ISOLATION, IDENTIFICATION OF BACTERIA FROM MANGROVE SOIL AND THEIR ANTIMICROBIAL ACTIVITY

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Abstract

The soil samples were collected from mangrove area at Shwe-thaung-yan Township, Ayeyawady Region. Isolation of bacteria was done by serial dilution method and cultured on Nutrient Agar (NA) medium (Atlas, 1993). As the results, total of seven bacterial strains (M-1 to M-7) were obtained and tested by agar well diffusion method with five kinds of test organisms. Four strains showed different levels of antimicrobial activities. Especially M-7 showed the best antifungal activity on *Candida albicans.* Therefore, M-7 was selected and identified by colony morphology, gram staining, spore staining, microscopical characters and biochemical reactions. According to the results, M-7 was characterized as the genus *Pseudomonas* sp.

Keywords: Mangrove Soil Bacteria, Antimicrobial activity, Identification, *Pseudomonas* sp.

Introduction

Mangrove are coastal wetlands mainly found at the intertidal zones of estuaries, deltas, creeks, lagoons, marshes of tropical and subtropical latitudes (Sahoo K, Dhal NK. 2009).

A vast suite of bacterial genera and functional types exist in mangrove soils and on above ground roots, but data are limited (Kathiresan and Bingham, 2001).

Antimicrobial agents play the most important role in the treatment of bacterial infections (Hacioglu, 2011) and wide spread efforts have been carried out by many scientists in order to screen for novel antibiotic producing microbes (Oskay, *et al.,* 2004).

Natural products having novel structures have been observed to possess useful biological activities, soil is a natural reservoir for microorganisms and their antimicrobial products (Dancer, 2004).

The bacteriologist must separate the mixed population into the separate components or isolate the organism desired. In general bacteria are classified both on the basis of what they do and of what they look like. Observations of the morphology of the cells, of their staining properties of the colonies they form on agar, and of the physiological or biochemical behavior of pure cultures of bacteria are two important tools in the study, identification, and classification of these minute forms of life (Alongi, 1988).

Therefore, the aim and objectives of the present study were to isolate the bacteria from mangrove soil, to study the antimicrobial activities of isolated bacteria and to observe the identification of selected bacterial strain based on their colony characters, gram staining, spore staining, cell morphology and biochemical reactions.

Materials and Methods

Soil samples were collected from mangrove soil of Shwe-thaung-yan Township, Magyi tidal Creek, Ayeyawady Region. The isolation of mangrove soil samples were carried out at the (BDC), Pathein University.

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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 from soil.

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

Figure 1 Location map of Shwe-thaung-yan Township in Ayeyawady Region

Gram Staining Method (Collins, 1965)

A smear of bacterial cells was prepared on a clean glass slide and the smear was then allowed to air-dry followed by a mild heat fixation. Crystal violet solution was added onto bacterial smear and incubated for one minute. The smear was washed with water Mordant Gram's iodine Solution was then added on bacterial smear and incubated for one minute. The smear was decolonized by washing with 95% ethyl alcohol and rinsed with water. Finally, safranin was used as counter for one minute and washed with water. Cell were then air dried and studied under microscope.

Spore Staining

Spore staining of isolated microbes were carried out according to the procedures described by Cruickshank 1968.

Preliminary Study on Antimicrobial Activities of Isolated Bacteria

The isolated soil bacteria were inoculated into seed medium and incubated for 1 day at room temperature. After one day, the seed culture was transferred into the fermentation medium and carried out by static culture. Then, the fermented broth was used to check the antimicrobial activity by agar well method (Collins, 1965). Agar well having (8 mm in diameter) were utilized for antimicrobial activity.

Agar Well Method (Collins, 1965)

1 day old culture test broth (0.2 mL) was added to 25 mL warm of assay medium (glucose 1.0 g, peptone 0.3 g, yeast 0.2 g, DW 100 mL, agar 1.8 g) and thoroughly mixed and poured into plate. After solidification, the agar was left to set. Cork borer was used to make the wells (8 mm in diameter). And then, the fermented broth (20 µL) was carefully added into the well and incubated at room temperature for 24-48 hours. The diameter of the zones of inhibition around each well was measured and recorded after 24-48 hours incubation.

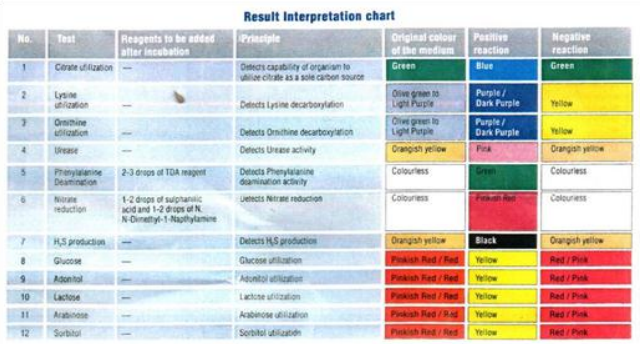
Test Organisms

Bacillus subtilis IFO 90571, *Candida albicans* NITE 09542, *Escherichia coli* AHU 5436, *Agrobacterium tumefaciens* NITE, 09678, *Bacillus pumilus* IFO 90571 were obtained from NITE (National Institute of Technology and Evaluation, Kisarazu, Japan) and Institute of fermentation Osaka, Japan.

Identification of Isolated Bacteria

The identification of isolated bacterial strains were carried out by their colony morphology, gram staining methods (Woodland 2004 and Collins, 1965), spore staining (Cruickshank, 1968) and biochemical tests such as catalase test (Salle 1948) oxidase test (Dickey & Kelman, 1988), motility test (Prescott, 2002), aerobic/anaerobic test (Prescott, 2002), methyl red (MR) test (Bisen and Verma, 1998), Voges-Proskauer (VP) test, citrate utilization (Atlas, 1993), urea hydrolysis (Christenson, 1946), hydrogen sulphide (H_2S) (Cowan, 1975), phenylalanine (PPA) test (Atlas, 1993) and NaCl salt tolerance (2%-10%) (Atlas, 1993), carbohydrate fermentation (Cowan, 1975) using seven different sugar, starch hydrolysis (Pelezar and Chan, 1972) of using the powder of soluble starch, tapioca and wheat powder, rice, sticky rice, glue, corn and potato; using KB002Hi Assorted™ Test Kit (India) were observed. In the proteolytic activity, gelatin and casein hydrolysis, esterase activity, and potato slice test were also studied.

Figure 2 KB 002 Hi Assorted™ Biochemical Test Kit



Result Interpretation chart

No.	Test	Reagents to be added after incubation	Principle	Original colour of the medium	Positive reaction	Negative reaction
1	Citrate utilization	—	Detects capability of organism to utilize citrate as a sole carbon source	Green	Blue	Green
2	Lysine utilization	—	Detects Lysine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
3	Ornithine utilization	—	Detects Ornithine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
4	Urease	—	Detects Urease activity	Orangish yellow	Pink	Orangish yellow
5	Phenylalanine Deamination	2-3 drops of TDA reagent	Detects Phenylalanine deamination activity	Colourless	Green	Colourless
6	Nitrate reduction	1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-1-Naphylamine	Detects Nitrate reduction	Colourless	Pinkish Red	Colourless
7	H_2S production	—	Detects H_2S production	Orangish yellow	Black	Orangish yellow
8	Glucose	—	Glucose utilization	Pinkish Red / Red	Yellow	Red / Pink
9	Adonitol	—	Adonitol utilization	Pinkish Red / Red	Yellow	Red / Pink
10	Lactose	—	Lactose utilization	Pinkish Red / Red	Yellow	Red / Pink
11	Arabinose	—	Arabinose utilization	Pinkish Red / Red	Yellow	Red / Pink
12	Sorbitol	—	Sorbitol utilization	Pinkish Red / Red	Yellow	Red / Pink

Figure 3 Result interpretation chart

Results

The total of seven bacterial strains such as M-1, M-2, M-3, M-4, M-5, M-6 and M-7 were isolated from the mangrove soils of coastal area. The results showed that the colonies morphology of those isolated strains (M-1 to M-7) were small and moderate in sizes; circular, irregular, entire in margins; cream, white and pale yellow in color; raised and flat in elevation and form; shiny, pale green and dull in pigments on agar, respectively. The results of colony morphology and cell morphology for the isolated bacterial strains were shown in Table 1-2 and Figure 4, 5. The antimicrobial activities were shown in Table 3 and Figure 6-9. The biochemical tests for the selected bacterium M-7 were shown in Table 4 and Figure 10-18.

Table 1 Colony morphology of the isolated bacteria

Isolated strains	Size of colony	Margin	Color	Elevation and form	Pigment on agar
M-1	Small	circular	white	flat	pale
M-2	Small	entire	cream	raised	shiny
M-3	Small	circular	white	raised	shiny
M-4	Small	circular	white	flat	shiny
M-5	Small	circular	cream	raised	shiny
M-6	Moderate	irregular	white	raised	dull
M-7	Small	circular	cream	raised	shiny

Table 2 Cell morphology of the isolated bacteria

Isolated strains	Gram staining	Cell morphology
M-1	+	cocobacilli
M-2	+	cocobacilli
M-3	-	short rod
M-4	-	short rod
M-5	-	short rod
M-6	-	short rod
M-7	-	short rod

positive = + negative = -

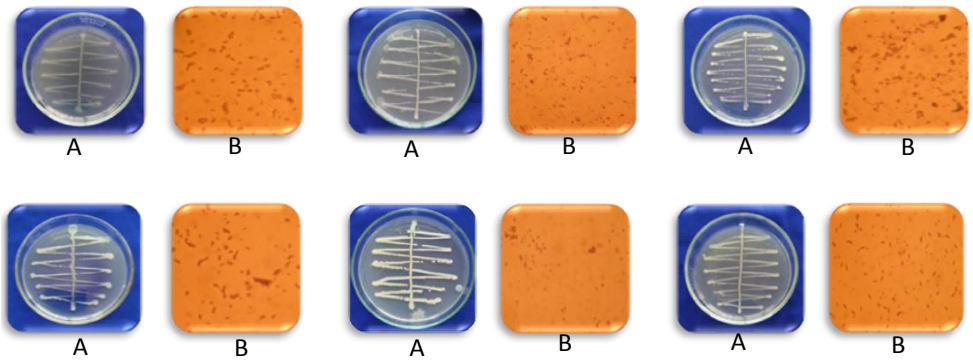


Figure 4 Cultural characteristics and cell morphology of M-1 to M-6
A = Culture (Streaks method), B = Cell morphology

Figure 5 Cultural characteristics and cell morphology of M-7

Antimicrobial activities of Isolated Bacterial strains

Seven isolated bacteria (M-1 to M-7) had antimicrobial activity and remaining 3 isolates (M-3, 4, 5) could not produce antimicrobial metabolites. These results were displayed in Figure (6-9) and Table 3. Among them, antifungal activity of isolated bacterium M-7 showed the maximum inhibitory zone against *Candida albicans.*

Table 3 Antimicrobial Activities of 7 Isolated Bacteria Against Five Test Organisms

No.	Isolated Bacteria	*Candida albicans*	*E. coli*	*Bacillus subtilis*	*Bacillus pumilus*	*Agrobacterium tumefaciens*
1	M-1	24.77 mm	23.28 mm	21.63 mm	20.03 mm	22.66 mm
2	M-2	16.60 mm	14.05 mm	15.21 mm	15.51 mm	13.35 mm
3	M-3	-	-	-	-	-
4	M-4	-	-	-	-	-
5	M-5	-	-	-	-	-
6	M-6	16.72 mm	14.48 mm	14.73 mm	15.45 mm	12.90 mm
7	M-7	**25.37 mm**	21.09 mm	19.21 mm	23.63 mm	19.24 mm

(+) = Activity present, (-) = No activity, Agar well size = 8 mm

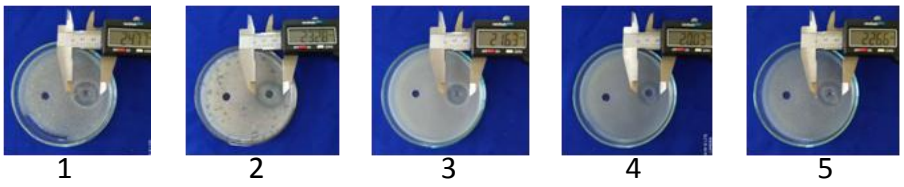


Figure 6 Antimicrobial activities of M-1 on five test organisms

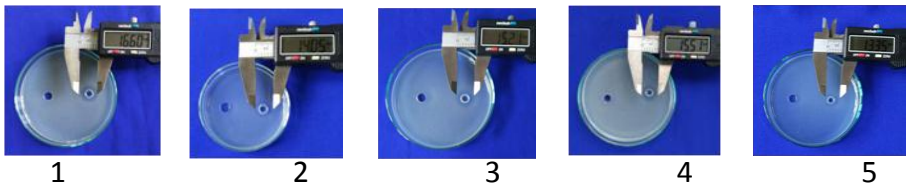


Figure 7 Antimicrobial activities of M-2 on five test organisms

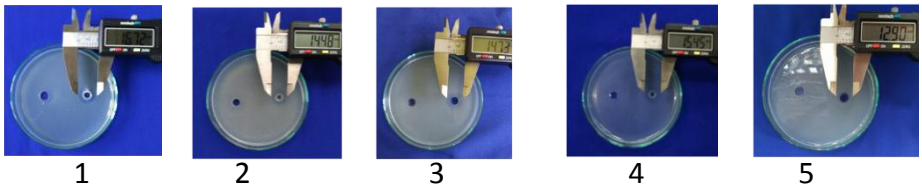


Figure 8 Antimicrobial activities of M-6 on five test organisms

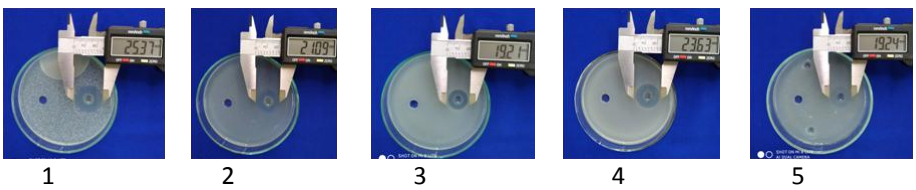


Figure 9 Antimicrobial activities of M-7 on five test organisms

1. *Candida albicans* 2. *Escherichia coli*
3. *Bacillus subtilis* 4. *Bacillus pumilus*
5. *Agrobacterium tumefaciens*

Table 4 Biochemical tests for the selected bacteria M-7

No.	Biochemical tests	Results
1	Catalase	+
2	Oxidase	-
3	Motility	+
4	Methyl Red (MR)	-
5	Voges-Proskauer (VP)	-
6	Citrate	+
7	Urea hydrolysis	+
8	Hydrogen Sulphide (H_2S)	+
9	Phenylalanine (PPA)	+
10	Salt tolerance NaCl (2% - 10%)	+
11	Carbohydrate fermentation (eight different	+
12	Starch hydrolysis	+
13	Gelatin hydrolysis	-
14	Casein hydrolysis	-

positive = + negative = -

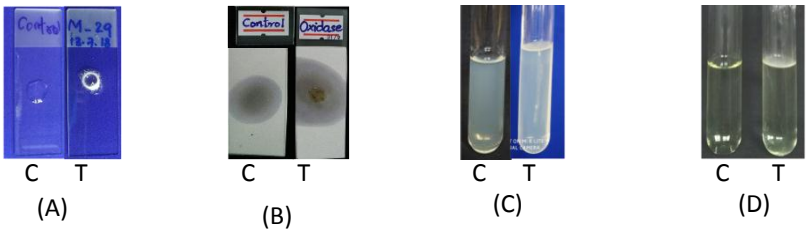


Figure 10 Biochemical tests of selected bacterium M-7 (A) Catalase Test (B) Oxidase Test (C) Motility Test (D) Aerobic/Anaerobic Test

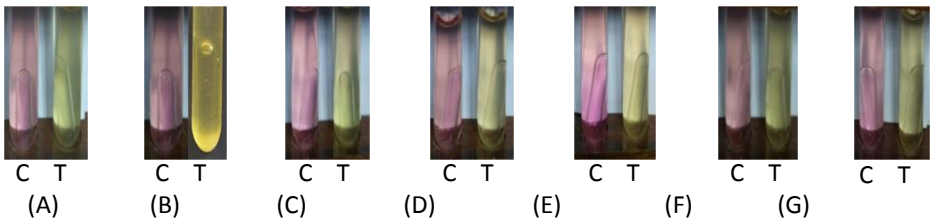


Figure 11 Carbohydrate fermentation for selected bacterium M-7

(A) Sucrose (B) Dextrose (C) Maltose (D) Arabinose
(E) Galactose (F) Xylose (G) Lactose

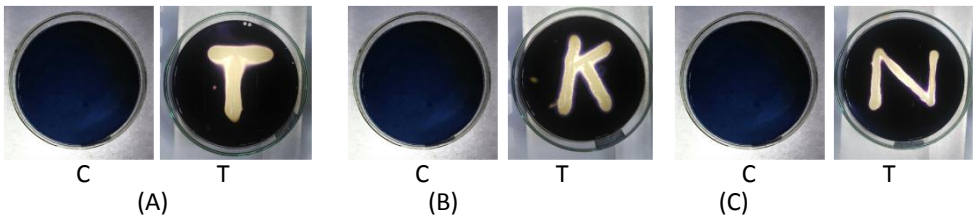


Figure 12 Starch hydrolysis tests of selected bacterium M-7
(A) Rice (B) Sticky Rice (C) Wheat

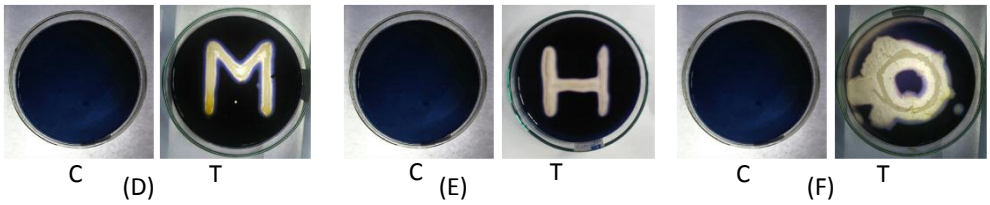


Figure 13 Starch hydrolysis tests of selected bacterium M-7
(D) Glue (E) Soluble starch (F) Potato

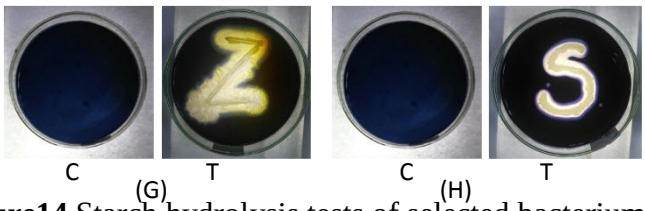


Figure14 Starch hydrolysis tests of selected bacterium M-7
(G) Corn (H) Tapioca

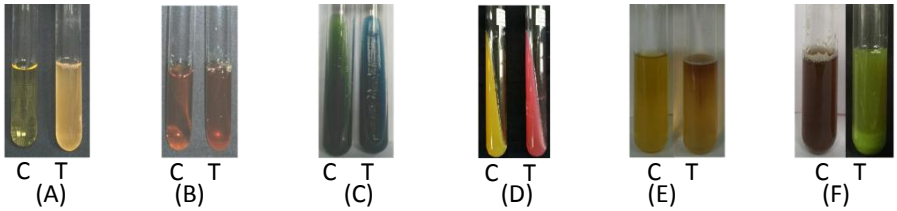


Figure 15 Biochemical tests of selected bacterium M-7
(A) Methyl Red (MR) Test (B) Voges Proskauer (VP) Test
(C) Citrate utilization Test (D) Urea hydrolysis Test
(E) Hydrogen Sulfide (H2S) production (F) Phenylalanine (PPA)

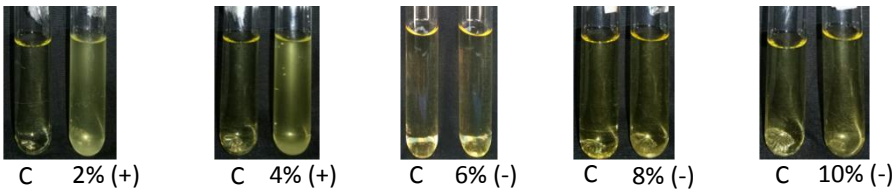


Figure 16 NaCl salt tolerance of selected bacterium M-7

Control **Treatment**

Figure 17 KB 002 Hi Assorted^{TM} Biochemical Test Kit of M-7

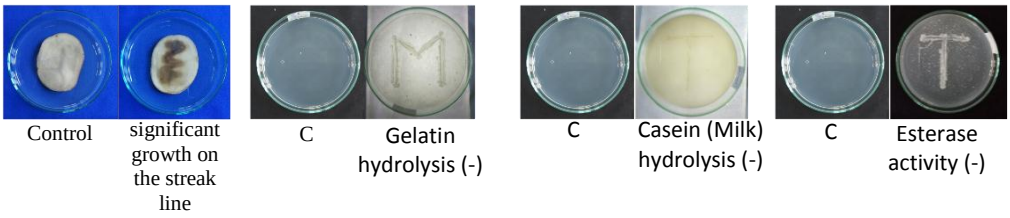


Figure 18 Biochemical test of selected bacterium M-7

Discussion and Conclusion

Mangrove ecosystems are rich in bacterial flora. Fertility of the mangrove water results from the microbial decomposition of organic matter and recycling of nutrients. Among the microbes, the bacterial population in mangroves is many-fold greater than the fungi. In tropical mangroves, bacteria and fungi constitute 91% of the total microbial biomass, whereas algae and protozoa represent only 7% and 2% respectively (Alongi D.M., 1988).

For the past 50 years antibiotics have revolutionized medicine by providing cures for formerly lie threatening diseases. Many marine free-living inhabiting marine bacterial have been shown to produce secondary metabolites that display antibacterial properties (Burgess JG *et al.*,1991).

In the present study, the total of seven bacterial strains were isolated and identified from the mangrove soil of magyi coastal area.

Seven isolated bacteria (M-1 to M-7) could display the antimicrobial activity inhibiting the five test organisms with agar well diffusion method and the remaining 3 isolates (M-3, 4, 5) could not produced antimicrobial metabolites. Among them, M-7 showed different levels of antimicrobial activities on five test organisms. M-7 showed the higher antifungal activity (25.37 mm and 23.63 mm) against *Candida albicans* and *Bacillus pumilus* than the other strains. Therefore, selection of M-7 was carried on further experiments.

Antibiotics and other bioactive compounds have been isolated from microorganisms in different environment (Charousova *et al.*, 2017; Devi *et al.*, 2017)

In the microscopical and biochemical characters, M-7 was gram-negative and short rod, catalase positive, aerobe, acid was produced in the sugar (dextrose, fructose, sucrose, maltose, arabinose, galactose, xylose and lactose) fermentation and gas was produced in the sugar sucrose, dextrose, galactose and xylose.

And then, M-7 can hydrolyze various starch sources (rice, sticky rice, wheat, soluble starch, tapioca powder, glue, potato and corn) respectively, H_2S was produced and motility present, Methyl red (MR) and Voges-Proskauer (VP) tests were negative, Phenylalanine (PPA) positive, citrate utilization positive, oxidase test negative, urease test positive, gelatin and casein hydrolysis negative; M-7 can tolerate in 2% and 4% NaCl salt concentration except 6%, 8% and 10% ; grown on potato slice.

These characters were performed according to the Bergey's Manual of Determinative Bacteriology by Breed *et al.*, 1957. Based on the obtained results of different biochemical tests, M-7 was classified as belonging to the genus *Pseudomonas* sp.

The genus *Pseudomonas* is the most heterogeneous and environmentally significant known bacterial group and includes mobile gram-negative aerobic rods, extended in all nature and characterized by its high metabolic versatility given by a complex enzymatic system. In addition, *Pseudomonas* species tend to be predominant among the bacteria associated with plants rhizosphere. Jaharamma *et al.,* 2009, Opasola *et al.,* 2011.

Juliana *et al.,* 2013 reported that in the nutrient agar, the colonies of *Pseudomonas* sp were circular, convex, entire margin, without or with pigmentation , which varied between-brown and pale yellow, some shinny.

It was concluded that the present study was the isolation bacteria from mangrove soil samples and the antimicrobial activity of isolated bacteria on five test organisms. In order to identify the selected bacterial strain M-7, biochemical tests were carried out and this bacterium can be noted as the *Pseudomonas* sp.

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