

Production and optimization of antibacterial metabolites from endophytic fungi *Nigrospora* sp. ML#3

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ABSTRACT

Ailanthus excelsa Roxb. is one of the most important plant which has a specific place in Ayurveda and phytotherapy. The tribal people generally used this plant for anthelmintic, anti-fertility, rejuvenation and anti-malarial against *Plasmodium falciparum*. In the present study total four endophytic fungi were isolated from the leaves of medicinal plant *A. excelsa* for the production of antibacterial bioactive compounds against five pathogenic bacterial strains. Various cultural conditions like media, pH, temperature and quarantine period was also optimized for the maximum production of antibacterial bioactive compounds from the potent fungus. After each optimizing step, the extracts were tested for antibacterial activity by agar well diffusion method and the best condition was selected for further process.

INTRODUCTION

Endophytic microorganisms are those that survive inside the living tissues of plants without causing any harmful effects or damages and have symbiotic relationships with host plants (Specian *et al.*, 2012). These endophytes have an ability to produce a variety of secondary metabolites (Sandhu *et al.*, 2014a). Nevertheless, increasing levels of antibiotic resistance in both nonpathogenic and pathogenic bacteria has spurred the search for new antibiotics to manage diseases (Petrini, 1991). Since the population has been increased, this was not possible to afford plant-based medicine. Due to the increasing demand of medicine and destruction of medicinal plants, a huge work carried out in the field of endophytes for producing bioactive compounds that can be used in the treatment of diseases (Onifade, 2007). Endophytes are the synthesizers inside plants that produce bioactive compounds with low toxicity toward

higher plants (Owen and Hundley, 2004). Endophytes provide an extensive variety of bioactive secondary metabolites with unique structure, synthesized via various metabolic pathways *i.e.* polyketide, isoprenoid, amino acid derivatives (Tan and Zou, 2001).

They have ability to produce a range of secondary metabolites, providing researchers with numerous leads for compounds of antibacterial, antifungal, anticancer, anti-malaria, antioxidant, anti-diabetic and agro-chemical significance, with possible development as new drugs (Strobel, 2003; Gunatilaka *et al.*, 2006). Sandhu *et al.* (2014b) isolated the endophytic fungi from *Calotropis procera* (Linn.) R.Br. of Jabalpur region, and tested their antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus epidermidis*.

The aim of the present investigation was to study the antibacterial activity and effect of various parameters to gain a high amount of antibacterial bioactive compounds from *Nigrospora* sp. ML#3 isolated from *A. excelsa* Roxb. This plant is commonly known as Maharukh belonging to family Simaroubaceae and the genus is native from southern India.

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MATERIALS AND METHODS

Collection of plant sample

Healthy and mature leaves sample of *A. excelsa* were collected from Hitkarini Engineering College campus, Jabalpur (MP) India in November 2014. The plant sample were placed in sterile plastic bags and brought to the laboratory for isolation of endophytic fungi.

Isolation of the endophytic fungi

Isolation of endophytic fungi was done according to the methods described by Peterni 1986, with some modifications. Firstly, the plant samples were immersed in 70% ethanol for 1 minute followed by 4% NaOCl for 2 minutes and then sample were rinsed in sterile distilled water. The surplus moisture was blotted in a sterile Whatman no.1 filter paper. Then the four sterilized samples were placed in each Petri plates containing PDA medium amended with Streptomycin. After that, Petri plates were incubated at 26±1°C in fungal incubator for 5 to 7 days. The Petri plates were monitored every day to check the growth of endophytic fungal colonies. The isolates so obtained were transferred on Potato Dextrose Agar slants and preserved at 4°C for further studies.

Morphological identification of isolated fungi

The endophytic fungal isolates were identified based on macroscopic and microscopic characteristics such as color, shape and size of spores, hyphal features and reproductive structures (Subramanian, 1971; Domsch *et al.*, 2007).

Test organisms

The cultures of test bacteria *Bacillus subtilis* MTCC441, *Escherichia coli* MTCC1679, *Klebsiella pneumoniae* MTCC4032, *Salmonella typhimurium* MTCC733 and *Staphylococcus aureus* MTCC96 were obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. The organisms were preserved at 4 °C in the presence of glycerol (15 %, v/v) for longer periods.

Cultivation and extraction

The fungal isolate was grown in 50 ml Potato Dextrose Broth (PDB) in 250 ml Erlenmeyer flask at 26±1°C in incubator for 14 days under stationary conditions. The broth cultures were filtered with sterile Whatman no. 1 filter paper to separate the filtrate and mycelia. The filtered broth was centrifuged at 6000 rpm in cooling centrifuged for 10 minute. After centrifugation, metabolite was extracted with the help of solvents like Hexane, Benzene, ethyl acetate and n-Butanol by solvent-solvent extraction procedure. Equal volume of solvent and metabolite were taken in the separating funnel and mixed well by vigorous shaking for 10 to 15 minutes. Separating funnel was allowed to settle until two immiscible layers were formed. The upper layer containing the extracted compounds was separated and collected in another flask. Then the solvent extracts were evaporated in Rotary Vacuum

Evaporator (Buchi R-300 Rotavapor, Buchi Co. Germany). The extract residue was dissolved in de-ionized water and stored at 4°C to be used as stock solution for antibacterial activity.

In vitro Antibacterial screening

For antibacterial activity, Agar Well Diffusion method was performed by standard process (Newyork *et al.*, 1972). Nutrient Agar media (NAM) plates were inoculated with overnight grown bacterial suspension of *Bacillus subtilis*, *Salmonella typhimurium*, *Escherichia coli*, *Streptococcus pyogenes* and *Klebsiella pneumoniae*. The agar wells were prepared by scooping out the media with a sterile cork borer (8 mm in diameter). After that, wells were filled with 80 µl of the fungal extract. Then the inoculated plates were incubated at 37°C for 24 hrs and the zone of inhibition was recorded by Himedia antibiotic Zone Scale. The zone of inhibition given by the secondary metabolites of fungal isolate was also compared with an antibiotic (streptomycin). Three replicates were carried out for every antibacterial activity test. Mycelia growth rate of the potent strain was determined as dry mycelial weight by drying the cell mass in an oven at 80°C overnight and expressed as dry weight of mycelia (mg/ 50ml).

Optimization of potent fungus for maximum production of bioactive compound

Effect of culture media

To select the suitable growth medium, the potent fungal isolate *Nigrospora* sp.ML#3 was grown in different growth media such as Asthana and Hawkers Broth, Czapek's Dox Broth, Potato Dextrose Broth, Richard's Broth and Sabouraud's Dextrose Broth. For bioactive metabolite production, the medium in which the isolate exhibited maximum antibiotic production expressed in terms of zone of inhibition was used as the optimized medium for further study.

Quarantine period

The time period for the maximum production of antibacterial compound from *Nigrospora* sp.ML#3 was studied from one to sixteenth days. The Erlenmeyer flasks containing Potato Dextrose broth was inoculated with fungi and incubated in fungal incubator in stationary condition. The mycelia free culture filtrate was extracted from the first day onwards and antibacterial activity was performed by applying agar well diffusion method.

Effect of pH

To check the effect of pH on the production of bioactive compound from *Nigrospora* sp.ML#3, pH ranges were adjusted from 3.0 to 9.0 in the basal medium. After incubation, the metabolites were separated through filter paper, their antibacterial activity was tested against test pathogen, and zone of inhibition was observed.

Effect of Temperature

Temperature plays a significant role in the production of metabolite from the fungi. Therefore, different temperature ranges

from 15 °C to 45 °C were taken for study the optimum temperature required for growth and production of bioactive metabolite yield. Basal medium was inoculated with 8 mm diameter of the culture disc and incubated for 14 days. After incubation, the dry mycelia weight and the antibacterial production were recorded.

Statistical analysis

Values were presented as means \pm SD of at least 3 replicate experiments. Differences between groups analyzed using ANOVA test significant $P < 0.05$ was considered to be statistical significant. Data analysis was conducted using Statistical Package for Social Sciences (SPSS 16.0 version).

RESULT AND DISCUSSION

Isolation of endophytic fungi

A total of four endophytic fungi were isolated from healthy leaves of *A. excelsa* Roxb, which were collected from Hitkarini engineering college Campus, Jabalpur (MP) India as shown in Table 1. Similarly, Sandhu *et al.* (2014c) isolated 15 species of endophytic fungus from *Bombex cebia* and *Argemone mexicana* successfully.

Table 1: Endophytic fungi isolated from *Ailanthus excelsa* Roxb.

Local Name	Botanical name	Plant Part	Isolated strain
Maharukh	<i>Ailanthus excelsa</i> Roxb.	Leaves	ML#1
			ML#2
			ML#3
			ML#4

Morphological identification of Endophytic Fungi

Slide culture techniques were used for identification of isolated endophytic fungi by using the standard protocol of Subramanian (1971); Domsch *et al.* (2007). The fungal strains were identified on the basis of macroscopic and microscopic characteristics as seen under the microscope as shown in Fig 1. The following endophytic fungi were identified:

Aspergillus niger (Tiegh.)

Moderately grown colonies, submerged mycelium, reverse colorless to pale yellow. Exudates limited or lacking with minute droplets, conidial heads large and black globes, conidiophores arising from the substratum varying from 200 μ m to several millimeters long and 10-20 μ m diameter, smooth vesicle globose, phialides borne directly on the vesicles in some species.

Alternaria sp.

Colonies expanding grey to olivaceous, powdery or fatty. Diagnostic characteristics of *Alternaria* genus include the

formation of chains of dark-colored, multi-celled conidia with beaks of tapering apical cells. Conidiophores unbranched, with one or few conidial scars. Conidia are ellipsoidal, with short, cylindrical beak.

Nigrospora sp.

Mycelium immersed on partly superficial, stroma none, state and hyphodia absent, conidiophores micro filamentous, branched flaxus colorless.

Curvularia lunata (Wakker) Boedjijn

Colonies effuse, brown, cottony, conidiophores are mononematous, macronematous, straight, often geniculate, sometimes nodose, brown usually smooth. Conidia solitary, acropleurogenous, simple, often curved, clavate, ellipsoidal, broadly fusiform with 3 transverse septa, dark brown, usually the end ones paler than the others, sometimes with dark bands at the septa, hilum scarcely, smooth-walled, middle septum not median, 20-28 X 8 - 14 μ m. Sandhu *et al.* (2014d) also isolated and identified 10 fungal species of endophytic fungi including *Aspergillus fumigates*, *Aspergillus japonicas*, *Aspergillus niger*, *Fusarium semitectum*, *Curvularia pallescens*, *Phoma hedericola*, *Alternaria tenuissima*, *Fusarium solani*, *Drechslera australien* and *Aspergillus repens* from medicinal plant *Ricinus communis*.

In vitro Antibacterial Activity

Antibacterial activity was carried out on the ethyl acetate extracts of the four fungal species against the five pathogenic bacteria. The antibacterial activity of the fungal extracts is shown in Table 2 and Fig 2. The values of zone of inhibition were highly variable, ranging between 05-20 mm. *Nigrospora* sp.ML#3 showed the maximum zone of inhibition against *K. pneumoniae* (20.26 mm), *E. coli* (17.27 mm), *B. subtilis* (15.47 mm) *S. typhimurium* (12.07 mm) and it gave minimum zone of inhibition against *S. pyogenes* (10.20 mm) and the zone of inhibition given by the antibiotic against test bacteria strain (streptomycin) was in the range of between 20.26 mm and 17.27 mm. Similarly, *Curvularia lunata* also showed good zone of inhibition against *K. pneumoniae* (16.37 mm), *E. coli* (15.18 mm), *S. pyogenes* (12.25 mm), *B. subtilis* (10.32 mm) and *S. typhimurium* (10.28 mm). Other fungi like *Aspergillus niger*, *Alternaria* sp. were also observed for their antibacterial activity. Similarly, Sandhu *et al.* (2014b) isolated 12 endophytic fungi from *Calotropis procera* (Linn.) R.Br. and evaluate their antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Staphylococcus epidermidis*.

Table 2: *in vitro* antibacterial activity against five pathogenic bacteria.

Name of pathogenic bacteria	Zone of inhibition (in mm)				
	<i>Aspergillus niger</i>	<i>Nigrospora</i> sp.	<i>Alternaria</i> sp.	<i>Curvularia lunata</i>	Streptomycin (1mg/ml)
<i>B. subtilis</i>	05.11 \pm 0.23	15.47 \pm 0.25	08.24 \pm 0.05	10.32 \pm 0.11	16.16 \pm 0.11
<i>E. coli</i>	10.43 \pm 0.19	17.27 \pm 0.31	18.11 \pm 0.15	15.18 \pm 0.05	19.20 \pm 0.10
<i>K. pneumoniae</i>	09.14 \pm 0.25	20.26 \pm 0.10	17.13 \pm 0.19	16.37 \pm 0.31	15.46 \pm 0.15
<i>S. typhimurium</i>	12.21 \pm 0.42	12.07 \pm 0.26	10.15 \pm 0.30	10.28 \pm 0.19	17.23 \pm 0.15
<i>S. pyogenes</i>	10.15 \pm 0.31	10.20 \pm 0.05	15.19 \pm 0.25	12.25 \pm 0.42	16.40 \pm 0.10

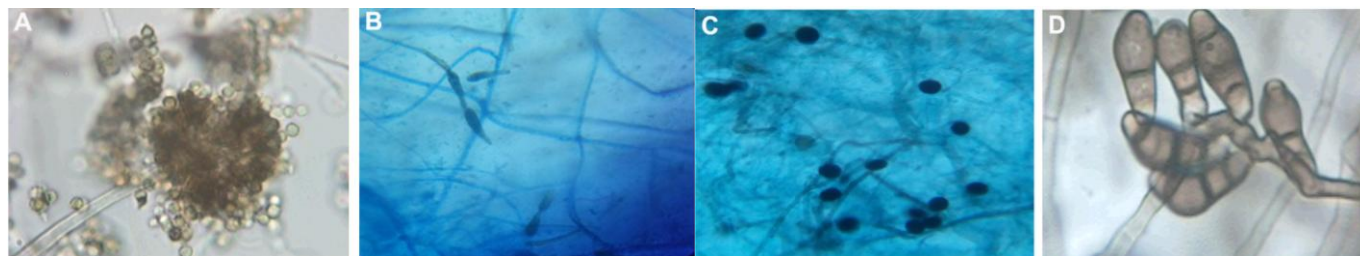


Fig. 1: Light microscopic (100X) observation of endophytic fungi (A): ML#1 - *Aspergillus niger* (B) ML#2 - *Alternaria* sp. (C) ML#3 - *Nigrospora* sp. (D) ML#1 - *Curvularia lunata*

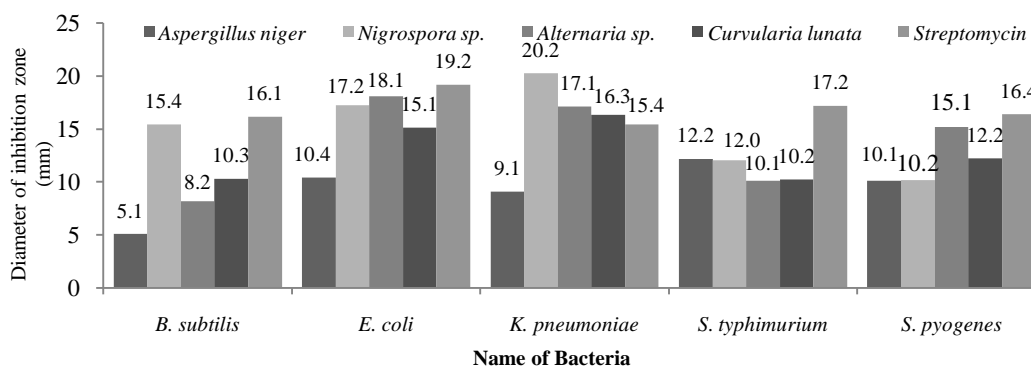


Fig. 2: *in vitro* antibacterial activity against five pathogenic bacteria.

Table 3: Effect of different quarantine period on biomass and bioactive metabolite production of *Nigrospora* sp ML#3.

Quarantine days	Dry weight of mycelia (g/50ml)	Zone of inhibition (in mm)				
		<i>K.pneumoniae</i>	<i>S. typhimurium</i>	<i>B.subtilis</i>	<i>S. pyogenes</i>	<i>E. coli</i>
3	0.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00
4	0.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00
5	0.07±0.34	08.5±0.20	09.53±0.15	00.00±0.00	00.00±0.00	00.00±0.00
6	0.13±0.19	10.33±0.25	09.36±0.25	00.00±0.00	11.16±0.11	00.00±0.00
7	0.18±0.15	11.10±0.25	10.36±0.11	00.00±0.00	12.43±0.15	00.00±0.00
8	0.31±0.26	13.10±0.20	12.60±0.11	00.00±0.00	12.40±0.10	00.00±0.00
9	0.46±0.25	13.26±0.20	14.63±0.15	00.00±0.00	13.66±0.26	09.30±0.15
10	0.59±0.31	14.61±0.10	16.53±0.25	00.00±0.00	13.20±0.17	09.30±0.25
11	0.62±0.10	16.56±0.05	17.05±0.10	12.60±0.20	14.40±0.26	12.53±0.25
12	0.68±0.15	19.30±0.20	17.73±0.23	18.36±0.15	15.56±0.41	15.23±0.05
13	0.67±0.36	13.23±0.15	14.40±0.26	16.40±0.10	12.46±0.11	18.66±0.32
14	0.61±0.25	13.36±0.15	12.36±0.25	14.33±0.19	10.33±0.15	15.40±0.26
15	0.45±0.05	09.50±0.23	08.36±0.32	08.43±0.15	07.50±0.29	11.23±0.21
16	0.19±0.26	05.51±0.19	00.00±0.00	00.00±0.00	00.00±0.00	07.25±0.32

Optimization of parameters for maximum production of bioactive compounds

Effect of quarantine period

The aim of present work was to optimize various physical parameters for maximum production of novel antibacterial bioactive compounds from the isolated fungal isolates. *Nigrospora* sp. was incubated for 16 days and each day it was observed for the antibacterial activity against pathogenic bacteria. It was found that it gave best result on 12th day of quarantine as shown in Table 3 and Fig 3. Bhattacharyya and Jha, (2011) detect the effect of quarantine period on the production of bioactive compound (Fumonisin B1) by *Fusarium moniliforme*. Similarly, Stinson, (2003) also observed similar results in the case of endophyte *Gliocladium* sp.

Effect of culture media

Five different basal media namely Saboraud's Dextrose Broth (SDB), Richard's Broth (RB), Potato Dextrose Broth (PDB),

Czapex Dox Broth (CDB) and Asthana and Hawker's broth (AHB) media were selected for maximum production of antibacterial bioactive compounds from *Nigrospora* sp. ML#3 and SDB was found to be the best media for maximum production of antibacterial bioactive compound against 5 pathogenic bacteria named *K. pneumoniae* (16.45 mm), *S. typhimurium* (12.53 mm), *S. pyogenes* (12.43 mm) and *E. coli* (11.56) followed by Potato Dextrose Media.

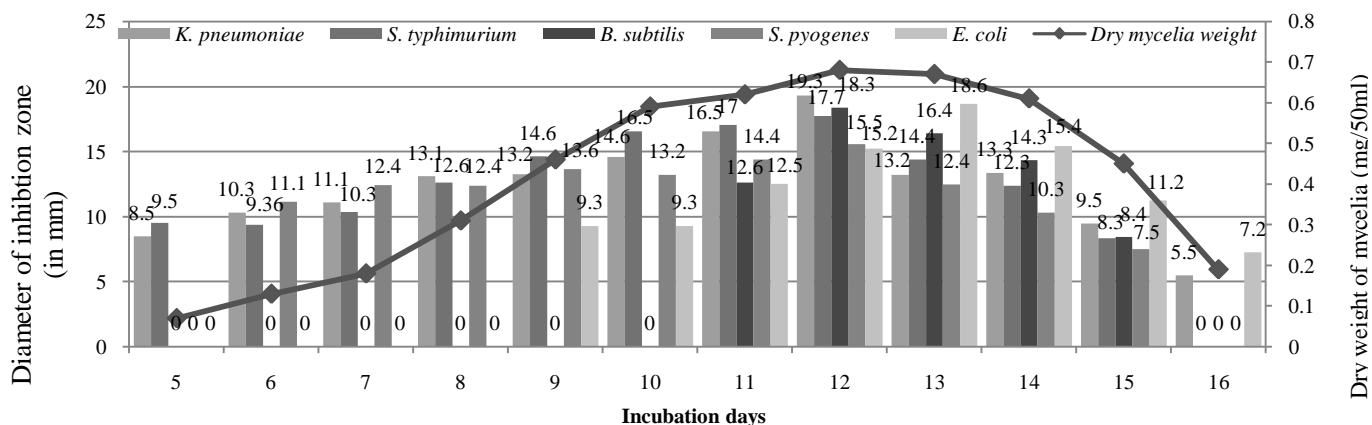
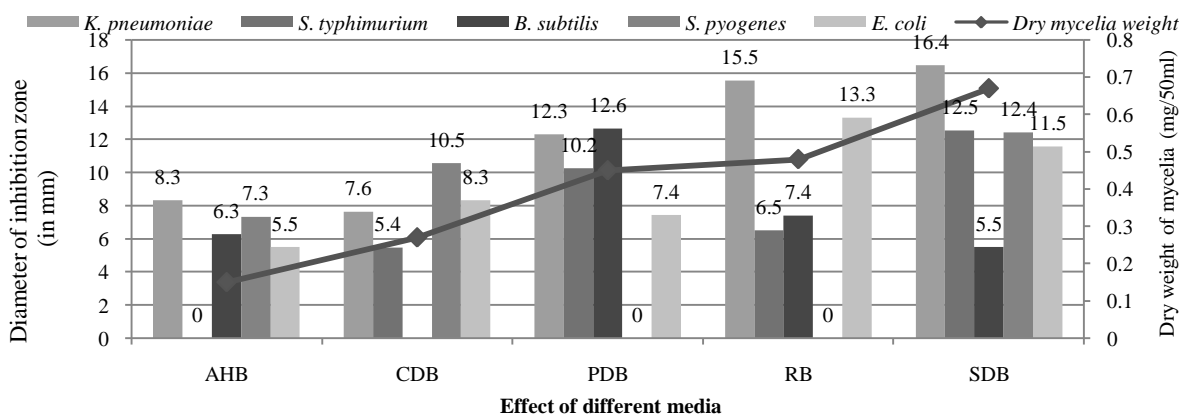
Maximum mycelial dry weight (0.67 g/50 ml) was also recorded in SDB medium with maximum zone of inhibition (16.45 mm) against *K. pneumoniae* as depicted in Table 4 and Fig 4.

Similarly, Zain *et al.* (2009) reported that the growth and bioactive metabolites production from *Penicillium janthinellum*, *Penicillium duclauxii* and *Aspergillus terreus* were extensively depended on the type of growth medium and further yeast extract showed the best mycelial growth and bioactive metabolite production.

Table 4: Effect of different growth media on biomass and bioactive metabolite production of *Nigrospora* sp. ML#3

Name of Media	Dry weight of mycelia (g/50ml)	Zone of inhibition (in mm)				
		<i>K. pneumoniae</i>	<i>S. typhimurium</i>	<i>B. subtilis</i>	<i>S. pyogenes</i>	<i>E. coli</i>
AHB*	0.15±0.15	08.33±0.25	00.00±0.00	06.30±0.17	07.33±0.20	05.50±0.10
CDB*	0.27±0.47	07.63±0.15	05.46±0.20	00.00±0.00	10.56±0.15	08.33±0.05
PDB*	0.45±0.25	12.30±0.10	10.26±0.15	12.66±0.25	00.00±0.00	07.43±0.11
RB*	0.48±0.26	15.53±0.25	06.53±0.11	07.40±0.30	00.00±0.00	13.30±0.17
SDB*	0.67±0.41	16.45±0.25	12.53±0.25	05.50±0.20	12.43±0.41	11.56±0.05

*Asthana and Hawker's broth (AHB); Czapek Dox broth (CDB); Potato Dextrose broth (PDB); Richard's broth (RB) and Saboraud's Dextrose broth (SDB)

**Fig. 3:** Effect of different incubation period on biomass and bioactive metabolite production of *Nigrospora* sp. ML#3**Fig. 4:** Effect of different growth media on biomass and bioactive metabolite production of *Nigrospora* sp. ML#3

Effect of pH

The production of antibacterial bioactive compounds from endophytic fungi was observed on different pH (3, 4, 5, 6, 7, 8 and 9). The result indicated that pH 7.0 was suitable for growth and maximum production of antibacterial bioactive compound from *Nigrospora* sp. ML#3. It showed highest zone of inhibition against *K. pneumoniae* (20.30 mm), *S. typhimurium* (17.50 mm), *B. subtilis* (14.26 mm), *S. pyogenes* (16.60 mm) and *E. coli* (13.46 mm) as depicted in Table 5 and Fig 5. Maximum mycelial growth (0.57 g/50ml) was also recorded at pH 7.0 and minimum growth and antibacterial activity was observed at pH 4.0. Similarly, Jain and Pudir, (2011) reported the maximum bioactive metabolite production by *Aspergillus terreus* in Potato Dextrose broth at pH 6.0. Gogoi et al. (2008) studied the influence of pH on the growth and production of bioactive metabolite by an endophyte *Hypocrea* sp. NSF-08 isolated from *Dillenia indica* Linn. in North-East, India. Similarly, Digrak and Eluk, (2011) also observed the highest

production of biomass by *F. equiseti* at pH 8, while maximum toxic metabolite was produced at the pH 5.

Effect of temperature

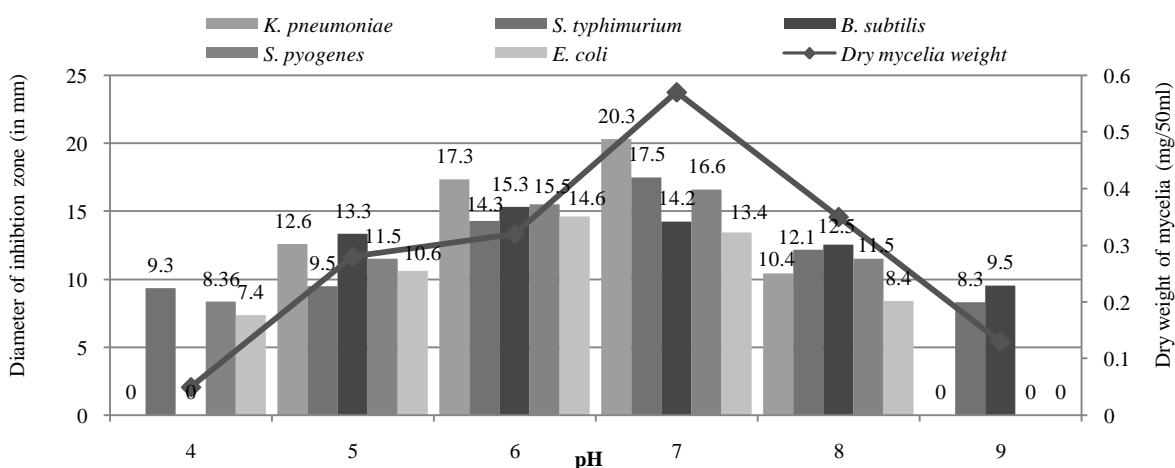
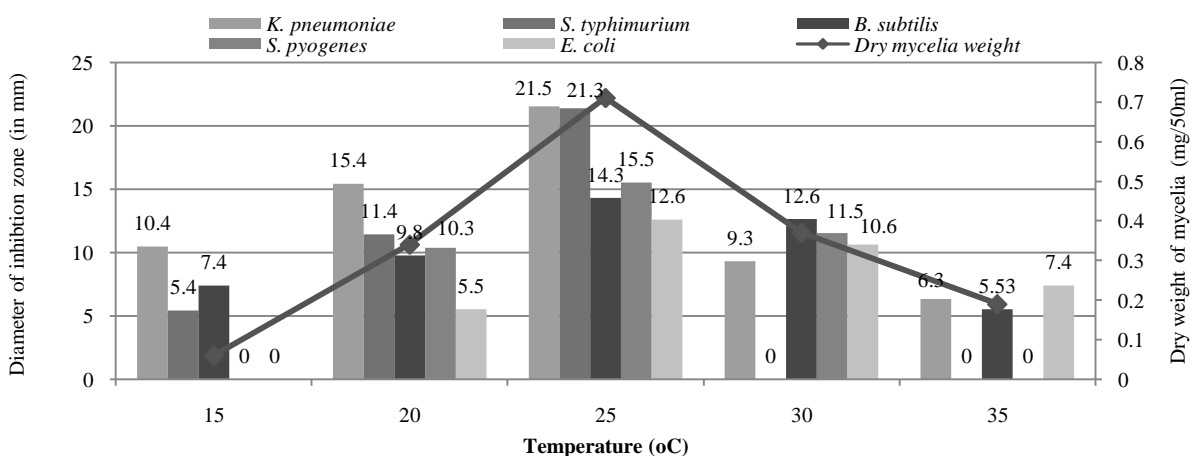
For maximum production of bioactive compounds from fungi *Nigrospora* sp. ML#3, it was incubated at different temperature (15, 20, 25, 30, 35, 40 and 45). Increase in quarantine period from 20°C to 30°C enhanced the growth and production of bioactive metabolite. Maximum mycelia growth (0.71 g/ml) and zone of inhibition was observed at 25°C against *K. pneumoniae* (21.53 mm), *S. typhimurium* (21.36 mm), *S. pyogenes* (15.50 mm) and *B. subtilis* (14.30 mm) as shown in Table 6 and Fig 6. However, minimum zone of inhibition was observed against *E. coli* (12.60 mm). Bhattacharyya and Jha, (2011) reported that, the increase of the incubation temperature from 25°C to 30°C enhanced the growth of mycelia and production of bioactive metabolite in *Aspergillus* strain.

Table 5: Effect of different pH on biomass and bioactive metabolite production of *Nigrospora* sp.ML#3

pH	Dry weight of mycelia (g/50ml)	Zone of inhibition (in mm)				
		<i>K. pneumoniae</i>	<i>S. typhimurium</i>	<i>B. subtilis</i>	<i>S. pyogenes</i>	<i>E. coli</i>
3.0	0.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00
4.0	0.05±0.19	00.00±0.00	09.36±0.15	00.00±0.00	08.36±0.15	07.40±0.20
5.0	0.28±0.25	12.60±0.10	09.50±0.20	13.33±0.20	11.50±0.10	10.60±0.17
6.0	0.32±0.31	17.36±0.15	14.30±0.10	15.33±0.05	15.50±0.10	14.63±0.25
7.0	0.57±0.24	20.30±0.26	17.50±0.10	14.26±0.15	16.60±0.20	13.46±0.15
8.0	0.35±0.15	10.43±0.11	12.16±0.11	12.56±0.05	11.53±0.05	08.43±0.20
9.0	0.13±0.10	00.00±0.00	08.33±0.25	09.56±0.05	00.00±0.00	00.00±0.00

Table 6: Effect of different temperature on biomass and bioactive metabolite production of *Nigrospora* sp.ML#3

Temp. (°C)	Dry weight of mycelia (g/50ml)	Zone of inhibition (in mm)				
		<i>K. pneumoniae</i>	<i>S. typhimurium</i>	<i>B. subtilis</i>	<i>S. pyogenes</i>	<i>E. coli</i>
15	0.06±0.37	10.33±0.15	05.46±0.15	07.43±0.05	00.00±0.00	00.00±0.00
20	0.34±0.15	15.43±0.23	11.43±0.11	09.80±0.10	10.36±0.15	05.56±0.05
25	0.71±0.23	21.53±0.25	21.36±0.15	14.30±0.02	15.50±0.10	12.60±0.10
30	0.37±0.25	09.30±0.10	00.00±0.00	12.63±0.15	11.53±0.15	10.63±0.34
35	0.19±0.37	06.36±0.05	00.00±0.00	05.53±0.11	00.00±0.00	07.40±0.26
40	0.03±0.10	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00	00.00±0.00

**Fig. 5:** Effect of different pH on biomass and bioactive metabolite production of *Nigrospora* sp.ML#3.**Fig. 6:** Effect of different temperature on biomass and bioactive metabolite production of *Nigrospora* sp. ML#3

CONCLUSION

A total four endophytic fungi *Aspergillus niger*, *Nigrospora* sp., *Aspergillus* sp. and *Curvularia lunata* was isolated from the medicinal plant *A. excelsa*. The endophyte *Nigrospora* sp. ML#3 has showed greater antibacterial activity against five pathogenic bacteria in this study. The maximum production of

the anti-bacterial metabolites by *Nigrospora* sp. was achieved by optimizing various parameters like media (SDB), quarantine period (12 days), temperature (25°C) and (pH 7) under aseptic conditions were found to be optimum for the maximal production of bioactive metabolite. These findings will assist in formulating a suitable culture medium for production of the antibacterial compound from *Nigrospora* sp. and further prospectus of this

study can be applied in drug production like antibiotics. Our study reports the optimization of still culture conditions for the maximum growth and production of bioactive secondary metabolite compounds from *Nigrospora* sp. ML#3.

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