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Research Article

IN-VITRO ANTIMICROBIAL STUDIES OF ISOLATED *MYROTHECIUM SPP* MRP001 AGAINST HUMAN PATHOGENS

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ABSTRACT
The isolated fungi *Myrothecium spp* MRP001 represent an important source of biologically active compounds. The maximum biomass production was optimized (pH 6.5, 96 h at 30 °C) using OFAT (one factor at time) in six different medias, After 96 h of growth, Tryptone Soya Broth (TSB) and Potato Dextrose Broth (PDB) shows maximum spore account (2×10^8 CFU/ml) of *Myrothecium spp* MRP001. Screening of biologically active metabolites obtained from 4-day old culture broth of the strain by Thin Layer Chromatography (TLC silica gel 60, 20 × 20, and 0.5 mm, Merck & Co, Inc) led to isolation of two fractions active against a wide variety of human pathogenic bacteria (*Escherichia coli, Salmonella Typhi, Klebsiella pneumoniae* and *Bacillus cereus*). The second active fraction was shows more antimicrobial activity and further purified by silica gel column chromatography. Among all the fungal extracts, methanol extract (6.5%) was found to have maximum extractive yield followed by the water (6.1%) and petroleum ether (5.7%) extract. The partially purified fraction exhibited good antimicrobial potential against human pathogenic bacteria using *In-vitro* plate assay. The purified metabolites were further concentrated using lyophilisation and 150 µg/ml dose confirmed to inhibit the growth of *Escherichia coli, Salmonella Typhi, Klebsiella pneumoniae* and *Bacillus cereus* respectively by MIC (Minimum Inhibition Concentration) test. The bio active metabolite was most stable at pH 7 and temperature 40 °C/30 min. These results are useful for further investigation of the fungus in the future for microbial disease treatment.

Key Words: *Secondary Metabolites, Submerge Fermentation, Myrothecium Spp. MRP001, Escherichia Coli, Salmonella Typhi, Klebsiella Pneumonia, Bacillus Cereus*

INTRODUCTION
Secondary metabolites are compounds produced by an organism. Development of multiple resistant microbes revealed the need to search for new and novel antimicrobials (Wise, 2008). Fungi produce large numbers of secondary metabolites, some of which are important in industry. Many fungi express secondary metabolites that influence competitive outcomes. The compounds are expressed along with enzymes necessary for extracellular digestion.
Some of the compounds released by fungi influence the organisms that interact with fungi leading to an anthropocentric interpretation of function in the fungus. Some metabolites, referred to as toxins, are compounds that have the potential to kill an organism at concentrations we might use. The activity of the metabolites in biological conditions may differ.
Many fungal endophytes produce secondary metabolites and some of these compounds are antifungal and antibacterial which strongly inhibit the growth of other microorganisms (Gunatilaka, 2006). These organisms have proven to be an unusually rich source of novel natural products (Heider *et al.,* 2003) and constitute a valuable source of bioactive secondary metabolites (Li *et al.,* 2000) some of them displayed antibiotics against pathogens and tumour cells to different degrees. Primary and secondary metabolites may be confused. Primary metabolites may accumulate as staling products slow growth of the fungus, or

as essential nutrients are removed from the growth medium. Regulatory compounds may also appear as growth slows, and they can be confused with secondary metabolites (Abdel-Wahab *et al.,* 2007).
Secondary metabolites are generally produced following active growth, and many have an unusual chemical structure. Some metabolites are found in a range of related fungi, while others are only found in one or a few species. The restricted distribution implies a lack of general function of secondary metabolites in fungi (Oh *et al.,* 2003).
Clear reasons exist for studying secondary metabolites. Many have been found to have use in industry and medicine. Indeed, six of the twenty most commonly prescribed medications for humans are of fungal origin (Schulz *et al.,* 1999). These metabolites have been subjected to combinatorial chemistry following growth in selective media. Some metabolites are toxic to humans and other animals. Yet others can modify the growth and metabolism of plants. Interestingly, the most important secondary metabolites seem to be synthesised from one or a combination of three biosynthetic pathways: polyketides arising from Acetyl Coenzyme A, mevalonate pathway that also arises from Acetyl Coenzyme A, and from amino acids. In addition, genes for the synthesis of some important secondary metabolites are found clustered together, and expression of the cluster appears to be induced by one or a few global regulators (Schulz *et al.,* 2002).
Some of the 'global regulators' are also involved with sporulation and hyphal elongation. Thus the expression of secondary metabolites may be a normal part of and occur at a predictable point in the life cycle of some fungi (Strobel *et al.,* 1998).

MATERIAL AND METHODS

Screening and Isolation of Biological Material

The screening of microorganisms was done against various human pathogenic bacteria and antimicrobial activity found in isolated fungal strain was further confirmed to be *Myrothecium spp* MRP001. The strain *Myrothecium spp* MRP001 which was used for this study was isolated from soil of PDKV region of Akola District. The stock culture of strain was maintained on a potato dextrose agar (PDA) slants. Slants were inoculated, incubated at 28 °C for 7 days and then stored at 4 °C.

Media and Fermentation Conditions

The microorganism was initially grown on PDA medium in a Petri dish, and then transferred to the culture medium (Potato Dextrose Broth, PDB) by punching out 5 mm disc of 48 h old culture with a sterilized self-designed cutter. Various process parameters influencing maximum biomass production were optimized. The optimization was done using OFAT (one factor at time). The effect of growth temperature (20 to 40°C), initial pH (pH 3 to 10), and substrate concentration on fungal biomass synthesis were determined by growing the organism under Submerge fermentation. The mycelial biomass was harvested and filters through muslin cloth and centrifuged at 10,000 rpm at 4 °C for 20 min (Papaspyridi *et al.,* 2010).

Process Development (Antimicrobial Compound Production in Different Media)

Myrothecium spp MRP001 growth and antimicrobial production were evaluated in six different synthetic media after 96 h of incubation. Two millilitres of *Myrothecium spp* MRP001 over night culture were inoculated in 100 ml of following synthetic media: Mueller Hilton (MH), Plate count broth (PCB), Tryptone Soya Broth (TSB), Rogosa and Sharpe (MRS), Potato dextrose broth (PDB) and Fred Waksman Basic 77 broth (WF). The cultures were incubated for 96 h at 30 °C on rotary shaker (120 rpm). Total counts, constituted by vegetative cells and spores, and were assessed after 96 h of incubation using Heamocytometer by the standard serial dilution method (Kobayashi *et al.,* 1996 and Petrini *et al.,* 1992). The experiments were done in triplicate.

Solvent Extraction Method

The cultural broth was extracted using the method of Accelerated Solvent Extraction (ASE) method. The extraction was conducted consecutively with acetone, dichloromethane (DCM), methanol (MeOH) and deionized water (DWR) (Ifeoma *et al.,* 2002). The extract showing antimicrobial activity against human pathogenic bacteria using *In-vitro* plate assay was loaded to silica gel column chromatography.

Isolation and Identification of Bio Active Compounds

The crude extract was subjected to silica gel (Merck 0.02-0.04mm) column chromatography. The elution was conducted with a step gradient CH2Cl2 / MeOH (100:0, 95:5, 90:10, 80:20, and 50:50) (Anastasiadi *et al.,* 2009). The different fractions were collected and again checked antimicrobial activity using *In-vitro* plate assay. Those fractions showing antimicrobial activity were pooled and concentrated using lyophilisation.

Thin layer chromatography (TLC) was used with the crude extract of *Myrothecium spp* MRP001 on silica gel (TLC silica gel 60, 20 × 20, and 0.5 mm, Merck & Co, Inc) with benzene: acetic acid (95:5) solvent system. The crude extract (30 µl) was spotted and the solvent front was allowed to run for approximately 16 cm. The running lane was then dried thoroughly and the elution of compound was detected at 365 nm. The cut portions (1 cm × 2.5 cm) were scraped into micro centrifuge tubes and were extracted with 100% acetone. The silica residues were removed by centrifugation and the supernatant was transferred to a second set of micro centrifuge tubes. The individual metabolites were again spotted on TLC plate for confirmation. Each fraction was concentrated by evaporating off the acetone and tested for antifungal activity of individual metabolites by *In-vitro* plate assay.

The effective antifungal metabolite (*Rf* 0.35) from above was purified again by column chromatography (50 cm × 2 cm) packed with a slurry of silica gel (60-120 mesh) pre-activated at 120 °C for 4 h. Later, the column was successively eluted with hexane-benzene (1:3) and 25 ml fractions with a flow rate of 1 ml/min were collected. These fractions were distilled on water bath and monitored by TLC. The fractions of similar compositions were mixed together for further studies.

Biochemical Assays and Biomass Determination

Secondary metabolites concentrations were determined spectrophotometrically as described (Bystrykh *et al.,* 1996). Briefly, samples (50 μl) from micro-cultures were transferred into flat bottom 96-well MTPs, an equal volume 2 M KOH were added before a spectrum from 400–700 nm of each well was recorded with a SPECTRAmax Plus MTP reader (Molecular Devices, USA). The metabolite concentration was calculated using with a SPECTRAmax plus MTP reader (Molecular Devices, USA), a molecular mass of 643.56 assuming a 3 mm path length in the wells. Larger samples were measured in standard 1 cm path cuevettes. Cell dry weight (CDW) in micro-culture cultivations was determined by collecting the broth and the biomass attached to the walls. After determination of the total culture volume, the entire sample was filtered though a pre-weighted glass fibre filter (GF/A, Whatman). The filters were washed with 2 volumes of water and dried at 80 °C. CDW in shake flask cultivations was determined similarly by withdrawing 10 ml samples from the broth after biomass attached to the wall had been resuspended, followed by the filtration, washing and drying steps. CDW could not be determined in all samples of the bioreactor cultivations because at later stages in the cultivation extensive growth on the walls and in the headspace interfered with representative sampling (Mosmann, 1983 and Ohno *et al.,* 1991).

In-vitro Antibacterial Assay

Antibacterial activity was carried out against human pathogenic bacteria (*Escherichia coli, Salmonella Typhi, Klebsiella pneumoniae* and *Bacillus cereus*) by agar well diffusion method. Muller Hinton agar plates were prepared and wells were made by using gel puncture (Bauer *et al.,* 1966). The test culture were swabbed aseptically and inoculated on the surface of nutrient agar so as to make a lawn. This was allowed to 10 min for the agar surface to dry before making the wells. One ml of fungal filtrate was mixed with one ml of solvent from which various concentrations and loaded in the wells using micropipette and one well was loaded with respective solvent as control (NCCLS, 2000; Hirota *et al.,*

1991). All the plates were done in triplicate. Plates were incubated for 20 hrs at 37 °C. The zone of inhibition was observed around the well. This indicates whether the test organism is resistant (no zone of inhibition) and sensitive (clear zone of inhibition) (Schulz *et al.*, 1995).

MIC (Minimum Inhibitory Concentration) Test

The solvent extract of microbial samples which shows the significant antimicrobial activity will be selected for determination of MIC. A stock solution of 1 mg/ml prepared. This will be serially diluted to obtain various ranges of concentrations between 5 µg/ml to 500 µg/ml. 0.5 ml of each of the dilutions of different concentrations will be transferred into sterile test tubes, 0.5 ml of test organism previously adjusted to a concentration will be introduced. A set of test tubes containing broth alone will be use as control. All the test tubes and control will be then incubated at optimized condition. After the period of incubation, the tube containing least concentration of extract showing no visible sign of growth will considered as the minimum inhibitory concentration (Deutsches Institut für Normung, 1999 and Ringertz, *et al.*, 1997).

RESULTS AND DISCUSSION

The experiments described in this paper were designed to achieve a preliminary understanding of the production, antimicrobial activity and chemical nature of compounds by *Myrothecium spp* MRP001.

Isolation and Screening of Fungi from Soil

The isolated fungi was confirm as *Myrothecium spp* MRP001. The six different associate fungi like *Aspergilus spp, Hypocrea spp, Trichoderma spp, Fussarium spp* and unidentified fungi were isolated from the soil of PDKV region of Akola District, Maharashtra, India. The different isolated fungi were tested for anti microbial activity against human pathogenic bacteria. The significance activity was found with *Myrothecium spp* MRP001.

Antibacterial Activity of Isolated Fungi

Methanol extract of *Myrothecium spp* MRP001 shows promising results by exhibiting maximum activity against four different human pathogenic bacteria (*Escherichia coli, Salmonella Typhi, Klebsiella pneumoniae* and *Bacillus cereus*). The zone of inhibition using *Myrothecium verrucaria* MRP001 culture filtrate was measured with *Escherichia coli, Salmonella Typhi, Klebsiella pneumoniae* and *Bacillus cereus* as 20, 18, 24 and 15 mm respectively. The culture filtrate of *Myrothecium spp* MRP001 shows maximum antibacterial activity with *Klebsiella pneumonia* followed by *Escherichia coli, Salmonella Typhi* (Table1).

Table 1: Antibacterial activity of isolated fungi *Myrothecium spp.* MRP001

Micro organisms	Zone of Inhibition (mm)
Escherichia coli	20
Salmonella Typhi	18
Klebsiella pneumoniae	24
Bacillus cereus	15

Fermentation Condition and Process Development

The maximum biomass production was optimized (pH 6.5, 96 h at 30 °C) using OFAT (One Factor at Time). The production of antimicrobial compound was tested in six different media; Mueller Hilton (MH), Plate count broth (PCB), Tryptone Soya Broth (TSB), Rogosa and Sharpe (MRS), Potato dextrose broth (PDB) and Fred Waksman Basic 77 broth (WF), and the resulting culture were check for their inhibitory activity against human pathogenic bacteria listed in Table. After 96 h of growth, Tryptone Soya Broth (TSB) and Potato dextrose broth (PDB) shows maximum spore account (2×10^8 CFU/ml) of *Myrothecium spp* MRP001 using Haemocytometer.

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Purification and Characterization of Bioactive Metabolites

Among all the fungal extracts, methanol extract was found to have maximum extractive yield followed by the water and petroleum ether extract (Figure 1). Methanol and aqueous extract were found to be maximum alcohol soluble and water soluble extractive values (Figure 2).

Screening of biologically active metabolites obtained from 4-day old culture broth of the strain by Thin Layer Chromatography (TLC silica gel 60, 20 × 20, and 0.5 mm, Merck & Co, Inc) led to isolation of two fractions active against a wide variety of human pathogenic bacteria (*Escherichia coli, Salmonella Typhi, Klebsiella pneumoniae* and *Bacillus cereus*). Further separation was followed by silica gel (Merck 0.02-0.04mm) column chromatography and second fraction shows maximum antibacterial activity using *In-vitro Plate* Assay.

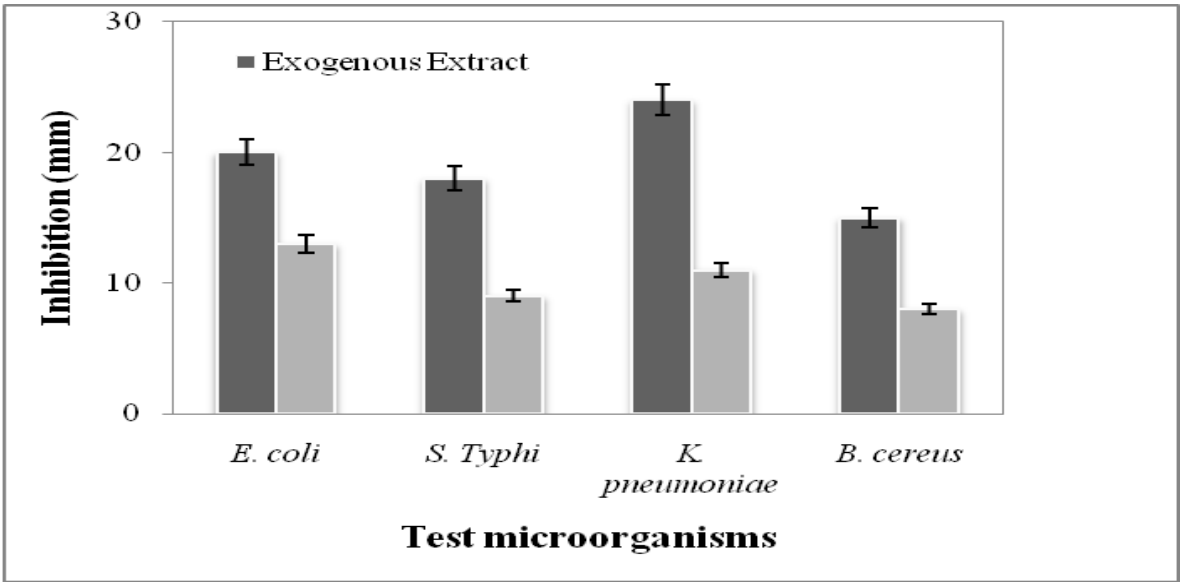

Figure 1: Antimicrobial activity of isolated extract against Human pathogens

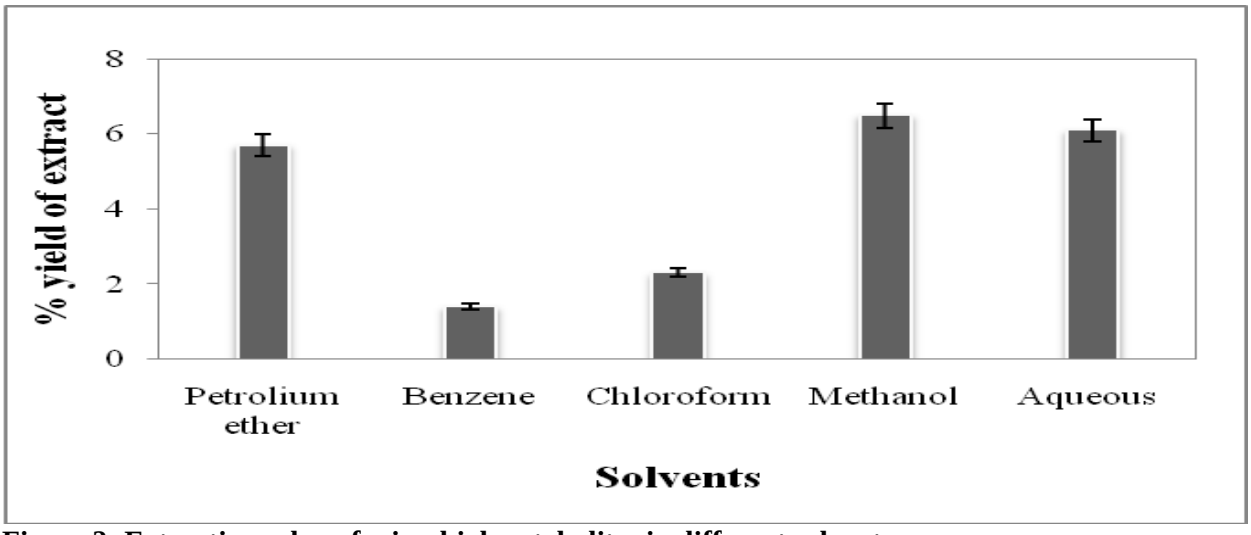

Figure 2: Extractive value of microbial metabolites in different solvent

MIC Determination

Antibacterial activity was determined against human pathogens *Escherichia coli*, *Salmonella Typhi*, *Klebsiella pneumoniae* and *Bacillus cereus* (Table2). The MIC of methanol extract showed appreciable growth inhibition with MIC of 150 (Figure 3).

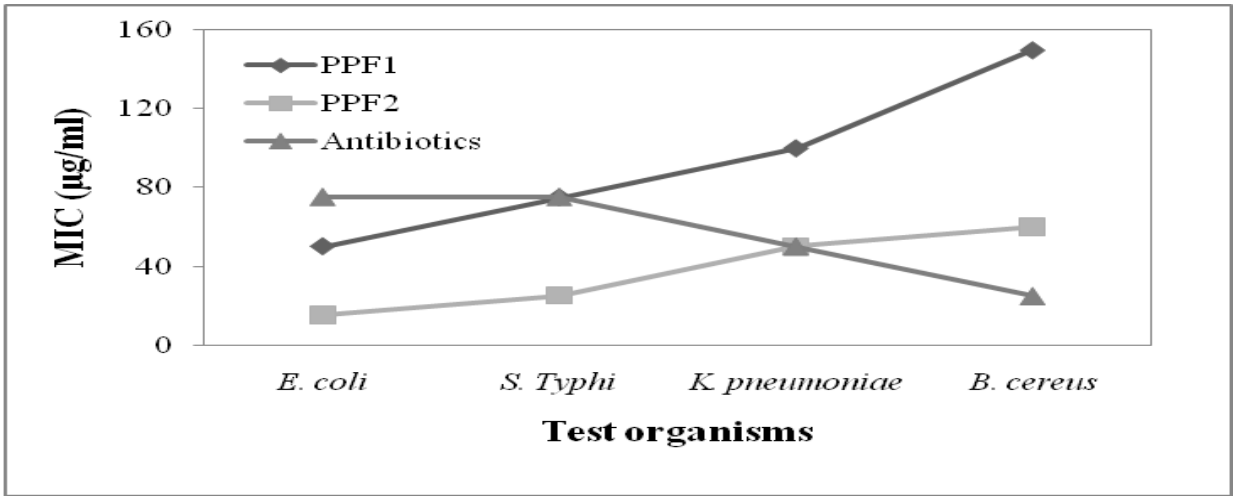

Figure 3: MIC of *Myrothecium spp.* MRP001; PPF1 (Partially purified fraction 1), PPF2 (Partially purified fraction 2) and Antibiotics (tetracycline reference drug) against human pathogens

Table2: MIC of *Myrothecium spp.* MRP001 fungal extract

Test organisms	**MIC (µg/ml)**		
	PPF1	PPF2	Antibiotics
Escherichia coli	50	15	75
Salmonella Typhi	75	25	75
Klebsiella pneumoniae	100	50	50
Bacillus cereus	150	60	25

PPF1- Partially Purified Fraction 1, PPF2- Partially Purified Fraction 2 and Antibiotics- tetracycline (reference drug)

Factor Effecting Antimicrobial Activity

The different factors effecting antimicrobial activity were studied such as enzymes, temperature and pH. The significant high in antimicrobial activity was at pH 7 and at temperature 40 °C/30 min (Table3).

Table 3: Factors affecting antimicrobial activity of *Myrothecium spp.* MRP001 against *Escherichia coli, Salmonella Typhi, Klebsiella pneumoniae* and *Bacillus cereus*

Treatment	Residual activity (%)*			
	Escherichia coli	***Salmonella Typhi***	***Klebsiella pneumoniae***	***Bacillus cereus***
Enzymes				
Lipase	35	18	51	18
Trypsin	20	31	34	29
Proteinase K	17	23	0	14
Chymotrypsin	0	6	5	4
Pepsin	18	41	56	14
Pronase E	16	48	12	5
Heat				
40 °C/30 min	93	100	75	100
60 °C/30 min	88	85	71	93
80 °C/30 min	38	40	50	45
100 °C/30 min	22	29	0	34
120 °C/30 min	16	0	0	20
pH				
3	30	20	42	70
4	40	35	50	70
5	45	65	55	75
6	90	100	75	90
7	100	100	90	100
8	60	70	100	60
9	40	40	85	30
10	30	35	70	30

**Residual activity, based on average ratio among inhibition areas, compared with antimicrobial activity before treatment*

DISCUSSION

The isolated fungus identified as *Myrothecium spp* MRP001 from soil from the soil of PDKV region of Akola District, Maharashtra, India. These bio active metabolites can be used as new role in human microbial disease treatments. In the present studies results of *In-vitro plate* assay is antagonistic to test pathogens.

MIC of isolated fractions was determined against human pathogens. The sensitivity test was performed by micro dilution. The edge of zone of inhibition correlates with the MIC for that particular bacterium. The present investigation confirms that there is antibacterial activity of isolated fraction as compare to reference antibiotics.

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