Isolation of Bacteria from Tobacco Cultivated Soils at Gaung-Say-Kyun Village

Khine Khine Oo[1], Mar Mar Aung[2]

Abstract

The soil samples were collected from tobacco cultivated soil at Gaung-say-kyun village in Hinthada Township, Ayeyarwady Region. The upper surface, the upper part of soil sample from the depth of 2 inches and 4 inches were taken from this location. Isolation procedure was undertaken by serial dilution method. Isolated microbes were cultured on agar plate and sub-cultured to obtain pure culture. The morphological characters and some biochemical activities were studied. According to the results of morphological characters and biochemical test of isolated strains were identified as K-1 (*Azomonas* sp-1), K-2 (*Azomonas* sp-2) and K-3 (*Azomonas* sp-3).

Keywords: tobacco cultivated soil, Gaung-say-kyun village, *Azomonas* sp

INTRODUCTION

The soil is one of the main reservoirs of microbial life. The most numerous organisms in soil are bacteria. Typical garden soil has millions of bacteria in each gram. Each gram of organically rich soil may contain more than 2 billion bacteria. The number and types of organisms present in soil strongly depend on the availability of nutrients and water, pH and the temperature of the environment. Aerobic organisms are most common and are concentrated in the surface layers unless the soil is highly aerated (Alexander, M. (1961)).

Bacteria are the most numerous of the culture able soil microorganisms. Their numbers are often as high as $1x10^8$ per gram of soil. Bacteria have a wide variety of shapes, sizes, and functions. Some require free-molecular oxygen, some can live without oxygen, and some (facultative anaerobic) can live with or without oxygen. Their sizes usually range from about 0.2 to 3 μm (Alexander, M. (1961)).

Bacteria live in almost any habitat. In general, bacteria tend to do better in neutral pH soils than in acid soils. In addition to being among the first organisms to begin decomposing residues in the soil, bacteria benefit plants by increasing nutrient availability. (https://www.sare.org/Learning-The-Living-Soil/Soil-Microorganisms)

MATERIALS AND METHODS

Study site

Table 1. Soil Samples from Study Sites

Soil No.	Collected Site	Location	Collecting Amount	Collected Date
S.1	The upper surface of soil sample from A	17˚39′09.7″N 95˚27′40.6″E	20g	2.12.2018
S.2	The upper part of soil sample from the depth of 2inches from A	17˚39′09.7″N 95˚27′40.6″E	20g	2.12.2018
S.3	The upper part of soil sample from the depth of 4inches from A	17˚39′09.7″N 95˚27′40.6″E	20g	2.12.2018

A = tobacco cultivated soils at Gaung Say Kyun village

[1] Demonstrator, Department of Botany, Hinthada University
[2] Lecturer, Department of Botany, Hinthada University

Collection of soil sample and soil analysis

The soil samples were collected from tobacco cultivated soils at Gaung Say Kyun village, Hinthada Township. The upper surface, the upper part of soil sample from the depth of 2 inches and 4 inches were taken from this field. The soil samples were added in the plastic bag. And then, the plastic bag with the soil samples were air dried under shade for six hours and unnecessary debris was sorted out and removed. Then, the soil particles were sieved with clean sieve of 1 mm square mash and finally capped in a clean sterile flask.

Calculation for soil analysis

Procedures

The amount of 10g soil sample was weighed and transferred into a beaker containing 90ml of water. In the beaker the mixture of soil sample was stirred with glass rod for 30 minutes. After stirring, this suspension was placed settle at cool and dry place for 24 hours. After 24hours, settlement of soil mixture was measured by a ruler. In these suspension the under layer was sand, middle layer was silt, upper layer was clay, outermost layer was organic material. The above respective layer was measured. Finally, the measurement for soil composition was calculated by means of USDA Texture Triangular. (https://www.nrcs.usda. gov/wps/detail/soils/ survey/)

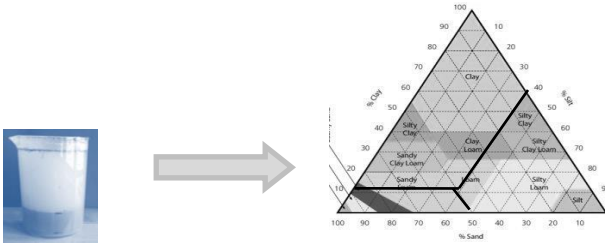


Fig 1. USDA Texture Triangular Isolation of bacteria from soil sample

Screening Procedure

Preparation of soil sample by Serial Dilution Method

Serial dilution techniques of Alexander (1961), Pelezar and Chan (1972) and Atlas (1988) with slight modification were applied in the screening and isolation of microbes from the soil sample of the upper surface, the upper part of soil sample from the depth 2 inches and 4 inches of soil from tobacco cultivated soils at Gaung Say Kyun village, Hinthada Township.

An appropriate amount (1 g) 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 1:1,000, 1:10,000, 1:1,000,000 and 1:10,000,000 in separate test tubes. Prepared Potato Glucose Agar (PGA) medium was used for isolation and maintenance of pure culture. Then 1 ml of soil solutions poured into sterile petridishes using sterile pipette each under aseptic condition.

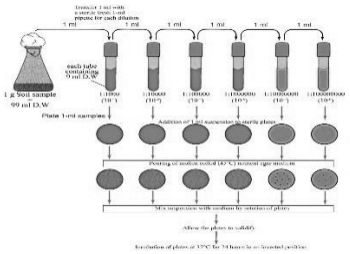


Fig 2. Serial Dilution Method (Collins 1965 and Pelczer and Chan, 1972)

Isolation of Pure Culture by Pour Plate Methods

About 15 ml of PGA media was separately distributed into test tubes. The test tubes were plugged with nonabsorbent cotton wool and sterilized by autoclaving them at 15 pounds pressure per square inch for 15 minutes at 121 ˚C. The sterilized mediums contained in each of the test tubes were cooled down to about 45˚C and separately poured into the petridishes containing the respective soil solution. The inoculated plates were shaken clockwise and anti-clockwise direction for about 5 times so as to make uniform distribution of the soil solution in the medium.

When the agar was solidified, the inoculated plates were inverted and incubated at room temperature for 48 hours. The individual colonies developed on the PGA media that was separately streak over the sterile PGA media contained in another set of separate sterile petridishes by inoculating loop under aseptic condition. The second set of inoculated plates were again incubated at room temperature for 3 days. The appeared microorganisms were subcultured on the sterilized test tube containing the same medium. To obtain pure culture, subculture was made three to four times. Then the pure cultures of isolated microbial strains were kept in specific slants at 4˚C in the refrigerator for the study of morphological and biochemical characters.

Medium used for isolation of bacteria

PGA Medium (Potato Glucose Agar)

Potato	-20 g
Peptone	-0.3 g
Glucose	-2.0 g
Agar	-1.8 g
D.W	-100 ml
pH	-±6.5

(After autoclaving Nystatin were added to the medium.)

The potatoes were not peeled but scrubbed and cleaned. The amount of 200g cubes were weighed out and rinsed rapidly in running water. It was added into 1 liter water and boiled about one hour until soft, mashed and squeezed the pulp as much as possible through a fine sieve. Agar 20g was added into it and boiled till dissolved. The amount of 20g of dextrose was added into the potato pulp and stirred till dissolved. Then the medium was made up 1 liter and sterilized at 15 p.s.i, for 20 min.

GYA Medium (Glucose Yeast extract Agar)

Glucose -0.3g

Yeast Extract -0.2g

KH_2PO_4 -0.1g

$MgSO_4$ -0.05g

KNO_3 -0.1g

Agar -1.8g

D.W -100ml

pH ±6.5

(After autoclaving Nystatin were added to the medium.)

Test for Morphological Characters

1. Gram staining test for bacteria

Actively growing culture (24 hours) was used for Gram staining. Small drop of sterilized water was added on a clear microscopic slide. A young colony was picked with a cool sterile loop from the agar medium and smeared the colony onto the grease free glass slide. The smear was heat fixed on the slide by passing the slide 4 times through a Bunsen flame. The crystal violet was flooded onto the slide and set a slide for 60 seconds. The slide was rinsed under running water and drained. Then, it was flooded again with iodine solution and set a slide for 60 seconds and wash with 95% ethanol for 30 seconds and then blotted to dry. It was counter stained with safranin for 10 seconds, then rinsed with water and dried. The slide was examined under light microscope at x100 magnification using oil immersion lens. (Christain Gram, 1884)

2. Endospore staining test for bacteria

The bacterial smear was prepared in the usual way and fixed by passing the slide 20 times over a flame. The endospore stain used in the research work was malachite green solution and safranin a counter stain. The smear was covered with malachite green and allowed it to react for 10 minutes on water bath. It was necessary to cover the smear with malachite green solution to avoid dryness to smear. The slide was rinsed with tap water for 10 seconds and then stained with safranin for 1 minute. It was rinsed with water, blot dry and examined under immersion. (Santra *et al*., 1998)

3. Test for Motility activity

The compositions of medium were peptone-10g, sodium chloride-5g, agar-5g and distilled water-1000ml. This medium was sterilized by autoclaving at 15 psi at 121 °C for 15 minutes and the isolated strains were inoculated into stabbing medium ¾ of the way to the bottom of the tube. During growth, motile bacteria will migrate from the line of inoculation to form a dense turbidity in the surrounding medium and non-motile bacteria will grow only along the line of the inoculation. (Woodland, 2004)

4. Test for Oxygen requirement activity

Nutrient broth was prepared with distilled water and mixed thoroughly into test tubes. It was autoclaved for 15 minutes at 15 psi at 121 °C. After autoclaving, the test tube was cool

down at room temperature. The isolated strains were taken by loop and it was dipped into the medium. After 24-hours the microbes were observed, occupying different regions of the medium which reflect their pattern of oxygen usage. (Prescott, 2002)

5. Test for Starch hydrolysing activity

The isolated microorganisms were inoculated in sterilized 5ml liquid medium containing soluble starch 1.5 g, K_2HPO_4-0.2g, $MgSO_4$-0.15g, distilled water-100ml for 24 hours at room temperature. Then diluted iodine solution was poured slowly into the liquid medium. (May-Suan Lee, 2022)

6. Test for Catalase activity

A few drops of 3% H_2O_2 solution was placed on the glass slide. A part of a 24 hours old colony was removed using a cooled sterile loop from agar medium. Bacterial colony was mixed with H_2O_2 solution. (R.C.Dubey, 2002)

7. Test for Potato soft rot activity

About 7-8mm thick slices from washed, alcohol-flamed, peeled potatoes were cut and each slice was placed in a petridish. Sterile distilled water was added to a depth of 3-4mm. A small niche was made in the center of each slice. A spot inoculation had brought about with a loopful of bacterial culture and incubated for at least 24hours. Determination had made whether the slice decayed beyond the point of inoculation. (Fontem. 1995)

8. Test for Oxidation activity

The basal medium containing glucose and phenol red indicator was used. Pure culture of bacteria was incubated into conical flask for 24-48 hours. If the medium in the anaerobic conical flask turned yellow, then the bacteria were fermenting glucose. If the medium didn't change color, and the open conical flask turned blue, then the organism neither fermented, nor oxidized glucose. (Mc Govern, 2004)

9. Test for Urea activity

Urea broth medium included urea-2g, yeast extract-.01g, KH_2PO_4-0.9g, Na_2HPO_4-0.9g, phenol red-0.001g, distilled water-100ml. The sterilized urea broth was inoculated with isolated bacteria and then incubated for 10 days at 30°C. The broth from the incubator was observed its color. (Christerison, 1946)

10. Test for Nitrate activity

After autoclaving the nitrate broth (KNO_3 -1.0g, beef extract-3.0g) was inoculated with a suitable culture and incubated at 37 °C for 48 hours. A bacterial plate was dispensed 3 drops of reagent (zinc chloride-2.0g, starch-0.4g, potassium iodide-0.2g, distilled water-100ml and 1drop of sulphuric acid). One drop of culture was transferred to it. (R.C.Dubey, 2002)

11. Test for Triple Sugar

The broth medium of glucose, sucrose and lactose was prepared. After autoclaving, small loop of bacteria was added to each broth medium. The broth cultures were incubated for 24-72 hours at room temperature. The changes color of broth medium was observed. (Acharya Tankeshwar, 2022)

12. Test for Methyl Red activity

Isolated microorganisms were inoculated in sterilized 5ml liquid medium (glucose 0.05g, peptone-0.05g, NaCl-0.05g, NaOH-0.05g, distilled water-100ml, pH-7.5) for two to five

days. After inoculation, the broths were taken from the incubator and 5 drops of methyl red reagent was added to the broth. Changes the color was observed. (Tille P.M.2014))

RESULTS

Soil analysis

According to USDA Texture Triangular method, classify a Gaung- say-kyun soil sample were 9% clay, silt 51%, and 38% sand. First located 9% on the clay axis, and drawn a line horizontally from left to right. Next, located 51% on the silt axis, and drawn a line going down diagonally to the left. Finally located 38% on the sand axis, and drawn a line going up diagonally to the left. The intersection was in a region called Silty Loam.

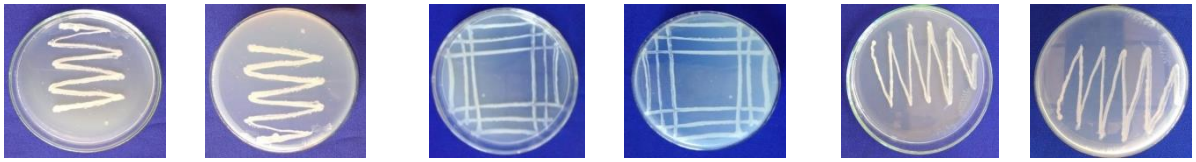
Table 2. Soil analysis

Collected soil	Soil pH	Soil texture			
		Sand %	Silt %	Clay %	Organic material
Gaung- say-kyun	± 6.5	38	51	9	2

Designation of isolated strains by Serial dilution

Table (3) Isolated bacteria from three different soil samples

Soil No.	Isolation bacteria
S.1	K-1
S.2	K-2
S.3	K-3



Front view (K-1) Reverse view (K-1) Front view (K-2) Reverse view (K-2) Front view (K-3) Reverse view (K-3)

Fig 4. Isolated pure culture of bacteria K-1, K-2, K-3 on PGA medium (3 days)

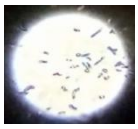
K-1 K-2 K-3

Fig 5. Isolated pure culture of bacteria preserved on PGA medium

Characteristic of isolated microbes

1. Gram staining test

After treating with Gram staining test, color of bacterium change to dark purple is Gram-positive and while change to red or pink color is Gram-negative. So, K-1, K-2 and K-3 showed the Gram-positive bacteria.

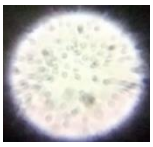
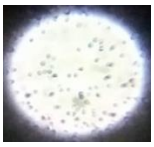
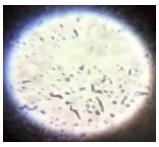


K-1 (Gram positive) K-2 (Gram positive) K-3 (Gram positive)

Fig 6. Gram staining test

2. Endospore staining test

The spores will not appear green so they do not produce endospores formation.

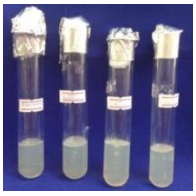
K-1 **K-2** **K-3**

Fig. (7) Endospore staining test

3. Motility activity test

A positive motility test is indicated by a diffuse zone of growth flaring from the line of inoculation. A negative motility test is indicated by growth confined to the stab line.

K-1, K-2 and K-3 showed positive motility.

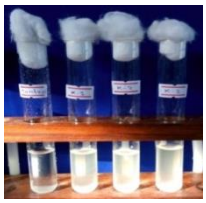


Control K-1 K-2 K-3

Fig 8. Motility activity test

4. Oxygen requirement activity test

Microorganisms have different oxygen requirements. In a static culture, microorganisms occupy different regions of the medium reflecting their pattern of oxygen usage.



Control K-1 K-2 K-3

Fig 9. Oxygen requirement test

5. Starch hydrolysing activity test

If the liquid medium changes the purple color, the microorganisms cannot hydrolyze the starch. If the color does not change, the microorganisms can hydrolyze the starch. So, K-1 and K-3 showed negative reaction and K-2 showed positive reaction.

Control K-1 K-2 K-3

Fig 10. Starch hydrolyzing activity test

6. Catalase activity test for bacteria

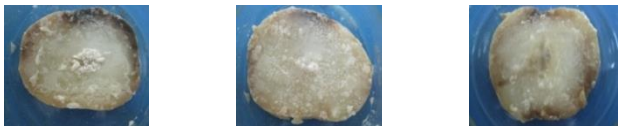
If some bubble appears within 20 seconds, the organism showed positive catalase activity. Absence of bubble showed negative catalase activity. So, K-1, K-2 and K-3 showed positive catalase activity.

K-1 K-2 K-3

Fig 11. Catalase activity test

7. Potato soft rot activity test

Potato soft rot test, positive reaction is decaying of potato beyond the point of inoculation. All microorganisms are decaying of potato. So, K-1, K-2 and K-3 showed positive reaction.



K-1 K-2 K-3

Fig 12. Potato soft rot activity test

8. Oxidation activity test

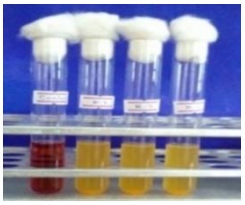
It used phenol red and produces deep yellow color of the medium showing oxidation reaction. So, K-1, K-2 and K-3 showed positive reaction.

Control K-1 K-2 K-3

Fig 13. Oxidation activity test

9. Urea activity test

All cultures that give positive test i.e., purple red color are discarded. So, K-1, K-2 and K-3 showed negative reaction.



Control K-1 K-2 K-3

Fig 14. Urea activity test

10. Nitrate activity test

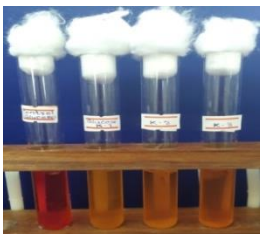
Appearance of blue color indicated the production of nitrate. If no blue color develops, occurrence of nitrate to nitrite reduction or all the nitrate so formed must have been further converted to other product. Therefore, K-1, K-2 and K-3 showed positive reaction.

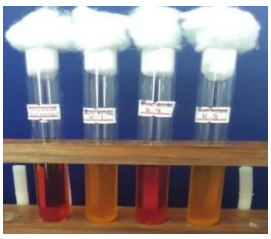
Control K-1 K-2 K-3

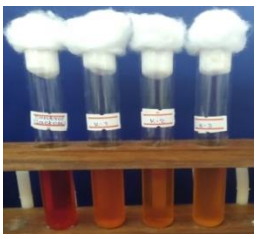
Fig 15. Nitrate activity test

11. Triple Sugar test

A positive reaction is indicated, if the color of the medium changes to yellow. So, K-1, K-2 and K-3 showed glucose and lactose are positive. K-1 and K-3 showed sucrose is positive but K-2 showed negative.







Glucose - Control K-1 K-2 K-3 Sucrose -Control K-1 K-2 K-3 Lactose- Control K-1 K-2 K-3

Fig 16. Triple Sugar test

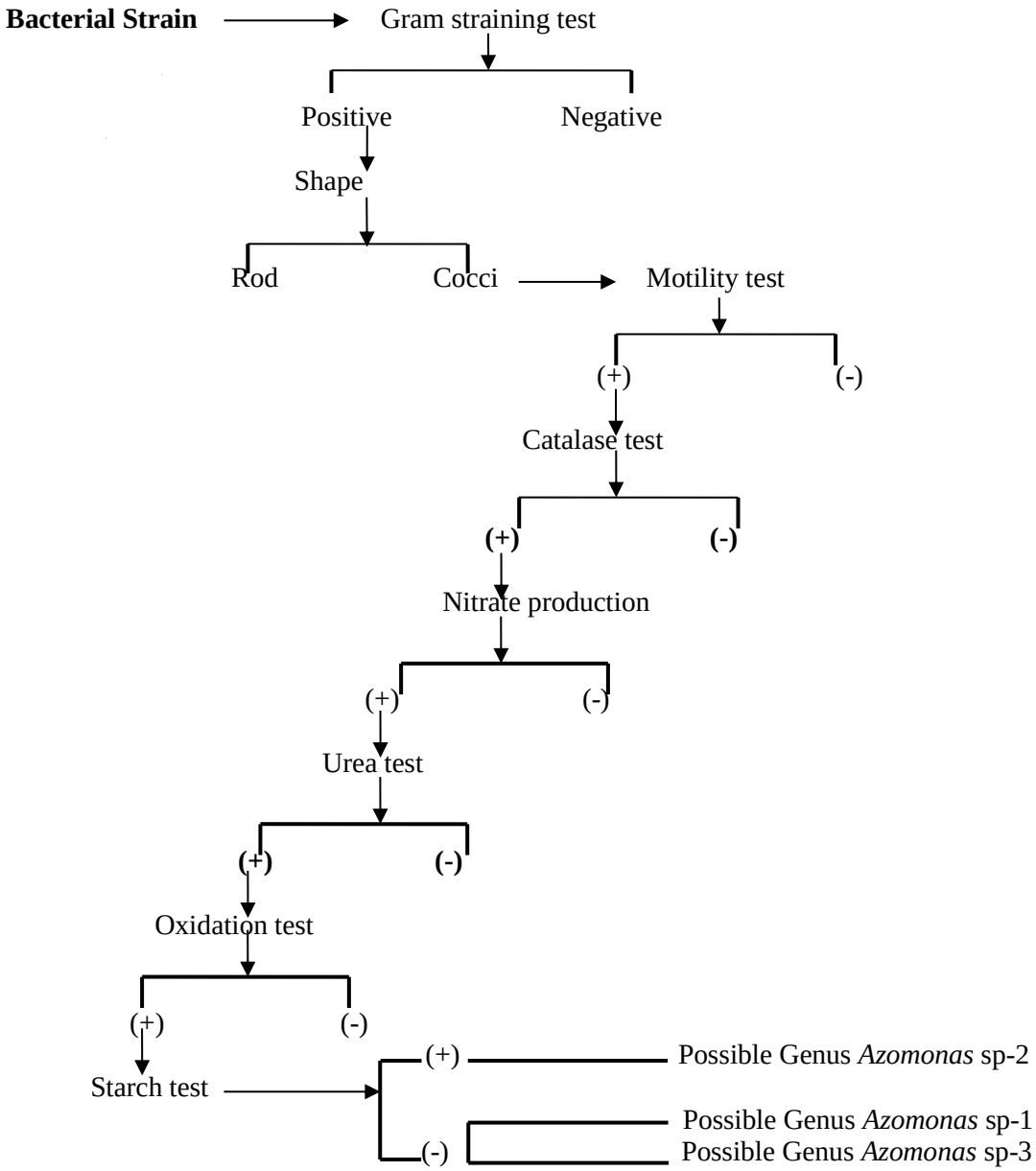
12. Methyl Red activity test

A positive reaction is indicated, if the color of the medium changes to red within a few minutes. If the color of medium changes to orange , that indicate a negative. So, K-1 showed negative reaction and K-2 and K-3 showed positive reaction.

Control K-1 K-2 K-3

Fig 17. Methyl Red activity test

A Dichotomous key leading to the Identification of Genus *Azomonas* sp (K-1, K-2, K-3) (according to Harley and Prescott, 2002)

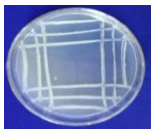


Morphological characters of *Azomonas*

Azomonas species are typically motile with polar or peritrichous flagella, oval to spherical and secrete large quantities of capsular slime. Cells occur singly, in pairs. Do not produce endospores formation. They are distinguished from Azotobacter by their inability to form cysts, but like Azotobacter, they can biologically fix nitrogen under aerobic condition. Found in soil and water.

Morphology of isolated bacteria

Microscopical character of *Azomonas* sp-1(x100)


Morphology of isolated bacteria


Microscopical character of *Azomonas* sp-2(x100)


Morphology of isolated bacteria

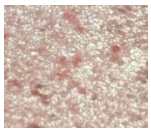

Microscopical character of *Azomonas* sp-3(x100)

Fig 18. Cell morphology of *Azomonas* sp

DISCUSSION AND CONCLUSION

The study was carried out to investigate the effect of isolated bacteria from tobacco cultivated soils at Gaung-say-kyun village in Hinthada Township. Isolation procedure was undertaken by serial dilution method using PGA medium. K-1 bacterial strain was isolated from the upper surface, K-2 was isolated from the upper part of soil sample from the depth of 2 inches and K-3 was isolated from the upper part of soil sample from the depth of 4 inches. Based on the biochemical activity test results of Dichotomous key, K-1, K-2 and K-3 were assumed *Azomonas* sp-1, *Azomonas* sp-2 and *Azomonas* sp-3 (according to Harley and Prescott, 2002).

The tobacco cultivated soil were selected *Azomonas* spp. these bacteria were used biofertilizer for plants growing and agricultural fields. So, these bacteria are applied to manure and improve the cultivated land.

Acknowledgements

My greatful acknowledgement and heartfelt thanks to Dr Theingi Shwe, Rector and Dr Yee Yee Than and Dr Cho Kyi Than, Pro-Rectors of Hinthada University for their permission to continue this work. I offer my gratitude and most sincere thanks to Dr. Khin Thu Zar Myint, Professor and Head, Dr. Aye Aye Mar, Professor, Department of Botany, Hinthada University, for her overall guidance, wise suggestion and continuous of this work.

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