Isolation, identification and characterization of *Rhizobium* from hyacinth bean (*Lablab niger*) root nodule and study its effect on plant growth and nitrogen fixation ability

A DISSERATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Dedicated to my parents, brothers, husband and my supervisor who always support and inspire me

DECLARATION

This is to declare that the research work reported in this thesis titled **"Isolation, identification and characterization of *Rhizobium* from hyacinth bean *(Lablab niger)* root nodule and study its effect on plant growth and nitrogen fixation ability"** by Khadiza Nasrin Yshita, has been carried out under the supervision of Professor Dr. M. Mahboob Hossain, Microbiology Program, BRAC University in partial fulfillment of BSc. in Microbiology, at BRAC University, Dhaka. It is further acknowledged that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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List of abbreviation

BNF=Biological Nitrogen Fixation

YMA= Yeast Mannitol Agar

IAA= Indole acetic acid

EPS= Exopolysaccharide

GPA= Glucose peptone agar

CFU: Colony Forming Unit

spp.: Species

Abstract

Symbiotic systems for biological nitrogen fixation (BNF) in agriculture are most promising. Accordingly, the present study was conducted for the isolation, identification, and characterization of nitrogen-fixing bacterial strains *Rhizobium* from the legume plant hyacinth bean which grows widely in almost every area of Bangladesh. Nitrogen fixation is the reduction of N_2 (atmospheric nitrogen) to NH_3 (ammonia) which is made possible by the enzyme nitrogenase. Strains of root-nodulating bacteria were isolated from root a nodule of *Lablab niger* known as hyacinth bean. After confirmation test with yeast mannitol agar (YMA), isolates were collected for morphological and biochemical characterization. For nitrogen fixation test, healthy seeds of hyacinth bean were used for pot trials. Previously sterile soil of one pot (marked with green colour) was inoculated with *Rhizobium* sp. isolated and identified in the present study and another pot (marked in white colour) was not inoculated with bacteria. Four seeds were sown in each pot and two seeds were germinated on green pot whereas one of four seeds was germinated on white pot. After forty-five days of seed sowing, the plant grown in a pot with *Rhizobium* showed nodulation. Plants from the same pot were healthier than the plant of other pot (white).

1. Introduction:

1.1 Biological Nitrogen Fixation

Nitrogen is required by all living organisms for the synthesis of proteins, nucleic acids, and other nitrogen-containing compounds. The earth's atmosphere contains almost 80% nitrogen gas. It cannot be used in this form by most living organisms until it has been fixed, that is reduced (combined with hydrogen), to ammonia because it is unavailable in its most prevalent form of atmospheric nitrogen. Green plants, the main producers of organic matter, use this supply of fixed nitrogen to make proteins that enter and pass through the food chain. Microorganisms (the decomposers) break down the proteins in excretions and dead organisms, releasing ammonium ions. These two processes form part of the nitrogen cycle. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as on affects the human health (Vitousek, 1997).

In soil, two types of nitrogen bacteria are available. They are classified into the symbiotic and the non-symbiotic (free living) forms. Symbiotic (e.g. *Rhizobium*, *Bradyrhizobium*) and non-symbiotic (e.g. Azotobacter, Acetobacter etc.) grow in the root nodules and the rhizosphere of many legumes. Free-living non-symbiotic bacteria cannot fix nitrogen. Symbiotic bacteria are able to fix atmospheric nitrogen.

Biological nitrogen fixation (BNF) is the cheapest and environment-friendly procedure in which nitrogen fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into the soil (Franche et al., 2009). Nutrient enrichment of soils by nitrogen-fixing symbiotic bacteria present in legumes have been known for centuries. Scientific demonstration of this symbiosis was started in the 19th century and it established the facts that the bacteria present in nodules on legume roots are responsible for fixing atmospheric nitrogen (Zsbrau, 1999). Nitrogen fixing bacteria are able to fix atmospheric nitrogen under different conditions independently, in loose association with other organisms, or in strict symbioses with them, such as in the *Rhizobium*-

legume plant symbiosis which is being considered the most efficient type of association between diazotrophic microorganisms and plants.

1.2 *Rhizobium sp.* and nodulation

Rhizobium sp. is a well-known group of bacteria that acts as the primary symbiotic fixer of nitrogen. The Rhizobia are broadly classified as fast- or slow-growing based on their growth on laboratory media. Rhizobia are further classified according to their compatibility with particular legume species. *Rhizobium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into the soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers et al., 2003). These bacteria at first infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. Sets of genes in the bacteria control different aspects of the nodulation process. One *Rhizobium* strain can infect certain species of legumes but not others. Specificity genes determine which *Rhizobium* strain infects which legume. Even if a strain is able to infect a legume, the nodules formed may not be able to fix nitrogen. Such rhizobia are termed ineffective. Effective strains induce nitrogen-fixing nodules. Effectiveness is governed by a different set of genes in the bacteria from the specificity genes. Nod genes direct the various stages of nodulation.

The initial interaction between the host plant and free-living rhizobia is the release of a variety of chemicals by the root cells into the soil. Some of these encourage the growth of the bacterial population in the area around the roots (the rhizosphere). Reactions between certain compounds in the bacterial cell wall and the root surface are responsible for the rhizobia recognizing their correct host plant and attaching to the root hairs. Flavonoids secreted by the root cells activate the nod genes in the bacteria which then induce nodule formation. The whole nodulation process is regulated by highly complex chemical communications between the plant and the bacteria. Once bound to the root hair, the bacteria excrete nod factors. These stimulate the hair to curl. Rhizobia then march into the root through the hair tip where they induce the formation of an infection thread. This thread is constructed by the root cells and not the bacteria and is formed only in response to infection. The infection thread grows through the root hair cells and penetrates other root cells nearby often with the branching of the thread. The bacteria multiply

within the expanding network of tubes, continuing to produce nod factors which stimulate the root cells to proliferate, eventually forming a root nodule. Within a week of infection, small nodules are visible to the naked eye. Each root nodule is packed with thousands of living *Rhizobium* bacteria, most of which are in the deformed form known as bacteroids. Portions of plant cell membrane surround the bacteroids. These structures, known as symbiosomes, which may contain several bacteroids or just one, are where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops (Dilworth and Parker, 1969).

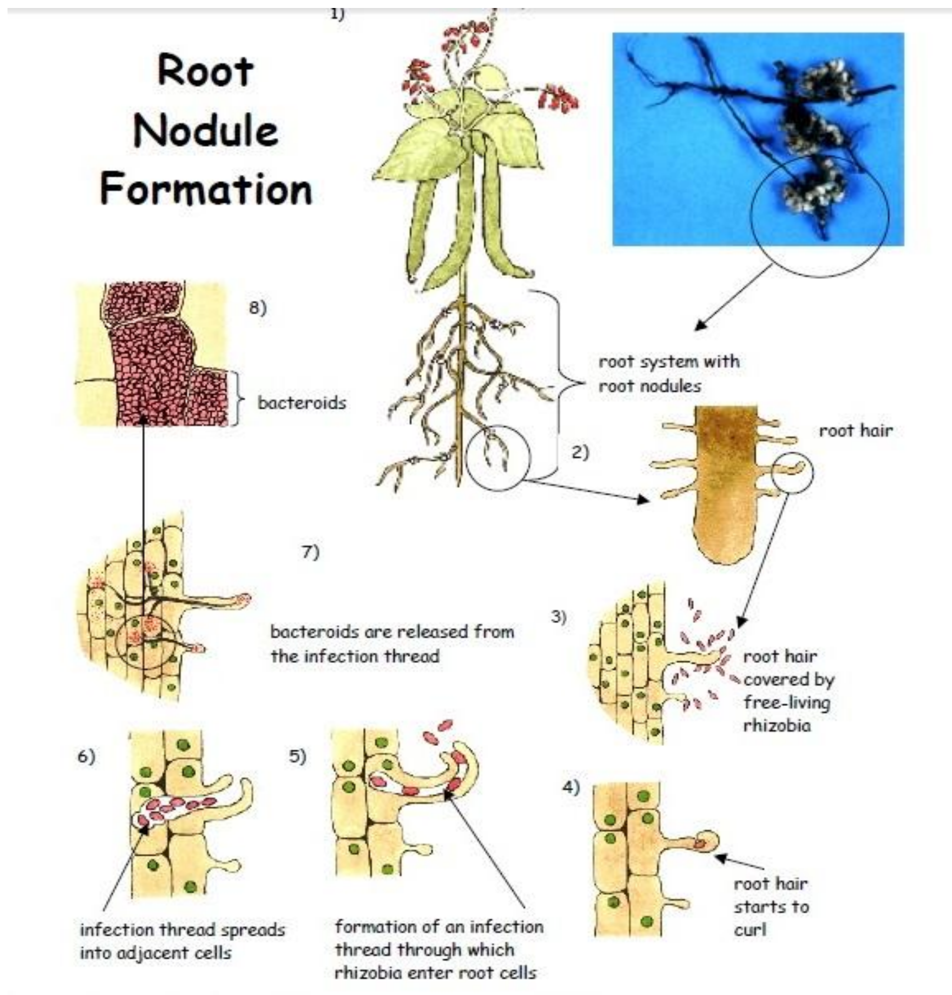

FIGURE 1.2: Formation of root nodules

The legume-*Rhizobium* interaction is the result of specific recognition of the host legume by *Rhizobium*. Various signal molecules that are produced by both *Rhizobia* and the legume confer the specificity (Phillips, 1991). Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection (Olivares et al., 1984). It also protects the cell from desiccation and predation and helps in nitrogen fixation by preventing high oxygen tension (Jarman et al., 1978).In addition, *Rhizobium* strains secrete growth hormones like indole acetic acid (IAA), which shows positive influence on plant growth and also plays an important role in the formation and development of root nodules (Nutman,1977). Hence, the production of EPS and IAA are considered as important traits of plant growth promoting Rhizobacteria. Moreover, *Rhizobium* species also have other various enzymatic activities. These benefits of this species were identified and measured biochemically by various researchers (Kumari et al, 2009). Although symbiotic nitrogen fixation by legumes is generally the dominant source of nitrogen input in the soil for imparting fertility but also avoid soil stresses, such as temperature, acidity, and salinity which pose a severe yield constraint in obtaining plant growth and development (Lawson et al, 1995). Additionally, symbiotic N_2 fixation can be limited either by lack of effective *Rhizobium* strain or if the environment is not favorable.

1.3 Literature review

The literature on the study of *Rhizobium* on field bean is inadequate. Conversely, obtainable information concerning effect of *Rhizobium* on nodulation, plant growth, and nitrogen fixation has been reviewed this chapter.

Legumes such as beans, peas, nuts etc have been used in agriculture since ancient time and legume seeds or pulses were the first source of human food and their domestication. Legume plant posses a unique ability to establish symbiosis with nitrogen-fixing bacteria of the family Rhizobiaceae. The bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Rinorhizobium and Mesorhizobium* (Martinez Romero, 2003; Willems, 2006) are able to form nodules on their host plants inside of which they fix-nitrogen.

Symbiotic nitrogen fixation by legumes is generally the dominant source of nitrogen input in the soil for imparting fertility but also avoid soil stresses, such as temperature, acidity, and salinity which pose a severe yield constraint in obtaining plant growth and development (Lawson et al., 1995).

Legumes have the ability to form a symbiotic relationship with soil bacteria capable of trapping nitrogen gas (N_2) from the atmosphere and converting it into ammonia, which can be used by the plant for growth, development and seed production. Atmospheric nitrogen is converted to ammonia by the nitrogenase enzyme in a process known as biological nitrogen fixation (BNF) (Postgate, 1998; Bhatia et al., 2001). The capacity of legumes to fix atmospheric nitrogen gives them an advantage over non-leguminous crops when grown on soils low in nitrogen (N). As such, they are an integral part of most small-landholder cropping systems (Bhatia et al., 2001). The need to improve the productivity of legumes as a global source of dietary protein, however, has made it vital to understand the factors that influence nitrogen fixation (Schulze, 2004).

BNF is a symbiotic relationship between specific nitrogen-fixing bacteria (rhizobia) and legume plants (Caetano-Anolles and Gresshoff, 1991). Upon perception of flavonoids exuded by legume roots, rhizobia activate nod genes which induce production of Nod factors (Burdman et al., 1997). Nod factors are calcium-dependent proteins that promote attachment of the rhizobia to root hair surfaces (Smit et al., 1987). Subsequent curling of the root hairs encloses the attached rhizobia (Heidstra et al., 1994), which induce hydrolysis of the root hair cell wall. Invagination of the plasma membrane by the plant forms an infection thread within which rhizobia multiply and penetrate host cells (Ridge et al., 1985). Once inside the parenchyma cells of the root, rhizobia, and infected cells proliferate forming a nodule initial. Whether the nodule initial develops into a functional nodule is determined by the plant and a host of environmental factors. Within the successful nodule, rhizobia are surrounded by a peri bacteroid membrane where they differentiate into bacteroids and begin fixing atmospheric nitrogen (Hedtke and Newcomb, 1977).

Rhizobium sp. is a well-known group of bacteria that acts as the primary symbiotic fixer of nitrogen. *Rhizobium* bacteria stimulate the growth of leguminous plants and they are able to fix

atmospheric nitrogen into the soil by interacting symbiotically with leguminous plants (Kiers et al., 2003).

In many regions of the world where beans are grown, nitrogen fixation is limited by unfavorable soil conditions and temperature and water stress. Temperature has been reported to affect nodulation, survival, and persistence of rhizobial strains in soil. Depending on their natural habitat, tolerance of rhizobia to temperature varies across various strains (Mohammadi et al., 2012).

Soil temperature, physical and chemical composition, moisture content in soil varies within small areas and these variations affect the populations of the soil inhabitants. Therefore, differences in response towards salinity, pH and temperature are expected. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Hashem et al. (1998) proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand by decreasing survival and proliferation of rhizobia in the soil and rhizosphere or by inhibiting very early symbiotic events, such as colonization, thus directly interfering with root nodule formation.

The salt inhibitory concentrations are varied among strains. Kassem et al., (1985) observed that strains of *Rhizobium* are able to grow in the presence of 4.5% NaCl; similarly Kucuk et al. (2006) reported that some Rhizobial isolates were grown under 4.5% NaCl. This study, therefore, reported the isolation of strains highly tolerant to high salt concentrations. Salt tolerant rhizobia may have the potential to improve the yield of legumes under salinity stress (El-Mokadem et al., 1991).

Legumes and rhizobia can be extremely sensitive to low soil pH (Corea and Barneix, 1997). Very few species survive in soils at pH below 5.0 (Mohammadi et al., 2012). Some *Rhizobium* strain may grow on lower pH range between pH 4.5 to pH 9.5 reported by Jordan (1984). The fact that different strains of the same species may vary widely in their pH tolerance has been demonstrated previously (Glenn and Dilworth, 1994; Correa and Barneix, 1997). Some Rhizobial isolates can be shown more sensitive to low pH than their host and this affects the

establishment of the symbiosis, limiting the survival and persistence of the rhizobia (Zahran, 1999). The performance of some clover-*Rhizobium* symbiosis under acidic conditions is the best when the Rhizobial strains were isolated from acidic soils (Zahran, 1999). Furthermore, Zahran (1999) attributed failure of nodulation at pH below 5.0 to failure of rhizobia to survive in such acidic soils. More than 23% of the soils for bean production in East Africa have pH at or below 5.0 (Beebe et al., 2012).The optimum pH range for rhizobial growth is between 6.0 and 7.0 below which very few rhizobia are capable of growing (Brockwell et al., 1991; Graham et al., 1994). Therefore, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic conditions may help the establishment of the symbiosis and also may improve the acid tolerance of legume. The ability of isolates to utilize a broad range of carbon substrates is also related to the survival of these isolates under acidic environments. Rhizobia are capable of metabolizing different carbon sources so that the products ameliorate the environmental pH (Glenn and Dilwarth, 1994). Under acidic conditions the catabolism of organic acids and amino acids leads to alkalinisation (Ibekew et al., 1997) and this buffering action may help in the establishment of the legume in acidic soils.

Soil nutrient concentrations also affect legume hosts, soil bacteria, and their symbiotic relationships (Mohammadi et al., 2012). Low phosphorus levels in acidic soils, for example, delay nodulation and infection of primary roots (Mullen et al., 1988). Aluminum and manganese toxicity and low levels of calcium inhibit the growth of rhizobia and nodulation at soil pH less than 5 (Alva et al., 1990; Bordeleau and Prevost, 1994; Campo, 1995). During the initial stages of symbiosis, rhizobia secrete calcium-dependent proteins to promote attachment onto root hair surfaces (Gage, 2004). The low concentration of calcium ions in acidic soils, however, inhibits attachment of rhizobia onto root surfaces (Cartano-Anolles et al., 1989). Numerous studies have documented the negative impact of high nitrate concentration in soil on nodulation and nodule weight (Malik et al., 1987; Muller and Pereira, 1995; Abaidoo et al., 1990; Streeter, 1984).

According to Jordan, (1984), the maximum temperature reported for *Rhizobium* is 28˚-30°C. However, the temperature range is highly strain dependent for genus *Rhizobium* (Jordan, 1984). Additionally, temperatures greater than 40˚ C inhibit infection of root hairs by rhizobia (Hungaria and Franco, 1993) and nitrogen fixation (Long, 2001). Moreover, nitrogenase activity

was lower when common beans formed nodules at 35˚C (Piha and Munns, 1987). In their study, Michiels et al. (1994) attributed ineffectiveness of *Rhizobium* symbiosis at temperatures greater than 40˚ C to deletion of the symbiotic plasmids. Other studies with clover rhizobia have demonstrated that certain strains are able to grow at various temperatures in artificial cultures, with growth response up to 41°C (Giddens et al., 1982). Nevertheless, survival under higher temperatures does not mean efficiency in nitrogen fixation. Rhizobial strains obtained from hot and dry environments that grew up to 45°C lost their ineffectiveness (Zahran 1999). The fact that high soil temperatures usually results in the formation of ineffective nodules, several strains of rhizobia have been reported to be heat tolerant and to form an effective symbiosis with their host legumes. Low temperatures also limit nitrogen assimilation (Gibson, 1965), photosynthetic assimilation, malate production, and nitrogenase activity (Bordeleau and Prevost, 1994).

1.4 Objectives

The bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Rinorhizobium* and *Mesorhizobium* (Martinez Romero, 2003; Willems, 2006) which are collectively referred to as Rhizobia, are strictly aerobic, rod-shaped cells, 0.5-0.9 μm x 1.2-3.0μm in size, non-spore forming, cells stain Gram-negative, fast growing, contains the enzyme nitrogenase, motile by a single polar flagellum or two to six peritrichous flagella, chemoorganotrophic, colonies are white pigmented, circular, convex, translucent, raised, and host specific.

The present study was carried out with following objectives:

- Isolation of *Rhizobium* strain from hyacinth bean (*Lablab niger)* root nodule
- Identification of isolated strain
- Study the characteristics of isolated strain
- Observing the effects of *Rhizobium* on plant growth and the ability of nitrogen fixation.

2. Material and method

2.1 Isolation of Root Nodulating Bacteria:

Plants were collected from Madhabdi upozilla, Narsingdi and uprooted carefully and nodules were collected from the roots. Then nodules were washed with sterile water followed by surface treatment with 95% alcohol and again with sterile water. The washed nodules were surface sterilized with 0.1% mercuric chloride for 2-3 minutes and again washed for at least 10 times with sterile water as to remove the traces of mercuric chloride. The nodules then transferred into sterile pastel half filled with sterile water and crushed with a sterile mortal to obtain a milky bacterial suspension. After serial dilution suspensions were spread on Yeast extract mannitol (YEM) agar plates and incubated for 2-3 days at 28°C.

2.2 Identification

Identification of isolated strain was carried out based on morphological and biochemical characteristics.

2.2.1 Morphological Characters

For physiological characteristics, isolated root-nodulating bacteria were streaked over YEM agar plates. Plates were kept at 28°C for 48 hours and their characteristics were observed.

2.2.2 Gram staining and Spore staining

Two colonies were taken from 48 hours old culture plate to perform gram staining and spore staining.

2.2.3 Biochemical Characters

Biochemical tests were conducted to observe biochemical characteristics of *Rhizobium* bacteria.

1) Catalase Activity

Isolates of 48 hours old culture were flooded with hydrogen peroxide to observe the release of bubbles of oxygen around the bacterial colonies according to Graham and Parker (1964).

2) Oxidase Activity

Few drops of p-aminodimethylaniline oxalate were added on the surface of isolates on YEM agar to observe the production of colour according to Kovaks (1956).

3) Acid from Glucose

Mannitol in the YEM agar was replaced by the equal amount of glucose and bromothymol blue (25 mg/l) were added to it, the modified media used to observe the Change in colour around the colonies.

4) Starch Hydrolysis

Starch hydrolysis test was performed to determine the ability of microorganisms to use starch as a carbon source (De Oliverira, 2007). The starch medium was inoculated with *Rhizobium* and analyzed for starch utilization. Iodine test was done to determine the capability of microorganisms to use starch. Drops of iodine solution (0.1 N) were added on 24 hours old cultures grown in petri plates and incubated for 48 hours. Formation of a clear zone around the colonies will indicate utilization of starch.

5) Growth on Glucose Peptone Agar

Streaking with isolated strains was done on Glucose Peptone Agar (GPA) plates. The presence of growth was observed after 48 hours according to Vincent (1970).

6) Urea Hydrolysis

YEM broth altered with 2% (w/v) urea and 0.012% phenol red to check the urea hydrolysis. The broth was inoculated with log phase cultures and incubated for 48 hours at 28˚C to observe for the production of colour according to Lindstrom and Lehtomaki (1988).

7) Gelatin Hydrolysis

Log phase cultures from YEM broth was swab on the surface of
YEM agar plates containing 0.4% (w/v) gelatin to examine gelatinase activity.
The plates were incubated at 28°C for 7 days (Sadowsky et al., 1983).

8) Citrate Utilization

By replacing mannitol from YEM agar with an equal amount of sodium citrate and bromothymol blue (25mg/l), citrate utilization ability was determined. The plates with modified media were inoculated with bacteria and then incubated for 48 hours (Koser, 1923) at 28°C.

9) Growth in Presence of 8% KNO_3

Bacteria were allowed to test for the ability to grow in the presence of 8% KNO_3 in YEM broth for a 7 days incubation period at 28°C (El Idrissi et al., 1996).

10) NaCl (2%) Tolerance

YEM agar media amended with 2% NaCl (w/v) were inoculated with bacteria and growth were observed after 48hours incubation.

11) Screening for N-fixing bacteria

To study nitrogen fixation ability of the bacteria, the isolates were grown in Jensen's medium which is an N-free medium and kept for 48 hours in a microbial incubator at 28˚C.

12) Salt, pH and Temperature Tolerance

The ability of the isolated *Rhizobium* strain to grow at different concentrations of salt were tested by streaking them on YEM medium containing 0.5%, 1.0%, 1.5%, 2.0%, 3%, 3.5%, 4% 4.5%. 5 %(w/v), NaCl.
Differences in pH tolerance were tested in YEM agar by adjusting the pH to 4.0, 5.0, 6.0, 7.5, 8.0 and 9.0. All the plates were incubated at 28°C for 72 hours and YEM medium plates used as controls.

Differences in the range of growth temperature were investigated by incubation of bacterial cultures in YEM agar at 34°C, 37°C, and 40°C. Control plates were incubated at 28°C for 48 hours.

2.3 Effect of *Rhizobium* strain on plant growth and fixation of atmospheric nitrogen

To observe the effect of *Rhizobium* strain on plant growth along with atmospheric nitrogen fixation ability, all the process (inoculums preparation, seed preparation, and inoculation) were done in the microbiology laboratory of BRAC University. At first, two pots where soils being placed were cleaned up with tap water then with mildly warm water and further with sterile distilled water. Soils were sterilized by autoclaving at 121˚c for 15minutes.

2.3.1 Inoculum preparation

Rhizobium inoculant was prepared by using broth culture. Yeast mannitol broth was prepared on Erlenmeyer flask and was sterilizes at 121˚c for 15 minutes. Then the liquid medium was kept for cooling. After cooling down, a small amount of *Rhizobium* strain was transferred aseptically from the agar medium to liquid medium with the help of a sterile inoculating needle. The flask containing broth and strain was then placed on the shaker at 28˚C under 120 rpm for three days to enhance the growth of Rhizobium. After three days, growth was observed on the flask and it was taken out from the shaker for inoculation process.

2.3.2 Seed preparation

Before placing, seeds were washed with tap water afterward washed up with distilled water for 5 minutes and again with sterile distilled water for 2 minutes. Seeds were kept at laminar air flow for few minutes to become dry.

2.3.3 Inoculation process:

Broth culture poured onto the few amount of sterile soils and mixed with hands by using sterile hand gloves. Sterile soils mixed with *Rhizobium* strain were placed on green pot whereas white pot contained only sterile soil but no *Rhizobium* strain. Seeds were then placed in the pots by maintaining a certain amount of distance between them. Both pots contained four seeds and were kept in a place full of sun light for proper growth. Seed sowing was done on October 18th, 2015.

3. Results

3.1 Sampling

Study was conducted with hyacinth bean root nodules. Samples were collected from Baburhat Greenfield School & College, Madhabdi upozilla, Narsingdi district. *Rhizobium* isolation was done in microbiology laboratory of BRAC University. Bacteria were isolated and maintained on Yeast mannitol agar medium for regular laboratory works and stored at low-temperature refrigerator for long term storage.

3.2 Morphological characteristics:

Isolated bacteria were streaked over YEM agar plates. After incubation at 28˚-30˚ C for 48 hours, it was observed that isolates form milky white, circular, translucent, glistening, convex, elevated and raised colonies. The margin was regular and 2-4 mm in diameter.

Figure 3.2 - Isolated bacterial colonies on Yeast extract mannitol medium

3.3 Gram staining and Spore staining:

After staining it was observed that isolates were non-spore forming and gram negative. Shapes of isolates were a rod.

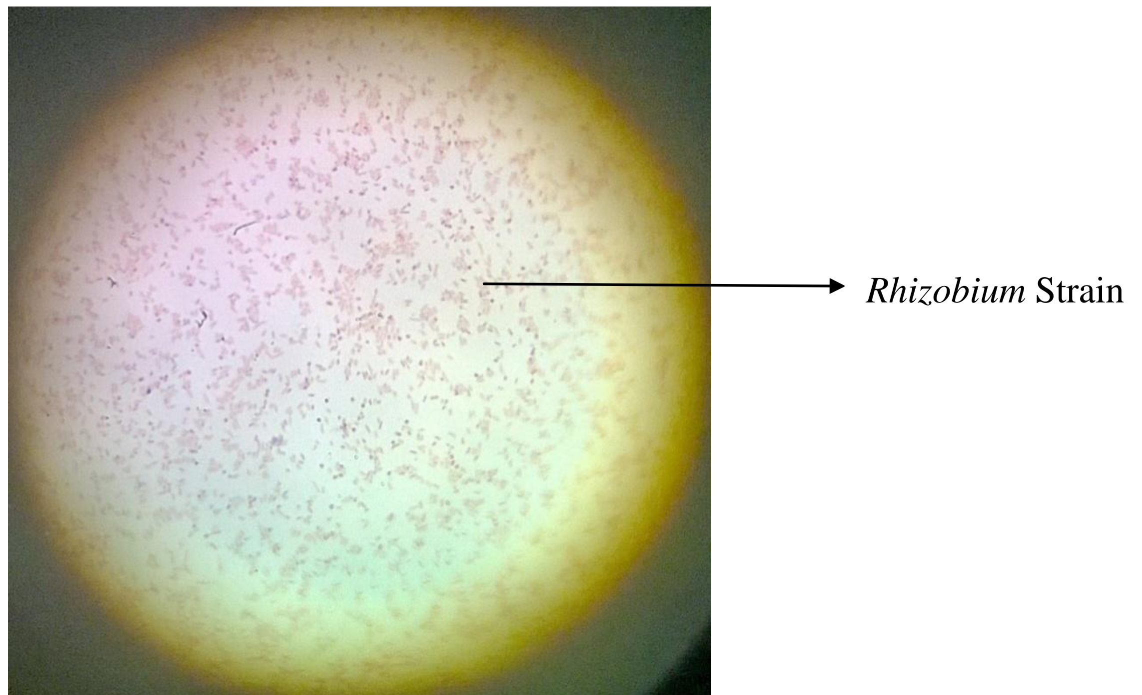

Figure 3.3- Figure: Morphology of isolates under microscope after gram staining

3.4 Biochemical Tests:

Isolates were studied for their biochemical characteristics. Strains of 48 hours old were used to perform different biochemical tests.

Test	Results
Gram staining	Negative
Spore staining	Non spore forming
Catalase test	Positive
Oxidase test	Negative
Acid from glucose	Positive Colour changes from green to yellow
Starch hydrolysis	Positive Clear zone observed around colonies
Growth on Glucose peptone agar	Positive Utilize glucose as the sole carbon source
Urea hydrolysis	Positive Colour changes from red to orange
Gelatin hydrolysis	Negative No clear zone formed around colonies
Citrate utilization	Negative No colour change
Growth in presence of 8% KNO3	Positive
NaCl 2% tolerance	Positive

Table 3.4.1- Results of different biochemical tests.

3.4.2: Screening for N-fixing bacteria

Isolates showed growth on Jensen's media which is a nitrogen free media. Experiment was conducted to observe its nitrogen fixing characteristics.

3.4.3 Salt, pH and Temperature Tolerance:

Isolates showed growth on 0.5%, 1%, 1.5% and 2% up to 4% (w/v) of NaCl concentration. No growth was observed on 4.5% and 5% NaCl concentration though temperature was 28˚C and pH was 7.

In the present study optimum, pH range for the growth of Rhizobia was between pH 6 to pH 7. No growth was observed in the medium with pH 3.5, pH 4, pH 8 and pH 9. Growth was observed at pH 6. pH 6.5 and pH 7.

No growth was seen on plates incubated at 34˚C, 37˚C, and 40˚C even at pH 7.

3.5 List of figures:

Figure 3.5.1: Catalase test for isolates with hydrogen peroxide

Figure 3.5.2: Oxidase test with p-aminodimethylaniline oxalate

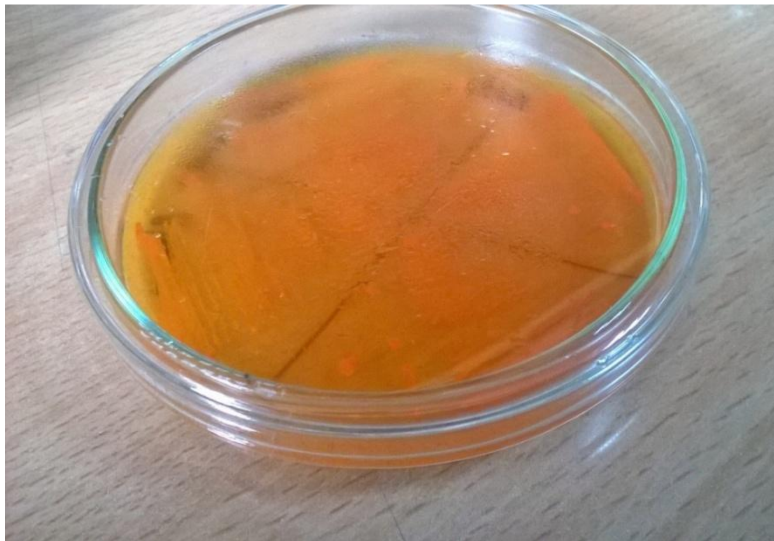

Figure 3.5.3: Changes of colour for acid production by bacterial isolate on modified YEM plate

Figure 3.5.6: Formation of clear zone indicated that bacteria were able to utilize starch for the period of Starch hydrolysis test

Figure 3.5.7: Growth on glucose peptone agar

Figure 3.5.8: Changes of broth colour during Urea hydrolysis test

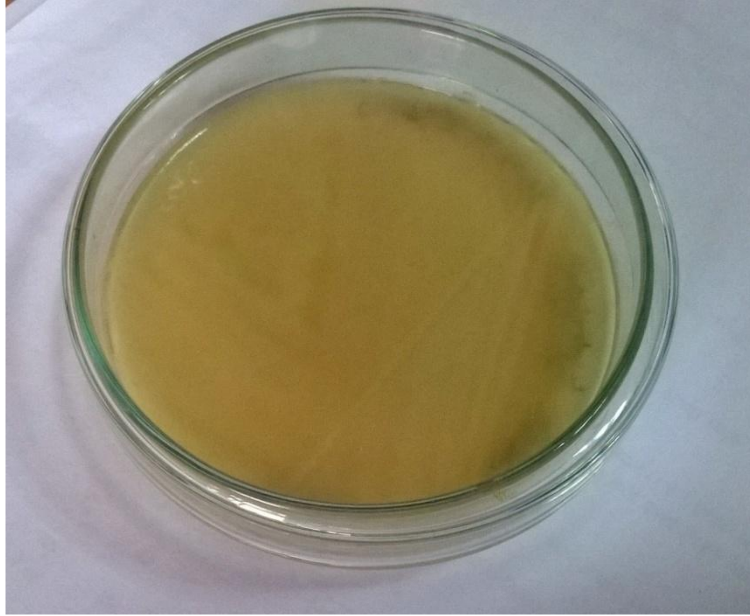

Figure 3.5.9: Gelatin hydrolysis test was performed to examine gelatinase activity of bacteria

Figure 3.5.10: Citrate utilization test was carried out to determine citrate utilization ability of isolates

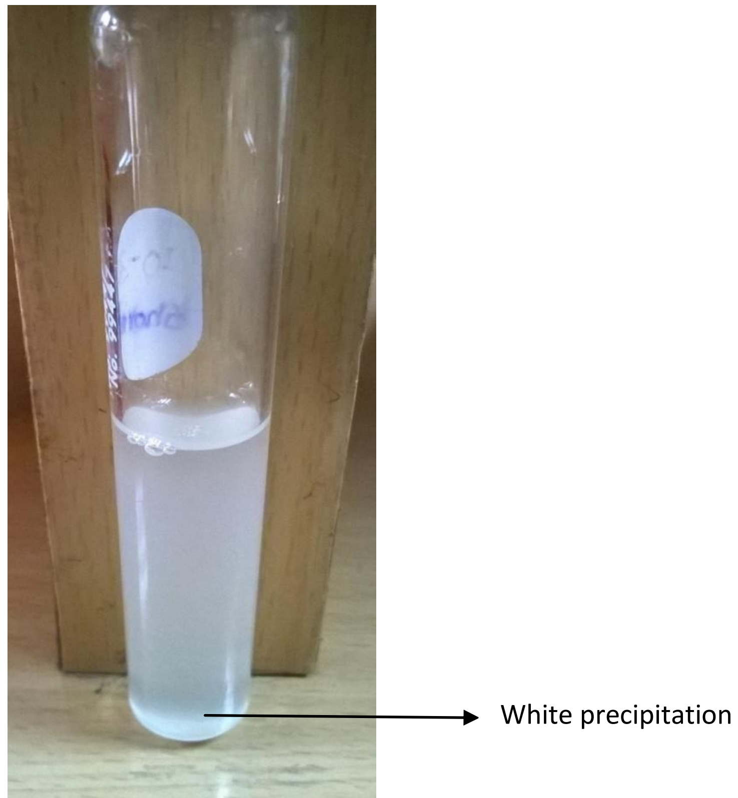

Figure 3.5.11: Turbidity was observed in YEM broth containing 8% KNO_3

Figure 3.5.12: Growth was observed in presence of 2% NaCl

Figure 3.5.13: Growth on nitrogen free media

3.6.1 Effect of *Rhizobium* strain on plant growth and fixation of atmospheric nitrogen:

Four seeds were sowing on each pot for pot trials. Two pots were used for this experiment. Green pot contained sterile soil along with Rhizobia and white pot contained only sterile soil. Forty five days later results were observed.

3.6.1.A Effect of *Rhizobium* strain on plant growth

After six days of seed sowing, one of the four seeds was germinated on the green pot which contained *Rhizobium* strain. Second seed germination was observed after twenty days of seed sowing on the same pot. At white pot (which did not contain bacterial strain), germination was observed after eleven days of seed sowing. Two plants were grown in green pot and only one plant showed growth on white pot. Growth measurement was done forty-five days later from seed placement day. The plant growth measurements were 85 cm and 40 cm on the green pot where on the white pot growth measurement of the plant was 80 cm.

Figure 3.6.1.A: Effect of *Rhizobium* strain on plant growth

3.6.1.B Fixation of atmospheric nitrogen

Plants were uprooted and washed up with running water to clean the soils which covered the roots. Roots were then dipped into fresh water for few minutes on a tray to remove the adhering soil particles. While plant roots were separated from soil, it was observed that root nodules were present on those plants collected from the soil contained bacterial strain but no root nodules were seen on the plant collected from soil without *Rhizobium* strain. This phenomenon suggests that *Rhizobium* strain present on the soil was able to fix atmospheric nitrogen otherwise root nodules would not be produced.

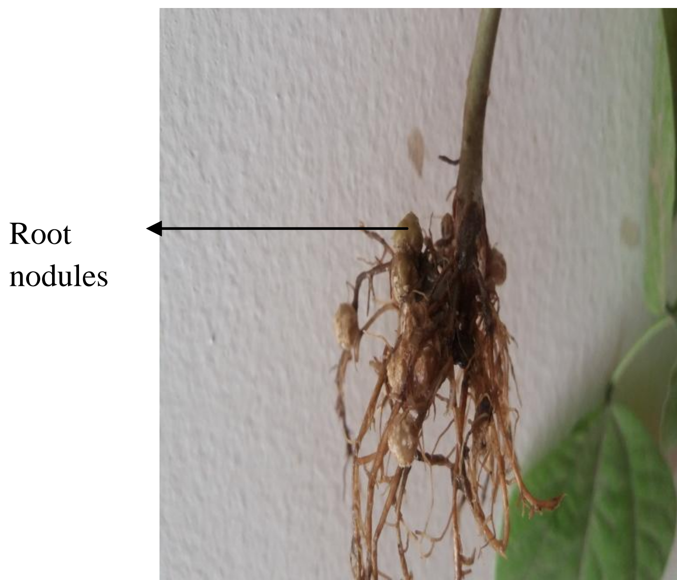

Figure 3.6.1.B(a): Plant with root nodules grown in soil inoculated with *Rhizobium*

Figure 3.6.1.B(b): Plant without root nodules grown in soil without *Rhizobium*

4. Discussion

In this study, strains of root-nodulating bacteria were isolated from the root nodules of hyacinth bean (*Lablab niger)* growing in different areas of Bangladesh. All the strains showed growth in three days and turned the yeast extract mannitol agar media containing bromothymol blue to yellow colour. It indicated that all were fast growers and acid producers (Alemayehu, 2009). The colonies were 2-4 mm in diameter, circular, translucent, glistening, elevated, convex with smooth edges, raised colonies. Microscopic examination revealed that the isolates were rod-shaped and gram negative in nature (Keyser, 1982; Anand and Dogra, 1991; Singh et al., 2008). Strains were also nonspore forming. In the present study, all isolates were Oxidase negative as experiment showed no colour change in the region of the colonies after addition of p-aminodimethylaniline oxalate on the surface of isolates (Kovaks 1956). The catalase test was positive. While isolates were flooded with hydrogen peroxide, the release of oxygen bubbles around bacterial colonies was observed. Urease test also showed positive results. In this experiment, YEM broth was altered with 2% w/v urea in company with 0.02% phenol red and inoculated with log phase culture. After 48 hours of incubation period, it was observed that colour of the broth changes from red to orange that is the indication of urea hydrolysis according to Lindstrom and Lehtomaki (1988). It means that experimenting microbes are able to use urea as a source of carbon and energy for growth. Strains were unable to utilize citrate (Lupwayi and Hague..1994). For this experiment, YEM media had to modify by replacing mannitol with an equal amount of sodium citrate and bromothymol blue (25 mg/l) and incubate. After finishing the incubation period colour remained blue. If strains were able to utilize citrate colour would be changed from blue to green. Our experimented Rhizobial cells did not produce gelatinase enzymes because clear zone formation was not seen around colonies. In the present study the isolate grown on modified YMA (contained 0.4% w/v) but did not show gelatinase activity. Negative gelatinase activity of *Rhizobium* was also observed by Hunter et al., (2007). Positive results were obtained from the starch hydrolysis assay as clear zone were present according to De Oliveria et al., (2007) which directed that investigational strains are able to use starch as a carbon source. Isolate in this study were able to tolerate 2% NaCl, which is in accordance with the characteristics of fast-growing *Rhizobium* (Holt et al., 1994). Present study indicated that

isolates showed growth on 0.5%, 1%, 1.5% and 2%, 2.5% (w/v) up to 4% of NaCl concentrations. No growth was observed at 5% NaCl concentration though the temperature was 28˚C and pH was 7. Growth rate decreased rapidly in high salt concentration. Some *Rhizobium* sp. are able to grow in the presence of 4.5% and 5% NaCl according to Kassem et al., (1985) and Kucuk et al. (2006). This study reported that the isolated strain is highly tolerant to high salt concentrations and have the potential to improve acquiesce of legumes in high salt concentration (El-Mokadem et al., 1991). Results also showed that optimum pH for Rhizobia strain was between pH 6 to pH 7. No growth was observed in the medium with pH 3.5, pH 4 and pH 9. Growth was also observed at pH 7.5. At pH 8 very few amount of growth was observed. This result indicated that experimented strain are not acid adapted as they were not capable of surviving at pH lower than 6.

Glenn and Dilworth, (1994); Correa and Barneix, (1997) reported that different strains of the same species may vary extensively in their capability of pH tolerance. Some Rhizobia can show more sensitivity to low pH than their host and this may hamper the symbiotic relationship between them and may perhaps limit the survival of *Rhizobium* (Zahran, 1999).

All isolates showed growth on temperature 26˚C, 27˚C, and 28˚C .No growth was seen on plates incubated at 34˚C, 37˚C, 38˚C according to Jordan (1984) and 40˚C though the pH was 7. This experiment indicated that strains are not heated tolerating. Moreover, temperature range depends highly on the strains of *Rhizobium* which are reported by Jordan (1984). Despite the fact that some strain may survive in high temperature but it does not represent that they are proficient in nitrogen fixation.

Indeed it is reported by Zahran (1999) that, some *Rhizobium* strains able to grow on higher temperature and formed nodules but the nodules became ineffective and the plant did not accumulate nitrogen.

The presence of the strains growing under stressed laboratory conditions in our study indicates their significance in contributing biologically fixed nitrogen to stressful ecosystems.

5. Conclusion

In this present study, *Rhizobium* sp. was isolated from the root nodules of leguminous plant hyacinth bean (*Lablab niger),* observed their morphological and biochemical characteristics by using different types of media and determined their nitrogen fixation ability and their effects on plant growth. Resulted strain is capable for nodulation and fixing the higher amount of nitrogen. Isolates also showed optimistic effects on plant growth. These findings may give us a prospect to explore extensive research and also using *Rhizobium* as a cheaper substitute for urea.

6. References

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Appendix

Instruments

Autoclave	SAARC
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
Oven, Model:MH6548SR	LG, China
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Refrigerator (4˚C), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Shaking Incubator, Model: WIS- 20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Weighing balance	ADAM EQUIPMENT™, United Kingdom