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Antimicrobial activity of bioactive molecules isolated from filamentous fungi

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Abstract

Various microorganisms display antagonism as a principle phenomenon for the production of secondary metabolites. The present study aims at employing this antagonistic property of fungi for the production of some useful bioactive secondary metabolites. 53 isolates were screened for the production of antimicrobials after the incorporation of lactose which triggered the production of secondary metabolites, out of which only 3 showed positive results against the test pathogens. These were identified as Aspergillus flavus var flavus, Acremonium cellulolyticus and Aspergillus chevialeri. The FTIR spectrum revealed the presence of various functional groups such as amines, amides, carboxylates, alcohols, alkanes, alkynes, nitro groups, etc. The knowledge of these components can be further employed for the production of pharmaceutically important products in due course of time

Keywords: Antagonism, bioactive metabolites, *Aspergillus, Acremonium*, FTIR.

Introduction

With estimated species ranging from 1.5 to 5.1 million species, fungi represent the second largest group of eukaryotic organisms on earth and owing to the diverse bioactive metabolites they produce, are known to inhabit almost every possible environmental niches of our planet[1,2]. These metabolites have major industrial applications as well as are well known to possess certain biological properties like anti-diabetic[3], anti-inflammatory, anti-haemolysis[4], anti proliferative[5] and can be employed for drug discovery[6] . Other fungal products and enzymes have also found uses in dying and food coloring industries[7] as well as natural decomposers of hydrocarbons[8]. Fungi are brilliant source of secondary metabolites, a fact that has been known to mankind since thousands of years. However, it was not until Alexander Fleming discovered the antibiotic Penicillin in 1929 (form penicillin F) and Florey and Chain developed it into a medicine in 1940 (Form penicillin G) that the scientific world drew its attention towards fungal secondary metabolites. Since then, tremendous efforts have been implied that have resulted in thousands of characterized compounds with a wide range of biological activity.

In recent years, antagonism has proved as a breakthrough in triggering secondary metabolism and has paved way in developing some extremely useful bizarre compounds[9]. Cultivation of fungi with other micro-organisms like *Lactobacillus* in presence of same substrates resulted in difference in morphology of fungus *Aspergillus nidulans* and its protein expression of two groups of proteins named LbuA (Lactobacillus up-regulated)[10] . Similarly a nuclear protein of *Aspergillus* named *laeA* act as a global regulator of secondary metabolism and affects the production of sterigmatocystin (carcinogen), penicillin (antibiotic), and lovastatin (anti hypercholesterolemic agent) gene clusters. Deletion of *laeA* blocks the expression of these metabolites whereas over expression of *lae A* triggers the increased expression of penicillin and lovastatin and subsequent product formation[11].

With the advancement in structure elucidation techniques, novel bioactive molecules are continuously being discovered. Hyphenated techniques like FTIR, NMR and GC-MS analysis have proved extremely useful in identifying such metabolites. In the present study we have chosen fungal colonies isolated from soil and studied the effect of lactose incorporated in the form of milk and whey and the secondary metabolites thus obtained.

Materials and methods

Collection of soil samples: The soil samples were taken from five different dairies of Raipur district, Chhattisgarh, India (21.25ºN and 81.63ºE). Older and recent both dairies were chosen owing to the fact that older dairy soil may contain acclimatized flora in comparison to the recent ones.

Isolation of fungi: The fungal microflora was isolated through serial dilution method. Potato Dextrose Agar (PDA) was employed as the culture medium supplemented with 10 µg/ml Amoxicillin powder to control bacterial contamination. The pure colonies were then subcultured and maintained at 4ºC for further analysis.

Study of effect of milk products on growth of fungi: The fungal plugs were cut from pure culture plates and inoculated into a combination of 50 ml Potato Dextrose Broth and 25 ml

milk and another combination of 50 ml Potato Dextrose Broth and 25 ml whey. The cultures were kept in stationary condition for 7 days and then a constant shaking at 180 rpm was provided again for 7 days.

Extraction of secondary metabolites from fungal cultures: The filtered supernettant was collected and treated with Tween 80 for spore destruction and then filtered again[12]. The filtrate was then treated with Ethyl Acetate, Chloroform and Methanol in the ratio 3:2:5[13]. Organic layer was collected separately and kept at 50-60ºC in hot air oven until all the liquid got evaporated leaving only the crude extract behind. The stock solution was prepared by dissolving the crude extracts in methanol at $1x10^5$ µg/ml concentration.

Antibiotic sensitivity against pathogenic bacteria: The antibacterial activity was assessed against Gram positive and Gram Negative bacteria. The following organisms were chosen *Salmonella enterica* (MTCC-3219), *Listeria monocytogenes* (MTCC-1143), *Micrococcus luteus* (MTCC-7950), *Bacillus subtilis* (MTCC-1789), *Staphylococcus aureus* (MTCC-7443), *Escherischia coli* (MTCC-3221) and *Klebsiella pneumoniae* (MTCC-9544).

FTIR Analysis: Fourier Transform Infrared Spectrum was obtained by adding potassium bromide (KBr) to the extract in the ratio 1:100 and then compressed to semi transparent disc. The spectra were recorded over wavelength 400- $4000cm^{-1}$ using Perkin Elmer Germany FTIR Spectrophotometer.

Molecular Identification: Out of 53 isolates obtained from soil only 3 exhibited tremendous activity against the test bacterial strains and hence were proceeded for identification. The identification of fungi were done by comparing their ITS regions with the available NCBI data and closest neighbor were assigned.

Results and discussion

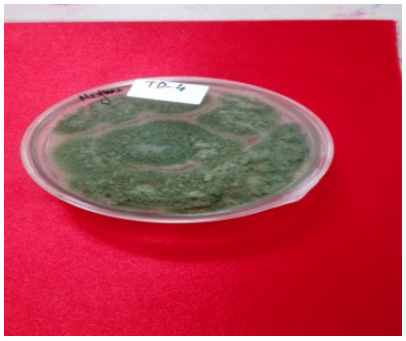
Isolation of fungi: Fungi were isolated from soil of various dairies through serial dilution method and the pure cultures were then inoculated into milk and whey (Figure-2).

Extraction of secondary metabolites: The fungal colonies inoculated into milk and whey containing Potato Dextrose Broth exhibited the production of secondary metabolites after an incubation of 14 days at static and shaking conditions. The crude extracts were then isolated and dissolved in methanol serving as stock solutions.

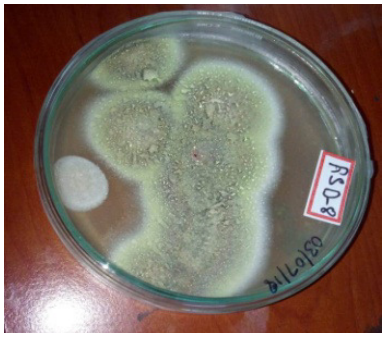
Antibacterial activity of methanolic extracts: The extracts were then tested for antibacterial activity against various pathogens and the extracts of three fungal colonies exhibited immense activity against the test organisms. These were later identified as *Acremonium cellulolyticus, Aspergillus chevialeri* and *Aspergillus flavus var flavus.* The extracts also seem to be capable of inhibiting some of the test pathogens even more than the reference antibiotic (Table-1, Figure-3).

FTIR analysis: The fourier transform infra red analysis described various peaks for all the three extracts pertaining to different functional groups (Figure-4,5,6, Table-2,3,4). The identification of peaks was done referring to available literatures[14,15]. Various functional groups have already proven to be components of various known antimicrobial compounds**.** The spectrum also exhibited presence of some unknown compounds, the further analysis of which can lead to discovery of some novel compounds that may possess some of the antimicrobial characteristics.

Molecular Identification: A total of 53 isolates were screened for antimicrobial secondary metabolite production. Only 3 isolates displayed satisfactory results and were chosen for further analysis. The isolates were identified as *Aspergillus flavus* var *flavus, Aspergillus chevialeri* and *Acremonium cellulolyticus.* All the identifications were done by National Centre for Microbial Resources (NCMR), Pune, Maharashtra, India.



Aspergillus flavus var flavus



Acremonium cellulolyticus

Aspergillus cheviliari

Figure-1: Fungal cultures.

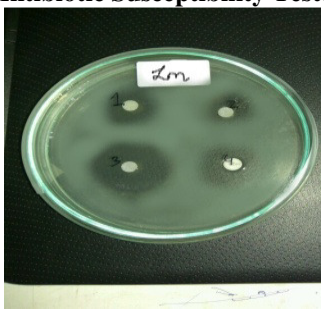
Figure-2: Inoculation of Fungal Cultures in Milk and Whey.

Antibacterial essay against pathogens

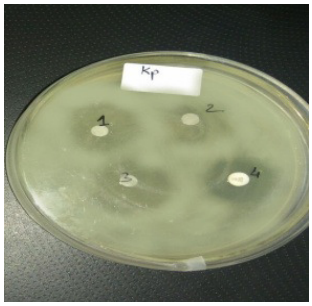
Table-1: Diameter of zone of inhibition in mm ± sd.

Pathogens	*Acremonium cellulolyticus*	*Aspergillus chevialeri*	*Aspergillus flavus var flavus*	Streptomycin
Listeria monocytogenes	23.6± 4.3	11.0± 2.6	30.6± 3.2	23.9± 4.2
Klebsiella pneumonia	24.2± 5.0	24.2± 2.0	23.5± 3.0	19.6± 2.0
Bacillus cereus	27.7± 3.0	10.6± 3.5	27.7± 4.0	21.4± 3.5
Staphylococcus aureus	35.8± 2.8	11.4± 4.2	25.2± 2.0	25.4± 3.0
Salmonella enteric	22.6± 3.0	12.2± 3.2	22.6± 3.6	17.4± 2.4
Bacillus subtilis	28.3± 2.4	9.7± 3.5	22.8± 3.2	15.3± 3.0
Escherishcia coli	30± 3.2	8.3± 3.0	29.6± 5.2	23± 3.0

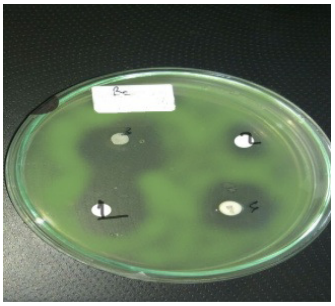
Antibiotic Susceptibility Test:



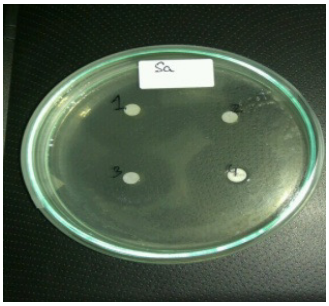
Listeria monocytogenes



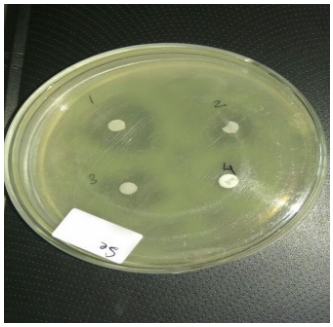
Klebsiella pneumonia



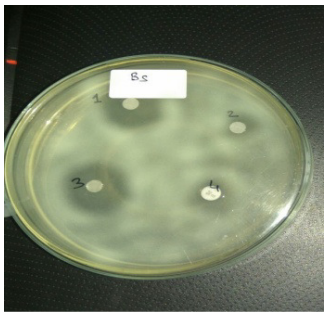
Bacillus cereus



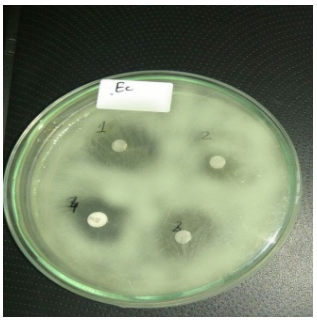
Staphylococcus aureus



Salmonella enteric



Bacillus subtilis



Escherischia coli

Figure-3: Antibiotic susceptibility test against various pathogens. Disc 1)*Acremonium cellulolyticus,* Disc 2) *Aspergillus chevialeri,* Disc 3) *Aspergillus flavus,* Disc 4) *Streptomycin.*

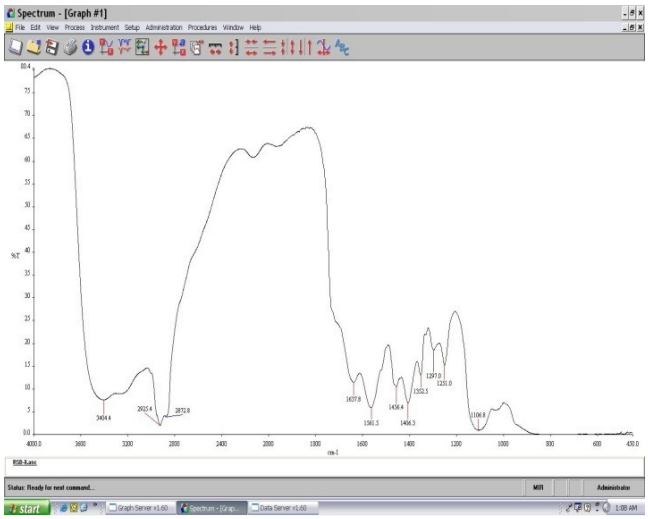


Figure-4: FTIR analysis of methanolic extract of *Acremonium cellulolyticus.*

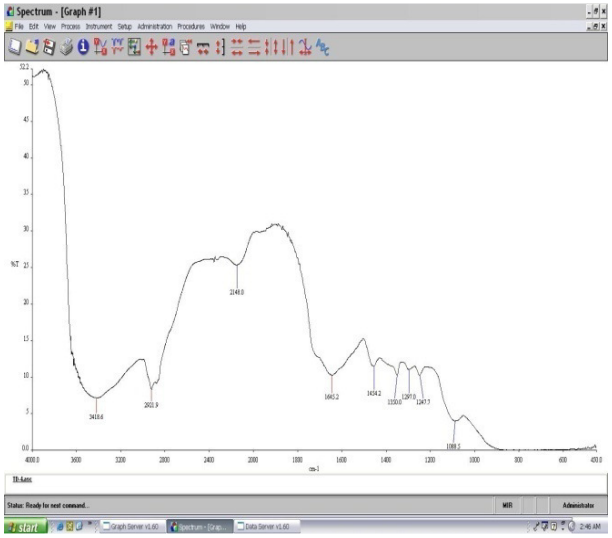


Figure-5: FTIR analysis of methanolic extract of *Aspergillus chevialeri.*

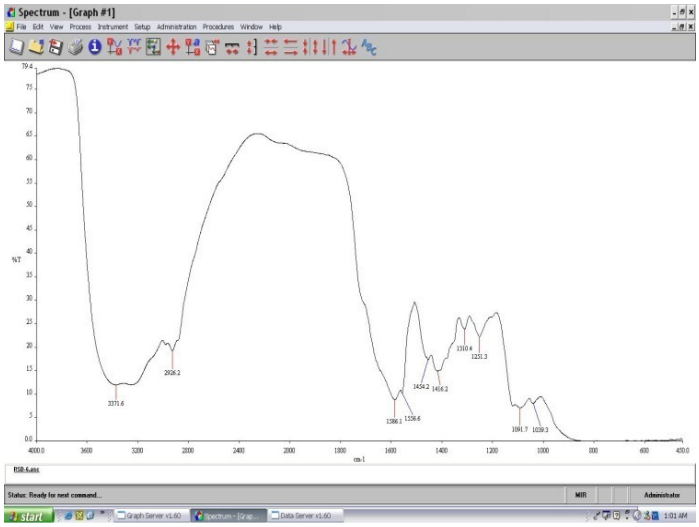


Figure-6: FTIR analysis of methanolic extract of *Aspergillus flavus var flavus.*

Table-2: Peak values of FTIR analysis of *Acremonium cellulolyticus*.

Peak values(cm^{-1})	Representing Bond	Functional group assignment	Group frequency.
3404.4	O-H	Normal polymeric –OH stretch	3200-3400
2925.4	-CH	Methylene –CH asymm.	2915.2935.
2872.8	-	Unknown	-
1637.8	-	Organic nitrate	1620-1640
1561.5	$-NO_2$	Nitro group	1500-1650
1456.4	$-CH_3$	Methyl –CH asymm.	1430-1470
1406.3	NH_3	Ammonium ions.	1390-1430
1352.5	-	Aromatic nitro compounds	1310-1390
1297.0	$-NO_2$	Nitro compounds	1250-1400
1251.0	$-NO_2$	Nitro compounds	1250-1400
1106.8	SO_2	Sulfones	1100-1150

Table-3: Peak values of FTIR analysis of *Aspergillus chevialeri.*

Peak values (cm^{-1})	Representing Bond	Functional group assignment	Group frequency.
3371.6	-NH	Amines	3300-3500
2926.2	-CH	Methylene –CH asymm.	2915-2935
1586.1	$R\text{-}COO^-$	Carboxylates	1550-1610
1556.6	$R\text{-}COO^-$	Carboxylates	1550-1610
1454.2	$-CH_3$	Methyl –CH asymm.	1430-1470
1416.2	$-NH_3$	Ammonium ions	1390-1430
1310.4	-	Aromatic nitro compounds	1310-1390
1251.3	-	Unknown .	-
1091.7	C-F	Aliphatic fluoro compounds	1000-1150
1039.3	C-F	Alkyl fluorides.	1000-1150

Table-4: Peak values of FTIR analysis of *Aspergillus flavus var flavus*.

Peak values (cm^{-1})	Representing Bond	Functional group assignment	Group frequency
3418.6	O-H	Normal polymeric O-H stretch	3400-3500
2921.9	-CH	Methylene –CH asymm.	2915-2935
2148.0	-	Unknown	
1645.2	R-CO-NR'R	Amides	1630-1690
1454.2	$-CH_3$	Methyl –CH asymm.	1430-1470
1350.0	$R\text{-}COO^-$	Carboxylates	1300-1430
1297.0	$-NOO^-$	Nitro groups	1250-1300
1247.7	C-OH	Alcohols	1150-1260
1088.5	-C-OR	Ethers	1085-1150

Conclusion

Microorganisms exhibit antagonism as a common phenomenon and very few studies have applied this for the production of secondary metabolites. The formation of clear zone of inhibitions shows the presence of some strong antimicrobial compounds in the extracts. The FTIR analysis also proves presence of secondary metabolites with tremendous biological activities. The structural elucidation of these compounds can prove extremely useful through different drug discovery techniques.

Acknowledgement

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