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EFFECT OF VARIOUS PHYSIOCHEMICAL PARAMETERS ON ANTIMICROBIAL ACTIVITY OF *ASPERGILLUS TERREUS*

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ABSTRACT

Aspergillus terreus isolated from soil showed broad spectrum antimicrobial activity against different pathogenic microorganisms including MRSA. Optimization of physiochemical parameters revealed the optimum period of incubation for best antimicrobial activity to be 5 days. Shaking conditions were found to be better than static cultures. The antimicrobial potential of the fungus was optimally best between pH 5-7. Incubation temperature of 25°C found to be optimum for growth as well as antimicrobial activity. Starch as a carbon source at a concentration of 1% was best for antimicrobial production. Yeast extract and soyabean meal (1%) were found to be the best nitrogen rich sources for maximum biomass and antimicrobial activity. Extraction with different organic solvents demonstrated ethyl acetate to be the best for maximum antimicrobial activity against the entire microorganisms used in the study.

KEYWORDS: Antimicrobial, *Aspergillus*, optimization, Soil fungi

INTRODUCTION

Antibiotics represent the medically important group of secondary metabolites, which have been useful in our battle against infectious diseases. A number of antibiotics have been isolated from different fungi such as *Aspergillus oryzae, Penicillium janczewskii, Penicillium canescens*[1], *Aspergillus fumigatus*[2] and *Myrothecium cinctum*[3] etc. Fungi are historically important sources of secondary metabolites and they continue to provide new chemical entities with novel biological properties.[4] They provide a rich source of compounds for therapeutic applications including antibacterial[5,6], antifungal[7], antiviral[8],

immunosuppressants and cholesterol-lowering agents.[9, 10] Further, bacterial resistance is spreading throughout the world, especially in all health care associated pathogens revealing the steadily decreasing potencies of prevalent antibiotics[11] thus necessitating the discovery of novel compounds, modification of already existing antimicrobial stock, be it from fungi, actinomycetes or any such natural resources. Soil holds an enormous biodiversity that can be screened for antibiotic production. Because of huge expenditure on synthetic molecules with effective antimicrobial properties, natural products are still a worth promise.[12] Although, many fungi have been listed to possess anti1microbial activity, still there is a need to explore more such organisms for their antimicrobial potential to solve the problem of emerging strains of resistant microorganisms. The present study was thus planned to isolate fungi from soil samples collected from different regions of Punjab, India and to screen them for their antimicrobial activity. As the various factors like temperature, pH, incubation period, carbon and nitrogen sources play a major role in the production of antimicrobial agents, so in the present study one of the soil fungal isolate has been used for optimization of such factors to enhance the production of antimicrobial agents.

MATERIALS AND METHODS

Isolation of fungi: The fungus was isolated using yeast extract glucose agar (YGA) medium from the soil samples collected from Punjab (30˚ 4’ N 75˚ 5’ E).[13] The culture so obtained was maintained by regular subculturing and also preserved in 10% glycerol stock at -70°C.

Fungal extract preparation: The fungus was grown on YGA plates for 6-7 days from which four discs (8mm) of fungal mycelia were used to inoculate 50 ml YPDS broth containing (g/l): yeast extract 4, peptone 10, dextrose 10, starch 10 and pH 6, taken in 250 ml flask. After 5 days of incubation at 25°C under shaking conditions, the contents were filtered through Whatman filter paper no 1 and the filtrate obtained was used for testing antimicrobial activity.

Test organisms: The reference strains of bacteria: *Staphylococcus aureus* (MTCC-740) *Staphylococcus epidermidis* (MTCC-435), *Klebsiella pneumonia* sub sp. *pneumoniae* (MTCC-109), *Escherichia coli* (MTCC-119), *Shigella flexneri* (MTCC-1457), *Pseudomonas aeruginosa* (MTCC-741), *Salmonella typhimurium*1 (MTCC-98), *Salmonella typhimurium* 2 (MTCC-1251) and *Enterococcus faecalis* (MTCC-439) and two yeast strains: *Candida albicans* (MTCC 227) and *Candida tropicalis* (MTCC 230) used for testing their sensitivity to fungal extract were obtained from Microbial Type Culture Collection (MTCC), Institute of

Microbial Technology (IMTECH), Chandigarh, India. The cultures were maintained on nutrient agar slants, subcultured regularly and stored at 4°C and also preserved in 10% glycerol stock at -70°C.

Inoculum preparation: A loopful of isolated colonies was inoculated into 5ml nutrient broth and incubated at 37°C for 4h. The turbidity of actively growing bacterial suspension was adjusted to match the turbidity standard of 0.5 Mc Farland units prepared by mixing 0.5ml of 1.75% (w/v) barium chloride dihydrate ($BaCl_2$) to 99.5ml of 0.18M (v/v) sulphuric acid with constant stirring. The bacterial suspensions so prepared were used for testing their sensitivity to fungal extract.

Screening of fungal extract for antimicrobial activity: The plates containing Muller Hinton agar medium were spread with 0.1ml of the bacterial inoculum. Wells (8mm diameter) were cut out from agar plates using sterilized stainless steel cork borer and filled with 0.1ml of the fungal extract. The plates were incubated at 37°C for 24h and diameter of resultant zone of inhibition, if any, was measured. Experiments were run in duplicates for each combination of extract and Microbial strain.

Fungal isolate, HT-66 subsequently identified as *Aspergillus terreus* was giving better inhibition zone and selected further to optimize various physiochemical parameters for maximum antimicrobial activity.

Effect of incubation period: Fifty ml of YPDS broth medium taken in 250 ml was inoculated with four discs (8mm) obtained from seven days grown fungal culture and incubated at 25°C under shaking conditions. The culture were processed at different time intervals. Initially, from 5th – 10th day, the duplicate set of flasks was processed after every 24 hr and after 10 days, the processing of the cultures was carried out at 5 days interval upto 30 days.

Effect of shaking conditions: In order to see the effect of shaking on antimicrobial activity, the fungus was grown under static and different shaking conditions at 100 RPM, 150 RPM, 200 RPM, and 250 RPM at 25 °C for 5 days.

Effect of temperature and pH: The antimicrobial activity of the fungus was checked in the culture broth obtained from the organism grown at different temperatures (25°C, 30°C, 35°C,

40°C, 45°C) under shaking conditions for 5 days while to study the effect of pH, the fungus was grown at different pH values (3-9) at 25°C at 200 RPM.

Effect of media components and their concentrations: The experiment was carried out in 4 sets.

The set I used YPDS-I medium, lacking starch but amended with dextrose at different concentrations.
The set II used YPDS-II medium, lacking dextrose but amended with starch at different concentration.
In set III (YPDS-III) both the carbon sources were retained as such while peptone was added at different concentrations with no yeast extract.
In set IV (YPDS-IV) different concentrations of yeast extract were used with no peptone.

Effect of different carbon and nitrogen sources: To study the effect of different carbon and nitrogen sources, total carbon sources i.e. dextrose and starch in YPDS medium were replaced by a single carbon source (dextrose, starch, sucrose, lactose and glycerol) at a concentration of 1%. Similarly, to work out the effect of nitrogen sources in the YPDS medium, yeast extract and peptone were replaced with one of the nitrogen rich sources (malt extract, yeast extract, urea, casein, ammonium dihydrogen phosphate, sodium nitrate, , ammonium chloride, ammonium nitrate, ammonium sulphate and potassium nitrate) at a concentration of 2%.

Thermostability of the bioactivity of fungal extract

The extract obtained from the fungus grown on YPDS medium at 25°C for five days was heated at 50°C, 60°C, 70°C, 80°C, 90°C, 100°C for one hr and assayed for the residual antimicrobial activity, with respect to untreated control.

Fractionation of extracts: Fifty ml of the culture broth obtained from the fungus grown on YPDS medium at 25°C for five days, was extracted with different organic solvents viz diethyl ether, chloroform, butanol, hexane, ethyl acetate in the ratio of 1:1 (v/v). The organic phase was separated and the solvent was then evaporated to dryness under vaccum and the residue obtained was reconstituted in 1 ml dimethyl sulfoxide (DMSO) and used to determine antimicrobial activity.

RESULTS

The isolated fungus was identified as *Aspergillus terreus* by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India (Accession no NFCCI 2556). Extracellular culture broth of the selected fungus showed broad spectrum antimicrobial activity against eleven microorganisms out of thirteen tested including MRSA with the inhibition zone ranging from 15 to 19 mm

Effect of different growth media on antimicrobial activity of *Aspergillus terreus*

With YPDS medium, *Aspergillus terreus* (HT66) showed maximum zone of inhibition, ranging from (15 - 19 mm) followed by yeast extract (13 - 16 mm), potato dextrose medium (13 -14mm) Malt extract medium showed antimicrobial activity against *S. aureus* and *C. albicans* with zone of inhibition 16 and 14 mm, respectively. Czapek dox's medium supported the least antimicrobial activity against *S. aureus* and *C. albicans* with zone of inhibition 15 and 12 mm, respectively (Fig 1).

Effect of incubation period on antimicrobial activity of *Aspergillus terreus*

Aspergillus terreus was found to be quite effective as it inhibited all the tested organisms except *C. tropicalis*. The maximum antimicrobial activity was observed on 5^{th} day of incubation which remained more or less stable till 9^{th} day and then declined. *C. albicans* was the most sensitive organism to extracellular culture broth of *Aspergillus terreus* showing inhibition zone of 20 mm. Biomass increased and was maximum at 30th day of incubation (Fig 2).

Effect of shaking conditions on antimicrobial activity of *Aspergillus terreus*.

The antimicrobial activity of *Aspergillus terreus* was better expressed under shaking conditions as there was no activity in static conditions. Of the thirteen microorganisms tested , *Aspergillus terreus* showed antimicrobial activity against eleven microorganisms *as K. p neumoniae* 2 and *C. tropicalis* were found to be resistant .No activity was observed at 100 rpm, and the activity increased with the increase in rpm. Best antimicrobial activity was observed at 200 rpm with an inhibition zone ranging from 15 mm to 20 mm and the activity remained same till 250 rpm. Biomass of the fungus also increased with the increase in the RPM and found to be maximum (20.35 mg/ml) at 250 rpm (Fig 3).

Effect of pH on antimicrobial activity of *Aspergillus terreus*

Aspergillus terreus when grown at different pH showed no antimicrobial activity at pH 3-4 and the activity increased from pH 5 which remained more or less stable upto pH 7 and then declined upto pH 10, so the pH optima lies between pH 5- 7. There was no significant difference in the antimicrobial activity from pH 5 to pH 7. Biomass of the fungus increased from pH 3-7 and remained stable till pH 9 (Fig 4).

Effect of incubation temperature on antimicrobial activity of *Aspergillus terreus*

Aspergillus terreus showed no growth at 15°C but picked up some growth at 20°C which increased with increase in temperature and reached its maximum at 30°C. Maximum antimicrobial activity was found at 25°C which remained more or less stable till 30°C and then declined. (Fig 5).

Effect of media components and their concentration on antimicrobial activity of *Aspergillus terreus.*

The maximum antimicrobial activity was observed at 1% dextrose which remained more or less stable upto 6% and then declined. In experimental setup II 1% starch gave maximum antimicrobial activity which remained more or less stable upto 6% and then declined. Further in experimental setup III and IV, 1% peptone and 1% yeast extract showed maximum antimicrobial activity which remained more or less stable upto 4% and then declined. However, biomass increased with increase in concentration of nitrogen sources. Thus, to work out the combined effect of carbon and nitrogen sources, these were respectively tested at 1 % concentration.

Effect of different carbon sources on antimicrobial activity of *Aspergillus terreus*.

Aspergillus terreus showed maximum antimicrobial activity as well as biomass when starch was used as a carbon source with a zone of inhibition ranging from 16 -20 mm and order of antimicrobial activity in different carbon sources was as follows starch> dextrose > sucrose > lactose > glycerol >maltose. No antimicrobial activity was found when maltose was used as a carbon source (Table 1).

Effect of different nitrogen sources on antimicrobial activity of *Aspergillus* terreus

Aspergillus terreus showed the maximum activity in yeast extract, soyabean meal and peptone based medium followed by malt extract, casein and ammonium sulphate. Of the different nitrogen sources used, ammonium chloride, ammonium dihydrogen phosphate,

sodium nitrate and ammonium nitrate did not support any antimicrobial activity. *C. albicans* was found to be the most sensitive organism and showed sensitivity to *Aspergillus terreus* grown in almost all the nitrogen based medium.

Solvent extraction and antimicrobial activity of *Aspergillus terreus.*

Culture broth from *Aspergillus terreus* when extracted with different solvents revealed ethyl acetate to be the best to elute the components responsible for antimicrobial activity followed by butanol > chloroform > hexane. *C. albicans* was found to be the most sensitive organisms. Ethyl acetate extract showed the maximum range of zone of inhibition of 24-37mm followed by butanol (20mm-35mm), chloroform with zone of inhibition ranging from 16-25mm. Further, Diethyl ether and hexane showed the least zone of inhibition ranging from 13-16mm and 14-17 mm respectively (Table 2). *C tropicalis* remained the resistant organism throughout the study. Further ethyl acetate was selected as an organic solvent for extraction of antimicrobial components from *Aspergillus terreus* for further studies.

Table1. Effect of different carbon sources on antimicrobial activity of *Aspergillus terreus*

	Dextrose	**Starch**	**Sucrose**	**Lactose**	**Glycerol**	**Maltose**
Biomass (mg/ml)	9.4	11.78	10.04	3.34	5.56	4.5
Microorganism		Zone of inhibition (mm)				
E. faecalis	16±0	16±1.4	15±0	-	-	-
S. aureus	15.5±0.7	15±1.4	15±1.4	15±1.4	15.5±0.7	-
S. epidermidis	16±0.7	16±1.4	16.5±0.7	16±1.4	16.5±0.7	-
E. coli	16±0	16.5±0.7	15.5±0.7	-	-	-
K. pneumoniae 1	17.5±0.7	18±0	17.5±0.7	15±1.4	15±1.4	-
P. aeruginosa	16±1.4	16±1.4	15±0	-	-	-
Sh. flexneri	16±1.4	16.5±0.7	16.5±0.7	-	-	-
Salm. Typhimurium 2	17.5±0.7	17.5±0.7	16±0	-	-	-
MRSA	17±0	18±0	16±1.4	-	-	-
C. albicans	20±0	21.5±0.7	19±0	15±0	14±1.4	-

Values are expressed as Mean ± Standard Deviation of three determinations

Table 2. Solvent extraction and antimicrobial activity of *Aspergillus terreus*

Solvents	BUT	DE	EA	Chl	Hex
Microorganisms		**Zone of Inhibition (mm)**			
E. faecalis	30.75±0.35	14.5±2.1	29.5±0.7	20.5±2.1	14.5±0.7
S. aureus	20±1.4	14.5±2.1	24±0	19.5±0.7	14.5±0.7
S. epidermidis	21.5±2.1	13.5±0.7	25±0	18±0	14.5±0.7
E. coli	23±1.4	14.5±0.7	25.5±0.7	17±1.4	14.5±0.7
K. pneumoniae 1	27±0	14.75±0.35	30.5±0.7	20±1.4	14.75±1.0
K. pneuminiae 2	25±1.4	0	25.5±0.7	13.5±2.1	0
P. aeruginosa	27.5±2.1	16±0	28.5±0.7	20.5±0.7	15±0
Sh. flexneri	27.5±0.7	16.5±0.7	27±0	16±1.4	16.5±2.1
Salm. Typhimurium 1	20±1.4	0	21.75±0.35	14.5±2.1	13.75±0.35
Salm. Typhimurium 2	22±1.4	16.5±0.7	27±0	20±0	17±0
C. albicans	35±1.4	12±1.4	37.5±0.7	25.5±0.7	11±0
C. tropicalis	0	0	0	0	0
MRSA	24.5±0.7	13.75±0.35	26.75±0.35	14.75±1.0	14±1.4

But- Butanol, DE- Diethyl ether, EA- Ethyl acetate, Chl- Chloroform, Hex- Hexane

Values are expressed in terms of mean ± Standard deviation

Figure legends

Fig 1 Effect of different growth media on antimicrobial activity of *Aspergillus terreus*

Fig 2 Effect of incubation period on antimicrobial activity of *Aspergillus terreus*

Fig 3 Effect of shaking conditions on antimicrobial activity of *Aspergillus terreus.*

Fig 4 Effect of pH on Antimicrobial Activity of *Aspergillus terreus*

Fig 5 Effect of incubation temperature on Antimicrobial activity of *Aspergillus terreus*

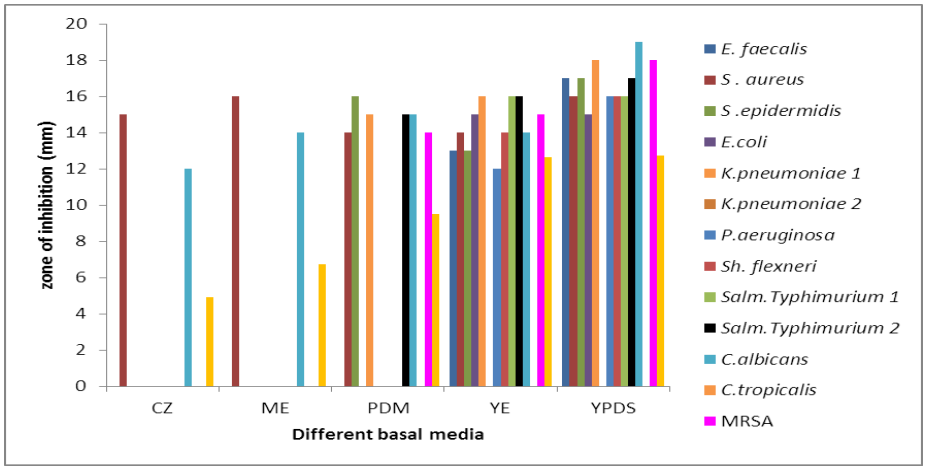

Fig 1 Effect of different growth media on antimicrobial activity of *Aspergillus terreus*

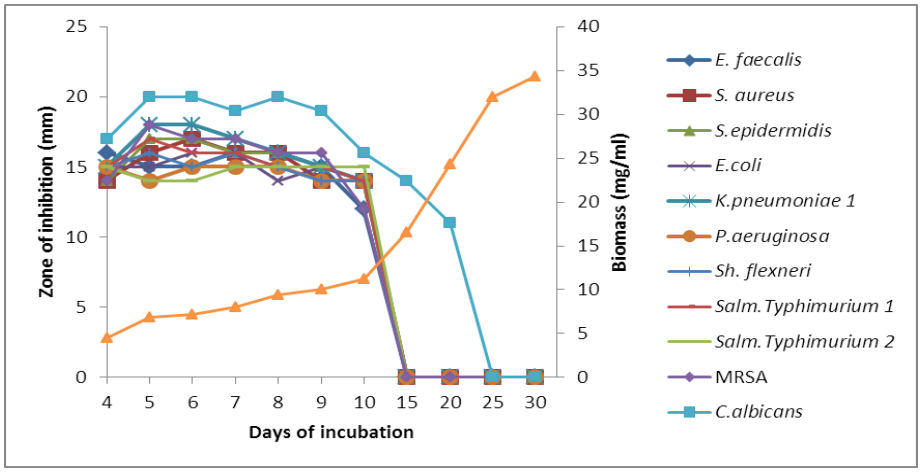

Fig 2 Effect of incubation period on antimicrobial activity of *Aspergillus terreus*

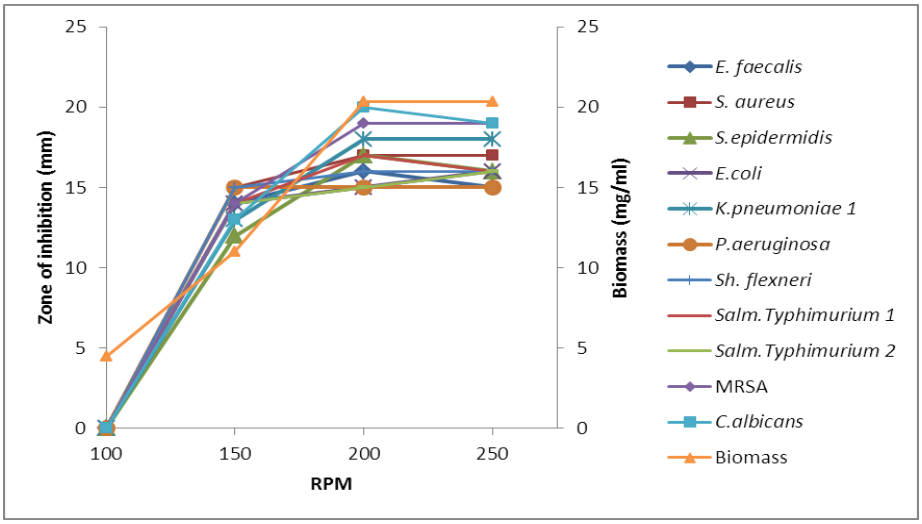

Fig 3 Effect of shaking conditions on antimicrobial activity of *Aspergillus terreus.*

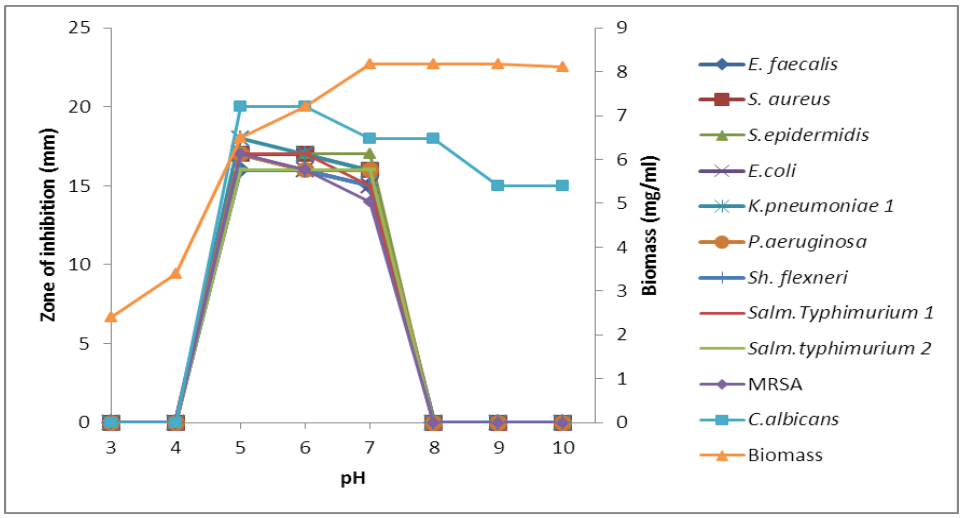

Fig 4 Effect of pH on Antimicrobial Activity of *Aspergillus terreus*

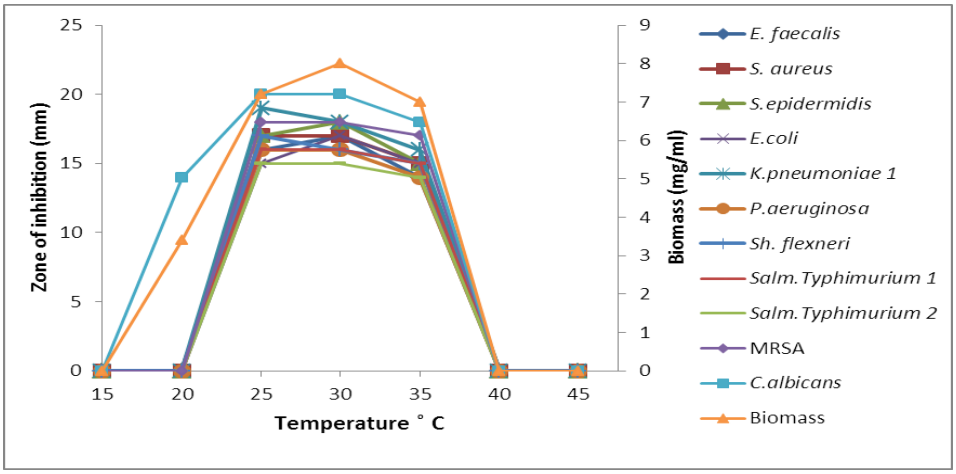

Fig 5 Effect of incubation temperature on Antimicrobial activity of *Aspergillus terreus*

DISCUSSION

Microorganisms have been used as a source for the production of variety of bioactive metabolites. Nutritional and physiological factors like different media, stationary or shake conditions, pH of the medium, temperature, and incubation period have a great influence on metabolite production and these conditions are generally taken into account for enhanced production. The yield of bioactive metabolites can sometime be substantially increased by the optimization of such factors used for the growth of microbes; a good understanding of such factors in the biosynthesis of metabolites may lead to better exploitation of such resources.[14]

Shaking conditions were found to be the best for antimicrobial activity which might be due to the requirement of aeration for better production of antimicrobial agents by this fungus.[15] The optimum period of incubation for antimicrobial activity was found to be 5 days and subsequent decline in antimicrobial activity after 10th day could be due to exhaustion of nutrients available for the fungi to produce such bioactive compounds.[16, 17]

The antimicrobial potential of the fungi was optimally best between pH 5-7 which may be attributed to better metabolite production under such pH conditions and is in consonance with earlier studies.[13,18] This shows that pH of the culture media is also one of the determining factors for the biosynthesis of secondary metabolites. Rubini *et al*.,[19] reported the growth and production of antimicrobial agent at neutral pH. The pH is related to permeability characteristics of the cell wall and membrane and thus the ion uptake or loss to the nutrient medium. No activity and low biomass was detected at pH extremes which may be due to delayed metabolite production caused by delayed mycelial growth or due to a reduced production of bioactive metabolites under such pH extremes.

The growth of *Aspergillus terreus* at different temperatures revealed 25°C to be the optimum for growth and metabolite production. Low temperature may slow down the metabolic activity and high temperature may kill the organism and/or inactivate the responsible antimicrobial compound.[17] The present results are in consonance with the previous studies reporting 25°C to be the optimum temperature for growth and metabolite production by different fungi.[20] During the microbial fermentations, the carbon and nitrogen sources not only act as major constituents for building of cellular material, but also as important energy sources.[21] Changes in the nature and concentration of carbon and nitrogen sources have been reported to influence the production of secondary metabolites by fungi. Starch at a concentration of 1% was best for antibiotic production in consonance with earlier studies. On development of fermentation medium where polysaccharides or oligosaccharides were found to be better than monosaccharide, such as dextrose for antibiotic production.[22] This might be due to its suppressive effect on production of secondary metabolites and is in consonance with other studies.[23] Other carbon sources that can readily serve as growth substrates, often repress secondary metabolites. Among nitrogen sources, yeast extract and soyabean meal (1%) were found to be the best for maximum biomass and antimicrobial activity in consonance with earlier studies.[24] Extraction with different organic solvents demonstrated ethyl acetate to be the best for maximum antimicrobial activity against the entire microorganisms used in the study. There may be many components with different polarities which might be responsible for antimicrobial activity of the fungal extract obtained with different solvents. Our observations are in consonance with earlier studies where ethyl acetate have been found to be better extractant for bioactivity.[25]

CONCLUSION

The above study suggests *Aspergillus terreus* as a potential candidate offering a better scope for the production, purification and isolation of broad spectrum antimicrobial compound. These findings will facilitate the scale up and further purification to ascertain the compound/s responsible for antimicrobial activity, which can be exploited for pharmaceutical applications.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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