Isolation and Characterization of *Rhizobium* spp. from the Root Nodules of *Phaseolus mungo* L.

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Abstract

The nodules are colonized by a bacteria, that is capable of fixing nitrogen. In this study, the genus *Rhizobium* spp. was isolated from the root nodules of *Phaseolus mungo* L. The plant samples were collected from the legume cultivated field of Aeik Pyat Village, Hinthada Township. This study was carried out in the Microbiology Laboratory of the Department of Botany, Hinthada University. The isolation of bacterial strains was carried out by the method of washing sterilization. The isolated bacteria from pure culture was selected and its cultural characteristics of colony, cell morphology and biochemical characterization were studied. Cell morphology of *Rhizobium* spp. was rod-shaped, gram negative, non-sporulating, motile and aerobic bacteria. According to the morphological characters tests and biochemical characterization tests, the *Rhizobium* spp. was determined to be a nodulated, colonizing bacteria.

Keyword: *Rhizobium*, nodule, nitrogen fixation

Introduction

Microorganisms are living organisms, they are ubiquitous and live in familiar settings such as soil, water, food and plant roots. Soil microorganisms constitute the world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. This microbial diversity significantly enhances the rates of carbon and nitrogen cycles in the ecosystem. Biological nitrogen fixation is carried out by either symbiotic or free living prokaryotic, it is well documented that biological nitrogen fixation mediated by nitrogenase enzymes is a process important to the biological activity of soil. *Rhizobium* is a soil habitat, gram negative bacterium which is associated symbiotically with the roots of leguminous plants. In the process of symbiosis, results in Biological Nitrogen fixation in which atmospheric nitrogen is converted into ammonia and is subsequently available for plants. In turn, plants provide nutrients to the bacterium (Datta *et al*., 2015).

In leguminous plants, breakdown of these legumes by bacteria during ammonification actually returns excess not utilized by the plant to the surrounding soil (Simon and Amare, 2014).

The effectiveness of *Rhizobia* populations in fixing nitrogens correlated with soil fertility status where acidic soils contained less effective *Rhizobia* strains. This research work aimed, isolated and characterization of nitrogen from nodules sample collected from legume cultivated field at Aeik Pyat Village, Hinthada Township. The objectives of this research is to isolate the bacteria from the root nodules of *Phaseolus mungo* L. and to find out the activities of root nodule bacteria. Biochemical characterization of the isolated strain was done for the identification of *Rhizobium* on the basis of different biochemical tests viz., including potato soft rot activity test (Fontem, 1995), starch hydrolyzing activity test, gelatin hydrolyzing activity test, catalase activity test, nitrate reduction activity test, phenylanine deaminase activity test, oxidation activity test, urease activity test, methyl red activity test, triple sugar test (Aryal, 2018), and H_2S gas production test (Cowan, 1975).

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Materials and Methods

Study Area

The plant samples were collected from the legume cultivated field at Aeik Pyat Village, Hinthada Township from December 2018 to February 2019. Fresh and healthy root nodules were selected for the present study. This study was conducted in the Microbiology Laboratory of the Department of Botany.

Isolation procedure of root nodule strain

Procedure of washing method

The roots of black gram plant were brought to the laboratory and washed in running tap water to remove adhering soil particles. Healthy pin, unbroken and firm root nodules were selected and washed in water. Then, the nodules were immersed in 3-5 % H_2O_2 for 5 minutes and repeatedly washed in sterile water for 3-4 times to get rid of the sterilizing agent. The nodules were placed in 70% ethyl alcohol for 3 minutes, repeatedly washed in sterile water and then dried on sterile filter paper. Finally, the nodules were placed on plates containing PGA media (potato extract 100 ml, glucose 2 g, agar 1.8 g, pH ±6.8). Inoculation plates were incubated for 24-48 hours at room temperature in an inverted position (Pagan *et al*., 1995).

Pure culture

The streak plate technique was used by the pure culture method for colony observation and selection of strains.

YEM medium (K_2HPO_4 0.125 g, NaCl 0.025 g, Mannitol 2.5 g,Yeast Extract 0.25 g, Sterilized water 100 ml, Agar 1.8 g, $MgSO_4$ 0.05 g, pH ± 6.5)

Characterization of isolated root nodule strains

The identification of the isolated bacterial strain was carried out using the morphological tests viz., gram staining test (Gram, 1884), endospore formation test (Santra *et al*., 1998), motility test (Woodland, 2004) and oxygen requirement test (Prescoot, 2002), and biochemical activities which include potato soft rot activity test (Fontem, 1995), starch hydrolyzing activity test, gelatin hydrolyzing activity test, catalase activity test, nitrate reduction activity test, phenylanine deaminase activity test, oxidation activity test, urease activity test, methyl red activity test, triple sugar test (Aryal, 2018), and H_2S gas production test (Cowan, 1975).

Results

In this study, a single strain was isolated from the root nodules of *Phaseolus mungo* L., legume cultivated field of Aeik Pyat Village, Hinthada Township. The sample was carried out by the method of washing sterilization. An Isolated microbe of pure culture was selected and studied, its cultural characteristics of colony, cell morphology and biochemical characterization.

Scientific classification

Scientific Name - *Phaseolus mungo* L.

Common Name - Black gram

Myanmar Name - Mat-pe

Family Name - Fabaceae

Outstanding Characters of *Phaseolus mungo* L.

Erect, fast-growing annual, herbaceous legume reaching 30-100 cm in height, stem slightly ridged covered with brown hair and much branched from the base. Leaves are large, trifoliate with a purplish tinge; petiloles are long; leaflets long and ovate. Inflorescences are borne at the extremity of a long peduncle, cluster of 5 to 6 flowers. Flowers bisexual, papilionaceous, small; bracteoles linear to lanceolate, exceeding the calyx. Calyx 5 sepals, campanulate. Corolla yellow, 5 petals: 2 wings petals, 2 keel petals spirally coiled with a terminal horn-like appendage. Stamens 10 in number (9 united and 1 free), diadelphous. Ovary 1-celled. Fruit cylindrical erect pod, hairy, buff to dark brown at maturity.

Habit

Nodules

Fig 1. *Phaseolus mungo* L.

Morphological Tests of an isolated bacterial strain

Gram staining test

According to the gram staining test, the colour change after each step, is based on the difference in the composition of the cell wall. Colour of bacteria changed to purple is gram positive or pink or red colour changed with gram negative.

The isolated bacterial cells were showed a pinkish purple colour. So, the isolated bacterial cells indicated the gram negative group.

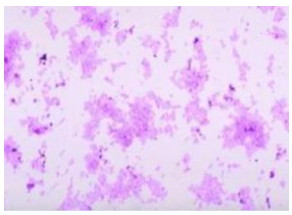


Fig 2. Cell morphology of the gram staining test

Endospore formation test

In this test, the morphology of bacterial cells indicated the green in colour under the microscope of positive reaction. Negative reaction not a change in colour.

The isolated bacterial cells given a negative reactions.So, the isolated bacteria showed no endospore formation.

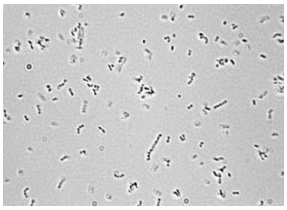
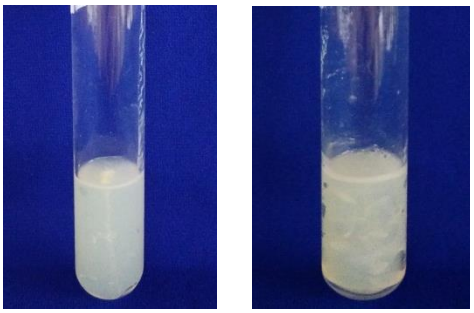


Fig 3. Endospore formation test

Motility test

Motile bacteria move about with flagella. Non-motile bacteria lack of flagella. Motile bacteria give diffuse hazy growths that spread throughout the solid medium rendering it slightly opaque but not transparent. Non motile bacteria give growth confined to the stab-line, which has sharp margins and leaves the surrounding medium clearly transparent.

The isolated bacteria gave hazy growth. So, the isolated bacteria had the ability to motile.



(A) = Control

(B) = Positive

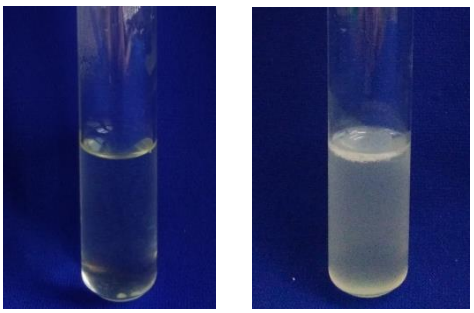
(A) (B)

Fig 4. Motility Test

Oxygen requirement activity test

Microbes have different oxygen requirements. Oxygen concentration is the highest level in aerobic bacteria, lowest in anaerobic bacteria and moderate in facultative anaerobic bacteria.

The isolated strain showed the region of the highest oxygen concentration level, so the isolated bacteria was aerobic.



(A) = Control

(B) = Positive

(A) (B)

Fig 5. Oxygen requirement activity test

Table 1. Morphological characters of isolated bacterial strain

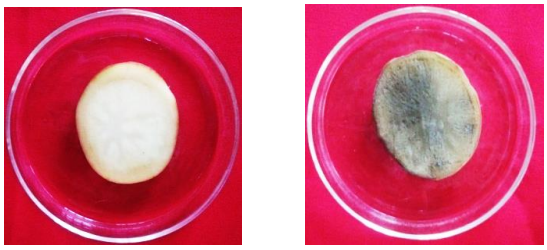
Strain	Colony color on YEM medium	Gram staining Test	Endospore formation	Motility	Aerobic/ Anaerobic	Color
Phaseolus mungo L.	Semitaneously, Raised and mucilaginous	Ve -	-	+	Aerobic	Pinkish purple
(+) = Positive						(-) = Negative

Biochemical characterization of isolated bacterial strain

1. Potato soft rot activity test

Positive reaction is decaying of potato beyond the point of inoculation.

The isolated bacterial strain showed decaying of potato.



(A) = Control

(B) = Positive

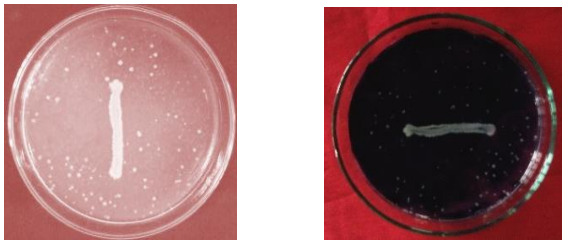
(A) (B)

Fig 6. Potato soft rot activity test

2. Starch hydrolyzing activity test

In a positive reaction, clear zone around the inoculated area indicated starch hydrolyzing test. In a negative test, there was no clear zone formation.

The isolated bacterial strain did not appear in the clear zone formation. So, this strain was not produced amylase enzyme.



(A) = Control

(B) = Negative

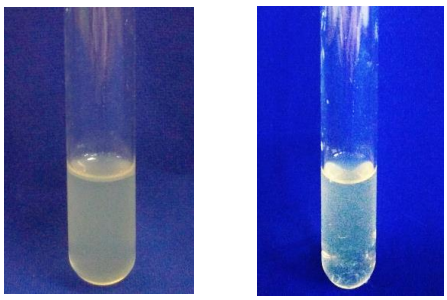
(A) (B)

Fig 7. Starch hydrolyzing activity test

3. Gelatin hydrolyzing activity test

In a positive reaction, it indicated partial liquefaction of purple in colour. In a negative reaction, indicated partial liquefaction of with no change in colour.

The isolated bacterial strain was showed a negative reaction.



(A) = Control

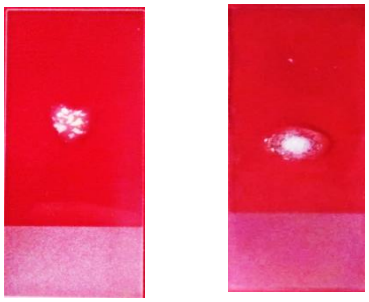
(B) = Negative

(A) (B)

Fig 8. Gelatin hydrolyzing activity test

4. Catalase activity test

In a positive reaction, bubbles appeared within 20 seconds. The isolated bacterial strain showed a positive reaction because of the bubbles.



(A) = Control

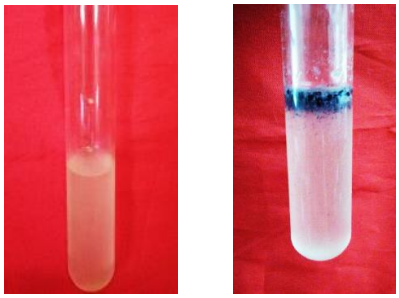
(B) = Positive

(A) (B)

Fig 9. Catalase activity test

5. Nitrate reduction activity test

Positive reaction indicated blue colour ring on the surface of broth medium. Negative reaction showed no ring structure. The isolated bacterial strain showed a positive reaction so this strain produced the nitrate reductase enzyme.



(A) = Control

(B) = Positive

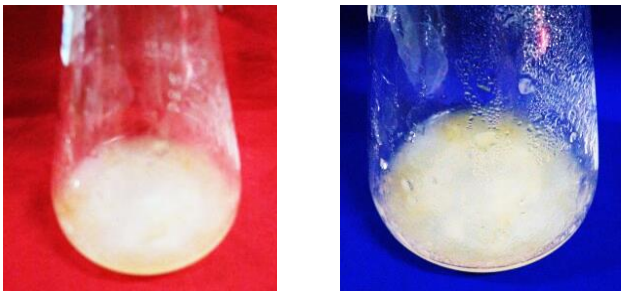
(A) (B)

Fig 10. Nitrate reduction activity test

6. Phenylalanine deaminase activity test

Formation of green colour in 1-5 minutes indicated the production of phenylalanine deaminase enzyme in positive reaction. Colour was not changed in this test indicated negative

reaction. The isolated bacterial strain showed negative reaction so, this strain was not produced phenylalanine deaminase enzyme.



(A) = Control

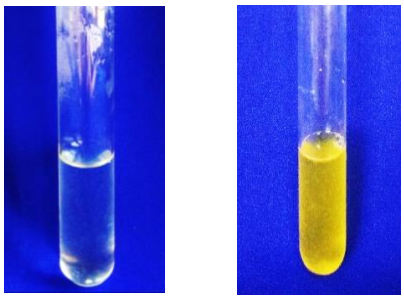
(B) = Negative

(A) (B)

Fig 11. Phenylalanine deaminase activity test

7. Oxidation activity test

In a positive reaction, the strains attacked the glucose molecules showed white precipitate region. Negative reactions indicated the clear region. The isolated bacterial strain showed a positive reaction. So, this strain was activite in oxidation in the fermentation process.



(A) = Control

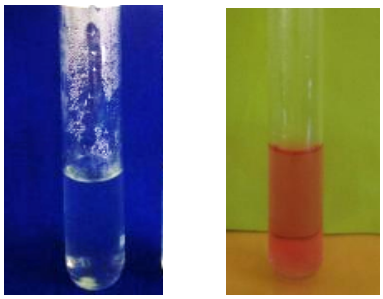
(B) = Negative

(A) (B)

Fig 12. Oxidation activity test

8. Urease activity test

In a positive reaction, the broth medium changed to brick red colour. In a negative reaction, change to colour did not occur. The broth medium changed to brick red colour. So, the isolated bacterial strain produced the urease enzyme.



(A) = Control

(B) = Negative

(A) (B)

Fig 13. Urease Activity Test

9. Methyl red activity test

In a positive reaction, the broth medium indicated a layer of cherry red in colour. In the negative reaction, it indicated the layer of yellow in colour. The broth medium showed a

positive reaction. So, the isolated bacterial strain produced large quantities of organic acids such as lactic, formic and succinic acid.

(A) = Control

(B) = Positive

(A) (B)

Fig 14. Methyl red activity test

10. Triple sugar activity test

Glucose, sucrose and lactose digestion tests are biological techniques utilized in microbiology to determine the way a microorganisms metabolizes a carbohydrate.

If the medium in a positive reaction indicated a change in yellow in colour. The isolated strain showed a change to yellow. So, this strain showed glucose and sucrose tests were strongly positive and the lactose test was weakly positive.

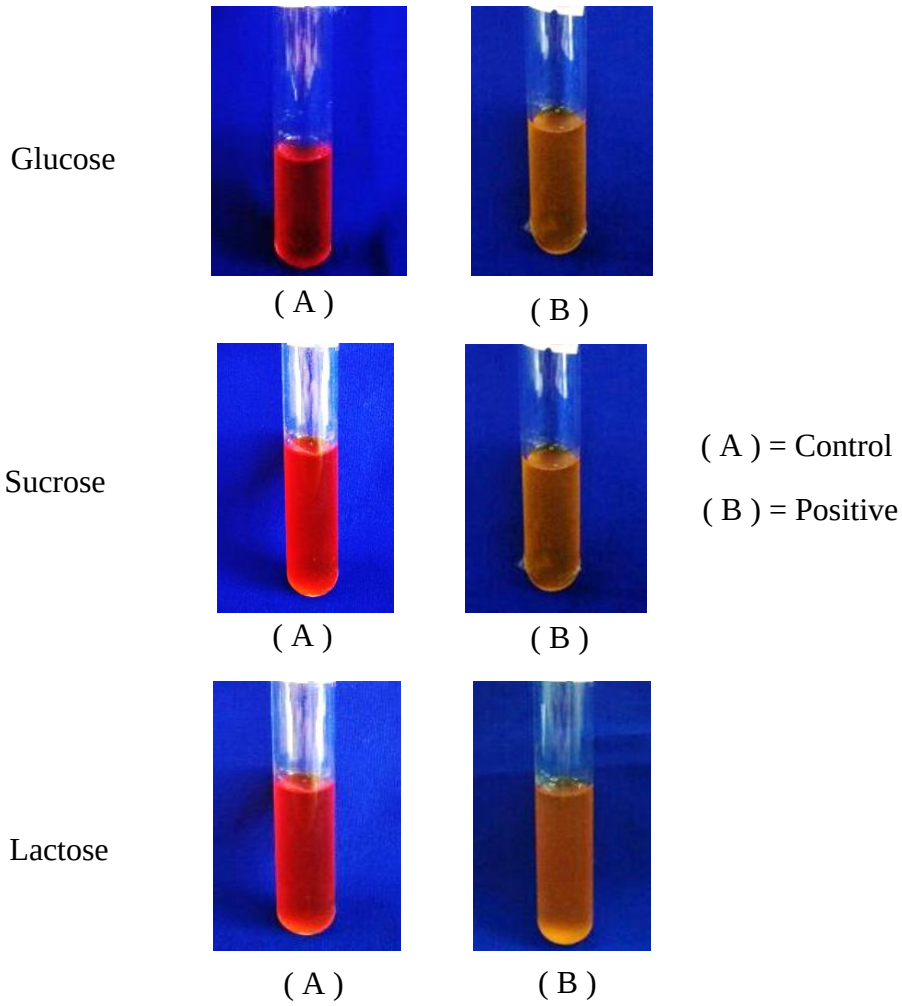
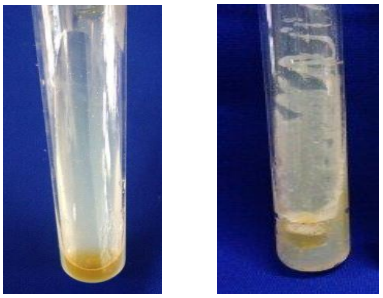


Fig 15. Triple sugar activity test

11. H_2S gas production test

In a positive reaction, the solid medium turned into black colour. In a negative reaction, change to colour did not occurr. The isolated bacterial strain showed a negative reaction so, this strain did not produce H_2S gas.



(A) (B)

(A) = Control

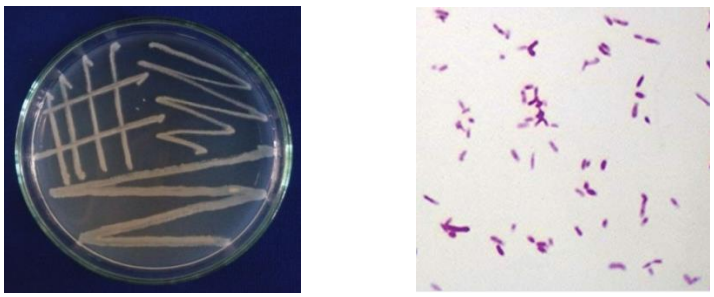
(B) = Negative

Fig 16. H_2S gas production test

Table 2. Biochemical test of an isolated bacterial strain

Strain	Poato Soft Rot Activ-ty Test	Starch Hydrol-yzing Activity Test	Gelatin Hydrol-yzing Activit y Test	Catal ase Activi ty Test	Nitrate Redu-ction Activity Test	Phen-yla lami-ne deamina se activity Test	Oxidat-ion Activity Test	Urease Activity Test	Meth yl Red Test	Triple Suger Test	H_2S Gas Prod-uction Test
Phaseo-lus mungo L.	+	-	-	+	+	-	+	+	+	+	-
(+) positive reaction							(-) negative reaction				

Culture characters and cell morphology of an isolated bacterial strain



Colony cultural Cell morphology x 100

Fig 17. Photomicrograph of the culture of colony and cell morphology of isolated bacterial strain

According to Bergery's Manual of Determinative Bacteriology and biochemical tests, the isolated strain was assumed to be in genus *Rhizobium* (Frenk, 1889).

Scientific Classification

Proteobacteria

Alpharoteobacteria

Rhizobiales

Rhizobiaceae

Rhizobium

Rhizobia are symbiotic diazotrophs that form a symbiotic association with legumes.

Colony circular, convex, semitransulcent, raised and mucilaginous; usually 2-4 mm in diameter within 3-5 days on YEM medium. *Rhizobium* is a gram negative, rod-shaped cell, non-sporulatingrod. Motile by a single polar flagellum. Bacteria colonize plant cells with root nodules.

Discussion and Conclusion

Nitrogen is an essential element for plant growth and development which is supplied by mutual symbiosis of rhizobia in cultivated legume plants. Biological nitrogen fixation could help to enhance agricultural productivity and ensure food security. Bacteria of Genus *Rhizobium* and related genera can interact with host plants in a process of nodulation. The bacteria induce the formation of an organ known as a nodule, on the roots of a plant.

In this study, a single strain was isolated from the root nodules of *Phaseolus mungo* L., legume cultivated field of Aeik Pyat Village, Hinthada Township. The sample was carried out by the method of washing sterilization. Isolated microbes of pure culture were selected and studied, their cultural characteristics of colony, cell morphology and biochemical characterization. According to the Bergery's Manual of Determinative Bacteriology (Holt *et al*., 1994) and biochemical tests, the isolated strain can be noted as the genus *Rhizobium*.

Rhizobium belongs to the Rhizobiaceae family, symbiotic diazotroph. It forms endosymbiotic associations with legumes. The cell morphology of the *Rhizobium* spp. strain is a gram- negative, rod-shaped and non-sporing form. *Rhizobium* spp. can infect the roots of leguminous plants leading to the formation of lumps or nodules where nitrogen fixation takes place. About 90% of legume species are types of root nodules and rhizospheres. Biochemical tests are performed by various workers for the identification of rhizobial isolates (Vishal and Abhishek, 2014).

The morphological characteristics of the isolated bacterial strain indicated positive reactions (Table 1). So, the isolated strain is defined as gram negative bacteria. The isolated bacterial strain was found to be gram negative, non-sporulating, rod shaped, motile and aerobic. In 2018, Vimala Gandhi *et al*. reported that all the strains isolated from the root nodules of *Arachis hypogaea* L. and *Phaseolus mungo* L. were found to be gram negative, rod shaped and non-motile. The bacterial strain grown on YEM medium produced small to medium sized colonies. This finding is in close aggrement with Vincent (1970). Among them, nitrate reduction and methyl red test showed rapidly positive reactions, therefore the root nodule strains are exactly assured of the genus *Rhizobium*.

Biological nitrogen fixation plays great by diminishing input of the hazardous chemical fertilizer in the field and contribute to the development in sustainable agriculture, which is necessary for the agriculture based under developed country.

This study showed the presence of nitrogen fixing bacteria on root nodules of *Phaseolus mungo* L. The *Rhizobium* isolated in this study could find potential for development

in sustainable agriculture as a biofertilizer which helps in soil fertilization without applying hazardous chemical fertilizer. However, further studies should be undertaken into the utilization of carbon and nitrogen sources, antimicrobial activities, and fermentation studies of the genus *Rhizobium* which can be isolated from the root nodules of *Phaseolus mungo* L.

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References

Atlas, Ronald M. (1993). **Handbook of Microbiological Media,** CRC Press, London.

Collins, C.H. (1984). **Microbiological Methods**, Fifth edition, Filmset and printed in England by Butter and Tanner Ltd. Frome and London.

Cowon, S. T, I. G. Holt, J. Liston, R. G. E. Murray, CF. Niver, A.W. Ravin and RY. Stanier. 1974. **BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY**, English Edition.

Datta, A., Singh, R. K., Kumar, S. and S. Kuumar. 2015. **An Effective and Beneficial Plant Growth Promoting Soil Bacterium *Rhizobium*: A Review**. Ann Plant Sci, 4(1),933-942.

Fontem, D.A. 1995, **Yield of Potato as influenced by Crop Sanitation and reduced fungicidal Treatments.** Tropicultura, 13(3) 99102.

Gram, H. C., 1884. Translation is also at: Brock, T. D. Pioneers in Medical Laboratory Science. Vol. 2: 185-189.

Holt J. G., N. R. Kreig, P. H. A. Sneath, R. J. T. Staley and S. T. Williams. 1994. **Bergey's Maual of Determinative Bacteriology**, 9th edition. Baltimore; Published by Williams and Wilkins, A. Wavely Company, 787.

Oke, V. 1999. **Bacteroid Formation in the *Rhizobium* legum symbiosis**.

Pagan, J. D., J. J. Child, W. R. Scowcraft and A. H. Gibson. 1975. **Nitrogen Fixation by *Rhizobium* Cultured on the Defined Medium.** Nature, 254:406-407.

Prescott, H. 2002. **Laboratory Exercises in Microbiology**. McGraw-Hill Companies.

Santra, S.C., T.P. Charterjiee and A.P., Das. 1998. **College Botany Practical Vol. II**. New Central Book Agency (P)Ltd.

Tyagi, A., Kumar, V. and A. Tomar. 2017. **Isolation, Identification, Biochemical and Antibiotic Sensitivity Characterization of *Rhizobium* strains from *Vigna mungo* (L.) Hepper, *Cier arietinum* (L.) and *Vigna radiata* (L.) Wilczek in Muzaffarnagar, Uttar Pradesh, India**. Int J Curr Microbial App Sci, 6, 2024-2035.

Vimala Gandhi, S., S. Suresh and A. Deb. 2018. **Isolation and Identification of Rhizobium species from Root Nodules of *Arachis hypogaea* L. and *Vigna mungo* (L.) Hepper in Tamil Nadu, India**. International Journal of Agricultural Sciences and Natural Resources, 10.

Vincent, J. M. 1970. **A Manual for the Practical Study of Root-Nodule Bacteria**. IBP Hand Book, Oxford; Blackwell Scientific Publication, Vol. 15.

Vishal K. and C. Abhishek. 2014. **Isolation and Characterization of *Rhizobium leguminosarum* from Root Nodule of *Pisum sativum* L**. Journal of Academia and Industrial Research (JAIR). Vol 2.

Woodland, J. 2004. **Bacteriology**, 2nd Ed. NWFHS Laboratory Procedure. Manual.

Websites

Aryal,2018, https://microbiologyinfo.com/category/biochemical-test/