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OPTIMIZATION OF FERMENTATION CONDITIONS, EXTRACTION OF CRUDE EXTRACT AND IDENTIFICATION OF SOIL FUNGUS MM-34

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Abstract

In the present study, twenty different soil samples were collected from twenty different places of Belu-Gyun, Chaung-Zon Township, Mon State. Totally 37 fungi were isolated from 20 soil samples. Among these 37 isolates, MM-34 was found to show maximum antibacterial activity, in compare to other isolates. Thus, soil fungus MM-34 was selected for the study of effects of pH on fermentation. Initial pH of 5.0 was the best for fermentation conditions. Maximum antibacterial activity (31.01 mm inhibitory zone) against *Micrococcus luteus* was observed in pH 5.0. Different temperature such as 20°C, 25°C, 30°C, 35°C and 40°C was studied for maximum production of antibacterial activity by soil fungus MM-34. Maximum antibacterial activity by fungal isolate MM-34 was recorded at temperature 30°C (30.52 mm inhibitory zone) against *Micrococcus luteus.* In the study of static and shaking culture, static culture was optimal condition for MM-34 fermentation (30.68 mm inhibitory zone). Based on the all optimum fermentation conditions, the selected soil fungus MM-34 was carried out by fermentation period. It was observed that the culture which were incubated for 6 days shows maximum antibacterial activity (34.84 mm inhibitory zone) against *Micrococcus luteus.* According to the R_f values, ethyl acetate is suitable for the extraction of crude extract from the fermented broth. Crude extract of ethyl acetate was observed antibacterial activity (26.19 mm inhibitory zone) against *Micrococcus luteus*. The TLC plates were developed in the solvent of chloroform and chloroform-methanol mixture (9:1, 8:2, 7:3, 6:4, 4:6 and 3:7) and Hexane only and Hexane-ethyl acetate mixture (9:1, 8:2, 7:3, 6:4, 5:5, 4:6 and 3:7). Based on the morphological and microscopical characters, soil fungus MM-34 was identified as *Penicillium* sp.

Keywords: Antibacterial activity, Optimization, *Penicillium* sp.

Introduction

Soil is a naturally occurring loose mixture of mineral and organic particles, considered as one of the most suitable environments for microbial growth (Nejad *et al.,* 2013). The dominant microorganism in all soils is fungi. Fungi are an abundant group in acid soil because acidic condition is not promoting the growth of bacteria and actinomycetes. Many new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidant compounds having pharmaceutical, industrial and agricultural importance are isolated characterized from soil fungi (Stobel and Daisy, 2003).

Antimicrobial substances or antibiotics are now referred to compound produced by microorganisms, or to a similar compound which inhibits other microorganisms at low concentration. The most well-known antibiotics produced by fungi are penicillins, cephalosporins and fusidic acid (Denyer *et al.,* 2004). In 1928, penicillin, the first antibiotic was incidentally discovered by Sir Alexander Fleming who isolated *Penicillium notatum*, which produced Gram positive bacteria killing compound. This discovery was the starting of the attention of secondary metabolites produced by microorganisms (Taylor *et al.,* 2003) and fungi became the interesting source of bioactive compounds since then.

Penicillium is a genus of ascomycetous fungi and has an important role in various natural processes. The wide and ubiquitous presence of the *Penicillium* species has been researched in several studies. According to a comprehensive literature analysis *Penicillium* is one of the most

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common fungi occurring in various environments such as soil, air and extreme environments (temperature, salinity, water deficiency and pH) and is also associated with plants and specific food products. Due to its huge diversity and existence in extreme environments there is great potential in using it for various environmental, biotechnological and industrial applications (Yadav *et al.,* 2018).

The aim and objectives of this research were to study the optimum fermentation conditions viz., pH, temperature, static and shaking conditions of fermentation and fermentation period, to select the suitable solvent for the extraction of crude extract and to identify the selected soil fungus MM-34.

Materials and Methods

Isolation of fungi

The isolation of microorganisms was taken by feeding method (Hayakawa, and Kobayashi, 2005). To prepare the isolation medium, glucose 1.0 g, yeast extract 0.7 g, K_2HPO_4 0.01 g, KNO_3 0.02 g, agar 1.8 g were placed in a 250 mL conical flask. The transfer medium was glucose 1.2 g, yeast extract 0.8 g, K_2HPO_4 0.01 g, $MgSO_4$ 0.01 g, agar 1.8 g.

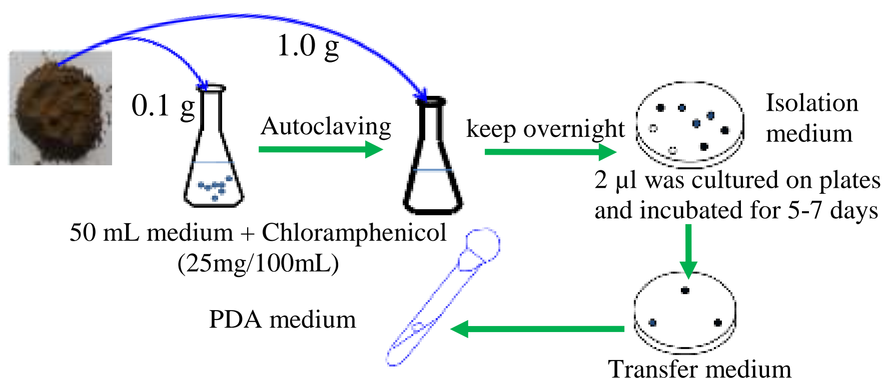


Figure 1 Feeding method (Hayakawa, and Kobayashi, 2005)

Effect of pH on antibacterial activity of soil fungus MM-34

Effect of different pH viz. 3, 4, 5, 6, 7, 8 and 9 was examined on the antibacterial activity of selected fungal isolates MM-34. The fermentation broth was adjusted to the desired pH by adding 1 N HCl or 1 N NaOH. The antibacterial activity was checked by paper disc diffusion assay method.

Effect of temperature on antibacterial activity of soil fungus MM-34

The optimum temperature for antibacterial activity was measured by incubating the fermentation broth at temperature 20℃, 25℃, 30℃, 35℃ and 40℃. The antibacterial activity was checked by paper disc diffusion assay method.

Effect of Static and Shaking Condition

To determine the effect of static and shaking on antibacterial activity of fungus MM-34, culture flasks (250 mL flask containing 100 mL fermentation medium) were incubated as static cultures as well as incubated in a rotatory shaker at 150 rpm.

Figure 2 Comparison with static and shaking cultures of MM-34 fermented broth

Paper chromatography to extract the crude extract of soil fungus MM-34

Paper chromatography was carried out to extract the crude extract from the fermented broth. The purpose of paper chromatography is to extract the crude extract using suitable solvent systems: 100% hexane, n-butanol saturated with water, 100% toluene and ethyl acetate saturated with water were used as solvents. The paper was chromatographed in each solvent. Then, bioautography was done to check the antibacterial activity of each. Each paper was placed on assay agar plates. After one hour, they were peel off and kept at over one night. Finally based on R_f value, optimum solvent will be chosen.

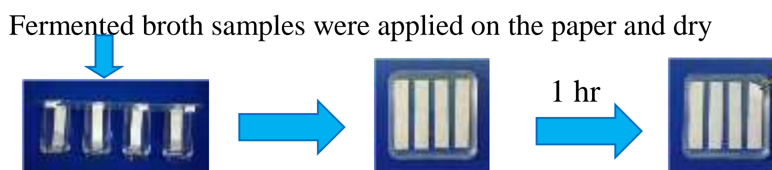


Figure 3 Preparation of paper chromatography

Extraction of crude extract from fermented broth of soil fungus MM-34

Based on PPC result, the fermented broth was carried out by extraction. The broth culture was filtered to separate the mycelia and the filtrate. To the filtrate added to extraction with ethyl acetate in the same volume, shaked well for half an hour and kept for 5 minutes until the two clear immiscible layers was formed. The upper layer of ethyl acetate containing the bioactive component was separated using a separating funnel. The antibacterial activity of the concentrated crude extract of ethyl acetate against *Micrococcus luteus* was checked by paper disc diffusion assay method.

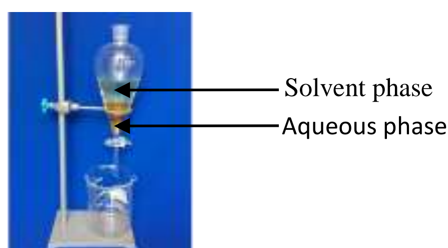


Figure 4 Extraction of crude extract from fermented broth of soil fungus MM-34 with ethyl acetate

Thin layer Chromatography and Bioautographic Assay

The obtained crude extract of ethyl acetate (20 µL) were applied on the TLC plate and allowed to dry. The TLC plates were developed in the solvents of Hexane and Hexane-Ethyl Acetate mixture (9:1, 8:2, 7:3, 6:4, 5:5, 4:6 and 3:7) and chloroform and chloroform-methanol mixture (9:1, 8:2, 7:3, 6:4, 4:6 and 3:7). Then, bioautography was done to check the antibacterial activity of each. Each TLC plate was placed on assay agar plates, then the plates were incubated for 24 hours.

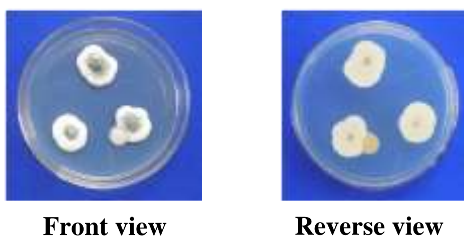
Identification of soil fungus MM-34

Soil fungus MM-34 was identified up to the genus level on the basis of their morphological characteristics such as colony morphology, color and growth pattern. The morphological character of the soil fungus MM-34 was investigated by direct microscopic examination of water-agar medium. The cultures were examined microscopically after 5-9 days incubation, using light microscopy.

The fungal strain was identified by Fungi Imperfecti, Barnett, 1969.

Results

Morphology and its antibacterial activity of isolated soil fungus MM-34



Front view **Reverse view**

Figure 5 Morphology of isolated soil fungus MM-34

Figure 6 Antibacterial activity of isolated soil fungus MM-34

Study on the effect of pH on the fermentation

The effect of pH on antibacterial activity of the fungal isolate MM-34 was tested using liquid culture at different pH levels (pH 3 to 9). Maximum antibacterial activity was obtained at pH 5.0 (31.01 mm clear zone) (Table 1, Figure 7).

Table 1 The effect of pH on the fermentation

pH	Activity (Clear zone, mm)
3	27.30
4	28.08
5	**31.01**
6	30.88
7	29.08
8	27.31
9	27.14

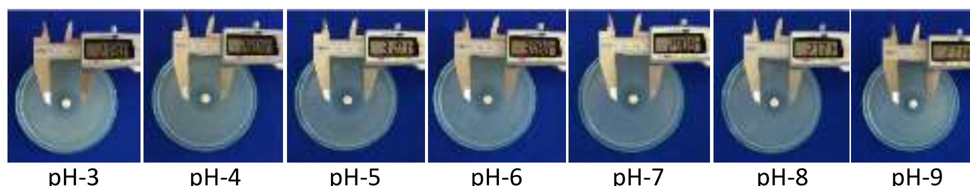


Figure 7 The effect of pH on the antibacterial activity of soil fungus MM-34

Study on the effect of temperature on the fermentation

In this study, the effect of temperature on the antibacterial activity of fermentation was done by carrying out the fermentation at five different temperature 20℃, 25℃, 30℃, 35℃ and

40ºC. The maximum antibacterial activity was observed at 30ºC (30.52 mm clear zone) (Table 2 and Figure 8).

Table 2 The effect of temperature on the fermentation

Temperature	Activity (Clear zone, mm)
20ºC	14.63
25ºC	24.14
30ºC	**30.52**
35ºC	20.64
40ºC	16.50

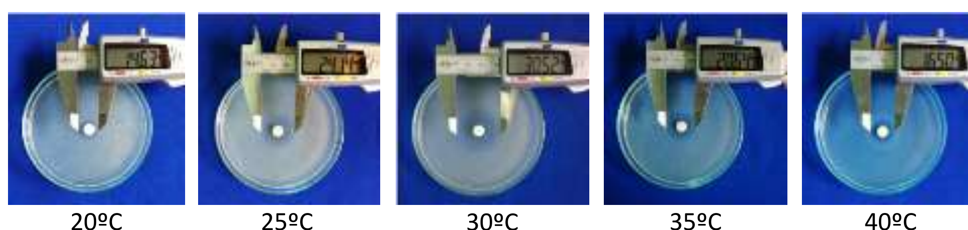


Figure 8 The effect of temperature on the antibacterial activity of soil fungus MM-34

Effect of Static and Shaking Condition

In this investigation, it was found that the antibacterial activity of static culture (30.68 mm) was higher than that of shaking culture (26.65 mm) as shown in Figure 9 and Table 3.

Table 3 Comparison with Static and Shaking Culture of MM-34 against *Micrococcus luteus*

Fermentation condition	Activity (Clear zone, mm)
Static culture	**30.68**
Shaking culture	26.65

Figure 9 Comparison with static and shaking culture of MM-34 against *Micrococcus luteus*

Effect of fermentation period on the antibacterial activity of soil fungus MM-34

Based on the result of the optimum fermentation conditions, fermentation period was carried out. Soil fungus MM-34 showed the highest antibacterial activity (34.84 mm, inhibitory zone) against *Micrococcus luteus* at 6 days fermentation period (Table 4, Figure 10).

Optimum fermentation conditions for antibacterial activity
Ages of inoculum = 84 hr seed culture
Sizes of inoculum = 15%
pH = 5.0
Temperature = 30°C
Suitable fermentation condition = static

Fermentation medium FM-3
Potato 2.0 g
Yeast extract 1.0 g
$MgSO_4$ 0.001 g
DW 100 mL

Table 4 Effect of fermentation period on antibacterial activity of soil fungus MM-34

Fermentation period	Activity (Clear zone, mm)
3	19.80
4	26.21
5	29.09
6	**34.84**
7	30.00
8	26.90
9	25.53
10	20.58

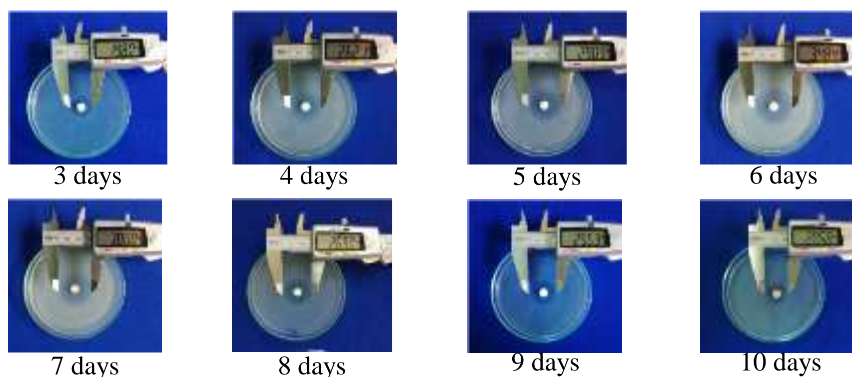


Figure 10 Effect of fermentation period of antibacterial activities against *icrococcus luteus*

Paper chromatography to extract the crude extract from fermented broth of soil fungus MM-34

In the present study, it was observed that R_f values are 0.00 in 100% hexane, 0.86 in n-butanol, 0.06 in 100% toluene and 0.96 in ethyl acetate. According to the R_f value, solvent ethyl acetate is more suitable for the extraction of crude extract than the other solvent (Figure 11).

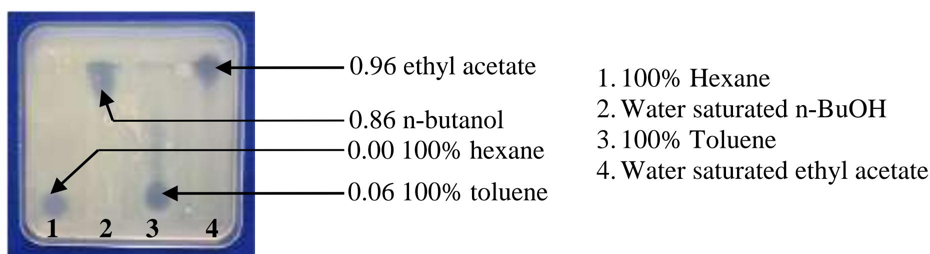


Figure 11 Paper Chromatography of antibacterial activity against *Micrococcus luteus*

Extraction of crude extract from fermented broth of soil fungus MM-34

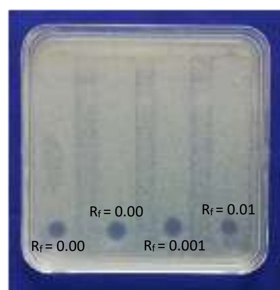
According to the results, crude extract could be extracted with ethyl acetate. Therefore, crude extract from fermented broth was extracted with ethyl acetate in the equal volume and ethyl acetate was concentrated. Antibacterial activity of ethyl acetate extract was 26.19 mm against *Micrococcus luteus* (Figure 12).

Extract

Figure 12 Antibacterial activity of crude extract of ethyl acetate against *Micrococcus luteus*

Thin layer chromatography and bioautographic overlay assay

Based on the TLC results (R_f values) (Figure 13) it was found that hexane-ethyl acetate solvent system was suitable for the separation of compound by silica gel column chromatography.

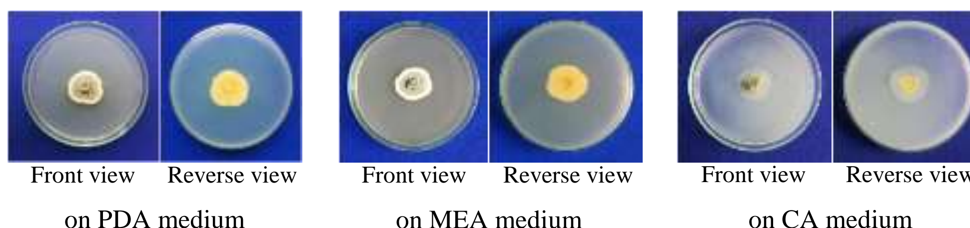


1. Hexaneonly
2. Hexane-ethyl acetate mixture (9:1)
3. Hexane-ethyl acetate mixture (8:2)
4. Hexane-ethyl acetate mixture (7:3)

1. Hexane-ethyl acetate mixture (6:4)
2. Hexane-ethyl acetate mixture (5:5)
3. Hexane-ethyl acetate mixture (4:6)
4. Hexane-ethyl acetate mixture (3:7)

Figure 13 Thin layer chromatography with Hexane-ethyl acetate mixture

Identification of soil fungus MM-34



Front view Reverse view Front view Reverse view Front view Reverse view

on PDA medium on MEA medium on CA medium

Figure 14 Colony morphology of soil fungus MM-34 (7 days old cultures)

Figure 15 Colony morphology of soil fungus MM-34 (7 days old cultures)

Table 5. Colony morphological colour of MM-34 (7 days old cultures)

No.	Medium	Upper surface	Reverse surface
1	PDA	bluish-gray green at center and white at periphery, exudates present (2.7 × 2.7 cm)	pale yellow at center and white at periphery
2	MEA	dull green at center and white at periphery (3.0 cm × 2.6 cm)	pale yellowish green at center and pale yellow at periphery
3	CA	thin, white at center and thick, white at periphery (2.7 cm × 2.5 cm)	white
4	OA	light green at center and 3-concentric white at periphery, exudates present (2.5 cm × 2.3 cm)	pale yellow at center and white at periphery
5	YEA	pale red at center and white at periphery, exudates present (3.0 cm × 2.4 cm)	pale yellow

PDA - Potato Dextrose Agar, MEA - Malt Extract Agar
CA - Corn Agar, OA - Oatmeal Agar,
YEA - Yeast Extract Agar

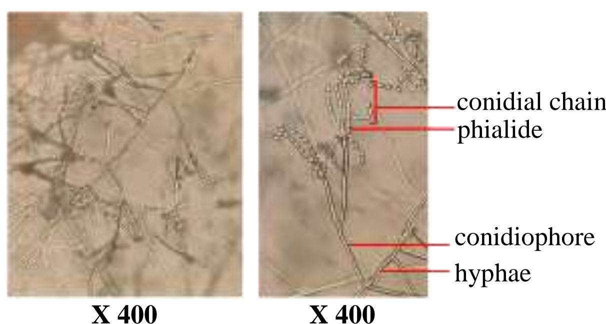


Figure 16 Photomicrograph of soil fungus MM-34

Morphological Characters of MM-34

Colonies are slow growing, produces filamentous, flat, radially sulcate colonies. These colonies are bluish-gray-green at center and white at the periphery. Reverse surface are pale yellow at center and white at periphery. Production of exudates observed at the center of the front surface.

Microscopical Characters of MM-34

Hyphae septate, conidiophores are hyaline, erect, branched, terminated by flask-shaped phialides, chains of single-celled conidia are produced in basipetal succession from a specialized conidiogenous cell called a phialide, 2-3. Conidium elliptical, catenulate.

Key to the genus *Penicillium* (Barnett, 1969)

A1 Mycelium coenocytic, septa infrequent or absent; conidia present -- (conidial PHYCOMYCETES)

* A2 Mycelium not coenocytic, with frequent septa; conidia normally present, except in a few genera ------- (FUNGI IMPERFECTI) -- B1

* B1 Conidia and conidiophores not produced within a pycnidium or acervulus ----- (MONILIALES)-- C2

B2 Not parasitic on small, soil-inhibiting animals ------------------------------(MUCORALES)

C1 Conidia more or less coiled or spirally curved, hyaline or dark ---------------------- (parts of Moniliaceae, Demaliaeae and Tuberculariaceae)

* C2 Conidia not coiled --- D1

* D1 Both conidia and conidiophores (if present) hyaline or brightly colored; conidiophores not united into sporodochia or synnemata (Moniliaceae) -------------------------------------E1

D2 Conidiophores forming a sporodochium

* E1 Conidia 1 celled, globose to short cylindrical -- F2

E2 Conidia more or less globoid, aquatic

F1 Conidiophores absent or reduced to phialides or peg-like sterigmata

* F2 Conidiophores present, although sometimes short --G2

G1 Cells of conidiophore not differing greatly from the catenulate conidia

* G2 Conidiophore and its branches distinct from conidia --------------------------------------H1

* H1 Conidiophores simple or sparingly branched; phialides, if present, not tightly clustered --- I1

H2 Conidiophores branched; conidia formed acropetally --------------------------------Morilia

* I1 Conidia catenulate -- J2

I2 Conidia not catenulate

J1 Phialides or conidia borne on swollen portion of conidiophore

* J2 Swollen fertile cells not present -- K1

* K1 Conidia borne on phialides, in basipetal chains -- L2

K2 Conidia chains not on definite phialides

L1 Conidiophores more or less in a layer; conidia in compact columns -- Melarrhizium --Myrothecium

* L2 Conidiophores not in layer; conidia usually in loose chains -----------------------------M1

* M1 Phialides in brush-like group, not divergent, not tapering -------------------------------- N2

M2 Phialides divergent, loose, tapering to a tube ----------- Paecilomyces --5----------- Spicaria

N1 Conidia truncate at base ---Scopulariopsis

* N2 Conidia globose to ellipsoid, not truncate at base ------------------------------ *Penicillium*

In the literature references, the characters of soil fungus MM-34 was identified as *Penicillium* sp.

Kingdom	Fungi
Phylum	Deuteuromycota
Class	Hyphomycetes
Order	Moniliales
Family	Moniliaceae
Genus	*Penicillium*

Discussion and Conclusion

In the course of investigation, 37 fungi were isolated from twenty different soil samples which were collected from Belu-Gyun, Chaung-Zon Township, Mon State. Feeding method was used in the isolation of soil fungi. Among 37 soil fungi, MM-34 showed the maximum antibacterial activity (36.35 mm) against *Micrococcus luteus.* Therefore, MM-34 was selected for further investigation. The present investigation was carried out to determine the optimum culture conditions required for the maximum antibacterial activity of soil fungus MM-34.

In this study, the various effects of pH utilization to the culture broth strongly influenced the growth and biosynthesis of active component by MM-34. The pH of the medium determines the rate and amount of growth and other life processes (Lilly and Barnett, 1951). The pH level of the growth medium has a marked effect on secondary metabolite production with synthesis falling rapidly either side of an optimal level. The hydrogen or hydroxyl ion concentration may have a direct effect on cell, or it may act indirectly by varying the degree of dissociation of substances in the medium.

Therefore, the change of pH is also important for the enzyme activity of microorganisms, for the intermediate products, their dissociation and solubility (Rizk *et al.,* 2007). In the present investigation, pH 5.0 is the best for the maximum antibacterial activity by MM-34 fungus suggesting the acidophilic characteristics of the isolate. Similar result had been reported earlier by Jiicheng *et al.,* (2008). It was shown that maximum antibiotics production was obtained at acidic pH.

Parameter optimization is one of the key parts of microbial production system. Temperature variation was also studied as one of the parameters for antibacterial metabolite production, a temperature range of 20ºC-40ºC was studied for maximum antibacterial activity of soil fungus MM-34. In this investigation, the highest antibacterial activity of soil fungus MM-34 was observed at 30ºC (30.52 mm clear zone). Fungi grown at different temperatures revealed 30ºC to be the optimum for maximum antibacterial activity of the selected soil fungus MM-34.

Incubation temperature is known to influence directly the overall growth and development of any organisms. It affects the physiology and subsequently the synthesis of various metabolites (Pandey *et al.,* 2005). Gunasekaran and Poorniammal (2008) have reported highest secondary metabolite production at a temperature of 30ºC in their study. Bhattacharyya and Jha (2011) and Kok and Papert (2002) also obtained maximum production of antimicrobial metabolite at 30ºC. In the present results were in agreement with those mentioned by Gunasekaran and Poorniammal (2008); Bhattacharyya and Jha (2011); Kok and Papert (2002).

In the course of investigation, the isolated soil fungus MM-34 exhibited the antibacterial activity of static culture (30.68 mm) was higher than that of shaking culture (26.65 mm). The fungi behaved in a different manner under static and shaking conditions. The analysis of the results for

antibacterial potential of soil fungi assay demonstrated static culture conditions to be more suitable for soil fungi MM-34 as compared to shaking cultures whereas shaking conditions exhibit different growth pattern in static and shaking condition varying from compact pellets to dispersed mycelium which strongly affect the production of secondary metabolites. The pellets were observed under shaking conditions while mycelial mat was seen under static conditions.

The low antibacterial activity (26.65 mm) produced by pellets under shaking conditions. This supports the earlier contention of various researchers who have used static conditions. Some group of fungi, static conditions were found to be better as reported by earlier researchers (Khadoor *et al.,* 2007; Nicoletti *et al.,* 2007; Rubini *et al.,* 2005). The result of the present work agree with Khadoor *et al.,* 2007 Nicoletti *et al.,* 2007 and Rubini *et al.,* 2005. In the present study, the isolated soil fungus MM-34 was observed on static culture was optimal condition for fermentation.

The optimum period of fermentation for antibacterial activity of soil fungus MM-34 was found to be 6 days and subsequent decline in antibacterial activity after 10th days could be due to exhaustion of nutrients available for the fungi to produce such bioactive compounds. In consonance with earlier studies where the time course for the production of antimicrobial agent differs according to the strain and cultivation conditions (Miao and Qian, 2005; Khaddor *et al.,* 2007).

And then, preliminary studies of paper chromatography was required to extract the crude extract from the fermented broth. Four kinds of different solvents were used to observe the optimum extraction ability of secondary metabolites. Hexane and Toluene, non-polar solvents, were utilized in this paper chromatography. The Rf values of hexane and Toluene were 0.00 and 0.06 respectively.

The crude extract could be extracted with ethyl acetate according to the R_f value of paper chromatography bioassay. Therefore, solvent ethyl acetate is suitable for the extraction of crude extract from the fermented broth. For the purification of active compounds from crude extract of ethyl acetate will be subjected to column chromatography.

Penicillium colonies are initially white, later to green or bluish green color. The colony surface appears flat. They are commonly called the blue or green moulds because they produce enormous quantities of greenish, bluish or yellowish spores which give them their characteristic colours. The penicillia produce conidia from conidiophores that branch near the apex, forming a brush-like structure. Conidia are globose to subglobose, smooth-walled and are produced in basipetal succession from the phialides (Royer and Lobuglio, 2004); fungus MM-34 has 2-3 phialides. Similary *Penicillium pimiteouiense* has 2-3 phialides (Peterson *et al.,* 1999), based on these Characters fungus MM-34 was assumed as genus *Penicillium.*

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