Isolation and Identification of Endophytic Bacteria from the Leaves of *Evolvulus nummularius* L.

La Ring[1]

ABSTRACT

The present research deals with isolation of endophytic bacteria from the leaves of *Evolvulus nummularius* L. The plant samples were collected from Mandalay University campus, Mandalay Region. This research work was carried out at Microbiology Laboratory of Botany Department, University of Mandalay from November 2019 to January 2020. The bacterial strains LR 1, LR 2, LR 3 and LR 4 were isolated from leaves of *Evolvulus nummularius* L. by using Nutrient Agar (NA) medium. These bacterial strains were identified based on their colony morphology and biochemical tests such as gram staining test, catalase test, motility, methyl red test, Voges-Proskauer test and starch hydrolysis test. The resulted endophytic bacteria were confirmed as *Bacillus* sp. (LR 1, LR 2, LR 3 and LR 4). The colony and cell morphology of four bacterial strains were described and also presented by photomicrograph.

Keywords: *Evolvulus nummularius* L.., Endophytic bacteria, Morphological, Biochemical

INTRODUCTION

Evolvulus nummularius L. known as *Convolvulus nummularius* belongs to the family Convolvulaceae (Pavithra *et al.* 2009). *Evolvulus nummularius* is a native of tropical America and Africa, but has been widely introduced throughout the tropics. Although it is known to occur in Malaysia, Thailand, Vietnam and Laos (Chua 2016). *Evolvulus nummularius* is a widely used ethno medicinal plant of North-East India. The whole plant of *E. nummularius* is used as a medicine for hysteria, to cure burns, cuts, wounds and scorpion stings**.** This plant has antihelminthic activity, wound healing activity, poor sedative and anticonvulsant properties (Susmita *et al.* 2016). *Evolvulus alsinoides* L. is used mainly in traditional medicine of East Asia. The plant is used in Ayurveda as a brain tonic in the treatment of neurodegenerative diseases, asthma and amnesia (Goyal & Singh 2005) as cited by (Rupanjali 2012).

Microorganisms are considered that fungi and bacteria could dwell within plant tissues without causing any apparent harm. Endophytes produce a variety of antimicrobial compounds. Numerous literatures would show peptides, quinones, phenols, alkaloids, steroids, terpenoids and flavonoids, as well as a host of enzymes such as hydrolases, chitinases, laminarinases and glucanases, as main antimicrobial compounds responsible for pathogen inhibition. (Vijay & Alan 2014). Endophytic bacteria belongs to the class of endosymbiotic microorganisms, ubiquitous among plants that establish in between and within the spaces of all plant parts and not causing any plant disease (Dinesh & Annapurna 2017). The role of endophytic microorganisms in plants can be divided into two categories based on types of activity: growth promotion and disease control (Prasad & Sunayana 2014). Endophytic bacteria are of biotechnological and agronomic interest because they can enhance plant growth and improve the nutrition of plants, and they can also control pests and plant diseases (Vijay & Alan 2014). Endophytes can be a promising source of bioactive compounds, and should be continuously isolated, characterized, and investigated for the discovery of lead bioactive

[1] Assistant Lecturer, Department of Botany, University of Mandalay

compounds which can be employed in agriculture, medicine, and industries (Nawed & Ramesh 2015).

Bacterial endophytes from *Evolvulus nummulurius* L. was not yet studied. In this study, characterised and identified bacterial endophytes from surface-sterilised leaves of *Evolvulus nummularius* L. obtained in Mandalay University Campus. The aims and objectives of this study are to isolate the different endophytic bacteria from leaves of *Evolvulus nummularius* L. and to identify and characterize the different endophytic bacteria by using morphology and biochemical tests.

MATERIALS AND METHODS

The present study was carried out at the Microbiology laboratory, Department of Botany, University of Mandalay from October 2019 to January 2020. The plant samples were collected from the Mandalay University Campus.

Fleshy collected leaves were washed slow running water for 15 minutes. Samples were cut into about 1cm pieces. Samples were sterilized by immersing the sample in 70% ethanol for 1 minute, sodium hypochloride for 3-4 minutes. The sterilized leaf sample were dried in the laminar flow cabinet and then placed on the nutrient agar medium supplemented with antifungal agents. Plates with tissues are sealed using parafilm tape and incubated at 28 ˚C ± 2˚C in order to recover the maximum possible colonies of bacterial colonies were selected and are repeatedly streaks in order to achieve bacterial isolates.

The morphological and microscopical characters of isolated strains were observed by statement of Bergey's Mannual of Determinative Bacteriology (Breed *et al*. 1958); Gram Staining (Atlas 1993), Catalase Test (Dickey & Kelman 1988), Motility Test (Cruickshank *et al*. 1968), Methyl Red Test (Aneja 1996), Voges-Proskauer Test (Atlas 1993), Starch Hydrolysis Test (Aneja 1996).

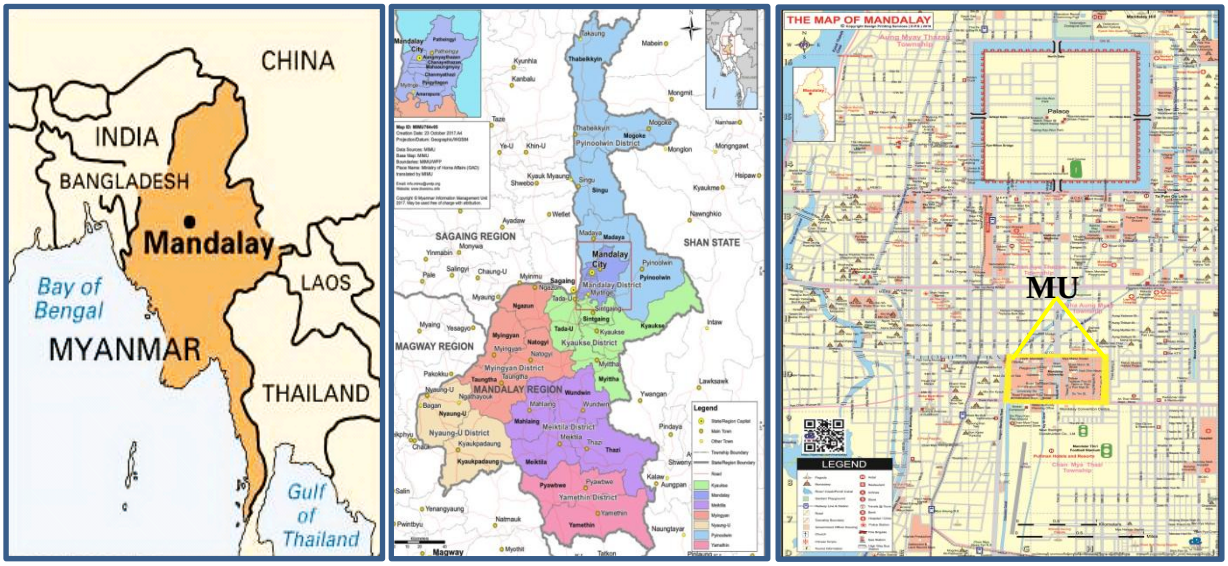

Figure 1. Location map of sample collection site

RESULTS

Four different kinds of bacterial strains were observed from *Evolvulus nummularius* L.. The bacterial strains were named as LR 1, LR 2 LR 3 and LR 4. The colonies morphology of isolated strains were creamy, yellow-white, circular or irregular, small, smooth, raised, dull, opaque and mucilaginous with entire edge. Cells are rod-shaped. The colonial and microscopic morphology and biochemical activities of isolated bacteria were presented in Table 4-2 and Figure 2-10.

Table 1. Colony Characteristics of Isolated Bacteria

Strains	Size of Colony	Margin	Colour	Elevation	Appearance
LR 1	moderate	entire	Yellow-white	raised	dull
LR 2	small	undulate	white	Flat	shiny
LR 3	moderate	entire	creamy	raised	dull
LR 4	large	curled	white	Flat	dull

Table 2. Biochemical Test for Characterization of Isolated Bacteria

Biochemical tests	LR1	LR 2	LR 3	LR 4
Gram staining	+	+	+	+
Catalase	+	+	+	+
Motility	+	+	+	+
Methyl red	–	–	–	–
Voges-Proskauer test	–	–	–	–
Starch hydrolysis	+	+	+	+

+ = Positive reaction - = Negative reaction

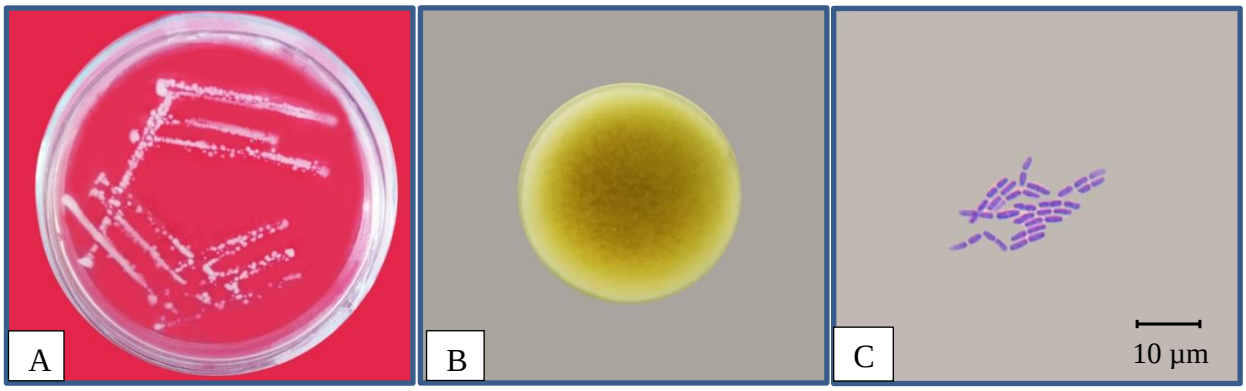

Figure 2 Colony and cell morphology of LR 1

A. Colonies on nutrient agar (NA) medium

B. Single colony

C. Photomicrograph of cells

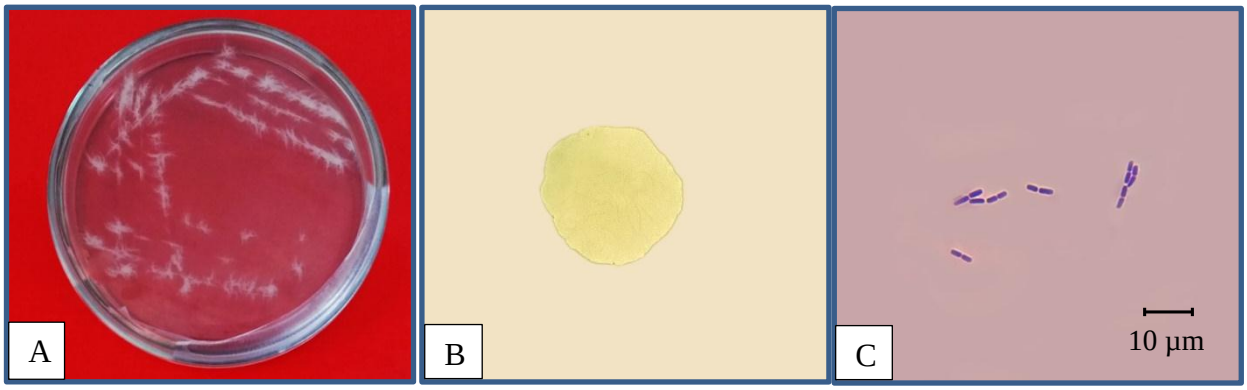

Figure 3 Colony and cell morphology of LR 2

A. Colonies on nutrient agar (NA) medium

B. Single colony

C. Photomicrograph of cells

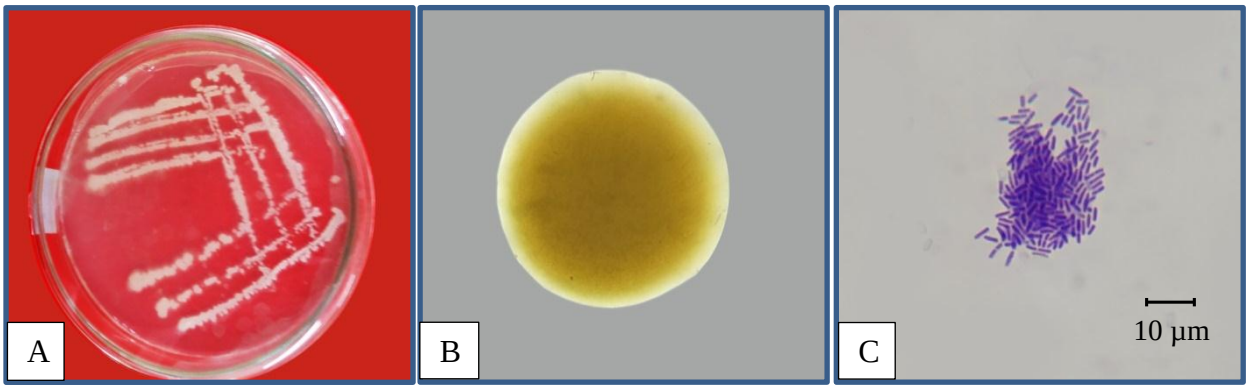

Figure 4 Colony and cell morphology of LR 3

A. Colonies on nutrient agar (NA) medium

B. Single colony

C. Photomicrograph of cells

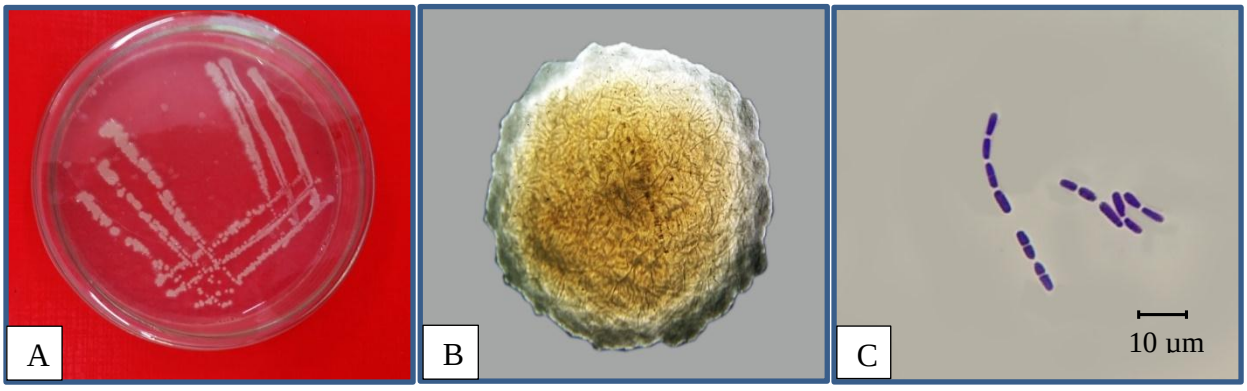

Figure 5 Colony and cell morphology of LR 4

A. Colonies on nutrient agar (NA) medium

B. Single colony

C. Photomicrograph of cells

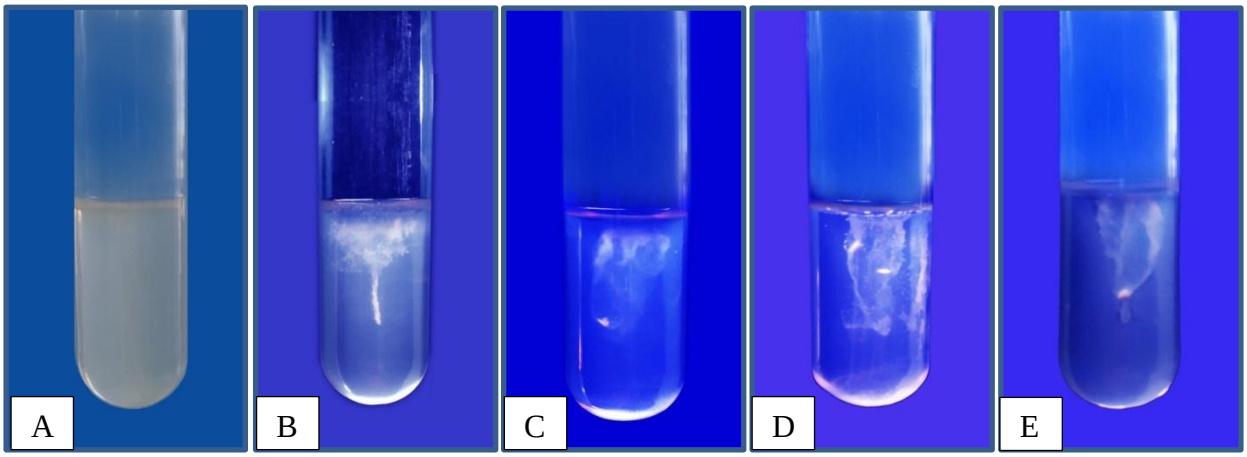

Figure 6. Motility Test

A. Control, B. LR 1 Positive, C. LR 2 Positive, D. LR 3 Positive, E. LR 4 Positive

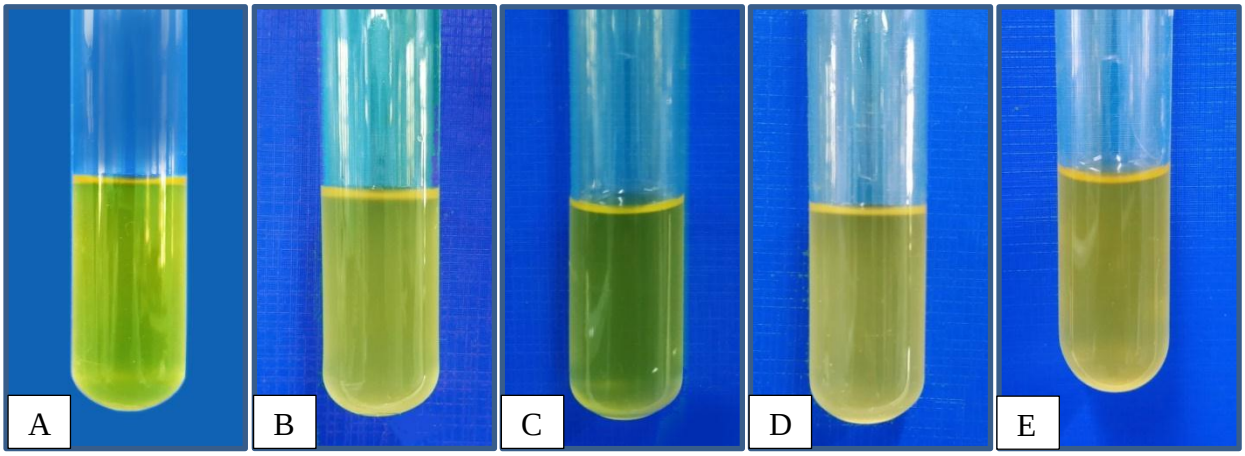

Figure 7. Methyl Red Test

A. Control, B. LR 1 Negative, C. LR 2 Negative, D. LR 3 Negative, E. LR 4 Negative

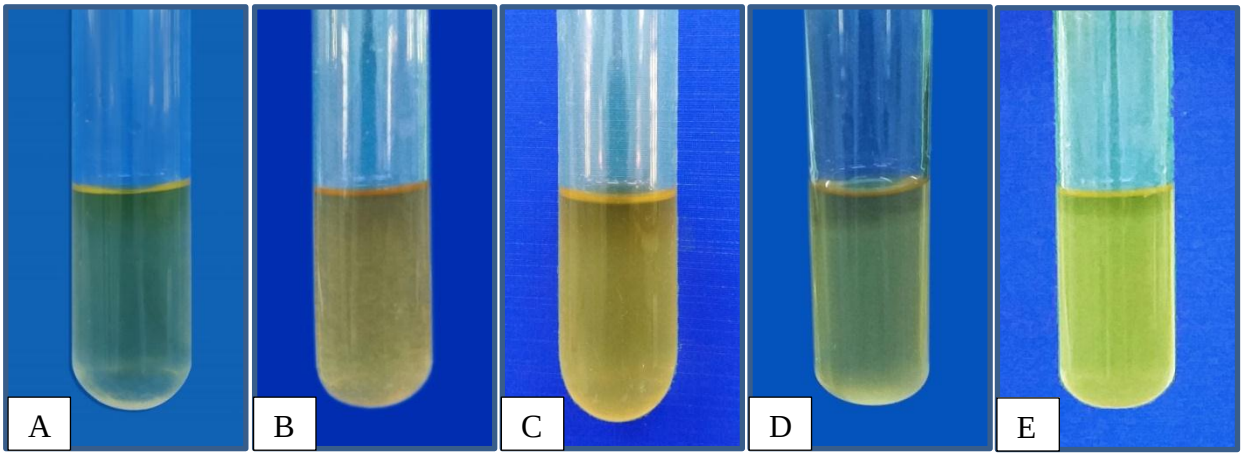

Figure 8. Voges-Proskauer Test

A. Control, B. LR 1 Negative, C. LR 2 Negative, D. LR 3 Negative, E. LR 4 Negative

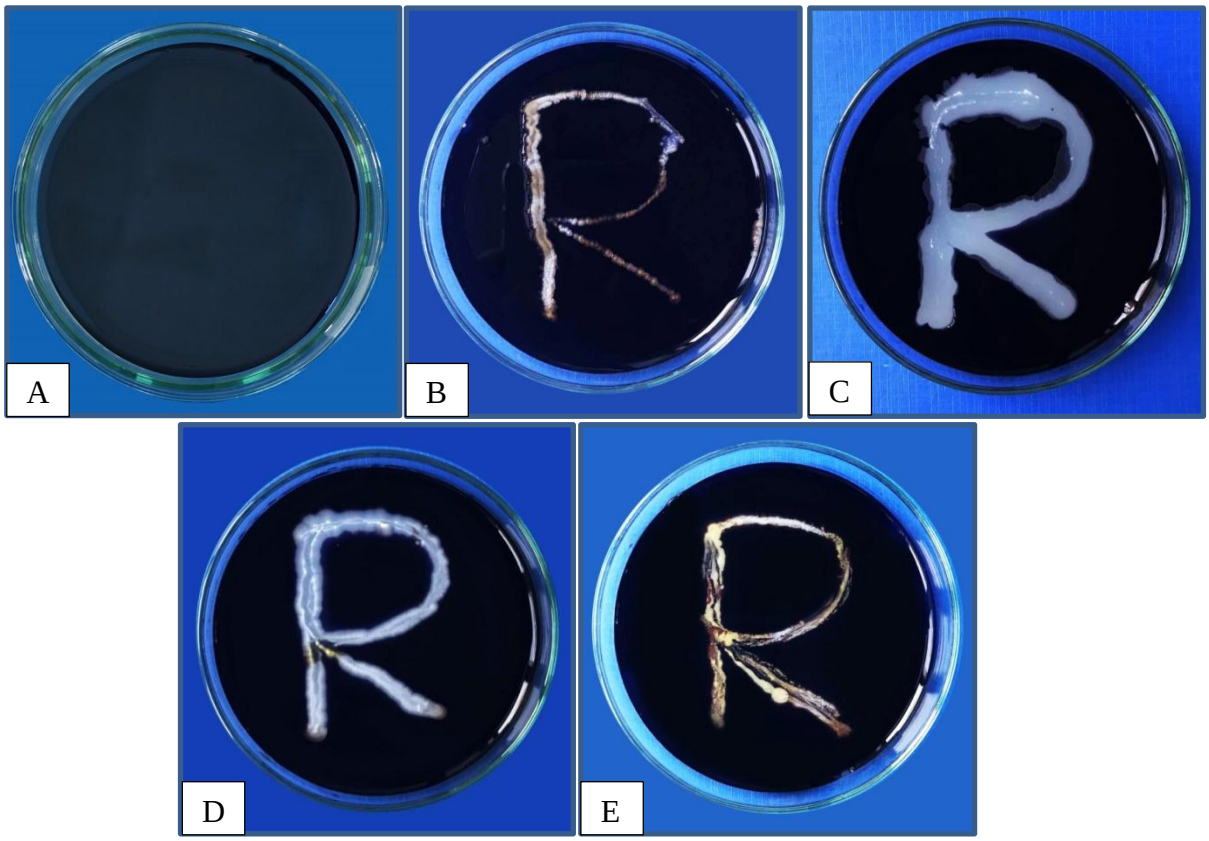

Figure 9. Starch Hydrolysis

A. Control, B. LR 1 Positive, C. LR 2 Positive, D. LR 3 Positive, E. LR 4 Positive

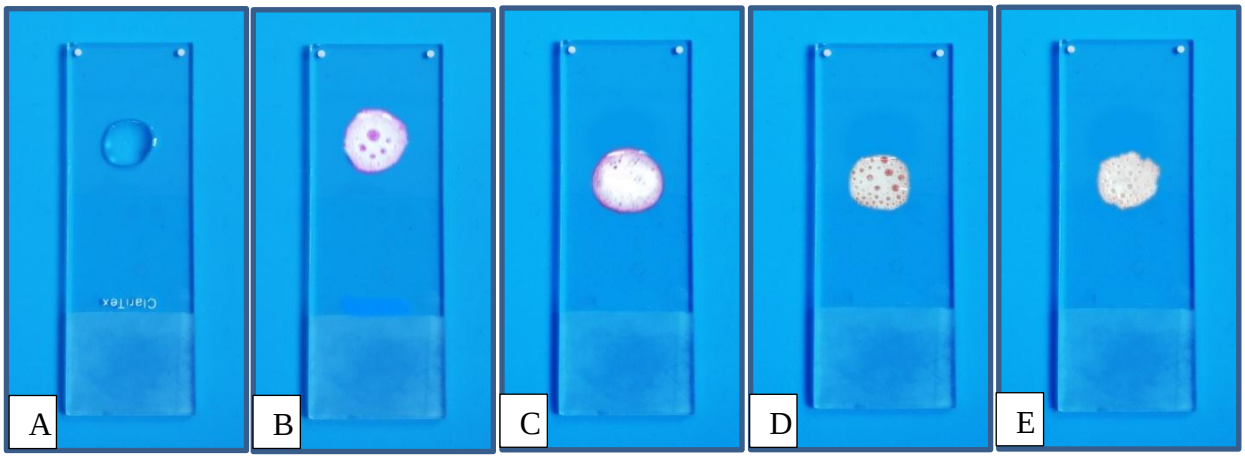

Figure 10. Catalase Test

A. Control, B. LR 1 Positive, C. LR 2 Positive, D. LR 3 Positive, E. LR 4 Positive

Discussion and Conclusion

In the present study, *Evolvulus nummularius* L. samples were collected from Mandalay University campus, Mahar Aung Myay Township, Mandalay Region. The specimens were conducted at the Microbiology Laboratory, Department of Botany, University of Mandalay. The bacterial strains were isolated by Nutrient Agar (NA) medium.

Phenotypic characteristics such as microscopic features, gram reaction, motility, catalase, and oxidase activity of all the isolated strains were determined by using standard procedures. Each of isolated strains was characterized to genus level by morphological examination (shape, colonies colur, cell size, gram staining and motility test) and biochemical (catalase, starch hydrolysis, methyl red, voges-proskauer) examination. Results were analyzed by Bergey's manual of Determinative Bacteriology (Breed *et al.* 1958). Four kinds of bacterial strains were isolated from this study, namely *bacillus* sp. (LR 1, LR 2, LR 3 and LR 4)

In that study, *Bacillus* spp. was the endophytic bacterium with a higher frequency in leaves of *Evolvulus nummularius* L, with four species. The isolated bacterial strains LR 1, LR 2, LR 3 and LR 4 were whitish colour, large, smooth, opaque, slightly convex with undulate edge. Cells are rod shaped, usually 1.0 µm - 2.5 um by 4.0 - 8.5 µm, gram positive, motile, methyl red test negative, voges-proskauer test negative and optimal temperature 28˚C - 50˚C.

Peter (1996) stated that the genera of family Bacillaceae are 0.7 -1.0 µm by 2.0 – 6.0 µm. *Bacillus* species are rod-shaped, endospore-forming aerobic or facultatively anaerobic, Gram-positive bacteria. The family Bacillaceae, consisting of rod-shaped bacteria that form endospores, has two principal subdivisions: the anaerobic spore-forming bacteria of the genus *Clostridium*, and the aerobic or facultatively anaerobic spore-forming bacteria of the genus *Bacillus.*

Colonies are large (2 - 7mm) with a frosted-glass appearance, some species may produce mucoid or smooth or raised wrinkly colonies. Colonies are cream to light yellow with no zone of egg. All *Bacillus* species are motile with the exception of *B. anthracis* and *B. mycoides* (Peter 1996). According to (Peter 1996) all of the isolated strains were confirmed as *Bacillus* sp.

Endophytes are generally isolated for following good reasons such as for their characterization, for studying population dynamics and diversity, use of microbial inoculants to improve plant growth and plant health, and as sources of novel biologically active secondary metabolites. Endophytic bacteria living in plant tissues deprived of doing substantive harm or gain benefit other than residency (Nawed & Ramesh 2015).

Endophytes have a great potential in future for sustainable agriculture since they could be used in a range of environmental and biological conditions. The study of endophytic bacteria is a challenging field of research, from a fundamental as well as an applied focus.

Future challenges are dependent on identifying, delineating, dissecting, and defining the mechanisms where by hosts and their symbionts accomplish this curious lifestyle. Defining these biological mechanisms will ensure the present and future successful technological applications of microbial endophytes.

The first attempts to use endophytic bacteria for the improvement of pest control or phytoremediation processes have been promising, but considerable research efforts are required to optimize the practical applications. More knowledge of the population dynamics and activity of endophytic bacteria in their host plants is required. The potential of the isolated endophytic bacteria to promote plant growth and their biocontrol potential in diseases that affect the aerial parts of the important plants for human diet will be addressed in future studies.

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References

Aneja, K. R. 1996. Experiment in microbiology, plant pathology; tissue culture and mushroom cultivation. Wishwa Prakashan New Age International Lmited.

Atlas, R. M. 1993. Microbiological media. Boca Raton Ann Arobor, London, Tokyo.

Breed, R. S., E. G. D. Murry& N. R. Smith. 1958. Bergey's manual of determinative bacteriology, 7th Edition. The Williams and Wilkins Company: Baltimore. 1268pp.

Chua, K. S. 2016. New record of the creeping plant *Evolvulus nummularius* in Singapore. Singapore Biodiversity Records 2016: 161. National University of Singapore.

Cruickshank, R., J. P. Duguid & R. H. A. Swain. 1968. Medical microbiology. The English Language Book Society and E. & S. Livingstone LTD. Great Britain; 11th Edition.

Dickey, R. S. & A. Kelman. 1988. Caratovora or soft rot group. In laboratory guide for identification of plant pathogenic bacteria 2nd Edition. pp 81-84.

Dinesh, K. M. & K. Annapurna. 2017. Endophytes: Crop Productivity and Protection. Sustainable Development and Biodiversity 16. Volume 16. Botany Department, M.L. Sukhadia University, Udaipur, Rajasthan, India.

Nawed, A. & C. Ramesh. 2015. Endophytic Bacteria: Isolation Procedure from Various Medicinal Plants and Their Preliminary Characterization. Asian Journal of Pharmaceutical and Clinical Research. Vol 8.

Pavithra, P.S., N. Sreevidya1, S. V. Rama. 2009. Antibacterial and antioxidant activity of methanol extract of Evolvulus nummularius. Department of Biotechnology, Institute of Technology Madras, Indian.

Peter, C. B. T. 1996. Medical Microbiology – Bacillus. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston.

Prasad, M.P. & D. Sunayana. 2014. Identification and characterization of Endophytic bacteria from fruits like Avacado and Black grapes. International Journal of Current Microbiology and Applied Science. Volume 3. Department of Biotechnology, Sangenomics Research Labs, Domlur Layout, Bangalore, India.

Rupanjali, S. 2012. Studies on Bioactivities of *Parkia javanica (Lamk.) Merr.* and *Evolvulus nummularius (L.) L.*: Department of Human Physiology Faculty of Science. Tripura University. India.

Susmita, S., D. Bijit, B. M. Jhinuk, R. C. Parichita, S. Partha, G. Biplab and K. S. Samir. 2016. Antibacterial Activity of *Evolvulus nummularius* Against Standard ATCC Gram Positive and Gram Negative Strains: Studies on MIC, MBC, Growth Curve Analysis and ROS Generation. International Journal of pure and applied Bioscience. India.

Vijay, C. V & C. G. Alan. 2014. Advances in Endophytic Research. Institute of Medical Sciences. Banaras Hindu University.