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ORIGINAL RESEARCH ARTICLE

Isolation, Identification and Production of Encapsulated *Bradyrhizobium japonicum* and Study on their Viability

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Abstract

Rhizobium, a nitrogen-fixing bacteria is the essential feature of leguminous plants which is essential for the regeneration of nutrient-deficient soil. This study was aimed to isolate, identify, mass culture and immobilize *Bradyrhizoium japonicum* in encapsulated form and test their viability. Root nodules were sterilized, grinded and cultured aseptically in YEMA media containing Congo red. The obtained colon was sub-cultured to get a pure culture and different biochemical tests were conducted which proved *Bradyrhizobium japonicum* as the slow-growing species. The test shows a positive result of catalase production and nodulation test whereas the pH tolerance test shows more tolerance to the acidic pH. Similarly, *Bradyrhizaobium japonicum* can tolerate 1% and 2% NaCl concentration and it doesn't show resistance to the penicillin disc of 10mg. The mass culture and encapsulation with sodium alginate adding sucrose as nutrient proved the simplicity for handling. Altogether 548 beads were prepared from the 100ml of the cultured broths which were viable for more than 190 days at 1%, 2% and 3% sucrose concentration but less viable at 5% and 10% sucrose concentration under room temperature.

Keywords: *Bradyrhizobium*, encapsulation, immobilization, viability, Legumes, symbiotic bacteria

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Introduction

A distinctive characteristic of the majority of legumes is their ability to enter into a nitrogen-fixing symbiosis with a distinct group of soil bacteria collectively called root nodules bacteria or the *Rhizobia* [1,2]. The *Rhizobia* reduce the atmospheric nitrogen into ammonium which is termed as the biological nitrogen fixation and is more advantageous in the perspective of soil quality. The productivity and sustainability of agriculture throughout the globe are being significantly enhanced through nitrogen fixation from effectively nodulated legumes [3]. However, only certain combinations of legumes and Rhizobia result in the formation of effective nitrogen-fixing nodules even though many moderately effective and ineffective combinations may and do arise. Thus the *Bradirhizobium japonicum* is host specific and nodulate only the species of soybean. Apart from direct benefit from effective nitrogen fixation [4] legumes and *Rhizobium* provides added value in weed, pathogen and insect control when rotated with crop in farming system [5] together with improving soil structure and increasing soil organic matter content [6].

The importance of legume crops to world production and compelling needs to exploit the nitrogen fixing potential of those crops have focused attention on technologies for the production of more effective legume inoculants. Most legume inoculants have been prepared by adsorbing broth culture of selected *Rhizobia* on a suitable carrier such as peat, clays, charcoal, lignite, cellulose powder, various powdered crop residues or soil compost mixtures. In 1979, Dommergues *et al.* [7] proposed to entrap rather than adsorb *Rhizobium* cells by incorporating the bacteria in a polymeric gel. The encapsulation of the inoculants with polyacrylamide maintained the suitable moisture content. These formulations of immobilized cells protect the microorganism against the environmental stresses and release them to the soil gradually when the polymers are degraded [8]. Increasing the efficiency of the use of available soil nitrogen can meet the additional Nitrogen demand by making cereal plants capable of fixing its own nitrogen through close

association with diazotrophic bacteria will pay off in term of increasing cereal production and helping resource poor farmers as well as saving the environment [9].

Symbiotic Nitrogen fixation is an important source of nitrogen and the various legumes crops and pasture species often fix as much as 200-300 kg Nitrogen per hectare [10]. Globally, symbiotic nitrogen fixation has been estimated to amount to at least 70 million metric tons of nitrogen per year [11]. In 1990, world consumption of fertilizer Nitrogen is 88 million tones and apart from the consumption of nonrenewable energy sources, environmental pollution from fertilizer Nitrogen escaping the root zones is high because in many cases Nitrogen fertilizers are not used efficiently by crops [10]. Therefore biological nitrogen fixation is an important and integral component of sustainable agricultural system. Furthermore, biological nitrogen fixation from legumes offers more flexible management than fertilizer nitrogen because the pool of the organic Nitrogen becomes slowly available to non-legumes species [10]. Concomitant with Nitrogen fixation, the legumes in rotation offers the control of crop disease and pests [3,12]. The Bellagio conference on N_2 fixation [13] acknowledged that with the decline in the price of manufactured fertilizer in 1990s, biological nitrogen fixation with legumes and Rhizobia, was most likely to remain in extensive rather than intensive agricultural systems. Thus the present study is emphasized for the mass production and immobilization of Rhizobial inoculants in the most effective and cost effective ways of encapsulation.

Rhizobia are the gram negative, rod shaped, aerobic and heterotrophic soil bacteria, which includes genera *Rhizobium*, *Bradyrhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium,* and *Azorhizobium*, which are able to form symbiosis with leguminous plants. They are facultative symbionts that have adapted to persist for long period in soil in a free living state if the suitable legume host is absent [2]. They could form the specialized organs, called nodules, on roots or stems of their hosts. Rhizobia inside nodule could reduce atmospheric nitrogen and make it available to the plant. Symbiotic rhizobia are common colonizers of the rhizosphere of both legume and non-legume plants and in addition to legumes they are also endophytes of several non-legumes like rice and maize [14]. However, non-symbiotic rhizobia can also be present in soil [15]. In the old system of classification the *Rhizobium* fall into two groups based on their growth characteristics i.e. fast growing and the slow growing *Rhizobium*. Fast growing *Rhizobium* are acid producers which develop pronounced turbidity in liquid media within 2-3 days and have the mean doubling time of 2-4 hours. The cells are rod shaped to pleomorphic, 0.5-0.9 microns in diameter and 1.2 to 3.0 micron long, and are motile by 2-6 peritrichous flagella. Whereas slow growing *Rhizobium* are alkali producing Rhizobia and require 3-5 days to produce moderate turbidity in liquid media and have the mean doubling time of 6-7 hours. The cells are predominantly rod shaped and motile by a single polar or sub-polar flagellum [16].

Since 1886, when it was discovered that bacteria caused the formation of the nitrogen-fixing nodules; then, the isolation of rhizobia from the nodules as pure cultures opened the way for artificial inoculation to replace the 'soil transfer' method, in which dry soil, from a location where the legume had been grown previously, was coated onto the seed just before sowing [17]. This dust method was modified to the "soil-paste or muddy water process", in which the soil was mixed with water before pouring over the seed [18]. The first commercial pure (agar) culture inoculants have been patented by Nobbe and Hiltner in 1896 [44]. Their patented culture was placed on the market under the name Nitragin, which consist of a pure culture of desired strain of rhizobia grown in flat glass bottle containing only a small amount of solid gelatin medium. This material was either to be applied to seed or mixed with soil and spread over the field [19]. Then, solid carrier such as soil or peat was first suggested in 1914 [17]**.** Present day inoculants production techniques have been changed from those of the early 1900s. Even many types of inoculant have been investigated; peat is the best carrier and is widely accepted in the inoculant industry. However, the challenge today is to

develop further improved inoculant formulations and methods of application.

In Nepal, Rhizobial inoculants has been used from few years. Rhizobial inoculants had been produced in soil science department of Nepal Agricultural Research Council. This was studied and conducted by Sanu kesheri Bajracharya. Powder inoculums were made in soil and goal mixture in 3:1 ratio. But for the research proposes liquid inoculums is being used. The work was performed under the supervision of Soil Science Department and Farmer Centered Agricultural Resource Management (FARM), Asian Bio-Technology and Bio- Diversity Sub–Program Nepal (Annual reports of Soil Science Department of Nepal Agricultural Research Council). Some research has been done on the effect of the peat based inoculums of the *Bradyrhizobium japonicum* on the *Glycine max* in the university researches.

Although rhizobia seem to be widely distributed in the soil, however soil in different places contains different strain of rhizobia and these rhizobia may not be effective for nitrogen fixation, and may not be appropriate for all legume. Some soil may have effective rhizobial strain, but the number of rhizobia is low or containing higher number of ineffective strain [20]. Inoculation of legume seed is a simple and practical means of ensuring effective nitrogen fixation. However, to answer the question "Is inoculation of seed necessary?" is critical, even the use of rhizobial inoculant is not necessary in that area. Therefore, Allen [21] has listed four indicators that, if positive, the inoculation would be beneficial i.e. the absence of the same or symbiotically-related legume in the Immediate past history of the land ; Poor nodulation when the same crop was grown on the land previously; when the legume followed a non-legume in the rotation and when the land was undergoing reclamation.

Rhizobial inoculants can be immobilized in different materials. The material for peat based carrier is obtained from a naturally occurring organic material. The supply of peat based organic material is limited. Even other solid materials such as lignite, charcoal, coir dust and compost of various agricultural wastes have been used instead of peat but their performance characteristics are not equivalent to peat based inoculants product [22]. Therefore it is important to immobilize the *Rhizobium* in any other suitable form as sodium alginate encapsulation. In solid and liquid inoculants three basic contaminant types were observed, such as bacteria, actinomycetes, and fungi. These include the possibilities of pathogenicity to human, animal, plant or rhizobia, which reduce the effectiveness of inoculant [23]. Thus it is necessary to immobilize the bacterial cells in the form of encapsulated beads made in aseptic condition which prevents the contaminants and well as preserved the cells for several months without losing their viability. Also the encapsulated beads are easy to handle, to use and to do packaging and distribute to the farmers.

Unbalanced use of chemical fertilizers had led to a reduction in soil fertility and to environmental degradation [24] and the cost of chemical fertilizers has increased so that it is unaffordable for farmer of developing country such as Nepal. As a consequent, there has recently been a growing level of interest in environmentally friendly sustainable agricultural practices including organic farming systems [25]. For example, *Rhizobium* and phosphate solubilizing microorganisms would reduce the need for N_2 and P chemical fertilizers and decrease adverse environmental effects. Therefore, in the development and implementation of sustainable agriculture techniques, bio-fertilization is of great importance in alleviating environmental pollution and the deterioration of nature [26]. A tightening of the agricultural N_2 cycling to reduce N losses and an increase of N_2 inputs through BNF to replace artificial fertilizer N_2 use can help achieve this goal while at the same time maintaining agricultural production and reducing greenhouse gas emissions and energy consumption for the production of artificial N fertilizers [27-29].

Materials and Methods

The materials used for the present study were root nodules of *Glycine max* (white seeded species) grown at the earthen pot. The seed of

Glycine max were taken from the market for the test. The necessary equipment and the chemicals required for the completion of the research were provided from the Biotechnology and Biochemistry unit of Central Department of Botany.

Preparation of YEMA media [30]: All the ingredients required for the preparation of the YEMA media except agar and Congo red were dissolved in the 950 ml of the sterilized water. Congo red was dissolved separately in the next conical flask in 50 ml of water and sterilized them separately. Then pH was maintained to 6.8-7.0. Agar was added in the mixture of the ingredients and sterilized in Autoclave at 121 degree Celsius and 15 lb. pressure for fifteen minutes. From the Autoclave, media was directly taken to the Laminar Air Flow Chamber and Congo red was mixed with the ingredients mixture and poured in the sterilized petri plates and allowed it to cool down. Finally the media was ready for the inoculation of the *Rhizobia*.

Isolation of *Bradyrhizobium japonicum*

Collection of root nodules: The roots of the soybean (white seeded species) were collected from Putalisadak, Kathmandu which were cultivated in the pots at the rooftops. The soil from the root was removed by washing with tap water. Then only the fresh, turbid, matured and pinkish colored nodules were selected and collected on the beaker. Only 0.2 gm. of nodules were taken for the present study.

Surface sterilization of the root nodules: Root nodules were rinsed with tap water to remove the soil particle followed by rinsing with detergent and few drops of tween-20 for 1 hour in the running tap water. Roots nodules were dipped in 95% ethanol for 5-10 seconds under laminar air flow chamber and transferred to 2.5% sodium hypochlorite for 2-4 minutes. Then rinsed with sterile water for five times.

Preparation of the inoculants: The root nodules were crushed in 1ml of sterile water in the test tube with the sterile glass rod. Then the solution was made 10 ml by adding sterile water. With the help of the pipette, 1 ml of the solution was taken in the next test tube and the final volume was made 10 ml by adding 9 ml of the sterile water to make 10^{-1} dilution of the solution. Similarly, the solution was serial diluted upto 10^{-6} by transferring 1ml solution from the former test tube to the next one. From each of the serial dilution, 0.5 ml solution was taken and inoculated in the YEMA media by spreading with the help of L-shaped glass rod. Finally, the plates were incubated at 30^0C in dark in inverted position for 4 days. To isolate the pure culture of *Rhizobia*, only red colony from 4th day cultures were taken with the inoculating loop and streaked in the YEMA media with Congo red and incubated at same condition as before.

Identification of *Rhizobium*:

The species of *Rhizobium* were identified on the basis of its host as well as some biochemical tests as mentioned below:

Catalase production test [31]: The dark red portion of 18 to 24 hours pure colony was picked with the help of an inoculating loop and placed in the clean glass watch. Then few drops of the 3% H_2O_2 were added over the organism on the watch glass with the help of the Pasteur pipette. The immediate emergence of bubbles shows the production of catalase.

pH tolerance test: YEM broth was prepared without adding the agar in the YEMA media and adjusted to different pH as 4.5, 7, 9 and 9.5 by adding HCl and NaOH. Then the media was sterilized and *Rhizobium* strain was inoculated and incubated for 14 days at 30 degree Celsius and observed the growth of the *rhizobia.*

NaCl tolerance test: YEMA plates with different concentration of NaCl (1%, 2%, and 4%) was prepared, sterilized and inoculated with *Rhizobium* and incubated for 14 days at 30 degree Celsius and observed the specific growth of the *Rhizobium.*

Penicillin resistance test (Kirby-Bauer Method) [32]: YEMA plates were prepared and placed right side up in an incubator at 37 ^{0}C for 10 to 20 minutes with the cover adjusted so that the slides are slightly opened. Each plates were labeled with the name of test organism to be inoculated. A sterile cotton swab was dipped into a test culture and removes excess inoculums by pressing the saturated swab against the inner wall of the beaker containing the test organism.

Using the swab, the entire agar surface was streaked horizontally and vertically to ensure a heavy growth over the entire surface. The culture plates were allowed to dry for about 5 minutes. Using the aseptic technique the penicillin disc was applied on the agar surface by using sterile forceps. Each disc were kept at least 15 mm from the edge of plate. Each disc were gently pressed down with the sterile forceps to endure that the disc adhere to the surface of the media. The plate cultures were then incubated in an inverted position for 24 to 48 hours at 30^0C. Finally all the plates were examined for the presence or absence of a zone of inhibition surrounding each disc.

Nodulation test [33]: The seeds of soya bean were taken and surface sterilized in running tap water followed by dipping in 95% ethanol for 1 minutes. Seeds were then washed with 6 consecutive washing with sterilized water. Then the earthen pots along with 1:1 ratio of sand and soil were sterilized in Hot Air Oven at 160^0C for three hours. The sticking solution was made by adding 10% sucrose in distilled water which was first heated and then cooled to make sticky. The Rhizobial inoculants of 4 days culture were added in the sticker solution to make slurry. The seeds of soybean were mixed in that slurry and stirred completely to make the inoculants attached on the seeds. They were then taken out and rolled on the $CaCO_3$ to maintain the alkalinity, the process is called pelleting. The seeds were then dried in the air and sown in the sterilized earthen pots at the depth of one inch. Similarly the seeds without inoculating the Rhizobia are also sown in the next pot. Finally the pots were watered and covered with transparent polyethylene sheet and tied around the pots. The polyethylene were made to have lots of holes for watering as well as for providing ventilation and kept in the green house. They were watered regularly and observed for the nodulation when the plant becomes 10-15 cm high. The presence of nodules in the inoculated plants and absence in un-inoculated plants shows the positive result of the respective Rhizobial strain.

Color change of BTB: YEMA plates containing BTB were prepared similarly as the YEMA plates with Bromothymol Blue and inoculated with test organism and incubated at 30^0 C and observed the color change after 4-5 days. The appearance of blue color shows that the rhizobial strain is slow growing and the appearance of the yellow color shows that the rhizobial strain is of fast growing type.

Mass production of *Rhizobium*

Starter culture of Rhizobium: YEM broth medium (100 ml) was prepared and autoclaved by transferring in a flask. Thereafter, pure *rhizobium* colony was transferred into sterilized YEM broth. Inoculated YEM broth was incubated at the water bath at 30^0C for four days. This was the starter culture of the Rhizobium.

Mass culture of Rhizobium: For the mass culture of *Rhizobium*, YEM broth was prepared in the large quantity in the conical flask and sterilized as mentioned before. The PH was maintained 6.5 to 7.0. Then the sterilized YEM broth was inoculated with the broth of starter culture prepared in advance. This was incubated for 3-4 days on the water bath at 30^0C. The culture was tested for the purity by inoculating in the YEMA plates staining with Congo red. The broth culture was then transferred to the large flask and incubated for 4-9 days for the bacterial growth.

Encapsulation of *Rhizobium* [34]: The *Rhizobium* were immobilized by encapsulating with sodium alginate along with different concentration of sucrose as their nutrient. Beads were prepared aseptically in laminar air flow chamber by using dropper and the micropipette. From the mass culture of Rhizobium of 4-9 days, 25ml of broth was taken in four different beaker. Then the 2% sodium alginate was weighted and mixed in the broth in each beaker. The sucrose concentration of 1%, 2%, 5% and 10% was added in different beaker and leveled them. The sterilized magnet was kept in the beaker and covered with the aluminum foil. Then the beaker was kept on magnetic stirrer at 250 rpm for 8 minutes in order to dissolve the sodium alginate and the sucrose. On the other hand the solution of the 0.2 M $CaCl_2$ was prepared in the 1 liter beaker. The solution of the inoculums, sucrose and the sodium alginate was allowed to settle down for few minutes so that the air bubbles get disappeared.

Table 1: Enumeration of organism by spread plate technique.

S.N	Dilution factor	Inoculums size(ml)	No. of colonies per plate	No. of organisms	Average no. of organisms	Number of c.f.u. per ml
1.	10^{-1}	0.5	339	33.9		
2.	10^{-2}	0.5	76	0.76		
3.	10^{-3}	0.5	17	0.017	578×10^{-2}	1156×10^{-2}
4.	10^{-4}	0.5	2	0.0002		
5.	10^{-5}	0.5	5	0.00005		
6.	10^{-6}	0.5	1	0.000001		

Then the mixture was dropped from about 30 cm height by using the blunt ended pipette and collected in beaker containing 0.2 M $CaCl_2$ solution. The rounded beads being formed in the beaker were

stirred regularly to prevent them from being attached with each other. After 30 minutes beads were formed which were taken out from the $CaCl_2$ solution by filtering with a muslin cloth and kept in the filter paper to be air-dried and left overnight. Finally, the air-dried beads were kept in the lead closed culture tubes for further use to test their viability.

Viability tests of the encapsulated beads of *Bradyrhizobium japonicum* (modified from [35]): The sodium alginate encapsulated beads hence prepared were stored in the airtight culture tube at room temperature for the further viability test. For the viability test YEMA media was prepared and sterilized as mentioned before. With the sterilized forceps the beads were inoculated in the surface of the media. The beads with different sucrose concentration were inoculated in different plates for testing the viability. Then they were incubated at 30^0C for 48 to 72 hours in the incubator. The plates were observed for the formation of the Rhizobial colony in the surface of the media. The same process was repeated at the interval of 2 weeks, 4 weeks, 6 weeks, 8 weeks, 10 weeks, and so on up to 7 months.

Results

Isolation and enumeration of the colonies

The *Bradyrhizobium* were isolated in the YEMA media and the number of colonies formed in the plates were enumerated and the average number of the cell forming unit were calculated by using following formula:

$$\text{Dilution factor} = \frac{\text{volume of the sample used}}{\textit{Total volume of sample and the diluents}}$$

Number of organism = dilution × number of colonies

$$\text{Number of cfu per ml} = \frac{\text{Number of organisms formed in average}}{\text{Inoculums size} \times \text{dilution}}$$

Here one colony is considered as one colony forming unit (cfu).

The enumeration by spread plate method shows random result where 1st dilution have more number of colonies and the 6th dilution have the lowest number of colonies but the other have the ascending number of the colonies except the 4th serial dilution which have the less number of the colonies than the 5th dilution which alter from the principle of the serial dilution. The calculation shows that altogether 1156×10^{-2}colony forming units are present in the 1 ml of the original sample obtained from the root nodules.

Shape, size, color and texture of organism on the plate: In the first plate many colonies were formed by the inoculation of the *Bradyrhizobium* inoculums which were irregular shaped and some were concentric and spreading. Some colonies were large enough and some were too small. The colony formed after re-streaked had shown smooth, the raised and convex shape at the place of the streak. The size of the colonies in average was 4-5 mm and the maximum colony was achieved in 6-8 days of culturing as it was noticed under visual estimation. The colonies were watery translucent with dark red rib like marking in the center of the streak. Their color was noticed pinkish red on the plate of re–streak.

Biochemical tests: Many biochemical tests have performed which confirmed the isolated bacterial strains as the *Bradyrhizobium japonicum*.

Table 2: Biochemical tests on *Rhizobium spp*

S.N	Biochemical tests	Result	Remarks
1.	Catalase production test	+ Ve	
2.	Penicillin resistance test	-Ve	
3.	**pH tolerance test**		
	pH 4.5	+Ve	
	pH 7	+Ve	Slow growing rhizobia
	pH 9	-Ve	
	pH 9.5	-Ve	
4.	**NaCl tolerance test**		
	1% NaCl	Extreme	
	2% NaCl	More	
	4% NaCl	Less	
5.	Color change of BTB	Yellow	
6.	Nodulation test	+ve	

+Ve = positive, -Ve= negative

Table 3: Number of beads formed from the 25ml of cultured solution in different concentration of sucrose.

S.N	% of soidum. alginate	% of sucrose	Calcium carbonate $CaCl_2$(M)	Beads per 25ml	Average beads
1.	2%	10%	0.2	169	
2.	2%	5%	0.2	127	
3.	2%	3%	0.2	126	137
4.	2%	2%	0.2	146	
5.	2%	1%	0.2	117	

Table 4: viability test of the encapsulated beads of *Rhizobium japonicum*

S.N	Periods of viability test (In Days)	Concentration of the sucrose 1%	2%	3%	5%	10%
1	7	+	+	+	+	+
2	20	+	+	+	+	+
3	50	+	+	+	+	+
4	75	+	+	+	+	+
5	100	+	+	+	+	+
6	120	+	+	+	+	+
7	145	+	+	+	+	+
8	170	+	+	+	-	-
9	190	+	+	+	-	-

*(+)means viable and (–) means not viable

Immobilization of Rhizobial strain: As the Rhizobial strain was immobilized by encapsulating in the beaded form with sodium alginate hardened by $CaCl_2$ and mixing the sucrose as the additives, the number of beads formed from every 25 ml of broth were enumerated and the beads formed per liter of the broth was calculated which is mentioned in the **Table 3**.

The number of beads formed from every 25 ml of the cultured broth was different. An average of 137 beads were formed from 25 ml of the solution.

Viability tests of the encapsulated beads: The encapsulated beads of the *Bradyrhizobium japonicum* were kept in the sealed bottle and they were tested periodically for the viability of the bacterial cells. The result of the viability test done up to 190 days is shown in the **Table 4**:

The result of the viability tests shows the diversified results. The beads prepared at 1%, 2% and 3% sucrose concentration had shown the viability up to six months. On the experiment done on 170th day and 190th day, the Rhizobial strain was absent and the zone clearance rings were observed around the beads having 5% and 10% sucrose concentration on the YEMA plates.

Discussion

Present study was carried on *Bradyrhizobium japonicum* and it was based on the *Rhizobium* present on the root nodules of the soybean species found in Nepal. Different methods and the materials were used for the isolation, identification, mass culture, immobilization and viability tests. The data obtained have been

discussed with the relevant information and the similar works carried out by the different investigators. Very few works have been done in Nepal but several works have been done by the foreign researcher. From the present study performed on the *Bradyhzobium japonicum*, varied responses were obtained.

For the identification of the bacterial species present in the root nodules of the soybean, different tests have been performed. Different biochemical tests performed for present study reveals that the strain of the *Rhizobium* under study was the slow growing species. The catalase production test of the *Bradyrhizibium japonicum* shows the positive result which is adjacent to the Rhizobial isolates of the alfalfa as in the biochemical characterization performed by Shahzed *et al*.[36]. It means that the Rhizobial isolates of the present study contain the catalase enzyme which decomposes the hydrogen peroxide to release oxygen. This conforms that the *Bradyrhizobium japonicum* is the cytochrome containing aerobic bacteria as described by Buchanan and Gibbons [37] that *Rhizobia* are aerobic bacteria utilizing oxygen as the terminal electron acceptor. Similarly the Rhizobial isolates of the present study shows the less resistance to the penicillin disc 10µg which indicate that penicillin is effective to the *Bradyrhizobium japonicum* which reduced the growth of the *rhizobium* showing the antimicrobial activity to rhizobia. The pH tolerance test performed for the present study shows that the rhizobial isolates can tolerate the low pH but cannot tolerate the high pH. It means that *Bradyrhizobium japonicum* is the acid tolerant species of the rhizobium. As Thornton & Davey [38]; Richardson & Simpson [39] mentioned that slight change in pH alone can significantly affect the growth of root nodule bacteria ,the *Bradyrhizobium* shows the high growth in pH 4.5 and 7 whereas it can't grow in pH 9 and and 9.5. The concentration of the sodium chloride also effects the growth and the survival of the *Rhzobium* species. As mentioned by Singleton *et al* [22] that increasing salt concentration may have detrimental effects on rhizobial population, the *Bradyrhizobium japonicum* grow well in the 1% and 2% NaCl but do not grow well in 4% NaCl concentration and it has also vital role in the cell viability for 7 weeks in the YEMA plates. Also the nodulation test shows the positive result of the present study confirmed the isolates as the *Bradyrhizobium japonicum* since it is host specific. The color change of BTB to yellow showed that it is the fast growing species but the all other results biochemical tests points it as the slow growing bacteria.

The mass culture of the Rhizobial isolates of the present study shows the dense growth of the bacteria at 7-9 days of the inoculation forming the dense mass at the surface of the YEMA broth. It also indicate that it is the slow growing species of *Rhizobium* since the fast growing species grow densely at 4-6 days of inoculation at the temperature of 30^0C.

The cultured mass of the *Rhizobium* was immobilized in the form of the encapsulated beads by using the sodium alginate extracted from algae as studied by Neely & Pettitt (1973) [40]. The preparation of encapsulated beads of *Rhizobium* was not easier and it has many limitations in its procedure. The missing of one step hampers severely the formation of beads. The height of dropping, time and rotation of magnetic stirrer are the most important factors. An average of 137 beads was prepared from 25ml of broth. Thus 548 beads per 100 ml can be prepared within the limitation of time and rotation of magnetic stirrer. The less rotation and the over rotation results in the deformation of beads. The large volume of the inoculant in the small beaker with small magnet could not dissolve the sodium alginate and hence the beads formation is effected which could not remain in the beaded form for the longer period at room temperature and dissolves itself. As Saiprasad (2001) [14] reported that Sodium alginate was the most accepted hydro-gel and frequently used as a matrix for the synthetic seeds because of its low toxicity, low cost, quick gelation and biocompatibility characteristics, it was used as the gelling agent along with the sucrose as the additives for their survival on the basis of study performed by Vincent [41] and found that 24-44% of cells suspended in a 10% sucrose solution

survived primarily drying whereas only 0.1 % survived when suspended in water. 2% of sodium alginate was found to be the best for the encapsulation which are hardened by 0.1 M $CaCl_2$ as noticed by Kierstan & Bucke (1977) [42]. When the beads encapsulated with sodium alginate were stored at the room temperature and tested for their viability, they showed the viable cells for six months. The air dried beads kept sealed in the culture tube have maintain their beaded structure for several months. The different sucrose concentration mixed as the additives for their survival have played the important role. In 1%, 2% and 3% sucrose concentration the cells were viable for 190 days of inoculation whereas in 5% and 10% sucrose concentration the cells were survived only for 145 days. Mcleod (1961) [43] had found that the incorporation of 10% sucrose in yeast Mannitol broth improved the survival on glass beads compared with un-amended broth cited by Vincent [41] but the survival of the *Bradyrhizobium japonicum* is less at higher sucrose concentration and vice versa. It shows that the sucrose at low concentration maintains the moisture content and support for the viability of the *rhizobium* whereas the higher concentration of the sucrose effects their survival after few months. Thus it can be said that beads of *Bradyrhizobium japonicum* prefers the lower concentration of the sucrose.

Conclusion

Findings of the present study carried on *Bradyrhizobium japonicum* concluded that it can be isolated, identified and encapsulated in the forms of beads which looks like chemical fertilizers found in the market. It also shows that *Bradyrhizobium japonicum* is slow-growing bacteria. Besides soil, peat, charcoal as the solid inoculants and the broth as a liquid inoculant, the rhizobial inoculants can be immobilized in the form of encapsulated beads by using 2% sodium alginate, 1-3% sucrose as additives and 0.1M $CaCl_2$ as the hardening substances. This maintains the moisture content of the beads as well as prevents the contaminants and preserved the cells for several months. This study concludes that the encapsulated beads with sucrose (1-3%) as the additives can be viable for more than 190 days whereas with the 5% and 10% sucrose cells survive only for five months. Also, they are easy for handling as well as can be viable for more than six months in the room temperature. Thus the rhizobial strain can be easily immobilized by using sodium alginate and sucrose as additive.

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