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Antibacterial Activity Assessment of Native Fungus Isolated from Chromite Mines of Sukinda, Odisha

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Abstract: ***The antibacterial activity assessment of the Penicillium sp. which are collected from chromium mines of Sukinda, Jajpur district of Odisha, have been studied against three pathogenic bacteria viz. Staphylococcus sp., Pseudomonas Sp. and Xanthomonas oryzae. The Penicillium fungus isolated from chromium contaminated soil was showing antibacterial activity, which were not observed by normal soil fungus. From the fungal extract different functional groups has been identified through FTIR analysis. The isolated fungal strains were showing antibacterial activities against collected bacterial pathogens due to presence of functional groups like alkane, alkyl halide, aldehyde , alkyne , amines and bioactive compounds like alcohol. Hence from this study, it can be concluded from the study that bioactive compounds found from the fungal isolates can be useful in the field of agriculture and act as a biocontrol agent against the above said pathogens.***

Keywords: Fungus; Chromium; Bioactive compounds; Antibacterial; Biocontrol agent

1. Introduction

Fungi are an extremely adaptable group of microorganisms and capable of growing under extreme conditions of pH, temperature, and a scarcity of nutrients. In addition, some reports have indicated filamentous fungi are showing high tolerance to Cr(VI) and can colonize on the sites that are contaminated with this pollutant [1],[2]. Mainly the filamentous fungus *Penicillium* species is significant not only due to its extensive presence but also because of their capability to produce mycotoxins and secondary metabolites [3], [4]. Thousands of Penicillium isolated and screened for their secondary metabolites and mycotoxin production [5]. The present investigation mainly focuses on Collecting soil sample from Chromium contaminated site Sukinda, Odisha, India, Isolating Fungal strain from collected soil sample which are showing antimicrobial activity ,extracting secondary metabolites from fungal strains, Examining the secondary metabolites against pathogenic bacteria, Collecting the fungal extract showing antibacterial activities and characterize through FTIR (Fourier transform infrared Spectroscopy) to confirm the functional groups present in the fungal extract. To the best of our knowledge current investigation represents the first time study on antibacterial activity of chromium resistant fungus isolated from mining area of Sukinda.

2. Literature Survey

In previous scientific studies, it has been already mentioned that pollution and high concentration of hexavalent chromium contamination has been affecting not only mine employees and inhabitants of the mining area but also microbes present in the soil. Several studies recommend all forms of Cr (VI) are considered as deadly chemical to workers however the most important pathway of proficient exposure to contaminant is inhalation and contact through skin [8]. When poisonous heavy metals accumulate in the soil to an anomalous point it causes spectacular modification in microbial composition and their actions, resulting production of bioactive compounds in response to stress condition [9],[10].

3. Materials and Method

3.1 Study area and Sample Collection

For our experiment, samples were collected from different sites of Chromium contaminated area of Sukinda, Jajpur district, Odisha. Samples like over burden, ore soil, sediment, waste water collected from 5 different mining area of Sukinda in a clean, sterilized, air tight sample bags.

3.2 Physio-chemical Parameters

The physio-chemical parameters including pH and Electrical Conductivity, moisture content, temperature, TDS of the collected samples were measured.

3.3. Isolation of Fungus

After serial dilution of the soil sample**,** 1 ml of each dilution were allowed on a PDA plate for spread plating and then incubated at 28℃-30℃ for 48-72 hours. After 3-4 days, fungal colonies were counted.

3.4. Screening of Fungus showing antibacterial activity

3.4.1 Primary Screening- Then for primary screening collected samples were inoculated on PDA plates by serial dilution method and incubated for 24-48 hours. After that zone of inhibition were observed. The fungal isolate showing antibacterial activity isolated and cultured in PDA slants at 4℃ and named as OSF (Ore Soil Fungus) and the bacteria isolates also cultured on NA slants and named as OSB (Ore Soil Bacteria).

3.4.2. Secondary Screening- Isolated pure fungal strains which were obtained from primary selection , cultivated on PDA plates. Then the fungal isolates were subjected to

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secondary screening via agar overlay technique. Soft NA (Nutrient Medium) which seeded with the bacteria that are inhibited by the fungus, were overlaid onto growing fungal isolates. 4 empty PDA plates were overlaid with soft NA used as controls. All the plates were incubated at room temperature for 24 hours. Then after 24 hours zone of inhibition was observed.

3.5. Identification of Fungal

Identification of fungal and bacterial isolates was done on the basis of their colony characteristics on media and microscopic characteristics after Lacto phenol cotton blue staining and Gram staining respectively. Detailed structure of collected fungal sample was studied through Scanning Electron Microscopy.

3.6 Extraction of secondary metabolites

Fungal isolates showing great antibacterial activity during secondary screening were selected and cultivated on PDB medium. Petri plates containing PDB medium supplemented with Streptomycin to prevent bacterial growth. After 8 days of growth fungal biomass filtered and centrifuged at 5000rpm for 20 minutes. Then pellets were collected, dried using hot air oven at 70^0C for 3hrs and then grinded using homogenizer before immersed with various solvents. For the extraction distilled water, hexane, benzene, chloroform, acetone, and methanol were used as solvents. After 4 days of immersion the crude extract were filtered and stored at 4^0C for further study.

3.7 Antibacterial Test

3.7.1 Collection of Test Bacteria

For antibacterial test, some pathogenic test bacteria were collected from Microbiology Lab, Orissa University of Agriculture and Technology. 3 pathogenic bacteria were collected, one gram +ve (*Staphylococcus* sp.,) and two gram –ve bacteria *(Pseudomonas* sp., *Xanthomonasoryzae)* and cultured in nutrient broth for further use.

3.7.2. Antibacterial Test

Then antimicrobial tests were carried out by agar well diffusion method. Then the plates were allowed to incubate at 37^0C for 24 hours. After 24 hours the observations were taken, by measuring the diameter of inhibition zone in millimetre and readings were recorded. Then Penicillium sp. from soil (without chromium) collected and also antimicrobial test was carried out.

3.8 Thin Layer Chromatography

0.1mg/ml of fungal crude extract was prepared by adding 0.1mg of crude extract and 100µl of hexane. Sample solvent was prepared in chamber by adding hexane and dichloromethane in ratio 1:3.5 in total of 25ml.Next 6µl of 1.0mg/ml crude extract was dropped twice on spot on silica plate. The TLC was then run until solvent reach on top. Then the TLC plates were dried and bands visualized under ultraviolet ray under laminar air flow. Rf values were calculated.

3.9 Identification of different functional groups by FTIR

FTIR analysis was carried out in ATR mode using model Perkin Elmer spectrum II. The separated bioactive compounds by the process of TLC are subjected to FTIR (Fourier Transform Infra-red spectroscopy) analysis to identify different functional groups present in fungal extract. This technique can identify different functional groups using an infra-red light source, by measuring absorptions.

4. Results

4.1 Physio-chemical Parameters

Various physio-chemical parameters of collected samples were measured. From the results it was confirmed that the collected soil was alkaline in nature as it varies from 7-8 and temperature varies from 35-40^0C.

4.2 Isolation of Fungus

From five different collected samples i.e. ore, soil, sediment, agricultural waste water, industrial effluents, 8 fungal isolates obtained and named as OSF1…OSF8 according to their collection site.

4.3. Screening for fungus showing antibacterial activity

4.3.1 Primary screening

After 72 hours, the plate containing ore sample showed formation of zone of inhibition. The fungus that showing great antibacterial activity, sub-cultured on a PDA slants. 4 such fungus were isolated as depicted in **Figure 1(A) & (B).**

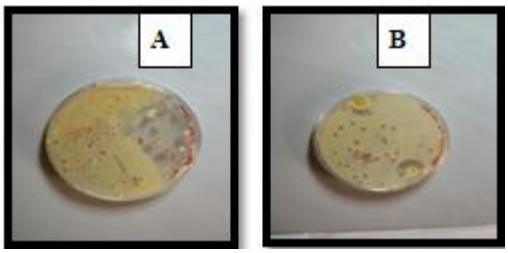

Figure 1 A & B: Primary Screening

4.3.2. Secondary screening

Secondary screening was carried out to confirm the antimicrobial activities possessed by selected fungal isolates from preliminary fungal isolates. In secondary screening our selected fungal isolates OSF were showing antibacterial activities against OSB.

4.4 Identification of fungus

4.4.1. Identification through staining

The isolates were identified on the basis of their results procured from lacto phenol cotton blue staining. It was found that all isolates belong to genus *Penicillium.* The microscopic images of penicillium sp. after lactophenol cotton blue staining represented in Figure 2(A). Then the morphological features of isolated fungal strain were further studied using scanning electron microscope. From the micrographs obtained from SEM showed that the presence of characteristic hyphae and spores of *Penicillium sp.* 2(B).

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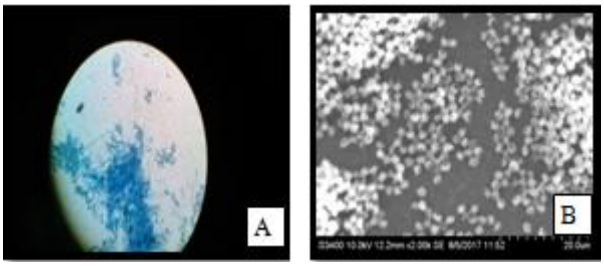
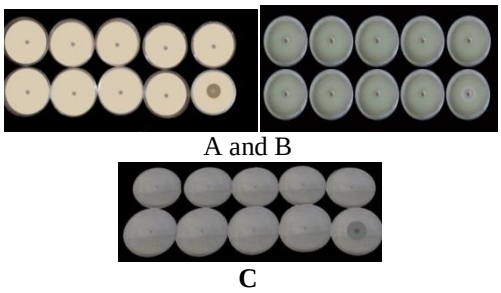


Figure 2 (A) & (B): Microscopic structure of fungal isolate after Lacto phenol cotton blue staining and Scanning Electron microscopic images of isolated fungal strain

4.5. **Extraction of secondary metabolites-** Our selected fungus which was grown in broth subjected to extraction after 8 days of growth. After extraction using various solvents, the colour change was observed in extracts.

4.6. **Antimicrobial tests-** From 8 fungal isolates collected were tested in vitro conditions against various pathogens collected from Microbiology lab, OUAT viz. *Pseudomonas* sp., *Xanthomonas oryzae*, *Staphylococcus sp.*, which are not observed by normal soil (without chromium) Penicillium sp..The result was recorded after 48-72 hours with measurement of the zone size of growth inhibition between the fungal extracts & the bacteria. It was observed that 4 fungal isolates were showing inhibition (Figure 3) and the measurements of zone of Inhibition against bacterial pathogens were represented in Table 1.



A and B

C

Figure 3: A- C -Effects of Hexane, Benzene, Chloroform, Acetone, Methanol extracts on *Staphylococcus sp.* *Xanthomonas oryzae* and *Pseudomonas sp.* (From left to Right); first row-controls, second row- fungal extracts

Table 1: Zone of Inhibition against bacterial pathogens

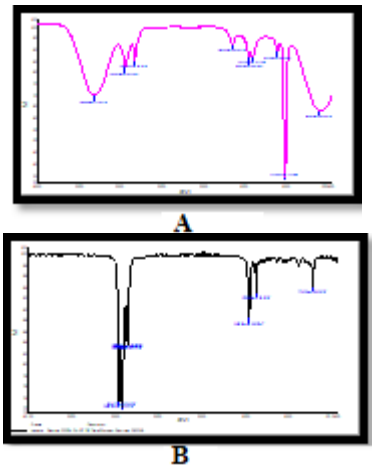
Fungal isolates	*Solvents Used For Extraction*	*Bacterial Strain to Which Our Fungal Extract Inhibits*					
		Pseudomonas sp.		*Xanthomonas oryzae*		*Staphylococcus sp.*	
		Zone of Inhibition (in MM)					
osf1	hexane	-	-	-	-	+	9±5
	Methanol	+	18±5	+	22±5	-	-
osf2	hexane	-	-	-	-	+	12±5
	methanol	+	16±5	+	19±5	-	-
osf3	hexane	-	-	-	_	+	8±5
	methanol	+	14±5	+	12±5	-	-
osf4	hexane	-	-	-	_	+	9±5
	methanol	+	12±5	+	16±5	-	-

4.7. Thin layer chromatography

Rf values were calculated from the bands obtained in thin layer chromatography of hexane and Methanolic extracts. Our Hexane extract after Tlc showing I band, & that band scrapped and collected. From Tlc of Methanolic extract of fungus 6 bands were obtained and that bands were also collected separately. Here, the distance travelled by the solvent is 6.5. Rf value of band I = 1.5/6.5=0.23, band II=1.8/6.5=0.27, band III=2.1/6.5=0.3, band IV=3.3/ 6.5=0.50, band V = 3.4/6.5=0.52 and band VI=4/6.5=0.61.

4.8. Identification of functional groups by FTIR analysis

Various functional groups present in hexane and methanolic solvent extracts of isolated fungus were analysed from graphs (Figure 4A &B) and peak absorptions by using IR spectrum library and depicted in Table 2 and 3. The infrared spectral differences, subsequent to the functional groups of the fungal extract were studied in the wavelength range of 500–3400cm−1. The peaks obtained from FTIR analysis was confirmed from the FTIR library provided by D'Souza and Kamat, 2017 and Salman et al., 2015 [6],[7]. Various functional groups were identified like alkane, alkyl halide, aldehyde, alkyne , amines and bioactive compounds like alcohol.



A

B

Figure 4 (A) & (B): FTIR analysis of Hexane and Methanolic extract respectively

Table 2: FTIR analysis of Methanolic extract

Absorption in cm^{-1}	*Functional group*	*Type of vibration*
3314.34	Alcohol	O-H $_{str.}$
2944.33	Carboxylic Acid	O-H $_{str.}$
2832.62	Aldehyde	C-H $_{str.}$
1449.29	Alkane	H-C-H $_{def.}$
1113.75	Alkyl Fluoride	C-F $_{str.}$
1022.10	Alkyl Fluoride	C-F $_{str.}$
616.71	Alkyl Fluoride	C-Cl $_{str.}$

Table 3-FTIR analysis of Hexane extract

Absorption in cm^{-1}	Functional group	Type of vibration
2968.56	Alkane	H-C-H $_{str.}$
2924.11	Alkane	H-C-H $_{str.}$
2808.77	Alkane	H-C-H $_{str.}$
1459.23	Alkane	H-C-H $_{str.}$
1378.34	Nitro Compound	N=O $_{str.}$
1037.77	Alcohol	C-O $_{str.}$
724.52	Alkyl Halide	C-X $_{str.}$

5. Summary and Conclusion

The fungal isolates were obtained from chromium contaminated soil, sediment & ore samples. From that, four isolates showed antibiosis against 3 pathogens –

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Xanthomonas oryzae and *Pseudomonas* sp. *Staphylococcus sp*. The fungal extracts were further analyzed for presence of bioactive compounds. Secondary metabolites production can be influenced by environmental and genetic factors. Fungus produces various secondary metabolites before sporulation time or after initial growth phase. The secondary metabolites may be beneficial or it may be toxic in nature and generally produce secondary metabolites in response to various environmental stress conditions like temperature, high pH, salt stress, heavy metal stress etc. Here, the secondary metabolite secretion of our fungus may be due to Cr stress. Presumably, the fungal cell receives many toxin stimulating signals from the environment. It appears that a common signal transduction pathway is partially responsible for development of natural product biosynthesis. Here, the induction may be from chromium to produce secondary metabolites. The objective of the present study is to evaluate the antimicrobial property of isolated fungus, which can be used to develop new antibiotics against disease causing pathogens in plants as well as in animals.

6. Future Scope

In future, the extracted fungal extract can be assessed and the exact active compound that is responsible for antibacterial activity can be determined.

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