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Characterization of a Biopigment from Newly Isolated Endophytic Fungus *Pezicula* sp. BDF9/1 and its Antimicrobial Activity

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Abstract: Endophytic microorganisms are found virtually in every plant on earth and they produce different secondary metabolites exhibiting a variety of biological activities. The aim of this study was to characterize a biopigment produced by endophytic fungi *Pezicula* sp. BDF9/1. The biopigment having molecular mass 449.3 was produced by liquid submerged fermentation of *Pezicula* sp. BDF9/1. The chemical structure of the compound was elucidated on the basis of different spectroscopic analysis (MS, IR, and ^{1}H NMR spectra). The compound was identified as 3,4-dihydro-2,6,8,9-tetrahydroxy-3-(1,3,4,6-tetrahydroxy-2,5-dioxoheptyl) anthracen-1(2H)-one. Probably this is the first report of production of this compound from an endophytic fungi, *Pezicula* sp. The antibacterial and antifungal efficacy of the produced red pigment was estimated and found that the pigment have noticeable antibacterial activity against *Aeromonas salmonicida, Aeromonas caviae, Bacillus cereus, Pseudomonas aeruginosa, Bacillus subtilis, Aeromonas hydrophila, Shigella flexneri, Proteus mirabilis, Escherichia coli* and antifugal activity against *Colletotrichum laginarium, Botrytis* sp., *Trichoderma* sp., *Fusarium* sp., *Pythium* sp., *Candita albicans*.

Key words: Endophytes, *Pezicula* sp., Biopigment, isolation, chemical chartaterization, Antimicrobial activity.

Introduction

Fungus residing in the healthy plant tissues for at least a part of its life cycle, without causing apparent harm to its host, is called endophytic fungus [4,28]. Now a day's worldwide interest in the production of pigments from natural sources is due to a serious safety problem with many artificial synthetic colourants [8]. These naturally occurring biopigments were widely used in different chemical, pharmaceutical, food, cosmetics and dye industries as food colouring agents , drug additives, silk and wool tanning agent , hypocholestemic agent etc. [9,27]. There are several reports of pigment production of plants, animals, and microorganisms [17,19,23], but each of them have some limitations, as well as their supply is limited to a season in year. Till now production of pigment from fungi is limited to certain genera such as *Penicillium, Paecilomyces, Fusarium, Laetiporus, Epicoccum*, *Aspergillus, Monascus* etc. and among them *Monascus* was mostly used [3,5,6,7,10,13,14,15,16,24,30,34]. In this regard new fungal genera having pigment producing ability will be an alternative because fungal pigments will be acceptable to the consumer because the natural pigments are associated with their image of being healthy and of good quality and showing high chemical stability with a unlimited production. Now a day's some fermentative food grade pigments are on the market: *Monascus* pigments, astaxanthin from

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Xanthophyllomyces dendror-hous [2], Arpink Red from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, β-carotene from *Blakeslea trispora.* and several medicinal drugs such as antibiotic penicillin from *Penicillium* sp., the immunosuppressant cyclosporine from *Tolypocladium inflatum* and *Cylindrocarpon lucidum*, the antifungal agent griseofulvin from *Penicillium griseofulvum* fungus, the cholesterol biosynthesis inhibitor lovastatin from *Aspergillus terreus* fungus, and β-lactam antibiotics from various fungal taxa [36,37]. Suryanarayanan *et al*. [31] discussed many fungal pigments with various chemical structures and their wide ranging biological activities and this reflects the high synthetic capability of fungi [32].

Therefore, the present investigation is an attempt to examine and illustrate the structural characterization of a pigment produced by an endophytic fungus *Pezicula* sp. BDF 9/1. An effort was also done for the screening of antimicrobial activity of isolated fungal pigment.

Materials and methods

Microorganism and culture media

Pezicula sp. BDF 9/1 an endophytic fungi was isolated from a plant *Melia azedarach*, collected from Paschim Medinipur District of West Bengal, India. The stock culture was maintained on potato dextrose agar slant. The inoculums were prepared in modified potato dextrose broth medium. The initial pH of the medium was adjusted to 5.0 before sterilization.

Identification of the endophytic fungi

The fungal molecular identification was confirmed by rDNA based molecular techniques. In brief, fungal genomic DNA was isolated and quality was evaluated on 1.2 % Agarose Gel, a single band of high-molecular weight DNA has been observed. A polymerase chain reaction (PCR) was performed using universal ITS4 and ITS5 primers. A single discrete PCR amplicon band was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with ITS4 and ITS5 primers using ABI-BigDye® Terminator v3.1 Cycle sequencing kit on ABI 3100 automated DNA sequencer. Consensus sequence of 450 nt was used for further analysis [35]. Sequences were submitted to GenBank (Gene Bank Accession Number KP234255). Sequences obtained in this study were compared to the GenBank database using BLAST. Sixteen sequences including BDF9/1 were selected and aligned using multiple alignment software program Clustal W.Distance matrix was generated using RDP (Ribosomal Database Project) database and the phylogenetic tree were constructed using MEGA 6 [33] (Molecular Evolutionary Genetics Analysis) .

Production of pigment

Pezicula sp. BDF 9/1 was initially grown on potato dextrose agar (PDA) medium in petri dishes at 23°C for 7 days and then transferred to the liquid potato dextrose broth culture medium by a sterilized inoculating needle. The culture was grown in 250 ml flasks containing 50 ml of potato dextrose broth medium at 23 °C on a rotary shaker (120 rpm) for 20 days. Experiments were performed at least in triplicate to ensure reproducibility.

Isolation and estimation of the red pigment

The culture broth was filtered through a filter paper (No.2; Whatman) and the supernatant fluid centrifuged at 10,000 g for 20 min. The concentration of red pigment was estimated measuring the absorbance of the culture filtrate at 500 nm (Spectrophotometer). Blank was prepared with uncultured broth.

Purification of the red pigment

The purification of the isolated red pigment was performed by column chromatography on SiO_2 (230-400 Å mesh) filtration column (65 x 2 cm) using ethyl acetate and hexane at a flow rate of 0.5 ml/min and elutes were collected in test tubes by an automated fraction collector. The purified red pigment was dried and stored in desiccators for further structural investigations.

LC-MS

The mass spectrum of the pigment was recorded on a LC-MSD-Trap-SL instrument. Mass spectrometry data are represented as a three-dimensional contour map. In this form, the mass-

to-charge, *m/z* is on the *x*-axis, intensity the *y*-axis, and an additional experimental parameter, time, is recorded on the *z*-axis, Interpreting mass spectra involves spectra with accurate mass. Two major peak of intensity (225.1, 449.3) indicate maximum mass per charge at the X-axis in this contour map.

^{1}H NMR spectroscopy

The ^{1}H experiment was recorded at 500 MHz, on a Bruker Avance DPX-500 spectrometer using 5-mm broadband probe. For NMR measurements Pigments was dried in vacuum for several days. The ^{1}H NMR spectra were recorded at 27 °C. Data were recorded using Bruker Topspin 3.1 software. Acetone was used as the internal standard.

IR Spectroscopic analysis

Pigment sample (2 mg) was mixed with highly purified KBr (200 mg) and a pressure generated sample-KBr disk was prepared for IR analysis. The IR spectrum was recorded on a Perkin- Elmer model L1600300 Spectrum Two LiTa IR Spectrophotometer in the range 4000-400 cm^{-1}.

Antimicrobial activity

Antibacterial activity of the pigment

Antibacterial activity was evaluated against *Aeromonas salmonicida*, *Aeromonas caviae*, *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Proteus mirabilis* and *Escherichia coli*. The antibacterial activity was carried out using Mullar Hinton agar medium. In petri dishes a cup boar was made up in middle and poured with 2µl suspension of red pigment. Then the bacterial pathogen was streaked out from the boar side. The distance between boar & streaks were observed.

Antifungal activity of the pigment

Antifungal properties of red pigment obtained from *Pezicula sp.* were estimated against several plant pathogenic fungal strains like *Colletotrichum laginarium*, *Botrytis sp.*, *Trichoderma sp.*, *Fusarium sp.*, *Pythium sp.* and *Candita albicans*.

Results and discussion

Identification of Pezicula sp. BDF 9/1

The mycelium of the organism looks white at first than light brown with lots of aerial hyphae. The reproductive characteristics of the organism had aseptate, hyaline hyphae; asexual conidiospore, single celled conidia cylindrical with both ends blunt (data is not shown here).

The rDNA gene sequence data of fungi BDF9/1 were deposited as entry KP234255 in GeneBank. Strain BDF9/1 showed 91 % sequence similarity with *Pezicula* sp (Fig 1). A BLAST search of the data base indicated a close genetic relationship with other *Pezicula* sp. [1,21,26,29]. The phylogenetic tree was constructed using MEGA 6 [33] (Molecular Evolutionary Genetics Analysis). The evolutionary history was inferred using the Neighbor-Joining method [25]. The evolutionary distances were computed using the Kimura 2-parameter method [18,20]. DNA sequences were deposited in Gene Bank under accession number KP234255. The isolated endophytic fungus was deposited with the access code number NFCCI -3339-*Pezicula* sp. in National Fungal Culture Collection of India, Pune, Maharashtra - 411004, India.

Purification and structure determination of Pigment

The endophytic fungi *Pezicula* sp. BDF9/1 was grown in PDB for 20 days. The fermented broth was filtered and centrifuged. After extraction with ethyl acetate, the resulting organic layer was washed with H_2O (three times). The organic phases were combined, dried and evaporated to afford a red residue (3.26 g). The red crude was fractionated by column chromatography on SiO2 (230-400 A mesh) using ethyl acetate and hexane as eluent afforded a light red oil (0.50 g) (Fig 2). After preliminary characterization the fractionated product was further purified by column chromatography on SiO2 using ethyl acetate and hexane as eluent to give red oil (20 mg). Two fractions of pigment (Fr-I and Fr-II) were collected. Fr-I and Fr-II were eluted between 20-30 and 50-60 tubes, respectively. The major fraction, Fr-I was considered for further investigation.

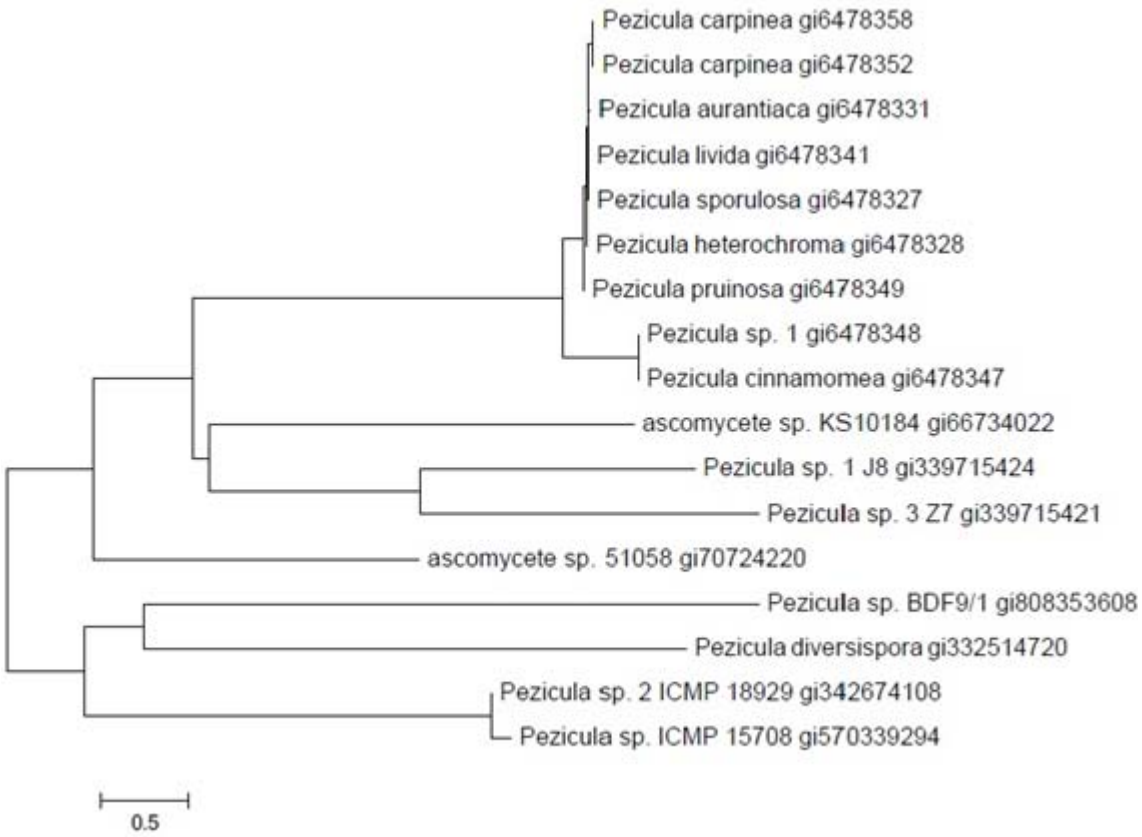

Fig. 1. Evolutionary relationships of *Pezicula* sp. BDF9/1 and some related fungi

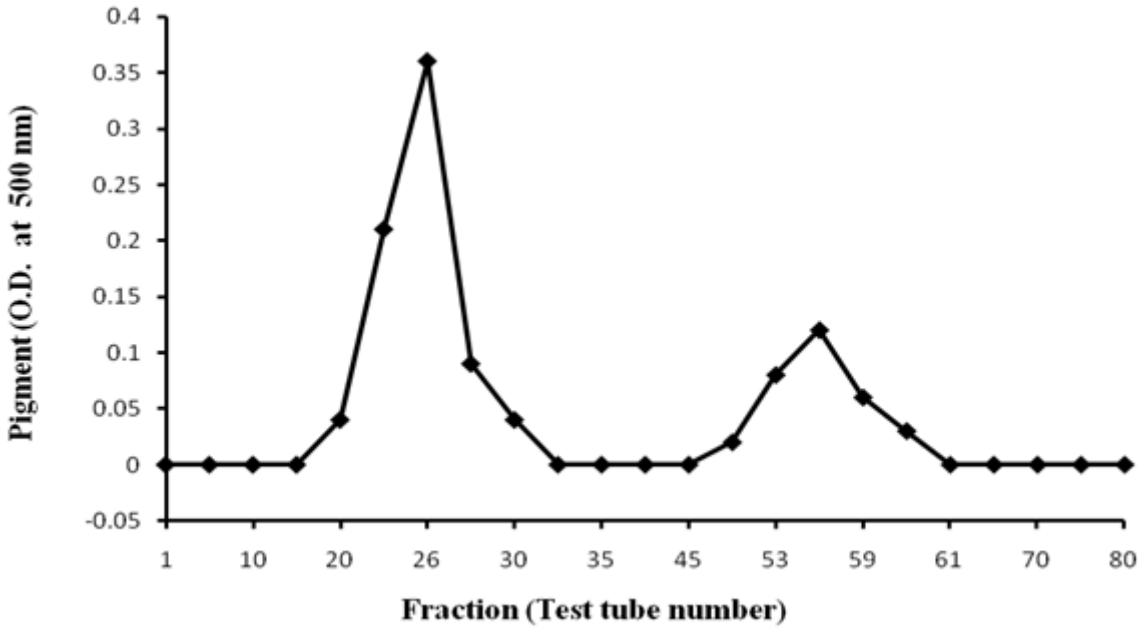

Fig. 2. Gel filtration chromatograms of the pigment produced by the endophytic fungus *Pezicula* sp. BDF9/1.

Analytical analysis

The IR spectrum (Fig 3) of the compound showed peaks at range 3400-3600 cm^{-1} are due to O-H stretch. Strong peak at 2954 cm-1 for C-

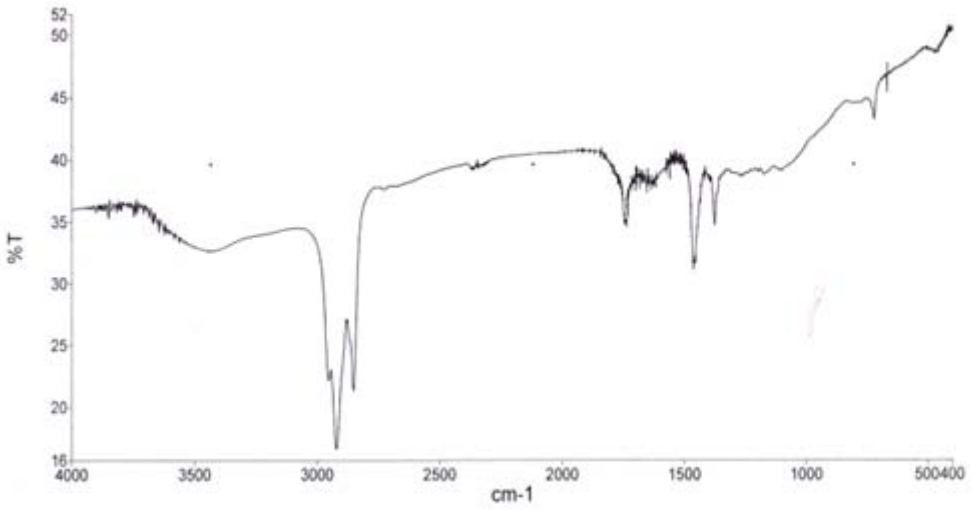

Fig. 3. FT-IR Spectrum of the biopigment isolated from *Pezicula* sp. BDF9/1.

H stretching in CH group and peaks around 1715-1675 cm-1 for C=O stretching in saturated aliphatic carbon and 6-membered cyclic ketone. Peaks around 1497 and 1461 may be obtained from Ph group.

The isolated structure has 22 hydrogen protons with 6 magnetically different environments. The ^{1}H NMR spectra showed peaks at the range of 12.0 -11.0 ppm conformed the four H-bonded protons (pink colour). Three aromatic protons (red colour) were observed at the range of 7.2-6.4 ppm, whereas two aromatic hydroxyl protons (blue colour) appeared at 5.6 and 5.0 ppm in ^{1}H NMR. The peaks at the range of 4.8 to 2.8 ppm are due to eight aliphatic protons of the compound. Two aliphatic hydroxyl protons (black colour) and one methyl group protons peaks were observed at around 2.0 ppm and 1.0 ppm respectively. ^{1}H NMR (CDCl3): δ 11.9 (s, OH, 1H), 11.5 (s, OH, 1H), 11.3 (s. OH, 1H), 11.0 (s, OH, 1H), 7.4 (Ar, 1H), 6.8 (Ar, 1H), 6.4 (Ar, 1H), 5.7 (OH, 1H), 4.9 (OH, 1H), 4.8 - 2.8 (aliphatic protons, 8H), 2.0 (OH, 2H), 1.0 (3H, CH3).

For characterization of the pigment, LC-MS data analysis of the experimental pigment shows two major peak of intensity (225.1, 449.3) indicate maximum mass per charge at the X-axis of the mass spectrum. So, 225.1 and 449.3 molecular mass compound found major amount in experimental pigment sample. The major compound identified as 3,4-dihydro-2,6,8,9-tetrahydroxy-3-(1,3,4,6-tetrahydroxy-2,5-dioxoheptyl)anthracen-1(2H)-one (449.3) (Fig 4).

MS: Molecular formula $C_{21}H_{22}O_{11;}$ $[M-1]^{-}$ = 449.3 (Fig 5).

Elemental analysis

Calcd. for C21H22O11: C, 56.00; H, 4.92; O, 39.08; Found. C, 55.06; H, 4.41; O, 37.87.

The chemical structure of the compound was elucidated on the basis of spectroscopic data. The molecular formula, $C_{21}H_{22}O_{11}$ of the anthracenone derivative was determined by MS spectra. The elemental analysis of the anthracenone derivative was confirmed that the compound contain only elements of carbon, hydrogen and oxygen. The

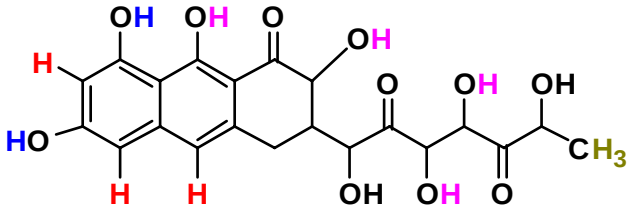

Fig. 4. Structure of the pigment 3,4-dihydro-2,6,8,9-tetrahydroxy-3-(1,3,4,6-tetrahydroxy-2,5-dioxoheptyl)anthracen-1(2H)-one

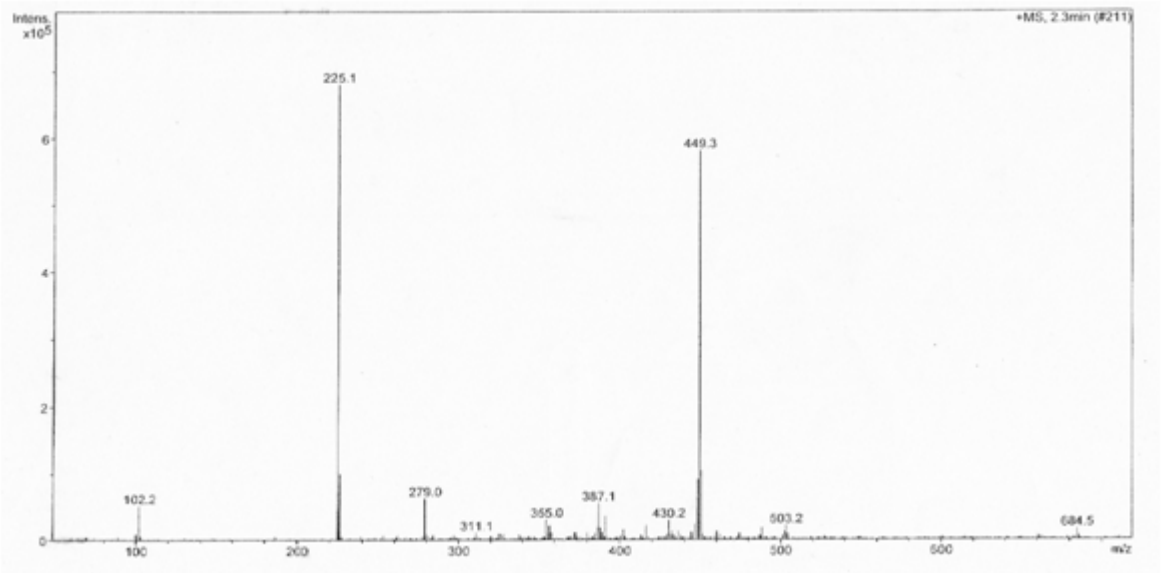

Fig. 5. Mass spectra of the biopigment isolated from *Pezicula* sp. BDF9/1.

IR spectrum of the compound suggested the presence of aliphatic cyclic system with aromatic ring, hydroxyl group and ketone group. In ^{1}H NMR spectra clearly show the presence of H-bonded protons (Fig 6). The peaks in the range of 7.2-6.4 ppm confirmed the presence of aromatic protons. The presence of many broad peaks indicated the presence of hydroxyl group with different magnetic field. Compound has many peaks at aliphatic region in ^{1}H NMR clearly confirmed the presence of aliphatic chain of the anthraquinone derivative. Till now, several bioactive compounds have been isolated from *Pezicula* sp. The antibiotic equisetin derivative [29], furofurandiones from *Pezicula livida* [21] and five fungicidal and hervicidal metabolites reported in 1995 [26] represent great potential in *Pezicula* fungi as a natural product source with diverse activities. Also cryptocandin and its related bioactive agents which out-standing antifungal activity against both human and plant pathogens were reported to be produced by *Cryptosporiopsis quercina*, the imperfect stage of *Pezicula cinnamomea* [28].

Antimicrobial activity

The screening of antimicrobial activity of iso-

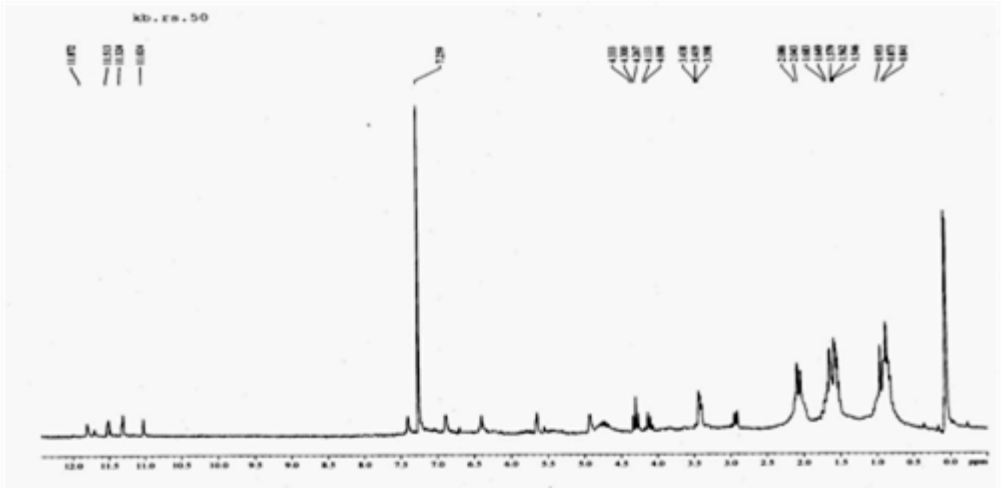

Fig. 6. ^{1}H NMR spectrum (500 MHZ, 27^{0}C) of the biopigment isolated from *Pezicula* sp. BDF9/1.

lated red pigment was carried out. It was found that the pigment showed antimicrobial activity against several pathogenic bacteria and fungi. The label of antimicrobial activity varies from organism to organism. The pigment was found most effective against almost all the tested fungal and bacterial strains (Table 1 and Table 2). The inhibition zone diameter varied from 3 mm to 7 mm. The maximum inhibition zone was found against *C. laginarium* and *Aeromonas caviae*. All fungal strains were more or less inhibited in presence of this red pigment.

Previously Visalakchi and Muthumary [37] evaluated antimicrobial activity of redish brown pigment produced by endophytic *Monodictys castaneae* SVJM139; and reported that the pigment significantly inhibited the growth of pathogenic *Staphylococcus aureus*, *Klebsiella pneumonia, Salmonella typhi* and *Vibrio cholera.*

Conclusions

Endophytes are rich sources of novel natural compounds with a wide spectrum of biological activities and a high level of structural diversity. From the present investigation we have tried to elaborate the molecular structure determination of the pigment produced by endophytic fungi *Pezicula* sp. BDF9/1 and its antibacterial, antifungal activity. The compound was identified as 3,4-dihydro-2,6,8,9-tetrahydroxy-3-(1,3,4,6- tetrahydroxy- 2,5-dioxoheptyl) anthracen -1(2H)-one. Further studies are going on to find out its toxicity against animal cells.

Acknowledgments

We are thankful to Dr. Asit Patra, Senior Scientist, National Physical Laboratory, New Delhi for structure elucidation of the compound and necessary suggestion.

Table 1.Antimicrobial activity against test pathogens

Bacterial Strain	Inhibition Zone (mm)
Aeromonas salmonicida	05
Aeromonas caviae	07
Bacillus cereus	04
Pseudomonas aeruginosa	04
Bacillus subtilis	05
Aeromonas hydrophila	03
Shigella flexneri	06
Proteus mirabilis	00
Escherichia coli	04

Table 2. Antifungal activity against test pathogens

Fungal Strain	Inhibition Zone (mm)
Colletotrichum laginarium	06
Botrytis sp.	04
Trichoderma sp.	05
Fusarium sp.	03
Pythium sp.	04
Candita albicans	03

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