ISOLATION OF *RHIZOBIUM* SP. FROM SOIL AND THEIR ANTIMICROBIAL ACTIVITY

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Abstract

Soil samples were collected from ten different places of Pyin-Oo-Lwin Township, Mandalay Region, Myanmar. These samples were cultured on Rhizobium Medium (RI) and Yeast Mannitol (YM) agar medium . A total of 50 bacterium colonies were isolated from these soil samples and 31 isolated were obtained from RI Medium and 19 isolated from YM Medium. Isolated bacteria were symbolized as KM. In the colony morphology, the isolated bacteria were small, medium and large in size and color were white, yellow, pale yellow, red and brown. The margins were entire, undulated, circled, lobate and rhizoid and the elevations were raised and flat. Cell morphology of isolated strains were studied by gram staining, colony characters and shape of cell. Thirty nine bacterial strains were short rod and four strains were cocci, seven strains were rod. All isolated bacteria were Gram-negative except ten other strains were positive. All these strains were tested for preliminary study of antimicrobial activity and these strains showed the different levels of antimicrobial activity against ten test organisms. Among them, nine isolates showed the different antimicrobial activity. Especially, KM-40 showed the highest antimicrobial activity (25.99 mm) followed by KM-22 (22.73 mm) on *Bacillus subtilis* respectively.

Key words: antimicrobial activity

Introduction

Microorganisms are present in natural ecosystem such as air, soil and water. They are also present on the himself and living animals and plants. Soil contains many types of microorganisms such as bacterial, actinomycetes, fungi and algae, which are important because they affect the physical, chemical and biological properties of soil. Microorganisms in soil are important because they affect the structure and fertility of different soils (Subba, 1999). Microbes are very small living organism, so small that most of

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them are invisible. Among the soil bacteria, unique group called Rhizobia has a beneficial effect on the growth of plant.

Rhizobium is the most well known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria can infect the roots of leguminous plants, leading to the formation of lumps or nodules 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. *Rhizibium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers *et al.*, 2003).

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).

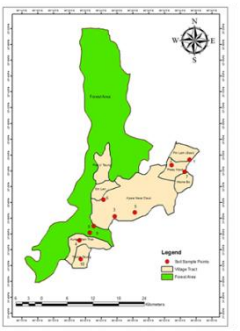
Soil bacteria called rhizobia are gram-negative capable to colonize the soil immediately surrounding roots under the influence of the plant 'rhizosphere' and reduce atmospheric nitrogen into the form available to plants through nitrogen fixation process.

Soil microorganisms specifically bacteria called rhizobia are able to colonise the rhizosphere, infect legume roots and biologically fix nitrogen in the soil through symbiotic process. Rhizobia are very important for crop production because they form symbiotic relationship with legume the process that converts atmospheric elemental Nitrogen (N_2) into accounting for 65% of the nitrogen currently utilized in agriculture. The aim and objectives of present research was to collect the soil samples from Phyin-Oo-Lwin Township, Mandalay Region, to isolate the Rizobium bacteria from these soil samples, to observe the colony morphology and cell shape of isolated bacteria by gram-staining and to study the preliminary study of antimicrobial activity on isolated bacteria with six test organisms.

Materials and Methods

Collection of soil samples

Ten different soil samples were collected from ten different places of Pyin-Oo-Lwin Township, Mandalay Region, Myanmar. This experiment was carried out at the laboratory of Biotechnology and Development Center of Pathein University.



Source-UTM 17.94-8, (Geography Dept. Pathein University)

Figure 1. Location map of Pyin-Oo-Lwin Township in Mandalay Region

Preparation of Glass wares

Pyrex glass wares were used throughout the experiments. The glass wares were treated with the chromosulphuric acid and wasted them with water. After air drying, they were sterilized in an autoclave at 15 psi and 121 Ċ at 15 minutes.

Table 1. Ten different soil samples collected at Pyin-Oo-Lwin Township

Soil samples	Collected places	Soil type	Soil pH	Location
Sample-1	Myaing Gyi	Silty clay	7.63	22' 5.434"N 96' 35.139"E
Sample-2	Pway Kauk	Clay	6.02	22' 4.73"N 96' 32.899"E
Sample-3	Nyaung Ni	Clay	5.94	21'58.221"N 96' 25.685"E
Sample-4	Kyauk	Silty clay	5.58	21' 55.207"E 96' 21.24"E

Soil samples	Collected places	Soil type	Soil pH	Location
Sample-5	Kywe Nwar	Silty clay	6.37	22' 0.355"N 96' 24.26"E
Sample-6	Si Thar	Silty clay	6.58	21' 58.221"N 96' 25.683"E
Sample-7	Htone Bo	Clay	5.97	22' 3.899"N 96' 34.045"E
Sample-8	War Bo Ye	Silty	6.37	21' 57.028"N
Sample-9	Aung Chan	Silty clay	5.46	21' 56.182"N 96' 22.569"E
Sample-10	Pyin Sar	Silty clay	6.58	21' 52.818"N 96' 21.386"E

Serial Dilutions Method of Soil Samples (Collins, 1964)

Serial dilutions of plating and streaking techniques described by Salle (1948), Collins (1964) and Pelezar and Chan (1972) were used for the isolation of bacteria species from soil. An appropriate amount (1 gm) of soil was introduced into a conical flask containing 99 mL of distilled water to make a soil-water dilution ratio of 1:100. The flask was then shaken for about 30 minutes in order to make the soil particles free from each other. This solution was then serially diluted into 10^{-1} to 10^{-5} dilution in separate test tubes and 1 ml each of the above dilutions was separately transferred into sterile petridishes under aseptic condition. A sterile pipette was used for each transfer.

The sterilized medium in conical flask was cooled down to about 45°C and separately poured into each of the petridish containing the respective soil dilution. The inoculated plates were shaken clock-wise and anti clock-wise direction for about 5 minutes so as to make uniform distribution of the bacterial inoculums. When the agar was solidified, the inoculated plates were inverted and incubated at room temperature for 24 hours. Various types of colonies developed on the inoculated plates. They were separately streaked over another set of petridishes containing the same sterile medium. Each of the discrete colonies visible in the second set of inoculated plates was separately transferred to sterile nutrient agar medium. The isolates were maintained in nutrient agar medium for further experimentations.

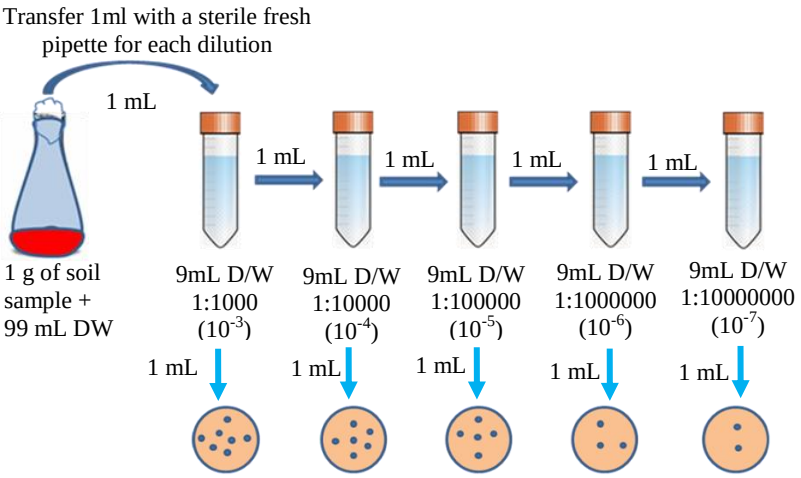


Figure 2. Serial dilution Method

Preparation of Agar Well Method

Isolated strains were subjected with antagonistic activities by agar well method. Cork borer was used to make the wells (8 mm in diameter) in the autoclaved basal antimicrobial test-medium. Wells impregnated with 24, 48 and 72 hours fermented broth (20 µL) were incubated at room temperature for 24-48 hours. After 24-48 hours of incubation, the clear zones were measured. Therefore, the diameter of clear zones has observed as potent activity shown by respective strain. Clear zones surrounding the test wells were indication of the presence of antimicrobial activities which inhibit the growth of the test organisms selectively (Collins 1965). The effectiveness of soil bacteria were rated according to modified Rukhsana Rating Scale (2011) as cited in Kyaut Kay Khaing (2012).

Isolation of pure culture from plate to slant

For pure culture from plate to test tube, about 100 mL of culture media were separately distributed in test tube. These test tubes were plugged with cotton wool and sterilized by autoclaving them at 15 pounds pressure per square inch for 15 minutes at 121ºC. The sterilized media were cooled down.

Each of the separate colonies on petridish was taken out to streak on the slant medium to obtain pure cultures (Atlas, 1993).

Preparation of culture media

Medium I **Rhizobium Medium RI** **(Atlas, 1993)**		**Medium II** **Yeast Mannitol Agar YM** **(Atlas, 1993)**	
$FeCl_3$	0.002 g	Mannitol	10 g
Yeast extract	10 g	Yeast extract	0. 4 g
K_2HPO_4	0. 5 g	K_2HPO_4	0. 5 g
$MgSO_4.7H_2O$	0. 2 g	$MgSO_4.7H_2O$	0. 2 g
NaCl	0. 2 g	NaCl	0.001 g
Agar	18 g	Agar	18 g
D/W	1000 mL	D/W	1000 mL
pH	6.8	pH	6.8

After autoclaving, Nystatin (1.5 mL) was added to the medium

Table 2. Tests organisms and Diseases

No	Tests organisms	Diseases
1	*Agrobacterium tumafaciens* NITE 09678	Plants diseases
2	*Aspergillus paracticus* IFO5123	Fruits diseases
3	*Bacillus subtilis* IFO 90571	Fever
4	*Candida albicans* NITE 09542	Alimentary tract, skin infection
5	*Micrococcus luteus* NITE 83297	Skin diseases
6	*Salmonella typhi* AHU 7943	Skin disease, food poison, wound infection, burns
7	*Staphylococcus aureus* AHU 8465	Food poisoning
8	*Escherichia coli* AHU 5436	Urinary tract infection, cholera, diarrhea and vomiting
9	*Pseudomonas fluorescens* IFO 94307	Rice spoilage
10	*Saccharomyces cerevisae* NITE 52847	Food diseases

Results

Isolation of bacterial from soil samples

Ten different soil samples were collected from Myain Gyi, Pway Kauk, Nyaung Ni, Kyauk Phyar Do, Si Thar, Kywe Nwar Dauk, Htone Bo, War Bo Ye, Aung Chan Thar and Pyin Sar villages in Pyin-Oo-Lwin Township, Mandalay Region. A total of 50 bacterial colonies were isolated from these soil samples. 31 isolated strains were obtained from medium 1 (RI) and 19 strains from medium 2 (YM). These results were shown in Table (1).

Colony Morphology of Isolated *Rhizobium* bacteria

The isolated bacteria were designated as KM 1-50. In the colony morphology, isolated bacteria were small, medium and large in size of colony and the color were white, yellow, pale yellow, red and brown.

The margin were entire, undulated and lobate. The elevation and form were raise and flat. The isolated bacterial strains were rod, short rod and cocci. Among them, 40 strains were gram-negative and 10 strains were gram-positive.

Antimicrobial activity of isolated bacterial strains

Some isolated bacterial strains were tested for antimicrobial activity to ten test organisms with agar well diffusion method and these strains showed different levels of antimicrobial activity. According to results, 2-3 days fermentation culture showed the highest activity (25.99 mm) in KM-40 and other strains were moderate activities (14.43-22.73 mm) respectively. These results were shown in table and figure.

Table 3. Colony morphology of isolated bacteria

Isolated bacteria	Size of Colony	Margin	Color	Elevation and form	Pigment on agar
KM-1	Large	Lobate	White	Flat	white
KM-2	Large	Entire	White	Flat	white

Isolated bacteria	Size of Colony	Margin	Color	Elevation and form	Pigment on agar
KM-3	Large	Undulate	White	Flat	white
KM-4	Large	Undulate	White	Flat	white
KM-5	Large	Entire	White	Flat	white
KM-6	Large	Entire	White	Flat	white
KM-7	Large	Entire	White	Flat	white
KM-8	Large	Entire	White	Flat	white
KM-9	Large	Entire	Pale	Flat	Pale
KM-10	Large	Undulate	White	Flat	white
KM-11	Large	Lobate	White	Flat	white
KM-12	Large	Lobate	White	Flat	white
KM-13	Medium	Entire	White	Flat	white
KM-14	Large	Entire	Yellow	Flat	Yellow
KM-15	Small	Entire	Yellow	Flat	Yellow
KM-16	Medium	Entire	Yellow	Flat	Yellow
KM-17	Small	Entire	Brown	Flat	Brown
KM-18	Medium	Entire	Red	Flat	Red
KM-19	Medium	Lobate	Yellow	Raised	Yellow
KM-20	Medium	Undulate	White	Flat	White
KM-21	Small	Lobate	White	Flat	white
KM-22	Small	Entire	Yellow	Raised	Yellow
KM-23	Small	Undulate	White	Flat	White
KM-24	Large	Lobate	White	Flat	White
KM-25	Large	Lobate	Yellow	Flat	Yellow
KM-26	Large	Entire	White	Flat	White
KM-27	Medium	Entire	Yellow	Flat	Yellow

Isolated bacteria	Size of Colony	Margin	Color	Elevation and form	Pigment on agar
KM-28	Small	Entire	Red	Flat	Red
KM-29	Large	Rhizoid	White	Flat	White
KM-30	Large	Rhizoid	White	Flat	White
KM-31	Medium	Undulate	White	Flat	White
KM-32	Large	Lobate	White	Flat	White
KM-33	Large	Entire	White	Flat	White
KM-34	Large	Undulate	White	Flat	White
KM-35	Large	Undulate	White	Flat	White
KM-36	Large	Undulate	White	Flat	White
KM-37	Medium	Entire	White	Raised	White
KM-38	Small	Entire	White	Raised	White
KM-39	Small	Entire	White	Raised	White
KM-40	Small	Entire	White	Raised	White
KM-41	Large	Entire	White	Flat	White
KM-42	Large	Undulate	White	Flat	White
KM-43	Medium	Curled	Yellow	Flat	Yellow
KM-44	Large	Rhizoid	White	Raised	White
KM-45	Large	Undulate	Yellow	Raised	Yellow
KM-46	Large	Lobate	Yellow	Flat	Yellow
KM-47	Large	Entire	White	Flat	White
KM-48	Large	Undulate	White	Raised	White
KM-49	Medium	Entire	Purple	Raised	Purple
KM-50	Large	Curled	Yellow	Raised	Yellow

Small < 2 mm (diameter)
Medium = between 2 mm and 5 mm (diameter)
Large > 5 mm (diameter)

Table 4. Cell morphology of isolated bacteria

Strain No.	Cell Morphology	Gram Staining	Strain No.	Cell Morphology	Gram Staining
KM-1	Short-rod	-	KM-26	Short-rod	-
KM-2	Cocci	-	KM-27	Short-rod	-
KM-3	Rod	-	KM-28	Short-rod	-
KM-4	Short-rod	-	KM-29	Short-rod	-
KM-5	Short-rod	-	KM-30	Short-rod	-
KM-6	Short-rod	-	KM-31	Short-rod	-
KM-7	Rod	+	KM-32	Short-rod	-
KM-8	Short-rod	+	KM-33	Short-rod	-
KM-9	Cocci	+	KM-34	Short-rod	-
KM-10	Rod	+	KM-35	Short-rod	-
KM-11	Short-rod	+	KM-36	Short-rod	-
KM-12	Short-rod	-	KM-37	Short-rod	-
KM-13	Short-rod	+	KM-38	Short-rod	-
KM-14	Short-rod	-	KM-39	short-rod	-
KM-15	Cocci	-	KM-40	Rod	+
KM-16	Short-rod	-	KM-41	Short-rod	-
KM-17	Short-rod	-	KM-42	Short-rod	-
KM-18	Cocci	-	KM-43	Rod	-
KM-19	Short-rod	-	KM-44	Rod	+
KM-20	Short-rod	-	KM-45	Short-rod	-
KM-21	Short-rod	-	KM-46	Short-rod	-
KM-22	Short-rod	-	KM-47	Rod	+
KM-23	Short-rod	-	KM-48	Short-rod	-
KM-24	Short-rod	-	KM-49	Short-rod	+
KM-25	Short-rod	-	KM-50	Short-rod	-

\+ = Gram-Positive
\- = Gram-Negative

KM-1-10 (Medium I, RI) | KM–11-20 (Medium I, RI) | KM-21-31 (Medium I, RI)

Figure 6. Pure culture of isolated bacteria from soil samples on the Rhizobium I medium

KM-1-10 (Medium II, YM) | KM-11-19 (Medium II, YM)

Figure 7. Pure culture of isolated bacteria from soil samples on the Yeast Mannitol Agar (YM)

Table 5. Antimicrobial activities of isolated bacterial strains on ten test organisms (2 days fermentation period)

Selected Strains	Test organisms and Antimicrobial activity (mm)									
	Test	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**
KM 1	14.19	-	-	9.70	16.00	11.92	15.02	16.00	12.00	13.87
KM 22	13.00	-	**22.73**	11.21	-	18.00	-	14.64	16.00	15.00
KM 28	16.00	-	-	-	15.00	14.28	14.99	14.65	14.69	17.52
KM 37	13.04	-	22.14	18.39	-	14.01	-	-	16.70	16.00
KM 40	-	20.01	**25.99**	19.14	20.42	20.02	-	18.00	19.00	21.80
KM 43	12.09	-	-	18.03	14.00	16.48	14.25	17.41	16.01	15.00
KM 45	-	-	-	17.30	14.52	16.39	14.43	18.16	16.60	17.01
KM 46	-	17.00	-	16.76	14.76	16.89	14.43	17.78	15.73	15.70
KM 50	-	22.00	-	20.00	20.15	16.11	-	17.22	20.52	18.13

Table 6. Antimicrobial activities of isolated bacterial strains on ten test organisms (3 days fermentation period)

Selected Strains	Test organisms and Antimicrobial activity (mm)									
	Test	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**	**Test**
KM 1	14.1	-	-	-	-	-	15.1	15.4	-	10.93
KM	10.9	-	11.2	9.70	-	14.2	-	14.5	12.6	-
KM	16.0	-	-	-	-	-	12.3	14.0	12.6	15.77
KM	-	-	-	18.0	-	-	-	-	-	-
KM	-	19.26	22.0	-	13.8	14.5	-	16.3	14.8	-
KM	-	-	-	15.0	11.7	14.0	13.3	14.0	15.1	14.24
KM	-	-	-	16.5	11.0	15.0	13.4	15.9	14.5	16.01
KM	-	-	-	15.2	12.9	13.0	13.0	15.4	14.1	15.43
KM	-	-	-	19.6	14.2	18.4	-	15.0	14.8	17.37

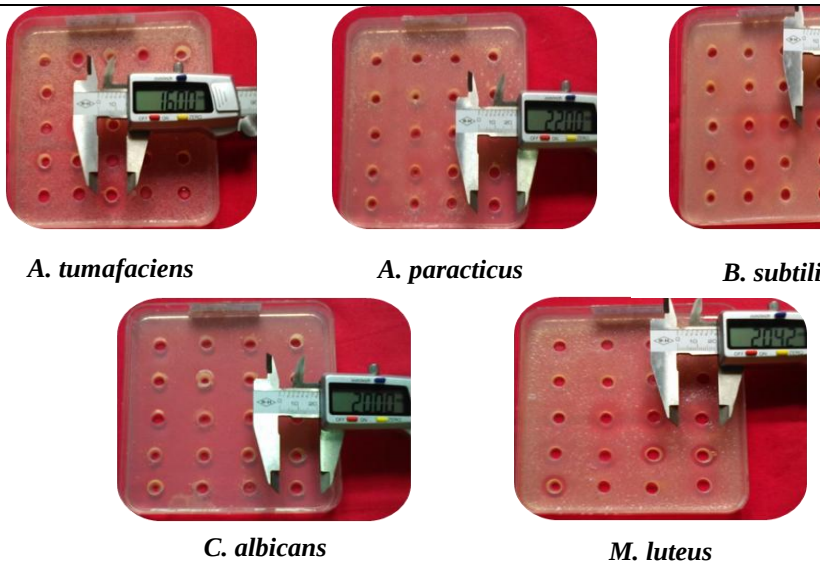


Figure 8. Antimicrobial activity of thirteen isolated bacteria on ten test organisms (2 days period)

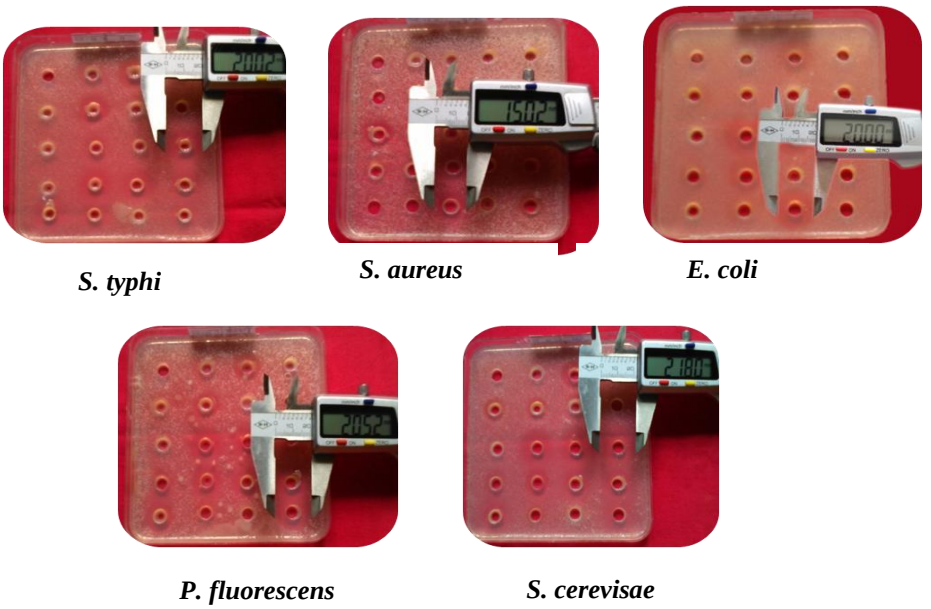


Figure 9. Antimicrobial activity of thirteen isolated bacteria on ten test organisms (3 days period)

Discussion and Conclusion

Ten different soil samples were collected from ten different places of Pyin-Oo-Lwin Township, Mandalay Region, Myanmar Freshly ten different soil samples were isolated by serial dilution method. These samples were cultured on RI medium and YM medium. A total of 50 bacterial strains were obtained. The isolated bacterial strains were designated as KM-1 to KM-50. In the colony morphology, the isolated strains were small, medium, large in size and color were white, pale yellow, yellow, brown, red and purple. The margins of colonies were entire, undulated, lobate, curled, rhizoid and the elevations were flat and raised. Some isolated bacterial strains were tested for antimicrobial activities on ten test organisms with agar well diffusion method

and these strains showed different levels of antimicrobial activities. According to the result, the highest activity was obtained in KM-40 (25.99 mm) and other strains also showed the moderate activities (14.43-22.73 mm) respectively.

Chitra *et al.*, 2013 reported that Rhizobium isolates showed antibacterial activity against *Streptococcus* and *E. coli*. Rhizobium isolates found to be the most potent strain and showed maximum diameter of inhibition zone ranges from 15 mm to 13 mm against *E. coli* and *Streptococcus* respectively. It is necessary Genes confirmation that all isolated strains must be further studied for exploitation such as biochemical characterization and fermentation procedures.

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