Isolation and Identification of Endophytic Fungus, *Fusarium* and its Antibacterial Activity on *Bacillus pumilus*

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Abstract

The endophytic fungus (F-05) from *Mucuna pruriens* Roxb. (Khwele-ya) belonging to Fabaceae grown in Pathein showed the best antibacterial activity (28.84 mm of inhibitory zone) on *Bacillus pumilus* among 8 endophytic microorganisms including 1 bacterium and 7 fungi. This fungus was identified as *Fusarium* sp. according to the macroscopical and microscopical characters. For the kinetic growth of F-05, the highest packed cell volume was 0.56 g at 96 hrs. Four days of inoculum was the best age (31.87 mm of inhibitory zone) and 20% of inoculum was the best size (36.11 mm of inhibitory zone) on *Bacillus pumilus* for fermentation. In the substitution of medium sources, the growth condition of the potato powder was the excellent and the best antibacterial activity (36.02 mm of inhibitory zone) among 9 different carbon sources. Although the growth condition of KNO_3 was the moderate among 9 different nitrogen sources, the antibacterial activity was the best (36.48 mm of inhibitory zone) on *Bacillus pumilus*. In the fermentation conditions on the different pH levels (pH-3 to 9), pH-5 showed the highest antibacterial activity (36.53 mm of inhibitory zone) on *Bacillus pumilus.* On the effects of different temperatures (20 ℃ to 35 ℃), 30 ℃ was the best (36.10 mm of inhibitory zone) for the production of antibacterial metabolite in the fermentation conditions on *Bacillus pumilus*. Then, the shaking condition was suitable for fermentation as 37.18 mm of inhibitory zone on *Bacillus pumilus*. The optimum fermentation period exhibited the best antibacterial activity (37.49 mm of inhibitory zone) on *Bacillus pumilus* for 2 days of fermentation of F-05.

Keywords: endophytic fungus, antibacterial activity, antibacterial metabolite

Introduction

Endophytes are viewed as a source of novel bioactive natural products because many of them occupy literally millions of unique biological niches (higher plant) growing in a variety of unusual environments. Based on the knowledge of the chemistry and biology of endophytes, the isolation of natural products can replace the existing synthetic drugs that provide the resistance to pathogens and safe environments (Gond *et al.*, 2011).

A fermentation is influenced by numerous factors including composition of medium source, pH level, temperature, incubation period (Chisti, 1999).

Fusarium spp. have been reported as endophytes from several plants for biological activity. Fungi are a good source for antimicrobial agents. Many new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidant compounds are reported pharmaceutical, industrial and agricultural importance from fungal endophytes (Tayung *et al*., 2011).

Therefore, the selected endophytic fungus or *Fusarium* sp. from *Mucuna pruriens* Roxb. (Khwele-ya) belonging to Fabaceae was investigated the production of antibacterial metabolite and identification. The aim and objectives of this research paper were to identify the endophytic fungus according to its macroscopical and microscopical characters and to investigate the optimum fermentation condition for the production of antibacterial metabolite.

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Materials and Methods

Isolation of endophytic microorganisms and their antimicrobial activity

The leaves of *Mucuna pruriens* Roxb. (Khwele-ya) belonging to Fabaceae grown in Pathein were collected for the isolation of endophytic miroorganisms. The leaves were washed in running tap water for 10 min. Plant parts were cut into small pieces. The surface of cut plant pieces were sterilized by socked in 95% alcohol for 15 seconds. Next, the surface of cut plant pieces into smaller pieces. The samples were dried on sterilized tissue paper. Then, the samples were cut pieces were incubated on nutrient agar plate for 2 days to 1 week at room temperature by four media (Medium-I, Glucose 1g, Polypeptone 0.3 g, Agar 1.8 g and DW 100 mL, Medium-II, Glucose 1 g, PDA 0.3 g, Agar 1.8 g and DW 100 mL, Medium-III, Glucose 1 g, Yeast extract 0.3 g, agar 1.8 g and DW 100 mL, Medium-IV, Glucose 1 g, Malt extract 0.3 g, Agar 1.8 g and DW 100 mL) (Suto, 1999).

The isolated endophytic microorganisms were incubated in seed medium (Glucose 1 g, Polypeptone 0.3 g, $CaCO_3$ 0.01 g and DW 100 mL) for 3 days and transferred into the fermentation medium (Glycerol 1.0 mL, Yeast extract 0.3 g, $CaCO_3$ 0.01 g and DW 100 mL) and then carried out for 3-12 days. Then, the fermentation broth was used to check the antimicrobial activity on 8 different test organisms by the paper disc diffusion assay (glucose 1.0 g, peptone 0.3 g, agar 1.8 g, distilled water 100 mL at pH 7.0) method (Ando, *et al.*, 2004).

Table 1. Different test organisms used for antimicrobial activity

No	Test organisms	Sources	Infections
1	*Escherichia coli*	AHU 5436	Diarrhea
2	*Micrococcus luteus*	NITE 83297	Skin disease
3	*Candida albicans*	NITE 09542	Candidiasis
4	*Bacillus subtilis*	IFO 90571	DNA topoisomerase I
5	*Staphylococcus aureus*	AHU 8465	Food poisoning, Methicillin Resistance
6	*Pseudomonas fluorescens*	IFO 94307	Rice disease
7	*Malasseia furfur*	AVU 0255	Dandruff, Seborrhea dermatitis
8	*Bacillus pumilus*	IFO 12092	Wound and burn infection, Fever

Identification of the selected endophytic fungus (F-05)

The macroscopical and microscopical characters of the selected endophytic fungus (F-05) were examined under light microscope for 7 days old culture on the nutrient agar medium. According to Barnett and hunter (1972), the selected endophytic fungus (F-05) was keyed out by its macroscopical and microscopical characters.

The kinetic growth, age and sizes of F-05

For the investigation of the kinetic growth of F-05, the dry weight of packed cell volume was measured the time course from 12 hrs to 120 hrs.

The seed culture of F-05 was undertaken for fermentation from 1 day to 5 days at room temperature as the age of inoculum optimization.

In the sizes of inoculum optimization, 4 days of seed culture was utilized as 5 % to 40 % for fermentation. Each fermentation was carried out 7 days and tested the antibacterial activity by paper disc diffusion assay on *Bacillus pumilus*.

The fermentation medium composition as carbon and nitrogen sources of F-05

For the growth conditions and the antibacterial metabolite production of F-05, 9 different carbon (rice powder, soluble starch, glucose, lactose, fructose, tapioca powder, sugar, potato powder, glycerol) and nitrogen (malt extract, bran, KNO_3, peanut, yeast extract, peptone, fish extract, meat extract, NH_4Cl) media sources were utilized for 7 days as the growth conditions and tested the antibacterial activity for 5 days by paper disc diffusion assay on *Bacillus pumilus* at room temperature.

The different pH levels and temperatures for fermentation of F-05

For the antibacterial metabolite production of F-05, pH 3 to 9 and temperature 20 ºC, 25 ºC, 30 ºC, 35 ºC were utilized at 5 days of fermentation for the antibacterial activity by paper disc diffusion assay on *Bacillus pumilus*.

The shaking and static conditions for fermentation of F-05

The shaking and static conditions of F-05 were inoculated for 5 days of fermentation at 100 spm and tested the antibacterial activity by paper disc diffusion assay on *Bacillus pumilus* at room temperature.

Results

One bacterium and 7 fungi were isolated from *Mucuna pruriens* Roxb. (Khwele-ya) belonging to Fabaceae grown in Pathein Township.

Outstanding Characters of *Mucuna pruriens* Roxb. (Khwele-ya) (Fabaceae)

Annual climbing Shrub. Leaves trifoliolate, pubescent. Inflorescences axillary, racemes, pendulous. Calyx campanulate-asymmetrical, sericeous-pubescent, the wings purple. Corolla papilionaceous, standard ovate, wings oblique-oblong. Stamens 10, diadelphous, filaments filiform, anthers dithecous. Carpel 1, ovary oblong, unilocular, style filiform, capitate. Pods oblong, covered with orange hairs. Seeds ellipsoid, brown with black spots.

Figure 1. Habit and inflorescence of *Mucuna pruriens* Roxb. (Khwele-ya)

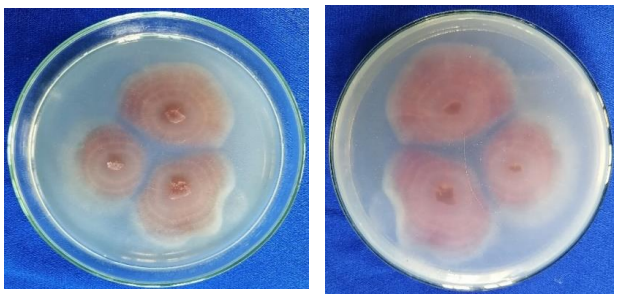
Table 2. No. of isolated endophytic microorganisms

Scientific Name	M-I	M-I	M-II	M-III	M-IV	Total
Mucuna pruriens Roxb.	B-01	F-01, F-02	F-03, F-04, F-05	F-06	F-07	8

Table 3. Antimicrobial activity of endophytic microorganisms

Used plants	Isolates	*Escherichia coli*	*Candida albicans*	*Bacillus subtilis*	*Staphylococcus aureus*	*Pseudomonas fluorescens*	*Malasseia furfur*	*Bacillus pumilis*	*Micrococcus luteus*
Mucuna pruriens Roxb.	B-01	+	-	-	-	+	-	-	-
	F-01	+	-	+	+	+	-	-	-
	F-02	+	-	+	+	+	-	-	-
	F-03	+	-	+	+	-	-	+	-
	F-04	+	-	+	+	-	-	+	-
	F-05	24.90 mm	-	15.50 mm	+	-	-	28.84 mm	-
	F-06	+	+	+	+	+	+	+	-
	F-07	+	-	+	+	+	+	+	-

According to the antimicrobial activity, 8 isolated endophytic microorganisms including one bacterium and 7 fungi showed the antimicrobial activity on the most of 8 different test organisms. Among them, F-05 had the best antibacterial activity (28.84 mm of inhibitory zone) on *Bacillus pumilus* for 5 days of fermentation.



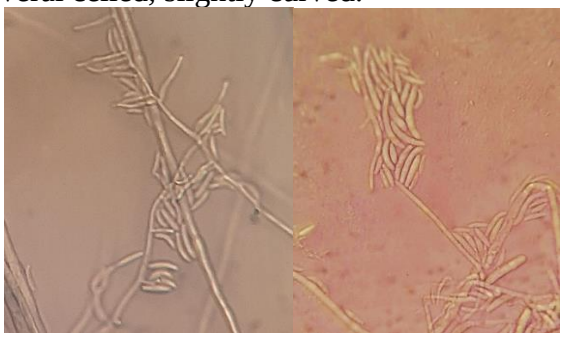
Front view Reverse view

Figure 2. Isolated endophytic fungus (7 days old culture of F-05) from *Mucuna pruriens* Roxb.

The macroscopical and microscopical characters of F-05

Mycelium white at margin and pink at center in the surface and reverse views, typically septate with frequent septa, conidia produced directly on mycelium on distinct conidiophores. Conidiophores typically compacted, slender and simple.

Conidia unbranched and hyaline, several celled curved at the pointed ends typically canoe-shaped and 3 to several celled, slightly curved.



Micrographs of *Fusarium* (F-05) (X400)

Figure 3. Microscopical characters of *Fusarium* (F-05)

Key for Identification (Barnett and Hunter, 1972)

1a. Conidia more or less coiled or spirally curved ----------2
1b. Conidia not coiled ----------12
12a. Conidiophores and conidia hyaline ----------13
12b. Conidiophores or conidia (or both) with dark pigment ------106
13a. Conidia 1-celled ----------14
13b. Conidia 2-celled ----------67
13c. Conidia 3 to several celled ----------75
75a. Conidia long-cylindrical ----------76
75b. Conidia slender or not cylindrical ----------77
77a. Conidia mostly phragmosporous, not branched----------78
77b. Conidia branched (staurosporous) ----------92
78a. Conidia not typically canoe-shaped ----------81
78b. Conidia canoe-shaped ---------- *Fusarium*

Classification of F-05

The isolated endophytic fungus (F-05) from *Mucuna pruriens* Roxb. (Khwele-ya) belonging to Fabaceae can be classified the genus as *Fusarium* belonging to family Tuberculariaceae, order Moniliales, class Deuteromycetes, division Deuteromycota and kingdom Fungi.

The effects of the kinetic growth, age and sizes of F-05

In the investigation of the kinetic growth of F-05, 0.56 g with dry weight was the best at 96 hrs.

Four days of inoculum was the best age (31.87 mm of inhibitory zone) on *Bacillus pumilus* for 5 days of fermentation at room temperature.

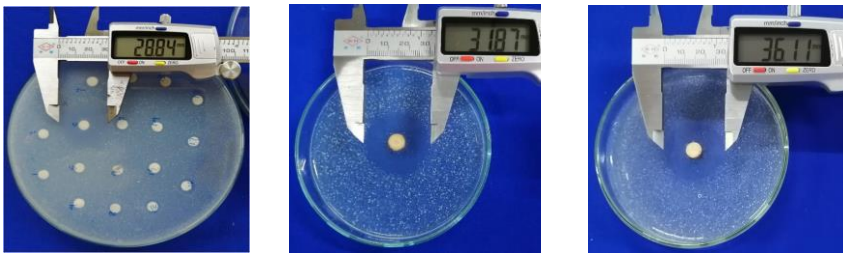
For the production of antifungal metabolite, 20% of seed culture (4 days age) was the best size (36.11 mm of inhibitory zone) among 5% to 40% for fermentation on *Bacillus pumilus* at room temperature.

The effects of carbon and nitrogen sources of F-05

The growth condition and the antibacterial activity of the potato powder were the excellent (36.02 mm of inhibitory zone) among 9 different carbon sources on

Bacillus pumilus at 5 days of fermentation for the production of antibacterial metabolite.

Although the growth condition of KNO_3 was moderate among 9 different nitrogen sources, the antibacterial activity was the best (36.48 mm of inhibitory zone) on *Bacillus pumilus* at 5 days of fermentation for the production of antibacterial metabolite.

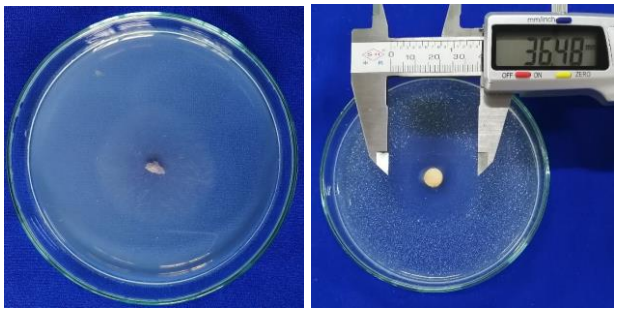


5 days of fermentation **4 days** **20%**

Figure 4. Antibacterial activity of F-05 on *Bacillus pumilus* by age and size

growth condition on potato antibacterial activity of **potato** on *Bacillus pumilus*

Figure 5. Growth condition and antibacterial activity of F-05 as carbon source



growth condition on KNO_3 antibacterial activity of KNO_3 on *Bacillus pumilus*

Figure 6. Growth condition and antibacterial activity of F-05 as nitrogen source

The effects of different pH levels and temperatures of F-05

In the different pH levels and temperatures for fermentation of F-05, pH 5.0 and 30ºC had the highest antifungal activity (36.53 mm and 36.10 mm of inhibitory zones) at 5 days on *Bacillus pumilus* for the production of antibacterial metabolite.

The effects of shaking and static conditions of F-05

The isolated endophytic fungus (F-05**)** exhibited the more antibacterial activity on the shaking condition (37.18 mm of inhibitory zone) than on the static condition (33.78 mm of inhibitory zone) on *Bacillus pumilus* at 5 days of fermentation for the antibacterial metabolite production.

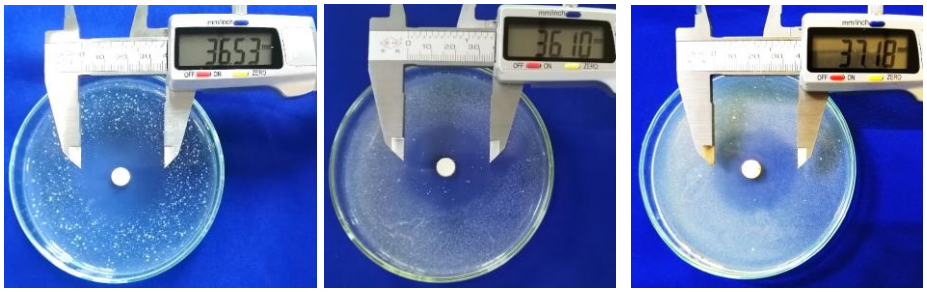


Figure 7. Antibacterial activity of F-05 on *Bacillus pumilus* by pH, temperature and shaking condition

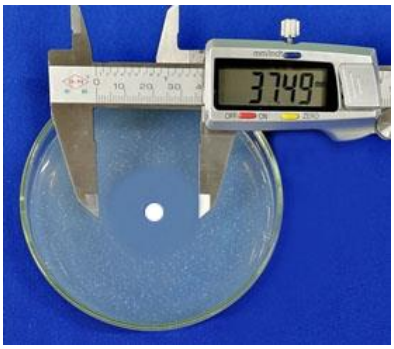


Figure 8. Antibacterial activity of F-05 on *Bacillus pumilus* by optimum fermentation condition

The optimum fermentation condition of F-05

The optimum fermentation period of F-05 showed the best antibacterial activity (37.49 mm of inhibitory zone) at 2 days of fermentation on *Bacillus pumilus* for the secondary metabolite production.

Discussion and Conclusion

An endophytic fungus isolated from bark of *Taxus baccata* was displayed the antimicrobial activity. The fungus was identified as *Fusarium solani* based on morphological and molecular characterization. The endophytic metabolite showed the antimicrobial activity against both bacterial and fungal pathogens. The optimum conditions for endophytic metabolite production were found to be 30±1°C, slightly acidic pH and incubation period of 10 days. The crude endophytic metabolite was separated into two fractions by column chromatography over silica gel (60–120 mesh) using a mixture of petroleum ether and ethyl acetate (20:1) as the eluent (Tayung *et al.*, 2011).

A total of 8 microorganisms from *Mucuna pruriens* Roxb. (Khwele-ya) were isolated. The selected endophytic fungus (F-05) was identified as *Fusarium* sp. by

macroscopical and microscopical characters. The endophytic fungus (F-05) had the best antibacterial activity at 2 days of the optimum fermentation condition with pH 5.0 at 30 ºC on *Bacillus pumilus* for the production of antibacterial metabolite.

An endophytic fungus isolated from root of tomato plant was displayed the antibacterial activity. The endophytic fungus was identified as *Fusarium solani* based on macroscopical and microscopical characterizations. The endophytic fungal extraction was carried out by ethyl acetate. The endophytic metabolite showed the antibacterial activity against *E. coli* (ATCC25922) and *S. aureus* (NCTC6571) by using a paper disc diffusion technique. The inhibition zones exhibited by fungal extract were 28 mm diameter on *E. coli* and 45 mm diameter on *S. aureus* (Hateet *et al.,* 2014).

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