Fermentation Optimization of Antibacterial Metabolite Produced By Fungus Kf-05 from Ayadaw Township Area

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Abstract

Eight different soil samples were collected at Ayadaw Township Area, Monywa District, Sagaing Region. The isolation of soil fungi was carried out by physical treatment dilution method. Fourteen fungi were isolated from eight different soil samples. Antibacterial activities of these fungi were evaluated by paper disc diffusion assay with seven test organisms. In the antibacterial study, eight fungal strains showed the antibacterial activity. Among them, the strain KF-05 showed more selective highly antibacterial activity against *Pseudomonas fluorescens*. Therefore, different fermentation parameters of KF-05 were studied by the fermentation period, proper age and size of inoculums, effect of various carbon and nitrogen sources, pH, temperature, fermentation medium on *Pseudomonas fluorescens*.

Keywords: Soil fungi, Antimicrobial activity, Fermentation

Introduction

Since microorganisms grow in unique and extreme habitats, they may have the capability to produce unique and unusual metabolites. Generally, the reason why they produce such metabolites is not known, but it is believed that many of these metabolites may act as chemical defense as an adaptation of fungi competing for substrates (Gallo *et al*., 2004). One gram of soil may harbor up to 10 billion microorganisms of possible thousands of different species (Rosello-Mora and Amann, 2001).

Fungi can produce a wide range of chemical compounds via secondary metabolism. These compounds are of major interest because of their potential application in medicine and biotechnology and as a potential source for new therapeutic agents and drug leads. However, under laboratory conditions, most secondary metabolism genes remain silent. This circumstance is an obstacle for the production of known metabolites and the discovery of new secondary metabolites (Wiemann and Keller, 2014).

This research paper aims to investigate the effect of fermentation, pH, temperature of selected fungus KF-05 on *Pseudomonas fluorescens*.

Materials and Methods

Preliminary Study on Antimicrobial Activities

Screening (or) Preliminary study for antibacterial activities was carried out by paper disc diffusion assay (Ando, K. 2014). Eight millimeter diameter of paper discs (Advantec, Toyo Roshi Kaisha Co., Ltd., Japan) were used for antibacterial assays. The isolated fungi were grown on GSY medium for 7 days at room temperature for sporulation. Then, agar block (4mm X 4mm) that contain fungal strain was inoculated into seed medium and incubated at 25°C for 3 days. Then, 20 mL of 3 days seed culture was transferred into the fermentation

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medium. Fermentation was undertaken at 25°C for 10 days. From 3 days to 10 days of fermentation period, fermented broth (20 µl) was put onto the paper discs daily and it was allowed to dry. Then, paper discs were placed on assay plates containing test organisms for 24-34 hr.

Antibacterial activity of isolated fungi against test organisms was carried out by the assay method. Assay plates were prepared by the following procedure.

1. 1% (1.5 x 10^8 / mL of spore suspension) of test organism was added to sterilized assay medium.
2. Sterilized assay medium that contain test organisms was poured into plates.
3. Assay plates were allowed to solidify.
4. After the solidification, the paper discs impregnated with samples (fermented broth) were placed onto it.
5. Assay plates were incubated for 24-36 hr at 28 to 30°C.

The clear zones (inhibitory zones) surrounding the test discs indicate that the presence of bioactive metabolites which inhibit the growth of test organisms.

Studies on Microbial Growth Kinetics of KF-05

The strain KF-05 was inoculated into the GYN medium and incubated for 132 hr. To recognize the growth of KF-05, the culture sample (5 mL) was taken in 12 hr interval. The sample (5 mL) was centrifuged at 2000 rpm for 30 minutes and Packed Cell Volume (PCV) was calculated. Then, Packed Cell Volume percent (PCV %) was recorded (Omura, 1985 and Crueger and Crueger, 1989).

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

Seed cultures of (54, 60, 66, 72, 78 and 84 hr) were utilized for the fermentation based on the results of microbial growth kinetics of KF-05. Fermentation was carried out 6 days and antibacterial activity was tested by paper disc diffusion assay.

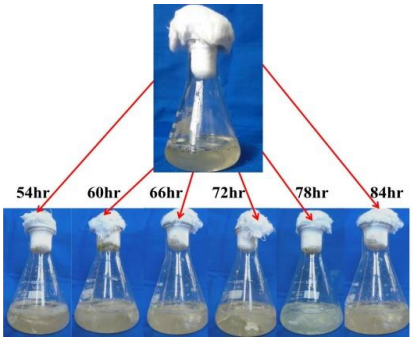


Figure 1. Effects of ages of inoculum of KF-05 on the fermentation

Effects of Sizes of Inoculum on the Fermentation

According to the results of the ages of inoculum of KF-05, (5%, 10%, 15%, 20%, 25% and 30%) of 72 hr seed cultures of KF-05 were utilized for the fermentation. Fermentation was carried out 6 days and antibacterial activity was tested by paper disc diffusion assay.

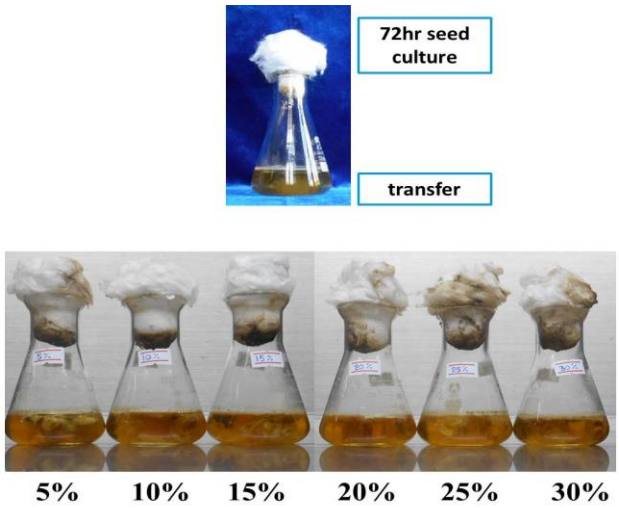


Figure 2. Effects of sizes of inoculum of KF-05 on the fermentation

Effect on the Carbon and Nitrogen Utilization of selected fungus (KF-05)

Optimal fermentations are very important for maximal productivity metabolites. In this study, carbon and nitrogen sources were employed in the fermentation for the production of antibacterial metabolites. Carbon sources such as potato powder, rice powder, dextrose, sucrose, soluble starch, glucose and glycerol were used. Nitrogen sources such as rice bran, KNO_3, meat extract, peptone, fish cake and yeast extract were also used.

Effects of Temperature on the Fermentation

In the investigation of the effects of temperature on the fermentation, fermentation media were kept in 22˚C, 25˚C, 28˚C, and 31˚C respectively. Bio-shaker was used to study in this case. The production of antibacterial metabolite was checked by paper disc diffusion assay method.

Effects of pH on the fermentation

Effects of different pH were used on the fermentation broth of KF-05 at pH 3,4,5 and 6 respectively. The different pH was adjusted by NaOH and HCL.

Results

Preliminary Study on Antimicrobial Activities

Preliminary study on antimicrobial activities of isolated fungi was carried out by paper disc diffusion assay. In this investigation, KF-03, KF-05, KF-09, KF-11 and KF-13 showed antimicrobial activities against test organisms. The fungus KF-05 showed the highest antimicrobial activity against *Pseudomonas fluorescens* (33.3 mm clear zone).

Table 1. Antibacterial activity of isolated fungi

Soil No.	Collected places	Isolated Fungi	Antibacterial activity
1	Watan	KF-01	No Activity
2	Minywa	KF-02	No Activity
		KF-03	**17.09 mm**
3	Myaynet	KF-04	No Activity
		KF-05	**33.3 mm** ***Pseudomonas fluorescens***
4	Nwamathin	KF-06	No Activity
5	Kanpyu	KF-07	No Activity
		KF-08	No Activity
6	Kyaukpyauk	KF-09	**20.00 mm**
		KF-10	No Activity
7	Magyisauk	KF-11	**20.01 mm**
		KF-12	No Activity
8	Zayit	KF-13	**18.05 mm**
		KF-14	No Activity

Studies on Microbial Growth Kinetics of KF-05

In the study of microbial growth kinetics, it was found that growth phase of the KF-05 was between 48 hr and 84 hr. According to Crueger and Crueger (1989), it was considered that ages of inoculum (54 hr, 60 hr, 66 hr, 72 hr, 78 hr and 84 hr) were used to optimize the fermentation.

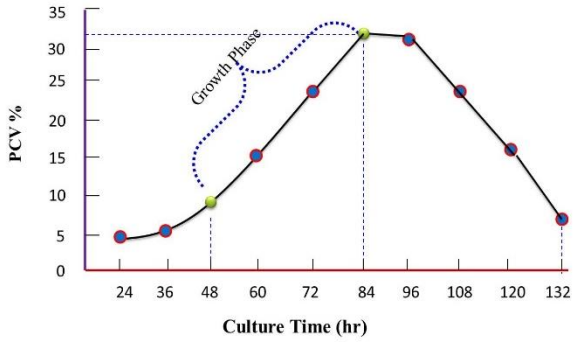
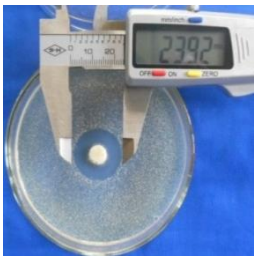


Figure 3. Microbial growth kinetics of KF-05

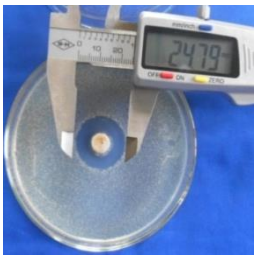
Effects of Ages of Inoculum on the Fermentation

In the study of ages of inoculum on the fermentation of KF-05, 54 hr seed culture showed 23.41 mm clear zone, 60 hr showed 23.92 mm clear zone, 66 hr showed 24.79 mm clear zone, 72 hr showed 25.07 mm clear zone, 78 hr showed 22.66 mm clear zone and 84 hr showed 17.50 mm clear zone respectively against *Pseudomonas fluorescens.* It was considered that 72 hr seed culture showed the best activity on *Pseudomonas fluorescens* than others seed culture. Therefore, 72 hr seed culture was selected for the fermentation.

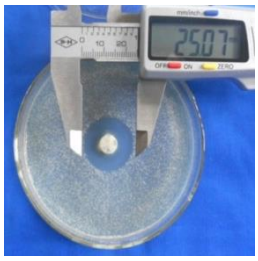
Activity of 54 hr age of culture



Activity of 60 hr age of culture

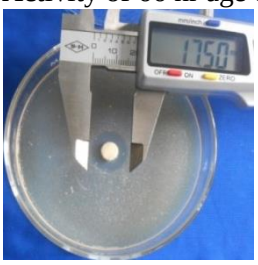


Activity of 66 hr age of culture



Activity of 72 hr age of culture

Activity of 78 hr age of culture

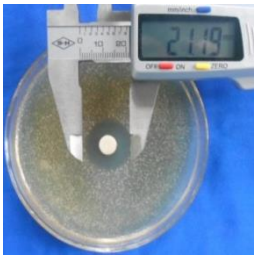


Activity of 84 hr age of culture

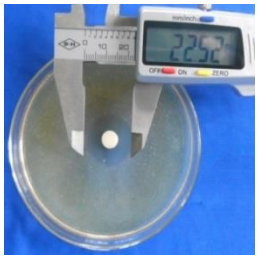
Figure 4. The effects of ages of inoculum on the fermentation

Effects of Sizes of Inoculum on the Fermentation

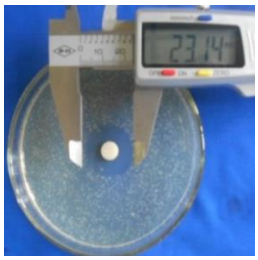
In the study of sizes of inoculum on the fermentation of KF-05, 5% size of culture showed 21.19 mm clear zone, 10% size of culture showed 22.52 mm clear zone, 15 % size of culture showed 23.14 mm clear zone, 20% size of culture showed 26.24 mm clear zone, 25% size of culture showed 25.97 mm clear zone and 30% size of culture showed 24.17 mm clear zone respectively against *Pseudomonas fluorescens.* It was considered that 20% size of inoculum gave the best activity on *Pseudomonas fluorescens*. Therefore 20% seed culture was selected for the fermentation.



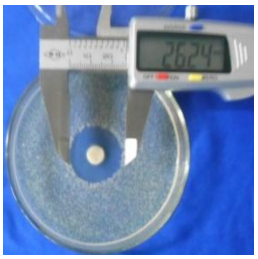
Activity of 5% size of inoculum



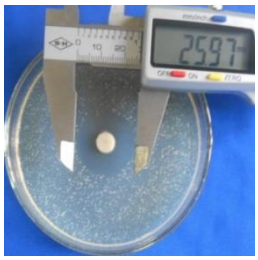
Activity of 10% size of inoculum



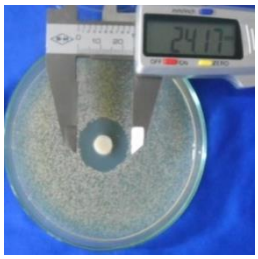
Activity of 15% size of inoculum



Activity of 20% size of inoculum



Activity of 25% size of inoculum



Activity of 30% size of inoculum

Figure 5. Effect of sizes of inoculum on the fermentation

Effects of Different Carbon Sources Utilization on the Fermentation

In the studies of different carbon sources utilization on the fermentation of KF-05, glucose, and glycerol gave the best activities on *Pseudomonas fluorescens.* Carbon sources utilized by potato powder showed 19.04 mm clear zone, rice powder showed 19.59 mm clear zone, sucrose showed 18.74 mm clear zone, soluble starch showed 12.56 mm clear zone, glucose showed 22.11 mm clear zone and glycerol showed 22.09 mm clear zone respectively.

Table 2. Effects of different carbon sources utilization on the fermentation

Carbon Source	Activity (Inhibitory Zone, mm)
Potato powder	19.04
Rice powder	19.59
Sucrose	18.74
soluble starch	12.56
Glucose	**22.11**
Glycerol	**22.09**

Effects of Different Nitrogen Sources Utilization on the Fermentation

In the studies of six different nitrogen sources utilization for the fermentation of KF-05, peptone and yeast extract gave the best activities on *Pseudomonas fluorescens*. Nitrogen sources utilized by rice bran showed 15.32 mm clear zone, KNO_3 showed 14.91 mm clear zone, meat extract showed 13.35 mm clear zone, peptone showed 28.02 mm clear zone, fish cake showed 16.6 2 mm clear zone and yeast extract showed 30.15 mm clear zone respectively against *Pseudomonas fluorescens.*

Table 3. Effects of different nitrogen sources utilization on the fermentation

Nitrogen Source	Activity (Inhibitory Zone, mm)
Rice bran	15.32
KNO_3	14.91
Meat extract	13.35
Peptone	**28.02**
Fish cake	16.62
Yeast extract	**30.15**

Media Optimization in the Fermentation Study

In the study of media optimization with six fermentation media, FM-1 showed the inhibitory zone 23.7 mm, FM-2 showed the 23.2 mm inhibitory zone, FM- 3 showed 25.6 mm inhibitory zone, FM- 4 showed 24.5 mm inhibitory zone, FM- 5 showed 30.4 mm inhibitory zone, FM- 6 showed 21.3 mm inhibitory zone respectively. It was determined that FM-5 showed the best activity on *Pseudomonas fluorescens*, FM-5 was selected for the production of antibacterial metabolite.

Table 4. Effect of media in fermentation study

Fermentation medium	Activity (Clear zone, mm)
FM-1	23.7
FM-2	23.2
FM-3	25.6
FM-4	24.5
FM-5	**30.4**
FM-6	21.3

Studies on the Effect of Temperature on the Fermentation

In the investigation of the effects of temperature on the fermentation, fermentation medium at 22˚C showed 18.29 mm clear zone, 20.19 mm clear zone at 24˚C, 30.91 mm clear zone at 26˚C and 24.38 mm clear zone at 28˚C respectively. Since the fermentation medium kept in 26ºC showed the best activity, it was considered that 26ºC is the best temperature for the fermentation.

Table 5. Effects of different temperature on the fermentation

Temperature (ºC)	**Clear zone (mm)**
22ºC	18.29
24ºC	20.19
26ºC	**30.91**
28ºC	24.38

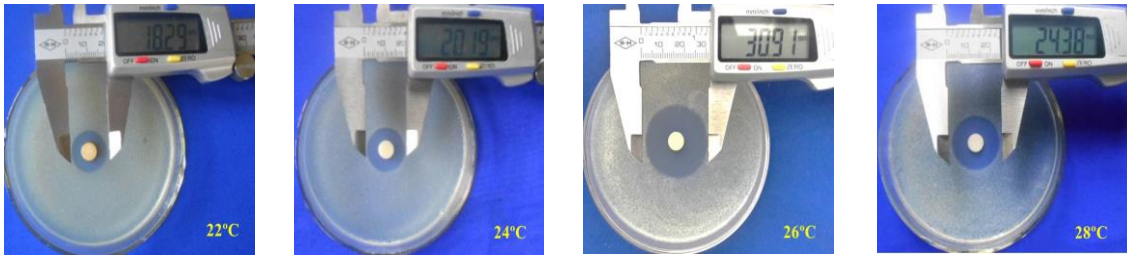


Figure 6. Effects of different temperature on the fermentation

The Effects of pH on the fermentation

In the study of the effects of pH for the fermentation of KF-05, it was found that the fermented broth with the adjusted pH exhibited the activities. However, pH 5.0 condition showed the best activity. Therefore, it was determined that pH 5.0 was suitable for the extraction of metabolite from the fermented broth.

Table 6. Effects of pH on the fermentation

pH	Clear Zone (mm)
3.0	17.46
4.0	18.60
5.0	**26.77**
6.0	16.25

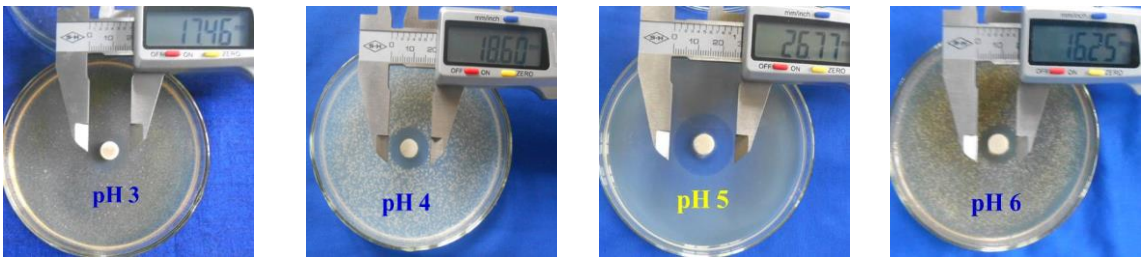


Figure 7. The effects of pH on the fermentation

Discussion and Conclusion

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 (Chin *et al*., 2012). Antibiotics have an important role in human health. Their necessity emerged from the spread of various diseases. As a result, scientists are trying to produce and discover more antibiotics (Rolain *et al*., 2000).

In this research, fourteen fungi were isolated from eight different soil samples collected at AyadawTownship Area, Monywa District, Sagaing Region. Soil samples were collected from six inches depth after removing the surface soil for the isolation of fungi. In general the majority of microbial population is found in the upper six to twelve inches of soil and the number decreases with depth (Cattle, et al., 2002). In the preliminary study of antimicrobial activities, five strains (KF-03, 05, 07, 11 and 13) showed activity against pathogenic microorganisms. Among them, strain KF-05 was selected for further studies because it showed more selective antibacterial activity against *Pseudomonas fluorescens*. This fungus KF-05 was isolated from Myaynet Village, Ayadaw Township, Monywa District. It was found that growth phase was between 48 hr and 84 hr. Based on the growth kinetics of fungus KF-05, 72 hrs ages and 20% size of inoculum were optimized for the production of metabolite. In the studies of different carbon and nitrogen sources utilization for the fermentation, carbon sources (glucose, glycerol,) and nitrogen sources (yeast extract, peptone) showed the best activities.

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*., 2005).

Based on the result (antibacterial activity) of carbon and nitrogen sources utilization on the fermentation, 6 different kinds of fermentation media were utilized in fermentation. According to the result (antibacterial activity), it was determined that FM-5 was the most suitable for the production of antibacterial metabolite.

In the studies of different temperature utilization on the fermentation, fermentation medium kept in 26ºC showed the best activities. Fermentation time is a very important factor, which affect the yield and quality of metabolites (Breidt *et al*., 1995).Time course of fermentation such as relationship between fermentation time and antibacterial activity, relationship between fermentation time and DCW%, and relationship between fermentation time and pH were investigated.

It was concluded that the present study revealed to observe the fermentation period of isolated fungus and to investigate the optimization of fermentation condition on KF-05 against *Pseudomonas fluorescens*.

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