FERMENTATION CONDITIONS FOR THE PRODUCTION OF ANTIBACTERIAL METABOLITE FROM THE ENDOPHYTIC FUNGUS, *ASPERGILLUS TURCOSUS*

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Abstract

Seventeen different kinds of selected mangrove leaves collected at Ma Gyi coastal area, Shwe Taung Yan Township, Ayeyarwady Region during monsoon period, 2016. The selected endophytic fungus, *Aspergillus turcosus* was isolated from *Bruguiera cylindrica* (L.) Blume (Byu-Kyet-Tet). In the investigation of antimicrobial activities, this endophytic fungus exhibited highly antibacterial activity on *Agrobacterium tumefaciens* IFO5431. Based on the growth kinetics of this fungus, it was found that 54 hr of ages and 10% of sizes of inoculum were suitable for the fermentation. The good production of antibacterial metabolite from the endophytic fungus was occurred in glycerol and corn powder as carbon sources and in peptone and beef extract as nitrogen sources against *Agrobacterium tumefaciens*. Five fermentation medium (FM-1, FM-2, FM-3, FM-4 and FM-5) were prepared for the fermentation study, according to the results of the effects of carbon and nitrogen sources utilization on the fermentation. It was observed that FM-1 medium was the most suitable for the production of antibacterial metabolite.

Keywords: antibacterial activity, *Agrobacterium tumefaciens*, *Aspergillus turcosus*, *Bruguiera cylindrica*, endophytic fungus, Ma Gyi coastal area, mangrove leaves.

Introduction

In developing countries, numerous communities have been using local plants in different ways to treat various diseases including gastroenteritis. Previous study on secondary metabolites of endophytic fungi from mangrove revealed that the fungi produced antibiotic, including griseofulvin, which commonly found in *Penicillium griseofulvum* (Strobel, 2002).

A clear understanding of microbial growth is necessary if the large-scale may be properly managed. Suitable ages and sizes of inoculum were crucial for the production of primary and secondary metabolite (Omura, 1985 and Crueger, 1989).

Many of the endophytic fungal strains have attracted special attention because they have the capability of producing different colour pigments with high chemical stability (Tan and Zou, 2001).

The physical and chemical parameters like pH, temperature, incubation period, carbon and nitrogen sources and amino acid plays a major role on production of bioactive compounds and antimicrobial agents (Gunasekaran and Poorniammal, 2008).

The objectives of this research are to investigate the fermentation conditions for the production of antibacterial metabolite against *Agrobacterium tumefaciens*, and to optimize fermentation to get the highest yields of antibacterial compound. Therefore, the fermentation optimization was investigated for the production of antibacterial metabolite from the endophytic fungus, *Aspergillus turcosus*.

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Materials and Methods

Sample collection and isolation of endophytic fungus

The selected endophytic fungus, *Aspergillus turcosus* was isolated from *Bruguiera cylindrica* (L.) collected at Ma Gyi coastal area, Shwe Taung Yan Township, Ayeyarwady Region during monsoon period, 2016.

Use of Test Organisms on the Antibacterial Activity

In the investigation of antibacterial activities of endophytic fungus *Aspergillus turcosus*, the test organisms *Agrobacterium tumefaciens* IFO5431 was used.

Studies on Microbial Growth Kinetics of *Aspergillus turcosus*

Microbial Growth Kinetics of *Aspergillus turcosus* was carried out by the methods of (Omura, 1985, Crueger and Crueger, 1989). The fungus *Aspergillus turcosus* was inoculated into 100 mL of GYN medium and incubated for 132 hrs. The culture sample (5 mL) was checked in 12 hrs intervals for the growth. The sample (5 mL) was centrifuged at 2000 rpm for 30 minutes and Packed Cell Volume (PCV) was calculated.

GYN MEDIUM

Medium Composition (g/L)

Glucose	10 g
Yeast Extract	2 g
NZ amine type A	3 g
pH	6.5

Effects of Ages of Inoculum on the Fermentation

Based on the results of microbial growth kinetics of the fungus, seed cultures of (24, 30, 36, 42, 48, 54, 60, 66, 72, 78 hrs) were utilized for the fermentation. Fermentation was carried out 8 days and antibacterial activity was tested by paper disc diffusion assay.

Effects of Sizes of Inoculum on the Fermentation

For the suitable fermentation conditions, the sizes of inoculum were also investigated. According to the results of the ages of inoculum of fungus, (5 %, 10 %, 15 %, 20 %, 25 % and 30 %) of 54 hrs seed cultures of this fungus were utilized for the fermentation. Fermentation was carried out 8 days and antibacterial activity was tested by paper disc diffusion assay.

Effects of Different Carbon Sources Utilization on the Fermentation

In this study, 54 hrs of ages and 10 % of sizes of inoculum were utilized for the fermentation with basal medium. Various carbon sources such as glucose, wheat, sucrose, soluble starch, glycerol, corn powder, rice powder and potato powder were used to study the different carbon sources utilization on the fermentation of fungus. Fermentation was carried out 8 days and 100 mL of fermentation media were prepared for carbon sources test. Each carbon source (2.0 g) was added to 100 mL basal fermentation medium.

Figure 1 Effects of ages of inoculum of fungus on the fermentation

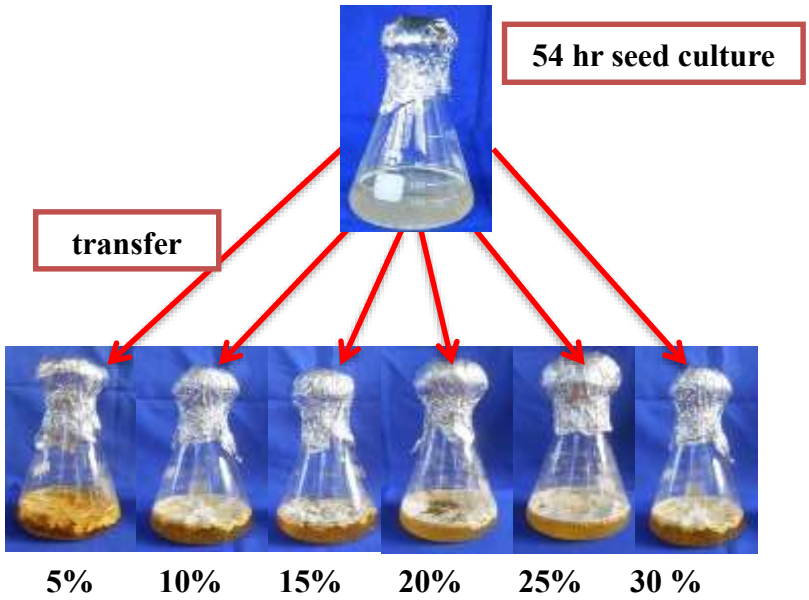

Figure 2 Effects of sizes of inoculum of fungus on the fermentation

Basal fermentation medium used in carbon sources test

Basal Medium (For Carbon sources)	
Medium Composition (g/ L)	
Yeast Extract	**10 g**
KNO_3	**2 g**
$MgSO_4$	**0.01 g**
K_2HPO_4	**0.01g**
pH	**6.5**

Effects of Different Nitrogen Sources Utilization on the Fermentation

Nitrogen sources like yeast extract, peptone, potassium nitrate, fish, peanut and beef extract were employed to study the different nitrogen sources utilization on the fermentation of fungus. Fermentation was carried out 8 days and 100 mL of fermentation media were prepared for nitrogen sources test. Each nitrogen source (0.5 g) was added to 100 mL basal fermentation medium.

Basal fermentation medium used in nitrogen sources test

Basal Medium (For Nitrogen sources)	
Medium Composition (g/ L)	
Glucose	**10 g**
Glycerol	**10 mL**
$MgSO_4$	**0.01 g**
K_2HPO_4	**0.01g**
pH	**6.5**

Establishment and Selection of Media for the Fermentation

Five fermentation medium (FM-1, FM-2, FM-3, FM-4 and FM-5) were prepared for the fermentation study, according to the results of the effects of carbon and nitrogen sources utilization on the fermentation of fungus. In the preparation of five fermentation medium, suitable carbon sources (Glycerol and Corn powder) and suitable nitrogen sources (Peptone and Beef extract) were utilized as suitable ratios and compositions. Fermentations were carried out for 8 days in the Rotary shaker and the activities were checked by paper disc diffusion assay method with 8 mm diameter paper discs.

Five fermentation medium prepared for the fermentation study Medium Composition (g/L)

FM-1	
Glycerol	10 mL
Peptone	8 g
K_2HPO_4	0.1 g
$MgSO_4$-$7H_2O$	0.1 g
pH	6.5

FM-2	
Glycerol	10 mL
Beef extract	8 g
K_2HPO_4	0.1 g
$MgSO_4$-$7H_2O$	0.1 g
pH	6.5

FM-3	
Corn powder	10 g
Peptone	8 g
K_2HPO_4	0.1 g
$MgSO_4$-$7H_2O$	0.1 g
pH	6.5

FM-4	
Corn powder	10 g
Beef extract	8 g
K_2HPO_4	0.1 g
$MgSO_4$-$7H_2O$	0.1 g
pH	6.5

FM-5	
Glycerol	5 mL
Peptone	5 g
Corn powder	5 g
Beef extract	5 g
K_2HPO_4	0.1 g
$MgSO_4$-$7H_2O$	0.1 g
pH	6.5

Results

Studies on Microbial Growth Kinetics of fungus *Aspergillus turcosus*

In the microbial growth kinetics study, as shown in Figure 3 and Table 1, it was found that the lag phase was between 24 hrs and 36 hrs. Growth phase was between 36 hrs and 72 hrs. It was observed that the growth of fungus declined after 84 hrs. According to Crueger and Crueger (1989), it was considered that ages of inoculum (42 hrs, 48 hrs, 54 hrs, 60 hrs, 66 hrs and 72 hrs) were used to optimize the fermentation.

Table 1 Microbial growth kinetics of fungus *Aspergillus turcosus*

Culture Time (hr)	PCV of 5 mL	PCV %
24	0.20	4
36	0.32	6.4
48	0.54	10.8
60	0.78	15.6
72	1.15	23
84	1.32	26.4
96	1.29	25.6
108	1.20	24
120	1.15	23

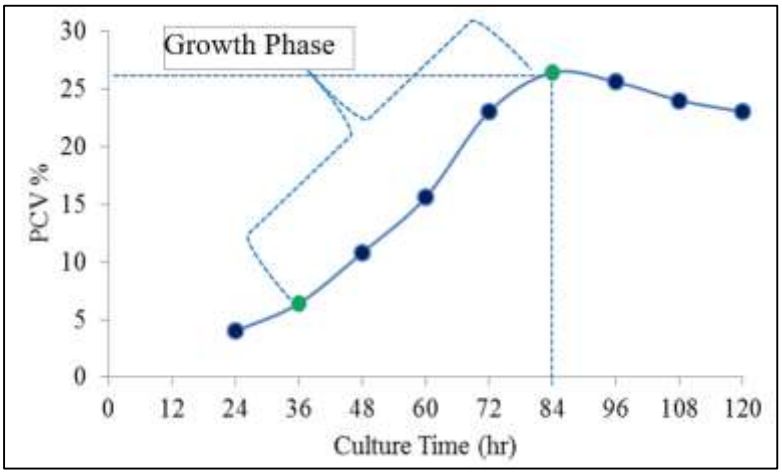

Figure 3 Microbial growth kinetics of fungus *Aspergillus turcosus*

Effects of Ages of Inoculum on the Fermentation

In the investigation of the age of inoculum, ten different hours of 24 hrs,30 hrs, 36 hrs, 42 hrs, 48 hrs, 54 hrs, 60 hrs, 66 hrs, 72 hrs and 78 hrs were used and the results showed the inhibitory zone of 14 mm, 15 mm, 15 mm, 17 mm, 18 mm, 22 mm, 20 mm, 18 mm, 15 mm and 15 mm, respectively against *Agrobacterium tumefaciens* (Table 2).

Table 2 Effect of ages of inoculums on the fermentation

Culture time (hrs)	Activity (Clear zone, mm)
24	14
30	15
36	15
42	17
48	18
54	**22**
60	20
66	18
72	15
78	15

Effects of Sizes of Inoculum on the Fermentation

In the investigation of the sizes of inoculum, 5%, 10%, 15%, 20%, 25% and 30% as six different percentages were used and the results showed the inhibitory zone of 20.06 mm, 22.53 mm, 21.96 mm, 21.06 mm, 21.72 mm and 21.06 mm, respectively against *Agrobacterium tumefaciens* (Table 3).

Table 3 Effect of sizes of inoculums on the fermentation

Sizes of Culture %	Activity (Clear zone, mm)
5	20.06
10	**22.53**
15	21.96
20	21.06
25	21.72
30	21.06

Effects of Different Carbon Sources Utilization on the Fermentation

In the investigation of different carbon sources utilization on the fermentation of fungus, various carbon sources such as glucose, wheat, sucrose, soluble starch, glycerol, corn powder, rice powder and potato powder were used and the results showed the inhibitory zone of 12.25 mm, 11.17 mm, 13.61 mm, 12.80 mm, 18.42 mm, 15.48 mm, 13.74 mm and 12.08 mm respectively against *Agrobacterium tumefaciens.* The activities were tested by paper disc diffusion assay method with 8 mm diameter paper size as shown in Table 4.

Effects of Different Nitrogen Sources Utilization on the Fermentation

In the investigation of different nitrogen sources utilization on the fermentation of fungus, yeast extract, peptone, fish extract, peanut cake and beef extract were employed and the results showed the inhibitory zone of 13.20 mm, 20.90 mm, 12.70 mm, 14.57 mm, 11.90 mm, respectively

against *Agrobacterium tumefaciens.* Only one source, Potassium nitrate gave no activity on *Agrobacterium tumefaciens*. The activities were tested by paper disc diffusion assay method with 8 mm diameter paper size as shown in Table 5.

Table 4 Effects of different carbon sources utilization on the fermentation

Carbon sources	Activity (Clear zone, mm)
Glucose	12.25
Wheat	11.17
Sucrose	13.61
Soluble Starch	12.80
Glycerol	**18.42**
Corn powder	15.48
Rice powder	13.74
Potato powder	12.08

Table 5 Effects of different nitrogen sources utilization on the fermentation

Nitrogen sources	Activity (Clear zone, mm)
Yeast extract	13.20
Peptone	**20.90**
Potassium nitrate	No Activity
Fish extract	12.70
Beef extract	14.57
Peanut cake	11.90

Establishment and Selection of Media for the Fermentation

In the study of media selection for the fermentation, five kinds of fermentation media were used. According to the results of antibacterial activity, fermentation medium FM-1 showed the highest inhibitory zone of 23.56 mm against *Agrobacterium tumefaciens* than that of other media (Table 6 and Figure 4.). Therefore, FM-1 was selected for the production of antibacterial metabolite.

Table 6 Effect of media in fermentation study

Fermentation medium	Inhibitory zone (mm)
FM-1	**23.56**
FM-2	11.57
FM-3	19.58
FM-4	No Activity
FM-5	11.30

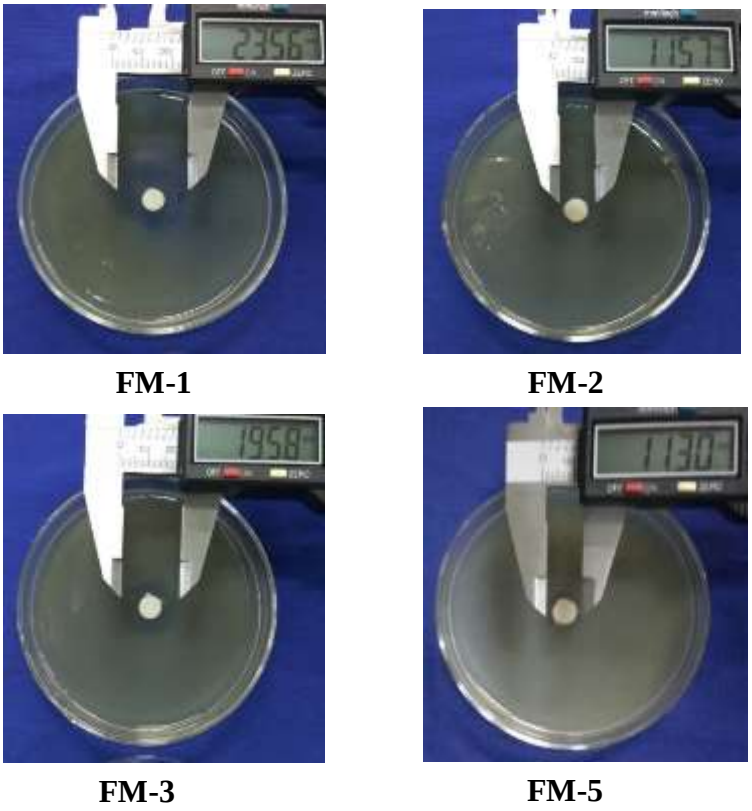

Figure 4 Antibacterial activities of the endophytic fungus *Aspergillus turcosus* on the fermentation media FM-1, FM-2, FM-3 and FM-5

Discussion and Conclusion

In the investigation of microbial growth kinetics of *Aspergillus turcosus*, it was observed that growth phase (trophophase) was between 36 hr and 72 hr. This result is in accordance with the statement of Crueger & Crueger, 1989.

Based on the growth kinetics of this fungus, it was determined that 54 hr of age of inoculum and 10 % of size of inoculum were suitable for the production of metabolite. In the study of carbon and nitrogen sources utilization on the fermentation, the results showed that organic nitrogen sources such as peptone support growth and good production of antibacterial metabolite. Inorganic salts (such as potassium nitrate) can be used as nitrogen sources for biocontrol agents, which are able to assimilate ammonium and to reduce nitrate. These inorganic nitrogen sources probably contain only the nutrients that satisfy no more than minimal requirement for growth and production of antibacterial metabolite (Gibbins 1978). However, the growth obtained for endophytic fungus *Aspergillus turcosus* was lower in potassium nitrate and no activity was shown in the fermentation.

The type of carbon source promotes growth of endophytic fungus influenced by the capacity of the microorganism to use the available nitrogen source (Gibbins 1978). In general, glycerol and corn powder were good carbon sources and peptone was good nitrogen source in fermentation media.

Medium formulation is necessary for each fermentation process. It is necessary to optimize each and every component of fermentation media by varying the concentration of media constituents in order to achieve maximum antibiotic production. The purpose of media optimization is to support efficient growth of microorganisms. Different combinations of medium constituents and sequences of optimized fermentation conditions need to be investigated to

determine growth conditions that produce biomass that is physiologically best suited for antibiotic production (Antal *et al*., 2012).

Based on the result of carbon and nitrogen sources utilization on the fermentation, 5 different kinds of fermentation media were established and utilized in fermentation. The result obtained in the study of 5 fermentation media showed that fermentation medium FM-1 was the most suitable for the production of antibacterial metabolite. Natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. The large proportion of natural products in drug discovery has stemmed from the diverse structures and the intricate carbon skeletons of natural products (Chen *et al*., 2003).

The present study was carried out on the antibacterial activity of marine microbes from mangrove plants in the coastal region of Myanmar. But there is still a need for the extensive study of marine microbes and their relationships to their environment.

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