Preliminary Study on Isolation of Endophytic microbes from Some Insulin Plants and Their Biochemical Activities

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Abstract

In the course of isolation, six endophytic fungi and two endophytic bacteria were isolated from the fresh leaves of some insulin plants belonging the family Cucurbitaceae, Asteraceae, Rubiaceae, Apocynaceae and Lamiaceae, which was carried out by the surface sterilization method (Ando and Inaba, 2004). Plant samples were collected from Hinthada Township, Ayeyarwaddy Region. Isolated endophytic microbes were designated as ET-1, ET-2, ET-3, ET-4, ET-5, ET-6, ET-7 and ET-8. These isolated endophytic microbes are cultured on agar plate and sub-cultured to obtain pure culture. The morphological characters and some biochemical activities were studied. According to the references, the microscopically examinations of genus of isolated endophytic microbes were suggested to be *Fusarium* sp., *Trichosporon* sp., *Aspergillus* sp., *Trichothecium* sp., *Micrococcus* sp., and *Pseudomonas* sp. In the fermentation study, it was recorded that 72 hours old culture and size of inoculum 25% at three days fermentation periods were found to be the best for antimicrobial activity. These zones indicate the presence of the bioactive compounds which inhibit the growth of test organisms. Eight endophytic microbes isolated strains were subjected in a preliminary study to find out the antimicrobial activity. In this research, eight endophytic microbes strains were selected as based on antimicrobial activity against seven test organisms by using paper disc diffusion method.

Keywords: Isolation, identification, endophytic fungi and bacteria, insulin plants, biochemical activities

Introduction

Insulin plant is one of the folk medicine used for the treatment of diabetes. Today public interest for the use of herbal medicine due to all effects associated with synthetic drugs. Plants have been source of diverse medicinal therapeutic agents (Anonymous, 1967 & Perry, 1980).

Some insulin plants have been identified and studied using scientific approaches. These plant are used in the treatment of many diseases including diabetes worldwide. Myanmar has rich of traditional medicinal plants for the treatment of diabetes. The popular plants including which will believed to relieve diabetes in Myanmar. Diabetes is the name used to describe a metabolic condition of having higher than normal blood sugar levels. Insulin plants are an important medicinal plant in Southeast Asia. (https://www.diabetes.co.UK)

The present research paper consists of eight species belonging to five families of some insulin plants. Such species were described in detail with their outstanding characters. Plants have been used as antimicrobial agents because of their antimicrobial traits. The microbes that isolated from plant parts are endophytes. Though the meaning of the term "endophytes" varies depending on the researching, it can be defined the endophyte as microbes living inside the healthy plants. (Scott and Lori, 1996)

Any chemical substance inhibiting the growth or causing the death of microorganisms is known as an antimicrobial agent. Many substances are antimicrobial agents, but very few of them are a potential chemotherapeutic agent,

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describes the chemical that are used to kill or inhibit the growth of microorganisms already established in the tissue of the body (Cruickshank, 1975).

The fermentation process is an important tool for production of secondary metabolite from endophytic microbes because of the ease of increasing production by environmental and genus manipulation (Demain, 2000). Optimal fermentation conditions such as proper ages and sizes of inoculum are very important for maximal productivity of metabolites (Omura, 1985).

According to Davis and Stout, (1971) diffusion method of paper disc with fermented broth is allowed to diffuse into an agar medium during the growth of microorganisms. A continuous concentration gradient between the disc and its peripheral area result in clear zone. The significance of the zone can be assessed by several types of criteria. It can be compared the zone produced by control with susceptible organisms tested under identical conditions. (Bryan, 1980)

In the present day, different types of microorganisms are studied in the aspect of microbial activity. Their partial identification as well as their biochemical significance in antimicrobial properties were investigated.

Materials and Methods

Study site

- Hinthada Township is situated in the Ayeyarwaddy Region.
- Hinthada Township is situated between North Latitude 17° 15' and 17° 39'.
- East longitude 95° 10 ' and 95° 35 ' with an area of 378.695 square miles.

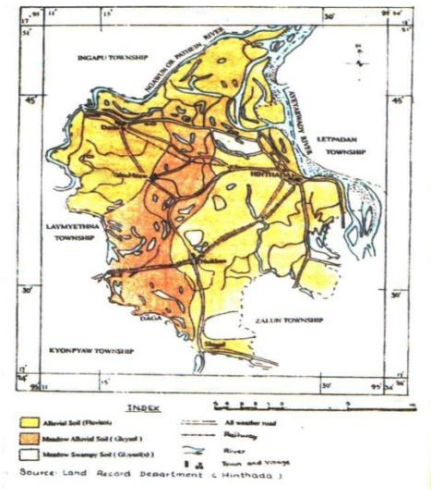


Fig. 1 Plant samples collected area (location map of Hinthada Township)

Collection of plant sample

The specimens used in this research were collected from Hinthada Township, Ayeyarwaddy Region. The vegetative and reproductive parts of the fresh specimens were identified by using available literatures such as Hooker, 1885; Backer, 1965; and Kress, *et al.*, 2003.

Table.1 Some insulin plants used for the isolation of Endophytic Microbes

No.	Plant Sources	Myanmar Name	Family
1.	*Momordica charantia*L.	Kyet-hinga	Cucurbitaceae
2.	*Gynura procumbens (Lour.) Merr.*	Pyarr-hmee	Asteraceae
3.	*Morinda citrifolia* L.	Ye-yo	Rubiaceae
4.	*Catharanthus roseus*(L.)*G.Don.*	Thinbaw-mahnyo	Apocynaceae
5.	*Orthosiphona ristatus (Blume)Miq.*	Thagya-mageik	Lamiaceae

Isolation procedures of endophytic microbes

Surface sterilization method (Ando and Inaba, 2004)

The endophytic microbes isolated from leaves of some insulin plants with following procedures.

The leaves were washed in running tap-water for 15 minutes. The leaves were cut into small pieces of 6×6 mm. The leaves were sterilized by immersed in 95% ethanol for 15 seconds. The samples were rinsed in sterile distilled water. Then, the samples were excised into smaller pieces of 5×5 mm and dried on sterilized paper. These pieces were placed on agar plates and then incubated for 3 to 7 days at room temperature.

The PGA medium and CzapekDox agar medium are used for isolation of endophytic microbes.

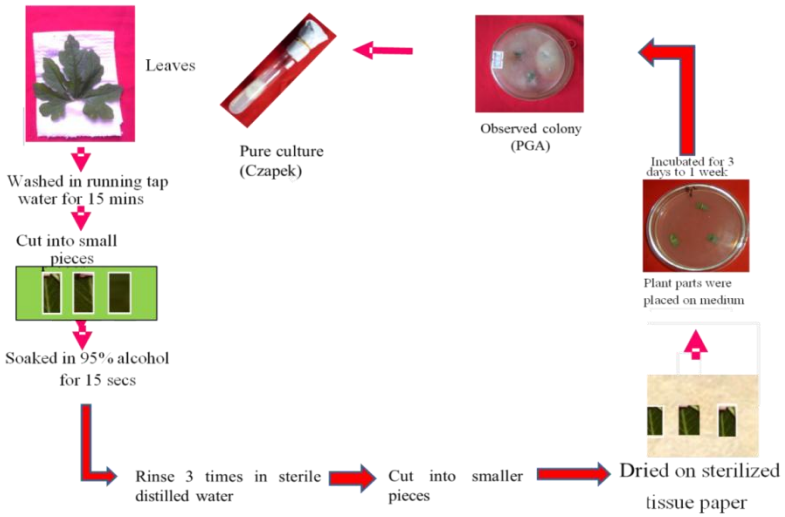


Fig. 2 Isolation procedure of endophytes from plant parts (Ando, 2004)

Morphological and microscopical characters of isolated endophytic microbes strains (Barnett, 1969)

Eight isolated endophytic microbe's strains grown on slant culture were transferred into the plates containing Potato – 20 g, Peptone – 0.3 g, Glucose – 2.0 g, Agar-1.8 g, Distilled water-100 mL (PGA) medium. In this medium, Chloramphenicol and Nystatin were used to inhibit for fungi and bacteria. Then, these plates were incubated at 30°C for 3-7 days. Colony forms, cell morphology of isolated endophytic microbes strains was recorded and they were identified according to the references of Barnett 1969.

Preliminary study on the growth Activity of Isolated Endophytic Microbes

7 days old selected microbes was inoculated into 50 mL conical flask containing 25 mL of sterilized seed medium and incubated for 3 days. 25 mL of seed medium was transferred to 25 mL of fermentation medium. The fermentation was carried out for 4-7 days. After the end of fermentation, the fermented broth was used to determine the activity against test organisms by paper disc diffusion assay.

Seed Medium		**Fermentation broth medium**		**Assay medium for test organism**	
Glucose	- 1 g	Glucose	- 1.5 g	Sucrose	- 10.0 g
Soluble starch	- 0.5 g	Yeast extract	- 0.5 g	Yeast extract	- 3.0 g
Yeast extract	- 0.3 g	polypeptone	- 0.3 g	NaCl	- 5.0 g
KNO_3	- 0.05 g	K_2HPO_4	- 0.1 g	Agar	- 1.8 g
K_2HPO_4	- 0.001 g	D.W	- 100 mL	D.W	- 100 mL
D.W	- 100 mL	pH	- ± 6.5	pH	- ± 6.5
pH	- ± 6.5				

Antimicrobial activities by paper disc diffusion assay (Davis and Stout, 1971)

1. The isolated fungi were grown at 27˚C for 7 days on PGA medium for sporulation.
2. The isolated fungi were inoculated on seed medium and incubated at 27˚C for 3 days.
3. 25 mL of seed culture was transferred into the fermentation medium and incubated at 27˚C for 7 days.
4. 20 µL of fermented broth was put on paper disc and place on assay plate test organisms.

BR-BDC-Screening Media (2004)

The test microorganisms were supported by Microbiology laboratory of Yangon University. Each test organism was cultured on synthetic low nutrient medium. G.P.A (for bacteria), G.Y.A (for fungi) Synthetic low nutrient agar medium (Ando *et.al*. 2004).

G.P.A (for bacteria)		**G.Y.A (for fungi)**	
Glucose	- 1.0 g	Glucose	- 1.0 g
Polypeptone	- 0.3 g	Yeast extract	- 0.3 g
KNO_3	- 0.1 g	Agar	- 1.8 g
Agar	- 1.8 g	D.W	- 100 mL
D.W	- 100 mL	pH	- ± 6.5
pH	- ± 6.5		

Table.2 Test organisms and their diseases (Madigan and Martinko, 2005)

No	Test organisms	Diseases
1	*Agrobacterium tumefaciens*	- Plant tumor cell
2	*Aspergillus flavus*	- Bronchitis
3	*Bacillus subtilis*	-Fever,
4	*Candida albicans*	-Skin infection, vaginal candidiasis, alimentary tract infection
5	*Escherichia coli*	-Cholera, diarrhea and vomiting, urinary tract infections
6	*Pseudomonas aeruginosa*	-Urinary infection, lung disease pneumonia
7	*Staphylococcus aureus*	-Boils, Food poison, skin disease

Results

Isolation of endophytic microbes

The endophytic microbes were isolated from the leaves of some insulin plants by the method of surface sterilization (Ando and Inaba, 2004). The morphological characters were observed by the method of Barnett, 1969, Ando and Inaba, 2004.

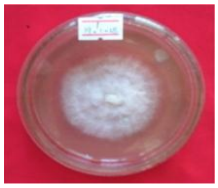
Table.3 Endophytic fungi and bacteria isolated from Five kinds of some Insulin plants

No.	Plant Sources	Isolated plant parts	Isolated endophytic microbes	
			Fungi	Bacteria
1.	Kyet-hinga	Leaf	ET-1,ET-2	-
2.	Pyarr-hmee	Leaf	ET-3	ET-7, ET-8
3.	Ye-yo	Leaf	ET-4	-
4.	Thinbaw-mahnyo	Leaf	ET-5	-
5.	Thagya-mageik	Leaf	ET-6	-

Isolated endophytic microbes were identified and described to the genus level according to the (Domsch, 1980 and Buchanan & Gibbons, 1974).

Microscopical character of ET-1

Colonies fast growing, reaching 5.6-8.0 cm diameter in 7 days at room temperature on Czapek medium, aerial mycelium loosely cottony, white at the agar surface. Conidiophores richly branched with rather short and broadly ending. Non-proliferating phialides are present. Micro conidia predominantly produced globes to broadly ellipsoidal, usually one celled.

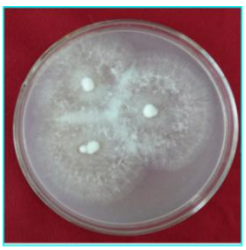
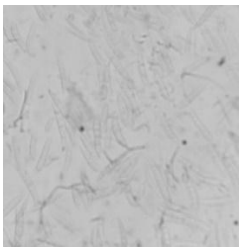



Morphology of colony Phtotomicrograph × 400

Fig. 3 ET -1 *Fusarium* sp

Microscopically character of ET-2

Colonies very fast growing, reaching 8 cm diameter in 7 days at room temperature on Czapek medium, white to pink on the agar surface. Conidiophore abundantly branched with short and wide phialides. Conidia broad, falcate, with a short sub terminally slightly constricted and almost pointed apical cell, 1-4 septa present.

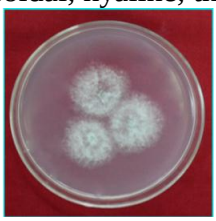



Morphology of colony Photomicrograph × 400

Fig. 4 ET -2 *Fusarium* sp.

Microscopical character of ET-3

Colonies slow growing, reaching 1-2 cm in diameter about 7 days at room temperature on Czapek medium, white to cream, smooth. Arthroconidia cylindrical to ellipsoidal, blastoconidia subglobose are attached by a narrow but distinct scar. Large, globose, ellipsoidal, hyaline, thick walled cells.

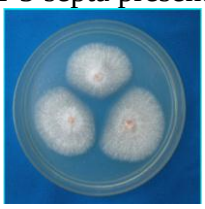



Morphology of colony Photomicrograph × 400

Fig. 5 ET -3 *Trichosporon* sp

Microscopical character of ET-4

Colonies fast growing, reaching about 6 cm diameter in 7days at room temperature on Czapek medium. Aerial mycelium floccose, whitish to buff brown at the agar surface.Sporodochia absent, conidiophores scattered in the aerial mycelium, loosely branched; phialides slender, cylindrical, conidia fusion, almost straight, with slightly bent, 1-5 septa present.

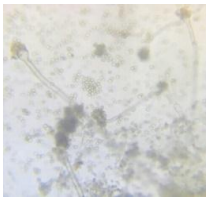



Morphology of colony Photomicrograph × 400

Fig. 6 ET -4 *Fusarium* sp

Microscopical character of ET-5

Colonies reaching, less than 1-1.5 cm, diameter after 1 week at room temperature on Czapek medium, olive-green. Conidiophores long, hyaline at smooth walled with hemisphere vesicle, conidial heads columnar, conidia narrowly ellipsoidal, echinulate, uninucleate.

Morphology of colony Photomicrograph × 400

Fig. 7 ET -5 *Aspergillus* sp.

Microscopical character of ET-6

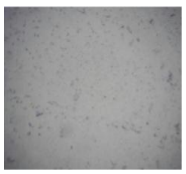
Colonies reaching 2-9 cm diameter in 7 days at room temperature on Czapek medium, pinkish. Conidiophores erect often with three septa in the lower part. Conidia ellipsoidal to pyriform with an obliquely prominent truncate basal scar.


Morphology of colony Photomicrograph × 400

Fig. 8 ET -6 *Trichothecium* sp.

Microscopical character of ET-7

Colonies cream colour, cells spherical, 0.5-3.5 μm in diameter, occurring singly or in pairs and characteristically dividing in more than one plane to form irregular clusters, tetrads or cubical packets.Usually non-motile. No resting stages known. Gram-positive.

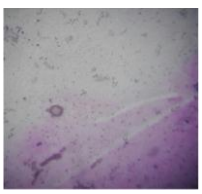



Morphology of colony Photomicrograph × 400

Fig. 9 ET -7 *Micrococcus* sp.

Microscopical character of ET-8

Colonies white colour,cells single, straight or curved rods,but not helical. Dimension, generally 0.5-1 μm in diameter.Motile by polar flagella; monotrichous or multitrichous. Do not produce sheaths. No resting stages known. Cell wall gram-negative type.

Morphology of colony Photomicrograph × 400

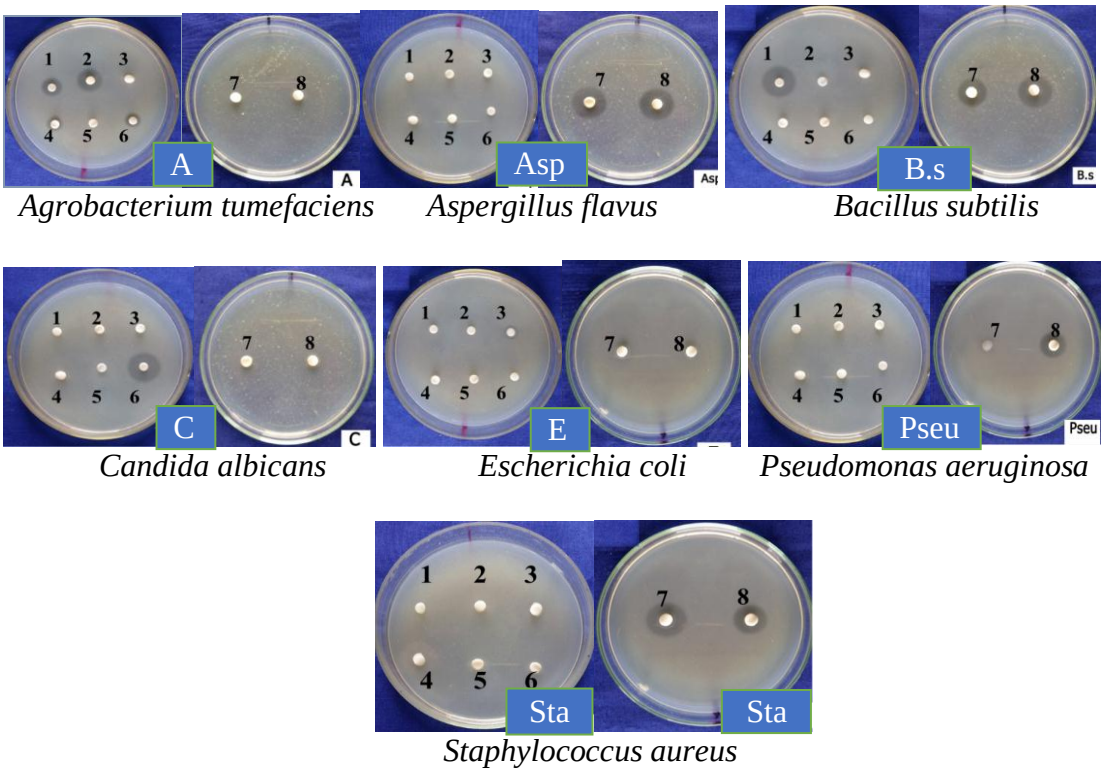
Fig. 10 ET -8 *Pseudomonas* sp.

Antimicrobial Activities of Endophytic Microbes

Determination of age of culture and size of inoculum and fermentation period

According to the production on preliminary study on antimicrobial activity the optimal age of culture was determined by used 3-7 days old culture of 6 kinds of fungi and 2 kinds of bacteria. The optimal size of inoculum was also determined by using 5 %, 10 %, 15 %, 20% and 25 % of isolated microbes.

Study on the Antimicrobial Activities



Agrobacterium tumefaciens *Aspergillus flavus* *Bacillus subtilis*

Candida albicans *Escherichia coli* *Pseudomonas aeruginosa*

Staphylococcus aureus

Fig .11 Antibacterial activities shown by isolated endophytic microbes from some insulin plants

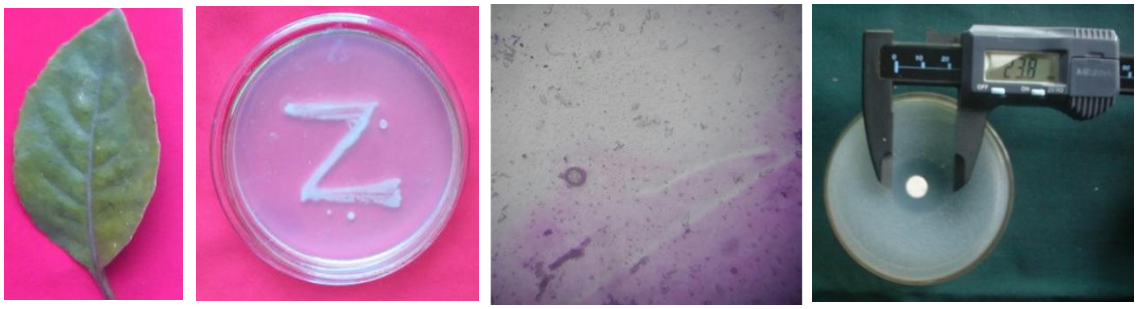
Table 4. Antimicrobial Activities of Isolated Endophytic Microbes Against Seven Test Organisms at 3 days Fermentation

No	Test organisms	ET-1	ET-2	ET-3	ET-4	ET-5	ET-6	ET-7	ET-8
		Fusarium sp.	*Fusarium* sp..	*Trichosporon* sp.	*Fusarium* sp.	*Aspergillus* sp.	*Trichothecium* sp.	*Micrococcus* sp.	*Pseudomonas* sp.
1	*Agrobacterium tumefaciens*	12.8 Mm	17.4 mm	-	8.7 mm	-	10.1 mm	-	-
2	*Aspergillus flavus*	-	-	-	-	-	-	19.1 mm	19.1 mm
3	*Bacillus subtilis*	19.2mm	-	-	-	-	-	17.3 mm	**23.8 mm**
4	*Candida albicans*	-	-	-	-	-	18.9mm	-	-

5	*Escherichia coli*	-	-	-	-	-	-	-	-
6	*Pseudomonas aeruginosa*	-	-	-	-	-	-	8.4 mm	14.8 mm
7	*Staphylococcus aureus*	-	-	-	-	-	-	17.3 mm	15.1 mm

(Paper disc – 6 mm) size of clear zone in millimeter (mm)

Selected Antimicrobial Active Bacteria



Pyarr- hmee leaf Cell morphology *Pseudomonas* sp. *Bacillus subtilis* against ET-8

Fig.12 Antibacterial activity of endophytic bacterial against ET-8

Discussion and Conclusion

In the isolation of endophytic microbes from some insulin plants were collected from Hinthada Township area. In this result, the eight endophytic microbes were isolated from five insulin plants. Among them, six endophytic fungi were isolated from *Momordica charantia* L.(Kyet-hinga),*Gynurapro cumbens*(Lour.) Merr.(Pyarr-hmee), *Morinda citrifolia* L. (Ye-yo), *Catharanthus roseus* (L.)G.Don. (Thinbaw-mahnyo), *Orthosiphon aristatus* (Blume) Miq. (Thagya-mageik) and only two endophytic bacteria have isolated from *Gynura procumbens* (Pyarr-hmee), which was carried out by the method of surface sterilization (Ando and Inaba, 2004).

Eight endophytic microbes of pure culture were selected and preliminarily studied their taxonomical characters, morphology of microbes and biochemical activities were described. According to the literature review, isolated endophytic microbes were identified by using the references keys as *Fusarium* sp., *Trichosporon*sp., *Aspergillus*sp., *Trichotheciums*p., *Micrococcus* sp., and *Pseudomonas* sp.

Fusarium sp. were isolated from the leaves of Kyet-hinga and Ye-yo, characterized as colonies very fast growing, aerial mycelium loosely cottony and conidiophore abundantly branched. *Trichosporon* sp. was isolated from the leaves of Pyarr-hmee and characterized as colonies slow growing, arthroconidia cylindrical to ellipsoidal, blastoconidia subglobose and attached by a narrow but distinct scar. *Aspergillus* sp. was isolated from the leaves of Thinbaw-mahnyo and characterized as conidiophores long, hyaline at smooth wall with hemisphere vesicle. *Trichothecium* sp. was isolated from the leaves of Thagya-mageik and characterized as pinkish, conidia ellipsoidal to pyriform with an obliquely prominent truncate basal scar. *Micrococcus* sp. was isolated from the leaves of Pyarr-hmee and characterized as cells spherical and gram -positive. *Pseudomonas* sp. was isolated from the leaves of Pyarr-hmee and characterized as curved rods and gram -negative.

In the fermentation study, the highest antimicrobial activities was recorded at three days fermentation period with 25 % size of inoculum and 72 hours ages of

culture of endophyticmicrobes are isolated and their antimicrobial activities was detected against *Agrobacterium tumefaciens, Aspergillus flavus, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.*

In the screening antimicrobial producing microorganisms' eight strains (6 fungi and 2 bacteria) were isolated from five kinds of insulin plants. Among them,*Pseudomonas* sp. (bacteria) showed more highly selective antibacterial activity (23.8mm of inhibitory zone) especially against *Bacillus subtilis* (Table.9). So, this strain microbes had the effective antimicrobial activity in this research. Therefore, ET-8 indicating that compound acts as bacteriocidal metabolite.The present studies on antibacterial metabolite with expectations which have the potential to provide a therapeutic agent to the treatment and control of bacterial infection with *Bacillus subtilis.*

It is concluded that screening of drug resistant strains of human and plant pathogen is essential. They are somehow applied in industrial production of various products such as enzymes, antibiotics, organic acids, hormones and pharmaceutical for the benefit of Myanmar people.

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References

Ando, K., M. Suto and S. Inaba, 2004.**Sampling and isolation methods of fungi.**Workshop at University of Pathein, Ayeyarwady Region.

Ando, K. and S. Inaba. 2004. **Workshop on Taxonomy and Identification of Fungi**, Pathein University Biotechnology Development Centre.

Anonymous. 1967**. Myanmar Indigenous medicinal plants**: plants with reputed hypoglycemic action Myanmar Medicinal Research Institute.

Backer, C. A. Bakhuizen. 1965. **Flora of Java**. Vol. II. The Netherland: V.V.P. Noordhoff-Groningen Company.

Barnett, H.L. 1969.**Illustrated Grenera of Imperfectie fungi**, 4th Ed. BurgressPublishing Company.

Bryan, L.E.1980. **Bacterial resistance and susceptibility to chemotherapeutic agents** (Ambridge University press, London)

Buchanan, R.E., +&N.E. Gibbons 1974. **Bergey's Manual of Determinative Bacteriology**. 8th Ed.

Crueger,W., and A.Crueger. 1989: **Methods of fermentation in Biotechnology**, A Textbook of Industrial Microbiology, Internal Student 2 nd Ed; 64-74

Cruickshank, R.J.P., B.P. Dugid, Marmion and R.H.A. Swain. 1975. **Medicinal Microbiology** Churchill Living Stone Ltd., London.

Davis, W.W. and T.R. Stout. 1971. **Dis Plate Method of Microbiological Antibiotic Assay**. Applied Microbiology.Vol 22, No.4.

Demain, AI.2000; **Pharmaceutically active secondary metabolites of microorganisms**. AppI Microbial biotechnol 52; 455-468.

Hooker, J.D. 1885. **Flora of British India,** Vol. IV. L. Reeve & Co. Ltd.

Madigan, M.J., Martinko (editor) 2005. **Brack Biology of Microorganisms**, 11th Ed.Prentice Hall.

Nite, 2004-2005. **Medium for fermentation to produce the metabolites**.

Omura, S. 1985. **Microbial growth kinetics and secondary metabolites**, J. fermentation Technology, 46, 134-140.

Scott, C.R. and M.C. Lori. 1996. **Endophytic Fungi in Grasses and Woody plants.** 31-65.

Selim, K.A., *et al.* 2012. **Biology of Endophytic Fungi.**Current Research in Environment & Applied Mycology 2(1), 31-82, Do 10.5943/cream/2/1/3.

Strobel G., 2003.**Endophytes as sources of bioactive products**, microbes and Infection. 5:535-544.

Toita, F &Suto. 1998. **Laboratory Method,** Hokkaido University, Japan.

Websites

https://www.diabetes.co.UK