ISOLATION AND ANTIMICROBIAL ACTIVITY OF BACTERIA FROM MANGROVE SOIL AND BIOCHEMICAL CHARACTERIZATION OF SELECTED BACTERIUM (ZM-7)

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Abstract

Mangrove soil samples were collected from four different station of U-To Creek at Shwe thaung yan Township (Ma-Gyi), Ayeyawady Region. These samples were cultured on Nutrient Agar (NA) and American Type Culture Collection (ATCC) medium. A total of 26 bacterial colonies were obtained and 14 strains from Nutrient Agar (NA) and 12 isolates from (ATCC) medium. Isolated strains were designated as ZM 1 to 26. These isolated strains were tested by using ten different test organisms from one day to three days old culture. Five strains showed the antimicrobial activity. Among them, ZM-7 showed the highest antimicrobial activity (24.34 mm) on *Escherichia coli* and (22.63 mm) on *Candida albicans.* Therefore, ZM-7 was selected and characterized by morphological, microscopial, Gram staining and biochemical characteristics. In the colony morphology, ZM-7 was medium in size, entire, creamy, flat and creamy glistening. In the microscopical and biochemical characteristics, ZM-7 was Gram positive and short rod, catalase positive, aerobes and acid was produced in the sugar fermentation and gas did not produce. According to the results, ZM-7 was characterized as the possible genus *Bacillus.*

Keywords: Soil Bacteria, Antimicrobial activity, Biochemical characterization

Introduction

The microorganisms of mangrove are essential in the productivity, conservation, and rehabilization of mangrove ecosystem (Holguin *et al.*, 2001). Mangrove ecosystem shows diversity of microbes such as bacteria, fungi, actinomycetes etc. Bacteria includes various types like nitrogen fixing bacteria, phosphate soilubilizing bacteria, sulphate reducing bacteria, photosynthetic anoxygenic bacteria, methano bacteria, enzyme producing bacteria (Sahoo and Dhal, 2009).

Microbial research always involves the isolation and identification of microorganisms, strain preservation testing for biological activity and fermentation practice. Microbial fermentations have also been developed for the production of a wide range of pharmaceutical products (Mansi and Charlie, 2003).

The most commonly used biochemical tests involve the observation of whether or not a growth of the bacterium in liquid nutrient medium will ferment particular sugar such as glucose, lactose or mannitol. Then acid and gas may be produced which may be detected by a change in colour of un indicator dye present in the medium. Other tests determine whether the bacterium produces particular end products (eg. indole, H_2S, nitrite) when grown in suitable culture media. Many enzyme activities (such as catalase, ureas, gelatinase) are frequently measured to aid in the identification of bacteria (Mitruka and Mary, 1977).

Therefore, the present study was carried out the isolation and identification of bacterial strains from the mangrove soil. The aim and objectives of this study were to isolate the bacterial strains of mangrove soil and to identify those bacterial strains based on their colony morphology, gram staining, microscopical characters and biochemical reactions.

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Materials and Methods

Study area and collection of soil samples

Soil samples were collected from four different station of U-To-Creek, Shwe-Thaung Yan Sub-Township (Ma-Gyi) Ayeyawady Region. Soil smaples were collected from 0-3 cm, 1-6 cm and under 6 cm deep from each of these stations using a sterile spatual. Then, it was brought to the Biotechnological Development and Resource Centre (BDRC) and soil was analyzed by Department of Agriculture (Land Use).

Isolation of Soil Bacteria

Isolation of the bacteria from collected soil samples was done by serial dilution method. Salle 1948; Collins 1965 and Pelezer and Chan 1972, as soon as possible after soil collection in fields.

Preliminary Study on Antimicrobial Activity of Isolated Bacteria (NITE 2005)

The isolated soil bacteria were inoculated into seed medium and incubated for 3 days at 27ºC. Seed culture were transferred to the fermentation medium. After three days, the pre-culture (1%) was transferred into the fermentation medium (glucose 1.0 g, peptone 0.5 g, yeast extract 0.5 g, $MgSO_4$ $7H_2O$ 0.01 g, K_2HPO_4 0.01 g, $CaCO_3$ 0.01 g, DW 100 mL at pH 7.0 and carried out for 3-7 days and evaluated by agar well diffusion method.

Screening of Antimicrobial Activity by Agar Well Method

(Collins, 1965)

1 day old culture test broth (0.2 mL) was added to 25 mL warm assay medium (glucose 1.0 g, peptone 0.3 g, KNO_3 0.1 g, DW 100 mL, agar 1.8 g) and thoroughly mixed and poured into plate. 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 measured and recorded after 24-48 hours incubation.

Test Organisms

Bacillus subtilis IFO 90571, *Candida albicans* NITE 09542, *Staphylococcus aureus* AHU 8465, *Escherichia coli* AHU 5436, *Pseudomonas fluorescens* IFO 94307, *Agrobacterium tumefaciens* NITE, 09678, *Aspergillus paraciticus* IFO-5123, *Micrococcus luteus* NITE-83297, *Saccharomyces cerevisiae* NITE 83297 and *Salmonella typhi* AHU-7943 were obtained from NITE 2005 (National Institute of Technology and Evaluation, Kisarazu, Japan) and PRD-Pharmaceutical Research Departmen (Ministry of Industry).

The identification of isolated bacterial strains were carried out by using their colony morphology, gram staining methods (Dubey and Maheshwari, 2002), and biochemical tests which include the motility test (Tittsler and Sandholzer, 1936), methyl red test (Aneja, 1996), sugar fermentation test (sucrose, lactose, maltose) (Atlas, 1993), nitrate reduction test (Dickey and Kelman, 1988), starch hydrolysis test (Aneja, 1996), catalase test (Dickey and Kelman, 1988), oxidase test (Dickey and Kelman, 1988), oxygen requirement (aerobic/anaerobic) (Prescott, 2002), citrate utilization test (Atlas, 1993), Voges-Proskauer VP test (Cruickshank, 1963), Urea test (Woodland, 2004), respectively.

Results

The total of 26 bacterial strains (ZM-1 to 26) were isolated from the mangrove soils. The antimicrobial activity of these strains were tested by using ten different test organisms. Five strains showed the activity on *Aspergillus paraciticus, Candida albicans, Escherichia coli, Saccharomyces cerevisiae.* Among them, ZM-7 showed the highest antibacterial activity on *E. coli.* Therefore, ZM-7 was selected and identified by colony morphology, Gram-staining and Biochemical characteristics. In the colony morphology, ZM-7 was medium in size, entire, creamy, flat and creamy glistening. In the microscopical and biochemical characteristics, ZM-7 was Gram positive and short rod, catalase positive, aerobes and acid was produced in the sugar fermentation and gas did not produce. According to the results, ZM-7 was characterized as the possible genus *Bacillus.* These results were shown in Table 1, 2 and Figure 1, 2.

Table 1 Isolated Bacteria from Soil Samples

Soil Sample No.	Nutrient Agar (NA)	American Type Culture Collection (ATCC) medium
S - 1	ZM 1 – 3 = 3	ZM 1 – 2 = 2
S - 2	ZM 4 – 5 = 2	ZM 3 – 6 = 4
S - 3	ZM 6 – 9 = 4	ZM 7 – 9 = 3
S - 4	ZM 10 – 14 =	ZM 10 –12 = 3 /12

Table 2 Colony and Cell Morphology of Isolated Bacteria

Isolated Bacteria	Size of Colony	Margin	Color	Elevation and Form	Cell Morphology	Gram Staining
ZM - 1	Medium	Entire	Pale	Flat	Cocco-bacilli	-
ZM - 2	Medium	Entire	C ream	Flat	Rod	+
ZM - 3	Medium	Entire	White	Raised	Rod	+
ZM - 4	Large	Entire	White	Flat	Cocco-bacilli	-
ZM - 5	Medium	Filamentous	White	Flat	Short-rod	-
ZM - 6	Large	Entire	White	Flat	Rod	-
ZM - 7	Large	Undulate	White	Flat	Short-rod	+
ZM - 8	Medium	Entire	White	Raised	Cocco-bacilli	-
ZM - 9	Large	Entire	White	Flat	Short-rod	-
ZM - 10	Large	Entire	Creamy	Flat	Rod	-
ZM - 11	Medium	Entire	Creamy	Flat	Rod	-
ZM - 12	Medium	Entire	Creamy	Flat	Rod	-
ZM - 13	Large	Filamentous	White	Flat	Rod	-
ZM - 14	Large	Entire	White	Convex	cocco-bacilli	-
ZM - 15	Large	Undulate	White	Flat	Rod	+
ZM - 16	Large	Entire	White	Flat	Rod	-
ZM - 17	Small	Entire	White	Flat	Rod	-

Isolated Bacteria	Size of Colony	Margin	Color	Elevation and Form	Cell Morphology	Gram Staining
ZM - 18	Large	Entire	White	Flat	Rod	-
ZM - 19	Small	Filamentous	White	Flat	Short-rod	-
ZM - 20	Large	Rhizoid	White	Flat	Rod	-
ZM - 21	Small	Entire	C ream	Flat	Rod	-
ZM - 22	Large	Filamentous	White	Flat	Short-rod	-
ZM - 23	Large	Curled	White	Flat	Rod	-
ZM - 24	Large	Filamentous	C ream	Flat	Rod	-
ZM - 25	Large	Entire	White	Flat	Rod	+
ZM - 26	Large	Undulate	White	Raised	Rod	-

Small < 2mm diameter/ between 2mm and 5mm diameter
Large > 5mm diameter + = Gram positive - =Gram negative

Table 3 Carbohydrate Fermentation of Selected Strain ZM-7

Sugar	Responces	Acid production	Gas production
Glucose	Yellow colour changes in medium	+	-
Maltose	Yellow colour changes in medium	+	-
Xylose	Yellow colour changes in medium	+	-
Galactose	Yellow colour changes in medium	+	-
Fructose	No change in colour	-	-
Arabinose	No change in colour	-	-
Lactose	No change in colour	-	-

+ = acid and gas was produced
- = acid and gas was not produced

Table 4 Biochemical Characteristics of Selected Strain ZM-7

No	Biochemical tests	Responses	Results
1	Urea hydrolysis test	No change in colour	(-)
2	Mannitol salt broth test	No change in colour	(-)
3	Nitrate reduction test	Medium change from pale yellow to orange	(+)
4	Methyl red test	Medium remain red	(-)
5	Voges proskaucer test	Medium change from yellow to red	(+)
6	Citrate utilization test	Medium change from green to blue	(+)
7	H_2S production test	Medium change from white to black	(+)
8	Catalase test	Release free oxygen gas bubble	(+)
9	Oxidase test	A little change in colour	(+)
10	Hanging slide test	No motility	(-)
11	Arginine hydrolysis	No change back to purple	(-)
12	Gelatin hydrolysis	No clear zone around the colony	(-)
13	Potato plug	Growth in streak line	(+)
14	starch hydrolysis		
	(i) Soluble starch	Clear zone around the streak line	(+)
	(ii) Tapioca powder	Clear zone around the streak line	(+)
	(iii) Sticky rice powder	Clear zone around the streak line	(+)

No	Biochemical tests	Responses	Results
	(iv) Wheat powder	Clear zone around the streak line	(+)
	(v) Rice	Clear zone around the streak line	(+)
15	Caesin hydrolysis	Clear zone around the colony	(+)
16	Esterase activity	No colour change in medium	(-)
17	Salt tolerance test		
	(i) 2% NaCl	Highest growth	(+)
	(ii) 4% NaCl	Highest growth	(+)
	(iii) 6% NaCl	Moderate growth	(+)
	(iv) 8% NaCl	Moderate growth	(+)
	(v) 10% NaCl	Poor growth	(+)

\+ = Gram positive - = Gram negative

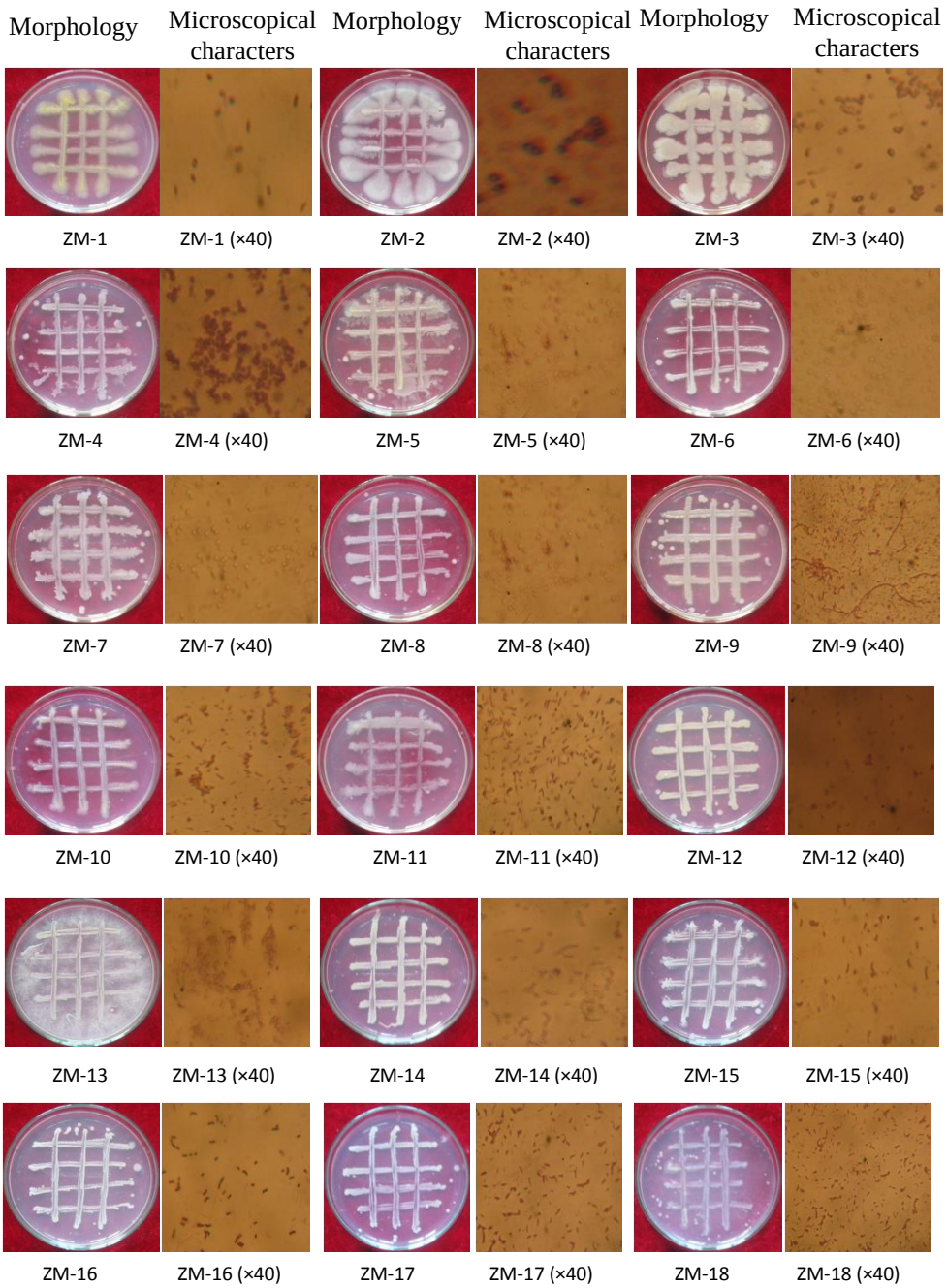


Figure 1 Cultural Character and Cell morphology of Isolated Bacteria ZM-1 to ZM-18

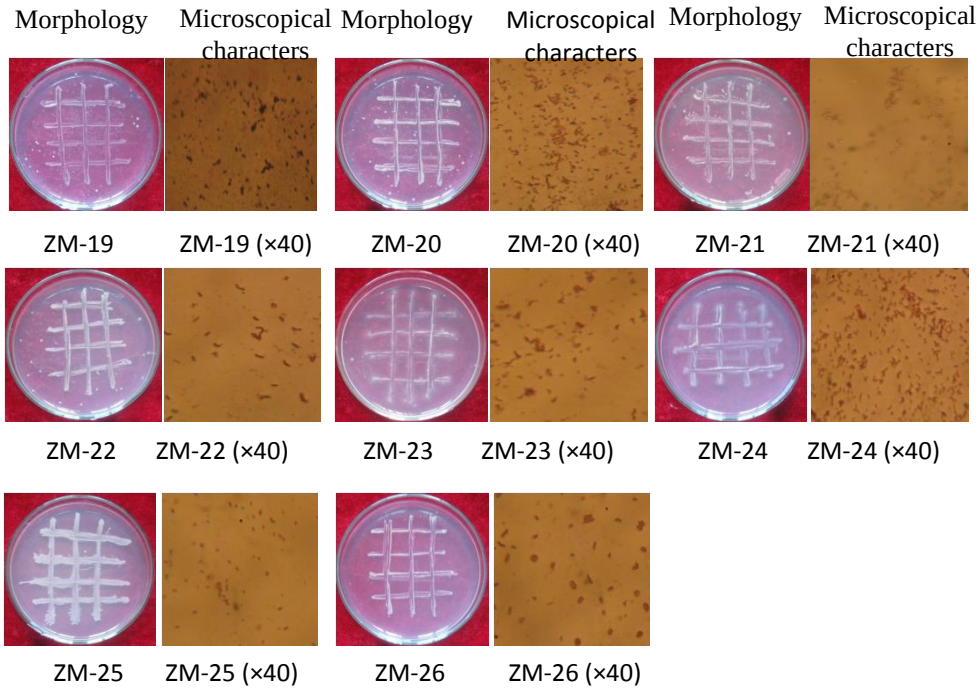


Figure 2 Cultural Character and Cell morphology of Isolated Bacteria ZM-19 to ZM-26

Antimicrobial Activity of Isolated Bacterial Strains from Mangrove Soil

Fourteen isolated bacteria (ZM-1, 2, 3, 4, 5, 6, 10, 11 and 14) could not produce antimicrobial metabolites. Five isolates (ZM-7, 8, 9, 12, 13) had antimicrobial activity and ZM-7 showed the highest antibacterial activity (24.34 mm) on *E. coli*, followed by (22.63 mm) on *Candida albicans.*

Table 6 Antimicrobial Activity of Five Selected Bacteria

No.	Isolated bacteria	Test Organisms and Antimicrobial Activity (mm)			
		Aspergillus paraciticus	***Candida albicans***	***Escherichia coli***	***Saccharomyces cerevisiae***
1	ZM-7	16.03 mm	22.63 mm	**24.34 mm**	14.45 mm
2	ZM-8	12.22 mm	-	18.30 mm	11.77 mm
3	ZM-9	-	-	-	15.00 mm
4	ZM-12	9.43 mm	-	10.75 mm	-
5	ZM-13	-	-	-	13.00 mm

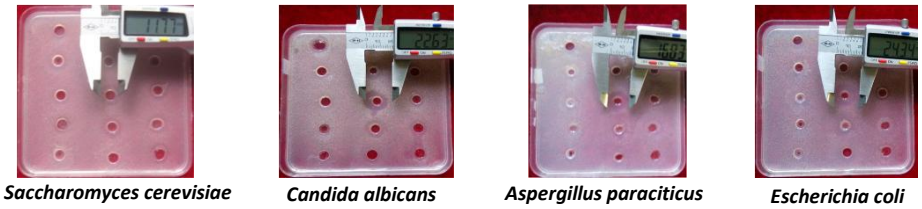
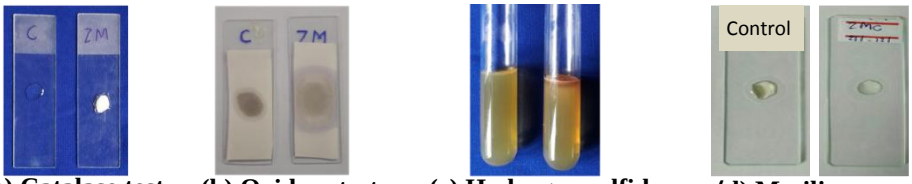


Figure 3 Antimicrobial Activity of Five Selected Bacteria



(a) Catalase test (positive) **(b) Oxidase test (positive)** **(c) Hydrogen sulfide test (positive)** **(d) Motility test (negative)**

Figure 4 Biochemical Characteristics of Selected Bacterium (ZM-7)

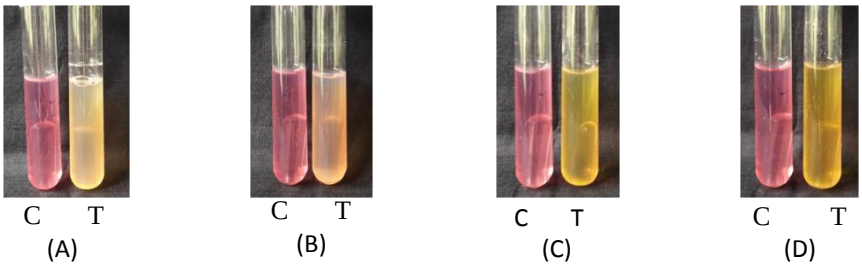


Figure 5 Carbohydrate Fermentation Test of Selected Bacterium (ZM-7) A. Glucose, B. Maltose, C. Xylose, D. Galactose (All positive)

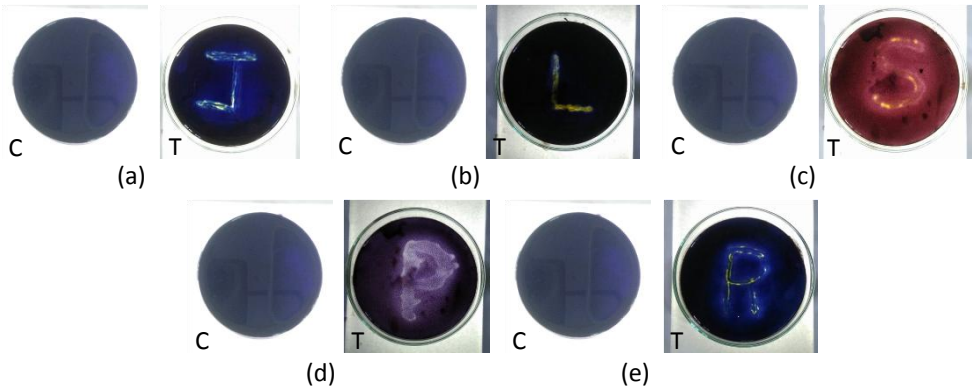


Figure 6 Starch Hydrolysis Test of Selected Bacterium ZM-7 (a) Soluble starch (positive), (b) Tapioca powder (positive), (c) Sticky rice (positive), (d) Rice (positive), (e) Wheat flour (positive)

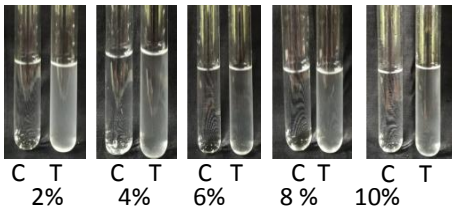


Figure7 NaCl Tolerance Test of Selected Bacterium ZM-7 (2% highest growth), (4% highest growth), (6% moderate growth), (8% moderate growth), (10% poor growth)

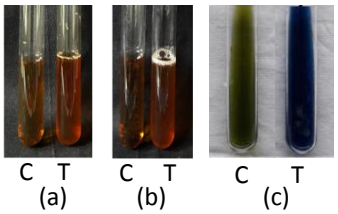


Figure 8 Biochemical Characteristics of Selected Bacterium ZM-7 (a)Methyl red (negative), (b)Voges Proskucer (positive), (c) Citrate utilization (positive)

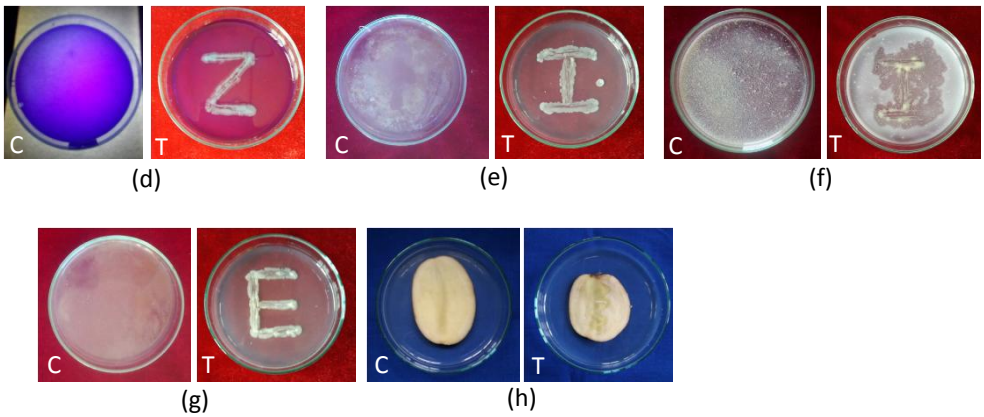


Figure 9 Biochemical Characteristics of Selected Bacterium ZM-7
(d) Arginine hydrolysis (negative), (e) Gelatin hydrolysis (negative),
(f) Caesin hydrolysis (positive), (g) Esterase (negative),
(h) Potato plug (positive)

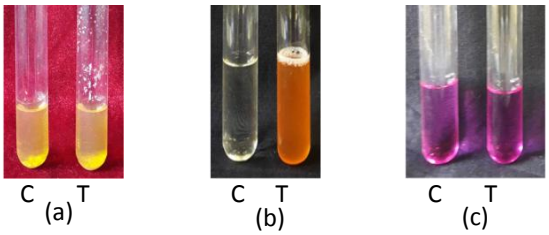


Figure 10 Biochemical Characteristics of Selected Bacterium ZM-7
(a) Mannitol test (negative), (b) Nitrate reduction (positive),
(c) Urea hydrolysis (negative)

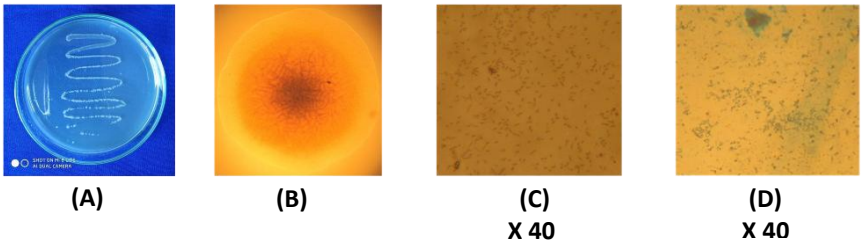


Figure 11 (A) Colony morphology (B) Single colony (C) Gram staining
(D) Spore staining of selected bacterium ZM-7

Discussion and Conclusion

In this study, 26 strains were isolated from four different samples collected from shwe-thaung yan coastal area (Ma-Gyi). Two different media were employed and it was found that 14 strains was got from NA medium and 12 strains from ATTC medium. These isolated bacteria were designated as ZM-1 to ZM-26. In the colony morphology, ZM-1 to ZM-26 were small, medium and large in size of colony and the color were cream, white, pale yellow and creamy. In the margin, ZM-1 to ZM-26 were entire, filamentous, undulate, rhizoid and curled. In the elevation and form, all strains were flat and raised and they produced pigments. Bhant, 2003

reported the bacterial colonies which were moist, small, with regular margin and were translucent from mangrove soil. Twenty isolates were found to be gram negative and 6 strains were gram positive. The results were in agreement with general rules of Haglund, 2003 that the proportion of Gram negative bacteria is much higher than the proportion of Gram positive bacteria in the ocean.

All strains were tested by using ten test organisms. Among them, strain ZM-7, showed the highest activity against *Aspergillus paraciticus, Candida albicans*, *Escherichia coli*, *Saccharomyces cerevisiae* at 2 days old culture. Therefore, ZM-7 was selected for futher study.

Mitruka and Mary, 1997 reported that in artificial classification, organisms are grouped together in a key. The first step in bacterial identification is necessary to obtain the organisms in pure culture. According to the biochemical characteristic, ZM-7 was short rod and Gram positive, spore present, catalase positive, oxidase positive, H_2S positive and motility negative, acid was produced in the sugar (glucose, maltose, xylose and galactose) except fructose, arabinose and lactose, gas not produced. In the starch hydrolysis, ZM-7 can hydrolyse soluble starch, tapioca powder, sticky rice, rice powder and wheat flour and can tolerate in NaCl 2%, 4%, 6%, 8% and 10% respectively, Methyl red negative, Voges Proskucer (VP) positive, citrate positive, esterase, arginine and gelatine hydrolysis, casein hydrolysis positive, potato plug positive, mannitol and urea hydrolysis negative, nitrate reduction positive. These characters were similar to the previous research of Buchanan 1974 and ZM-7 was classified as the genus *Bacillus* sp. Similarly observation was reported by Park *et al.*,2003 who isolated *Bacillus* spp. and identified from rotating biological contractor based on their biochemical properties Joshi *et al.*, 2007 identified *Bacillus* and studied its biochemical characteristics as well.

Thus, it would be concluded that the present findings of those isolated bacterial strains (ZM-7) can be noted as the *Bacillus* bacterial strain. Those bacterial strain would be isolated from the soil and identified as *Bacillus* spp. However, further study should be undertaken for the antimicrobial activities and biocontrol agent by using the effective bacterial strain.

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