FERMENTATION STUDIES OF BIOACTIVE FUNGAL STRAIN MM4 ISOLATED FROM THE LEAVES OF *PSIDIUM GUAJAVA* L.

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Abstract

In this study, bioactive strain MM4 isolated from the leaves of *Psidium guajava*L. was utilized to investigate optimal fermentation conditions such as carbon and nitrogen sources, various culture media, age of inoculum, size of inoculum and pH utilization. In carbon sources, sucrose, starch and glycerol were the best whereas yeast, meat, malt and soybean were good nitrogen sources. In antimicrobial activity, sucrose, glucose, starch and mannitol in carbon sources showed high activity against *Candida albicans, Escherichia coli* and *Malassezia furfur* while in nitrogen sources, yeast extract, meat extract, and soybean indicated very high activity against *Candida albicans*and *Escherichia coli.* In the investigation of morphological characters on eleven media, fermentation media 9, 7 and 10 were good to produce antimicrobial metabolites from strain MM4. Antimicrobial activity of eleven media, fermentation medium 9 showed highest activity against test organisms. Two days old age of inoculum showed highest activity against test organisms. According to the results, size of inoculum (1.5%) and pH 4 with two days old age of inoculum were the best for extraction of the bioactive compounds from fermented broth.

Keywords*: Bioactive strain,optimal fermentation, bioactive compounds*

Introduction

Endophytes are microorganisms that include bacteria and fungi living within plant tissues without causing any immediate negative effects (Kumar and Sagar, 2007). Fungi are considered as a good natural source for a production of bioactive secondary metabolites that contain different bioactive agents including antibiotics, antitumors, and antioxidants (Elaasser *et al*., 2011). Microbial fermentation is the basis for the production of a wide range of pharmaceutical products, targeting practically any medical indication. Optimization of the fermentation conditions of the endophytic fungus may

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lead to the development of an economically process for the production of bioactive compounds (Puri *et al*., 2005). The large numbers of known bioactive compounds of microbial origin are currently produced by fermentation (Parkinsan, 1994). Parton and Willis (1989) studied strain preservation, inoculum preparation and development for fermentation of active strains. In this research work, optimal fermentation conditions and various media of endophytic fungal strain MM4 were carried out for extraction of the bioactive compounds.

Materials and Methods

Collection of plant samples

The plant samples were collected from Nyaung-Hna-Pin area, Hmawbi Township and studied their outstanding characters by Backer and Bakhuizen, 1968 and Hooker, 1885.

Fermentation Studies

Utilization of carbon and nitrogen sources of strain (MM 4) (Monaghan *et al*., 1999)

In this research, morphological characters of strain MM 4 were studied by using various carbon and nitrogen sources. Carbon sources are sucrose, glucose, starch, mannitol and glycerol whereas nitrogen sources are yeast extract, meat extract, malt extract, oat meal and soy bean. Basal media for finding out suitable carbon sources contained yeast extract 0.3%, K_2HPO_4 0.01%, $MgSO_4$ 0.01%, $CaCO_3$ 0.01% and for nitrogen sources the basal medium consisted glycerol 1.0%, K_2HPO_4 0.01%, $MgSO_4$ 0.01%, $CaCO_3$ 0.01%.

Antimicrobial activity of strain MM 4 by using various carbon and nitrogen sources (Monaghan *et al*., 1999)

Fungal strain MM 4 grown on slant culture was transferred into 50ml flasks containing 25 mL of various carbon and nitrogen sources and incubated for ten days. The fermented broth was used to check antimicrobial activity by paper disc diffusion assay.

Morphological characters of strain MM 4 on eleven different media (Monaghan *et al*., 1999)

In this study, various media were employed for media optimization. A piece from fungal plate culture of strain MM 4 was inoculated on each of various media plates and incubated for 3-10 days.

Antimicrobial activity of strain MM 4 on eleven different media (Monaghan *et al*., 1999)

Fungal strain MM4 grown on slant culture was transferred into 50 mL flasks containing 25 mL of eleven different media. The fermented broth was used to check antimicrobial activity by paper disc diffusion assay.

Eleven different media

Eleven media were Polypeptone, Yeast medium (FM-1)/ Meat, Polypeptone, NaCL medium (FM-2)/Yeast, Malt, Glucose medium (FM-3)/ Glycerol, K_2HPO_4, $MgSO_4$, NaCL medium (FM-4)/ Oat meal medium (FM-5)/ Glycerol, K_2HPO_4 medium (FM-6)/ Soybean, Mannitol medium (FM-7) / K_2HPO_4, $MgSO_4$, NaCL medium(FM-8) Sucrose, Yeast extract medium (FM-9)/ Malt, Meat extract medium (FM-10) and Sucrose, Malt extract, Soluble strach medium (FM-11) as shown in Figure 1.

Figure 1. Eleven different media for media optimization

Age of inoculum of strain MM 4 (Strobel and Sullivan, 1999)

First fermentation (two days old) and second fermentation (three days old) seed cultures were transferred into 50 ml fermentation flasks containing 25 mL of sucrose/yeast extract medium. They were incubated for ten days. Then, these fermented broths were checked for their inhibitory activity by paper disc diffusion assay.

Size of inoculum of strain MM 4 (Monaghan *et al*., 1999)

The proper cultivation and transfer (size of inoculum) are essential for the production of bioactive metabolites. A piece from fungal plate culture of strain MM 4 was inoculated into 300 mL conical flask containing 100 mL of sucrose/yeast extract seed medium. These flasks were incubated at room temperature for 2 days. After two days, the seed cultures (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%) were transferred into seven conical flasks (300 mL) containing 100 ml of fermentation medium. The fermentation was carried out for 10 days.

pH utilization of strain MM 4 (Monaghan *et al*., 1999)

For the seed culture, a piece from fungal plate culture of strain MM 4 was inoculated into 300 mL of conical flask containing 100 mL of sucrose/yeast extract medium and then flasks were incubated at room temperature for two days. Seven 300 mL conical flasks containing 100 mL fermentation medium were adjusted at pH 4, 5, 6, 7, 8, 9 10 and autoclaved. After two days, the seed culture (1.5%) was transferred to each fermentation flask with pH 4 to 10 and fermentation was carried out for 3 days. After three days, seven fermentation flasks were checked their antimicrobial activity.

Table1. Test organisms and diseases

Test organisms	Code	Diseases
Bacillus subtilis	JAP- 0225025	It can cause dysentery, but at the first sign of diarrhea
Candida albicans	IFO- 1060	Skin infection, vaginal candidasis, alimentary tract infection urogenital infection.
Escherichia coli	ATCC- 25922	Cholera, diarrhea and vomiting, urinary tract infections
Salmonella typhi	ST-3/JEP-69	Typhoid, strong fever.
Staphylococcus aureus	ATCC- 12877	Skin disease, food poison, wound infection, burns, abscesses, blood stream infection, staphylococcal pneumonia
Malassezia furfur	AUW- 0255	Dandruff

Results

Figure 2. Habit of *Psidium guajava* L.

Scientific Name - *Psidium guajara* L.

English Name - Guava

Myanmar Name - Malaka

Family - Myrtaceae

Outstanding characters

Mostly shrubs to small tree; Leaves opposite and distichous, simple, petiolate, exstipulate;Inflorescence axillary, cymose; Flowers ebracteate, pedicellate, bisexual, actinomorphic, epigynous; Sepals 5, valvate, persistent; Petals 5, imbricate; Stamens numerous, polyandrous, filaments long and filiform, anthers dithecous, dorsifixed, introrse; Pistil 1, pentacrpellary, syncarpous, pentalocules, placentation axile, style long and simple, stigma capitates, ovary inferior; Fruits fleshy berry; Seed exalbuminous, embryo curved as shown in Figure 2.

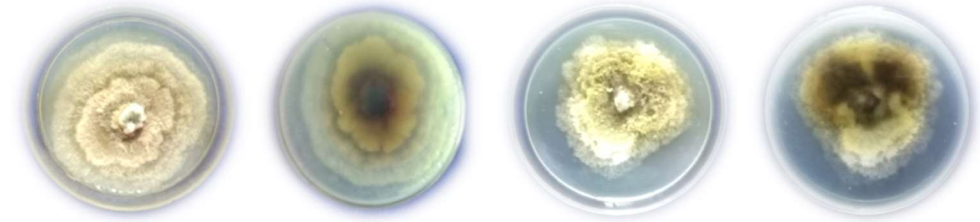
Utilization of carbon and nitrogen sources of strain MM4

Carbon utilization

Among carbon sources, sucrose, starch and glycerol were the best carbon sources whereas glucose and mannitol were also suitable for fermentation as shown in Table 2 and Figures 3 & 4.

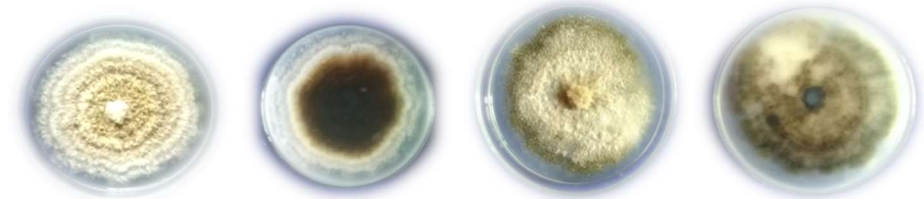
Table 2. Morphological characters of strain MM4 on various carbon sources

No.	Carbon source	Growth	Surface colour	Reverse colour
1	Sucrose	Good	White	Pale yellow
2	Glucose	Moderate	Cream	Light brown
3	Starch	Good	White	Dark brown
4	Mannitol	Moderate	Light gray	White
5	Glycerol	Good	White	Red



Surface view Sucrose Reverse view Surface view Glucose Reverse view

Figure 3. Strain MM4 grown on the plates of various carbon sources



Surface view Starch Reverse view Surface view Mannitol Reverse view

Surface view Starch Reverse view

Figure 4. Strain MM4 grown on the plates of various carbon

Nitrogen utilization

Among nitrogen sources, yeast extract, meat extract, malt extract and soy bean were the best nitrogen sources while oat meal was poor for fermentation as shown in Table 3 and Figures 5 & 6.

Table 3. Morphological characters of strain MM4 on various nitrogen sources

No.	Nitrogen source	Growth	Surface colour	Reverse colour
1	Yeast extract	Good	White	White
2	Meat extract	Good	Cream	Cream
3	Malt extract	Good	Pale yellow	Light yellow
4	Oat meal	Poor	Dark cream	Dark cream
5	Soybean	Good	Cream	Dark

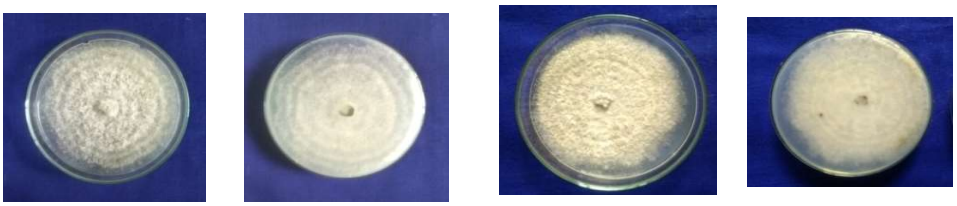


Figure 5. Strain MM4 grown on the plates of various nitrogen sources

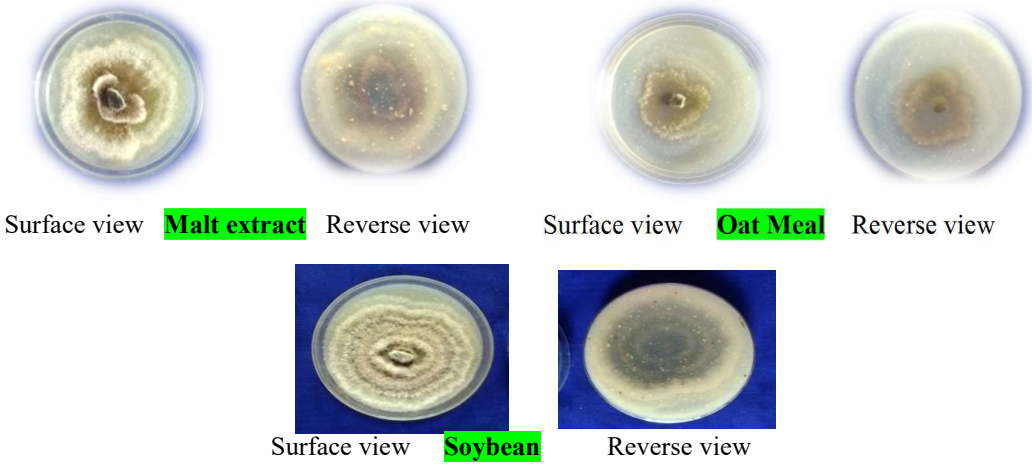


Figure 6. Strain MM4 grown on the plates of various nitrogen sources

Antimicrobial activity of strain MM 4 by using various carbon sources

Antimicrobial activity of strain MM 4 by using various carbon sources. Fermented broth of strain MM4 in sucrose medium showed high activity against *Candida albicans* seventh day (15cm).Strain MM 4 in glucose medium exhibited high activity against *Escherichia coli* at fourth day and *Candida albicans* seventh day (15cm).Strain MM4 in starch indicated high activity against *Escherichia coli* at fourth day(15cm).Strain MM 4 in mannitol showed high activity against *Escherichia coli, Malassezia furfur* at fourth day (15cm) as shown in Figure 7.

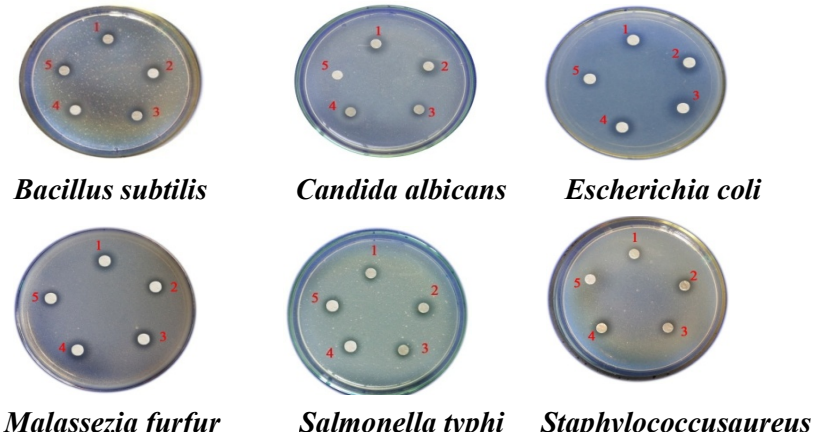
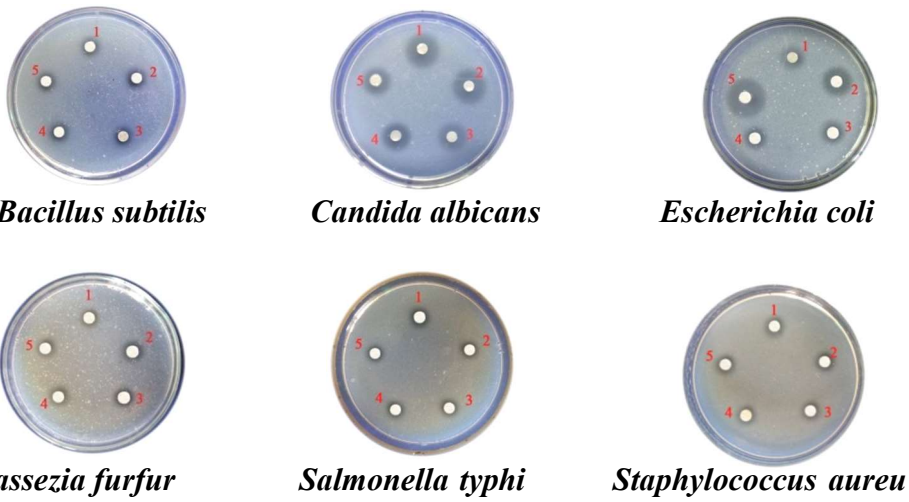


Figure 7. Inhibitory zones of fermented broths on carbon sources

Antimicrobial activity of strain MM 4 by using various nitrogen sources

Fermented broth of strain MM 4 in yeast extract showed very high activity against *Candida albicans* fourth day and *Escherichia coli* at eighth day (20cm). Strain MM 4 in meat extract indicated very high activity against *Candida albicans* at fourth day (20cm). Strain MM 4 in malt extract and oat meal exhibited high activity against *Candida albicans* at fourth day (18cm).Strain MM 4 in soybean indicated very high activity against *Escherichia coli* at fourth day (20cm) as shown in Figure 8.



1.Yeast extract 2. Meat extract 3. Malt extract 4.Oat meal 5. Soybean

Figure 8. Inhibitory zones of fermented broths on nitrogen source

Morphological characters of strain MM 4 on eleven different media

In the investigation of morphological characters of strain MM 4 on eleven media, fermentation medium 9, 7 and 10 media were good whereas fermentation medium 1, 2, 3 and 11 were moderate for fermentation. However, fermentation medium 6, 4, 8 and 5 were not good for fermentation to produce antimicrobial metabolites from strain MM 4 as shown in Table 4and Figures 9-11.

Table 4. Cultural characters of strain MM 4 on eleven different media

Medium	Growth	Surface view	Reverse view
1	Moderate	Light brown	Dark brown
2	Moderate	Brown	Dark brown
3	Moderate	White	Cream
4	Poor	Gray	Gray
5	Poor	Dark gray	Dark gray
6	Poor	White	White
7	Good	Cream	Yellowish red

Medium	Growth	Surface view	Reverse view
8	Poor	Light gray	Light gray
9	Good	Dark cream	Red
10	Good	Cream	Light red
11	Moderate	Light brown	Light brown

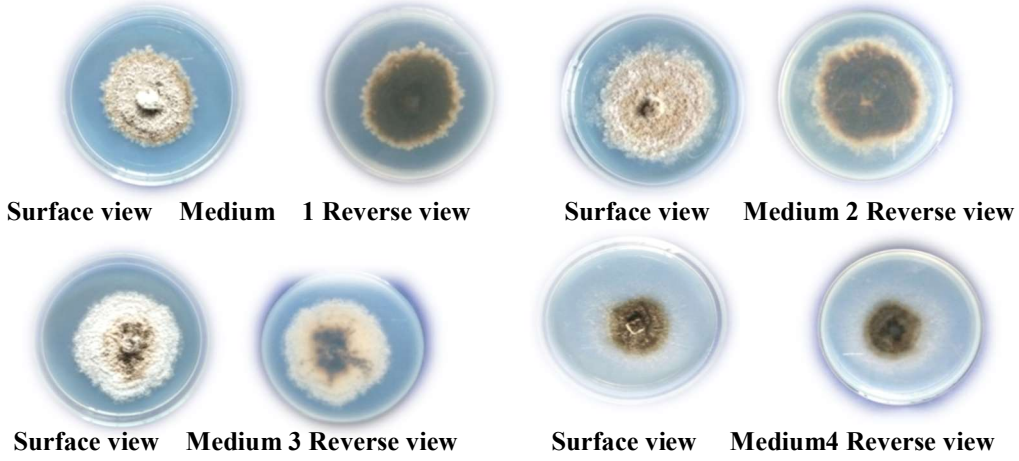


Figure 9. Strain MM 4 grown on the plates of media 1-4

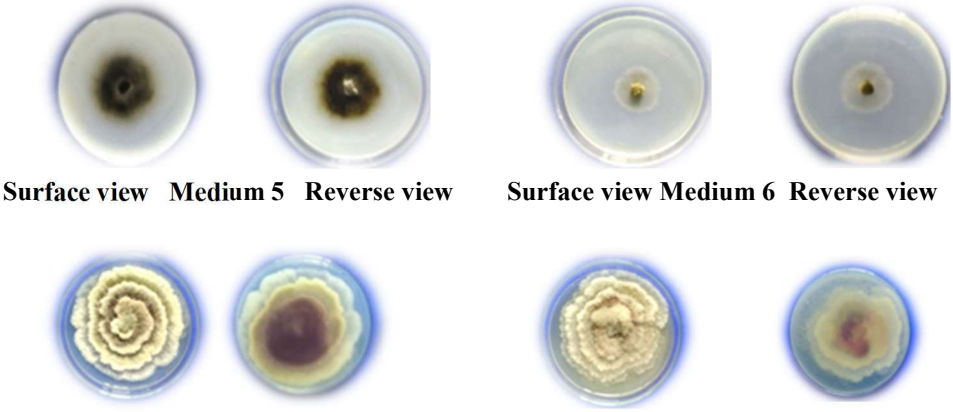


Figure 10. Strain MM 4 grown on the plates of media 5-8

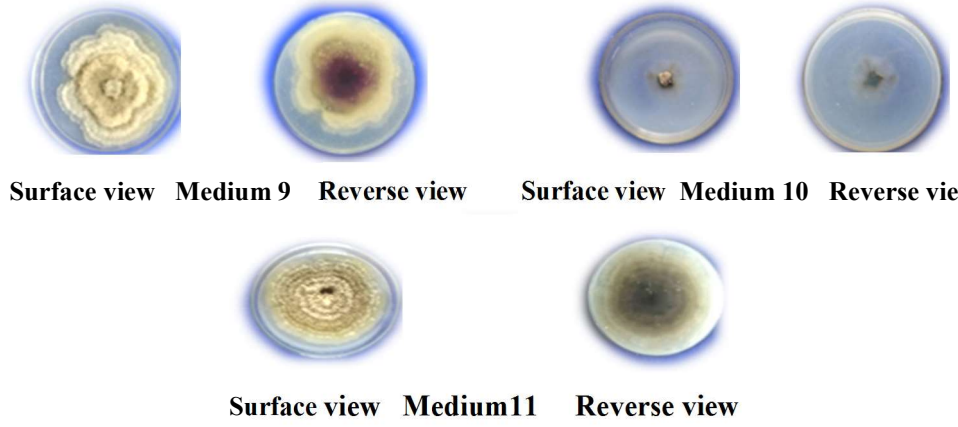


Figure 11. Strain MM4 grown on the plates of media 9-11

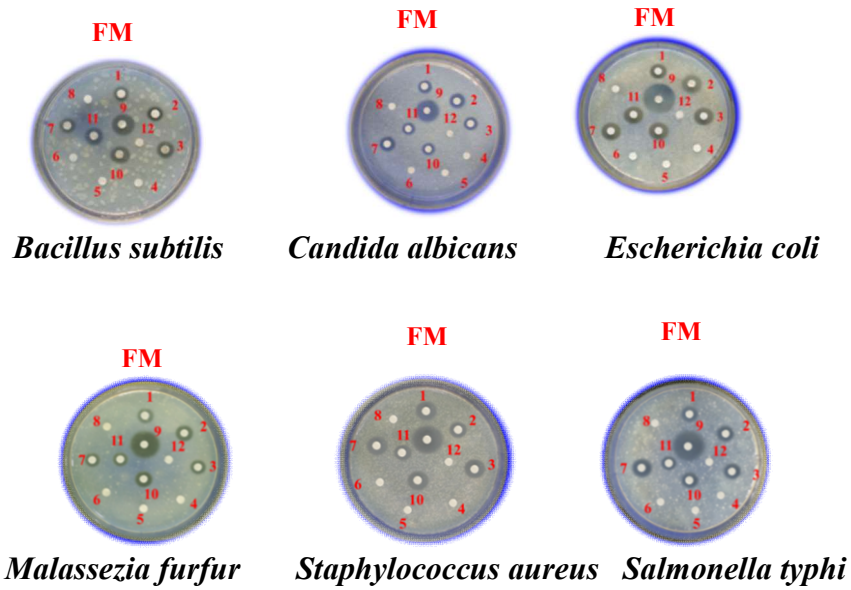
Antimicrobial activity of endophytic fungal strain (MM 4) by using eleven media

In this study, fermented broth of strain MM 4 in sucrose/ yeast (SY) fermentation medium (9) showed very high activity against *Bacillus subtilis, Candida albicans, Escherichia coli, Malassezia furfur, Salmonella typhi*, *Staphylococcus aureus.* Strain MM4 in fermentation medium 4, 5, 6 and 8 media indicated no activity and other media exhibited high activity against six test organisms. Therefore, among eleven media, sucrose/yeast (FM-9) medium was suitable for large fermentation as shown in Table 5 and Figure 12.

Table 5. Inhibitory zones (mm) of strain MM 4 on eleven different media

Media / T.O	FM 1	FM 2	FM 3	FM 4	FM 5	FM 6	FM 7	FM 8	FM 9	FM 10	FM 11
Bacillus subtilis	12	1	15	-	-	-	12	-	18	15	15
Candida albicans	13	15	12	-	-	-	15	-	20	12	12
Escherichia coli	13	15	15	-	-	-	15	-	22	15	15
Malassezia furfur	12	15	12	-	-	-	12	-	25	15	12
Salmonella typhi	15	12	15	-	-	-	15	-	20	15	12
Staphylococcus aureus	12	13	12	-	-	-	15	-	22	12	10

10 -12 mm = weak activity, 13 - 18 mm = high activity, >18 mm = very high activity (disc size = 6 mm), T.O = Test Organisms



FM – 1-11= Eleven fermentation medium 12- Control

Figure 12. Inhibitory zones of fermented broths on eleven different media

Fermentation studies of strain MM4

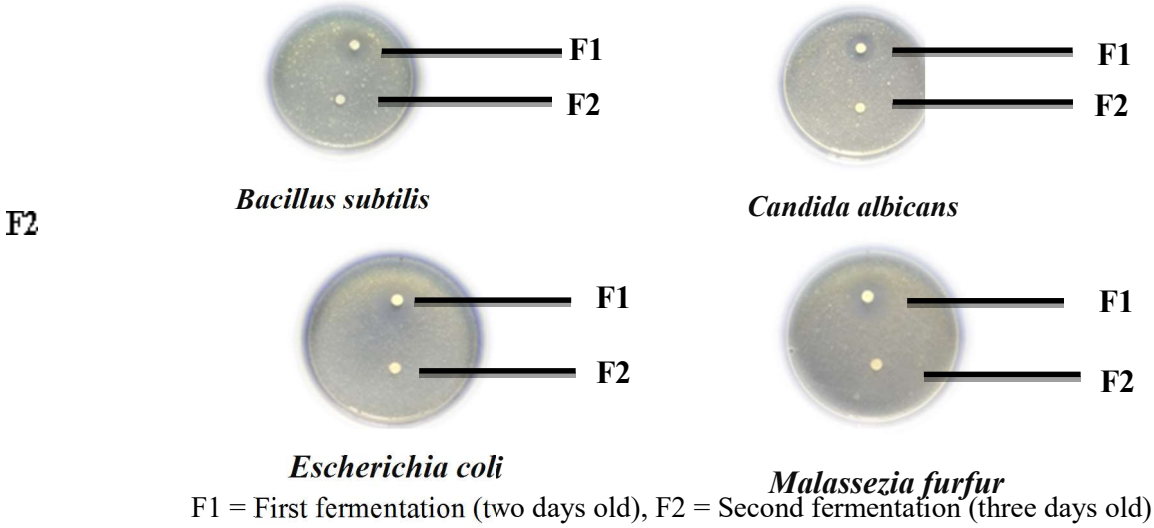
Age of inoculum

In this study, first fermentation (two days old) showed very high activity at seventh day of fermentation on *Baillus subtilis, Candida albicans, Escherichia coli* and *Malassezia furfur* and second fermentation (three days old) indicated weak activity. Therefore, the study of first and second fermentation, first fermentation showed the higher activity than second fermentation on *Bacillus subtilis, Candida albicans, Escherichia coli* and *Malassezia furfur* as shown in Table 6 and Figure 13.

Table.6. Inhibitory zones (mm) of strain MM4 for first fermentation

Days / T.O	1	2	3	4	5	6	7	8	9	10
Bacillus subtilis	-	16	14	22	15	20	**20**	12	18	18
Candida albicans	-	12	10	15	17	15	**18**	15	12	15
Escherichia coli	-	10	18	13	16	13	**20**	15	18	18
Malassezia furfur	-	14	16	15	15	13	**20**	18	13	18

10 -12 mm = weak activity, 13 - 18 mm = high activity, >18 mm = very high activity (disc size = 6 mm), T. O = Test Organisms



F1 = First fermentation (two days old), F2 = Second fermentation (three days old)

Figure 13. Inhibitory zones of age of inoculum

Size of inoculum

In the study of size of inoculum, among the seed culture (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%) **1.5** % of seed culture within five-seven days fermentation was suitable for the production of the bioactive compounds. Strain MM4 inhibited bioactivity against six test organisms but they indicated very high antimicrobial activity on *Bacillus subtilis, Salmonella typhi* and *Staphylococcus aureus* at seventh days (25 cm, 30 cm, 30 cm), *Candida albicans, Escherichia coli, Malassezia furfur* at fifth day (28 cm, 30cm, 30cm) as shown in Figure 14.

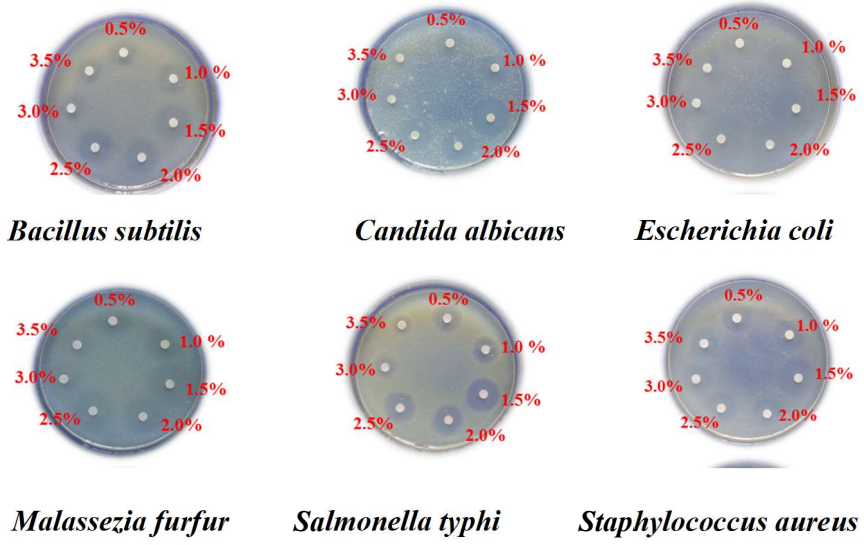


Figure 14. Inhibitory zones of size of inoculums

Effect of different pH on antimicrobial activity of strain MM 4

Among pH 4, 5, 6, 7, 8, 9 and 10 of fermented broths of strain MM4, pH 4 was the best for the extraction of the bioactive compounds from fermented broth.According to their results of inhibitory zones, strain MM4 showed high activity on *Bacillus subtilis, Escherichia coli, Malassezia furfur, Staphylococcus aureus* at pH 4 as shown in Table 7 and Figure 15.

Table 7. Inhibitory zones (mm) of different pH of strain MM 4

T.O \ pH	4	5	6	7	8	9	10
Bacillus subtilis	**15**	12	12	12	12	10	10
Candida albicans	10	10	12	12	12	10	10
Escherichia coli	**18**	15	12	12	15	10	10
Malassezia furfur	**18**	10	10	10	10	12	10
Salmonella typhi	12	15	10	10	10	10	10
Staphylococcus aureus	**18**	15	10	15	15	12	10

10 -12 mm = weak activity, 13 - 18 mm = high activity,>18 mm = very high activity (disc size = 6 mm)

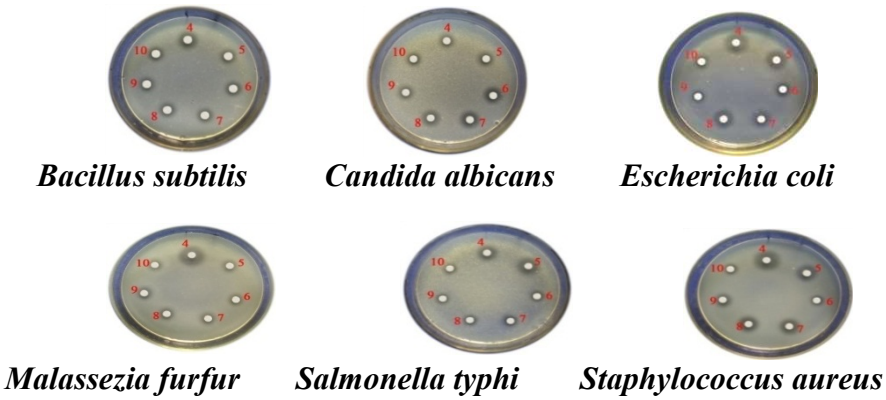


Figure 15. Inhibitory zones of pH utilization

Discussion and Conclusion

In this study, endophytic fungal strainMM4 was isolated from the leaves of *Psidium guajava* L. Fermentation studies of strain MM4 were investigated in order to produce its bioactive secondary metabolites. The large numbers of known bioactive compounds of microbial origin are currently produced by fermentation (Parkinsan, 1994). Among carbon sources, sucrose,

starch and glycerol were the best whereas glucose and mannitol were also suitable for fermentation.

Ritchie *etal.,* (2009) reported that sucrose was the most suitable carbon source for the growth of some isolates of ***Rhizoctoniasolani.*** Yeast extract, meat extract, malt extract and soybean were the best nitrogen sources for fermentation.

KyawtKyawtAung (2014) stated that sucrose and starch in carbon sources and yeast extract, meat extract and soybean in nitrogen sources were the best media for fermentation.The antimicrobial activity of strain MM4 in carbon sources, sucrose, glucose, starch and mannitol showed high activity against *Candida albicans, Escherichia coli* and *Malassezia furfur* whereas in nitrogen sources, yeast extract, meat extract and soybean indicated very high activity against *Candida albicans* and *Escherichia coli.*

The production of antimicrobial metabolites by fungi is also influenced by nutrients mainly carbon and nitrogen sources (EL-Banna , 2006).In morphological characters on various media, 9, 7 and 10 were good whereas media 1, 2, 3 and 11 were moderate for fermentation. In the present study, two days old of age of inoculum was the best for fermentation. It is in agreement with the statement of Yee Yee Thu (2006). In inoculum optimization, among the seed culture (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%) 1.5% of seed culture within five-seven days fermentation was suitable according to the results of their antimicrobial activity.

Yee YeeSoe (2014) reported that 1.5% size of inoculum for fermentation of bioactive strain showed highest activity against *Bacillus subtilis* .Optimal fermentation conditions such as proper age and size of inoculum are very important for the production of metabolites (Omura, 1985).

Festus *et al.,* (2015) isolated endophytic fungal strain from *Psidium guajava* L. leaves and reported that rice medium was the best for fermentation. In the screening of optimal pH for fermentation, pH 4 was the best for extration of bioactive compound according to the result of inhibitory zones of against six test organisms. Yee Yee Thu (2006) has reported that endophytic fungus isolated from *Chaetomium* sp. indicated high activity at pH 4.5.

In conclusion, the best fermentation medium for strain MM4 should consists of sucrose, glucose or starch for carbon sources, yeast extract or meat extract for nitrogen sources. The best fermentation conditions were 1.5% of two days old seed culture and pH 4 to produce bioactive metabolites from strain MM4.

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References

Backer C. A. and R. C. Bakhuizen. (1968). **Flora of Java, Vol. III. Angiospermae**, Families 191-238; Noordhoff N. V.-Graningen, Netherlands. p-162

Elaasser, M.M, M.M Abdel-Aziz, R. A El- Kassas. (2011). **Antioxidant, antimicrobial, antiviral and antitumor activities of pyranone derivative obtained from *Aspergillus candidus***. J Microbiol Biotech Res 1:5-17.

Festus,B.C.O, C.S. Nworu, A.Debbaba, C. O. Esimone, P. Proksch. (2015). **Two new cytochalasins from an endophytic fungus, KL-1.1 isolated from *Psidium guajava* leaves.** Phtochemistry letters 14 (2015) 51-55.

Hooker, SIR J. D., C. B., K. C. S. I. (1885). The Flora of British India.Vol. IV. L Reeve & Co., 5, Henrietta Street, Convent garden. London.

Kumar, S. and A. Sagar. (2007). **Microbial associates of Hippophaerham- noides** (Seabuckthorn). Plant Pathol. J. 6 (4): 299–305.

Kyawt Kyawt Aung, (2014). **Investigation of bioactive compound produced by endophytive fungal strain isolated from C*occiniaindica* Weight &Arn.** Ph.D Thesis; Department of Botany, University of Yangon.

Monaghan, R. L., M. M. Gagliardi., and S. L. Streicher.(1999).**Culture Preservation And Inoculum Develpoment, Manual Of Industrial Microbiology And Biotechnology**.2nd Edition, P 29-48

Parkinsan, D: (1994). **FermentousFungi.,In Methods Of Soil Analysis**,Part 2,329-350.

Parton, C. and P. Willis. (1989). **Strain preservation, inoculum preparation and development,** p. 39-64. In B.McNeil and L.M. Harvey (ed), Fermentation;a practical approach.IRL Press, Oxford,United Kingdom.

Puri, S. C., V. Verma, T. Amna, G. N. Qazi, M. Spiteller. (2005). An endophytic fungus from Nothapodytes foetida that produces camptothecin. J Nat Prod. 68(12), 1717-1719.

Ritchie, F., R. A. Bain, M. P. McQuilken. (2009). **Effects of nutrient status, temperature and pH on mycelia growth,sclerotial production and germination of *Rhizoctonia solani*from potato .**J Plant Pathol 91:589- 596.

Stroble, R.J. and G.R. Sullivan. (1999). **Experimental Design For Improvement Of Fermentations**, **Manual Of Industrial Microbiology And Biotechnology.**2nd Edition, P-80-102.

Yee YeeSoe, (2014). **Investigation of secondary metabolites produced by *Pseudomonas* sp. isolated from soil.** Department of Botany, University of Yangon.

Yee Yee Thu, (2006). **Novel Antimicrobial Metabolites Produced by *Trichoderma*sp., *Streptomyces* sp. and *Chaetomium* sp. Isolated from *Mimusops elengi* L., Soil and *Tamarix cananriensis* Willd**., Ph.D Thesis; Department of Botany, University of Yangon.