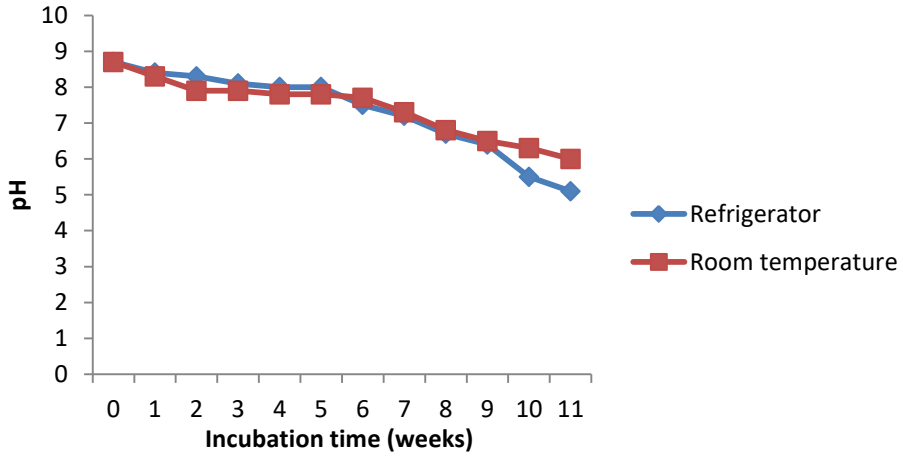


Figure 22: pH of *Rhizobium* species blended with farmyard manure at different temperature.

Population of *Rhizobium* in farm yard manure at both the temperature is nearly same. There is rise in first week of incubation and population is gradually decreased. Biomass is more in farm yard manure kept in refrigerator than kept in room temperature.

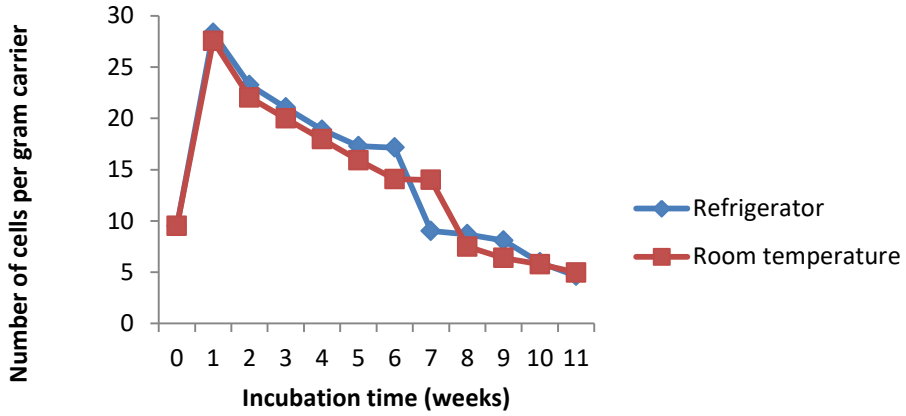


Figure 23: Biomass of *Rhizobium* species blended with farmyard manure at different temperature.

4.5 Survival rate of bacteria blended with different carrier and stored in two different temperature

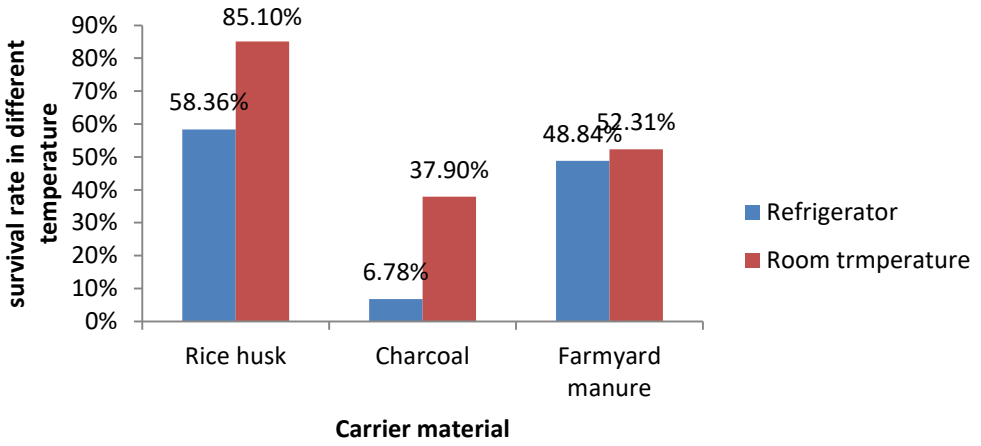


Figure 24: Survival rate of *Rhizobium* species in different carrier

Rice husk has the highest survival rate in both room temperature and refrigerator. The least survival rate is observed in charcoal stored in refrigerator. Farmyard manure has very little difference in survival rate at both temperatures.

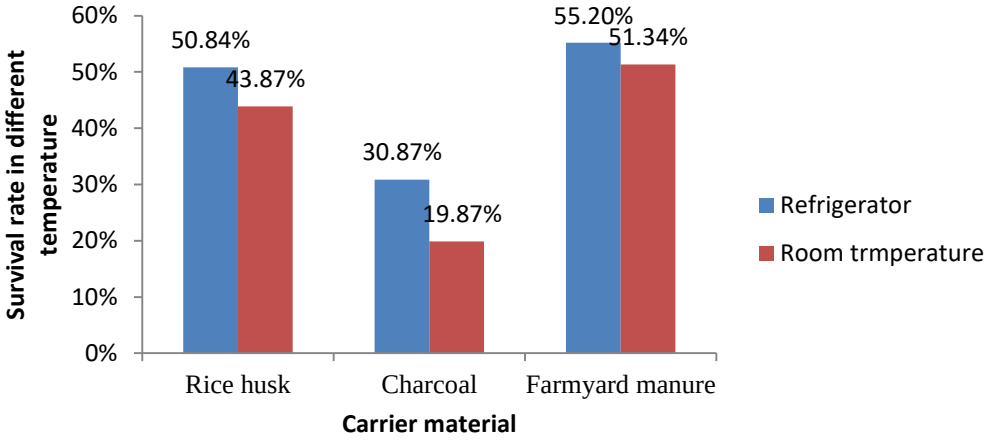


Figure 25: Survival rate of *Azotobacter* species in different carrier material

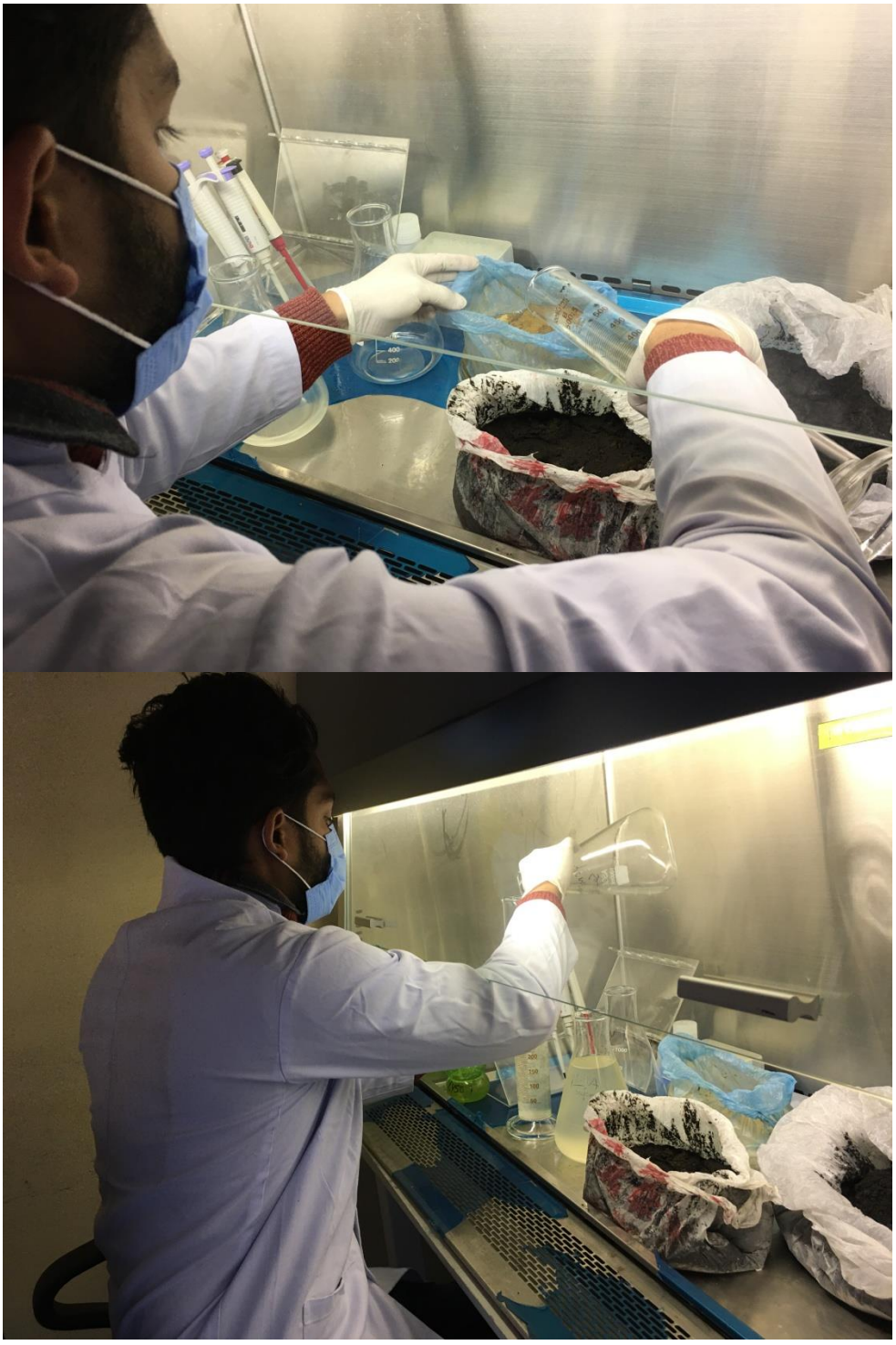
The survival rate of *Azotobacter* species is highest in farmyard manure in both temperatures and lowest is observed in charcoal in both temperatures. Survival

rate in rice husk is nearly equal in both temperatures with less than 5% difference. In the entire carrier material highest survival rate is observed in inoculants stored in refrigerator.

Photographs

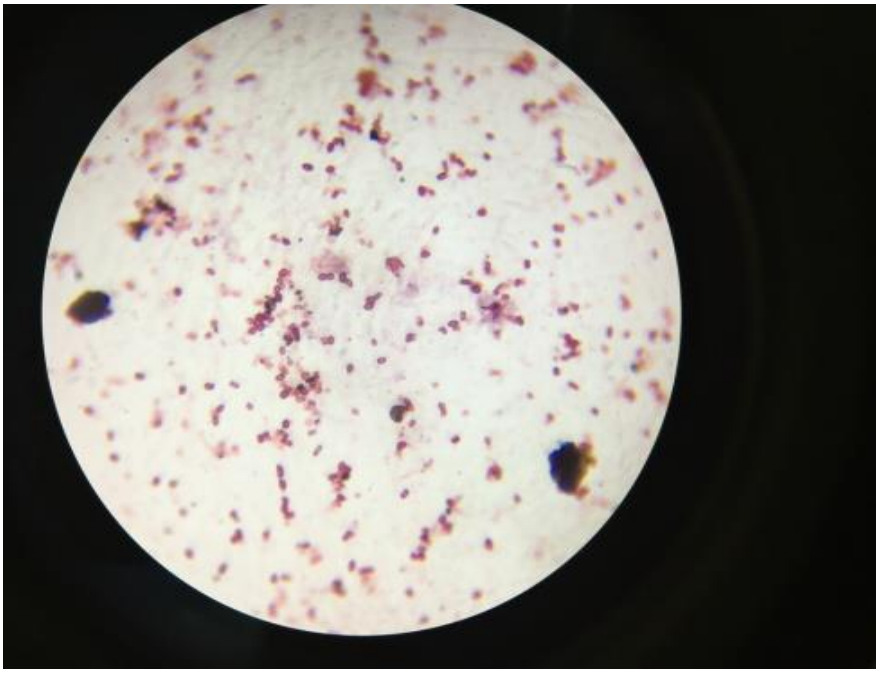
Photograph 1: Carbohydrate fermentation test of *Rhizobium* spp.

Photograph 2: Biochemical test of *Azotobacter* species

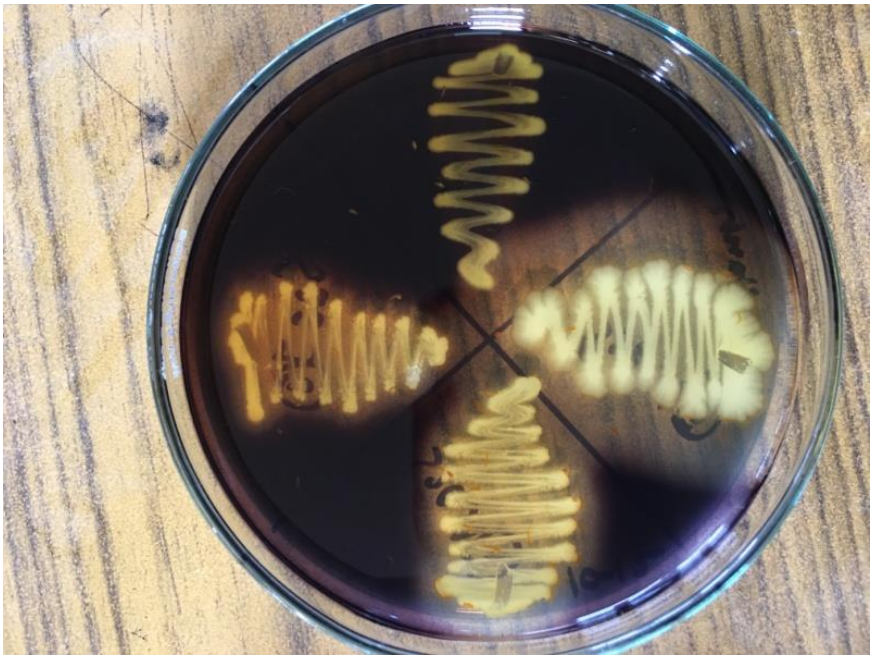


Photograph 3: Blending (mixing of carrier material with inoculums)

Photograph 4: *Azotobacter* culture in Ashybes media



Photograph 5: Microscopic view of *Azotobacter* species



Photograph 6: Starch hydrolysis of rhizobia

CHAPTER-V

DISCUSSION

Biofertilizers are living or dormant cells of efficient strains of microorganisms usually immobilized on a carrier that helps crops take up nutrients through interactions in the rhizosphere, resulting in increased yield. Biofertilizers outperform chemical fertilizers in a variety of ways. Through biological processes, biofertilizers can convert nutritionally important elements from unavailable to available form (Vessey 2003). Because some bacteria can fix atmospheric nitrogen both symbiotically and non-symbiotically, they are used in the production of biofertilizers. The use of these bacteria is limited due to storage and viability issues over long periods. As a result, carrier materials are used to extend their shelf life because they provide a favorable environment for their growth and survival.

A variety of carriers have been shown to improve inoculant survival and biological effectiveness by protecting bacteria from biotic and abiotic stresses (Phiromtan, Mala and Srinives 2013). A suitable carrier should be inexpensive, simple to use, mixable, packageable, and widely available. In addition, the carrier must allow gas exchange, particularly oxygen exchange, and have a high organic matter content and water holding capacity (Ben Rebah *et al.* 2007). This study focuses on the ability of three inoculant carriers to determine their suitability as alternatives to peat, using two common nitrogen-fixing bacteria i.e. *Rhizobium* (symbiotic nitrogen fixer) and *Azotobacter* (non-symbiotic nitrogen fixer) at two different temperatures.

Rhizobium was isolated from the root nodule of the pea plant and *Azotobacter* was isolated from the soil sample. These isolates were identified according to the Bergeys manual using the different morphological and biochemical tests.

Then the selected carrier materials were powered and passed through 0.063mm sieve to know the size. Carrier materials were sun-dried for 7 days and autoclaved for sterilization. Different methods were used for the sterilization of carrier materials to obtain the most suitable one without any effect on their quality. Abd El-Fattah et al., (2013) reported that steam sterilization by autoclaving is the most commonly used and has the superiority among all employed methods due to its low cost and its ability to allow a pure culture of inoculant to be prepared.

Physical properties were checked as these characteristics affect the shelf life of inoculant. Most of the evaluated carriers had a pH of about 7.0 thus adjusting the pH was not necessary as rice husk, charcoal, and farm yard manure had pH 8.2, 7.5, and 6.3 respectively. Carrier material with similar pH was used by M. Singh et al.,(2019) for powder formulations. According to Stephens & Rask, (2000) and Plant et al., (1993), the carriers should have near neutral or readily adjustable pH. All had a high water retention capacity for the optimal moisture potential of 40% for growth and survival of *Rhizobium* and *Azotobacter* inoculants as described by All,(1970); Thompson, (1980). In addition, preliminary studies of the growth of *Rhizobium* and *Azotobacter* have shown that these carriers were non-toxic for bacterial growth.

These identified species were mass cultured. The first isolated organism was cultured in a test tube of 10ml broth. After a week of incubation, these organisms were transferred to a conical flask of 1liter and kept in a shaker incubator until the growth reached 10^7 organisms.

These carrier materials were combined with an isolated microorganism that is *Rhizobium* spp and *Azotobacter* spp. 750ml of broth was mixed into 1kg of carrier material. In this experiment, inoculant is stored at room temperature and frozen to find out the survivability of microorganisms in the carrier. On week basis moisture content, pH, and the bacterial population were observed.

The moisture content of *Rhizobium* in freeze was obtained adequate within the 11^{th} week of storage. The highest moisture was obtained in farmyard manure with

55.3% and the lowest was obtained in charcoal 51.2% in the zero week that is data was taken as soon as packing was completed week whereas the lowest was obtained in rice husk 32.4% in the 11^{th} week. Carrier (*Rhizobium*) stored at room temperature had little change in moisture than stored in freeze. Moisture was adequate till 8^{th} week in rice husk and charcoal and 7^{th} week for farmyard manure with 26.5%, 26.4% and 26.9% respectively. After the 7^{th} and 8^{th} week moisture was good but not adequate. The lowest moisture was observed in rice husk 19.7% followed by farmyard manure 20.5%.

The moisture of *Azotobacter* is different than *Rhizobium*. Inoculant stored in freeze was great in moisture. They had more than 40% in the 7^{th} week. The least moisture was observed in rice husk that is 42.1% at the end of 7^{th} week. When the same inoculant was stored at room temperature shows variation, as the least moisture was seen in rice husk with 26.5%. In the 1^{st} week, the highest moisture was obtained in farmyard manure with 55.3% followed by rice husk 52%. (Gaind and Gaur 2004) also reported a reduction in the percent moisture content of the carrier with the increased incubation period. S. Gaind & Gaur, (1990) also had a similar decrease in the moisture.

The growth of *Rhizobium* stored in freeze is highest in farmyard manure with 9.5×10^7 CFU per ml followed by rice husk with 9.2×10^7 CFU per ml in the first week. At the end of the 11^{th} week, the highest growth is seen in rice husk with 5.37×10^7 CFU per ml and the lowest is observed in charcoal 0.59×10^7 CFU per ml, similar to earlier reports Ali et al., (2005) and Srinivasula et al., (2001). Initial microbial populations of those materials were higher than those reported by Novinscak et al., (2008). The growth in charcoal from the 10^{th} week is inadequate which makes charcoal the least useful in comparison with three carrier materials. It is different in room temperature though the highest growth is observed in farmyard manure with 9.5×10^7 CFU per ml followed by rice husk with 9.2×10^7 CFU per ml. growth in room temperature is more in compared with the freeze in the first few weeks. Open & Publisher, (2014) have also observed more bacterial growth in 30^0C compared to 4^0C.

At the end of the 11th week, the highest rhizobial growth is seen in rice husk with 7.83×10^7 CFU per ml followed by farmyard manure with 4.97×10^7 CFU per ml. n. Other authors obtained the same results working with slow and fast-growing rhizobia strains (Temprano, Albareda and Rodrı 2008)(Yardin, Kennedy and Thies 2000).

In *Azotobacter* inoculants highest growth is seen in farmyard manure with 8.55×10^7 CFU per ml followed by rice husk with 8.32×10^7 CFU per ml which is stored in freeze. A similar result was obtained by (Plant *et al.* 1993). At the end of the 7th week, the highest number of bacteria is observed in farmyard manure with 4.72×10^7 CFU per ml followed by rice husk with 4.23×10^7 CFU per ml the microbial population was less than those reported by many workers Raja Sekar & Karmegam, (2010). Taylor et al., (2007) found that populations of *Azotobacter chroococcum* did not reduce below 10^8 CFU/g within three months. *Azotobacter* inoculants stored at room temperature have the same result as that stored in freeze but the number of bacteria in the carrier is different. More number of bacteria is observed in a carrier that is kept in room temperature. Kennedy et al., (2015); Somarathne et al., (2013) have reported that there is a decrease in the population of bacteria when stored for a long period of time.

In the case of the bacteria, charcoal has the least number of bacterial counts in the first and last week stored in both the temperature. The bacterial population increased in first week of incubation and reached the peak with more than 25% but after 1st week population started decreasing. Similar to the result of (Hoben 2016). During the 2nd, 3rd and 4th week of storage the death rate was the smallest but greatly decreased from the 6th and 7th week in *Azotobacter*. A similar trend was also documented by Phiromtan et al., (2013).

The growth decreased from 2nd week to the third week in high numbers that is more than 20% and slowly decreased in the coming weeks in both bacterial inoculant. A similar result was obtained by K.C. Kirankumar & Rudresh, (2017)

The ph of *Rhizobium* inoculants stored in freeze shows that Rice husk was basic with pH 8.2 in the first week and turned acidic in week 7 with pH 6.5 and gradually turned acidic with pH 5.1 at the end of 11^{th} week. Charcoal and farmyard manure were basic with pH 8.7 each in the first week and gradually turned acidic in 8^{th} and 9^{th} week with pH 6.6 and 6.7 respectively. At the end of the 11^{th} week pH of charcoal was5.5 and pH of farmyard manure was 5.1. pH of *Rhizobium* inoculants stored at room temperature was similar to that of stored in freeze. The pH of rice husk, charcoal and farmyard manure had basic pH in first week and turned acidic in nature at the end of the 11^{th} week with pH 5.5, 5.7 and 6 respectively. In general fast-growing rhizobia strains produce acids from sugars after their growth in yeast extract-mannitol media, but it can vary depending on the strain and the composition of the culture medium (Stowers and Eaglesham 1984)

The pH of *Azotobacter* inoculants showed the same nature as that of *Rhizobium* inoculants. Rice husk, charcoal and farmyard manure were the basis in nature. Though *Azotobacter* inoculants were only stored for seven weeks, a change in pH was obtained. Rice husk and farmyard manure turned acidic with pH 6.5 and 6.7 respectively when stored in freeze. Charcoal in freeze showed neutral at the end of the 7^{th} week. The same result was obtained in *Azotobacter* stored at room temperature. Rice husk turned acidic but charcoal and farmyard manure remain neutral at the end of the 7^{th} week.

The highest survival rate of *Rhizobium* is observed in rice husk in both temperatures with 58.36% in refrigerator and 85.1% in room temperature. Abd El-Fattah et al. (2013) have also said that more bacterial population is obtained in room temperature. The lowest is obtained in charcoal with 6.78% when stored in refrigerator. Farmyard manure has a nearly equal percentage of survival rates in both the temperature with 48.84 in refrigerator and 52.31 in room temperature.

There are many factor that effect the growth of bacteria in carrier among them pH and moisture was tested and statistical analysis was performed. *Rhizobium*

blended with rice husk and stored in refrigerator has 59% of effect on CFU from pH and moisture remaining effect was from other different factor. This data shows the significant effect in CFU with significance value 0.018. Moisture had positive effect and pH had negative effect to the CFU. Similarly CFU of charcoal and farmyard manure showed significance difference though moisture effect was positive and pH was negative in charcoal and vice versa for farmyard manure. CFU in charcoal and farmyard manure was affected by 89.8% and 72.4% respectively.

In the other hand *Rhizobium* stored in room temperature had different result. CFU of rice husk had no significance influence but CFU of charcoal and farmyard had significance influence with significance value 0.143, 0.000 and 0.005 respectively. Moisture and pH in rice husk had only 35.1% effect in change of CFU but charcoal and farmyard manure moisture and pH had 88.9% and 68.6% effect in change of CFU. In all three carriers material moisture had negative effect and pH had positive effect to the CFU.

In case of *Azotobacter* species CFU of rice husk and charcoal showed significance difference with significance value 0.022 and 0.027 but farmyard manure showed insignificance with 0.057 significance values. Moisture and pH effected 78.3%, 76.4% and 68.1 % in rice husk, charcoal and farmyard manure respectively. Moisture had negative effect in rice husk and farmyard manure but positive effect in charcoal and pH had positive effect for all carrier material. *Azotobacter* stored in room temperature had different result. Rice husk had significance difference but charcoal and farmyard manure were insignificance with 0.003, 0.088 and 0.366 significance value. Moisture and pH effected 89.7%, 62.1% and 33.1% in respective carrier material. Moisture showed negative effect in rice husk and charcoal but positive effect in charcoal and pH had positive effect for all carrier material.

There is significant difference (asymp.sig =0.000) and (asymp.sig =0.026) in the CFU of *Rhizobium* spp for three different carrier material stored in refrigerator

and room temperature respectively. But in case of *Azotobacter* species significance difference was seen only in inoculants stored in refrigerator with (asymp.sig =0.046) and insignificant difference in room temperature with (asymp.sig =0.99) for three different carrier material.

The highest survival rate is seen in farmyard manure stored in freeze and the lowest is seen in charcoal. (Srinivasula *et al.* 2001) reported that the population of *Azotobacter vinelandii* A1 in rice husk carriers rise up to 128% from the initial population after storing at 30^0C.

In room temperature highest survival was obtained in farmyard manure with 51.34% followed by rice husk with 43.87%. High bacteria count is seen in farmyard manure in both temperatures with both organisms this is because the compost also had high clay mineral derived from adding clayey soil during the composting process which played a critical function in promoting physical and biochemical environment for the microbial population. The increase in the high specific surface area of plastic can promote adsorption of organic and inorganic substances, cation exchange capacity, and water holding capacity. In addition, clay particles also encourage microbial catabolism by increasing adherence and tolerance capacity of *Azotobacter* in the plastic under hot conditions (Barker and Rechcigl 2000). This finding is similar to the report of (Schachtman *et al.* 1998).

A high survivable rate is observed in rice husk in both temperatures with *Rhizobium* this is because of the low moisture content and bulk density, high porosity and water absorption capacity observed for rice husk are consistent with earlier reports (Taylor, Mansaray and Ghaly 2007). On the other hand, rice husk is composed of organic matter of 12 %, Nitrogen, 0.5 %, and low concentrations of minerals (Sitthaphanit *et al.* 2010). However, depending on the efficiency of the milling process, fresh rice husk as used in this study may also contain traces of starch and rice bran scraped from the grain.

A high survivable rate of growth is observed in carrier material stored in freeze than that stored at room temperature in *Azotobacter* species. Similar to the report of Bozida& Vladimir (1995) At the refrigerating temperature, the bacteria had lower metabolism and physiological activity which maintained high mineral contents and more available moisture than that stored over room temperature, similar to the report of (Phiromtan, Mala and Srinives 2013).

CHAPTER-VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Rice husk, however, demonstrated extraordinary potentials, particularly in respect of shelf life. Utilization of this material for this purpose will yield the additional benefits of its safe disposal thereby releasing more land for agriculture. These carriers have physicochemical properties that enable them to support the survival and viability of rhizobia and *Azotobacter* inoculants during storage. This may have contributed to the enhanced quality of the formulations. The rhizobia and *Azotobacter* strains population significantly decline over time regardless of the carrier material and storage temperature. A decline in population on prolonged incubation may be attributed to the depletion of nutrients, moisture and cell death.

6.2 Recommendations

- The research should be extended to molecular level by using different molecular techniques.
- The research can be extended for more time to view the survivability of microorganisms in the carrier material.
- Effect of biofertilizers on plants should be studied.
- Toxicity analysis of the biofertilizer needs to be performed.
- Several other carrier materials can be studied for their efficacy to the survival of microbial inoculants

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APPENDIX

APPENDIX A: Materials and reagent

A Glassware's and equipments

Pipettes

Cover slip

Petri plates

Conical flasks

Test tubes

L-Shaped dolly rod

Glass rods

Autoclave

Hot air oven

Microscope

Incubator

Test tube rack

Wash bottle

Burner

Refrigerator

B Media and chemicals

Ashybes agar

YEMA media

Nutrient broth

Simmons citrate media

MR-VP broth

Glucose

Fructose

Sucrose

Lysol

Glycerol

Sodium chloride

Sulfuric acid

Barium chloride

Catalase reagent(3%H_2O_2)

Alpha-napthol(5%)

Crystal violet

Gram's Iodine

Safranin

Kovacs reagent

Methyl red

Potassium hydroxide

Ashybes Agar

YEMa media

Nutrient broth

Simmons citrate media

MR-VP broth

Glucose

Fructose

Sucrose

APPENDIX B: Composition of media used

YEMA(yeast extract mannitol agar)

Mannitol	10g
K_2HPO_4	0.5g
$MgSO_4.7h_2O$	0.2g
Nacl	0.1g
Yeast extract	1g
Agar	15g
Distilled water	1liter

Ashbys media	**gram/liter**
Mannitol	20
Dipotassium phosphate	0.2
Magnesium sulohate	0.2
Sodium chloride	0.2
Potassium sulphate	0.1
Calcium carbonate	5.0

GPA media

Ingredients	**Grams / Litre**
Peptone	20.000
Dextrose (Glucose)	10.000
Sodium chloride	5.000
Agar	15.000

Nutrient Agar:

Ingredients	**Grams/Litre**
Beef Extract	3g
Peptone	5g
Agar	15g
Final pH	6.8+/-0.2

Nutrient Broth

Ingredients	Grams/Litre
Beef extract	1
Yeast extract	2
Peptone	5
Sodium chloride	5

MR-VP media:

Ingredients	Grams/Litre
Peptone	7
Dextrose	5
Dipotassiumphosphate	5

Simmons citrate:

Ingredients	Grams/Litre
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15

APPENDIX C: Stains and reagents used

1. Crystal Violet:

Crystal Violet	20g
Ethyl alcohol	95ml
Ammonium oxalate	9g
Distilled water	905ml

2. Gram's iodine:

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

3. 95% Ethyl alcohol:

Ethyl alcohol	95ml
Distilled water	5ml

4. Safranin:

Safranin	(2.5% safraninin 95% ethylalcohol)	10ml
Distilled water		100ml

5. Kovacs reagent:

Di methyl amino benzaldehyde	5g
Amyl alcohol	75ml
Conc. Hydrochloric acid	25ml

6. Methyl Red solution:

Methyl Red	0.05g
Ethyl Alcohol	28ml
Distilled water	22ml

7. VP reagent:

VP reagent–I

α-Napthol	5g
Ethyl alcohol	100ml

VP reagent–II

Potassium hydroxide	40g
Distilled water	100m

8. Hydrogen Peroxide Solution

Hydrogen peroxide	3ml
Distilled water	97ml

APPENDIX D: Characterization of bacteria

Table 1: Morphological characteristics of *Azotobacter*isolates

S.N	Sample code	Gram staining	Cell shape	Colony shape margin	Color	Consistency	Motility	Catalase
1	S1	Gram –ve	oval rod	circular, raised	Transparent	Dry	+	+
2	S2	Gram –ve	oval rod	smooth, opaque, raised	Transparent	Slimy	+	+
3	S3	Gram –ve	oval rod in chain	circular, smooth	Brown	Viscous	+	+
4	S4	Gram –ve	oval rod	raised, opaque	Brown	Viscous	+	+
5	S5	Gram –ve	small oval rod in clustered	opaque, confined	Milky white	Mucoid	+	+
6	S6	Gram –ve	oval rod	circular, raised	Creamy white	Dry	-	+
7	S7	Gram –ve	oval rod in chain	large, flat, smooth	Milky white	Mucoid	+	+
8	S8	Gram –ve	oval rod in clustered	circular, flat	Brown	Viscous	+	+
9	S9	Gram –ve	oval rod in chain	smooth, glistwning, raised	Creamy white	Mucoid	+	+
10	S10	Gram –ve	oval rod in clustered	Medium circular flat	Brown	Viscous	+	+
11	S11	Gram –ve	oval rod in chain	Medium circular flat	Brown	Viscous	-	+
12	S12	Gram –ve	oval rod mostly in pair	Medium circular flat	Brown	Viscous	+	+
13	S13	Gram –ve	small oval rod in clustered	Medium circular flat	Brown	Viscous	+	+
14	S14	Gram –ve	oval rod	Medium circular flat	Brown	Viscous	-	+
15	S15	Gram –ve	oval rod in chain	Irregular flat	Brown	Mucoid	+	+
16	S16	Gram –ve	oval rod	Irregular raised	Brown	Mucoid	+	+
17	S17	Gram –ve	small rod	large circular convex	Water droplet	Mucoid	-	+
18	S18	Gram –ve	small rod	large circular convex	Water droplet	Mucoid	+	+
19	S19	Gram –ve	small rod	large circular convex	Water droplet	Mucoid	-	+
20	S20	Gram –ve	oval rod in chain	Small circular flat	Brown	Dry	+	+

Table 2: Biochemical characteristics of *Azotobacter* isolates

S.N	Sample code	Citrate	Mannitol	Maltose	Glucose	Starch hydrolysis	Inndole	MR	VP
1	S1	+	+	+	-	+	+	+	-
2	S2	+	+	+	+	+	+	+	-
3	S3	+	+	+	+	+	+	+	-
4	S4	+	+	+	-	+	+	-	+
5	S5	+	+	+	+	+	+	+	-
6	S6	+	+	+	+	+	+	-	-
7	S7	+	+	+	-	+	+	+	-
8	S8	+	+	+	-	+	+	+	-
9	S9	-	+	+	+	+	+	-	+
10	S10	+	+	+	+	+	+	+	-
11	S11	-	+	+	-	+	+	+	-
12	S12	+	+	+	+	+	+	+	-
13	S13	+	+	+	+	-	-	-	+
14	S14	+	-	+	+	+	+	+	-
15	S15	+	+	+	+	+	+	-	+
16	S16	-	+	+	-	+	+	+	-
17	S17	+	+	+	+	+	+	+	-
18	S18	+	+	+	+	-	-	-	+
19	S19	+	+	+	+	-	-	-	+
20	S20	+	+	+	+	+	+	+	-

Table 3: Morphological characteristics of *Rhizobium* isolates

S.N	Sample code	Gram staining	Cell shape	Colony shape margin	Color	Consistency	Motility	Catalase	GPA
1	p1	Gram – ve	Small round	opaque, confined	White	Mucoid	+	+	-
2	p2	Gram – ve	Small oval	circular, raised	White	Slimy	+	+	-
3	p3	Gram – ve	Small oval	large, flat, smooth	White	Viscous	+	+	-
4	P4	Gram – ve	Small oval	circular, flat	Red	Dry	+	+	+
5	P5	Gram – ve	Small round	Circular, raised	Red	Dry	+	-	+
6	P6	Gram – ve	Small round	Circular raised	Milky white	Viscous	-	+	-
7	P7	Gram – ve	oval rod	Circular raised	Red	Dry	+	+	+
8	P8	Gram – ve	oval rod in chain	Circular smooth	White	Viscous	+	+	-
9	P9	Gram – ve	oval rod	Circular smooth	White	Mucoid	+	+	-
10	P10	Gram – ve	small rod	circular, smooth	White	Viscous	+	+	-
11	P11	Gram – ve	small rod	raised, opaque	White	Viscous	-	+	-
12	P12	Gram – ve	small rod	opaque, confined	Red	Viscous	+	-	-
13	P13	Gram – ve	oval rod in chain	circular, raised	Red	Dry	-	+	-
14	P14	Gram – ve	Small round	Circular raised	Creamy white	Viscous	-	+	-
15	P15	Gram – ve	Small round	Circular raised	Red	Mucoid	+	+	+
16	P16	Gram – ve	Small oval	circular, raised	Red	Dry	-	-	+
17	P17	Gram – ve	Small oval	smooth, opaque, raised	Red	Mucoid	-	-	+
18	P18	Gram – ve	Small oval	circular, smooth	Red	Mucoid	+	+	+
19	P19	Gram – ve	Small round	raised, opaque	Creamy white	Mucoid	-	+	-
20	P20	Gram – ve	Small round	opaque, confined	Red	Dry	+	-	+
21	P21	Gram – ve	oval rod	circular, raised	Red	Mucoid	-	-	+
22	P22	Gram – ve	oval rod in chain	large, flat, smooth	Creamy white	Mucoid	-	+	-
23	P23	Gram – ve	oval rod	circular, flat	Red	Dry	-	-	+
24	P24	Gram – ve	small rod	Circular, raised	Red	Mucoid	+	-	+
25	P25	Gram – ve	small rod	Circular raised	White	Dry	+	+	-
26	P26	Gram – ve	small rod	Circular raised	White	Mucoid	+	+	-
27	P27	Gram – ve	oval rod in chain	Circular smooth	White	Mucoid	+	+	-
28	P28	Gram – ve	Small round	Circular smooth	White	Mucoid	+	+	-

Table 4: Biochemical test of *Rhizobium* isolates

S.N	Congo red YAMA	Urea hydrolysis	2%NaCl	H_2s	Starch hydrolysis	glucose	fructose	mannitol	sucrose	Maltose
p1	-	+	-	-	-	+	+	+	+	+
p2	-	+	-	-	-	+	+	+	+	+
p3	-	+	-	-	+	+	+	+	+	+
P4	+	-	+	-	-	+	+	+	+	+
P5	+	-	+	-	-	+	+	+	+	+
P6	-	-	-	-	-	+	+	+	+	+
P7	+	+	+	-	-	+	+	+	+	+
P8	-	+	-	-	-	+	+	+	+	+
P9	-	+	-	-	-	+	+	+	+	+
P10	-	+	-	-	-	+	+	+	+	+
P11	-	-	-	-	-	+	+	+	+	+
P12	+	-	+	-	-	+	+	+	+	+
P13	+	-	+	-	-	+	+	+	+	+
P14	-	-	-	-	-	+	+	+	+	+
P15	+	-	+	-	-	+	+	+	+	+
P16	+	-	+	-	-	+	+	+	+	+
P17	+	+	+	-	-	+	+	+	+	+
P18	+	+	+	-	-	+	+	+	+	+
P19	-	+	-	-	-	+	+	+	+	+
P20	+	+	+	-	-	+	+	+	+	+
P21	+	+	+	-	-	+	+	+	+	+
P22	-	+	-	-	-	+	+	+	+	+
P23	+	+	+	-	-	+	+	+	+	+
P24	+	+	+	-	-	+	+	+	+	+
P25	-	+	-	-	+	+	+	+	+	+
P26	-	+	-	-	-	+	+	+	+	+
P27	-	-	-	-	-	+	+	+	+	+
P28	-	-	-	-	-	+	+	+	+	+

APPENDIX E: Statistical analysis

Correlation and regression analysis between pH/CFU and mc/CFU of *Rhizobium* species blended with three different carriers stored in two different temperatures

Relation of moisture and pH vsCFU of Rhizobiumspecies blended with rice husk stored in refrigerator.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.768[a]	.590	.499	1.66159	.590	6.488	2	9	.018

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-2.203	3.894		-.566	.585
	Moisture	.443	.205	1.154	2.163	.059
	Ph	-1.192	1.435	-.443	-.830	.428

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Rhizobium*species blended with charcoal stored in refrigerator.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.948[a]	.898	.876	1.09169	.898	39.713	2	9	.000

a. Predictors: (Constant), ph, moisture

fficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-19.557	2.665		-7.339	.000
	Moisture	.805	.156	1.386	5.170	.001
	Ph	-1.454	.779	-.500	-1.866	.095

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.851[a]	.724	.663	4.43981	.724	11.834	2	9	.003

a. Predictors: (Constant), moisture, ph

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Rhizobium*species blended with farmyard manure stored in refrigerator.

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.339	13.490		.099	.923
	Ph	9.862	2.492	1.509	3.957	.003
	Moisture	-1.346	.607	-.846	-2.218	.054

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Rhizobium* species blended with rice husk stored in room temperature.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.592[a]	.351	.206	5.57219	.351	2.430	2	9	.143

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-40.406	35.219		-1.147	.281
	Moisture	-.428	.605	-.636	-.707	.497
	Ph	9.933	7.649	1.168	1.299	.226

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Rhizobium* species blended with charcoal stored in room temperature.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.943[a]	.889	.864	1.26090	.889	36.060	2	9	.000

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-21.390	3.831		-5.583	.000
	Moisture	-.332	.113	-.805	-2.943	.016
	Ph	5.498	.928	1.621	5.922	.000

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Rhizobium* species blended with farmyard manure stored in room temperature.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.828[a]	.686	.616	4.46663	.686	9.831	2	9	.005

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-62.954	18.578		-3.389	.008
	Moisture	-.681	.315	-.915	-2.159	.059
	Ph	13.267	3.640	1.545	3.644	.005

a. Dependent Variable: tpc

Correlation and regression analysis between pH/CFU and mc/CFU of *Azotobacter* species blended with three different carrier stored in two different temperature

Relation of moisture and pH vs CFU of *Azotobacter*species blended with rice husk stored in refrigerator.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.885[a]	.783	.696	1.12772	.783	9.024	2	5	.022

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-13.041	7.391		-1.764	.138
	Moisture	-.524	.712	-.787	-.736	.495
	Ph	6.026	3.919	1.643	1.538	.185

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Azotobacter* species blended with charcoal stored in refrigerator.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.874[a]	.764	.669	1.46236	.764	8.085	2	5	.027

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-29.752	9.662		-3.079	.027
	moisture	.437	.360	.458	1.215	.279
	Ph	1.863	1.530	.459	1.218	.278

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Azotobacter* species blended with farmyard manure stored in refrigerator.

Model Summary

Mod	R	R	Adjusted R	Std. Error	Change Statistics

el		Square	Square	of the Estimate	R Square Change	F Change	df1	df2	Sig. F Change
1	.825[a]	.681	.554	3.99639	.681	5.344	2	5	.057

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-23.808	18.947		-1.257	.264
	moisture	-1.463	.815	-.939	-1.795	.133
	ph	13.322	4.609	1.512	2.891	.034

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Azotobacter* species blended with rice husk stored in room temperature.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.947[a]	.897	.856	1.68020	.897	21.834	2	5	.003

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-51.177	9.246		-5.535	.003
	Moisture	-.852	.202	-1.577	-4.222	.008
	Ph	12.684	2.168	2.184	5.850	.002

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Azotobacter* species blended with charcoal stored in room temperature.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.788[a]	.621	.470	2.46757	.621	4.104	2	5	.088

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	-25.578	15.643		-1.635	.163
	moisture	.143	.205	.300	.696	.517
	ph	3.111	2.509	.534	1.240	.270

a. Dependent Variable: tpc

Relation of moisture and pH vs CFU of *Azotobacter* species blended with farmyard manure stored in room temperature.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.575[a]	.331	.064	5.61627	.331	1.238	2	5	.366

a. Predictors: (Constant), ph, moisture

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-56.097	44.994		-1.247	.268
	moisture	-.027	.304	-.040	-.088	.933
	ph	7.965	6.019	.598	1.323	.243

a. Dependent Variable: tpc

Kruskal willis analysis of CFU of *Rhizobium* species blended with different carriers stored in two different temperatures

Relation between CFU of *Rhizobium* species stored in refrigerator.

Ranks

	types of tpc	N	Mean Rank
value of tpc	ricehusk	12	19.00
	charcoal	12	9.63
	fym	12	26.88
	Total	36	

Test Statistics[a,b]

	value of tpc
Chi-Square	16.127
Df	2
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: types of tpc

There is significant difference (asymp.sig =0.000) in the CFU of *Rhizobium* spp for three different carrier material stored in refrigerator.

Room temperature

Relation of CFU of *Rhizobium* species stored in room temperature

Ranks

	types of tpc	N	Mean Rank
value of tpc	Ricehusk	12	23.17
	Charcoal	12	12.00
	Fym	12	20.33
	Total	36	

Test Statistics[a,b]

	value of tpc
Chi-Square	7.285
Df	2
Asymp. Sig.	.026

a. Kruskal Wallis Test

b. Grouping Variable: types of tpc

There is significant difference (asymp.sig =0.026) in the CFU of *Rhizobium* spp for three different carrier material stored in room temperature.

Kruskalwillis analysis of CFU of *Azotobacter* species blended with different carrier stored in two different temperature

Relation of CFU of *Azotobacter* species stored in refrigerator.

Ranks

	types of tpc	N	Mean Rank
value of tpc	Ricehusk	8	10.75
	Charcoal	8	9.25
	Fym	8	17.50
	Total	24	

Test Statistics[a,b]

	value of tpc
Chi-Square	6.180
Df	2
Asymp. Sig.	.046

a. Kruskal Wallis Test

b. Grouping Variable: types of tpc

There is significant difference (asymp.sig =0.046) in the CFU of *Azotobacter* spp for three different carrier material stored in refrigerator.

Relation of CFU of *Azotobacter* species stored in room temperature.

Ranks

	types of tpc	N	Mean Rank
value of tpc	ricehusk	8	14.38
	charcoal	8	8.13
	fym	8	15.00
	Total	24	

Test Statistics[a,b]

	value of tpc
Chi-Square	4.625
df	2
Asymp. Sig.	.099

a. Kruskal Wallis Test

b. Grouping Variable: types of tpc

There is insignificant difference (asymp.sig =0.099) in the CFU of *Azotobacter* spp for three different carrier material stored in room temperature.