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Research article

ANTIBACTERIAL ACTIVITY OF SOME ISOLATED ENDOPHYTIC FUNGI FROM *MENTHE VIRIDIS*

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ABSTRACT: The aim of the present study was to isolate, identify and observe antibacterial activity of the endophytic fungi against some pathogenic bacteria as well as to optimize various parameters for maximum production of antibacterial bioactive compounds. Endophytic fungi were isolated from *Menthe viridis* collected from Khamariya, Jabalpur M.P. (India). Screening of endophytic fungi for *in-vitro* antibacterial activity against six pathogenic bacteria i.e. *Bacillus subtilis*, *Streptococcus pyogenes Escherichia coli*, Klebsiella *pneumoniae*, *Salmonella typhimurium* and *Enterococcus* sp. was seen by agar well diffusion method. The potent fungus was optimized for suitable media, pH, temperature and salt concentration to obtain the maximum production of bioactive compound. A total eight endophytic fungi *Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium solani*, *Aspergillus repens*, *Alterneria alternata*, *Alternaria* sp., *Phoma hedericola* and *Fusarium oxysporum* were isolated and evaluated with respect to their antibacterial activity against six pathogenic bacteria. In the present study, the fungal strain *Fusarium oxysporum* produced potent antibacterial bioactive compounds and may be commercially browbeaten for the development of novel drugs.

Key words: Endophytic Fungi, Bioactive Compounds, *Menthe viridis*, Optimization, Antibacterial activity

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INTRODUCTION

In the present era, the need for the discovery of new bioactive compounds for the development of drugs to target the microorganisms, which cause pathogenic effects on human beings, has increased. In the past, plants extracts were the only source for the treatment of various diseases, because they produce novel bioactive compounds. However, in recent, a great attention has turned towards endophytes as they demonstrate a great potential source for new bioactive compounds (Strobel, 2003). These are the microorganisms which live inside the plants without causing any harm to their host (White et al., 2001; Padhi et al., 2013). Approximately, all kinds of microorganisms like fungi, bacteria and actinomycetes are setup within plants as endophytes which protect their host from adverse conditions and infectious agents by producing bioactive compounds (Strobel et al., 2003; Sandhu et al., 2014). The novel bioactive compounds produced by the endophytes are not only important from the perspective of ecological system but also from biochemical and molecular standpoint.

A lot of endophytic fungi have been reported to produce novel antibacterial, antiviral, anti-inflammatory, antitumor, antifungal and other compounds belong to the flavonoids, alkaloids and terpenoids (Guo et al., 2007; Yu et al., 2010; Aly et al., 2011; Guiterrez et al., 2102). Sandhu et al. (2014) isolated the endophytic fungi from some medicinal plants of Jabalpur region, and tested their antibacterial activity against *E. coli, K. pneumoniae, S. pyogenes, S. typhi, B. subtilis* and *Enterococcus* sp. Similarly, Subbulakshmi et al. (2012) isolated 270 endophytic fungi of 20 different genera from leaf of three different types of gymnosperm plants. Along with the isolates, *Alternaria* sp., *Colletotrichum gloeosporioides*, *Pestalotiopsis* sp., *Fusarium* sp., *Pestalotiopsis* sp. were selected to study the production of antimicrobial compounds against bacterial and fungal pathogens. In another study, many endophytic fungi were isolated from the *Calotropis procera* and their antimicrobial activity was observed against a number of bacterial and fungal strains (Sandhu et al., 2014). The present study was carried out to isolate the endohpytic fungi from different plants of Jabalpur, M.P. (India) to observe their antibacterial activity and optimization of various parameters for maximum production of antibacterial novel bioactive compounds.

MATERIALS AND METHODS

Survey and Collection of endophytic fungi

In this work, the fungal strains were isolated from the medicinal plant Mint (*Menthe viridis*) collected from Khamariya region of Jabalpur, M.P. (India) in January-February months. Healthy and mature plants were carefully chosen for sampling. The plant material was brought to the laboratory in sterile bags and processed within a few hours after sampling. Fresh plant materials were used to reduce the chances of contamination. Different parts *i.e.* stems, leaves and roots were sampled for the isolation of endophytic fungal strains.

Isolation of Endophytic fungi

Endophytic fungi from the medicinal plants were isolated by using the protocol of Strobel et al. (2001) and by some modifications of Radu et al. (2002) method. The different plant parts such as leaves stem and roots were washed in the running tap water for 1-1.30 hours and then cut into small segments about 1-1.5 cm. The samples were surface sterilized by modified method of Dobranic et al. (1995). Then these samples were immersed in 70% ethanol for 1 minute followed by 4% sodium hypochlorite for 1- 2 minutes and then rinsed in sterile distilled water for 1 minute. After proper sterilization, the samples were placed on Whatman No.1 filter paper for removal of moisture and four samples were transferred in each PDA plate containing a pinch of antibiotic (Chloremphanicol). After inoculation, all the plates were incubated into the fungal incubator at 26ºC±1 for 5 to 7 days. The isolates so obtained were relocated on potato dextrose agar slants and preserve at 4^{o}C for further studies.

Calculation of colonizing frequency

The colonization frequency of endophytic fungi was also calculated as stated by Suryanarayanan et al. (2003). Colonization frequency of an endophyte species is equal to the number of segments colonized by a single endophyte divided by the total number of segments observed X 100.

$$\text{Colonizing frequency \%} = \frac{\text{Number of segments colonized by single endophyte}}{\text{Total number of segments observed}} \text{ X } 100$$

Identification of Endophytic fungi

The identification of fungi was done on the basis of their morphological characterization. Total eight fungal strains were identified according to their macroscopic features such as shape, growth and color of the cultured colonies as well as microscopic characteristics like structure of conidia, hyphae and spore size etc.

Fermentation and production of secondary metabolites

For the production of secondary metabolites, the fungi were grown on Potato Dextrose broth (PDB) and incubated at 26±1^{o}C for 7, 14 and 21 days respectively. After the corresponding days, the metabolites were alienated with the help of filter paper for testing their antibacterial activity against bacterial strain.

Test bacteria

Six pathogenic test bacteria such as *Klebsiella pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, *Enterococcus* sp., *Streptococcus pyogenes* and *Salmonella typhimurium* were obtained from MTCC, Chandigarh (India).

Test of antibacterial activity

The antibacterial activity of fungal metabolites was tested by using agar well diffusion assay (Lorian et al., 1996). In this method, wells were aseptically made in the seeded media and appropriate amount of the bioactive metabolites were dropped in the prepared wells and incubated at 37°C in bacteriological incubator for 24 hrs. Finally, plates were observed for zones of inhibition and their diameters were measured with the help of Hi-Antibiotic zone scale, Hi Media Laboratories Mumbai.

Optimization

In the present study, all experiments were carried out to detect the effects of various parameters like incubation period, media, temperature, pH and salt concentration for the maximum production of novel antibacterial bioactive compounds.

Effect of media

One of the objectives of this experiment was to select the suitable media for maximum production of bioactive compounds from *Fusarium oxysporum*. For this, five media *i.e.* Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB), Asthana and Hawker media (A&H), Muller Hinton media (MH) and Richard Broth media (RB) were used to analyze the effectiveness of antibacterial activity of isolated fungal strain.

Incubation period

Effect of incubation period on the production of bioactive compound was studied on *Fusariun oxysporum* for 20 days. 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

For the optimization of the pH of the media, the fermentation broth of six different pH values *viz.* (3, 4, 5, 6, 7, 8, 9 and 10) was prepared. For each pH value, 100 ml PDB was taken and inoculated with a disc of fungi and incubated for 11 days at 26±1^{o}C. After incubation, the metabolites were separated using filter paper and their antibacterial activity was tested against test pathogen by agar well diffusion method.

Effect of Temperature

The incubation temperature directly affects the overall growth and development of the organism and subsequently, the synthesis of various metabolites. Productions of bioactive antibacterial compounds were observed at different temperatures like 15^{o}C, 20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C and 40^{o}C.

Effect of NaCl concentration

To study the effect of salinity on the growth and production of antibacterial bioactive compounds, the fungal strain was grown in different concentration of NaCl salt ranging from 1g/l - 5g/l into the basal media.

RESULTS

Isolation and identification of fungi

In the current study, endophtyic fungi were isolated from the Mint (*Menthe viridis*) collected from Khamariya region of Jabalpur M.P. (India). 30 segments (12 leaves, 12 stem and 6 segments of root) of Mint (*Menthe viridis*) were processed for the isolation of endophytic fungi. Total eight endophytic fungi were isolated from which three belong to class Eurotiomycetes two from Sordariomycetes and three belong to Dothideomycetes as shown in Table 1.

Table 1: Endophytic fungi isolated from *Menthe viridis*

Plant parts	Fungal isolates	Class
Leaf	*Aspergillus fumigatus*	Eurotiomycetes
	Fusarium solani	Sordariomycetes
	Aspergillus repens	Eurotiomycetes
	Fusarium oxysporum	Sordariomycetes
Stem	*Aspergillus niger*	Eurotiomycetes
	Phoma hedericola	Dothideomycetes
Root	*Alterneria alternata*	Dothideomycetes
	Alternaria sp.	Dothideomycetes

Table 2: Colonizing frequencies of Endophytic fungi isolated from *Menthe viridis*

Name of Fungi	Plant parts	% frequency of colonization	No. of isolates
Aspergillus fumigatus	Leaf	25.00	3
Fusarium solani	Leaf	16.66	2
Aspergillus repens	Leaf	25.00	3
Alterneria alternata	Leaf	33.33	4
Aspergillus niger	Stem	33.33	4
Phoma hedericola	Stem	08.33	1
Alternaria sp.	Root	83.33	5
Fusarium oxysporum	Root	66.66	4

Identification and colonizing frequency of endophytic fungi

Isolated endophytic fungi were successfully identified on the basis of their macroscopic and microscopic characterization. These fungi are *Aspergillus fumigatus, Aspergillus niger, Fusarium solani, Aspergillus repens, Alterneria alternata, Alternaria* sp, *Phoma hedericola* and *Fusarium oxysporum* as depicted in Table 2 & Figure 1.

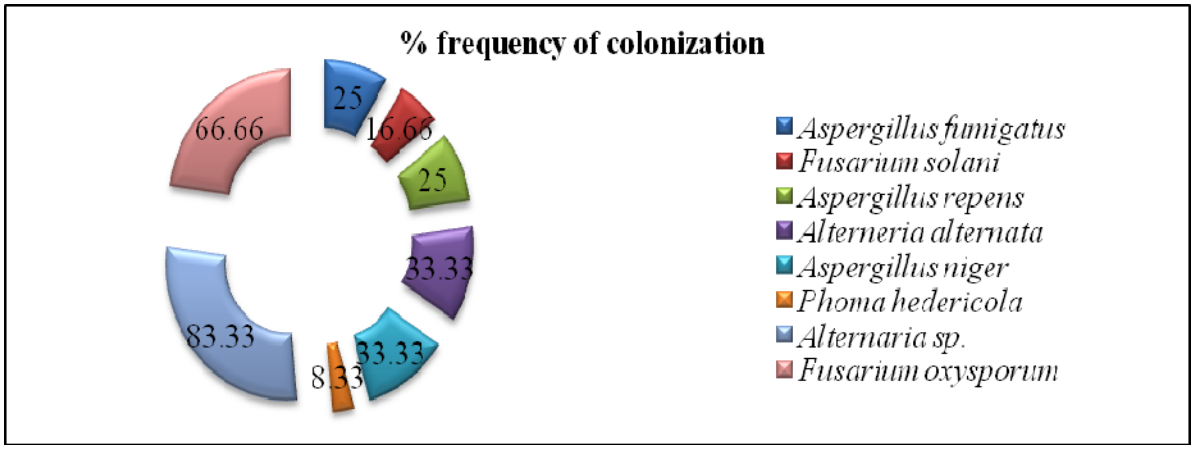

Figure 1: % of colonization frequency

Screening for antibacterial activity of fungi

For screening the antibacterial activity of endophytic fungi, all the strains were inoculated in PDB for 7, 14 and 21 days, incubated at 26±1°C in fungal incubator. Out of eight fungi, only two gave the maximum activity against the pathogenic bacteria on 14th day of inoculation (Table 3 & Figure 2). The maximum zone of inhibition was given by *Fusarium oxysporum* against *Bacillus subtilis* (25 mm), *Enterococcus* sp. (24 mm), *E. coli* (23 mm), *S. typhimurium* (21 mm) *S. pyogenes* (20 mm) and *Klebsiella pneumoniae* (18mm). Similarly, *Fusarium solani* showed maximum zone of inhibition against *B. subtilis* (23 mm), *E. coli* (21 mm), *S. typhimurium* (19 mm) *Enterococcus* sp. (16 mm), *S. pyogenes* (14 mm) and gave minimum zone of inhibition against *Klebsiella pneumoniae* (10 mm). *Phoma hedericola* also showed the better zone of inhibition against *S. typhimurium* (19 mm), *E. coli* (22 mm), *B. subtilis* (17 mm), *S. pyogenes* (15 mm) and similar zone of inhibition was shown against *Enterococcus* sp. and *Klebsiella pneumoniae* (13 mm). The other fungi like *Aspergillus fumigatus, Aspergillus niger, Aspergillus repens, Alternaria alternata* and *Alternaria* sp. also gave antibacterial activity against the pathogenic bacteria.

Table 3: Screening of endophytic fungi against six bacterial strains

Fungal strain	Zone of Inhibition					
	E. coli	***K. pneumoniae***	***S. typhimurium***	***S. pyogenes***	***B. subtilis***	***Enterococcus* sp.**
Aspergillus fumigatus	12	-	-	6	13	10
Aspergillus niger	9	-	11	8	11	7
Fusarium solani	21	10	19	14	23	16
Aspergillus repens	16	10	14	-	8	-
Alterneria alternate	11	14	-	10	13	-
Alternaria sp.	13	8	10	-	11	-
Fusarium oxysporum	23	18	21	20	25	24
Phoma hedericola	22	13	19	15	17	13

Effect of media

Five different basal media (depicted in Table 4 & Figure 3) namely Saboraud Dextrose medium (SDM), Richard Broth (RB), Potato Dextrose Broth (PDB), Muller and Hinton media (M&H) and Asthana and Hawker (A&H) were selected for maximum production of antibacterial bioactive compounds from *Fusarium oxysporum.* PDB was found to be the best media for maximum production of antibacterial bioactive compound against bacterial strain *E. coli* (24 mm), *Enterococcus* sp. (25 mm), *S. pyogenes* (22 mm), *B. subtilis* (26 mm), *K. pneumoniae* (23 mm) and *S. typhimurium* (24mm).

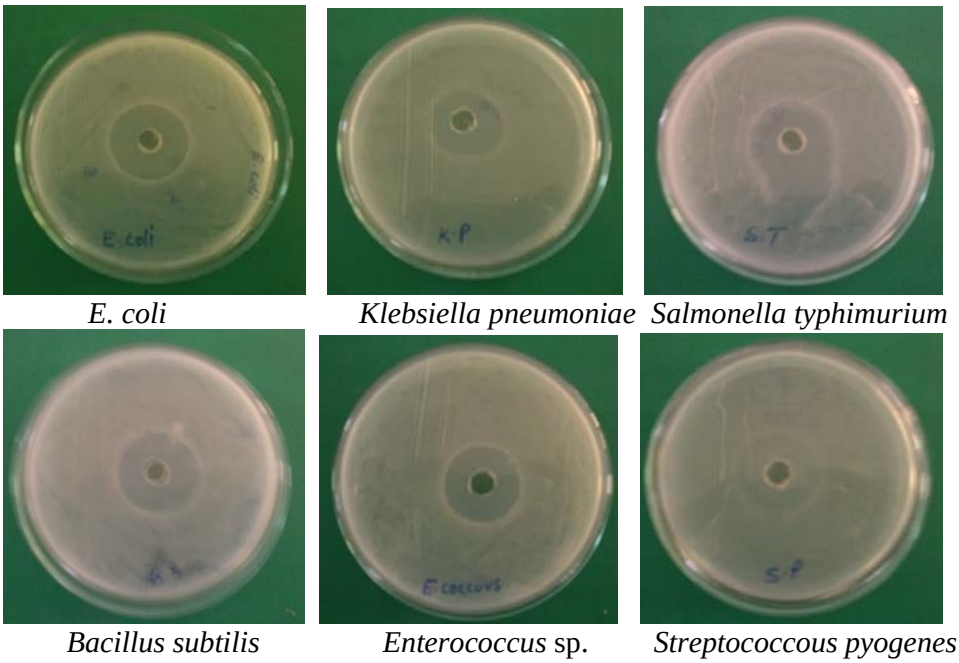

Figure 2: Zone of inhibition of endophytic fungi against the pathogenic bacteria strain

Table 4: Effect of media

Media	Zone of Inhibition(mm)					
	E. coli	***Enterococcus* sp.**	***S. pyogenes***	***B. subtilis***	***K. pneumoniae***	***S. typhimurium***
SDB	22	15	20	18	16	14
PDB	24	25	22	26	23	24
A&H	10	16	12	13	10	5
M&H	15	13	0	17	0	11
RB	12	10	8	11	5	0

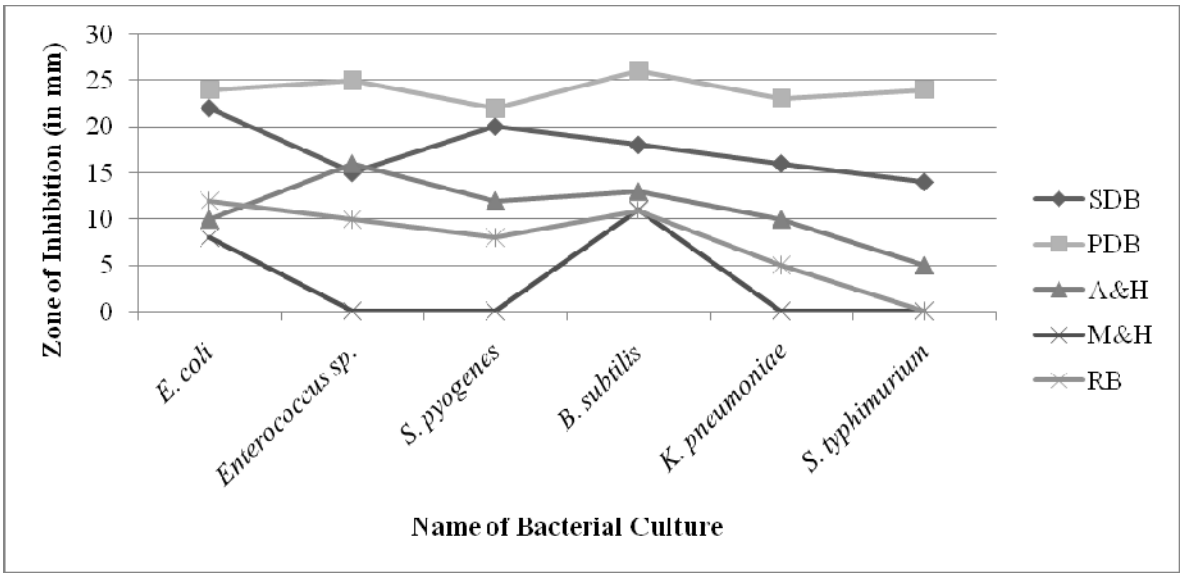

Figure 3: Effect of media

Effect of incubation period

The aim of present work is to optimize various physical parameters for maximum production of novel antibacterial bioactive compounds. *Fusarium oxysporum* was incubated for 20 days and each day, it was observed for the antibacterial activity against pathogenic bacteria. It was found that it gave best result on 11th day of incubation are shown in Table 5 and Figure 4.

Table 5: Effect of incubation period

Incubation Days	Zone of Inhibition (mm)					
	E. coli	*K. pneumoniae*	*B. subtilis*	*Enterococcus* sp.	*S. typhimurium*	*S. pyogenes*
5	-	-	5	-	-	-
6	5	-	5	-	-	-
7	10	7	8	10	-	-
8	11	9	9	12	10	11
9	12	15	11	13	16	15
10	17	16	18	24	22	21
11	26	20	27	26	23	24
12	25	19	25	25	20	23
13	23	17	24	23	20	21
14	20	17	22	20	19	16
15	15	14	20	17	18	10
16	12	11	11	10	15	9
17	8	-	10	-	-	-
18	-	-	5	8	-	-

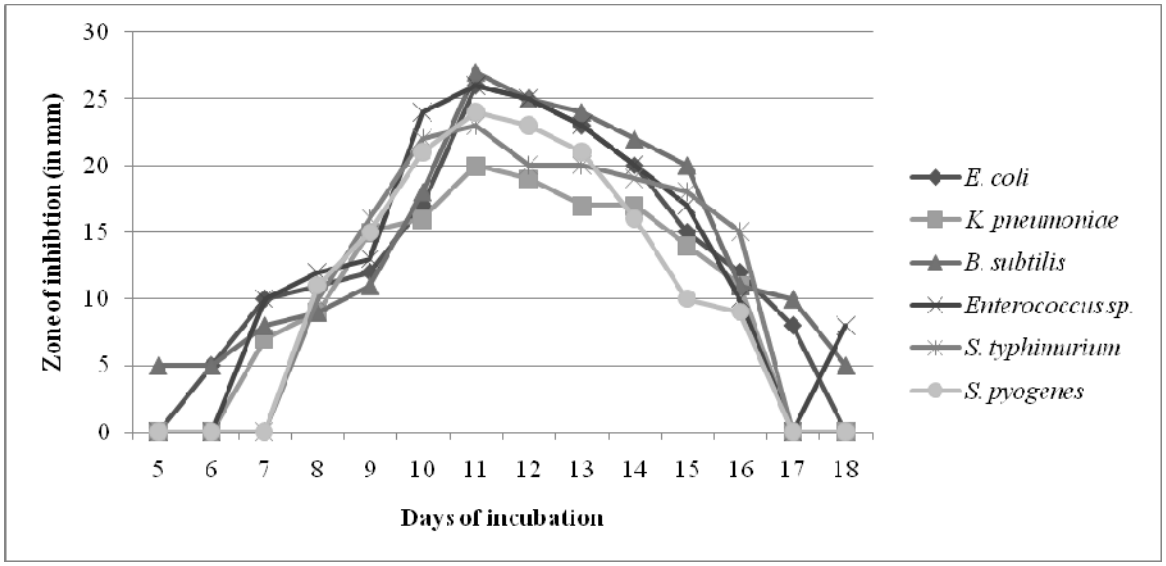

Figure 4: Effect of incubation period

Effect of pH

The production of antibacterial bioactive compounds was observed on different pH (3-10). The result indicated that pH 7 was suitable for growth and maximum production of antibacterial bioactive compound from *Fusarium oxysporum*. It showed highest zone of inhibition against *B. subtilis* (28 mm), *E. coli* (26 mm), *Enterococcus* sp. (24 mm), *S. pyogenes* (22 mm), *K. pneumoniae* (21 mm) and *S. typhi*murium (20 mm) as depicted in Table 6 & Figure 5.

Effect of Temperature

For maximum production of bioactive compounds from fungal strain different incubation temperature (15, 20, 25, 30, 35 and 40) were provided. Increase in incubation period from 20°C to 30°C enhanced the growth and production of bioactive metabolite. Maximum growth and zone of inhibition was recorded at 25°C. However, lower growth and minimum zone of inhibition was recorded at 15°C and 35°C (Table 7 & Figure 6).

Table 6: Effect of pH

pH	Zone of inhibition (in mm)					
	E. coli	*Enterococcus* sp.	*S. pyogenes*	*B. subtilis*	*K. pneumoniae*	*S. typhimurium*
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	17	11	10	15	12	13
7	26	24	22	28	21	20
8	20	15	13	10	16	14
9	7	-	5	10	10	13
10	7	-	-	5	-	-

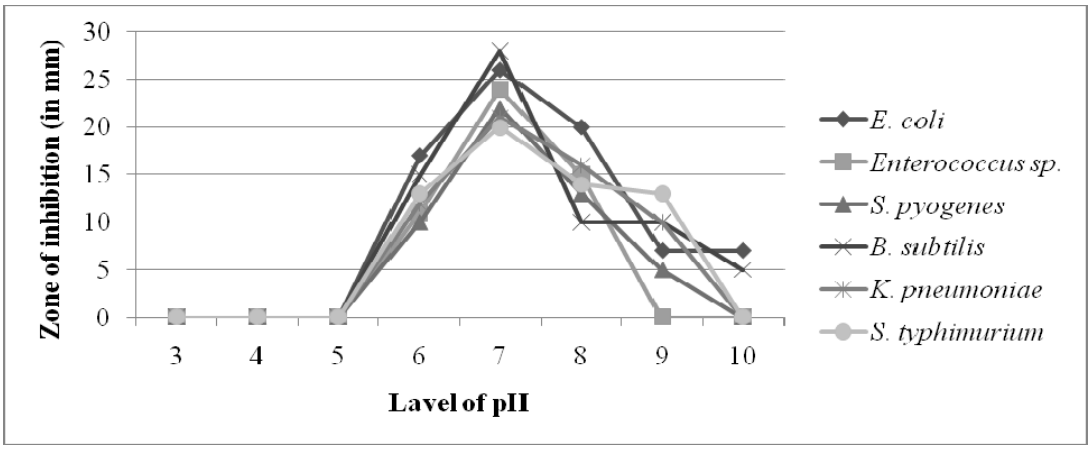

Figure 5: Effect of pH

Table 7: Effect of Temperature

Temperature (°C)	Zone of inhibition (in mm)					
	E. coli	*Enterococcus* sp.	*B. subtilis*	*K. pneumoniae*	*S. typhimurium*	*S. pyogenes*
15	0	0	7	5	0	0
20	10	13	15	11	5	0
25	26	24	27	20	21	18
30	17	13	20	12	0	11
35	10	0	5	0	0	0
40	0	0	0	0	0	0

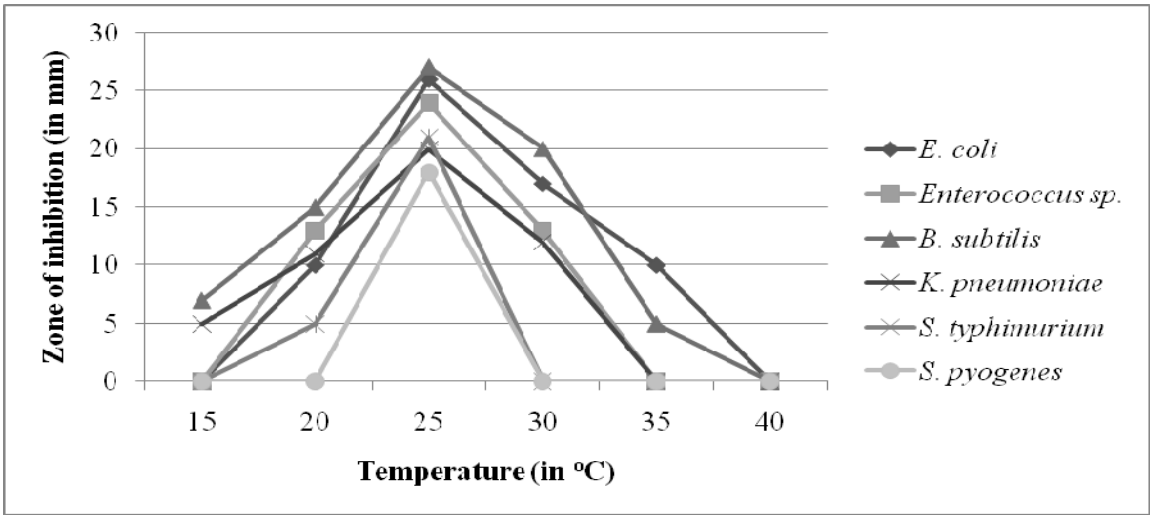

Figure 6: Effect of Temperature

Effect of NaCl concentration

It was observed that the NaCl concentration effected the growth and production of the antibacterial metabolite. The production of antibacterial metabolite was observed for the concentration of NaCl from 0.5 g/l to 5g/l. The fungi *Fusarium oxysporum* gave best result at 0.5 g/l to 2.5 g/l (Table 8 & Figure 7). The maximum zone of inhibition was observed at the concentration of 1.5 g/l against *Bacillus subtilis* (28 mm), *E. coli* (26 mm), *Enterococcus* sp. (24 mm), *S. pyogenes* (25 mm) and *Klebsiella pneumoniae* (22 mm) *S. typhimurium* (20 mm).

Table 8: Effect of NaCl concentration

Concentration of NaCl (gm/l)	Zone of inhibition (in mm)					
	E. coli	***S. typhimurium***	***Enterococcus* sp.**	***K. pneumoniae***	***S. pyogenes***	***B. subtilis***
0.5	13	15	7	10	5	12
1.0	11	17	10	13	18	10
1.5	26	20	24	22	25	28
2.0	20	11	17	15	12	19
2.5	12	9	10	5	0	12
3.0	0	0	0	0	0	0
3.5	0	0	0	0	0	0
4.0	0	0	0	0	0	0
4.5	0	0	0	0	0	0
5	0	0	0	0	0	0

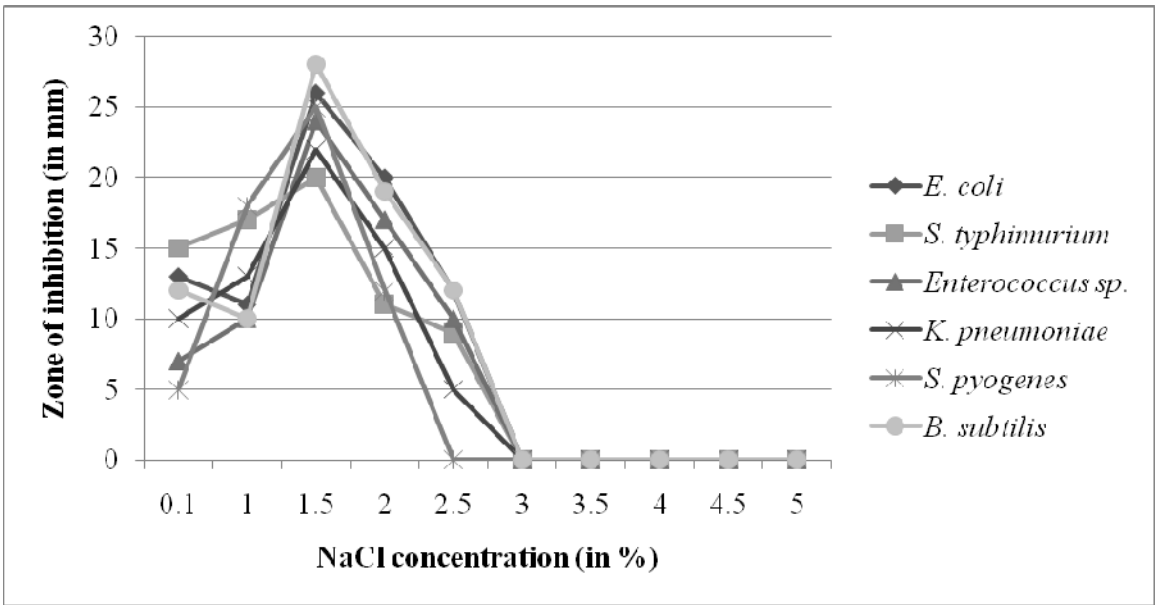

Figure 7: Effect of NaCl concentration

DISCUSSION

For few decades, the resistance of pathogens against drugs has been increased which has directly affected the discovery for new bioactive compounds. Therefore, a number of bioactive compounds are being extracted from plants, bacteria, fungi and algae and many more are in processing to fight against different types of drug-resistant pathogenic organisms. A huge work has carried out on fungi especially endophytic fungi which produce many novel bioactive compounds effective against pathogenic bacteria.

In the present study, 8 endophytic fungi namely *Aspergillus fumigatus, Aspergillus niger, Fusarium solani, Aspergillus repens, Alterneria alternata, Alternaria* sp., *Phoma hedericola* and *Fusarium oxysporum*, isolated from the mint plant (*Menthe viridis*) which was collected from Khamariya region of Jabalpur (India). All the fungi were of class Hypomycetes and Ascomycetes. Similarly, Min-Yuan et al. (2012) isolated 67 endophytic fungi from the twigs of medicinal plants of Lauraceae family and 89 from Rutaceae family in central and northern Taiwan and studied their taxonomical feature and antimicrobial activity against pathogenic organisms.

In other study, Sandhu et al. (2014) also reported 7 endophytic fungi from a religious plant *Saraca indica* belong to class Sordariomycetes, two from Ascomycetes, one from Eurotiomycetes and two fungi which do not produce any reproductive structures, as it produce sterile mycelia, was obtained and observe their antibacterial activity against 6 pathogenic. Kharwar et al. (2003) exploited the endophytic fungi *Chloridium* sp. for the production of Javanicin in liquid and solid media. It was isolated from *Azadirachta indica* and the compound produced showed strong antibacterial activity against *Pseudomonas* sp. (pathogenic bacteria).

The main aim of the present study was to optimize various culture conditions for maximum production of antibacterial compounds. The fungi were incubated for 20 days and the antibacterial activity was recorded after each day of incubation. It was found that it gave maximum antibacterial activity on 11th day of incubation. For media optimization, *Fusarium oxysporum* were grown on five different media. Potato Dextrose Agar media was found to be most suitable for its growth. *Fusarium oxysporrum* was also optimized at different pH and temperature and found that it gave maximum production of antibacterial bioactive compound at 25±1^{o}C and pH 7. In The present study, effect of NaCl concentration was also observed on *Fusarium oxysporum* and satisfactory growth and antibacterial activity was found within the concentration range of 1g/l to 3g/l. Similarly, Agastian et al. (2005) detected the effect of NaCl concentration on *Fusarium solani* and found a decline in the growth and production of antibacterial bioactive compound after 6 %. It gave its best activity at concentration of 3%. Gupte et al. (2002) optimized the pH condition for maximum production of antibiotic tetreane polyene from *S. arenae* var. *ukrainiana* and found that pH 6.5 was most suitable for a promising production of the antibiotic. Singh et al. (2012) tested *Streptomyces rimosus* 10792 over different pH and temperature ranges for maximum production of antibiotic and reported that pH 7.5 and 28^{0}C were suitable for the maximum production of the novel bioactive compound.

CONCLUSION

From the above study it was found that various parameters like temperature, incubation period, media, NaCl concentration and pH affects the growth and metabolites production from endophytic fungi *Fusarium oxysporum* isolated from the plant *Menthe viridis*. The 11th day incubation period has shown the maximum antibacterial activity as compared to other days. Different media also affects the growth and production of metabolites. The maximum production was observed in potato dextrose medium. The other parameters like temperature (25^{o}C), NaCl concentration (3gm/l) and pH (7) are also optimized for the maximum production of antibacterial metabolite.

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