ISOLATION AND ANTIMICROBIAL ACTIVITIES OF ENDOPHYTIC FUNGUS, NF-01 FROM *CROTON ROXBURGHIANUS* N.P.BALAKR.

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Abstract

A total of 20 endophytic fungi were isolated from three different plants, *Croton roxburghianus* N.P.Balakr (Thet-yin-gyi) , *Tadehagi triquetrum* (L.) H. Ohashi. (Lauk-thay), *Cassia siamea* L. (Mezali) collected from Pathein Township. In the investigation of antimicrobial activities of 20 endophytic fungi with ten kinds of test organism, *Agrobacterium tumefaciens, Aspergillus paraciticus, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens, Saccharomyces cerevisiae, Salmonella typhimurium* and *Staphylococcus aureus* were used for the test throughout the research studied. In this study, endophytic fungi NF-01(27.85mm) and NF-07(26.67mm) showed highly activity against *Bacillus subtilis.* Among them, fungus NF-01 isolated from *Croton roxburguianus* n.p.balakr. was screened for further investigation based on the results of maximum inhibition against *Bacillus subtilis*. In the investigation of carbon and nitrogen sources utilization, the excellent growth of NF-01 fungus was found on carbon sources such as glucose and glycerol; nitrogen sources were yeast extract and peanut cake.

Keywords: endophytic fungi, antimicrobial activity and *Bacillus subtilis.*

Introduction

Endophytic microorganisms are survived inside the living tissues of plants without causing any harmful effects or damages and have symbiotic relationships with host plants (Specian *et al*., 2012). These endophytes have an ability to produce a variety of secondary metabolites (Sandhu *et al*., 2014). Nevertheless, increasing levels of antibiotic resistance in both nonpathogenic and pathogenic bacteria has spurred the search for new antibiotics to manage diseases (Petrini, 1991).

Since the population has been increased, this was not possible to afford plant-based medicine. Due to the increasing demand of medicine and destruction of medicinal plants, a huge work carried out in the field of endophytes for producing bioactive compounds that can be used in the treatment of diseases (Onifade, 2007). Endophytes are the synthesizers inside plants that produce bioactive compounds with low toxicity toward higher plants (Owen and Hundley, 2004). Endophytes provide an extensive variety of bioactive secondary metabolites with unique structure, synthesized via various metabolic pathways i.e. polyketide, isoprenoid, amino acid derivatives (Tan and Zou, 2001).

Materials and Methods

Collection of plant samples

The plant samples were collected at different places in Pathein Area. These plant specimens were identified according to the available references and internet websites information.

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Table1 Plant used for isolation of endophytes

No.	Scientific Name	Family	Myanmar Name	Location
1	*Croton roxburghianus* N.P.Balakr.	Euphorbiaceae	Thet-yin-gyi	Yadanar street, Pathein Uni, Campus.
2	*Tadehagi triquetrum* (L.) H. Ohashi.	Fabaceae	Lauk-thay	Yadanar street, Pathein Uni, Campus.
3	*Cassia siamea* L.	Caesalpiniaceae	Mezali	Near BDC, Pathein Uni, Campus.

Isolation procedure of endophytes from plants (Tomita, 1998)

The plants were washed in running tap water for 15 mins. The plant leaves were cut into about 1cm pieces. Sterilize the surface of plant part by soaking it in 75% ethanol for 2mins. These parts were dried on sterilized paper and then they were placed on agar plates containing medium. The plates were incubated for 3days to 1 week at room temperature.

Preliminary Study for Antimicrobial Activities by Paper Disc Diffusion Assay (NITE, 2004)

The isolated fungi were grown at 25ºC for 5 days on Potato Glucose Agar medium. These isolated fungi were inoculated into seed medium and incubated at 25ºC for 3 days (Tomita, 1998)**.** Then, 10mL of seed culture were transferred into the fermentation medium. The fermentation was carried out for 10 days. The fermented broth (20µL) was used to examine the antimicrobial activity against test organisms by paper disc diffusion assay. Paper disc having eight-millimeter diameter (Advantee, Toyo Roshi Kaisha Co., Ltd., Japan) were utilized for antimicrobial assays. The assay medium was used for the antimicrobial activity test. One percent of test organisms was added to assay medium, then poured into plates. After solidification, paper disc impregnated with samples (fermented broth) were applied on the agar plates and the plates were incubated 24-36 hours at 25ºC.The appearance of clear zone (inhibitory zones) around the test disc indicates the presence of antimicrobial activity. The test organisms used in paper disc diffusion assay were as followed (Table 2).

Table 2 Test organisms and diseases used in antimicrobial activities

Test organisms	Code number	Diseases
Agrobacterium tumefaciens	NITE 09678	Plant disease, Crown gall disease and tumors.
Aspergillus paraciticus	IFO 5123	Fruits disease.
Bacillus subtilis	IFO 90571	Fruits and seeds disease.
Candida albicans	NITE 09542	Candidosis.
Escherichia coli	AHU 5436	Cholera, diarrhoea and vomiting, urinary tract infections.
Micrococcus luteus	NITE 83297	Skin disease.
Pseudomonas fluorescens	IFO 94307	Rice disease and pulmonary disease.
Saccharomyces cerevisiae	NITE 52847	Food spoilage, empyema, pneumonia, liver abscess, asthma and diarrhea.
Salmonella typhi	AHU 7943	Typhoid fever and food poisoning.
Staphylococcus aureus	AHU 8465	Skin disease, food poison, wound infection, burns, abscesses, blood stream infection, staphylococcal pneumonia.

In the investigation of carbon and nitrogen sources utilization, carbon sources such as glucose, sucrose, glycerol, soluble starch, oat and tapioca powder were employed whereas nitrogen sources such as peptone, yeast extract, malt extract, meat extract, peanut cake and sesame cake. The cultures for NF-01 were undertaken on plates containing these carbon and nitrogen sources for 6 days at 25ºC.

Effects of Carbon and Nitrogen Utilization

Carbon sources (1.0%) with basal medium		Nitrogen sources (1.0%) with basal medium	
Yeast extract	0.6%	Glucose	1.5%
Corn powder	0.6%	Glycerol	1.5%
K_2HPO_4	0.002%	K_2HPO_4	0.002%
$MgSO_4$	0.002%	$MgSO_4$	0.002%
$CaCO_3$	0.002%	$CaCO_3$	0.002%
pH	6.0	pH	6.0
DW	100mL	DW	100mL

Results

Isolation of endophytic fungal strains

Twenty fungal strains were isolated from the leaves of *Croton roxburghianus* N.P.Balakr, *Tadehagi triquetrum* (L.) H.Ohashi ,*Cassia siamea* L. In the investigation of antimicrobial activities of these endophytic fungi (NF-01 to NF- 10) showed antimicrobial activities. Among them, NF-01 and NF-07 (isolated from the plant leaves of *Croton roxburghianus* N.P.Balakr (Thet-yin-gyi)) were highly activity against *Bacillus subtilis* than the other fungi. Therefore, these strains were selected for further investigation of fermentation.

Table 3 Isolation of endophytic fungal strains

Strain	Source
NF-01 to 08	Leaves of *Croton roxburghianus* N.P.Balakr.(Thet-yin-ghi)
NF-09 to 14	Leaves of *Tadehagi triquetrum* (L.) H. Ohashi (Lauk-thay)
NF-14 to 20	Leaves of *Cassia siamea* L. (Mezali)

Table 4 Antimicrobial activities of isolated fungi

Test organisms / Endophytes	*Agrobacterium tumefaciens*	*Aspergillus paraciticus*	*Bacillus subtilis*	*Candida albicans*	*Escherichia coli*
NF-01	-	12.61mm	27.89mm	15.67mm	-
NF-02	-	10.81mm	24.46mm	12.22mm	-
NF-03	-	11.48mm	23.07mm	12.07mm	-
NF-04	-	14.24mm	23.07mm	12.80mm	-
NF-05	-	13.02mm	24.55mm	14.68mm	-
NF-06	-	-	21.52mm	11.98mm	-
NF-07	-	12.61mm	26.35mm	12.74mm	-
NF-08	-	-	20.64mm	11.36mm	-
NF-09	-	14.09mm	24.15mm	13.98mm	-
NF-10	-	-	25.37mm	11.98mm	-

Table 5 Antimicrobial activities of isolated fungi

Test organisms / Endophytes	*Micrococcus luteus*	*Saccharomyces cerevisiae*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Pseudomonas fluorescence*
NF-01	26.64mm	-	-	19.61mm	-
NF-02	25.66mm	-	21.53mm	20.46mm	-
NF-03	19.50mm	-	17.54mm	22.79mm	-
Nf-04	20.17mm	-	-	23.63mm	-
NF-05	23.36mm	-	19.38mm	21.50mm	-
NF-06	21.95mm	-	17.91mm	19.14mm	-
NF-07	18.92mm	-	17.94mm	21.67mm	-
NF-08	20.58mm	-	17.93mm	21.21mm	-
NF-09	22.52mm	-	19.87mm	22.47mm	-
NF-10	23.74mm	-	-	18.99mm	-

Table 6 Antimicrobial Activities of isolated fungi

Test organisms / Endophytes	*Agrobacterium tumefaciens*	*Aspergillus paraciticus*	*Bacillus subtilis*	*Candida albicans*	*Escherichia coli*
NF-11	13.61mm	13.52mm	12.81mm	-	-
NF-12	10.11mm	12.83mm	10.61mm	-	-
NF-13	14.28mm	14.05mm	11.34mm	-	-
NF-14	12.44mm	16.35mm	14.04mm	-	-
NF-15	12.02mm	16.89mm	13.42mm	-	-
NF-16	10.56mm	19.08mm	-	-	-
NF-17	16.61mm	18.42mm	12.71mm	-	-
NF-18	16.08mm	15.44mm	-	-	-
NF-19	14.09mm	13..21mm	14.22mm	-	-
NF-20	17.09mm	12.21mm	-	-	-

Table 7 Antimicrobial activities of isolated fungi

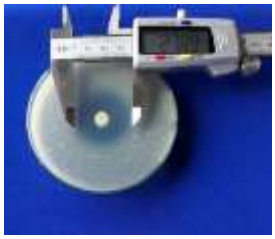
Test organisms / Endophytes	*Micrococcus luteus*	*Saccharomyces cerevisiae*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Pseudomonas fluorescence*
NF-11	20.43mm	22.43mm	19.55mm	-	-
NF-12	-	19.43mm	19.77mm	-	-
NF-13	15.74mm	23.44mm	22.09mm	-	-
NF-14	16.73mm	21.61mm	22.75mm	-	-
NF-15	18.39mm	19.71mm	19.75mm	-	-
NF-16	12.71mm	21.31mm	23.53mm	-	-
NF-17	20.94mm	18.69mm	21.31mm	-	-
NF-18	19.39mm	21.44mm	19.39mm	-	-
NF-19	18.77mm	17.09mm	-	-	-
NF-20	-	-	21.75mm	-	-

Figure 1 Antimicrobial activity of isolated fungi (NF-01 to 10) on *Bacillus subtilis.*

Table 8 Antimicrobial activity against *Bacillus subtilis*

Fermentation Period / Endophytes	2days	3days	4days	5days	6days	7days	8days	9days	10days
NF-01	-	21.51 mm	27.89 mm	27.66 mm	26.78 mm	23.72 mm	20.42 mm	-	-
NF-07	-	23.55 mm	26.35 mm	24.77 mm	22.49 mm	20.5 2mm	18.09 mm	-	-

Based on the results of antimicrobial activity test, it was found that 20 strains showed activities on six test organisms, among them, NF-01 and NF-07 showed highly activity against *Bacillus subtilis.*

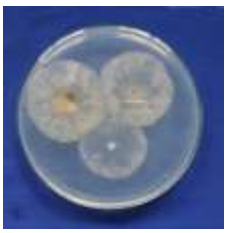


NF-01(27.89 mm)

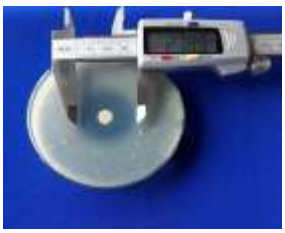
NF-07(26.35 mm)

Figure 2 Antimicrobial activity against *Bacillus subtilis*

Front view



Reverse view



Antimicrobial activity on *Bacillus subtilis*

Photomicrograph X 40

Figure 3 Morphology, Antimicrobial activity and Photomicrograph of selected fungus NF-01 against on *Bacillus subtilis*

Investigation of Carbon and Nitrogen Sources Utilization

In the study for the growth with carbon and nitrogen sources utilization, the excellent growth of NF-01 fungus was found on carbon sources such as glycerol, glucose and nitrogen sources such as yeast extract and peanut cake gave excellent growth. It was found that the good growth of NF-01 on carbon sources were sucrose and tapioca powder, nitrogen sources were peptone and meat extract. Carbon source were oat and soluble starch and nitrogen sources were malt extract and sesame cake gave poor growth. These results are shown in Table 9 and 11, Figure 4 and 6.

Table 9 Morphological Characters of NF-01 on Various Carbon Sources

Carbon Source	Growth	Colour
Glycerol	Excellent	White
Sucrose	Good	White
Glucose	Excellent	White
Oat	Poor	White
Tapioca powder	Good	White
Soluble starch	Poor	White

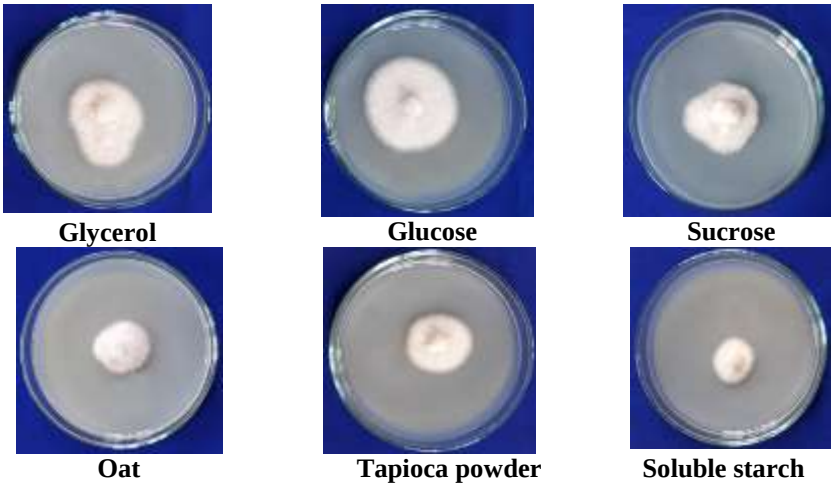


Figure 4 Morphological characters of NF-01 on various carbon sources

Table10 Effects of Different Carbon Sources Utilization against on *Bacillus subtilis*

Day / Sources	1 day	2 days	3 days	4 days	5 days	6 days
Glycerol	21.55 mm	24.60 mm	28.33 mm	34.21 mm	35.67 mm	**38.93 mm**
Sucrose	19.88 mm	23.53 mm	36.23 mm	36.88 mm	37.90 mm	**37.94 mm**
Glucose	**22.36 mm**	**23.60 mm**	**24.52 mm**	**38.23 mm**	**38.27 mm**	**42.52 mm**
Oat	20.17 mm	20.19 mm	21.07 mm	32.68 mm	34.65 mm	**35.11 mm**
Tapioca powder	19.74 mm	18.94 mm	23.16 mm	32.16 mm	33.36 mm	**34.04 mm**
Soluble starch	23.13 mm	25.16 mm	27.59 mm	29.72 mm	33.59 mm	**36.32 mm**

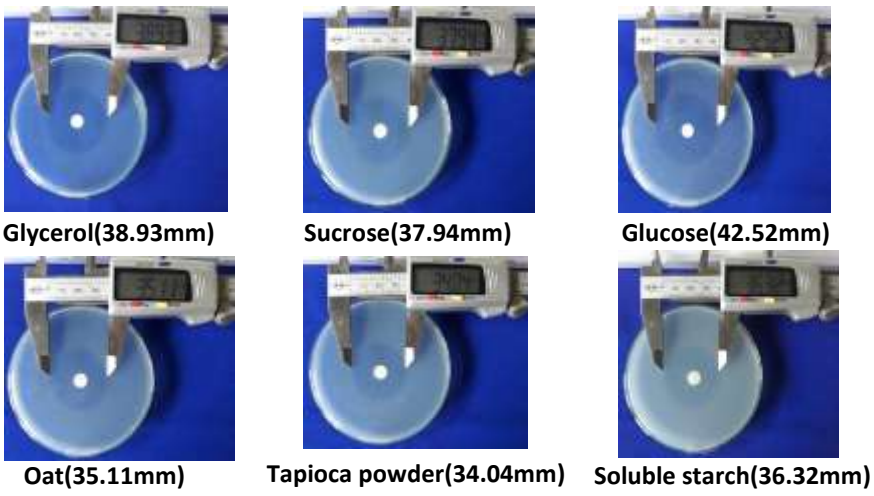


Figure 5 Carbon sources activities of fungus NF-01

Table 11 Morphological Characters of NF-01 on Various Nitrogen Sources

Nitrogen Source	Growth	Colour
Sesame cake	Poor	White
peptone	Good	White
Meat extract	Good	White
Malt extract	Poor	White
Peanut cake	Excellent	White
Yeast	Excellent	White

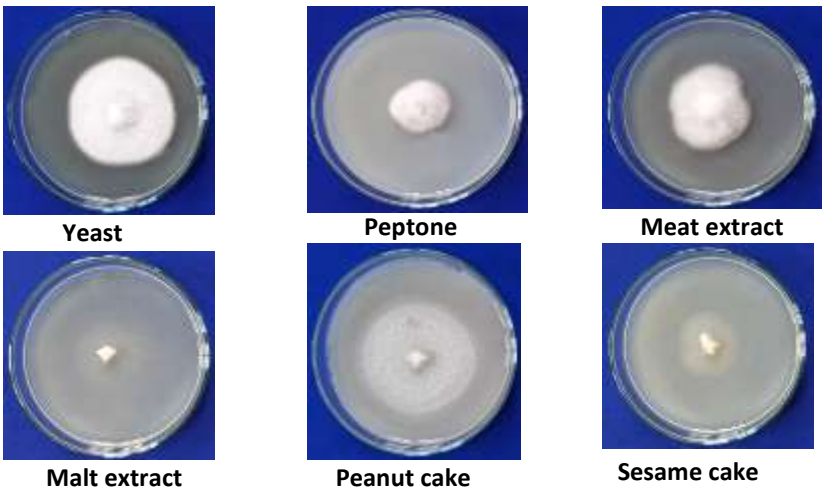
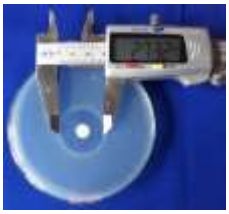


Figure 6 Morphological characters of NF-01 on various nitrogen sources

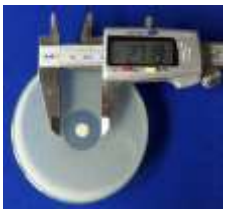
Table 12 Effects of Different Nitrogen Sources Utilization against on *Baciullus subtilis*

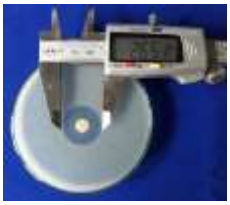
Day / Sources	1 day	2 days	3 days	4 days	5 days	6 days
Sesame cake	**29.31 mm**	27.32 mm	26.17 mm	26.32 mm	25.33 mm	24.03 mm
Peptone	**27.32 mm**	27.03 mm	24.79 mm	24.29 mm	22.48 mm	21.79 mm
Meat extract	**26.75 mm**	25.30 mm	24.07 mm	23.54 mm	23.36 mm	22.46 mm
Malt extract	**27.19 mm**	26.44 mm	25.36 mm	24.36 mm	23.48 mm	23.29 mm
Peanut cake	**26.51 mm**	25.73 mm	25.48 mm	25.25 mm	23.11 mm	21.08 mm
Yeast extract	**30.23 mm**	**29.14 mm**	**28.82 mm**	**26.73 mm**	**24.65 mm**	**22.65 mm**


sesamecake(29.31mm)


Peptone(27.32mm)


Meat


Malt extract(27.19mm)


Peanut


Yeast extract(30.23mm)

Figure 7 Nitrogen sources activities of strain NF-01

Discussion and Conclusion

During the study of the isolation of endophytic fungi from three different plants collected from Pathein Area. The isolation of endophytic fungi, 20 fungi were isolated. In the investigation of antimicrobial activities of these endophytic fungi (NF-01 to NF- 20) showed antimicrobial activities. Among them, NF-01 and NF-07 (isolated from the plant leaves of *Croton roxburghianus* N.P. Balakr (Thet-yin-gyi)) showed highly activity against *Bacillus subtilis.* Therefore, these strains were selected for further investigation of fermentation.

In conclusion, the isolation of endophytic fungi from three different plant leaves samples and screening them for antimicrobial activity by *Bacillus subtilis.* Among them, these active strains NF-01 showed highly activity than NF-07. Therefore NF-01 was selected for carbon and nitrogen sources utilization. In the investigation of carbon and nitrogen sources utilization, the excellent growth of NF-01 fungus was found on carbon sources such as glucose and glycerol; nitrogen sources were yeast extract and peanut cake.

The excellent growth of NF-01 fungus was found on carbon sources activity such as glucose (42.52mm) in 6 days fermentation period. In nitrogen sources activity yeast extract was (30.23mm) in 1 day fermentation period. Among them, these active strain NF-01 was selected further investigation of optimal fermentation conditions.

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