STUDY ON THE FERMENTATION CONDITIONS OF ENDOPHYTIC FUNGUS, MZF-2 FROM *MOMORDICA CHARANTIA* L.

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Abstract

Isolation of endophytic fungi was done by surface sterilization procedure from Cucurbiticeae family, *Mormordica charantia* L. (Kyet-hinga), *Cephalandra indica* Naud. (Kinmon), *Lagenaria siceraria* (Mol.) Standl. (Bu) and *Luffa acutangula* (L.) Roxb. (Hkawe). Agar well diffusion method was used for assay performance with ten kinds of test organisms. All isolated fungal strains showed antimicrobial activity except MZF-8, 11, 12, 13 and 15. Among them fungus MZF-2 isolated from *Momordica charantia* L. was screened for further investigations based on the results of maximum inhibition against *Candida albicans* NITE 09542. Different fermentation parameters were studied and included fermentation period, inoculum size, effect of various carbon sources and nitrogen sources, fermentation media, pH, temperature and agitation condition. 5th day and 4% size were found to be optimum time period of fermentation and inoculum size. In the growth of carbon sources, MZF-2 were excellent growth on corn and potato as well as malt extract showed the excellent result for nitrogen sources. Maximum antifungal activity was obtained when fermentation medium was supplemented with carbon source as glucose and peptone as nitrogen source. Concerntration of fermentation medium (FM-7) ingredients like each 1.0 g peptone, glucose and sucrose proved to be the best fermentation medium. And maximum bioactive metabolite productions occur in pH of 6, temperature at 25˚C and shaking culture with 100rpm speed.

Keywords: antimicrobial activity, antifungal metabolites.

Introduction

Endophytes are defined as microorganisms which inhabit inside of healthy plant tissues and are now considered as ubiquitous symbionts of plants from their surprisingly common detection from many species. Common

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endophytes include a variety of bacteria, fungi and actinomycetes and they can be isolated from wild or cultivated crops of either the monocots or dicots (Petrini, 1986).

Among the microbial group of the most frequently isolated endophytes are the fungi. Cucurbiticeae family is a major source of medicinal agents since ancient time. Both plants and fungi are known for producing a large number of chemically diverse secondary metabolites. The secondary metabolite is obtained by fermentative process. During fermentation, the organisms produce the antibiotic material, which can then be isolated for use as a drug (Fenical, 1993). Fermentation is a complex process, it not only depends on the performance and fermentation medium, also requires the suitable environmental conditions such as inoculation volume, medium capacity, fermentation time, temperature, agitation rate and initial pH . These factors may affect the antibiotics production (Martin and Demain, 1980).

World health problems caused by drug-resistant bacteria and fungi are increasing as a result an intensive search for newer and effective antimicrobial agents is needed. Endophytic fungi have received attention of the scientific community due to their capacity to produce novel bioactive compounds (Strobel *et al*., 2004). Therefore, the present study was carried out the isolation and fermentation studies for antifungal compounds produced from selected fungus. The aim and objectives of this study were to isolate the endophytic fungi and optimization of production parameter for antifungal metabolites.

Materials and Methods

Study area and collection of plant samples

These plant materials, *Momordica charantia* L.,*Cephalandra indica* Naud., *Langenaria siceraria* (Mol.) Standl. and *Luffa acutangula* (L.) Roxb. were collected from Pathein Township, Ayeyarwady Region from June to August, 2016. The identification of these plants were referred by (Flora of Hong Kong, 2009 and Hundley and Chit Ko Ko, 1987).

Isolation of endophytic microorganisms (Espinosa-Garcia, F. J. & J. H. Langenhein. 1991)

In the isolation procedure of endophytic microorganisms, the leaves were washed in running tap water for 15 minutes and were cut into about 0.3 cm pieces. Then, these parts were sterilized by soaking in 95% ethanol for 15 minutes. And again, these parts were cut into smaller pieces and dried on sterilized tissue paper. After drying these pieces were placed on Low Carbon Agar (LCA) medium plate and incubated at room temperature. When hypal tips grow out, they were transferred into Potato Glucose Agar (PGA) medium.

Screening for antimicrobial activities (NITE 2005)

The isolated fungi were grown on PGA medium at room temperature for 5 days. After incubation period, these fungi inoculated into the seed medium (glucose 0.5 g, peptone 0.3 g, yeast extract 0.3 g, $MgSO_4 7H_2O$ 0.01 g, K_2HPO_4 0.01 g, $CaCO_3$ 0.01 g, DW 100 mL at pH 6.5) for 3 days at room temperature. After three days, the seed medium (2%) was transferred into the fermentation medium (glucose 1.0 g, peptone 0.5 g, yeast extract 0.5 g, $MgSO_4 7H_2O$ 0.01 g, K_2HPO_4 0.01 g, $CaCO_3$ 0.01 g, DW 100 mL at pH 6.5) and carried out for 3- 10days and evaluated the antimicrobial activity by agar well diffusion method.

Screening of antimicrobial activity by agar well method (Collins, 1965)

1 day old culture test broth (0.2 mL) was added to 25 mL warm assay medium (glucose 1.0 g, peptone 0.3 g, KNO_3 0.1 g, DW 100 mL, agar 1.8 g) and thoroughly mixed and poured into plate. After solidification, the agar was left to set . Cork borer was used to make the wells (8 mm in diameter). And then, the fermented broth (20 µL) was carefully added into the well and incubated at room temperature for 24-48 hours. The diameter of the zones of inhibition around each well measured and recorded after 24-48 hours incubation.

Test organisms

Agrobacterium tumefaciens NITE 09678, *Aspergillus paraciticus* IFO5123, *Bacillus subtilis* IFO 90571, *Candida albicans* NITE09542 , *E. coli*

AHU5436, *Micrococcus luteus* NITE83297, *Pseudomonas fluorescens* IFO94307, *Saccharomyces cerevisiae* NITE52847, *Salmonella typhi* AHU 7943 and *Staphylococcus aureus* AHU8465, were obtained from NITE (National Institute of Technology and Evaluation, Kisarazu, Japan).

Study on the effects of sizes of inoculum on fermentation (Crueger, W., & Crueger, A. 1989)

The selected fungus MZF-2 was grown on PGA medium for 5 days at room temperature. After 5 days incubation period, this fungus was inoculated into 100mL seed medium. For the size of inoculum, seed culture (1%, 2%, 3%, 4%, 5%) were transferred into the each flask of 100 mL fermentation medium. All fermentation media were carried out 5 days and antifungal activity was investigated by agar well diffusion method.

Effect of carbon and nitrogen sources

Various carbon sources such as glucose, fructose, sucrose, galactose, dextrose, lactose, xylose, maltose, glycerol, soluble starch, tapioca powder, corn and various nitrogen sources as asparagine, KNO_3, malt extract, meat extract, NH_4Cl, NH_4NO_3. $NaNO_3$, $(NH_4)_2SO_4$, Peanut cake, Peptone, yeast extract, and urea were employed.

Effect of glucose and peptone concentration on fermentation medium

In this study six types of carbon concentration, fermentation medium FM1- glucose 1.5 g, FM2- glucose 2 .0 g, FM3- glucose 2.5 g, FM4- glucose 3.0 g, FM5- glucose 3.5 g, FM6- glucose 4.0 g were used . As well as six types of nitrogen concentration, fermentation medium FM7-peptone 1.0 g, FM8- peptone 1.5 g, FM9- peptone 2 .0 g, FM10- peptone 2.5 g, FM 11-peptone 3 .0 g, FM12- peptone 3.5 g were applied.

Effect of pH (Furtado *et al*., 2005)

The optimization of pH of the fermentation broth for antifungal metabolite production was done by carrying out the fermentation at seven different pH values viz. 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0. For each pH value,

desired pH by using either 0.1M NaOH or 0.1 M HCl was adjusted into fermentation medium.

Effect of incubation temperature (Cazar *et al*., 2004)

The optimization temperature for antifungal metabolite production was carried out at six different incubation temperatures viz. 20, 25, 30, 35, and 40 and 45°C. The fermentation medium was carried out 5 days and antifungal activity was studied by agar well diffusion method.

Comparision of static culture and shaking culture (Hassan and Bakhiet *et al*., 2017)

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 those fermentation medium was incubated under static condition without shaking. These shaking culture and static culture were compared by using agar well diffusion method.

Results

Isolation of endophytic fungi

A total of fifteen fungi were isolated from four selected species of Cucurbitaceae family. Isolated fungi were designated as MZF. Two isolates MZF-1 and 2 were obtained from *Momordica charantia* L. and the other strains (MZF 3 and 4) were isolated from *Cephalandra indica* Naud. Another strains (MZF 6-15) were obtained from *Luffa acutangula* (L.) Roxb. and MZF-5 was isolated from *Lagenaria siceraria* (Mol.) Standl.These results were shown in Table 1 and Figure 1.

Table 1. Isolated fungi

Scientific Name	Myanmar Name	English Name	Fungi
Momordica charantia L.	Kyet-hinga	Bitter gourd	MZF-1, MZF-2
Cephalandra indica Naud.	Kinmom	Wild snake gourd	MZF-3, MZF-4
Lagenaria siceraria (Mol.) Standl.	Bu	Bottle gourd	MZF-5
Luffa acutangula (L.) Roxb.	Hkawe	Ridge gourd	MZF-6 to MZF-15

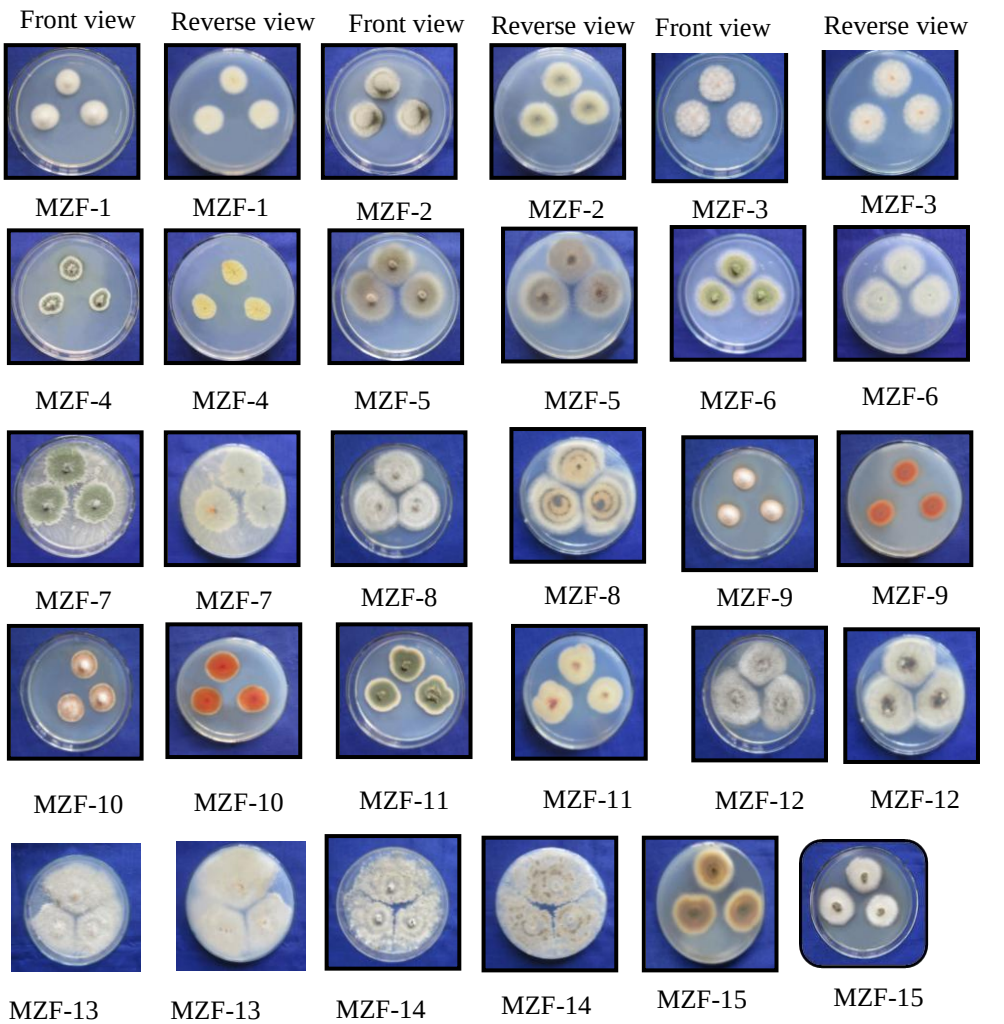


Figure 1. Morphologies of isolated fungi on PGA medium

Antimicrobial activities of isolated fungal strains

Ten isolated fungi (MZF-1, 2, 3, 4, 5, 6, 7, 9, 10 and 14) had antimicrobial activity and remaining five isolates (MZF- 8, 11, 12, 13,15) could not produce antimicrobial metabolites. MZF- 1 (21.77 mm) and MZF-4 (21.70 mm) in 5 days as well as MZF-7 (20.23 mm) and MZF- 14 (20.09 mm) in 6 days fermentation period played the highest activities on *Agrobacterium tumefaciens.* MZF-2 (23.95 mm) and MZF-9 (19.86 mm) had the best activities on *Candida albicans* in 5 days fermentation period. MZF- 3 (20.00 mm) and MZF-6 (18.96 mm) were the strongest activity against *Escherichia coli* in 6 days and 7 days fermentation period. MZF-5 (22.17 mm) and MZF-10 (19.19mm) showed the significant inhibitory zone on *Micrococcus luteus* in 5 day fermentation period. These results were displayed in Figure 2. Among them, antifungal activity of isolated fungus MZF-2 showed the maximum inhibitory zone against *Candida albicans.*

Table 2. Zone of inhibition (in mm) of isolated fungus MZF-2

Fermentation period	**Test organisms**									
	1	2	3	4	5	6	7	8	9	10
3 day	_	_	_	20.78	15.53	_	_	_	16.32	17.11
4 day	_	_	_	22.18	15.97	_	15.46	_	16.30	16.69
5 day	10.31	_	_	23.95	16.94	_	15.86	_	14.31	16.00
6 day	10.86	_	_	23.61	16.97	_	14.95	_	14.00	15.43
7 day	11.75	_	_	23.18	14.74	_	14.47	_	13.27	14.46
8 day	11.24	_	_	22.93	14.52	_	15.85	_	13.21	_
9 day	11.24	_	_	21.86	13.59	_	15.13	_	13.20	_
10 day	11.23	_	_	21.84	13.57	_	15.10	_	13.70	_

1 – *Agrobacterium tumefaciens*
2 – *Aspergillus paraciticus*
3 – *Bacillus subtilis*
4 – *Candida albicans*
5 – *Escherichia coli*
6- *Micrococcus luteus*
7 – *Saccromyces cereviciae*
8 – *Salmonella typhimurium*
9 – *Staphylococcus aureus*
10- *Pseudomonas fluorescence*

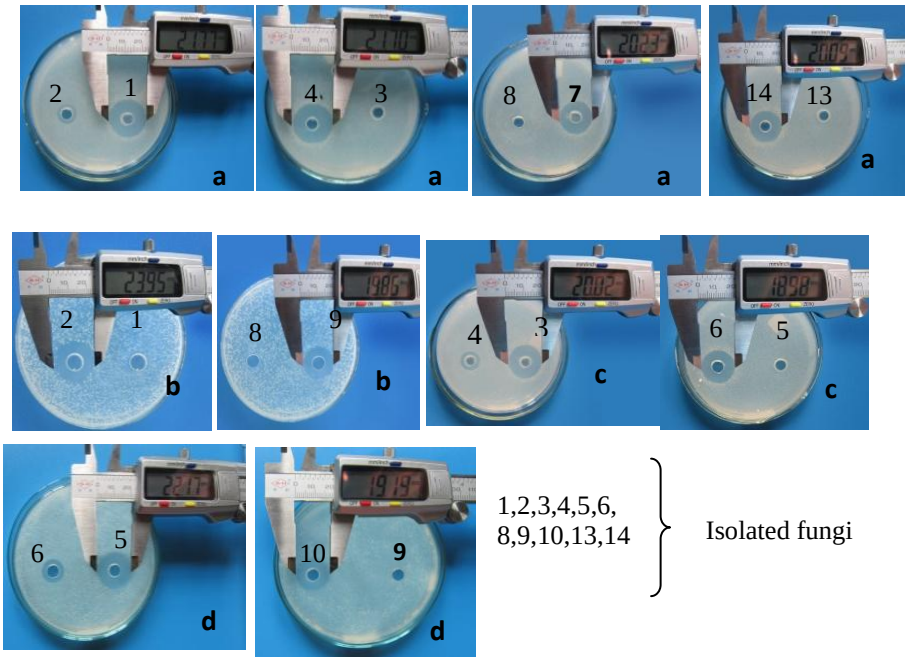


Figure 2. Anitmicrobial activity of isolated fungi against
(a) *Agrobacterium tumefaciens*
(b) *Candida albicans*
(c) *Escherichia coli*
(d) *Micrococcus luteus*

The effect of size of inoculum on the fermentation

In this research work, the effect of size of inoculum was studied by using 1% to 5 % inoculum. Using 4% inoculum showed significantly higher (25.54mm) than others, followed by 3% and 5% (22.32mm and 21.87 mm) respectively. Minimum inhibition zone was observed by using 1% (19.93mm) and 2% (20.81mm).

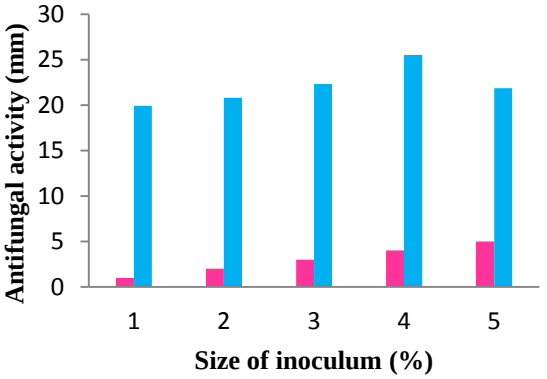


Figure 3. The effect of size of inoculum

Carbon and nitrogen sources utilization for growth

The carbon sources from corn and potato were excellent growth, moderate growth on glucose, sucrose, glycerol, soluble starch and tapioca powder, while other six carbon sources showed poor results. The excellent growth were found on malt extract, poor results on NH_4Cl, NH_4NO_3. $NaNO_3$, $(NH_4)_2SO_4$, urea and the left six nitrogen sources were good growth.

Table 3. Growth of MZF-2 on carbon and nitrogen sources

Carbon sources	Growth	Nitrogen sources	Growth
Glucose	Moderate (4.0cm)	Asparagine	Moderate (3.6cm)
Fructose	Poor (2.0cm)	KNO_3	Moderate (4.0cm)
Sucrose	Moderate (3.6cm)	Malt extract	Excellent (5.5cm)
Galactose	Poor (2.3cm)	Meat extract	Moderate (4.5cm)
Dextrose	Poor (1.6cm)	NH_4Cl	Poor (1.7cm)
Lactose	Poor (1.8cm)	NH_4NO_3	Poor (2.4cm)
Xylose	Poor (2.0cm)	$NaNO_3$	Poor (1.8cm)
Maltose	Poor (1.8cm)	$(NH_4)_2SO_4$	Poor (2.8cm)
Glycerol	Moderate (4.0cm)	Peanut cake	Moderate (3.6cm)
Soluble starch	Moderate (3.5cm)	Peptone	Moderate (3.5cm)
Tapioca powder	Moderate (3.5cm)	Yeast extract	Moderate (3.7cm)
Corn powder	Excellent (4.7cm)	Urea	Poor (2.0cm)
Potato	Excellent (4.5 cm)	_	_

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

Effect of carbon and nitrogen utilization on fermentation

The significant inhibition zone (24.09 mm, 23.81mm, and 22.04 mm) were obtained in glucose, sucrose and corn as amended media. Dextrose (22.00mm), xylose (20.80 mm), glycerol (20.51mm), and soluble starch (20.18 mm) showed moderate inhibition zone. Glactose (17.32 mm), maltose (18.54 mm), tapioca powder (12.84 mm) and potato (17.38 mm) were regarded as poor inhibition zone. Similarly, the addition of peptone displayed the greatest activity (22.91mm), followed by KNO_3 (22.38 mm), asparagine (22.32 mm), yeast extract (21.94 mm), meat extract (21.70 mm) and $NaNO_3$ (20.53 mm). There were no activities when fructose and lactose were used as carbon source and malt extract, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$ and peanut cake were used as nitrogen source. These results were shown in Figure 4 and 5.

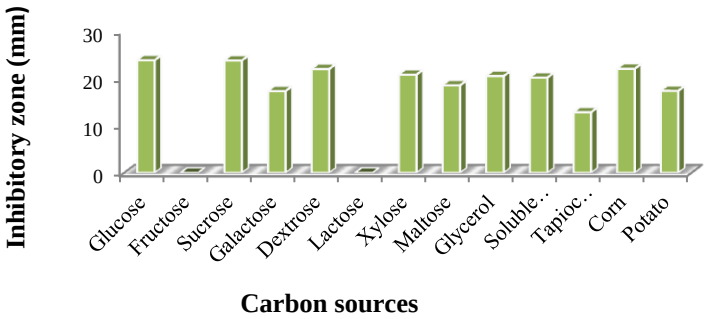


Figure 4. Effectof carbon utilization on fermentation

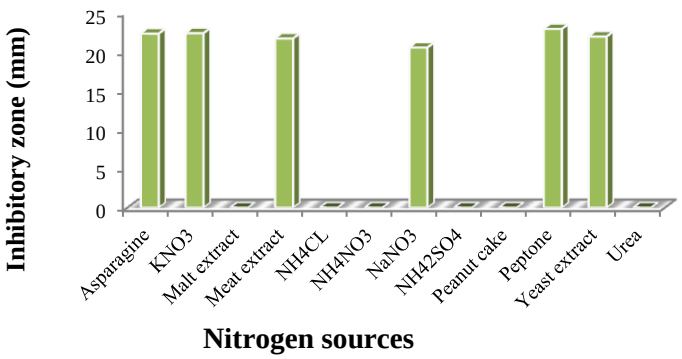


Figure 5. Effect of nitrogen utilization on fermentation

Effects of carbon and nitrogen concentration on fermentation medium

Various glucose concentrations were tested 1.5 g, 2.0 g, 2.5 g, 3.0 g, 3.5 g and 4.0 g. Glucose of 2.5 g concentration (FM 3) showed remarkable result 24.40 mm followed by glucose 2.0 g (FM 2) 23.92 mm and 1.0 g glucose (FM 1) 23.80 mm. Peptone concentrations (1.0 g, 1.5 g, 2.0 g, 2.5 g, 3.0 g, 3.5 g) were also studied. The addition of peptone at concentration of 1.0 g (FM 7) resulted in a maximum antifungal activity 25.42 mm, followed by (FM 8) in peptone 1.5 g 23.79 mm and (FM 9) in peptone 2.0 g 20. 00mm. There were no activity on concentration of peptone 3.0 g and 3.5 g. These datas were described in Figure 6 and 7.

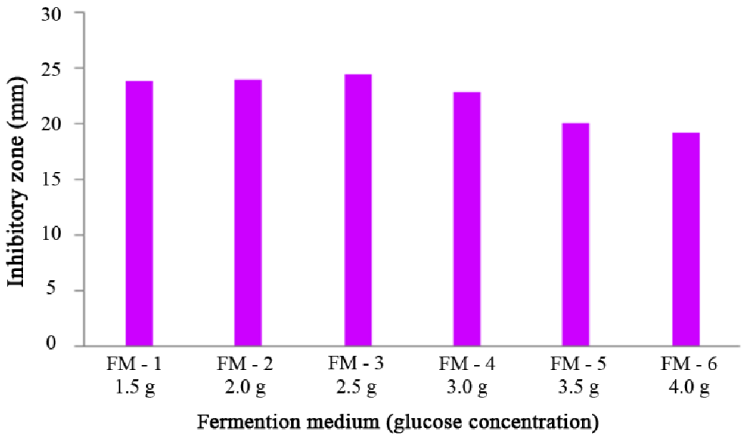


Figure 6. Antifungal activity of MZF-2 on fermentation medium with various glucose concentration

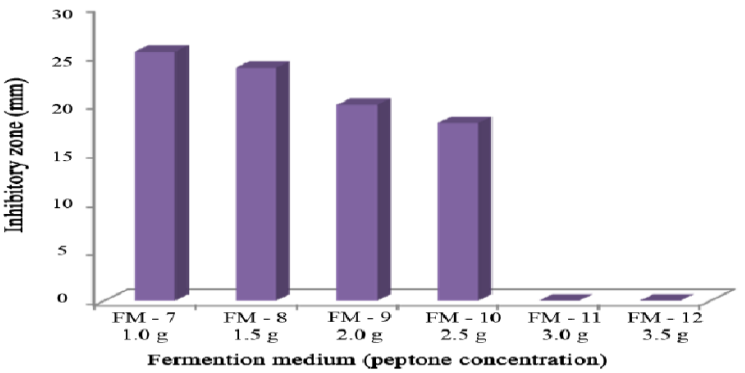


Figure 7. Antifungal activity of MZF-2 on fermentation medium with various peptone concentration

Effect of pH and temperature

The effect of pH and temperature were tested with different pH levels (pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) and different temperature ranges (20˚C, 25˚C, 30˚C, 35˚C, 40˚C and 45˚C). Maximum inhibitory zone was occurred in pH 6 (25.04 mm) and it was followed by pH 5 (22.53 mm) , pH 7 (21.90 mm) and pH 4 (21.03 mm). Under base conditions, minimum inhibitory zone was observed at pH 8, 9, 10 (19.85 mm, 18.52 mm and 17.60 mm) respectively. Maximum antifungal activity was recorded at 25˚C (24.62 mm) , followed by 30˚C (22.99 mm) and 35˚C (20.87 mm). And the antifungal activity completely inhibited at 45˚C. There was a gradual decrease in antifungal production when the temperature was increased from 25 ˚C to 40˚C.

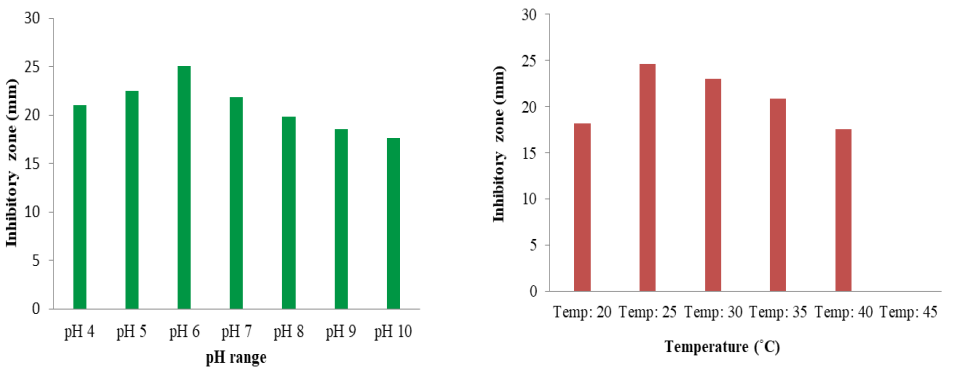
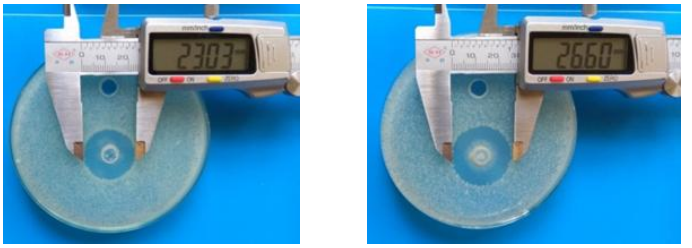


Figure 8. Effect of different pH and temperature

Comparision of static culture and shaking culture

When comparing the static culture and shaking culture of fermentation medium, antifungal activity from shaking culture is better than (26.60 mm) than that of static culture (23.03 mm).



Static culture Shaking culture

Figure 9. Comparisom of static culture and shaking culture

Discussion and Conclusion

Endophytic fungi from medicinal plants are relatively unexplored as potential source of novel species and novel natural products for medicinal and commercial exploitation. In present study, a total of fifteen endophytic fungi were isolated and screened for antimicrobial metabolite production. Ten isolated fungi (MZF-1, 2, 3, 4, 5, 6, 7, 9, 10 and 14) could display the antimicrobial activity inhibiting the test organisms and the remaining five isolates (MZF- 8, 11, 12, 13,15) could not produce antimicrobial metabolites. Similarily, Sarika *et al*., 2014 isolated total eight endophytic fungi from Cucurbiticeae family, *Momordica charantia* L. and those endophytic fungi showed maximum inhibition zone against *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis* and *Staphylococcus aureus*.Among the potent strains, antifungal activity of isolated fungus MZF-2 , isolated from *Momordica charantia* L. showed the maximum inhibitory zone of 23.94 mm against *Candida albicans* NITE 09542 in 5 days fermentation period.Therefore selection of MZF-2 was carried on further experiments.

Modifying fermentation parameters such as time, temperature, pH, and nutrients can help expanding the range of secondary metabolites (Pfefferle *et al*., 2000) . In determining the most suitable size for production antifungal compounds, 4% inoculum size reached the highest activity (25.54 mm) so that 4% size of inoculum regarded as the most suitable size. In investigation of the effect of carbon and nitrogen, addition of corn powder and potato as carbon

source were excellent growth and maximum inhibition zone reached up (24.09 mm and 23.81mm) in glucose and sucrose, followed by corn (22.04 mm). Addition of potato resulted excellent growth of the fungus but less bioactive metabolite production (17.38mm). Suja, 2013 aslo reported that zone of inhibition, 24 mm was obtained in sucrose supplemented media. Excellent growth and the maximum production of antifungal metabolite in MZF-2 was observed in the presence of malt extract and peptone (22.91 mm) as nitrogen source. The supplement of malt extract showed excellent growth of the fungus but did not show antimicrobial activity.

Fermentation medium (FM-7) showed significant result by using peptone (1.0 g), glucose (1.0 g) and sucrose (1.0 g). It has been reported that generally addition of glucose enhances the metabolite production, but its significance in many fermentation process decreases because at higher concentration, it has inhibitory effect (Hutter *et al*., 1982). Similarly, addition of peptone above 1 % decreased antifungal metabolite production in selected strain MZF-2. Therefore FM-7 was chosen as a selected fermentation medium. Maximum antifungal activity was found at pH 6 as the diameter of zone of inhibition was 25.04 mm. Similar result had been reported earlier by Nishihara *et al*., 2001 during the production of FR198248, a new antiinfluenza agent at pH value between 6.3 to 6.4 from *Aspergillus terreus.* Maximum inhibitory activity was recorded at the incubation temperature of 25˚C (24.62 mm). Antifungal metabolite production increased with the increase in temperature from 20 to 25 ˚C. However, as temperature was increased from 25 to 40 ˚C, there was a decline in antimicrobial metabolite production. According to these results, 25 ˚C is the most suitable temperature for the antimicrobial metabolite production. These results were in agreement with Jain and Pundir, 2011. In this study, under shaking culture, the diameter of inhibitory zone was more higher than under static culture. The result was in agreement with the description of Hassan *et al*., 2017 that the antibacterial compounds production of *Aspergillus fumigatus* showed the best result with 100 rpm shaking speed.

The present study concluded that the optimum conditions required for the production of bioactive metabolites by selected endophytic fungus MZF-2 were determined and metabolites showed better antifungal activity against human pathogen, *Candida albicans*.

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