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Isolation and Characterization of Endophytic Bacteria from the Leaves of *Carica papaya* L.

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Abstract

Endophytic bacteria were isolated from the leaves of *Carica papaya* L. (papaya). The leaf samples were collected from the Campus of University of Mandalay. This experiment was carried out at the Microbiology Laboratory, Department of Botany, University of Mandalay from December 2017 to August 2018. The six bacterial strains, TS 1 - TS 6 were isolated and characterized based on their colony morphology and biochemical tests. Each of isolates was characterized by the morphological characters (shape, colony, colour, cell size, gram staining, aerobic growth and motility) and biochemical tests (catalase, oxidase, starch hydrolysis, lysine decarboxylase, urea hydrolysis, sugar fermentation such as dextrose, glucose, manitol and sucrose, citrate utilization, methyl red, triple sugar iron (TSI) and nitrate reduction) were carried out. The isolated bacteria were confirmed TS 1 as *Mycobacterium* sp., TS 2 as *Bacillus* sp., TS 3 as *Staphylococcus* sp., TS 4 as *Micrococcus* sp. TS 5 as *Streptococcus* sp. and TS 6 as *Enterobacter* sp..

Keywords: *Carica papaya* L., Endophytic bacteria, Morphological, Biochemical

Introduction

Caricapapaya L. belongs to the family Caricaceae. Papaya is commonly known for its food and nutritional values throughout the world. The properties of papaya leaf and other parts of the plants are also well known in traditional system of medicine. Papaya leaf possess excellent medicinal properties for treatment of different ailments. The leaves of the papaya plants contain chemical compounds of carpain, substance which kill microorganisms that often intefere with the digestive function. Papaya leaves also play as acne medicine, increase appetite, ease menstrual pain, meat tenderizer and relieve nausea (Yogiraj *et al*. 2015).

Endophytes were isolated from medicinal plants showed bioactivity for broad spectrum of pathogenic microorganisms. Some commonly found endophytes are those belonging to the genera *Enterobacter* sp. Most of the endophytes isolated from plants are known for their antimicrobial activity. They help in controlling microbial pathogens in plants and animals (Dhanya & Padmavathy 2014). Endophytic bacteria colonize in plant and for competition from other microorganisms that they produce antimicrobial compounds and many enzymes. A variety of antibiotics, enzymes, anti-inflammatary, anticancerous, antifungal and biological control agents have been isolated from endophytic microorgarisms (Krishnan *et al*. 2012). Papaya leaf use for prevent oxidization of cholesterol, treating of gastrointestinal tract disease, nausea and morning sickness, weight loss, looting of body immunity, recovery of kidney, effect liver cancer cells, dengue fever treatment and menstrual irregularities in woman (Brij *et al*. 2013).

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Therefore, the present study was carried out the isolation and characterization of endophytic bacteria from the leaves of *Carica papaya* L. The aim and objectives of this study were to isolate the endophytic bacterial strains from papaya leaves and to study their colony morphological characters, gram staining, microscopical characters and biochemical activities.

Materials and Methods

The *Carica papaya* L. leaf samples were collected from the Campus of University of Mandalay. This experiment was carried out at the Microbiology Laboratory, Department of Botany, University of Mandalay from December 2017 to August 2018. The leaf samples were collected from these regions (Figure 1).

Fleshy collected leaves were washed slow running water for 15 minutes. Samples were cut into about 1 cm pieces. Samples were sterilized by immersing the sample in 70% ethanol for 1 minute, sodium hypochloride for 3 - 4 minutes, again 70% ethanol for 1 minute and then sterilize water for 3 - 4 minutes. The sterilized leaf samples were dried in the laminar air flow cabinet and then placed on the nutrient agar medium supplemented with antifungal agents. Plates with plant tissues are sealed using parafilm tape and incubated at 28ºC ± 2ºC in order to recover the maximum possible colonies of bacterial endophytes. The observation was made for 48 hours. After different bacterial colonies were selected and are repeatedly streaks in order to achieve bacterial isolates.

The characterization of isolated bacterial strains were carried out by using their colony morphology, gram staining method (Atlas 1993), and biochemical activities which include Catalase Test (Speck 1976), Oxidase Test (Dickey &Kelman 1988), Motility Test (Cruickshank *et al.* 1968), Starch Hydrolysis Test (Aneja 1996), Lysine Decarboxylase Test (Downes 2001), Urea Hydrolysis Test(Christensen 1946), Sugar Fermentation Test (Dextrose, Glucose, Manitol and Sucrose) (Atlas 1993), Citrate Utilization test (Sharma 2007), Methyl Red Test (Aneja 1996), Triple Sugar Iron Test (TSI) (Woodland 2004), Nitrate reduction (Shelle 1948), Aerobic Growth Test (Prescott 2002), Salt Tolerance Test (Atlas 1993).

Figure 1. Location map of sample collection site

Results

The total of six isolated bacteria such as TS 1, TS 2, TS 3, TS 4, TS 5 and TS 6 were isolated from the leaves of *Carica papaya* L. (papaya). The colonies morphology of those isolated bacteria TS 1 – TS 6 were small, moderate and puntiform in sizes; circular, irregular, entire in margins; creamy, pale yellow, white in color; flat in elevation and form; shiny and dull in pigments on agar. Those isolated bacteria were rod and coccus in their cell morphologies, aerobic and anaerobic, positive and negative in motility. The results of their colony morphology, cell morphology and biochemical activities were shown in Table 1 – 4 and Figure 2 – 22.

Table 1. Colony characteristics of six isolated bacteria

Strains	Size of Colony	Margin	Color	Elevation and Form	Appearance
TS 1	small	entire	creamy	flat	dull
TS 2	moderate	undulate	creamy	flat	shiny
TS 3	small	entire	pale yellow	flat	shiny
TS 4	small	undulate	creamy	flat	shiny
TS 5	puntiform	entire	white	flat	shiny
TS 6	small	entire	creamy	flat	shiny

Table 2. Cell morphology and physical characteristics of isolated bacteria

Strains	Gram Staining	Cell Shape	Physical Characteristics
TS 1	+	rod	aerobic
TS 2	+	rod	aerobic
TS 3	+	coccus	anaerobic
TS 4	+	coccus	anaerobic
TS 5	+	coccus	aerobic
TS 6	_	rod	anaerobic

positive reaction = + , negative reaction = –

Table. 3. Biochemical tests for characterization of isolated bacteria

	Isolated Bacteria					
Biochemical Tests	TS	TS 2	TS 3	TS 4	TS 5	TS 6
Catalase	+	+	+	+	+	+
Oxidase	_	_	_	+	_	_
Starch Hydrolysis	_	+	_	+	_	+
Lysine Decarboxylic	+	+	+	+	+	_
Urea Hydrolysis	_	_	+	+	+	_
Sugar Fermentation (Dextrose)	+A	+A	+A	+A	+AG	+A
Citrate Utilization	_	_	+	+	_	_
Methyl Red	_	_	_	_	_	+
Sugar Fermentation (Glucose)	_	_	_	+A	+A	+A
Sugar Fermentation(Manitol)	_	_	_	_	_	_
Sugar Fermentation (Sucrose)	_	+A	+A	_	+AG	+AG
Triple Sugar Iron	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+

positive reaction = + , negative reaction = – , +A = Acid, +AG = Acid with Gas

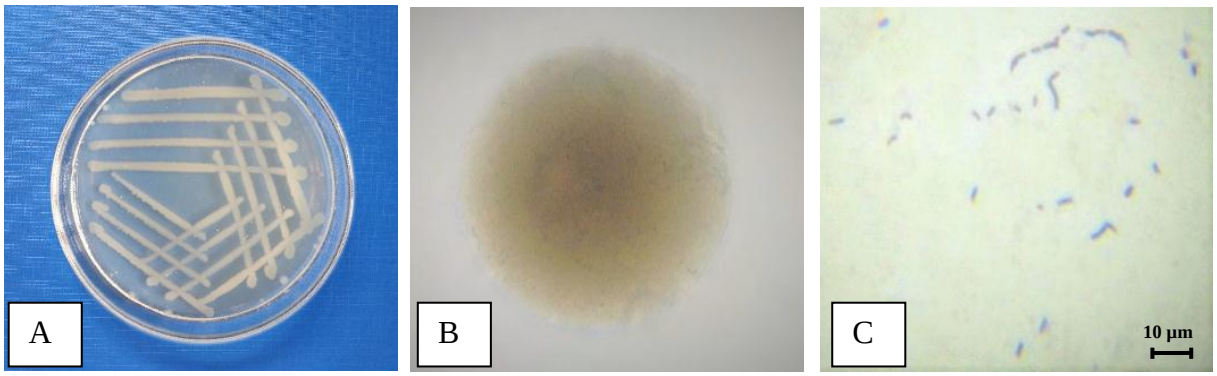

Figure 2. Colony Characteristics and Cell Morphology of TS 1

A. Colonies on streak plate,
B. Single colony
C. Photomicrograph of cells

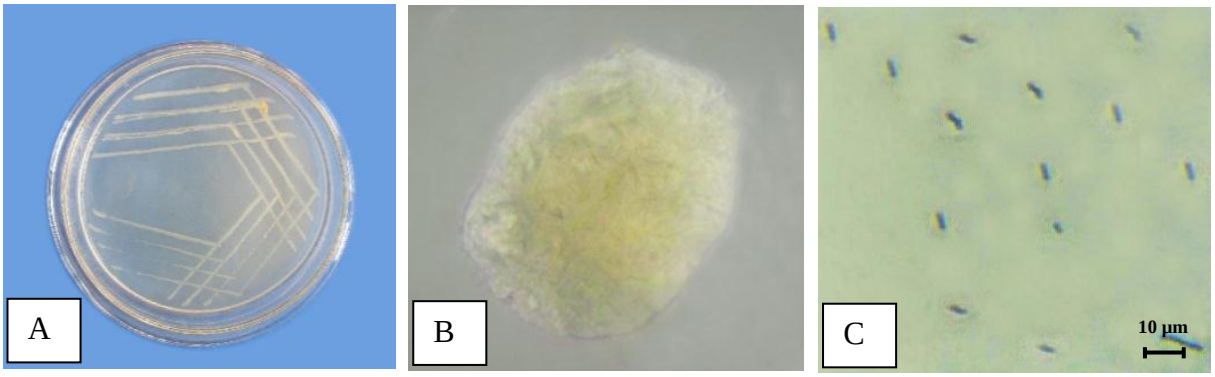

Figure 3. Colony Characteristics and Cell Morphology of TS 2

A. Colonies on streak plate,
B. Single colony
C. Photomicrograph of cells

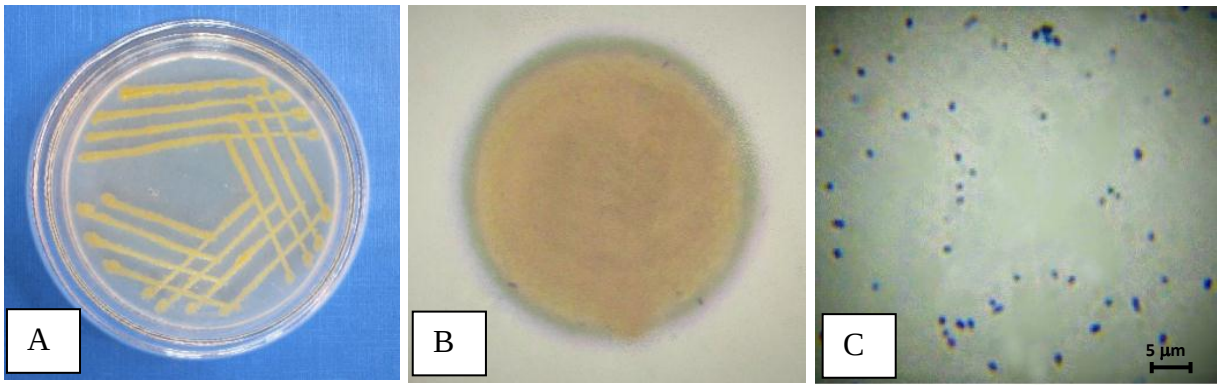

Figure 4. Colony Characteristics and Cell Morphology of TS 3

A. Colonies on streak plate,
B. Single colony
C. Photomicrograph of cells

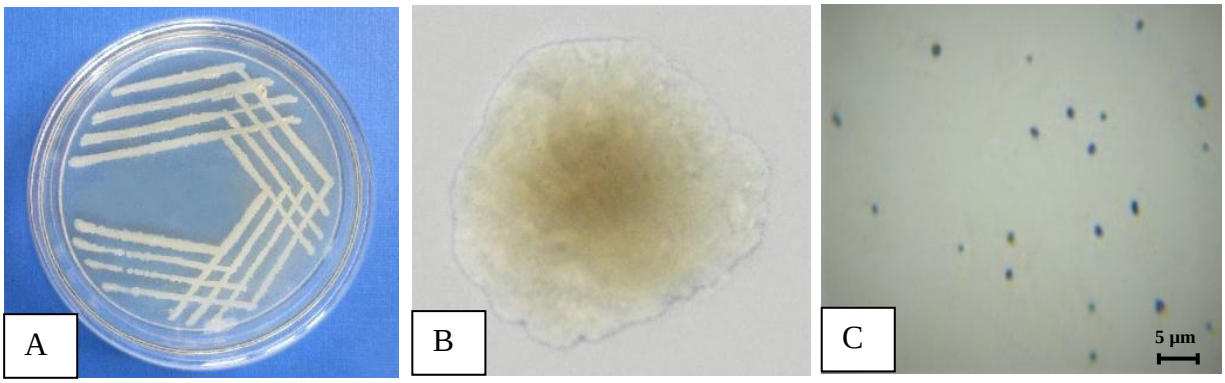

Figure 5. Colony Characteristics and Cell Morphology of TS 4

A. Colonies on streak plate
B. Single colony
C. Photomicrograph of cells

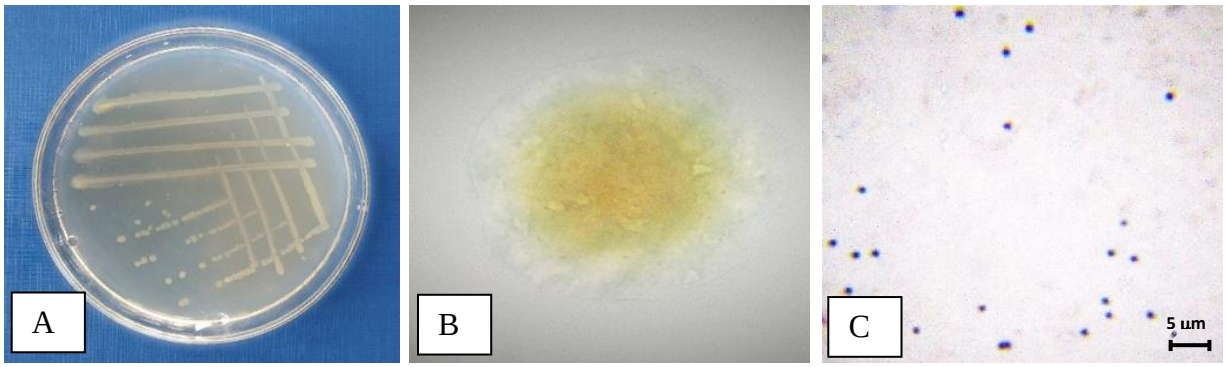

Figure 6. Colony Characteristics and Cell Morphology of TS 5

A. Colonies on streak plate
B. Single colony
C. Photomicrograph of cells

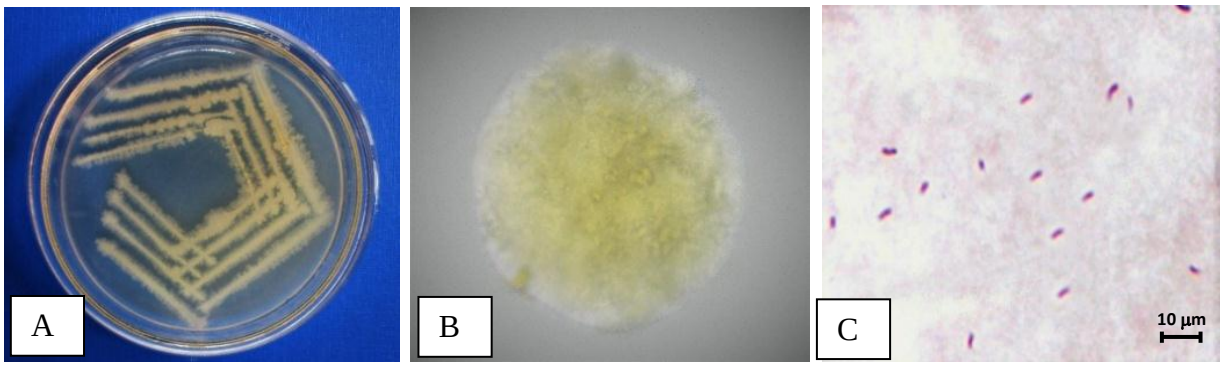

Figure 7. Colony Characteristics and Cell Morphology of TS 6

A. Colonies on streak plate
B. Single colony
C. Photomicrograph of cells

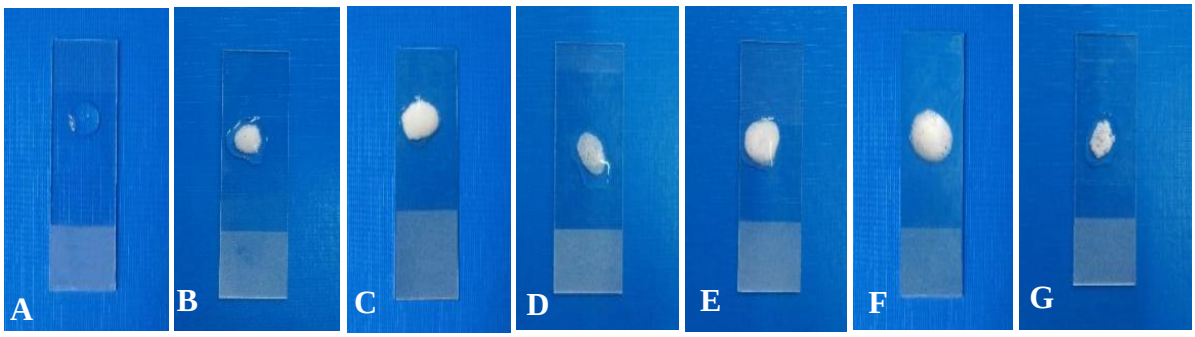

Figure 8. Catalase test

A. Control, B. TS 1 Positive, C. TS 2 Positive, D. TS 3 Positive

E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Positive

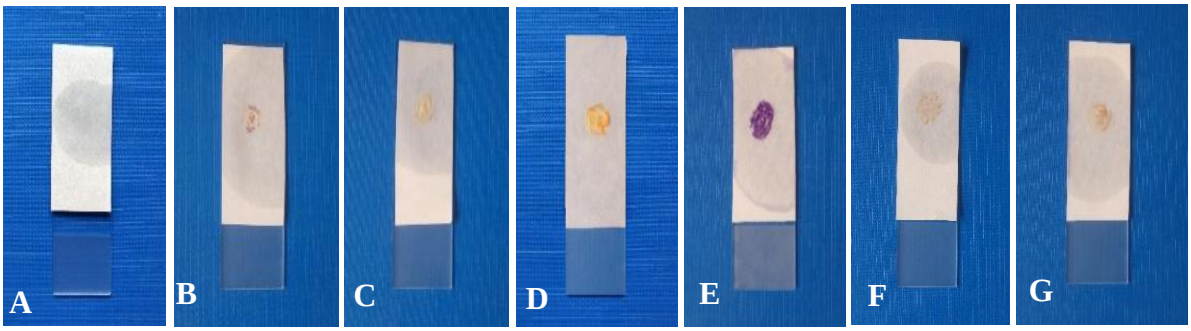

Figure 9. Oxidase test

A. Control, B. TS 1 Negative, C. TS 2 Negative, D. TS 3 Negative

E. TS 4 Positive, F. TS 5 Negative, G. TS 6 Negative

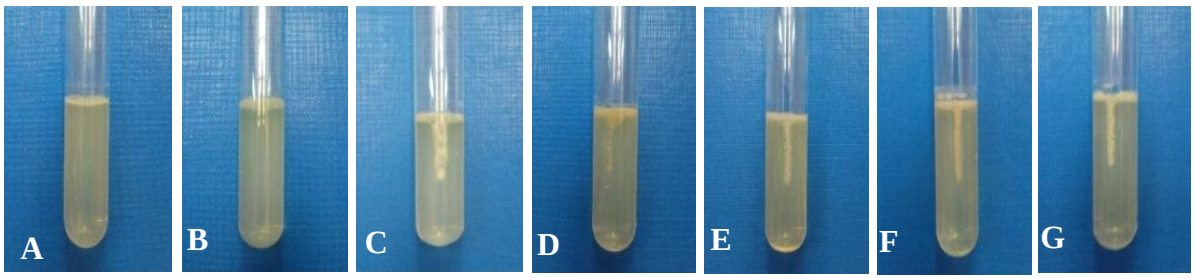

Figure 10.Motility test

A. Control, B. TS 1 Negative, C. TS 2 Positive, D. TS 3 Positive

E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Positive

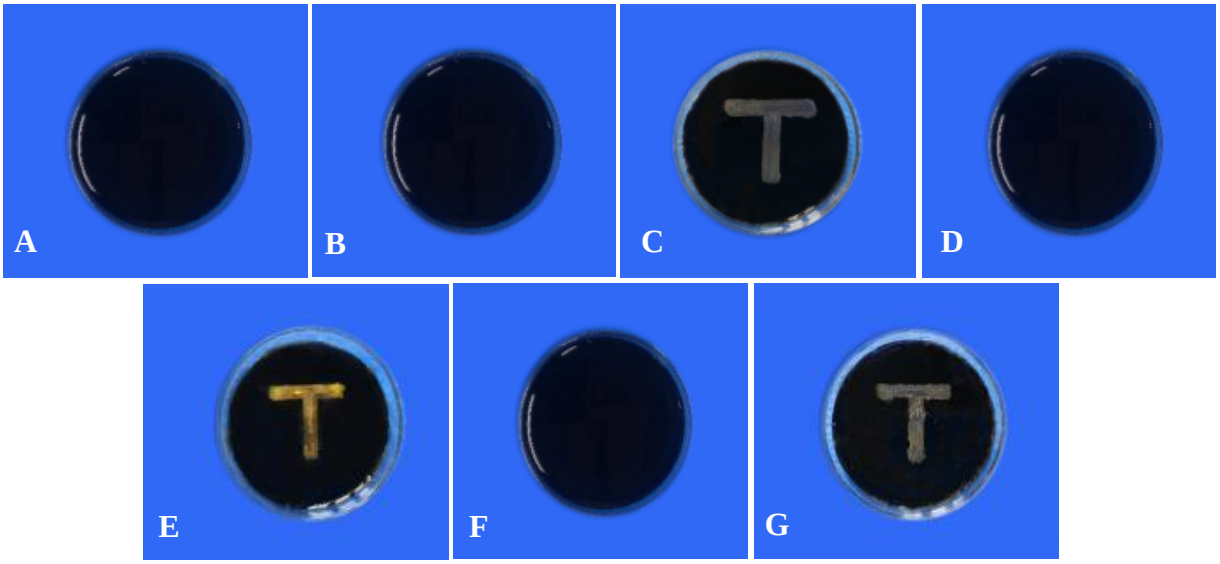

Figure 11. Starch hydrolysis test

A. Control, B. TS 1 Negative, C. TS 2 Positive,

D. TS 3 Negative, E. TS 4 Positive, F. TS 5 Negative,

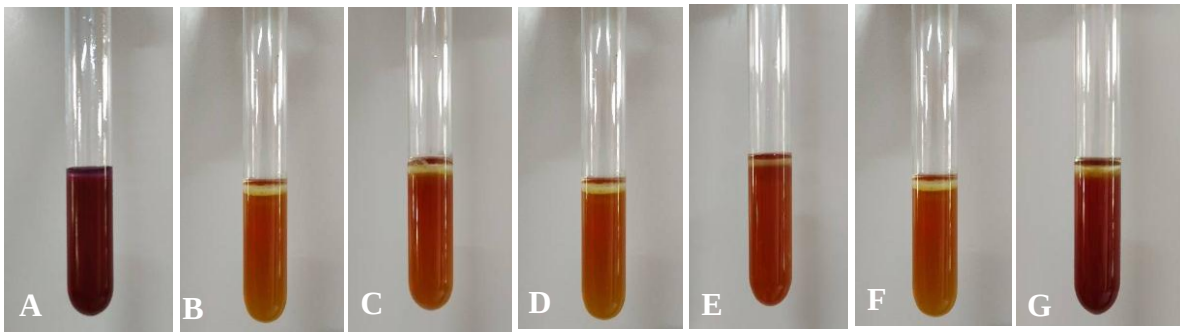

Figure 12. Lysine decarboxylase test

A. Control, B. TS 1 Positive, C. TS 2 Positive, D. TS 3 Positive

E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Negative

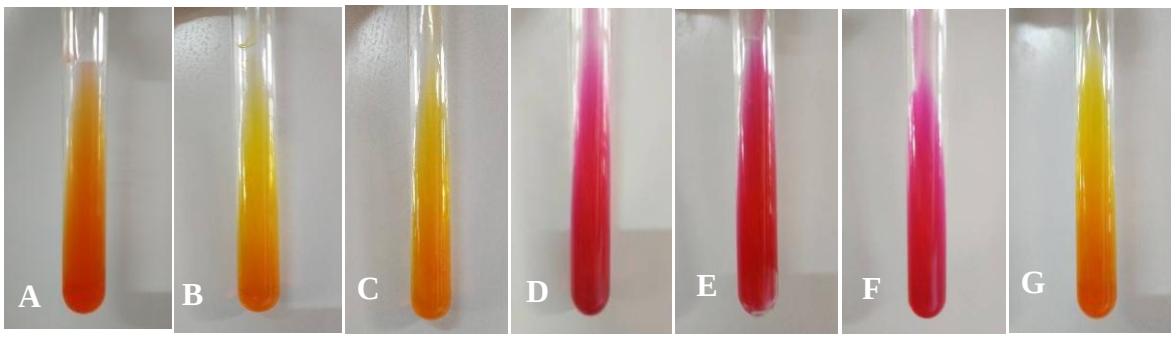

Figure 13. Urea hydrolysis test

A. Control, B. TS 1 Negative, C. TS 2 Negative, D. TS 3 Positive

E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Negative

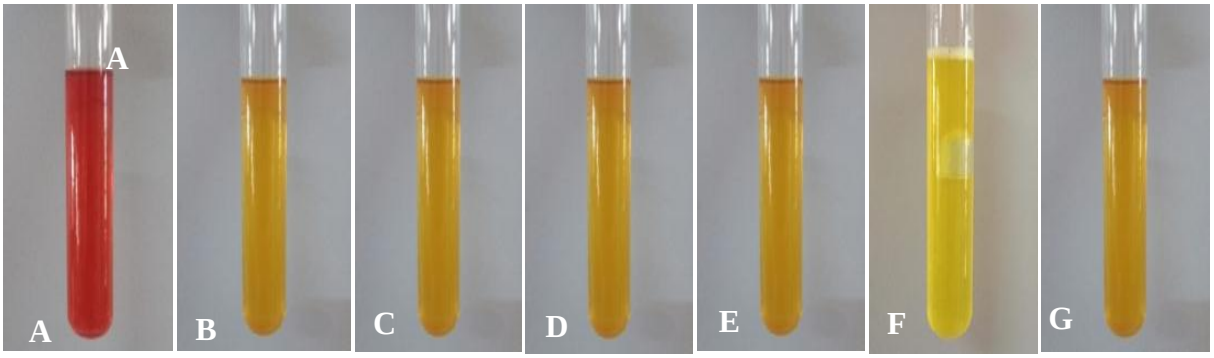

Figure 14. Sugar fermentation test (Dextrose)

A. Control, B. TS 1 Positive, C. TS 2 Positive, D. TS 3 Positive,

E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Positive

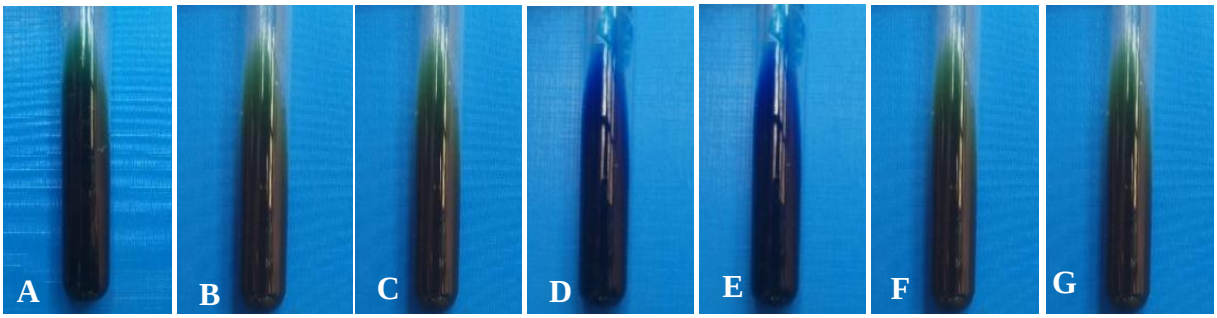

Figure 15. Citrate utilization test

A. Control, B. TS 1 Negative, C. TS 2 Negative, D. TS 3 Positive,

E. TS 4 Positive, F. TS 5 Negative, G. TS 6 Negative

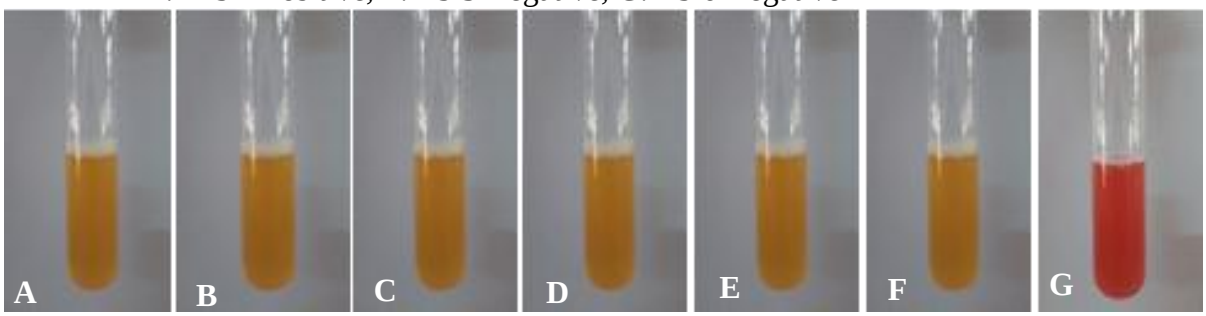

Figure 16. Methyl red test

A. Control, B. TS 1 Negative, C. TS 2 Negative, D. TS 3 Negative,

E. TS 4 Negative, F. TS 5 Negative, G. TS 6 Positive

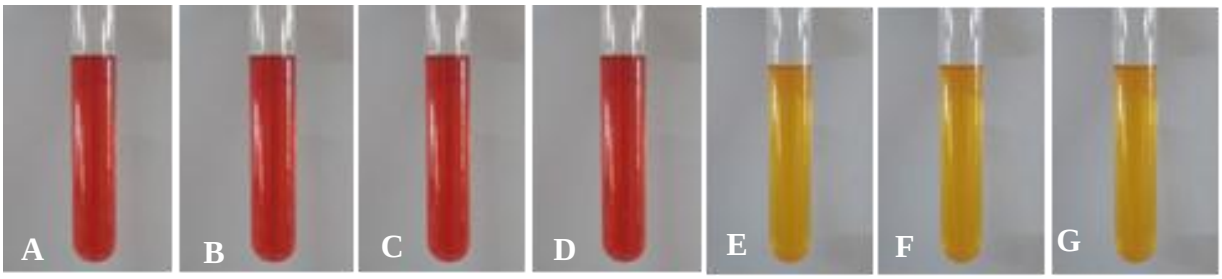

Figure 17. Suger Fermentation test (Glucose)

A. Control, B. TS 1 Negative, C. TS 2 Negative, D. TS 3 Negative,

E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Positive

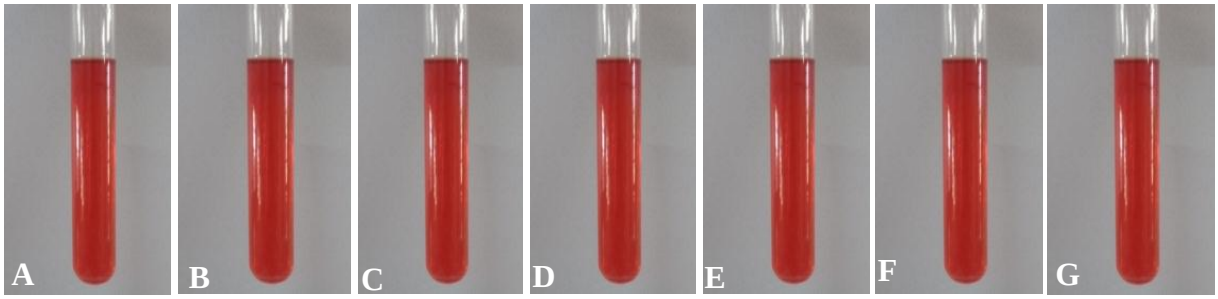

Figure 18. Sugar fermentation test (Maniton)
A. Control, B. TS 1 Negative, C. TS 2 Negative, D. TS 3 Negative,
E. TS 4 Negative, F. TS 5 Negative, G. TS 6 Negative

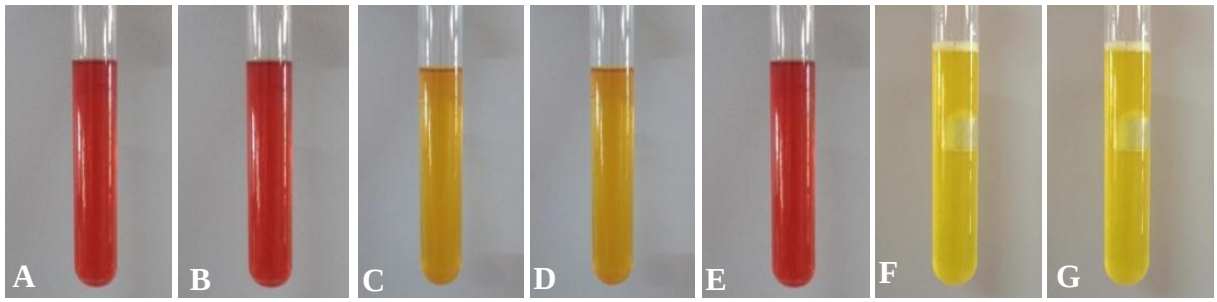

Figure 19. Suger fermentation test (Sucrose)
A. Control, B. TS 1 Negative, C. TS 2 Positive, D. TS 3 Positive,
E. TS 4 Negative, F. TS 5 Positive, G. TS 6 Positive

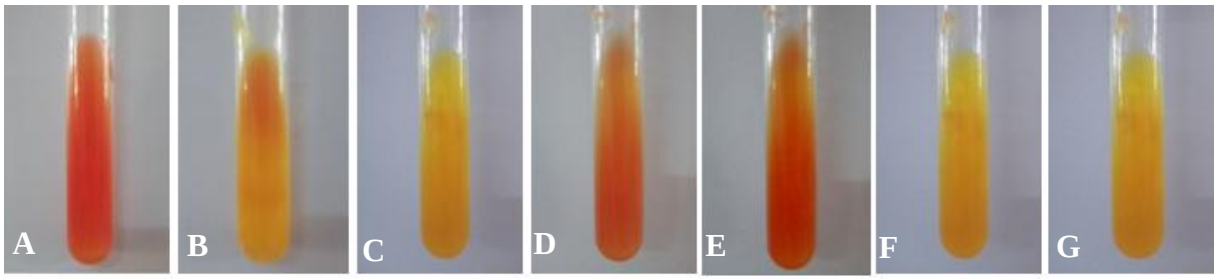

Figure 20. Triple sugar iron test
A. Control, B. TS 1 Positive, C. TS 2 Positive, D. TS 3 Positive,
E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Positive

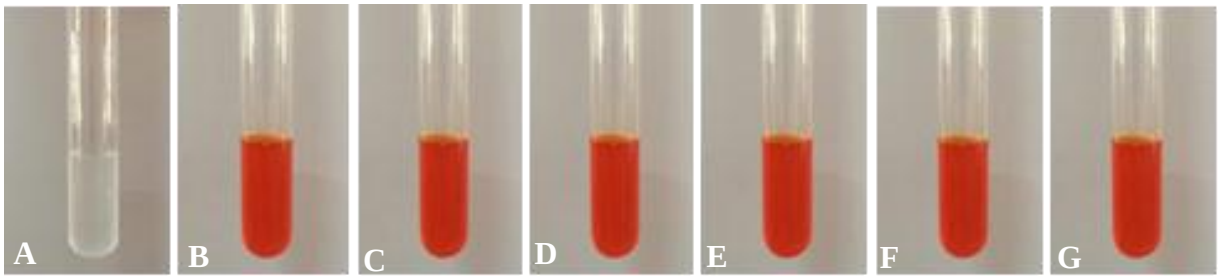

Figure 21. Nitrate reduction test
A. Control, B. TS 1 Positive, C. TS 2 Positive, D. TS 3 Positive,
E. TS 4 Positive, F. TS 5 Positive, G. TS 6 Positive

Discussion and Conclusion

Isolation and characterization of *Carica papaya* L. belong to to the family of Caricaceae were studied in this research.The leaf samples were collected from the Campus of University of Mandalay. This experiment was carried out at the Microbiology Laboratory, Department of Botany, University of Mandalay from December 2017 to August 2018. Each of isolated bacteria was characterized to genus level base on their characterization morphological characterization (shape, colony, colour, cell size, gram staining and motility test) and biochemical tests (catalase, oxidase, starch hydrolysis, lysine decarboxylase, urea hydrolysis, sugar fermentation, citrate utilization, methyl red, triple sugar iron, nitrate reduction) according to Breed *et al*. (1958).

The isolated bacterial strains were designated as TS 1, TS 2, TS 3, TS 4, TS 5 and TS 6. In order to characters the isolated strains, the isolated bacterial strains, the colony morphology, gram staining, microscopical characters and biochemical tests were carried out during the study. As the results, the colony sizes of TS 1 – TS 6 were small, moderate puntiform. Their margins were entire and undulate edges. Their colony color were creamy, pale yellow and white. The elevation of these isolated strains were flatted. The colony pigments were dull and shiny on agar medium.

All of the isolated bacterial strains TS 1 – TS 6 in their cell morphologies were gram negative and gram positive; rod and coccus; aerobic and anaerobic; motile and nonmotile. All the isolated strains showed positive results in catalase test and all strains are negative in oxidase except in TS 4. The isolated bacterial strains TS 2, TS 4 and TS 6 were positive expect in TS 1, TS 3 and TS 5 are negative. All isolated bacterial strains were positive except in TS 6 in lysin test. The isolated bacterial strains TS 1, TS 2 and TS 6 are negative expect in TS 3, TS 4 and TS 5 in urea test. In addition, acid and gas were produced from dextrose, glucose and sucrose, all isolated bacterial strains were negative expect in TS 6 in methyl red test, all are positive in triple sugar iron test and then all isolated bacterial strains were positive in nitration reduction test.

Those results were based on the characterization of colony morphology, microscopical characters and biochemical reactions from the isolated strains which were more or less the same characters of *Mycobacterium* sp., *Bacillus* sp., *Staphylococcus* sp., *Micrococcus* sp., *Streptococcus* sp. and *Enterobacter* sp..Mccarter and Hastings 1934 mentioned that *Mycobacterium*are gram positive, non motile, nitrate reduction is positive, obligate aerobes and fungus like bacteria. According to Mccarter& Hastings (1934) and Breed *et al*. (1958), the TS 1 strain was confirmed as *Mycobacterium* sp..

Breed *et al*. (1958) stated that the genera of family Bacillaceae are motile, gram positive, rod shaped, 0.7 - 1.0 µm by 2.0 - 6.0 µm, sometimes in chains, catalase positive, citrate was not utilized, nitrate was produced from nitrate, optimal temperature 32ºC - 37ºC. Lyngwi*et al*. (2014) mentioned that members of the aerobic spore forming *Bacillus* and related genera can be recovered from almost every niche in the environment. The gram positive bacteria form an important part of microbiota in many soils. According to Breed *et al*. (1958) and Lyngwi*et al*. (2014), the TS 2 strain was confirmed as *Bacillus* sp..

Breed *et al*. (1958) stated that the genera of family Staphylococcaceae are motile or non motile, gram positive cocci 0.5 - 1.5 µm in diameter and facultatively anaerobes. Citrate was utilized and nitrite was produced from nitrate, catalase positive and oxidase negative, starch was not hydrolyzed and optimal temperature is 26ºC - 37ºC. Gotz*et al*. (2006) mentioned that the genera of *Staphyloccus*are gram positive cocci, catalase positive and facultatively anaerobes. According to Breed *et al*. (1958) and Gotz *et al*. (2006), the TS 3 strain was confirmed as *Staphylococcus* sp..

Breed *et al*. (1958) stated that the genus *Micrococcus* appear mostly as cocci, diplococcic, the group was regarded as gram positive, some species were motile or show

motile varities, growth on agar usually abundant, citrate was utilized, nitrite was produced from nitrate, catalase positive, starch was hydrolysed, optimum temperature is 25ºC. The source of this genus is isolated from dairy products, presumably widely distributed in soil. Santhini (2009) mentioned that *Micrococcus* occurs in a wide range of environments including dust, water and soil. *Micrococcus* have gram positive, spherical cells ranging from about 0.8 - 1.0 µm in diameter and a typically arranged in cluster, methyl red test negative, citrate was utilized, oxidase and catalase positive and then glucose and sucrose are positive. According to Breed *et al*. (1958) and Santhini (2009), the TS 4 strain was confirmed as *Micrococcus* sp..

Breed et *al.* (1958) stated that the genera of family Streptococcaceae are motile, spherical or ovoid, occasionally rods, gram positive, 0.5 - 1.25 µm, citrate was not utilized, nitrite was produced from nirate, catalase positive, starch was not hydrolysed, oxidase negative and optimal temperature is 26ºC - 37ºC. The source of this genus isolated from serum and blood. Sharma *et al*. (2014) mentioned that *Staphylococcus* sp. was gram positive bacterium belonging to the phylum Firmicutes, family Staphylococcaceae and order Lactobacillales, motile, spherical or ovoid. According to Breed *et al.* (1958) and Sharma *et al*. (2014), the TS 5 strain was confirmed as *Streptococcus* sp..

Breed *et al*. (1958) stated that the genera of family Enterobacteriaceae are motile, non sporing straight rods, gram negative, 0.6 - 1.0 µm by 1.2 - 3.0 µm, citrate was not utilized, nitrite was produced from nitrate, catalase positive, oxidase negative, starch was hydrolyzed and optimal temperature is 37ºC. Grimont & Grimont. (2006) mentioned that *Enterobacter* sp. was gram negative motile, anaerobes, acid and gas produced from sucrose, catalase positive, nitrite was produced from nitrate and optimal temperature is 37ºC. According to Breed *et al*. (1958) and Grimont&Grimont. (2006), the TS 6 strain was confirmed as *Enterobacter* sp..

This study will be provided that the endopyhytic bacterial strains can be isolated from the leaves of *Carica papaya* L. endophytic bacteria are viewed as the outstanding sources of secondary metabolites, bioactive natural products and chemically novel compounds which are potentials for the expolotation in a wide varieties of medical, agriculatural and industrial areas.

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