CULTURAL CHARACTERISTICS AND ANTIMICROBIAL ACTIVITIES OF ENDOPHYTIC FUNGI FROM LEAVES OF TWELVE MEDICINAL PLANTS

Myo Htaik Aung[1], Zaw Lin Aung[2]and Kay Thi Mya[3]

Abstract

A total of 18 endophytic fungi were screened and isolated into pure culture from the leaves of 12 medicinal plants collected from Pathein University and Mawlamyine University Campus and Taung wine area, Mawlamyine. The isolates were designated as MHA-1 to MHA-18. Among them MHA-10 which was isolated from the leaves of *Dioscorea birmanica* Prain and Burkill. (Khat-Cho) showed the highest antibacterial activity (18.90 mm clear zone) against *Bacillus subtilis.* Thus MHA-10 was selected for further study of optimal fermentation parameter to produce antibacterial metabolites. It was recorded that 84 hrs ages of inoculums, 30% size of inoculums were found to provide the best antibacterial activity in fermentation medium-1. Maximum antibacterial activity (18.03 mm clear zone) was found when fermentation media was supplemented with glucose. Among the wide variety of nitrogen sources tested, peptone proved to be the most suitable for antibacterial activity (18.95 mm clear zone) of selected fungus MHA-10 against *Bacillus subtilis.* FM-1 gave maximum antibacterial activity (20.64 mm clear zone) against *Bacillus subtilis.*

Keywords: endophytic fungi, antibacterial activity

Introduction

Natural products from microbial origin have been a consistent source of novel lead molecules and recently several endophytes have been shown to possess the potentials to synthesize novel bioactive compounds that have found great use for drug discovery (Okoye, *et al.,* 2015).

It is estimated that there might be as many as one million different endophytic fungal species, however, only a handful of them have been described, which means investigating the metabolites of these endophytes can increase the chance of finding novel bioactive natural products (Petrini, 1991). Endophytes are microorganisms which live in close association with living plant tissues in a symbiotic relationship. Fungi form a major portion of the endophytic population (Strobel *et al.,* 2004).

Isolation of endophytic fungi from medicinal and other plants to produce biologically active agents for biological utilization on a large commercial scale is possible because they can easily culture in laboratory instead of harvesting plants and affecting the environmental biodiversity. Medicinal plant acts as a richest source of endophytic fungi.

Scientists have exploited endophytic fungi very much attention for detection of bioactive compounds in the form of antimicrobial activity, anti-cancer activity etc. (Yu *et al.,* 2010).

The aim and objectives of the present study were to isolate the endophytic fungi from the leaves of 12 medicinal plants, to determine the antimicrobial activities of these fungi and to optimize the fermentation conditions of the selected fungus.

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Materials and Methods

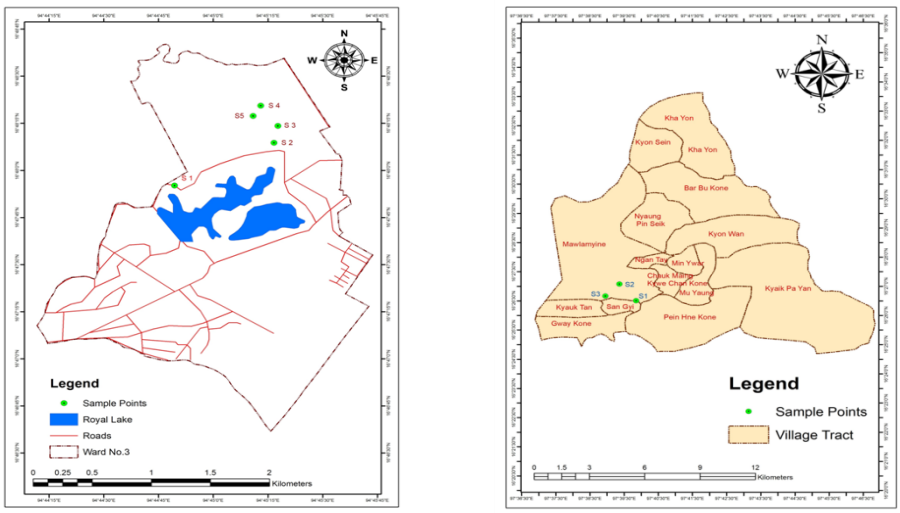
Isolation of Endophytic Fungi from Leaves of Medicinal Plants

Leaf samples were washed thoroughly in running tap water and air dried. The materials were then surface sterilized by immersing them in 70% ethanol for 1 min and rinsed in sterile distilled water for 1 min. And then the materials were immersed in 70% ethanol for 30 seconds and rinsed in sterile distilled water for 1 min and blot-dry. Then the leaf samples were dissected and place on petri-plates containing fungi isolation media.

Media Used for Isolation of Endophytic Fungi (Ando, 2004)

Isolation medium		**Transfer medium**	
Glucose	1.0 g	Glucose	1.2 g
Yeast extract	0.5 g	Yeast extract	0.7 g
$MgSO_4$	0.01 g	$MgSO_4$	0.01 g
K_2HPO_4	0.01 g	KNO_3	0.1 g
Agar	1.8 g	Agar	1.8 g
Distilled water	100 mL	Distilled water	100 mL

After autoclaving, chloramphenicol (25 mg/100mL) was added to the medium



(a) (b)

Source: Geography Department, Pathein University

Figure 1 (a) Map of Pathein University Campus and
(b) Map of Mawlamyine Showing Locations from
where Plant Samples were Collected

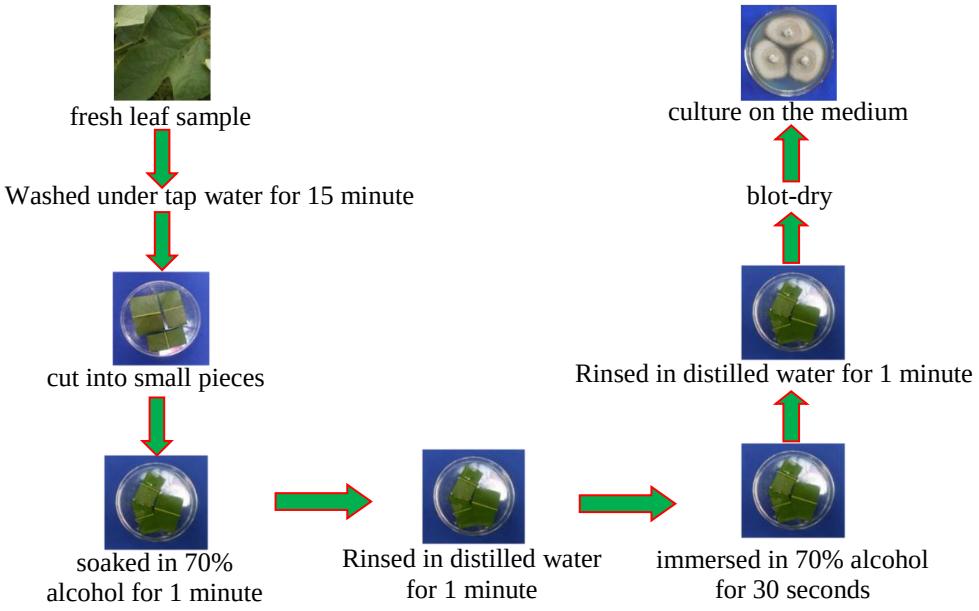


Figure 2 Procedure for isolation of endophytic fungi (Ando, 2004)

Table 1 Medicinal plants used for the isolation of the endophytes

No.	Botanical Name	Myanmar name	Family	Collected area
1	*Chrozophora rottleri* Geiseler Juss.	Joe-sar-kauk	Euphorbiaceae	Pathein University Campus
2	*Momordica cochinchinensis* (Lourein) Sprengel	Taw Sa byit	Cucurbitaceae	Pathein University Campus
3	*Gloriosa superba* L.	Si mi dauk	Colchicaceae	Mawlamyine
4	*Passiflora foetida* L.	Taw Su Ka	Passifloraceae	Pathein University Campus
5	*Spilanthes iabadicensis* A.H. Moore.	Shar-hton	Asteraceae	Mawlamyine
6	*Vernonia patula* (Dryand.) Merr.	Not known	Asteraceae	Pathein University Campus
7	*Cayratia japonica* (Thunberg) Gagnepain	Yin-naung	Vitaceae	Mawlamyine
8	*Dioscorea birmanica* Prain and Burkill	Khat-cho	Dioscoreaceae	Mawlamyine University Campus
9	*Hyptis capitata* Jacq.	Not known	Lamiaceae	Pathein University Campus
10	*Plantago major* L.	Akyawta-htaung	Plantaginaceae	Mawlamyine
11	*Tiliacora triandra* (Colebr.) Diels	Not known	Menispermaceae	Mawlamyine
12	*Kaempferia parviflora* Wall ex Baker	Nanwin-net	Zingiberaceae	Mawlamyine

Medium Used for Antimicrobial Activity Test (Ando, 2004)

Seed Medium		**Fermentation Medium**		**Assay Medium**	
Glucose	- 1.0 g	Glucose	- 1.5 g	Glucose	- 0.8 g
Yeast extract	- 0.5 g	Yeast extract	- 1.0 g	Peptone	- 0.7 g
MgSO4	- 0.001 g	MgSO4	- 0.001 g	KNO3	- 0.2 g
KNO3	- 0.1 g	KNO3	- 0.1 g	Agar	- 1.8 g
DW	- 100 mL	DW	- 100 mL	DW	- 100

Screening of Antimicrobial Activities of Effective Endophytic Fungi by Paper Disc Diffusion Assay (Tomita, 1988)

The isolated fungi were grown at room temperature for 5 days. The isolated fungi were inoculated on seed medium and incubated at room temperature for 3 days. Seed culture (20%) was transferred into the fermentation medium and incubated at room temperature for 10 days. 20 µL of fermented broth was put on paper disc. After drying, placed on assay plate containing test organism and incubated for 24 hours. In the present study, six microorganisms were used for antimicrobial activity.

Table 2 Test Organisms utilized for antimicrobial activities

No.	**Test Organisms**	**Disease**
1	*Aspergillus flavus* IFO3290	Aspergillosis
2	*Bacillus subtilis* KY-327	Fever
3	*Micrococcus luteus* NITE83297	Skin disease
4	*Escherichia coli* AHU5436	Diarrhoea
5	*Pseudomonas fluorescens* IFO94307	Rice disease
6	*Salmonella typhi* AHU7943	Typhoid fever and food poison

Effect of Age of Inoculum on the Fermentation of antibacterial activity by Isolated Endophytic Fungus MHA-10

Five days old culture of the selected fungus MHA-10 was inoculated into seed medium and then transfer to fermentation medium. Age of culture with 48 hrs, 60 hrs, 72 hrs, 84 hrs, 96 hrs, 108 hrs and 120 hrs were employed for fermentation. The antibacterial activity was checked by paper disc diffusion assay method.

Effect of Sizes of Inoculum on the Fermentation of Antibacterial activity by Isolated Fungus MHA-10

In this study 6%, 12%, 18%, 24%, 30% and 36% of 84 hrs of seed culture were employed for the fermentation. The antibacterial activity was undertaken by paper disc diffusion assay method.

Effect of Carbon Source Utilization on the Fermentation of Antibacterial Activity by Isolated Fungus MHA-10

To evaluate the effect of various carbon sources on the fermentation of antibacterial activity by isolated endophytic fungus MHA-10, different carbon sources such as glucose, sucrose, soluble starch, potato powder, tapioca powder, corn powder and rice powder were supplemented separately into the fermentation medium. 2 g of each carbon sources were added into basal medium.

Effect of Nitrogen Source Utilization on the Fermentation of Antibacterial Activity by Isolated Fungus MHA-10

To evaluate the effect of various nitrogen sources on the fermentation of antibacterial activity by isolated endophytic fungus MHA-10, different nitrogen sources such as yeast extract, peptone, KNO_3, NH_4Cl_2, NH_4SO_4, NH_4NO_3 and meat extract were utilized. 1 g of each nitrogen sources were added into basal fermentation medium.

Study on Medium Optimization for Fermentation

In the present study, 6 fermentation media was undertaken. Fermentation was carried out with 84 hrs age and 30% size of inoculums with six different media.

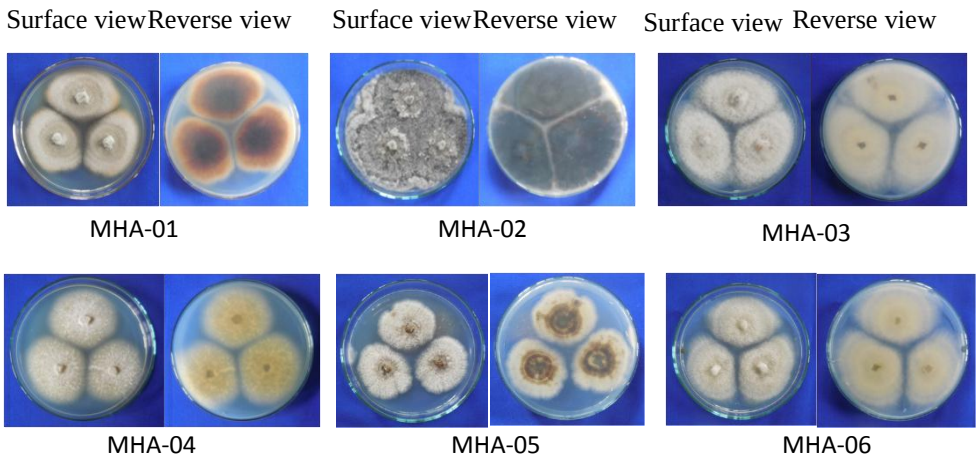
FM-1		**FM-2**		**FM-3**	
Glucos e	2.0 g	Glucose	2.0 g	Glucose	2.0 g
Yeast extract	1.0 g	Peptone	1.0 g	Yeast extract	0.5 g
$MgSO_4$	0.001 g	MgSO4	0.001 g	Peptone	0.5 g
KNO_3	0.1 g	KNO3	0.1 g	MgSO4	0.001 g
D/W	100 mL	D/W	100 mL	KNO3	0.1 g
				D/W	100 mL

FM-4		**FM-5**		**FM-6**	
Glucose	2.0 g	Glucose	2.0 g	Glucose	1.5 g
Yeast extract	0.7 g	Yeast extract	0.3 g	Yeast extract	0.5 g
Peptone	0.3 g	Peptone	0.7 g	Peptone	0.5 g
$MgSO_4$	0.001 g	$MgSO_4$	0.001 g	$MgSO_4$	0.001 g
KNO_3	0.1 g	KNO_3	0.1 g	KNO_3	0.1 g
D/W	100 mL	D/W	100 mL	D/W	100 mL

Results

Isolation of Endophytic Fungi from Leaves of Medicinal Plants

In the present investigation, 18 endophytic fungi were isolated from leaves of 12 medicinal plants.



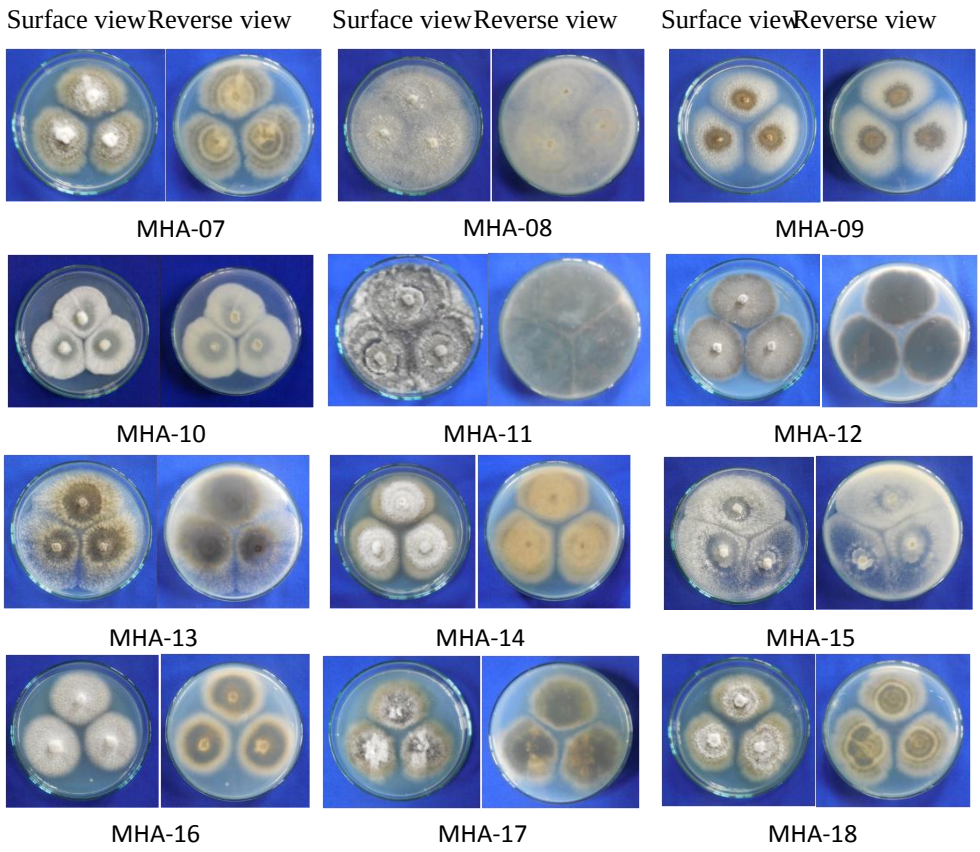


Figure 3 Morphology of isolated soil fungi MHA-01 to MHA-18

(7 Days old culture)

Table 3 Endophytic fungi isolated from the leaves of 12 medicinal plants

No.	Botanical Name	Isolates	Designated Number of endophytic fungi
1	*Chrozophora rottleri* Geiseler Juss.	1	MHA-01
2	*Momordica cochinchinensis* (Lourein)	1	MHA-02
3	*Gloriosa superba* L.	2	MHA-03, MHA-04
4	*Passiflora foetida* L.	1	MHA-05
5	*Spilanthes iabadicensis* A.H. Moore.	1	MHA-06
6	*Vernonia patula* (Dryand.) Merr.	1	MHA-07
7	*Cayratia japonica* (Thunberg) Gagnepain	2	MHA-08, MHA-09
8	Dioscorea birmanica Prain and Burkill	2	MHA-10, MHA-11
9	Hyptis capitata Jacq.	2	MHA-12, MHA-13
10	Plantago major L.	2	MHA-14, MHA-15
11	Tiliacora triandra (Colebr.) Diels	2	MHA-16, MHA-17
12	Kaempferia parviflora Wall ex Baker	1	MHA-18
	Total endophytic fungi	18	

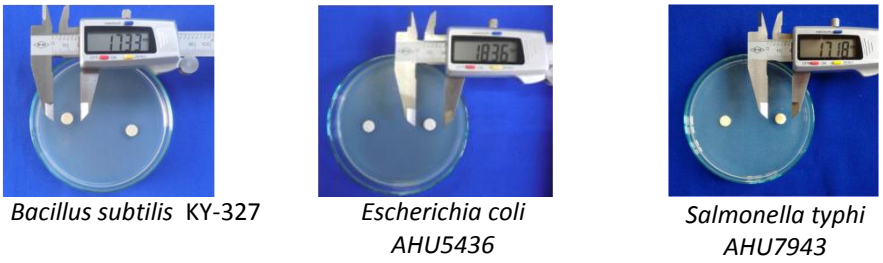
Screening of Antimicrobial Activities of Effective Endophytic Fungi by Paper Disc Diffusion Assay

In this study, two endophytic fungi, (MHA-09 and MHA-10) showed the antimicrobial activities on *Bacillus subtilis*, *E. coli* and *Salmonella typhi*. The endophytic fungus MHA-10 showed the highest activity on *Bacillus subtilis* (18.90 mm, inhibitory zone). This fungus MHA-10 was isolated from the leaf of *Dioscorea birmanica* Prain and Burkill.

Table 4 Antimicrobial activities of isolated fungi on six test organisms

Isolated fungi	Antimicrobial activities on test organisms (inhibitory zone, mm) (4 to 9 days)					
	Aspergillus flavus	***Bacillus subtilis***	***Micrococcus luteus***	***Escherichia coli***	***Pseudomonas fluorescens***	***Salmonella typhi***
MHA-09	-	17.33	-	18.36	-	17.18
MHA-10	-	18.90	-	17.64	-	18.74

(-) = No activity



Bacillus subtilis KY-327 | *Escherichia coli* AHU5436 | *Salmonella typhi* AHU7943

Figure 4 Antimicrobial activity of isolated fungus (MHA-09) against three test organisms

Bacillus subtilis KY-327 | *Escherichia coli* AHU5436 | *Salmonella typhi* AHU7943

Figure 5 Antimicrobial activity of isolated fungus (MHA-10) against three test organisms

Effect of Age of Inoculums on the Fermentation of antibacterial activity by Isolated Endophytic Fungus MHA-10

According to the table 5, it was shown that 84 hrs ages of inoculums was the best for fermentation.

Table 5 Effects of age of culture on fermentation of antibacterial activity by isolated endophytic fungus MHA-10

Seed culture (Time, hrs)	Activity (clear zone mm)
48	13.72
60	15.51
72	17.28
84	**18.80**
96	17.69
108	16.73
120	14.05

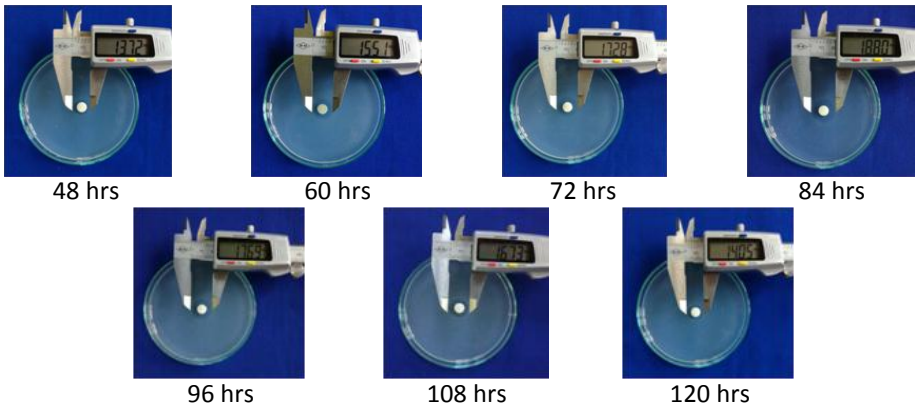


Figure 6 The effect of age of inoculum on fermentation of antibacterial activity by isolated endophytic fungus MHA-10 against *Bacillus subtilis*

Effect of Size of Inoculum on Fermentation of Antibacterial Activity by Isolated Endophytic Fungus MHA-10

According to the table 6, it was observed that 30% size of inoculums was the best for fermentation.

Table 6 Effects of size of inoculum on fermentation of antibacterial activity by isolated endophytic fungus MHA-10 against *Bacillus subtilis*

Size of inoculum	Activity (clear zone mm)
6 %	9.94
12 %	10.03
18 %	11.03
24 %	14.48
30 %	**17.06**
36 %	15.04

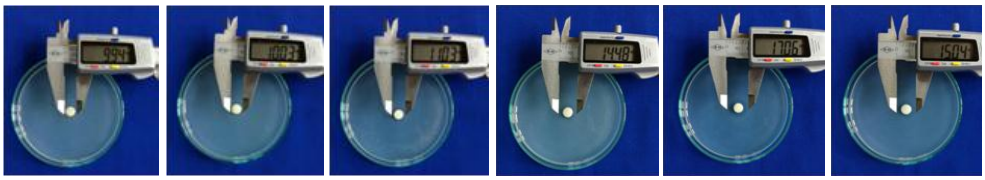


Figure 7 The effect of size of inoculum on fermentation of antibacterial activity by isolated endophytic fungus MHA-10 against *Bacillus subtilis*

Effect of Carbon Source Utilization on the Fermentation of Antibacterial Activity by Isolated Fungus MHA-10

Among the carbon source tested, glucose showed the highest antibacterial activity (18.03 mm clear zone) followed by sucrose (14.39 mm clear zone) against *Bacillus subtilis*, lowest activity was exhibited by corn powder (10.07 mm clear zone). (Table 7, Figure 8).

Table 7 Effects of carbon utilization on fermentation of antibacterial activity by isolated fungus MHA-10 against *Bacillus subtilis*

Carbon sources	Activity (clear zone mm)
Glucose	**18.03**
Sucrose	14.39
Soluble starch	12.79
Potato powder	13.46
Tapioca powder	10.19
Corn powder	10.07
Rice powder	12.88

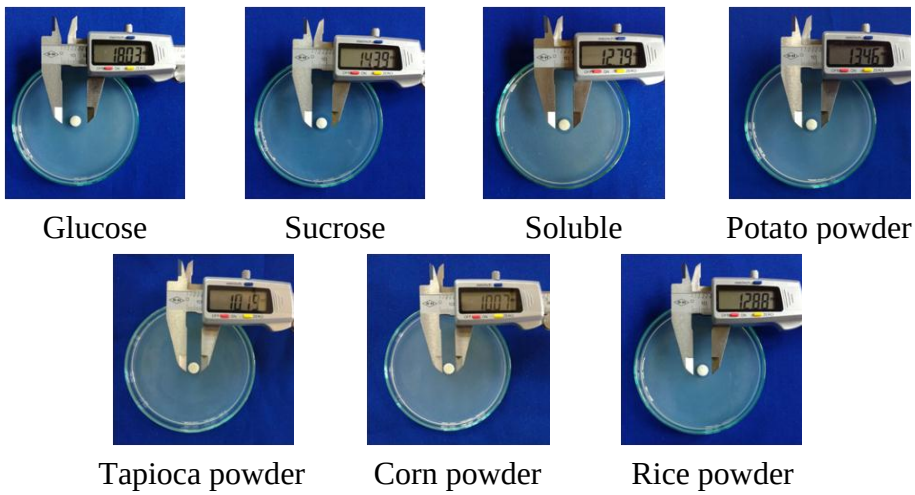


Figure 8 Effects of carbon utilization on fermentation of antibacterial activity by isolated endophytic fungus MHA-10 against *Bacillus subtilis*

Effect of Nitrogen Source Utilization on the Fermentation of Antibacterial Activity by Isolated Fungus MHA-10

Among the nitrogen source tested, peptone showed the highest antibacterial activity (18.95 mm clear zone) followed by yeast extract (16.34 mm clear zone) against *Bacillus subtilis*. Lowest activity was exhibited by potassium nitrate (KNO_3) (10.08 mm clear zone). (Table 8, Figure 9).

Table 8 Effects of nitrogen utilization on fermentation of antibacterial activity by isolated fungus MHA-10 against *Bacillus subtilis*

Nitrogen sources	Activity (clear zone mm)
Yeast extract	16.34
Peptone	**18.95**
KNO_3	10.08
NH_4Cl	14.19
NH_4SO_4	15.63
NH_4NO_3	14.43
Meat extract	15.28

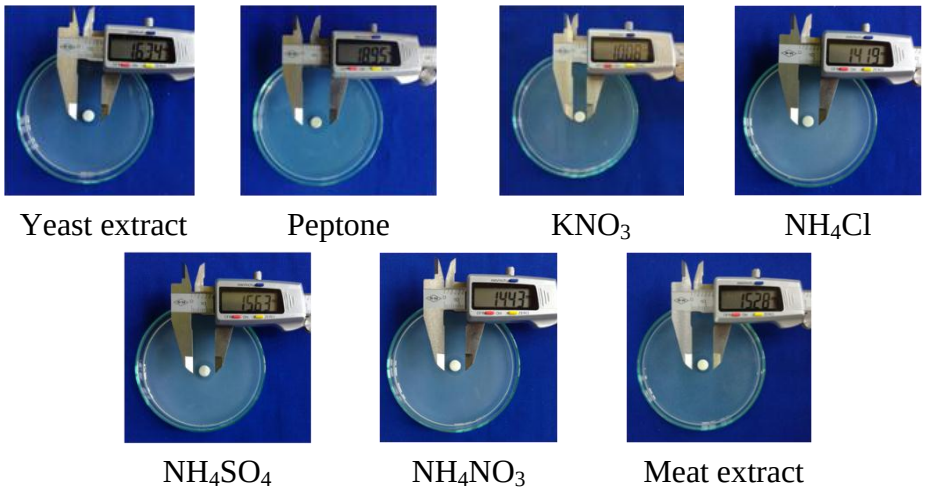


Figure 9 Effects of nitrogen utilization on fermentation of antibacterial activity by isolated endophytic fungus MHA-10 against *Bacillus subtilis*

Effect of Medium on Fermentation

FM-1 gave maximum antibacterial activity (20.64 mm clear zone against *Bacillus subtilis*).

Table 9 Effects of media on fermentation

Medium	Activity (clear zone mm)
FM-1	**20.64**
FM-2	13.77
FM-3	16.78
FM-4	13.17
FM-5	14.04
FM-6	14.45

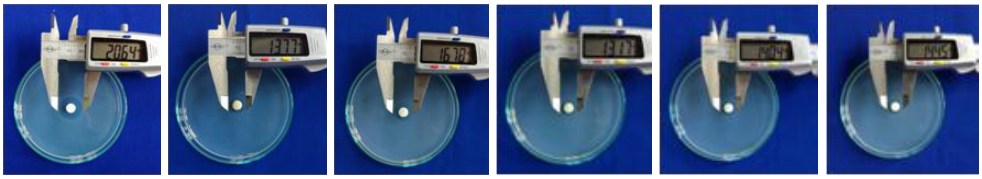


Figure 10 Effects of media on fermentation against *Bacillus subtilis*

Discussion and Conclusion

A total 18 endophytic fungi were isolated from leaves of 12 medicinal plants. Endophytic fungus MHA-01 was isolated from *Chrozophora rottleri* Geiseler Juss. Fungus MHA-02 was isolated from *Momordica cochinchinensis* (Lourein) Sprengel. Endophytic fungi MHA-03 and MHA-04 were isolated from *Gloriosa superba* L. Budhiraja *et al.,* (2012) isolated 22 endophytic fungi from different parts of *Gloriosa superba* L. Endophytic fungus MHA-05 was isolated from *Passiflora foetida* L. Kanjana *et al.,* (2019) isolated endophytic fungus *Chaetomium globosum* from *Passiflora foetida* L. Endophytic fungus MHA-06 was isolated from *Spilanthes iabadicensis* A.H. Moore. Endophytic fungus MHA-07 was isolated from *Vernonia patula* (Dryand.) Merr. Endophytic fungi MHA-08 and MHA-09 were isolated from *Cayratia japonica* (Thunberg) Gagnepain. Endophytic fungi MHA-10 and MHA-11 were isolated from *Dioscorea birmanica* Prain and Burkill. Endophytic fungi MHA-12 and MHA-13 were isolated from *Hyptis capitata* Jacq. Endophytic fungi MHA-14 and MHA-15 were isolated from *Plantago major* L. Farias *et al.,* (2018) isolated *Colletotrichum gloesporioides* from leaves of *Plantago major* L. Endophytic fungi MHA-16 and MHA-17 were isolated from *Tiliacora triandra* (Colebr.) Diels. Senadeera *et al.,* (2012) isolated endophytic fungus *Dothideomycete* sp. from *Tiliacora triandra* (Colebr.) Diels. Endophytic fungus MHA-18 was isolated from *Kaempferia parviflora* Wall ex Baker. Jankong (2004) isolated 36 endophytic fungi from *Kaempferia parviflora* Wall ex Baker.

The endophytic fungi MHA-10 has shown greater antibacterial activity against *Bacillus subtilis.* The maximum production of the antibacterial metabolites by MHA-10 was achieved by optimizing various parameters like age (84 hrs) and size (30%) of inoculums, and fermentation

media (FM-1) were found to be optimum for the maximal production of bioactive metabolite. Jain, 2010 reported that the variations in the fermentation environment often result in alteration in antibiotic production. Among the different carbon sources tested, glucose was the best carbon source for antibacterial activity. Similar results were shown by Tanseer and Anjum (2011) where glucose promoted the secondary metabolite production. The results were in good agreement with Haque *et al.,* (2014). Among the different nitrogen sources tested, peptone was the best nitrogen source for antibacterial activity. Peptone has been observed to be the best nitrogen sources by Reddy *et al.,* (2011). Peptone has been found to favour antibiotic production by Praveen (2008), Tanseer and Anjum (2011). These findings will assist in formulating a suitable culture medium for production of the antibacterial compound from endophytic fungus MHA-10.

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