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Molecular identification and profiling of volatile organic compounds (VOCs) of *Porostereum sp.* HGBS16 and *Neurospora sp.* PAMS29: Chambal ravine soil fungal isolates

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Abstract

The study is aimed at the molecular identification of ravine soil fungal isolates and their volatile organic compounds (VOCs) profiling. Chambal ravines of Morena, located at latitude 26º5′N and longitude 78º0′ E at an elevation of 177 meters. Ravine soil is marked for depleted nutrients. The isolates were identified by macroscopic and microscopic examinations followed by molecular identification the extracted fungal DNA was amplified for specific internal transcribed spacer primer (ITS1/ITS4). The products were sequenced and deposited in GenBank (NCBI), sequence similarity was checked and a phylogram was constructed. The isolates were identified and named *Porostereum sp.* HGBS16 and *Neurospora sp.* PAMS29. The VOCs/bioactive molecules were allowed to produce under static submerged fermentation. VOCs/bioactive molecules extracted with polar solvent and characterized by GCMS analysis. Besides playing an active role in communication, the obtained VOCs have other useful attributes of industrial and other beneficial uses. The prevailing compounds produced by *Neurospora sp.* PAMS29 is octasiloxane (50.32%) followed by the production of octadecane (42.67%) and cyclopentasiloxane (7.01%) whereas *Porostereum sp.* HGB16 displayed bicyclo (2.2.1) heptane-2-one (86.09%), followed by dodecane (6.09%) and tetradecane (4.05%). The VOC octadecane is reported as a pheromone, a chemical messenger which is useful for mating in fungi. The Presence of octadecane confirms that *Neurospora sp.* PAMS29 used Pheromones as the mating messenger. Both fungal extracts showed the presence of vitamin C under screening test and exhibited good DPPH free radical scavenging activity with 76.74±7.81 inhibition by *Porostereum sp.* HGB16 whereas *Neurospora sp.* PAMS29 showed 82.1±6.47 percent inhibition activity. Results showed that the VOCs produced by fungal isolates have the potential for industrial uses and can be used in body care products in place of synthetic polysiloxanes, though the D5 is already reported to be used in cosmetics. This study introduces new fungal strains and their VOCs to the microbial research domain. Simultaneously the isolates are producing vitamin C and also exhibited the DPPH free radical scavenging activities. Both isolates are aromatic therefore it can be used in the perfume industry. Concluding, this is the

first attempt at molecular identification of ravine soil fungal isolates and exploration of their VOCs. These results supported that VOCs are not waste products, they are very useful products at a certain level.

Keywords: Molecular identification, Chambal ravines, VOCs, DPPH free radical, Scavenging activity

1. Introduction

Soil is basic for all resources; it harbors the number of microbes that are an essential element of all biological reactions and ecological systems. The microbial flora of soil is varied according to the climatic conditions encountered by the region. Soil microorganisms are the major contributor to maintaining the sustainability of all life forms. Ravines are landforms due to extensive water erosion and soil loss; Chambal ravines are the most unexplored geographical distribution of soil [1,2].

Organic compounds of low molecular weight have high vapor pressure under encompassing environ, usually, they are water-insoluble and thus possess solubility in lipids [3]. VOCs from different biochemical origins belongs to different groups such as alcohol, esters, ether, aromatic compounds, terpenes (mono and sesuiterpenes), aldehydes, furans, ketones, compounds containing S and N element, and hydrocarbons are produced by fungi [4,5]. Around four hundred seventy-nine intermediate and final products of different metabolic pathways have been reported and identified from fungi, their emission plays the centric role in physiological and ecological roles for many organisms [5]. Communication is the base of survival and VOCs are recognized as communication signals in the microbial world which is a very notable aspect of the microbial environment. However, the molecular aspect of it is still unknown. It is believed that these VOCs are harmful toxic compounds. Besides this, the VOCs are useful such as they act as communication signals among the community simultaneously, they are reported as a good biocontrol agent against phytopathogens [6].

Taxonomically, *Porostereum sp.* belongs to Basidiomycota whereas *Neurospora sp.* comes under Ascomycota. A lot of research work has been conducted on *Neurospora crassa*, its half haploid cycle and easy-to-grow nature make it suitable for research studies therefore, it is known as model fungi for genetic studies whereas little research has been conducted on *Porostereum spadieceum* in the present study the molecular characterization of *Porostereum sp.* HGBS16 & *Neurospora sp.* PAMS29 was done simultaneously their VOCs profile is explored and their possible uses are described.

2. Research method

2.1. Sample collection and isolation

Soil samples were collected from the ravine soil of Morena, and fungal isolation was carried out by serial dilution method described by Sohhail [7], plates were incubated at 25±2°C for week pure cultures were obtained by subsequent inoculation of a single colony on potato dextrose agar plate supplemented with 1% streptomycin, thus pure cultures were maintained for further analysis [8].

2.2. Morphological and microscopic identification

Colony characteristics such as shape, size, color, pigmentation, and hyphae were observed and recorded. Lactophenol cotton blue staining was followed to analyze microscopic attributes; examination was performed by compound microscope under 40X resolution [8].

2.3. Molecular characterization

Seven days old pure fungal isolates were used for genomic DNA isolation, the kit method was followed to extract and amplify genomic DNA. Amplification of ribosomal internal transcribed spacer (ITS) was done by using primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3')

[9,7]. A montage PCR clean-up kit (Millipore) is used for the purification of PCR products. ABI PRISM genetic analyzer (Applied Biosystems) is used to sequence the amplicon [10,9], BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq, DNA polymerase (FS enzyme) (Applied Biosystems) was used. The fragments were analyzed to obtain a consensus sequence which is compared with the other related sequence by BLAST search, obtained sequence deposited to the Genbank (NCBI) [11,12,13]. MUSCLE 3.7 is used for multiple alignments [14] and the resulting aligned sequences were cured using the program Gblocks 0.91b. These Gblocks eliminate poorly aligned positions and divergent regions (remove alignment noise) [15]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis, and HKY85 as a Substitution model. The program Tree MEGA 7.0 was used for tree rendering [16].

2.4. Production & extraction of bioactive molecule/VOCs

The bioactive molecules were allowed to be produced under static submerged fermentation. 8 mm disc of each isolate was cut and inoculated into 50 ml of sabouraud's dextrose broth in an Erlenmeyer flask (250 ml), and the flasks were kept at 30±2°C for seven days after incubation After incubation the mycelium was filtered through whatmann filter paper no.1, and filtrate was centrifuged at 4000 rpm for 15 minutes [17,18] the supernatant (fermentation broth) was collected [18]. Thereafter the extraction was followed with n-butanol [6] at room temperature the obtained extract was allowed to evaporate in a vacuum evaporator.

2.5. VOCs detection by GCMS analysis

The fermented broth of each fungi *Porostereum sp*. HGBS16 & *Neurospora sp*. PAMS29 was extracted with organic solvent n-butanol, according to the extraction procedure of Siddiquee et al.[19]. The extracted VOCs were identified via GC-MS analysis. The gas chromatography-mass spectroscopy had carried out on TRACE 1300 GC, TSQ 8000 TRIPLE QUADRUPOLE MS fitted with TG 5MS (30m X 0.25mm, 0.25µm) column and S/SL Injector. The injector temperature had kept at 250˚C and the MS transfer line temperature had kept at 300˚C along with the ion source temperature at 230℃. The column temperature had programmed between 60˚-280˚C at 10˚C/min using helium as carrier gas at a carrier flow rate of 1 ml/min. Injection volume had 1.0µl prepared in DMSO having Split flow 1 ml/min. The mass spectra had taken at 75 eV with a mass scan range from m/z 40-500 amu. The individual constituents had been identified by comparing their mass spectra with those of standard using NIST (National Institute of Standards and Technology, U.S. Department of Commerce) compounds.

2.6. Screening of Vitamin C

To determine the presence of Vitamin C, the AOAC titrimetric method was used as explained by Nielsen [20]. 5 ml of each fungal extract was titrated with DCPIP reagent, appearance of distinct light pink color for more than 5 seconds confirms the presence of vitamin C, the experiment was performed in triplicates.

2.7. DPPH radical scavenging activity

The DPPH scavenging activity of the fungal extract was investigated by the Blois method described by Esmaeilli & Sonoboli [21] with minor modifications. A 1:1 ratio of fermentation broth was added to 1ml of o.1mM methanolic solution of DPPH and incubated for 30 min. in dark. Absorbance was recorded at 517 nm and the % inhibition activity was estimated from [(Ao-A1)/Ao] × 100, where Ao is the absorbance of the control (DPPH solution) and A1 is the absorbance of the extract/standard. The DPPH free radical scavenging activity curves were prepared and IC50 values were calculated.

3. Results and discussion

The fungal isolates were grown on an agar plate and their morphological and microscopic characteristics were recorded. The isolate *Porostereum sp.* HGBS16 showed moderate growth, the colony was transparent with white margins after seven days of incubation at 25±2 ℃ on the PDA plate (Figure 1), with margin white, thin, and incurved, underside smooth. During incubation, the fungal colony produces a characteristic fragrant

aroma. Basidia clavate, 4-sterigmata, 18.5 – 28.5 X 5.7 – 6.6 µm. Basidiospores are narrowly ellipsoid, slightly apiculate, smooth, thin-walled, hyaline 6.7 – 8.4 X 3.5 – 4.5 µm. Skeletocystidia developed in large numbers, originate from the subiculum, cylindrical, sub-hyaline becoming light brown with age, immersed or slightly projecting out of hymenium, walls thick, slightly encrusted at apices, 40.5 – 85.6 X 4.0 – 7.0 µm. Skeletocystidia is pseudocystidia not true cystidia. Skeletocystidia is the elongation of skeletal hyphae that run horizontally and curve into the hymenium. The morphological and microscopic attributes were shown in figure 1, whereas isolate *Neurospora sp.* PAMS29 is fast growing, on PDA attained colony diam of 4 – 5 cm in one day at 25ºC, orange color powdery substances deposited to the edges on the agar plate within three days of incubation (Figure 2) The culture produces a marked citrus smell when opening the lid of plate. Mycelium is a tangled mass that grows within and on the substratum. Mycelium is white when young and turns yellow at maturity. Hyphae branched 7.5 – 8.5 µm in diameter. Conidiation is initiated when some hyphae change their mode of growth from hyphal elongation to apical budding, they give rise to proconidial chains. Most conidia are produced by apical budding. Macroconidiophores branched, septate, produce many multinucleate (3 – 6 nucleate) bright orange/pinkish, orange moniliod macroconidia (blastoconidia), 4.5 – 8.5 µm. Arthroconidia multinucleate (3 – 6 nuclei) arises by fragmentation of conidiophores. Uninucleate conidia arise singly or in short chains from microconidiophores 2.3 – 3.6 µm. Ascomata immersed to semi-immersed, sub-globose, dark brown to black, beaked, wall membranous, 300 – 450 µm, ostiole conical. Asci club-shaped, cylindrical, contains 8 ascospores. Ascospores ellipsoid, dark brown to black, variously ornamented with characteristic ribs. The morphological and microscopic attributes were shown in figure 2.

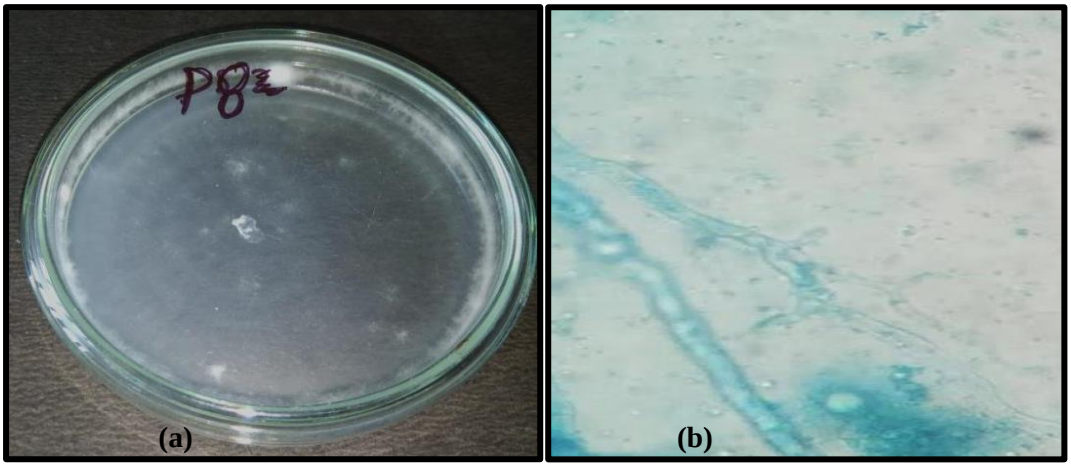


Figure 1. *Porostereum* sp. HGBS16 (a) Colony on PDA plate and (b) Mycelium (under 40X magnification)

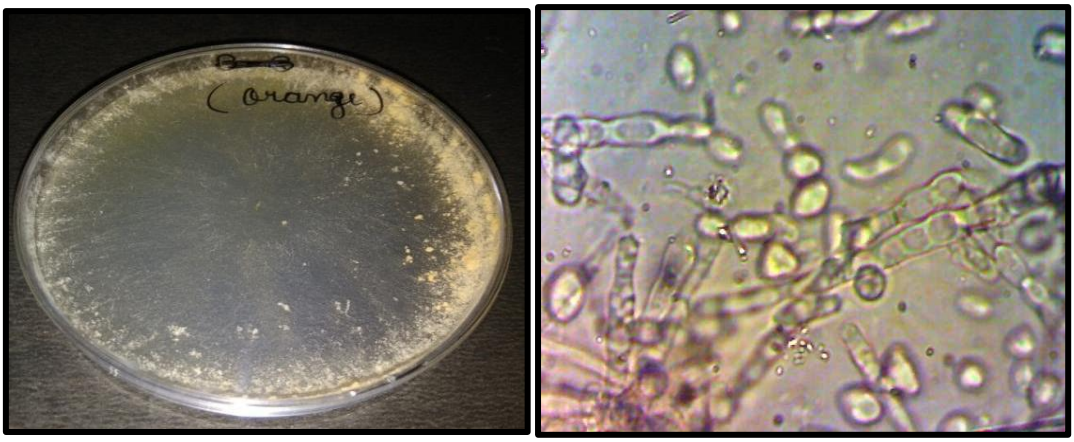


Figure 2. *Neurospora* sp. PAMS29 (a) Colony on PDA plate and (b) Conidiophore & conidia (under 40X magnification)

3.1. Molecular identification

The results obtained by morphological, microscopic identification were confirmed by PCR amplification of extracted DNA from these isolates with primers ITS1 and ITS4. The amplified ITS region of each *Porostereum sp.* and *Neurospora sp.* isolates were sequenced, and the obtained nucleotide consensus sequence was subjected to a similarity search by using the BLAST program, the results supported and confirmed the morphological and microscopic identification of obtained soil isolates. The comparison of ITS region (ITS1, 5.8SrDNA, and ITS4) was evaluated with the sequence previously deposited to the GenBank, the consensus sequence of *Porostereum sp.* HGBS16 showed the highest and nearest genetic similarity of 98% with the record sequence of *Porostereum spadiceum* TB30 from Madras, India MN184785. As well, close phylogenetic relationships also appeared with some *Porostereum spadiceum* isolates KP771706, MK269250, KF291007, and MW081306 (Figure 3).

Comparatively, the sequence obtained from *Neurospora sp*. PAMS29 with the other record sequence deposited to the GenBank revealed that the highest genetic similarity was 100% with *Neurospora crassa* OR74A XR_898035% (Figure 4).

The *Porostereum sp.* HGBS16 isolate identified and reported in this study also displayed genetic differences ranging 95–98% with the other *Porostereum spadiceum* isolates formerly identified, reported, and deposited in NCBI (Table 1 and Fig. 4). This newly identified *Porostereum sp.* PAMS29 can be more useful for future studies.

The results obtained from BLAST analysis revealed that isolate *Porostereum sp.* PAMS29 was genetically different from the other isolates previously registered in GenBank. The obtained consensus sequence of each *Porostereum sp.* PAMS29 and *Neurospora sp.* HGBS16 were recorded under the accession numbers MK156152, and MK204926 respectively. Polymerase chain reaction (PCR) is a highly accurate technology for the diagnosis of many organisms, including pathogenic and non-pathogenic fungi such as *F. solani*, *R. solani*, *Alternaria alternata*, and *Aspergillus* spp. [22,23], various researchers have analyzed the ITS region for fungal identification [24,25,26]. Therefore, PCR was used in this study to identify the *Porostereum sp.*HGBS16 and *Neurospora sp.* PAMS29 Chambal ravine soil fungal isolates.

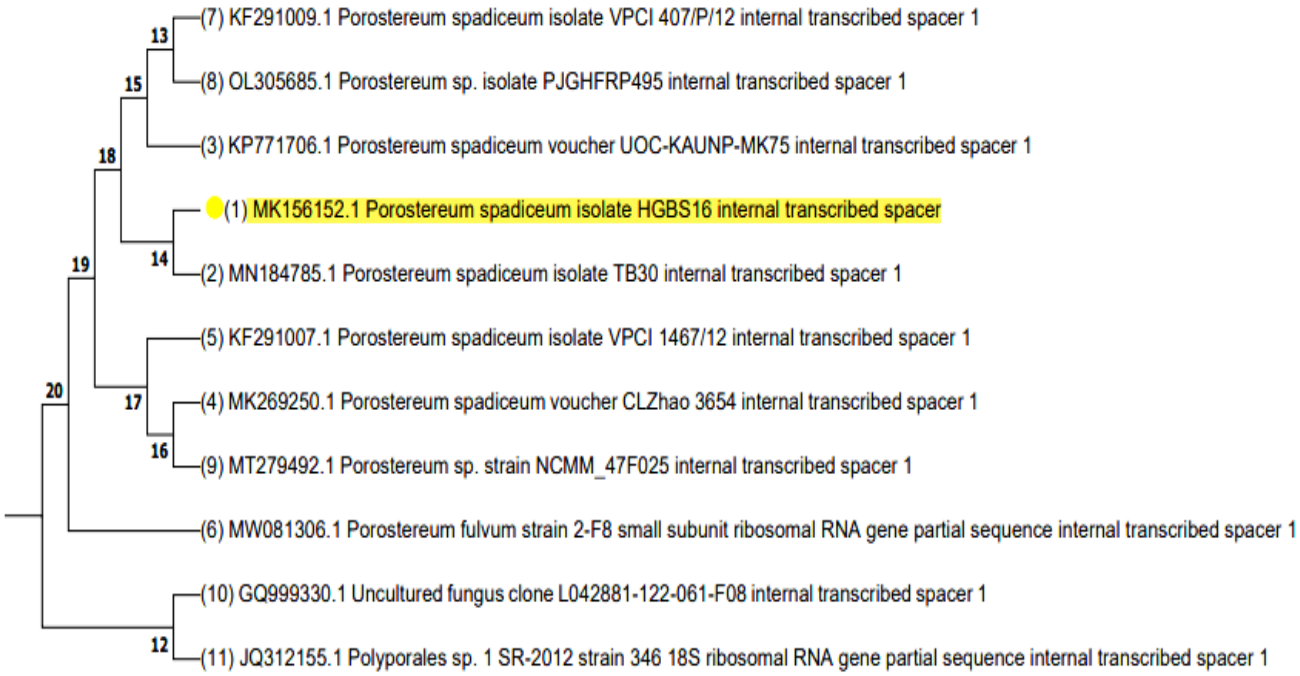


Figure 3. Phylogenetic tree of *Porostereum sp*. HGBS16

*Yellow color shows the query sequence

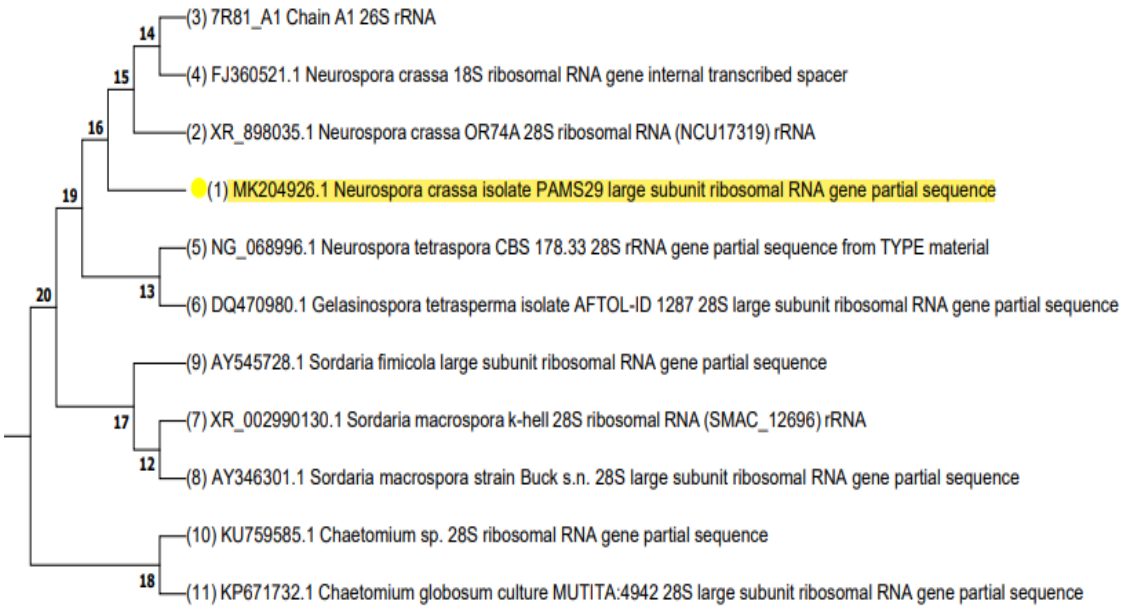


Figure 4. Molecular phylogram of *Neurospora* sp. PAMS29

*Yellow color shows the query sequence

The UPGMA algorithm was used to infer evolutionary history. The ideal trees were displayed, with a branch length sum of 0.10109714 & 0.03380163 respectively. The phylogenetic tree is displayed on a scale, with branch lengths (next to the branches) in the same units as the evolutionary distances used to build the tree. The evolutionary distances were calculated using the Maximum Composite Likelihood technique and are in base substitutions per site units. Eleven nucleotide sequences were examined. Gaps and missing data were removed from all positions. MEGA7.0 was used to perform evolutionary analysis.

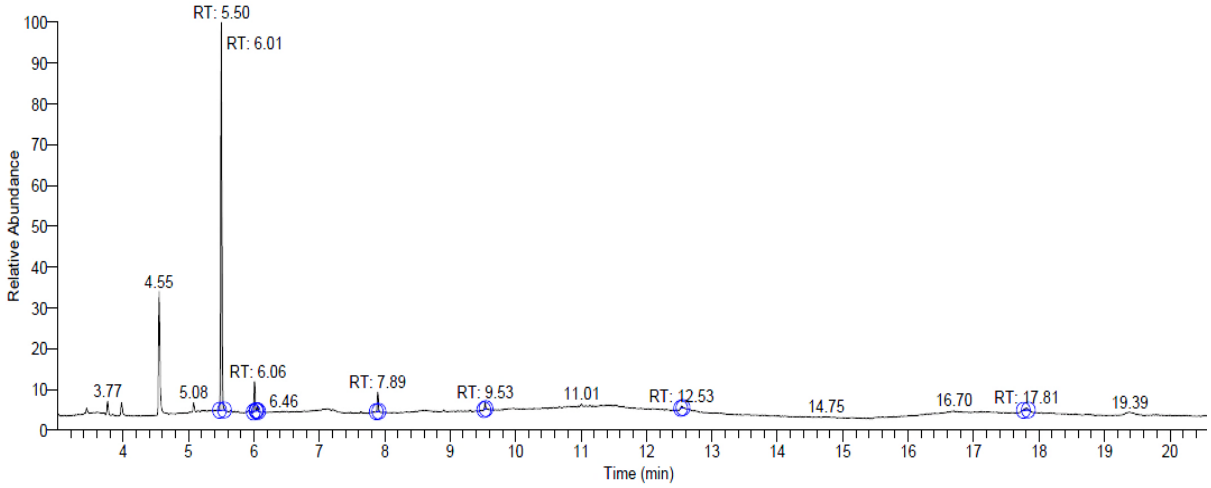
3.2. Production of Vitamin C

During titration the appearance of pink color confirms the presence of vitamin C.

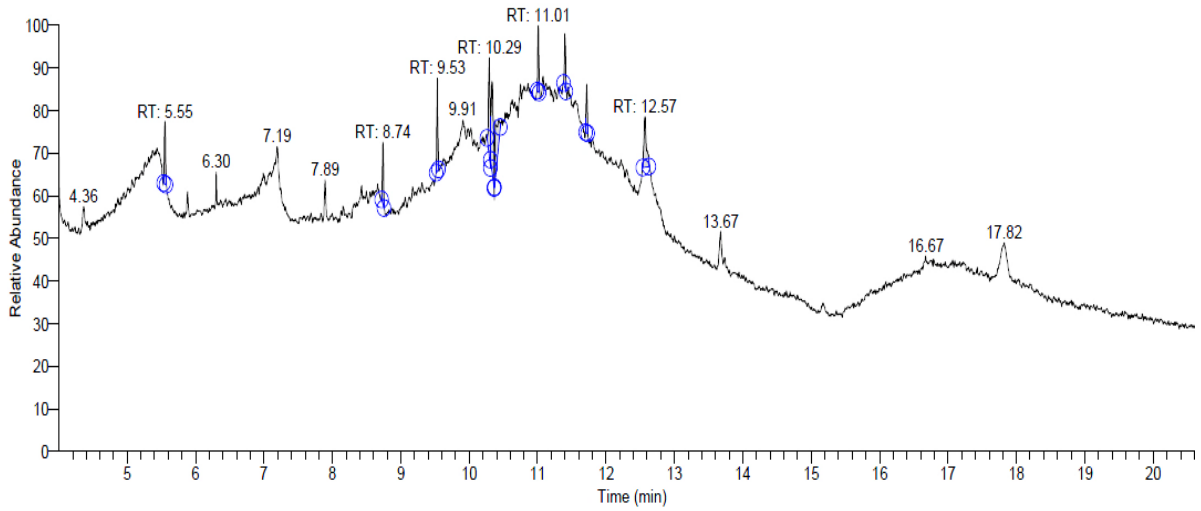
3.3. VOCs characterization by GCMS analysis

The presence of VOCs is characterized by GCMS analysis. The GCMS chromatogram of the fungal extract is displayed in figure 5. GCMS is one of the most adoptable techniques for VOCs/bioactive molecule identification [28]. Therefore, the VOCs/bioactive molecules were characterized by GCMS analysis as shown in figure 6. The interpretation of chemical constituents was based on retention time, mass spectra, molecular formula, molecular formula, and concentration percentage (area %) [28,29,6]. The National Institute Standard and Technology NIST database was used to evaluate the mass spectra of obtained fungal extract. The analyzed data revealed the presence of polysiloxane compounds. *Porostereum sp.* HGBS16 produced seven compounds. The major metabolite was Bicyclo (2.2.1) heptane-2-one (86.09%), the rests were dodecane (6.09%) and tetradecane (4.05%) whereas *Neurospora sp.* PAMS29 produced octasiloxane (50,32) followed by the production of octadecane (42,67) and cyclopentasiloxane (7.01%). recognized with the persistence of important biological activities in many applications. Similarly, Joel and Bhimba, [30] followed the GCMS analysis for bioactive molecule identification and reported 9-octadecanoic acid as the principal compound in *Neurospora crassa* but no clear evidence of *Neurospora sp.* VOCs is available. Since there was no report has been identified of VOCs of *Porostereum sp.* through GCMS analysis. This is the first report of VOCs production by Porostereum genera earlier studies discussed gibberellin production by genera Porostereum [31]. Hence many researchers have followed GCMS analysis for fungal VOCs/bioactive molecule identification they adopted GCMS for fungal bioactive molecule identification [32,33,34,27]. However, various scientists reported the presence of VOCs in another fungal extract like VOCs of *Trichoderma virens* was explored by Tabarestani [6], similarly, the role of

VOCs of bacterial strain *Serratia plymuthica* PRI-2C was explored by Schmidt et al. [35]. Similarly, Gao et al. [36] reported VOCs in *Aspergillus sp*. The obtained molecules are of industrial concern reported by various researchers such as octasiloxane and bicyclo (2.2.1) heptane-2-one reported as antimicrobial properties [37]. Similarly, n-octadecane is reported as an anti-corrosion agent, transformer oil, lubricant, solvent, paraffin, and chemical intermediate in organic synthesis, simultaneously it also acts as a chemical messenger in pheromones [38,39] Therefore, useful for mating in fungi. Rydberg [40] reported that Dodecane is used as a scintillator component, distillation chase, and solvent and also used as tributyl phosphate (TBP) diluent used in reprocessing of plants whereas tetradecane is used in organic synthesis and a kind of solvent in mixture with hexadecane also used as phase changing materials (PCMs) for air-conditioning, refrigeration and cool storage and standard for chromatographic analysis as well as act as an internal calibrator for silicate ion detection [40,41]. Pentasiloxane (D5) is a major compound of *Neurospora sp*. PAMS29 is widely used in cosmetics such as hair conditioners, skin care products, and so on and it is reported safe to be used in cosmetics [42]. It is concluded that both the fungal strains can produce a variety of VOCs/bioactive molecules of industrial interest. Though polysiloxanes are widely used in body care products and exclusively artificially synthesized there is a scope to separate the molecules and use them in place of chemically synthesized.



(a)



(b)

Figure 5. GCMS chromatogram of secondary metabolites produced by *Porostereum* sp. HGBS16 and *Neurospora* sp. PAMS29

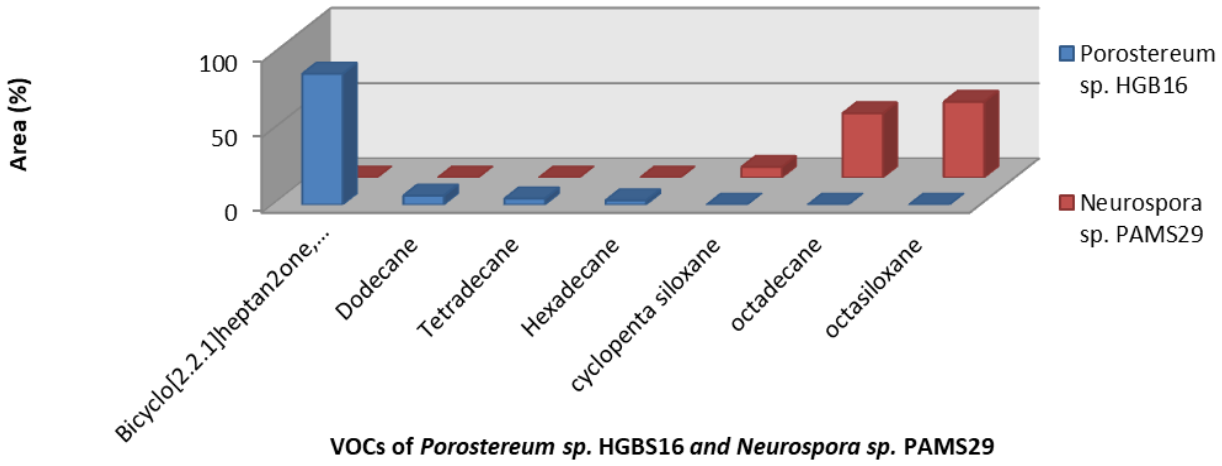


Figure 6. GCMS chromatogram of secondary metabolites produced by *Porostereum* sp. HGBS16 and *Neurospora* sp. PAMS29

3.4. DPPH assay

DPPH (1, 1-diphenyl-2-picrylhadrazyl) assay measures the radical scavenging activity, which reflects on antioxidant capacity. This method is widely used to evaluate antioxidant activity in a short time in comparison to other methods. In this study good DPPH free radical scavenging activity was exhibited by fungal extracts of *Porostereum sp.* HGBS16 and *Neurospora sp.* PAMS29. The activities were demonstrated in Figure 5. *Porostreum sp.* HGBS16 displayed maximum inhibition of free radicals at a concentration of 500µl/ml i.e., 76.74±7.81 percent, similar to the soil fungal isolate *Neurospora sp.* PAMS29 exhibited the maximum activity at the same concentration of 500µl/ml but the exhibited inhibition activity was 82.1±6.47 percent. Since a similar assay was not performed with the same fungal isolates though various have been reported free radical scavenging DPPH assay in other fungal strains like Sugiharto, [43] reported] 93.11 ± 0.03 and 14.20 ± 0.01 percent free radical scavenging activity in *A. charticola and R. oryzae* respectively, similarly, *Arora & Chandra*, [44] reported 89.8 percent DPPH activity in *Aspergillus fumigatus*. The obtained fungal strains showed promising antioxidant activity. Concluding, both the fungi *Porostereum sp.* HGB16 showed comparatively good antioxidant activity.

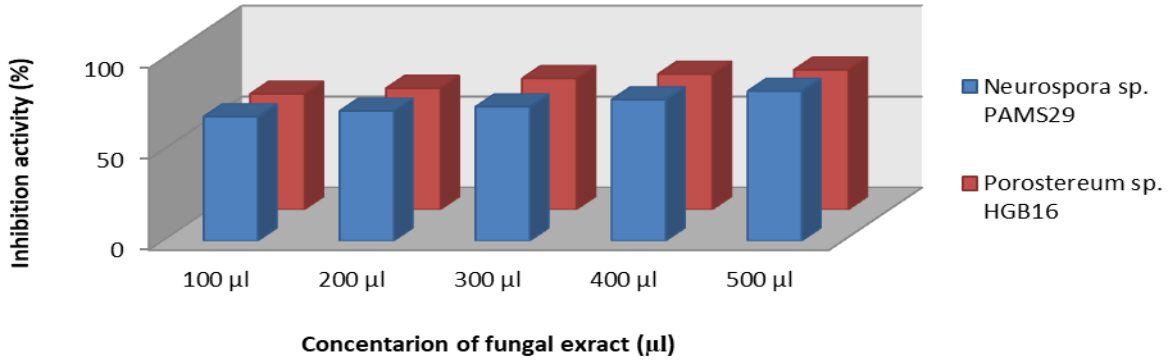


Figure 7. DPPH free radical scavenging activity of *Porostereum sp.* HGBS16 and *Neurospora sp.* PAMS29

4. Conclusions

The fungal isolates *Porostereum sp.* HGBS16 and *Neurospora sp*. PAMS29 (of Chambal ravine soil) has been isolated, identified and reported for the first time. Based on similarity index of ITS sequencing the isolated *Porostereum sp*. HGBS16 has the probability of a new strain of the genera *Porostreum*, further research is needed for confirmation. Volatile metabolites have been used in a variety of biological functions like

biocontrol and in communication (fungi-environment). Simultaneously, antibiotic, and immunosuppressive properties are also reported in some VOCs, it necessitates the monitoring of fungal VOC profiles. GC-MS shows the existence of VOCs in both fungal extracts. The VOCs are Bicyclo (2.2.1) heptane-2-one, pentasiloxanes, octasiloxanes, tetradecane, dodecane, and octadecane. Vitamin C and DPPH free radical scavenging activity was found in both fungal extracts. *Neurospora sp.* PAMS29 produced octadecane which acts as a pheromone messenger. Thus, both fungal strains can be employed as important fungal strains of industrial potential. The strains showed good antioxidants with strong free radical scavenging activity and the obtained VOCs have reported various industrial applications in body care products, conditioners (skin & hair), some of them are used as a lubricant and organic solvents. Both the fungal isolates are aromatic and therefore can be used in the perfume industry. To conclude, this is the first attempt at molecular identification of fungal isolates and exploration of their VOCs. The identified VOCs have numerous industrial applications. These results supported that VOCs are not waste products, they are very useful products at a certain level. The VOCs are significant chemicals in microbial communication hence may serve as a lingua franca in fungal interactions. The research suggests that VOCs have several industrial applications such as cosmetics, lubricants, etc. It is suggested that further research is needed for new species confirmation and possible applications of obtained VOCs and to evaluate DPPH activities.

Declaration of competing interest

The authors declare that they have no known financial or non-financial competing interests in any material discussed in this paper.

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