



Prevalence of fungal flora in the acidic environment of anthracite and lignite coal mines of Jammu.

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ABSTRACT

In the present study, coal samples from Moghla (Rajouri) and Kotla (Reasi) coal mines of Jammu province known for anthracite and lignite coal respectively were analyzed for their physical properties in order to assess their influence on the prevalence of acid tolerant and acidophilic fungi. The pH of lignite coal was found to be 2.5, whereas that of anthracite coal was 3.9. This revealed that both the types of coal have strongly acidic pH, which may favour the prevalence of both acid tolerant and acidophilic fungal species. The electrical conductivity of lignite coal was strong (3.50 dSm^{-1}), whereas that of anthracite coal was slight (0.803 dSm^{-1}). During the survey, a total of 33 fungal species belonging to 19 genera were recovered from these two types of coal. The recovered fungal species included two species of Zygomycetes, one species of Ascomycetes and a large number of mitosporic fungal species (30). Of these, 30 fungal species belonging to 17 genera were recovered from lignite coal, whereas 28 fungal species belonging to 16 genera were recovered from anthracite coal. They were all acid tolerant.

Keywords: *Physical properties, coal mines, anthracite, lignite, fungal species.*

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INTRODUCTION

Coal is physically and chemically a complex and heterogeneous material mainly consisting of organic and inorganic mineral constituents. There are over 861 billion tonnes of proven coal reserves world-wide and the biggest reserves are in U.S.A, Russia, China and India. According to an estimate, there are 44 major coalfields in India and the total reserves of all types of coal are 287 billion tonnes [1]. There are basically three major types of coal, namely anthracite, bituminous and lignite. Among these, anthracite is considered to be the oldest coal from geological perspective, which is hard and composed mainly of carbon with little moisture and volatile content. On the other hand, lignite is considered to be the youngest coal from geological perspective, which is soft, composed of high moisture and volatile content.

A number of bacteria and fungi are known to degrade the organic matter and cellulose content of coal by their enzymatic action [2]. In fact, different types of micro-organisms have co-existed with coal since its deposition millions of years ago. The microbes have survived in a dormant state, which slowed down their metabolism in order to survive in the unfavourable conditions of coal mines [3]. Microbial degradation of coal has been considered as an economic and effective way of transforming macromolecules into simpler, low molecular weight products [4].

The first pure culture of a microorganism growing on brown coal samples was obtained by Galle [5]. Later, the ability of microorganisms to attack low rank coal and modify its physico-chemical properties was reported by some other workers also [6, 7]. In addition, microbes consisting of bacteria, yeasts and filamentous fungi have also been tested for utilization of hard coal as sole source of carbon and energy [3]. Few researchers have even demonstrated that some of the filamentous fungi and Streptomyces have the ability to attack and solubilize the low rank coal [8, 9].

In India, very few studies have been conducted on the physical properties of coal and the prevalence of fungal flora in the coal mines [10]. So far, there is no such work from Jammu and Kashmir State, where a number of coal mines are scattered in different districts. In view of this, the present investigation was undertaken to study the physical properties and prevalence of fungal flora in the coal of Moghla mine at Kalakote (Rajouri district) known for anthracite coal and Kotla mine at Ransoo (Reasi district), known for lignite coal.

MATERIALS AND METHODS

Sampling of Coal

Samples were collected from two coal mines of Jammu Province viz., Moghla and Kotla coal mines situated in district Rajouri and district Reasi respectively. Moghla coal mine (altitude 600 m above m.s.l) is known for the anthracite type of coal and Kotla coal mine (altitude 466m above m.s.l) is known for the lignite type of coal. The samples were collected from these two coal mines with the help of sterilized spatula and were brought to the laboratory in pre-sterilized polythene bags for the isolation of fungal flora and analysis of coal for their physical characteristics.

Isolation of fungal species

Two methods viz., dilution pour plate method [11] and Warcup method [12] were used for the isolation of fungal flora. According to the dilution pour plate technique, 1 g of coal sample from the site was taken and transferred aseptically to 99 ml of sterile water in a 250 ml conical flask. The contents were stirred on a rotary shaker at 180 rpm for 30 minutes at room temperature. Then 1 ml of the final diluted suspension was poured in each sterilized petriplate, which was then plated with 15-20 ml of modified Czapek Dox agar (CDA) medium supplemented with Rose Bengal (0.1mg/100 ml) and streptomycin sulphate (50mg/1000ml). Each petriplate was then swirled to mix the inoculum properly. Five replicates were prepared and incubated at $28^{\circ} \pm 2^{\circ}\text{C}$ for 7 days or till proper growth of the fungal colonies was obtained. According to Warcup method, petriplate was prepared by pouring 10-15 ml of modified Czapek Dox agar (CDA) medium supplemented with Rose Bengal (0.1mg/100 ml) and streptomycin sulphate (50mg/1000ml) and a small amount of coal sample was dispersed over it. The petriplate was slightly swirled before the agar solidified. Five replicates were prepared and incubation was done at $28^{\circ} \pm 2^{\circ}\text{C}$ for 7 days or till proper growth of the fungal colonies was obtained.

Physical characterization of coal samples

Coal samples from Moghla and Kotla coal mines were analysed for their physical properties in order to assess their influence on the occurrence and distribution of fungal flora. The physical characterization of coal samples was done by following different determination methods. The pH of the coal was determined by following the potentiometric determination method. Weighed 20g of processed coal sample, transferred it to a 100ml beaker and added 50ml of distilled water. Stirred intermittently for about half an hour. Switched on pH meter, set the temperature compensation bob. Warmed for 10-30 minutes and adjusted the galvanometer pointer to zero. Dipped the electrodes in the buffer of known pH and adjusted the pH meter accordingly. Took reading of coal suspension on the pH meter after adjusting it to desired pH with a buffer of suitable pH.

Rating chart of pH:

Class	pH rating
Strongly acidic	<4.5
Moderately acidic	4.5-5.5
Slightly acidic	5.6-6.5
Neutral	6.6-7.5
Slightly alkaline	7.6-8.5
Moderately alkaline	8.6-9.5
Strongly alkaline	>9.5

The electrical conductivity was determined by conductivity meter. Electrical conductivity (EC) indicates the presence or absence of salts, but does not indicate which salt may be present. Set the temperature knob of conductivity meter and connected the cell to the water. Coal suspension made for the determination of pH is used for conductivity determination after keeping the suspension for more than 12 hours. Dipped the conductivity cell in the test solution and rotated the knob until the dark shadow is at its widest and noted the reading on the scale.

Electrical conductivity ratings:

Code	Class	EC (1:2) (dSm ⁻¹)
S1	Slight	<2.0
S2	Moderate	2 - 3
S3	Strong	3 - 4
S4	Very strong	> 4.0

Identification

For the purpose of identification, the recovered fungi were grown and made to sporulate on different culture media, such as potato dextrose agar medium (PDA), malt extract agar medium (MEA), Czapek yeast agar medium (CYA), potato sucrose agar medium (PSA) and water agar medium (WA). Relevant

literature and various taxonomic keys given by several workers were used for the identification purpose [13,14,15,16,17,18,19,20, 21,22,23,24,25,26,27].

Calculation of percentage colonization frequency, colony forming units and percentage distribution

Percentage colonization frequency (CF %), cfu and percentage distribution were calculated for each fungal species. Colony forming units (cfu /g) were calculated by following Parikh and Shah [28].

$$CF(\%) = \frac{\text{Number of coal samples colonised by a specific fungus}}{\text{Total number of samples studied}} \times 100$$

$$cfu/g = \frac{a \times d}{s}$$

where,

a = average number of colonies on the petriplate

d = dilution factor (100)

s = dry weight of the coal sample

$$Distribution (\%) = \frac{\text{Number of isolates of a fungus}}{\text{Total number of isolates}} \times 100$$

RESULTS AND DISCUSSION

During the period under study, 30 coal samples from two different sites viz., Kotla mine having lignite coal and Moghla mine having anthracite coal were collected and screened for associated fungal diversity. A total of 33 fungal species belonging to 19 genera were recovered from the two sites. Of these, 30 fungal species were recovered from lignite coal samples and 28 fungal species were recovered from anthracite coal samples (Figure 1). Among the recovered fungal flora from both the sites, Zygomycetes were represented by one species each of *Rhizopus* (*R. oryzae*) and *Syncephalastrum* (*S. racemosum*), which accounted for 6% of the recovered fungal species. Class Ascomycetes was represented by only one species belonging to genus *Ajellomyces* (*A. capsulatus*), which accounted for 3% of the recovered species, whereas mitosporic fungi represented the bulk (91%) of the recovered species (Figure 2). The fungal species of common occurrence included *Rhizopus oryzae*, *Syncephalastrum racemosum*, *Aspergillus niger*, *A. candidus*, *A. flavus*, *A. parasiticus*, *A. fumigatus*, *A. sulphureus*, *A. sydowii*, *Isaria farinosa*, *Metarhizium carneum*, *Phialemonium inflatum*, *Sagenomella griseoviridis*, *Penicillium griseofulvum*, *P. purpurogenum*, *Sarocladium strictum*, *S. implicatum*, *Acremonium roseolum*, *Curvularia lunata*, *C. brachyspora*, *C. pallescens*, *C. tsudae*, *Cladosporium cladosporioides*, *Fusarium incarnatum* and *Alternaria alternata*.

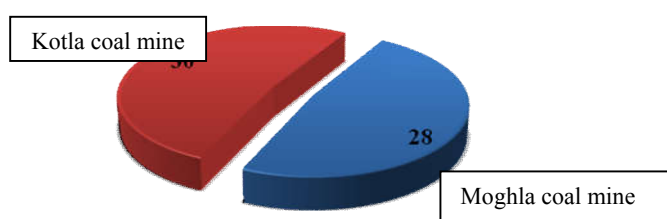


Figure 1. Total number of fungal species recovered from the investigated coal mines.

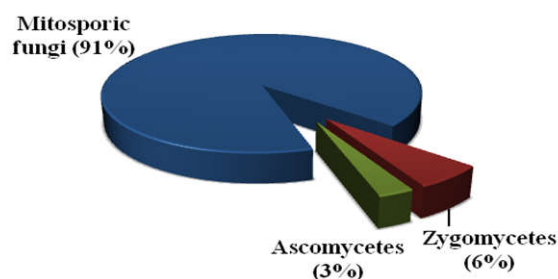


Figure 2. Representation of different fungal groups.

Assessment of Kotla mine (lignite coal)

A total of 30 fungal species belonging to 17 genera (*Acremonium*, *Aspergillus*, *Penicillium*, *Paecilomyces*, *Metarhizium*, *Phialemonium*, *Sarocladium*, *Sagenomella*, *Isaria*, *Curvularia*, *Cladosporium*, *Alternaria*, *Fusarium*, *Rhizopus*, *Syncephalastrum*, *Ajellomyces* and *Trichoderma*) were detected to be associated with the lignite coal of Kotla mine. *Aspergillus* was represented by maximum number of species (8) accounting for 26.6% of the total recovered species. *Curvularia* and *Penicillium* were represented by 13.3% and 10% of the recovered species respectively. These 3 fungal genera (*Aspergillus*, *Curvularia* and *Penicillium*) formed the bulk of the recovered mycodiversity, whereas species of other fungal genera [14] contributed 50.1% of the total fungal diversity (Figure 3). There are very few reports on the microflora associated with lignite coal (29). However, Prasanth *et al* [10] studied the air borne mycoflora near lignite mine in Tamil Nadu (India) and observed almost similar results, that is, *Aspergillus* was represented by maximum number of species followed in decreasing order by *Curvularia* and *Penicillium*. Mukherjee [30] investigated facultative fungal remains from miocene lignite coal of Neyveli, Tamil Nadu (India) and found the fossil fungal elements to have affinities with Hyphomycetes, Ascomycetes and Basidiomycetes. Highest colony forming units (cfu) were recorded again for *Aspergillus fumigatus* (14.5×10^2), whereas least cfu were that of *Ajellomyces capsulatus*, *Isaria farinosa* and *Syncephalastrum racemosum* (1.0×10^2). In this lignite type of coal, species of *Aspergillus* represented highest percentage distribution (43.3%) followed in decreasing order by species of *Curvularia* and *Penicillium*, which represented 11.84% and 11.1% respectively. Fungal genera with least distribution (between 0.37% to 5.55%) included *Acremonium*, *Paecilomyces*, *Metarhizium*, *Phialemonium*, *Sarocladium*, *Sagenomella*, *Isaria*, *Cladosporium*, *Alternaria*, *Fusarium*, *Rhizopus*, *Syncephalastrum*, *Ajellomyces* and *Trichoderma* (Table 1). Many of these genera recovered from lignite coal mine have allergenic species, which may cause health issues to the miners [31].

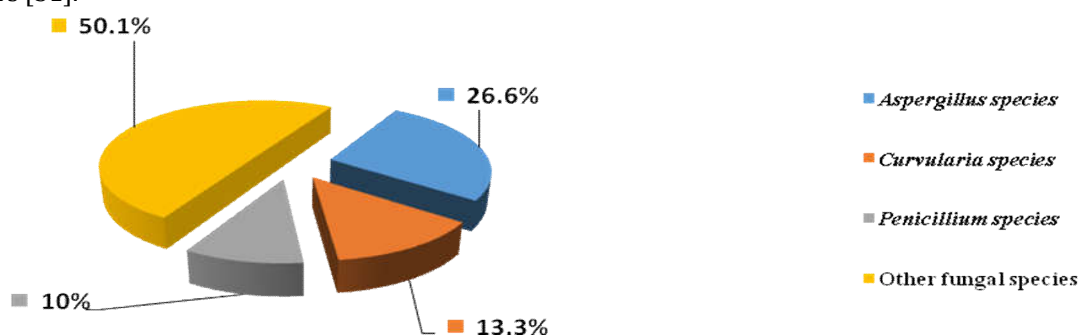


Figure 3. Percentage distribution of total fungal species recovered from lignite coal (Kotla mine).

Table 1. Fungal species recovered from lignite coal (Kotla mine).

Fungal species	Distribution %	CF (%)	cfu/g
<i>Acremonium roseolum</i>	2.59	40	3.5×10^2
<i>Ajellomyces capsulatus</i>	0.37	7	1.0×10^2
<i>Alternaria alternata</i>	5.55	5	3.5×10^2
<i>Aspergillus candidus</i>	1.85	20	2.5×10^2
<i>Aspergillus flavus</i>	8.14	67	11.0×10^2
<i>Aspergillus fumigatus</i>	10.7	87	14.5×10^2
<i>Aspergillus niger</i>	10.0	60	13.5×10^2
<i>Aspergillus nutans</i>	1.11	7	1.5×10^2
<i>Aspergillus parasiticus</i>	5.55	53	7.5×10^2
<i>Aspergillus sulphureus</i>	1.85	27	2.5×10^2
<i>Aspergillus sydowii</i>	4.07	53	5.5×10^2
<i>Cladosporium cladosporioides</i>	2.96	33	4.0×10^2
<i>Curvularia brachyspora</i>	1.85	27	2.5×10^2
<i>Curvularia lunata</i>	4.81	73	6.5×10^2
<i>Curvularia pallescens</i>	2.22	40	3.0×10^2
<i>Curvularia tsudae</i>	2.96	40	4.0×10^2
<i>Fusarium incarnatum</i>	2.22	27	3.0×10^2

<i>Isaria farinosa</i>	0.74	13	1.0×10^2
<i>Metarhizium carneum</i>	4.07	53	5.5×10^2
<i>Paecilomyces divaricatus</i>	1.11	13	1.5×10^2
<i>Penicillium griseofulvum</i>	6.66	80	9.0×10^2
<i>Penicillium purpurogenum</i>	3.33	47	4.5×10^2
<i>Penicillium restrictum</i>	1.11	20	1.5×10^2
<i>Phialemonium inflatum</i>	3.70	20	5.0×10^2
<i>Rhizopus oryzae</i>	1.85	33	2.5×10^2
<i>Sagenomella griseoviridis</i>	2.59	40	3.5×10^2
<i>Sarocladium implicatum</i>	1.11	7	1.5×10^2
<i>Sarocladium strictum</i>	1.85	20	2.5×10^2
<i>Syncephalastrum racemosum</i>	0.74	13	1.0×10^2
<i>Trichoderma koningii</i>	1.11	13	1.5×10^2

Assessment of Moghla mine (anthracite coal)

From anthracite coal of Moghla mine, 28 fungal species were recovered, which belonged to 16 genera viz., *Acremonium*, *Sarocladium*, *Aspergillus*, *Penicillium*, *Metarhizium*, *Phialemonium*, *Isaria*, *Sagenomella*, *Curvularia*, *Cladosporium*, *Alternaria*, *Fusarium*, *Rhizopus*, *Syncephalastrum*, *Stachybotrys* and *Trichurus*. Among the recovered fungal species, genus *Aspergillus* was again represented by maximum number of species (8), which accounted for 28.6% of the total recovered species, followed in decreasing order by *Curvularia* represented by 14.3% species. Other fungal genera (14) contributed 57.1% of the total mycodiversity in anthracite coal (Figure 4).

Recently, Yoltas *et al.* [32] also studied the microfungus flora of coal mine in Manisa (Turkey) and found *Aspergillus*, *Alternaria*, *Amorphotheca*, *Cladosporium*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, *Scopulariopsis* and *Trichoderma* as the commonly occurring fungal genera. Among the fungal species recovered from anthracite coal, *Aspergillus fumigatus* showed highest percentage colonization frequency (93%) and *Sarocladium strictum*, *Aspergillus candidus*, *Isaria farinosa*, *Syncephalastrum racemosum*, *Stachybotrys chartarum* and *Trichurus spiralis* were recorded with lowest colonization frequency (7%). Highest colony forming units (cfu) were recorded for *Aspergillus niger* (9.0×10^2), whereas least cfu were that of *Sarocladium strictum*, *Aspergillus candidus*, *Fusarium incarnatum*, *Metarhizium carneum*, *Isaria farinosa*, *Rhizopus oryzae*, *Syncephalastrum racemosum* and *Trichurus spiralis* (1.0×10^2). Further species of *Aspergillus* showed highest percentage distribution (52.91%) followed in decreasing order by species of *Curvularia* which represented 15.66% species (Table 2). Other fungal genera were detected to have least distribution (Figure 4).

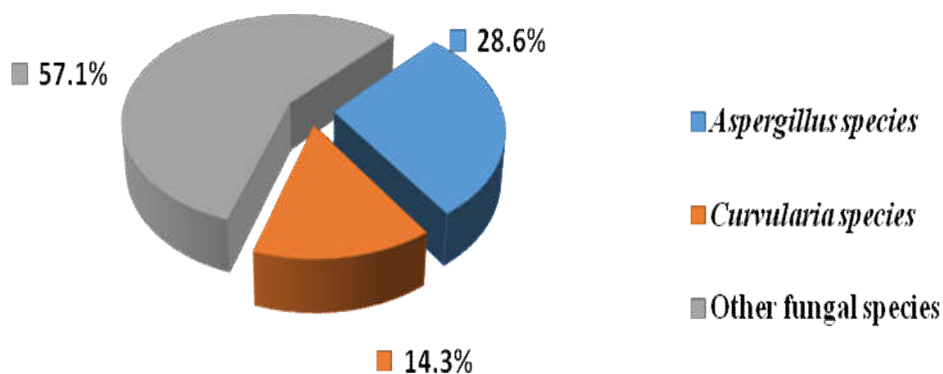


Figure 4. Percentage distribution of total fungal species recovered from anthracite coal (Moghla mine).

During the survey, both types of coal were detected to have a high number of *Aspergillus fumigatus* spores which are known agents of cystic fibrosis and allergic bronchopulmonary aspergillosis [33]. Spores of *A. fumigatus* also pose a serious risk for miners who also under the risk of occupational diseases related to inhalation of coal dust such as pneumoconiosis, silicosis and emphysema [32]. *Aspergillus flavus*, which was also found in good numbers in both the coal types, is also known to be an allergenic and causal agent of aspergillosis. Both these fungal species are even known producers of some mycotoxins and other secondary metabolites, which may cause disorders like irritation of the mucous membranes,

nausea, immune system deficiency, cancer and acute or chronic damages in liver and central nervous system [34]. In the present study, some species of *Penicillium*, *Cladosporium*, *Alternaria* and *Stachybotrys* were also detected from the coal mines are also reported in literature as possible allergenic species [35, 36, 37]. Further, association of *Stachybotrys chartarum* has also been reported as causal agent of idiopathic pulmonary hemosiderosis (IPH) in infants [38].

Table 2. Fungal species recovered from anthracite coal (Moghla mine).

Fungal species	Distribution %	CF (%)	cfu/g
<i>Acremonium roseolum</i>	2.61	27	3.5×10 ²
<i>Alternaria alternata</i>	5.88	40	3.5×10 ²
<i>Aspergillus candidus</i>	1.30	7	1.0×10 ²
<i>Aspergillus flavus</i>	11.1	80	8.5×10 ²
<i>Aspergillus fumigatus</i>	11.1	93	8.5×10 ²
<i>Aspergillus niger</i>	11.7	47	9.0×10 ²
<i>Aspergillus parasiticus</i>	5.22	40	4.0×10 ²
<i>Aspergillus sulphureus</i>	2.61	13	2.0×10 ²
<i>Aspergillus sydowii</i>	7.84	33	6.0×10 ²
<i>Aspergillus terreus</i>	1.96	13	1.5×10 ²
<i>Cladosporium cladosporioides</i>	1.96	13	1.5×10 ²
<i>Curvularia brachyspora</i>	3.26	20	3.0×10 ²
<i>Curvularia lunata</i>	7.18	20	2.5×10 ²
<i>Curvularia pallescens</i>	2.61	53	5.5×10 ²
<i>Curvularia tsudae</i>	2.61	20	2.0×10 ²
<i>Fusarium incarnatum</i>	1.30	20	2.0×10 ²
<i>Isaria farinosa</i>	1.30	7	1.0×10 ²
<i>Metarhizium carneum</i>	1.30	13	1.0×10 ²
<i>Penicillium griseofulvum</i>	6.53	67	5.0×10 ²
<i>Penicillium purpurogenum</i>	3.26	27	2.5×10 ²
<i>Phialemonium inflatum</i>	3.92	13	3.0×10 ²
<i>Rhizopus oryzae</i>	1.30	13	1.0×10 ²
<i>Sagenomella griseoviridis</i>	3.92	33	3.0×10 ²
<i>Sarocladium implicatum</i>	1.96	13	1.5×10 ²
<i>Sarocladium strictum</i>	1.30	7	1.0×10 ²
<i>Stachybotrys chartarum</i>	2.61	7	2.0×10 ²
<i>Syncephalastrum racemosum</i>	0.65	7	1.0×10 ²
<i>Trichurus spiralis</i>	1.30	7	1.0×10 ²

Analysis of coal samples for physical properties.

Analysis of coal samples showed that the pH of lignite coal was 2.5, whereas that of anthracite coal was 3.9. This revealed that both types of coal have strongly acidic pH, which may favour the prevalence of both acid tolerant and acidophilic fungal species. In the present investigation, some fungal organisms like *Aspergillus nutans*, *Ajellomyces capsulatus*, *Paecilomyces divaricatus*, *Penicillium restrictum* and *Trichoderma koningii* were recovered only from lignite coal whose pH was too low (2.5), whereas rest of the fungal organisms were recovered from both the types of coal having pH upto 3.9 (Table 3). Similar observations have also been recorded earlier by several researchers. [39-47] who found acidic habitats (pH<3) to harbour highly diversified microbial communities in which fungi represented most abundant and important component. The acid tolerant and acidophilic fungi usually cope with their environment by maintaining a relatively neutral pH by means of pumping protons out of the cell and by establishing a low proton membrane permeability [49]. In view of this internal pH regulation, a number of fungal species are able to exist in the acidic environment.

The electrical conductivity of coal samples was analyzed and as per the standard electrical conductivity ratings, the electrical conductivity of lignite coal was strong (3.50 dSm⁻¹), whereas that of anthracite coal was slight (0.803 dSm⁻¹). It was also observed that with an increase in coal conductivity, there was a corresponding decrease in pH (Table 3).

Table 3. Physical characteristics of lignite and anthracite coal samples.

Coal characteristics	Lignite coal	Anthracite coal
pH	2.5	3.9
Electrical conductivity(dSm ⁻¹)	3.50	0.803
Total species recovered	30	28
Fungal species of special occurrence	<i>Ajellomyces capsulatus</i> , <i>Aspergillus nutans</i> , <i>Penicillium restrictum</i> , <i>Paecilomyces divaricatus</i> and <i>Trichoderma koningii</i>	<i>Aspergillus terreus</i> , <i>Stachybotrys chartarum</i> and <i>Trichurus spiralis</i>
Fungal species of common occurrence	<i>Rhizopus oryzae</i> , <i>Syncephalastrum racemosum</i> , <i>Aspergillus niger</i> , <i>A. candidus</i> , <i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. fumigatus</i> , <i>A. sulphureus</i> , <i>A. sydowii</i> , <i>Isaria farinosa</i> , <i>Metarhizium. carneum</i> , <i>Phialemonium inflatum</i> , <i>Sagenomella griseoviridis</i> , <i>Penicillium griseofulvum</i> , <i>P. purpurogenum</i> , <i>Sarocladium strictum</i> , <i>S. implicatum</i> , <i>Acremonium roseolum</i> , <i>Curvularia lunata</i> , <i>C. brachyspora</i> , <i>C. pallescens</i> , <i>C. tsudae</i> , <i>Cladosporium cladosporioides</i> , <i>Fusarium incarnatum</i> and <i>Alternaria alternata</i> .	

CONCLUSION

In conclusion, the important findings arising out of this study are encapsulated below: Occurrence and survival of fungal species is dependent on the physical properties of their environment. From the present investigation, it is concluded that coal mine is an important habitat, which harbours a number of fungal organisms that have the ability to survive and multiply in an extreme environment with strong acidic pH. These fungi usually cope with their environment by maintaining a relatively neutral pH by pumping protons out of the cell and establishing a low proton membrane permeability. The fungal diversity in coal may also be attributed to high carbon content in coal as the fungal species are more efficient in utilizing coal as the sole source of carbon.

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