OPTIMUM FERMENTATION OF ENDOPHYTIC FUNGUS, *COLLETOTRICHUM* SP. AGAINST *PSEUDOMONAS FLUORESCENS*

Lwin Lwin Soe[1], Khaing Su Wai[2], Mya Htet Htet Aung[3]

Abstract

This research paper was carried out to isolate endophytic fungi from *Jatropha gossypifolia* L. (Myanmar name, Taw-kanako). In this study, endophytic fungus (*Colletotrichum* sp.) was successfully isolated from the leaves of *Jatropha gossypifolia* L. plant samples by the surface sterilization method. In the investigation of antibacterial activity, the paper disc diffusion method was used for assay performance with *the Pseudomonas fluorescens* test organism. According to the macroscopical and microscopical characters and references, the endophytic fungus was identified as *Colletotrichum* sp. The result showed that the optimum antibacterial activity against *Pseudomonas fluorescens* was obtained after a 5-day incubation period at pH 6.0 and a temperature of 29°C in shaking conditions. The time course of fermentation for optimum antibacterial activity against *Pseudomonas fluorescens* was observed with the dry cell weight (22%) at pH 6.0 of a 96 h fermentation period.

Keywords: *Jatropha gossypifolia*, endophytic fungus, surface sterilization method, antibacterial activity, fermentation, *Colletotrichum* sp., *Pseudomonas fluorescens*

Introduction

The Jatropha gossypifolia plant, a medicinal plant, has an upright growth habit, able to grow up to 1-4 m tall. The leaves of the plant are used for intermittent fevers, carbuncles, carbuncles, eczema, itches, sores on the tongues of babies, swollen mammae, stomach ache and venereal disease (Fatokun *et al*., 2016). Medicinal plants are known to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products. Endophytes are the microorganisms that are present in living tissues of various plants (root, fruit, stem, seed, Leaf, etc.), establishing mutual relationships without causing any symptoms of diseases. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. The endophytic fungi play the important physical and ecological roles in their host life (Sardul *et al*., 2014).

Isolation techniques for isolating effective new or interesting microorganisms from natural substrata are expected to emerge from the field research (Moncalvo, 1997). Seed culture must be made in order to have enough inoculums for a large fermenter. If a production fermenter is started with too little inoculum, growth is delay and the product formation rate can be unsatisfactory. The inoculum concentration for the production determines the number of stages of seed culture that are required (Gaden, 1959). Fermentation procedures have to be developed for the cultivation of microorganisms under optimal conditions and for the production of desired metabolites or enzymes by the microorganisms. The proper cultivation and transfer of inoculums are essential for the production of both primary and secondary metabolites. The physiological condition of the inoculum is crucial to the condition of the lag phage (Yamanè and Shimizu 1984). Time course and inoculum age are crucial factors in fungal metabolic activity and growth. The optimal incubation period and inoculum age for enzyme biosynthesis vary depending on the enzyme and substrate used (Smith *et al*., 1996).

The aim of this study is to determine the optimum fermentation of endophytic fungi from *Jatropha gossypifolia* L. to produce the highest antibacterial activity.

[1] Department of Chemistry, University of Mandalay
[2] Department of Chemistry, University of Mandalay
[3] Ministry of Education, Nay Pyi Taw

Materials and Methods

Isolation of Endophytic Fungi from *Jatropha gossypifolia* Plant

The Jatropha gossypifolia plant was collected from Magway Township, Magway Region, for the isolation of endophytic fungi. The Surface Sterilization Method (NITE, 2004) was used to isolate endophytic fungi from plant samples. Fresh leaves are washed, cut into small pieces, and sterilized using 75% ethanol and 5% sodium hypochlorite before being rinsed in sterile water. The sterilized pieces are then dried and placed on LCA medium for incubation. After 3–5 days, emerging fungal colonies are subcultured on PGA medium for purification. The pure strains are maintained in test tubes, and their morphological characteristics are studied for identification.

Microbial Growth Kinetics of Endophytic Fungus (*Colletotrichum* sp.)

Measurement of dried cell weight

Measurement of fungus dried cell weight was carried out by the method of Phay, 1997. At 12 h intervals, a 5.0 mL sample was taken from each flask. Then these samples were poured into 10 mL of ice-cold 5% TCA and placed for 1h in the ice bath. The mixture was filtered through a filter paper and washed with 15 mL of cold 5 % TCA. The residues were dried in a hot-air oven and the dry cell weight was measured. (TCA= Trichloroacetic acid).

The dry weight is calculated as follows:

Dry weight of fungus = (weight of filter paper + fungus weight) – weight of filter paper

Effect of age of inoculum on the fermentation of *Colletotrichum* sp.

The endophytic fungus (*Colletotrichum* sp.) was inoculated into the seed culture medium and incubated for 3 days at room temperature then transfer to fermentation medium at 12 h intervals. Ages of culture with 48 h, 60 h, 72 h, 84 h, 96 h, and 108 h were utilized for the production of antibacterial metabolites. The antibacterial activity of each age of inoculum against *Pseudomonas fluorescens* was carried out by the paper disc diffusion assay method. (Omura, 1985)

Effect of size of inoculum on the fermentation of *Colletotrichum* sp.

In this research work, 5%, 10%, 15%, 20%, 25%, and 30% of inoculum 96 h-of age of seed culture were utilized for the maximum production of antibacterial activity. Fermentation was carried out for days and the antibacterial activity of each size of inoculum was tested by paper disc diffusion assay (Crueger and Crueger 1989).

Study of the morphologies and growth of the endophytic fungus *Colletotrichum* sp. in various carbon and nitrogen sources utilization

The morphologies and growth of the selected fungus, *Colletotrichum* sp., were studied in various carbon and nitrogen sources in basal medium. Glucose, sucrose, potato, maltose, glycerol, and soluble starch were utilized as the carbon sources.

Peptone, KNO_3, urea, soybean, NH_4Cl, and yeast extract were utilized as nitrogen sources.

Effect of different carbon sources on the antibacterial activity of *Colletotrichum* sp.

In this study, the antibacterial activity of *Colletotrichum* sp. in various carbon sources such as glucose, sucrose, potato, maltose, glycerol, and soluble starch as much as 1% was added for the basal medium.

Effect of different nitrogen sources on the antibacterial activity of *Colletotrichum* sp.

The antibacterial activity of *Colletotrichum* sp.in various nitrogen sources, such as peptone, KNO_3, urea, soybean, NH_4Cl, and yeast extract as much as 1% added to the basal medium, was studied.

Medium Optimization for Fermentation and Production of Antibacterial Metabolites

Fermentation was undertaken with suitable conditions of 96 h of age and 25 % size of inoculum with six different medium of *Colletotrichum* sp., FM-1 to FM-6 (Table 1). Fermentation was carried out for 7 days, and an antibacterial activity test of each medium of *Colletotrichum* sp. was carried out every 24 h.

Table 1. Six Different Fermentation Media

	FM-1	**FM-2**	**FM-3**	**FM-4**	**FM-5**	**FM-6**
glucose (g)	1.0	1.5	1.5	-	-	-
peptone(g)	-	-	-	2.0	1.0	2.0
yeast extract(g)	1.0	1.5	1.0	1.5	1.0	2.0
NZ amine type A(g)	0.3	0.3	0.3	-	-	-
K_2HPO_4(g)	0.001	0.001	0.001	0.001	0.001	0.001
$MgSO_4.7H_2O$ (g)	0.001	0.001	0.001	0.001	0.001	0.001
$CaCO_3$(g)	0.02	0.02	0.02	0.1	0.02	0.1
distilled water (mL)	100	100	100	100	100	100
pH	6	6	6	6	6	6

Effect of pH of the fermentation medium on antibacterial activity

The effect of pH on the selected FM-2 for antibacterial metabolite production was done by carrying out the FM-2 at six different pH values, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Fermentation was carried out for 7 days and then the antibacterial activity of each medium was tested every 24 h-and the highest activity was recorded.

Effect of temperature of the fermentation on the antibacterial activity

The effect of six different temperatures, 25℃, 27℃, 29℃, 31℃, 33℃, and 35℃, was studied for the production of metabolite from FM-2. Fermentation was carried out at pH 6 for 7 days, and antibacterial activity at different temperatures of fermentation was tested every 24 h.

Comparison of antibacterial activity of static culture and shaking culture of *Colletotrichum* sp.

Using the selected medium FM-2**,** the capacity of 250 mL conical flask containing 100 mL of the fermentation medium was incubated on the shaker (100 rpm) for 5 days. At the same time, another fermentation medium (FM-2) was incubated under static conditions without

shaking for 5 days. The antibacterial activities of shaking culture and static culture were compared by using paper disc diffusion assay method (Hassan *et al*., 2017).

Time course of fermentation for the production of metabolite

It is necessary to know the time course of fermentation to produce the metabolite. Therefore, fermentation was conducted with 96 h ages and 25% size of inoculum at 29 ºC for 7 days using fermentation medium FM-2.

Results and Discussion

The Microbial Growth Kinetic of Fungus *Colletotrichum* sp.

According to Figure 1, it was observed that the growth phase is between 36 and 96 h. Based on this result, the age of inoculum (36, 48, 60, 72, 84, and 96 h) was investigated for the fermentation.

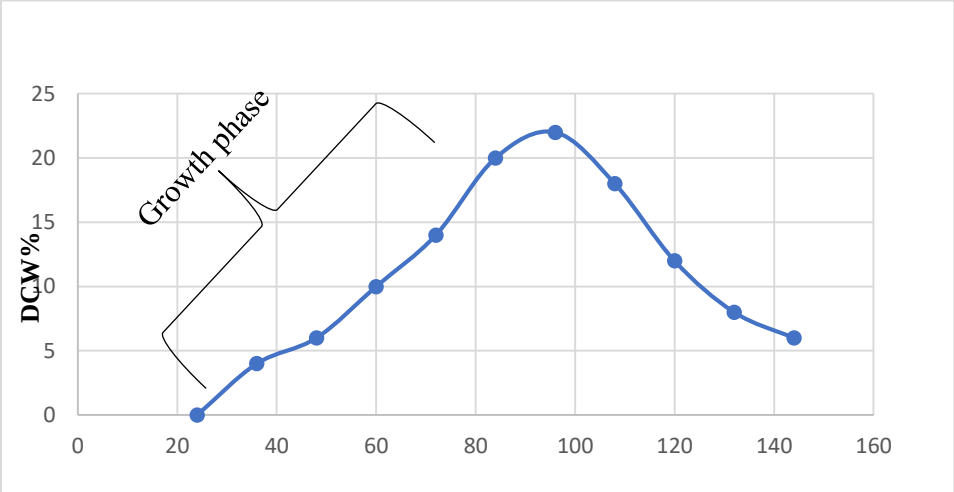
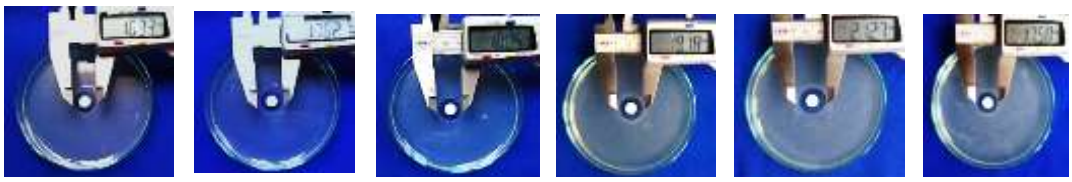


Figure 1. Microbial growth kinetic of fungus *Colletotrichum* sp.

Effect of age of inoculum on the fermentation

As shown in Figure 2 and Table 2, it was observed that the antibacterial activity of *Colletotrichum* sp. against *Pseudomonas fluorescens* reached the highest (21.17 mm) in 96 h age of inoculum.



48 h of age 60 h of age 72 h of age 84 h of age 96 h of age 108 h of ag

Figure 2. Effect of age of inoculum *Colletotrichum* sp. against *Pseudomonas fluorescens*

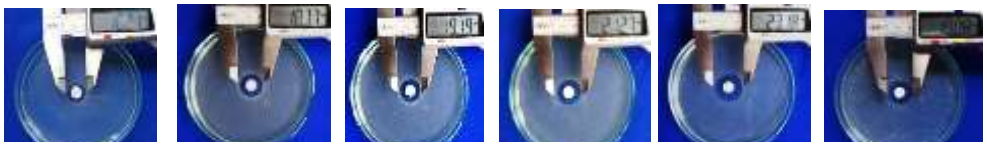
Table 2. Effect of Age of Inoculum on Fermentation of *Colletotrichum* sp. against *Pseudomonas fluorescens*

Sr. No	Age of inoculum (h)	Inhibition zone diameter (ID) (mm)
1	48	16.33
2	60	17.62
3	72	18.05
4	84	19.18
5	**96**	**21.17**
6	108	17.50

(Paper disc size= 8 mm)

Effect of size of inoculum on the fermentation

As shown in Figure 3 and Table 3, it was observed that 25% size showed the highest antibacterial activity and was the best for fermentation; high activity reached at a 5 - day period.



5 % 10 % 15 % 20 % 25 % 30 %

Figure 3. Effect of size of inoculum *Colletotrichum* sp. against *Pseudomonas fluorescens*

Table 3. Effect of Size of Inoculum on Fermentation of *Colletotrichum* sp. Against *Pseudomonas fluorescens*

Sr. No	Size of inoculum (%)	Inhibition zone diameter (ID) (mm)
1	5	16.21
2	10	18.77
3	15	19.19
4	20	21.27
5	**25**	**23.18**
6	30	19.08

(Paper disc size= 8 mm)

Morphologies and growth of *Colletotrichum* sp. in various carbon sources

The morphologies and growth of the selected fungus *Colletotrichum* sp. were studied by different carbon sources such as glucose, sucrose, maltose, glycerol, potato, and soluble starch. In this study, growth for carbon sources utilization, the excellent growth of *Colletotrichum* sp. fungus was found on glucose and sucrose as shown in Figure 4 and Table 4.

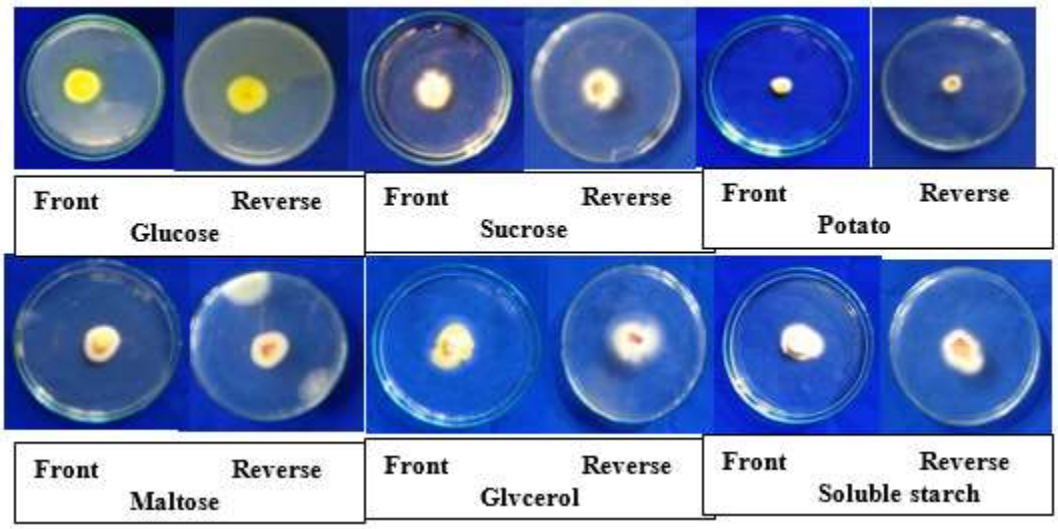


Figure 4. Growth of *Colletotrichum* sp. on various carbon sources

Table 4. Growth of *Colletotrichum* sp. on Various Carbon Sources

Sr. No	Carbon sources	Front colour	Reverse colour	Growth results
1	glucose	yellow	yellow	excellent
2	**sucrose**	**yellow**	**yellow**	**excellent**
3	potato	yellow	yellow	Poor
4	maltose	yellow and white	white	moderate
5	glycerol	pale yellow	white	moderate
6	soluble starch	pale yellow	white	moderate

1cm-2cm = poor growth, 3cm-4cm = moderate growth, > 4cm = excellent growth

Morphologies and growth of the *Colletotrichum* sp. in various nitrogen sources

Morphologies and growth of the endophytic fungus, *Colletotrichum* sp. were studied by different nitrogen sources such as yeast extract, urea, soybean, KNO_3, peptone, and NH_4Cl. In this study, growth for nitrogen sources utilization, the excellent growth of *Colletotrichum* sp. was found on peptone and yeast extract as shown in Figure 5 and Table 5.

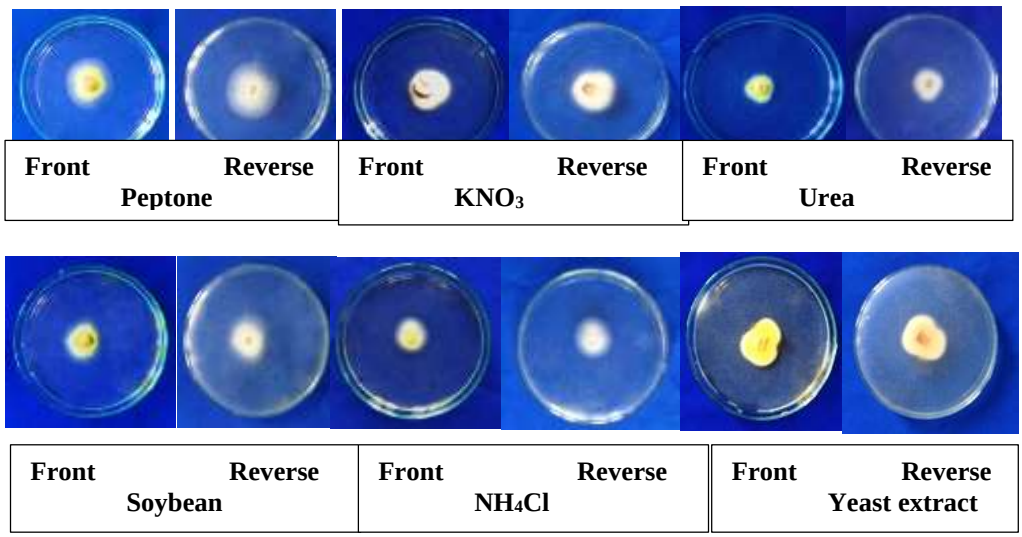


Figure 5. Growth of *Colletotrichum* sp. on various nitrogen sources

Table 5. Growth of *Colletotrichum* sp. on Various Nitrogen Sources

Sr. No	Nitrogen sources	Front colour	Reverse colour	Growth results
1	peptone	yellow and white	white	excellent
2	KNO_3	pale yellow	white	moderate
3	urea	green yellow and white	white	poor
4	soybean	green yellow and white	white	poor
5	NH_4Cl	yellow and white	white	poor
6	**yeast extract**	**yellow and white**	**white**	**excellent**

1cm-2cm = poor growth, 3cm-4cm = moderate growth, > 4cm = excellent growth

Effect of carbon sources on the antibacterial activity of *Colletotrichum* sp.

According to the results of the antibacterial activity of the *Colletotrichum* sp. using various carbon sources in the medium, using sucrose as a carbon source exhibited the highest activity (ID: 22.38 mm). Therefore, sucrose was selected for fermentation as shown in Figure 6 and Table 6.

Glucose **Sucrose** **Maltose** **Potato** **Glycerol** **Starch soluble**

Figure 6. Effect of carbon sources on the antibacterial activity against *Pseudomonas fluorescens* by paper disc diffusion assay

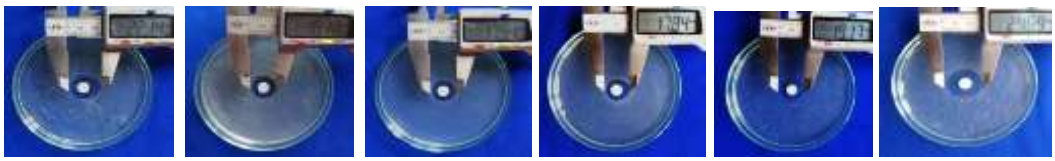
Table 6. Effect of Carbon Sources on the Antibacterial Activity of *Colletotrichum* sp.against *Pseudomonas fluorescens*

Sr. No	Carbon sources	Inhibition zone diameter (ID) (mm)
1	glucose	21.88
2	**sucrose**	**22.38**
3	maltose	20.97
4	potato	15.32
5	glycerol	20.76
6	starch soluble	20.12

Effect of nitrogen sources on the antibacterial activity of *Colletotrichum* sp.

According to the results of the antibacterial activity of the C*olletotrichum* sp. using various nitrogen sources in the medium, using yeast extract as a nitrogen source exhibited the

highest activity (ID: 24.69 mm). Therefore, yeast extract was selected for fermentation as shown in Figure 7 and Table 7.



Peptone **KNO_3** **Urea** **Soybean** **NH_4Cl** **Yeast extract**

Figure 7. Effect of nitrogen sources on the antibacterial activity against *Pseudomonas fluorescens* by paper disc diffusion assay

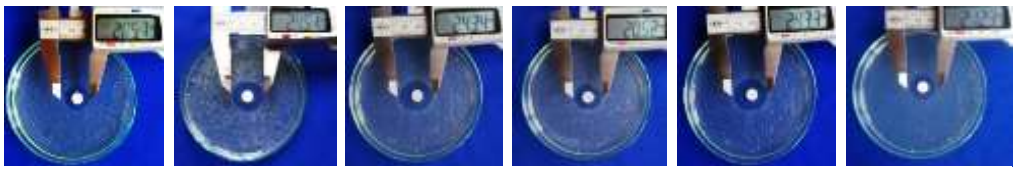
Table 7. Effect of Nitrogen Sources on the Antibacterial Activity of Colletotrichum sp. against *Pseudomonas fluorescens*

Sr. No	Nitrogen sources	Inhibition zone diameter(ID) (mm)
1	peptone	22.14
2	KNO_3	18.10
3	urea	17.51
4	soybean	17.84
5	NH_4Cl	19.17
6	**yeast extract**	**24.69**

(Paper disc size =8 mm)

Effect of various fermentation media

According to the results (Figure 8 and Table 8) of the antibacterial activity of the six fermentation media of *Colletotrichum* sp., the highest activity at a 5-day period in FM-2. Therefore, FM-2 was selected for the production of antibacterial metabolites.



FM-1 **FM-2** **FM-3** **FM-4** **FM-5** **FM-6**

Figure 8. Effect of various fermentation media of the antibacterial activity against *Pseudomonas fluorescens*

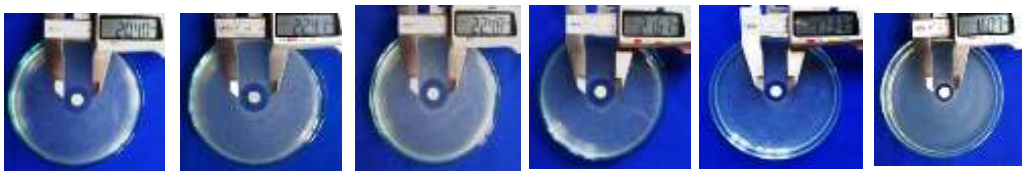
Table 8. Effect of Various Fermentation Media of *Colletotrichum* sp. against *Pseudomonas fluorescens* at 5 Day Periods

Sr. No	Fermentation medium	Inhibition zone diameter (ID) (mm)
1	FM-1	20.57
2	**FM-2**	**24.51**
3	FM-3	24.34
4	FM-4	20.52
5	FM-5	24.33
6	FM-6	21.23

(Paper disc size = 8mm)

Effect of Temperature of fermentation medium on the antibacterial activity

According to the results in Figure 9 and Table 9, 29 ºC was selected for fermentation and optimum activity was reached at a 5-day period.



25ºC **27**ºC **29**ºC **31**ºC **33**ºC **35**ºC

Figure 9. Effect of temperature of fermentation medium on the antibacterial activity against *Pseudomonas fluorescens* by paper disc diffusion assay

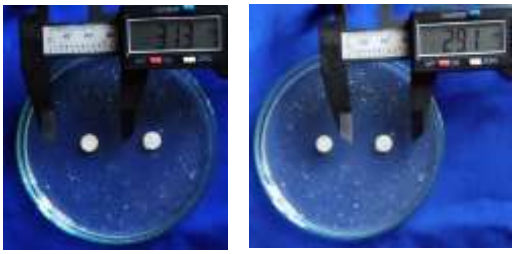
Table 9. Effect of Temperature of Fermentation Medium on the Antibacterial Activity against *Pseudomonas fluorescens*

Sr. No	Temperature (ºC)	Inhibition zone diameter(ID) (mm)
1	25	20.40
2	27	22.41
3	**29**	**22.48**
4	31	21.67
5	33	17.37
6	35	16.07

(Paper disc size= 8mm)

Effects of static and shaking culture of the fermentation conditions on antibacterial activity

In the comparison of the antibacterial activity of static and shaking fermentation conditions, the shaking fermentation showed higher activity (ID: 33.1 mm) than static fermentation (ID: 29.1 mm) against *Pseudomonas fluorescens* as shown in Figure 10 and Table 10.



shaking culture **static culture**

Figure 10. The antibacterial effect of static and shaking culture of *Colletotrichum* sp. against *Pseudomonas fluorescens*

Table 10. The Antibacterial Effects of Static and Shaking Culture of *Colletotrichum* sp.

Sr. No	*Colletotrichum* sp.	Inhibition zone diameter(ID) (mm)
1.	Shaking culture	33.1
2.	Static culture	29.1

Time course of fermentation for the production of antibacterial metabolites

In the study of the time course of fermentation, the fermentation period of 96 h, pH 6.0 and 22% DCW showed the highest activity (25.6 mm) than among others (Figure 11and Table 11)

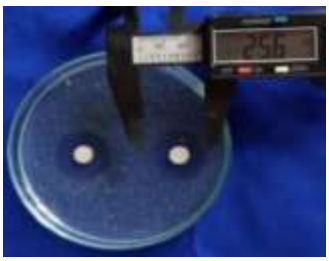


Figure 11. Antibacterial activity of *Colletotrichum* sp. against *Pseudomonas fluorescens* for 96 h fermentation

Table 11. Time Course of Fermentation for the Production for Antibacterial Metabolite against *Pseudomonas fluorescens*

Sr. No	Fermentation period (h)	pH	DCW %	Inhibition zone diameter (ID) (mm)
1.	36	6.5	4	16.4
2.	48	6.5	6	17.4
3.	60	6.4	10	18.0
4.	72	6.3	14	18.2
5.	84	6.2	20	24.5
6.	96	6.0	22	25.6

Sr. No	Fermentation period (h)	pH	DCW %	Inhibition zone diameter (ID) (mm)
7.	108	5.9	18	20.6
8.	120	5.7	12	19.0
9.	132	5.6	8	15.8
10.	144	5.6	6	11.5

(Paper disc size = 8 mm)

Conclusion

The endophytic fungus *Colletotrichum* sp., isolated from the leaves of *Jatropha gossypifolia* L., exhibited significant antibacterial activity against *Pseudomonas fluorescens*. Optimal fermentation conditions, including inoculum age, size, carbon and nitrogen sources, and fermentation media, were critical for metabolite production. The highest activity was observed with a 96-h-old seed culture (ID: 21.27 mm) and a 25% inoculum size (ID: 23.18 mm). Glucose and sucrose supported excellent growth among carbon sources, while peptone and yeast extract were the most effective nitrogen sources. FM-2 medium showed the highest antibacterial activity (ID: 24.51 mm). The optimal pH and temperature for fermentation were pH 6.0 (ID: 26.24 mm) and 29°C (ID: 22.48 mm), with maximum antibacterial activity and dry cell weight (DCW %) achieved at 96 h of fermentation. These findings highlight the importance of optimized fermentation conditions for metabolite extraction, isolation, and further characterization.

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References

Crueger W. and A. Crueger. (1989). *Biotechnology: A Textbook of Industrial Microbiology*. Sunderland, MA: 2nd Ed., Sinauer Associates, Inc.

Fatokun, O. T., O. Liberty, K.B. Esievo, S.E. Okhale, and O.F. Kunl. (2016). "Phytochemistry, Ethnomedicine and Pharmacology of Jatropha gossypiifolia L.: A Review". *Archives of Current Research International*, vol. 5(3). pp. 1-21.

Gaden, E. L., (1959). "Fermentation Process Kinetics". *J. Biochem. Microbio. Techn. Eng*, vol.1(4) pp.413-429.

Hassan, S. A. A., and S. E. A. Bakhiet, (2017). ''Optimization of Antibacterial Compounds Production by *Aspergillus fumigatus* Isolated from Sudanese Indigenous Soil**"**. *Int. Biol. Biomed. J.*, vol.3(4), pp.204-208.

Moncalvo, J. M. (1997). *Evaluation of Fungal Biological Diversity in the Tropics*. In Systematics Perspectives in Tropical Mycology. Janardhanan K.K., C. Rajendran, K.Natrajan, and D.L.HawksworthL (Eds.) Oxford: Science Publishers Inc., pp. 1-27.

NITE (National Institute of Technology and Evaluation). (2004). *Surface Sterilization and Baiting Method*. Tokyo.

Phay, N. (1997). *Highly Selective Antibiotics.* Doctoral Thesis, Faculty of Agriculture, Hokkaido University, Japan.

Sandhu, S.S., S. Kumar, and R.P. Aharwal. (2014). "Isolation and Identification of Endophytic Fungi from *Ricinus communis* Linn. and Their Antibacterial Activity**"**. *International Journal of Research in Pharmacy and Chemistry*, vol. 4 (3), pp. 611-618.

Smith, J.P., A. Rinzema, J. Tramper, E.E. Schlosser, and W. Knol. (1996). "Accurate Determination of Process Variables in A Solid-State Fermentation System"**.** *Process Biochem*., vol. 31 (7), pp. 669-678.

Yamanè, T. and S. Shimizu. (1984). *Fed-batch Techniques in Microbial Processes*. In Bioprocess Parameter

Control. Fiechter, A.(Editor). Berlin: 1st Ed., Springer-Verlag, pp. 147–194.