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ANTIFUNGAL SUSCEPTIBILITY PROFILING OF FUNGAL ISOLATES RECOVERED FROM PHARMACEUTICAL WASTE SITES AND THEIR CHARACTERIZATION AS SOURCE OF BIOACTIVE COMPOUNDS

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ABSTRACT: Background: The emergence of antimicrobial resistance (AMR) among different microbial species increases mortality and morbidity rates and makes healthcare services unaffordable. The pace in acquiring resistance to antimicrobial agents stresses the dire need for novel drug discoveries and defining new drug targets. **Objectives:** The present study aimed to isolate and identify the fungi from pharmaceutical waste sites, their antifungal profiling and characterization as potential antibiotic producers. **Methods:** Fungal isolates were primarily screened for antibiotic production following the giant colony method against standard bacterial pathogens and further screened to evaluate their capacity as extracellular antibiotic producers. Antifungal susceptibility testing to commonly prescribe antifungal agents was also performed for the recovered fungal isolates. **Result and Conclusion:** Total fifteen (n=15) fungal isolates recovered including *Aspergillus* sp., *Alternaria* sp., *Candida* sp., *Rhizopus* sp., *Geotrichum sp., Rhodotorula sp., Pyricularia sp., Trichosporon sp., Trichothecum sp.,* and *Fusarium sp*. Cell-free supernatant (CFS) obtained from *Geotrichum sp., Fusarium sp.* and *Trichosporon sp.* were extracellular antibiotic producers with maximum zone size of 22mm, 16mm and 14mm respectively against *Escherichia coli* MTCC-1687. CFS obtained between 15th - 18th days of fermentation was observed with maximum bioactivity. Antifungal susceptibility testing shown maximum resistance to amphotericin B and it was least for fluconazole. Multiple Antibiotic Resistance (MAR) score was ranged between 0.6-0.8 indicating high risk source of antibiotic contamination. The incidence of highly resistant strains among environmental fungal isolates describes the impact of a pharmaceutical waste laden environment on the selection of resistant species.

INTRODUCTION: Microbial byproducts or secondary metabolites have been used for centuries as a source of antibiotics for the treatment of various infectious diseases. Several groups of microbes, such as bacteria, fungi and actinomycetes produce antibiotics to kill or inhibit other competitive microbes. The pharmaceutical waste loaded with antibiotics is a prominent contributor to environmental antibiotic pollution and poses a significant environmental risk, mainly due to inadequate treatment and irresponsible disposal [1].

Microorganisms producing metabolites with antimicrobial potential were isolated from various environments. A well-known source of microbial diversity capable of producing bioactive secondary metabolites is soil [2]. However, the presence of different pharmaceutical chemicals which might promote microbial adaptability and evolution

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makes soil hotspots distinct around pharmaceutical waste sites. These sites harbor diverse chemical compounds, including active pharmaceutical novel ingredients, metabolites, and degradation products which may evolve microbes with distinct metabolic capabilities and may produce novel antimicrobial compounds. Food wastes, were also studied to contain numerous phenolic compounds, flavonoids and organic acids, known for their antimicrobial properties [3]. Numerous antibiotics including tetracycline and streptomycin have historically been derived from soil microorganisms [4]. Bacteria or fungi have evolved the ability to produce antimicrobial chemicals to both eradicate their coexisting microbial populations and to live in environments deficient in nutrients. The majority of antibiotics so far isolated were mainly produced by fungal inhabitants of the soil [5]. Fungi are well-known for their bioactive compounds and twenty of the best-selling medical drugs worldwide were derived from fungi [6]. Historical discoveries such as the isolation of penicillin from *Penicillium notatum* highlight the potential of fungi as sources of antimicrobial agents [7]. Simultaneously with the mass production of antimicrobials, the emergence of antimicrobial resistance (AMR) came into existence and the roots of drug resistance can be found in the theory of natural selection [8]. Indiscriminate use of antibiotics can impose strong selection pressure on microbial populations, leading to the survival of highly adaptable strains [9]. The problem of antimicrobial resistance can devastate a country's socioeconomic structure and healthcare system. This eventually deepens the socioeconomic difference by raising the expense of health care, lowering living standards, and shortening life expectancy.

Fungal infections generate significant mortality and morbidity, as well as a significant economic burden [10]. Serious fungal infections are caused by yeasts (*Candidiasis*, *Cryptococcosis*), molds (*Aspergillosis*, *Mucormycosis*), and dimorphic fungi (*Blastomycosis*, *Coccidioidomycosis*, and *Histoplasmosis*). Commonly prescribed antifungal agents such as fluconazole, ketoconazole, clotrimazole, miconazole etc. are used in clinical settings to eradicate fungal infections. The emergence of antifungal-resistant strains poses a significant global health challenge, necessitating the exploration of novel sources for antimicrobial agents [11]. In order to develop pharmacologically active compounds, modern researchers emphasize the need to investigate unique niches including rainforests, marine sponges, mangroves, and endophytes to address the issue of antimicrobial resistance in the future [12]. The pharmaceutical industry has become increasingly interested in discovering novel compounds and designing new drug targets and repurposing of existing drugs to overcome the challenge of drug resistance. Keeping in view, the present study aimed to explore the microflora around pharmaceutical waste sites as potential source of antimicrobial compounds. In addition, the antifungal susceptibility profiling was performed to determine the resistance pattern among fungal inhabitants of such specific sites.

MATERIAL & METHODS:

Study Site and Sampling: Soil samples (10g each) were aseptically collected from four pharmaceutical waste dumping sites in district Solan, Himachal Pradesh (India). The collected samples were shifted in sterile and cool conditions to the research laboratory, Department of Microbiology, Himachal Pradesh University (Shimla) and further processed to obtain pure fungal cultures.

Fungal Isolation and Identification: Soil samples were serially diluted 10-fold in physiological saline. 100µl aliquots of appropriate dilutions (10^{-2}–10^{-6}) were plated out aseptically on Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), Yeast Mannitol Agar (YMA) and Yeast Extract Peptone Dextrose Agar (YEPDA) plates (Hi-Media, Mumbai). The plates were incubated at 25°C for 3-7 days until visible growth was observed. Sub-culturing was done to obtain purified growth and cultural characteristics such as shape, size, color, elevation and appearance were observed. Morphological characteristics were evaluated by microscopic analysis of Lactophenol Cotton Blue (LCB) stained preparations under 40X magnification. The purified fungal cultures were stored in 10% glycerol (v/v) stocks and kept at -20°C for further use.

Screening for Antimicrobials Production:

Primary Screening: The fungal isolates were preliminary screened by giant colony method for the production of metabolites with inhibitory

potential against test bacterial strains [13]. The fungal isolates were inoculated on Muller Hinton Agar (MHA) plates by making a straight streak line in the middle of the plate. The plates were then incubated at 25°C for 72 hours to stimulate the fungal growth. Once the luxuriant growth of each fungal isolate was achieved, the cultures of test organisms were inoculated perpendicularly close to each fungal isolate. The standard bacterial strains used as test organisms in the primary screening assay were *Escherichia coli* MTCC-1687, *Staphylococcus aureus* MTCC-96, *Pseudomonas aeruginosa* MTCC-1688 and *Salmonella typhi* MTCC-98. The plates were then incubated at 37°C for 24 hours. The inhibition spectrum against the test organisms was recorded as the distance of growth inhibition from the central streak line of fungal growth.

Secondary Screening: The fungal isolates were further evaluated for extracellular production of antimicrobial compounds following the methodology described by Omeike *et al.*, 2019 and Synytsya *et al.*, 2017 [14,15]. Preliminary screened fungal isolates were selected and inoculated on different liquid media namely Yeast Extract Dextrose Broth (YEDB) and Tryptone Soy Broth (TSB), to induce the extracellular production of antimicrobials. Fermentation in these media was carried out in a thermostat-regulated shaker incubator at 150 rpm for 29 days at 30°C. 1ml aliquot from the fifth fermentation day onwards was taken out in micro-centrifuge tube and centrifuged at 9000 rpm for 15 minutes at 25°C to obtain cell-free supernatant (CFS). In-vitro antibacterial activity of CFS was tested against the standard bacterial strains used in the study.

Antibacterial Assay: The bioactivity of CFS was determined using the agar well diffusion method described by the Clinical and Laboratory Standards Institute (CLSI) [16]. Bacterial inoculum (1ml) was adjusted to 10^5cfu/ml (0.5 McFarland's standard) and seeded over Muller-Hinton agar (MHA) medium. CFS (100 µl) was added into appropriate wells that had been bored into the plate using a 6 mm diameter pre-sterilized cork-borer. The bioactivity of CFS was measured by clear zones of inhibition after being incubated at 37°C for 18–24 hours. The bioactivity of CFS from each consecutive fermentation day was tested in triplicates to determine the statistical mean values with standard errors. Additionally, CFS with maximum bioactivity was varied in the range of 80µl - 120µl volume to determine the change in their bioactivity potential. The statistical Fisher's test (F-Test) was used to determine the significance level between decreasing CFS volume and increasing bioactivity potential and vice versa.

***In-vitro* Antifungal Susceptibility Testing (AFST):** Antifungal susceptibility profiling of all recovered fungal isolates was performed following standard Kirby-Bauer disc diffusion method. Commercially available antibiotic discs namely Hexa-Antimyco-01 (HX104) (Hi-Media, Mumbai) were utilised for the purpose. It comprises six different antifungal discs of 6 mm diameter and each with different concentrations namely Amphotericin-B (AP) (100 units), Nystatin (NS) (100 units), Clotrimazole (CC) (10mcg), Ketoconazole (KT) 10mcg), Fluconazole (FLC) (25 mcg) and Itraconazole (IT) (10mcg). These six antifungal agents belong to three different antibiotic classes i.e. triazoles (fluconazole and itraconazole); polyene (amphotericin B and nystatin); and imidazole (clotrimazole and ketoconazole). Inoculums were prepared using 24 hours old culture grown on Sabouraud Dextrose Agar (SDA) and 4-5 colonies were suspended in 5ml normal saline and the turbidity should be adjusted to yield 1 x 106 - 5 x 106 cells /ml (i.e. 0.5 McFarland standard). Muller Hinton Agar (MHA) plates were prepared by adding 2% Glucose + 0.5 mcg/ml Methylene Blue Dye for carrying out susceptibility of antifungal discs. Lawn culture was prepared on MHA plates and allows the plates to dry for 15 minutes with lid in place. Apply the discs using sterile applicator, invert the plates and incubate at 35°C for 24-48 hours. The diameter of zone of inhibition were recorded and interpreted following CLSI M44-A2 protocol [17].

Multiple Antibiotic Resistance (MAR) Index: The MAR index was computed for all the isolates using the formula MAR = A/B, where "A" denotes the number of antifungal agents to which the test isolate has shown resistance and "B" represents the total number of antifungal agents tested [18]. Isolates with intermediate resistance (I) were taken as resistant (R) as a whole for calculating the MAR index.

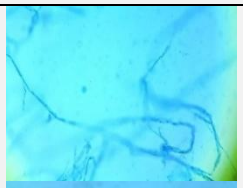
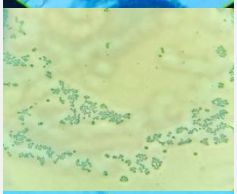
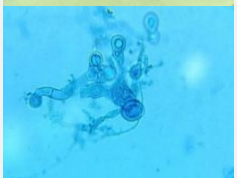
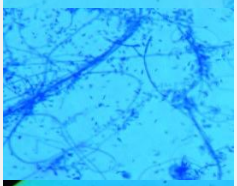
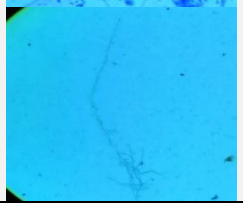
RESULTS:

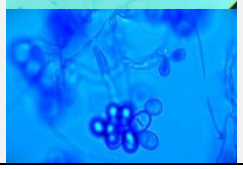
Identification of Fungal Isolates: A total of fifteen (n=15) fungal isolates belonging to ten different genera were recovered from soil samples collected from pharmaceutical waste dumping sites. Fungal isolates were identified as *Aspergillus* sp. (n=3), *Alternaria* sp. (n=2), *Candida* sp. (n=2), *Rhizopus* sp. (n=2) and one isolate each of *Geotrichum sp., Rhodotorula sp., Pyricularia sp., Trichosporon sp., Trichothecum sp.,* and *Fusarium sp*. All isolates were identified on the basis of microscopic and cultural characteristics. The details of the cultural characteristics, morphology and genus identified are presented in **Table 1**.

Primary Screening for Antibiotic Production: All fungal isolates were tested against standard test bacterial strains by giant colony method. During primary screening, only 53% (8/15) fungal isolates were found to have antibacterial production against test organisms. The zone of inhibition was observed only against *Escherichia coli* MTCC-1687 but not for other test organisms selected.

The details of fungal isolates selected during primary screening are as follows: M2C3 (*Geotrichum sp.*), P2C2 (*Fusarium sp.*), P1C2 (*Rhizopus sp.*), P2C3 (*Aspergillus sp.*), M1C1 (*Candida sp.*), P2C5 (*Rhodotorula sp.*), W1M1 (*Trichosporon sp.*) and P1C1 (*Aspergillus sp.*). The selected fungal isolates were investigated further for their potential as antibiotic producers. The bioactive compounds from selected fungal isolates showed significant results against *E. coli* MTCC 1687 only, hence selected for further assessment.

TABLE 1: IDENTIFIED FUNGAL ISOLATES WITH THEIR CULTURAL CHARACTERISTICS AND MORPHOLOGY

Isolate no.	Genera	Cultural characteristics	Microscopic view & characteristics	
W1M2	*Alternaria* sp.	Green hyphae with black terminals and white margins	Ellipsoidal, pale coloured, smooth walled conidia, with hyphal mass	
P2C4	Aspergillus sp.	White coloured hyphae with dark black-green colour spores and white margins	Septate hyphae, Uniseriate and columnar conidial head, flask shaped vesicles	
P2C5	*Rhodotorula sp.*	Slimy yeast like growth with pink pigment production	Yeast cells	
P1C3	Candida sp.	Small, raised and creamy white coloured colonies	Yeast like appearance, pseudohyphae formation	
P2C2	*Fusarium sp.*	White coloured colonies with aerial mycelium	Tapered end two celled macroconidia, septate hyphae	
M2C3	*Geotrichum sp.*	Flat, creamy white colonies	Hyaline septate hyphae, brick shaped conidia	

M1C2	*Pyricularia sp.*	White coloured colony with spores	Pear shaped spores	
P2C6	*Rhizopus sp.*	Cottony and aerial mycelia of black colour	Presence of stolons and rhizoids, single sporangiophores	
W1M1	*Trichosporon sp.*	Septate hyphae, yeast like growth on PDA plate	Hyaline septate hyphae, oval arthroconidia	
M2C2	*Trichothecum sp.*	Flat pink coloured filamentous colonies	Two celled spores, septate hyphae	

Antibacterial assay of CFS: The cell-free supernatant (CFS) from all the selected fungal isolates (8/15) was obtained from fifth fermentation day onwards consecutively up to the 26th day of fermentation. The bioactivity of all consecutive CFS (100 µl) was determined following the antibacterial assay against *Escherichia coli* MTCC-1687. From the selected fungal isolates, three namely *Geotrichum sp.*, *Fusarium sp.* and *Trichosporon sp.* with isolate numbers M2C3, P2C2 and W1M1 respectively, were found to be extracellular antibiotic producers. The maximum diameters of inhibition zones were recorded for M2C3, P2C2 and W1M1 were 22mm, 16mm and 14mm respectively **Table 2**. Maximum bioactivity was observed for the CFS obtained between 15th - 18th day of fermentation for all the three fungal isolates and it was gradually reduced as the fermentation progresses **Fig. 1**. In contrast, rest of the fungal isolates (5/8) showed bioactivity with zone of inhibition diameter of ≤10mm and were regarded as weak producers **(Table 2)**. It was also observed that after 23rd day of fermentation, the bioactivity of CFS was declined to <10mm for all the fungal isolates. Variation in CFS volume from the baseline (100µl) showed marginal effects on the bioactivity potential but a slight increase in zone of inhibition was observed with decreasing volume **Fig. 2**. However, the difference between the variances for baseline volume (100µl), 80µl (lower volume) and 120µl (higher volume) was statistically insignificant as the $F_{(calculated)} < F_{(critical)}$ with a p-value > 0.05 **Table 3**.

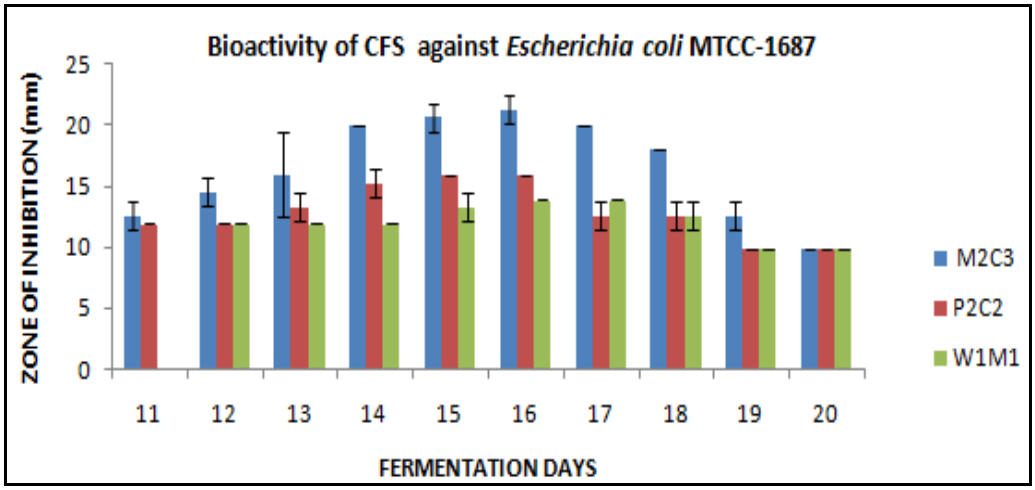


FIG. 1: THE BIOACTIVITY OF CFS EXPRESSED AS ZONE OF INHIBITIONS (Y-AXIS) AGAINST THE CFS OBTAINED BETWEEN 11TH - 20TH FERMENTATION DAYS (X-AXIS) FOR THREE FUNGAL ISOLATES (M2C3, P2C2 & W1M1)

Antifungal Susceptibility Profiling: Commercially available antifungal discs belong to three classes were tested against all the isolated fungi and their susceptibility profile was recorded. All isolates were sensitive to fluconazole and found to be most effective antibiotic in this study. Maximum resistance was observed against amphotericin B (100%) followed by clotrimazole, ketoconazole, nystatin (93.33% each) and itraconazole (40%). 7/15 (46.67%) fungal isolates were found sensitive to itraconazole while 13.33% were with intermediate resistance. Overall, triazoles were most effective and polyene class was found least effective against the isolated fungi **Fig. 3**. Antibiogram showing the susceptibility pattern of fungal isolates is given in **Table 4**.

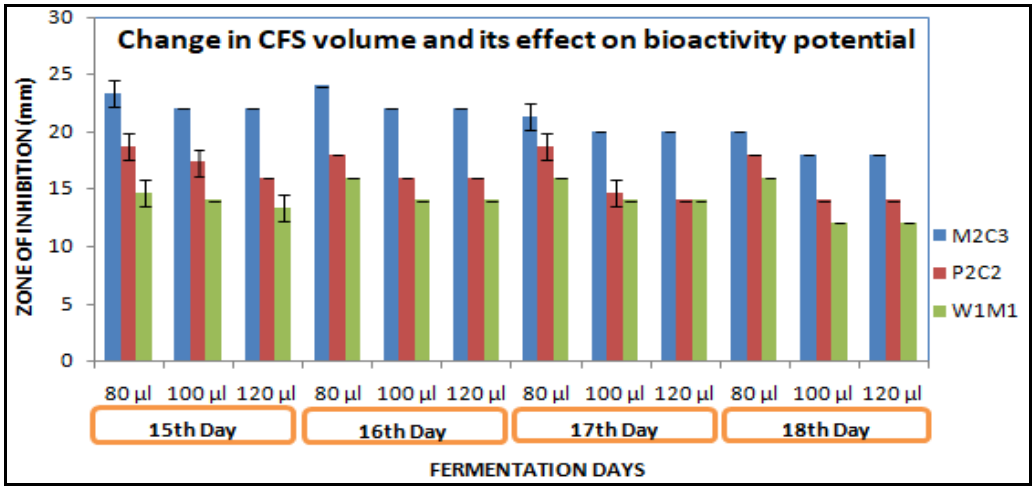


FIG. 2: VARIATION IN VOLUME OF CFS OBTAINED FROM CONSECUTIVE FERMENTATION DAYS AND ITS EFFECT ON BIOACTIVITY POTENTIAL AGAINST *ESCHERICHIA COLI* MTCC-1687

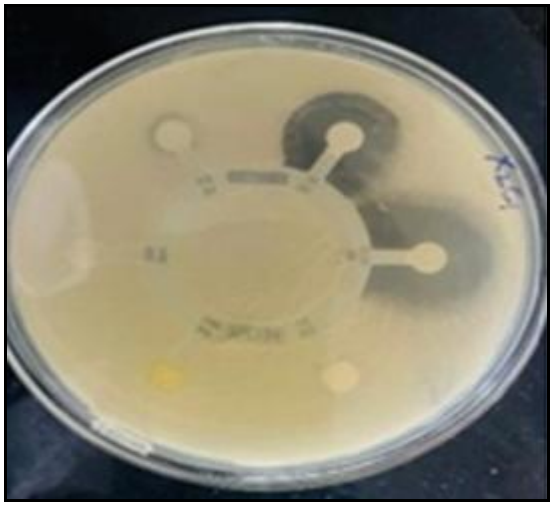


FIG. 3: ISOLATE (*FUSARIUM SP.)* SHOWING SUSCEPTIBILITY TO FLUCONAZOLE AND ITRACONAZOLE AND RESISTANCE TO ALL OTHER ANTIBIOTICS TESTED

MAR Index: Multiple Antibiotic Resistance (MAR) score of 0.8 was observed for 53% of the fungal isolates whereas it was 0.6 for rest of the isolates. Phenotypic antibiogram along with MAR index is presented in **Table 4**.

DISCUSSION: Antimicrobial resistance becomes a major challenge to the medical fraternity over the past few decades due to the elevated incidence of treatment failures. Modern healthcare system has emerged with new challenges like reduced efficacy for several antimicrobials, increased duration of treatment, and longer hospital stays which pose catastrophic costs on patients. For better clinical outcomes, different therapeutic modalities have been adopted such as combination therapy or new drug delivery techniques. Microorganisms have been the prime source for most important antimicrobial agents ever developed. Penicillin, chloramphenicol, streptomycin, erythromycin, gentamicin and rapamycin are some of the key antibiotics from microbial origin. Increased incidences of antimicrobial resistance have been reported from all over the world. Indiscriminate use of antibiotics, ease in accessibility over the counter and their promotion as growth boosters in agricultural and animal husbandry sectors are some of the major drivers of developing antimicrobial resistance. Microorganisms evolve over time under the influence of antimicrobial selection pressure and proliferate as resistant organisms. To combat this situation, there is an urgent need to find inventive antimicrobial compounds or develop newer therapeutic strategies so that life saving potential of antibiotics could be preserved for

future generations. The present study aimed to explore microbial sources for novel compounds and to assess their potential as antibiotics. Soil samples from the pharmaceutical waste sites were collected for the purpose. Fungi namely *Aspergillus* sp., *Alternaria* sp., *Candida* sp., *Rhizopus* sp., *Geotrichum* sp., *Rhodotorula* sp., *Pyricularia* sp., *Trichosporon* sp., *Trichothecum* sp., and *Fusarium* sp. were isolated and identified from the collected soil samples. Similarly, fungal genera *Penicillium, Aspergillus, Fusarium, Trichoderma, Mucor,* and *Rhizopus* were isolated from pharmaceutical sludge [14] and microbial contamination of some pharmaceutical products have also been reported from Nigeria [19]. In the present study, eight isolates of different fungal genera were found to be antibiotic producers in primary screening and the zone of inhibition was only observed against *Escherichia coli* MTCC-1687. The zones of inhibition have not been observed against other test organisms selected in this study namely *Staphylococcus aureus* MTCC-96, *Pseudomonas aeruginosa* MTCC-1688 and *Salmonella typhi* MTCC-98. In contrast, Omeike*et al.* in 2019 observed the antimicrobial activity of the cell free supernatant (CFS) of *Geotrichum* sp. against *S. aureus* with a zone of inhibition of 32mm but not for *E. coli* [14]. Standard bacterial strains with no zone of inhibition might be intrinsically resistant to the antimicrobial compounds secreted by the fungal isolates in primary screening; hence zones of inhibition may have not been appeared.

TABLE 2: ANTIBACTERIAL ASSAY SHOWING BIOACTIVITY (ZONES OF INHIBITION) OF CELL FREE SUPERNATANT AGAINST *ESCHERICHIA COLI* MTCC-1687

Fermentation day	Zones of inhibition [1] (mm)							
	M2C3 (*Geotrichum sp.*)	**P2C2 (*Fusarium sp.*)**	**W1M1 (*Trichosporon sp.*)**	**P1C2 (*Rhizopus sp.*)**	**P2C3 (*Aspergillus sp.*)**	**M1C1 (*Candida sp.*)**	**P2C5 (*Rhodotorula sp.*)**	**P1C1 (*Aspergillus sp.*)**
5th	<10	<10	<10	<10	<10	<10	<10	<10
6th	<10	<10	<10	<10	<10	<10	<10	<10
7th	<10	<10	<10	<10	<10	<10	<10	<10
8th	<10	<10	<10	<10	<10	<10	<10	<10
9th	<10	<10	<10	<10	<10	<10	<10	<10
10th	<10	<10	<10	<10	<10	<10	<10	<10
11th	12	12	<10	<10	<10	<10	10	<10
12th	16	12	12	<10	<10	<10	10	<10
13th	20	14	12	<10	10	<10	10	10
14th	20	16	12	<10	10	<10	10	10
15th	22	16	14	<10	10	<10	10	10
16th	22	16	14	<10	10	<10	10	10
17th	20	14	14	<10	<10	<10	<10	10
18th	18	14	12	<10	<10	<10	<10	10
19th	12	10	10	<10	<10	<10	<10	10
20th	10	10	10	<10	<10	<10	<10	<10
2st	10	10	<10	<10	<10	<10	<10	<10
22nd	10	<10	<10	<10	<10	<10	<10	<10
23rd	<10	<10	<10	<10	<10	<10	<10	<10
24th	<10	<10	<10	<10	<10	<10	<10	<10
25th	<10	<10	<10	<10	<10	<10	<10	<10
26th	<10	<10	<10	<10	<10	<10	<10	<10

TABLE 3: F-TEST TO DETERMINE THE STATISTICAL SIGNIFICANCE OF VARYING BASELINE VOLUME OF CELL FREE SUPERNATANT OBTAINED BETWEEN 15TH – 18TH FERMENTATION DAYS

	Lower volume (80µl)	Baseline volume (100µl)	Higher volume (120µl)
Mean	18.72222222	16.5	16.27777778
Variance	8.720634921	10.82857143	11.23492063
Observations	36	36	36
df	35	35	35
F (calculated)	1.241718238		1.037525652
P(F<=f) one-tail	0.262638583		0.456921768
F Critical one-tail	1.757139526		1.757139526

TABLE 4: ANTIBIOGRAM SHOWING SUSCEPTIBILITY PATTERN OF FUNGAL ISOLATES AS RESISTANCE (RED), INTERMEDIATE (ORANGE) AND SENSITIVE (GREEN)

Isolate No.	Identified genus	Antibiotic classes*						MAR score
		Triazoles		Polyenes		Imidazoles		
		FLC	IT	AP	NS	CC	KT	
M1C1	*Candida sp.*	Green			Red			0.833…
M1C2	*Pyricularia sp.*	Green			Red			0.833…
M2C2	*Trichothecum sp.*	Green			Red			0.833…
M2C3	*Geotrichum sp.*	Green			Red			0.666…
P1C1	*Aspergillus sp.*	Green			Red			0.666…
P1C2	*Rhizopus sp.*	Green			Red			0.666…
P1C3	*Candida sp.*	Green			Red			0.666…
P2C1	*Alternaria sp.*	Green	Yellow		Red			0.833…
P2C2	*Fusarium sp.*	Green			Red			0.666…
P2C3	*Aspergillus sp.*	Green			Red			0.833…
P2C4	*Aspergillus sp.*	Green		Red		Yellow		0.666…
P2C5	*Rhodotorula sp.*	Green			Red			0.833…
P2C6	*Rhizopus sp.*	Green			Red			0.666…
W1M2	*Alternaria sp.*	Green			Red			0.833…
W1M1	*Trichosporon sp.*	Green	Yellow		Red			0.8333…

During secondary screening, three isolates namely *Geotrichum sp.*, *Fusarium sp.* and *Trichosporon sp.* were confirmed as extracellular antibiotic producers and maximum bioactivity was observed from the cell-free supernatant obtained between 15^{th} – 18^{th} days of fermentation. Similarly, a study from Basrah (Iraq) reported the antimicrobial bioactive compounds from fungus *Drechslera halodes* and tested against *S. aureus* and *E. coli* with zones of inhibition 30mm and 25mm respectively [20]. Another study reported sixty-one filamentous fungal strains of *Aspergillus* sp. with antimicrobial action against *Candida albicans*, methicillin-resistant *S. aureus* (MRSA), beta-lactamase producing *E. coli*, and *Enterococcus faecalis* [21]. Similar studies with ethanolic extracts of fungal isolates showing antagonistic effects on *Bacillus cereus, Enterococcus faecalis, Micrococcus luteus, Staphylococcus epidermidis, Enterobacter cloacae* and *Pseudomonas aeruginosa* have also been reported from Czech Republic[15] and Pakistan [6]. In our study, the gradual decrease in bioactivity after 18^{th} fermentation day can be related with the growth curve and might be due to the over accumulation of some toxic secondary metabolites during the end of stationary phase, hence impacted the antibiotic yield **Table 2** & **Fig. 1**. A sharp decline in the bioactivity of CFS after 23^{rd} fermentation day may also related to the feedback inhibition due to the over accumulation of antimicrobial compound itself. A study on feedback inhibition of biosynthesis of an antibiotic aurodox (X-5108) from *Streptomyces goldiniensis* have also been reported [22]. In the present study to speculate about the minimum inhibitory concentration (MIC) of antimicrobial compounds, change in bioactivity was viewed by changing the volume of CFS by 20µl on both sides. Increasing volume showed minimal effect on the bioactivity while it was slightly increased with decreasing volume, hence, hypothesized that the baseline volume have the minimum inhibitory concentration. However, the exactness of this fact was statistically determined using Fisher's test (F-Test) and was not found statistically significant as the difference between the sample variances (F-value) was lower than the corresponding F-critical value for degree of freedom 35 and p-value > 0.05 **Table 3**. However, further investigation with varying wide range of sample volumes and even the change in sample concentration level could better explain the statistical significance. Further, they may generate unique outcomes when production medium and other fermentation-related aspects shall be optimized.

Antifungal susceptibility profiling using Hexa-Antimyco-01 antibiotic discs (HX104) (Hi-Media, Mumbai) was also performed in this study. Antifungals are few in number contrary to the numerous classes of antibiotics used to treat bacterial infections. It consists of three main categories, namely, azoles, echinocandins and polyenes. Azoles are further classified under the class imidazoles and triazoles. In this study, antifungal resistance pattern showed maximum

resistance towards amphotericin B (100%), clotrimazole, nystatin, ketoconazole (93% each) and 40% to itraconazole. Fluconazole was found to be the most effective antifungal with 100% sensitivity followed by itraconazole (46%). Hence, triazoles can be the choice of drugs to treat fungal infections in this region of the country. Similar findings reported less susceptibility to Amphotericin B among clinical fungal isolates than environmental isolates [23–25]. According to Hadrich *et al*. 2012, *Aspergillus* has gained resistance to azoles since 1990, with a dramatic surge in the resistance statistics of *A. fumigates* [26]. Similarly, the resistance rate of *Candida* sp. to azole was 7.8% to 66.66% while the same for *Aspergillus* sp. was 30% [27].

The MAR index ranged between 0.6 - 0.8 in our study indicating the resistance paradigm is shifting towards higher side among environmental fungal isolates. It further indicates the release of potent drugs through pharmaceutical waste into the nearby environment which serve the basis for development of antimicrobial resistance. MAR index values greater than 0.2 also indicates the high risk source of antibiotic contamination [18, 28]. The MAR score in our case seems to be very high and the reason could be the selection of the study site because pharmaceutical waste release in the environment without any treatment may lead to the creation of a resistant gene pool and transfer of resistant genes among different microbial genera.

CONCLUSION: Based on the antifungal susceptibility pattern observed, presumptions can be made out that the pharmaceutical waste dumping sites contributing in developing resistance. The occurrence of antimicrobial resistance among environmental isolates is a threat to public health. Dissemination of such highly resistant strains through water bodies can lead to major disease outbreaks. Hence, pharmaceutical pollution should not be overlooked and render ineffective before environment release. The continuous supervision and characterization of such environmental strains is therefore essentially required. Microbial flora inhabiting such unusual environment can be a source of novel drugs as the microbes continuously evolving under the influence of antimicrobial compounds. This study has provided a preliminary data on the antimicrobial compounds secretion by microflora from pharmaceutical waste sites; however, it can be further investigated with optimized fermentation parameters and compounds purification methods to declare possible novelty.

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