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Development, Optimization & Application of Corn Cobs as Cost Effective Matrix for Immobilization of Fungal Strains *Paecilomyces sinensis* and *Geotrichum pseudocandidum*

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Abstract: **Fungal strains *Paecilomyces sinensis* and *Geotrichum pseudocandidum* were successfully immobilized on cheap yet effective support matrix, corn cobs. Immobilization was analysed by obtaining growth of the immobilized fungi from induced corn cob on solid media and further confirmed by FTIR analysis. Immobilization was successfully optimized on various temperatures and for different time duration. Activities of economic importance, performed by selected fungi were heavy metal absorption (in water and on solid medium), synthetic dye removal, enzyme production, were also performed efficiently by them in their immobilized form. Proposed work is an inclination towards immobilization of fungi via waste material appropriate for their growth and activity. Other fungi of economic importance may be immobilized using other matrices. The work suggests a cost-effective technique in order to increase industrial production.**

Index Terms: **Atomic Absorption Spectrophotometer (AAS), Bio-Remediation, Fourier-Transform Infrared (FTIR) spectroscopy, Immobilization, Synthetic dye removal.**

I. INTRODUCTION

Fungi are ubiquitous microorganisms that are continuously being researched upon and utilized for production of many commercially important products like antibiotics, enzymes and organic acids. The application of fungi has not been limited till production, rather these being applied as an agency for bio-remediation, for example, detoxification of coal wastes, sewage sludge and heavy metal contamination (Baldrian, 2003; Spina, 2012). Mere use of the fungal mycelium will never benefit the purpose of application because of the exposure to the environmental stress. For such applicative purposes fungi need to be associated with a support matrix, since the mycelium can never be used due to obvious drawbacks (Rodriguez-Couto, 2008). This particular step of providing a support matrix is one of the most important procedure since it affects the measure of fungal activity directly. The selection of the support biomass/ matrix is crucial. Studies have proposed obtaining a better performance by the microbe associated as compared with the free microorganism (Gao, 2010). Such scaffolding studies have impacted the perspective of research on utilizing the best support matrix for the purpose of immobilization. The selection of support matrix makes the process more advantageous and economic with continuous progress in the technology, which includes use of system repeatedly (Spina, 2012: 175-180). Such scopes in the field make the efforts of the researchers noteworthy.

Immobilization is the imprisonment of all types of biocatalysts including enzymes, cellular organelles, animals and plant cells in a distinct phase that allows exchange with but is separated from the bulk phase or the external environment. Immobilization has a wide range of application in many industries like biotechnology, pharmaceutical, environmental, food and biosensor industries. As stated before, Immobilization can affect the purpose directly therefore it becomes the key aspect of the process of optimization of the commercial product (Sheldon, 2007). Immobilization is a good practice but requires constant research and raw material (support matrix) which brings extra financial load.

Utilization of fungi for bioremediation has been proven significant since the contamination of water bodies with heavy

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metal is a major environmental problem all over the world (Svobodová, 2018).

'Heavy metals' is an umbrella term used to describe more than dozen elements that are either metals or metalloids having densities above 5g/cm^3 (Adriano, 2005). Examples of heavy metals include cadmium, lead, mercury, copper, nickel, and manganese. Generally, heavy metals are present naturally in trace amount in our soil and are also an essential part of all living organisms. The Anthropogenic sources of heavy metal contamination are mainly associated with certain industrial activities which include disposal of untreated wastes and agricultural practices with consideration to use of chemical fertilizers and pesticides. Other than these major sources coal fired power generation plants, municipal incinerators also contribute in increase of heavy metals in natural components, both biotic and abiotic (Rattan, 2002).

Immobilization of fungal biomass is an important but expensive technique. It will become more advisable and practical, if waste material is used as a matrix. Corn is a major crop plant, every part of which is utilized except the cob. Corncobs which are generally used as fuel creating air pollution and global warming. Thus, a matter of primary concern is the economical and efficient utilization of these corn cobs for a purpose. In this study corn cobs were induced with fungi *Paecilomyces sinensis* and *Geotrichum pseudocandidum* as adsorbent material for the immobilization of fungal biomass. Immobilization of fungal biomass in corn cobs were confirmed by fungal colony formation showing similar macro and microscopic features of mother culture on potato dextrose agar medium (PDA). Immobilization of both fungi in corn cob was further confirmed by FTIR analysis.

Studies were carried out with immobilized fungi in induced corn cobs, to analyze bio-remediation by absorption of heavy metals (Cu and Pb) using atomic absorption spectrophotometer (AAS), qualitative assessment of amylase enzyme production and absorption of synthetic dyes.

II. MATERIALS AND METHODS

A. Isolation of Material

Fungal strain of *Paecilomyces sinensis* (MCC No.1224) and *Geotrichum pseudocandidum* (MCC No. 1167) were obtained from the stock cultures of Laboratory of Department of Botany and Microbiology, St. Aloysius' College Jabalpur (M.P.). Both the cultures have been isolated and identified as endophytes of medicinal tree *Oroxylum indicum* (L.) Vent. and has been already deposited to NCCS (National Centre for Cell Science, Pune).

Corn cobs were collected from local farmers, cut into small pieces, soaked in distilled water overnight and then sterilized in autoclave in screw cap bottles (Fig. 1).

Fig. 1: Sterilized corn cobs as adsorbent for immobilization

B. Immobilization of fungal biomass (P. sinensis and G. pseudocandidum) on corn cobs

For the immobilization purpose, potato dextrose broth was prepared and autoclaved, then inoculated with the isolated fungus *P. sinensis* (Fig. 2) and *G. pseudocandidum* (Fig.3) and with the sterilized corn cobs (Fig.4). PDA broth was kept in the BOD incubator for 7 days.

Fig. 2: Pure culture of *Paecilomyces sinensis*

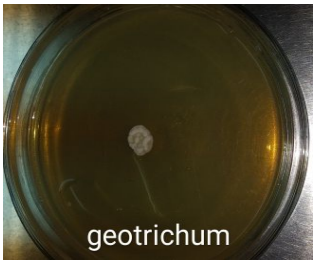


Fig.3: Growth of *G. pseudocandidum* on PDA

Fig.4: Sterilized corn cobs inoculated in potato dextrose broth

C. Optimization of immobilization of fungi on various temperatures and after various time periods

Immobilization of fungus *P. sinensis* and *G. pseudocandidum* was optimized on different range of temperatures and for different time intervals. Both parameters were optimized on the basis of colony diameter developed from them. To observe effect of temperature sterilized corn cobs were incubated in fungal broth on different temperature and observation were taken after 24 hrs. Range of temperature was taken near by the optimum temperature (28^0C). The study was carried out to get maximum growth of fungi from cob induced with fungi. Similarly, time duration is also an important factor. For present work organic waste material has been taken as matrix, so there may be possibilities to spoil of matrix after some hours of induction. To check the viability of fungi and stiffness of matrix, colony diameter form after time intervals (6, 12, 18, 24 and 38 hours) definite induced cob has also been observed (Awad, 2016).

D. Confirmation of immobilization of fungal biomass on corn cob: on solid media

To confirm that the fungi were immobilized on corn cob, potato dextrose media was poured in sterilized Petri plates and allowed to solidify. A piece of induced and un-induced corn cobs were inoculated on solidified PDA plates under aseptic conditions in laminar air flow, separately, and then these plates were kept in BOD incubator for 2-3 days (Elegbede & Lateef, 2018).

E. Confirmation of immobilization of fungal biomass on corn cobs by Fourier-Transform Infrared (FTIR) spectroscopy (Kowalczuk, 2019)

Dried composite of fungal mycelium and mycelium induced corn cob, were macerated using sterilized mortar and pestle. Samples of 1 mg were mixed with 100 mg of spectroscopic grade KBr, HiMedia and analyzed against KBr background. The FTIR spectra were determined between 4000 and 400 cm^{-1} using a Shimadzu IR 00158 with the following parameters: Spectral resolution 4 cm^{-1}, 20 scans min^{-1}, encoding interval 1 cm^{-1}, Happ-Genzel apodization and scanning speed 2.8 mms^{-1}

F. Application of immobilized fungi

1) Heavy metal absorption from water

Solutions of different concentrations of lead and copper i.e. 1, 3 and 5ppm have been prepared. Each set of solutions (of different concentrations) were inoculated with induced cobs, un-induced cobs and fungal mycelium (Contreras-Cortés, 2020). One set was kept as control. Experiment was designed in triplicate for each sample. Each sample was observed after 72 hours under Atomic Absorption Spectrophotometer (AAS).

2) Determination of tolerance of heavy metals on solid medium

To determine the heavy metal tolerance of immobilized fungi, PDA medium was poured in sterilized Petri plates allowed to solidify, and then 10-30 ppm heavy metal solutions were poured on each plate separately (Muñoz, 2012). Cu and Pb were the choice of heavy metals. Then each Petri plate was inoculated with mycelium and induced cob, on different concentrations of heavy metals, to compare the difference in growth. Petri plates with PDA medium (without any heavy metal solution) and inoculated with induced cob and mycelium separately were used as controls.

3) Screening for Amylase production

To check whether the induced fungus (*P. sinensis* and *G .pseudocandidum*) produces amylase, a piece of induced cob was inoculated on solidified potato dextrose agar medium plates under aseptic conditions in laminar air flow and then these plates were kept in BOD incubator for 3-4 days and then tested for amylase production.

4) Decolourization of synthetic dyes (Casieri, 2008).

Diluted concentrations of dyes namely phenol red, fuchsine (basic), malachite green, ferrochrome black T, safranin were filled in screw cap bottles and then each dye inoculated with induced and un-induced corn cobs and then observed after 24 hours of inoculation.

III. RESULTS

A. Confirmation of immobilization of fungal biomass on corn cobs–

After 3-4 days of incubation green colored fungal colony with powdery appearance was appeared on PDA from induced cob (Fig. 5). This was exactly similar to mother culture of *P. sinensis* (Fig. 2) and white colored colony was appeared on PDA from induced cob (Fig. 6) which was exactly similar to mother culture of *G. pseudocandidum* (Fig. 3).

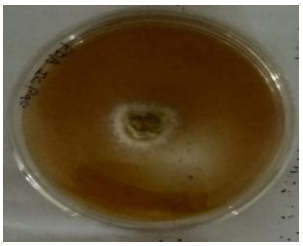


Fig. 5: Growth of *P.sinensis* from Induced Cob on PDA medium

Fig. 6: Growth of *G. pseudocandidum* from induced cob on PDA

Same fungal colony was not appeared from un-induced cob. Formation of fungal colony around induced cob (IC) was a sign of induction and immobilization of fungi. Even area of fungal colony was larger, arose from IC than area of fungal colonies developed from point inoculation from mother cultures. Immobilization has been confirmed further by various experiments. The ICs were capable to work even after 3-4 sub-cultures for each functioning described below.

B. Optimization of immobilization of fungal mycelium P. sinensis and G. pseudocandidum in corn cob on various temperatures and after various time periods.

Fungal growth on provided temperature was measured in the form of colony diameter (given in Table-1) developed on the PDA. It was observed that the optimum temperature on which fungal colony with largest diameter grown was different for both fungi. It was 28^0C for *P. sinensis* with the colony diameter form 11.1cm. Optimum temperature observed for maximum growth of *G. pseudocandidum* was 28.5^0C with the colony diameter of 10.2cm.

C. Confirmation of immobilization of fungal biomass by FTIR-

Four dominant spectral windows showing C-H, O-H stretching region, amide I, amide II and polysaccharide regions were identified displaying characteristic variable bands at 3400-3200 cm^{-1}, 2900-2850 cm^{-1}, 2350-2215 cm^{-1}, 1750 cm^{-1}, 1625-1600 cm^{-1}, 1582-1547 cm^{-1}, 1375-1315 cm^{-1} and 900-725 cm^{-1}. The strong absorption at 1052.43 cm^{-1} that appeared in the FT-IR spectrum suggested that the monosaccharide in dried mycelium of *Paecilomyces sinensis* has a pyran structure. The

Table 1: Optimization of immobilization of fungi *P. sinensis* and *G. pseudocandidum* in corn cob on various temperatures and after various time periods.

Temperature (^{0}c)	Time (hours)	Colony diameter (cm.)	
		P. sinensis	***G. pseudocandidum***
26	-	-	-
28	-	11.1	9.4
28.5	-	9.3	10.2
29	-	7.5	7.5
29.5	-	-	2.1
30	-	-	-
-	6	-	-
-	12	9.3	10.5
-	18	11.2	10.7
-	24	11.8	10.8
-	36	11.0	10.5

absorption band at 874.55 cm^{-1} indicated that the glucoside bond in the dried mycelium was a betalinkage (Lu, 2007). Analysis of IC samples was directly proportional to the spectra of *Paecilomyces sinensis* confirming the induction and immobilization of fungi in corn cobs (Fig. 7)

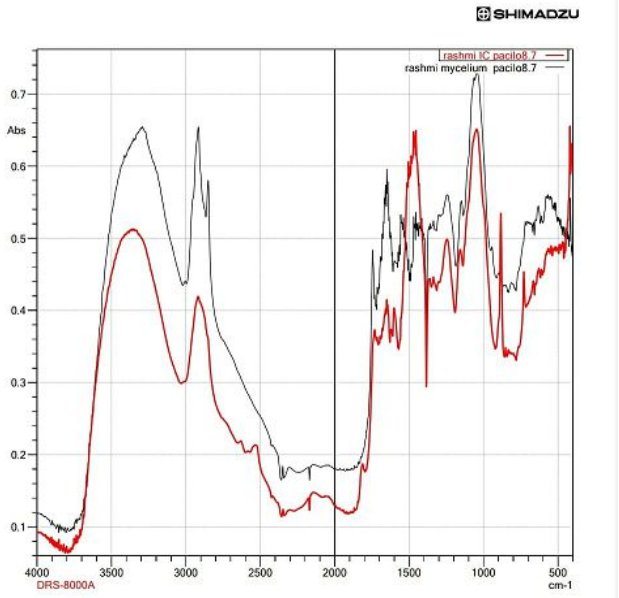


Fig. 7: FT-IR Spectrum of Paecilomyces sinensis mycelium (Black) & Paecilomyces induced cob (Red). Similar peaks confirming the induction of mycelium in the cob.

D. Heavy metal absorption from water

Heavy metal concentrations were analyzed by using Atomic absorption spectrophotometer (AAS). IC cob of 8-10 days aged was kept in the different concentrations (1, 3 and 5 ppm) of heavy metals.

Table 2: Determination of heavy metal ($PbNO_3$) absorption with *P. sinensis* and *G .pseudocandidum* in induced cob (IC) by Atomic Absorption Spectrophotometer (AAS).

Std/ Sample name	Weight (gms)	Volume (ml)	Dilution factor	Absorbance	Concentration (ppm)
Std 1	-	-	-	0.275	1.0000
Std 2	-	-	-	0.459	2. 0000
Std 3	-	-	-	0.462	3. 0000
IC 1	1.00	1.00	1.00	0.043	0. 0000
IC 2	1.00	1.00	1.00	0.102	0.0000
IC 3	1.00	1.00	1.00	0.094	0.0000

Table 3: Determination of heavy metal ($CuSO_4$) absorption with *P. sinensis*and *G. pseudocandidum* in induced cob (IC) by Atomic Absorption Spectrophotometer (AAS).

Std/ Sample name	Weight (gms)	Volume (ml)	Dilution factor	Absorbance	Concentration (ppm)
Std 1	-	-	-	1.398	1.0000
Std 2	-	-	-	1.872	3. 0000
Std 3	-	-	-	2.012	5. 0000
IC 1	1.00	1.00	1.00	0.112	0. 0300
IC 2	1.00	1.00	1.00	0.258	0.0800
IC 3	1.00	1.00	1.00	0.331	0.1100

It is clear from table-2 & 3; that concentrations of heavy metals Pb and Cu (Fig. 8) were readily decreased when treated with IC.

A B

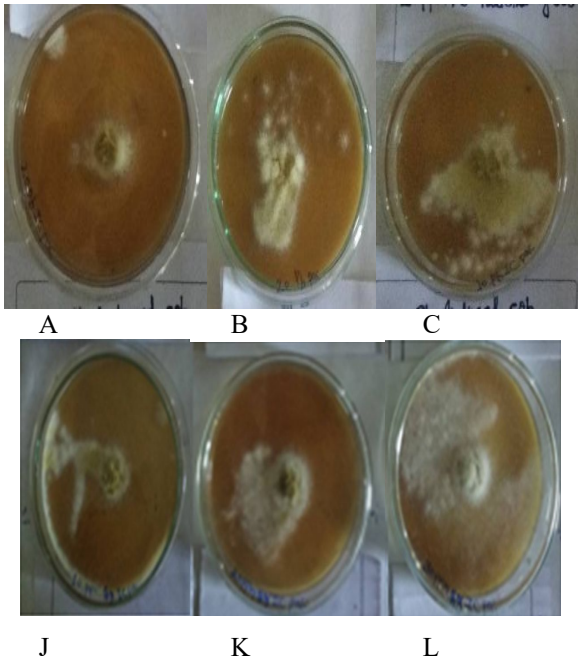
Fig. 8: Heavy metal solutions inoculated with mycelium, un-induced cob and induced cob
A. $PbNO_3$ (1, 3 and 5ppm) B. $CuSO_4$ (1, 3 and 5 ppm)

All heavy metal concentrations were also kept with un-induced cob remained unchanged. Fungus *P. sinensis* was capable to absorb heavy metal from water. But in immobilized form, capacity of absorbance was enhanced as the fungi were provided with natural nutrition from corn cobs. No absorbance from un-induced cob was an indication, that corn cob used in itself was not capable to absorb heavy metal.

E. Determination of tolerance of heavy metals on solid medium

When IC inoculated on solid PDA containing different concentrations of Pb and Cu gave fungal colonies of different diameters. It is clear from figures (Fig. 9; Fig.10) that area of diameter of fungal colony developed from IC was increased with increasing concentrations of heavy metals. In immobilized form as well, fungi showed tolerance against both heavy metals.

The results have also been tabulated for better comprehension of the differences in table 4.



A B C

J K L

Fig 9: Growth of *P. sinensis* from IC on PDA containing heavy metal:

$PbNO_3$.-A.10 ppm; B. 20 ppm & C.30 ppm
CuSO4- J.10 ppm; K. 20 ppm & L.30 ppm

A B C

G H I

Fig. 10: Growth of *G. pseudocandidum* from IC on PDA containing heavy metal:
$PbNO_3$.-**A**.10 ppm; **B**. 20 ppm &**C**.30 ppm
$CuSO_4$- J.10 ppm; K. 20 ppm & L.30 ppm

Table 4: Determination of tolerance of heavy metals of immobilized fungi (*P. sinensis*and *G. pseudocandidum*) measuring fungal colony diameter on heavy metal containing PDA.

Culture name	Heavy Metal	Concentration (ppm)	Sample	Fungal colony diameter (cm)
P. sinensis	$PbNO_3$	10	M	11.2
		20		17.2
		30		18.9
		10	IC	11.9
		20		18.9
		30		26
	$CuSO_4$	10	M	0.9
		20		11.9
		30		14
		10	IC	8.75
		20		14.4
		30		22.5
G. *Pseudocandidum*	$PbNO_3$	10	M	10.2
		20		12
		30		14
		10	IC	10.6
		20		12.9
		30		15.2
	$CuSO_4$	10	M	10
		20		12.2
		30		13
		10	IC	10.6
		20		12.9
		30		14.2

F. *Screening for Amylase production*

Immobilized microbes are readily used for the purpose of industrial production. In present work both the immobilized fungi were capable to produce enzyme amylase (Fig. 11 and Fig. 12).

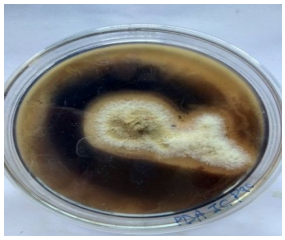


Fig. 11: Amylase enzyme production from *P. sinensis*developed from induced cob

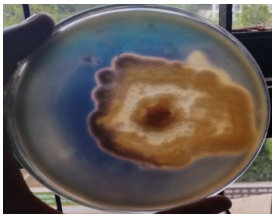
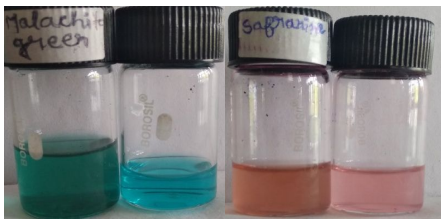


Fig. 12: Amylase enzyme production from *G. pseudocandidum* developed from induced cob.

G. *Decolourization of synthetic dye*

Some fungi are readily used for the purpose of stain removal. They must need their optimised immobilised forms, so that they can be used again and again for functioning. In the present work, it was observed that fungus *P. sinensis* in its immobilized form was capable to absorb dye from water sample (Fig. 13).

A B



C D

Fig. 13: A-D: Decolourization of different dyes after 24 hours with *P. sinensis*
A. Fuchsin (Basic) Dye; **B**. Eriochrome Black T Dye
C. Malachite Green Dye; **D**. Safranine Dye

The fungus in its immobilized form was ready to use approximately 10-12 times for different purposes described. The induced cob with immobilized fungi appearance was like capsules. They can be stored in distilled water for long period of time without taking any other nutrition from outside in the form of media. The were-

1. Convenient for transport.
2. Easy application
3. More efficient in immobilized form in comparison to
4. Cost effective
5. Eco- friendly.

IV. DISCUSSION

Immobilization is an important tool to use the beneficial microbes in secure mode and repetitively. Generally, matrices used for immobilization are not economic. There is an imperative need to search the material for microbial immobilization, which is economic, safe, easy to handle and environment friendly. Corn cob is a waste material in our country, which is used as a raw material for industrial production of many products (Kalieva, 2017). Such significant importance of the immobilization has been the driving force for the present research.

In the present work, a protocol was developed to successfully immobilize the fungi *P. sinensis and G. pseudocandidum* using corn cobs as a matrix. Previously some other fungi have also been immobilized using matrix obtained from raw material (Yesilada, 2018). Studies of Immobilization of white rot fungi on sorghum (Zahmatkesh, 2018), *Penicilllum citrinum* on chitosan (Rocha, 2012), *Funalia trogii* on adsorbents prepared from apricot stone (Birhanli, 2013), *P. Chrysosporium* on bagasse as a support matrix (Mohammadi & Nasernejad, 2009) and demonstration of biomethylation of metalloids by various fungi (Gadd & Sayer, 2000) have significantly impacted the research in this direction. The effect on the properties & characteristics of the fungi in immobilized form is the major point of analysis.

The present work focuses not only on immobilization of the isolated fungi but their functioning in induced form was analysed and found to be better than the mycelial forms, similar to work of Yesilada *et al*. 1997, where the biodegradation yields were found to be enhanced after immobilization which supports the result of present study.

The use of FTIR technique has been proven quite significant to establish the efficient immobilisation of fungi in the corn cob. Studies on FTIR (Charumathi & Das, 2012; Kowalczuk & Pitucha, 2019) have been a guiding line and provide future prospects of studying site specific reactions can further support the study more efficiently. The simplicity of sample preparation, avoidance of chemical (i.e. costs and environmental impact), reliability, short measurement times (<1 min) makes FTIR technique suitable for a large scale screening of fungal samples and for routine analysis. These facts also encourage the possibility of developing FTIR spectroscopy as a reliable method for rapid identification of fungal pathogens. There is a great potential of FTIR microscopy for an easy and rapid discrimination and identification of various fungi genera (Salman, 2010).

The FTIR confirmed immobilization of the fungi in the corn cob was also screened for amylase enzyme production which reflects that the matrix is suitable for the fungi to exhibit the characteristics.

The confirmation of immobilization by FTIR is also supported by the data obtained employing the AAS instrument. Atomic absorption spectrometry (AAS) analysis has been a significant step to justify reduction in concentration of heavy metals. The technique is sensitive and has been utilized by many researchers. Literary works on bioremediation analysis using AAS technique have a wide time span. A thorough studies on the instrument and its application in different time periods in the late 1977 till present time (Huet & Puchooa, 2017; Sperling, 1977) and as recent as in research (Kamarudheen, 2020) & review on AAS instrument (Akash & Rehman, 2020) have been the basis of present study.

Many fungi have been experimented with that show the potential of bioremediation. One such basidiomycetous fungi (*Merulius aureus* syn.*Phlebia* sp.) and a deuteromycetous fungus (*Fusarium sambucinum*) have been considered as fungi with bioremediation potential (Malaviya & Rathore, 2007). Other works include use of *ladosporeum perangustum*, *Penicillium commune*; and *Fusarium equiseti* was employed for tannery wastewater bioremediation, Different species of *Paecilomyces* ,i.e., *Paecilomyces* lilacinus was also utilized for bioremediation (Sharma & Malaviya, 2016).

White-rot fungi are renowned for their remarkable potential to degrade a wide range of organic pollutants and thus find a special status in bioremediation studies (Varma, 2015). White rot fungi and their enzymes (laccase, lignin peroxidase, and Mn peroxidase) can be used to bioremediate various xenobiotics and

wastewaters, decolorize, and detoxify textile dyes in water bodies originating from textile manufacturing facilities (Yesilada, 2018)

The textile dyes, when released untreated, significantly compromise the aesthetic quality of water bodies. These pollutants increase biochemical and chemical oxygen demand (BOD and COD), impair photosynthesis, inhibit plant growth, enter the food chain and many such ecosystem deteriorating effects have been observed (Lellis, 2019). This problem is also being tackled through the immobilization of potent fungi.

The different fungal strains have also been tested for Treatment of real textile wastewater, and obtained results reflect a significant intrinsic variability in terms of decolorization yields (Srivastava & Thakur, 2006).

The availability of variety of biomass and their metal binding potential makes it a economical and sustainable option for developing effluent treatment process for removal, recovery of heavy metals (Bishnoi & Garima, 2005) and decolorization of textile dyes (Kurade, 2019).

The results of FTIR analysis and AAS analysis in the present work are satisfying and provide a strong foundation for further research and future prospects regarding the employment of *Paecilomyces* and *Geotrichum* as organisms for bioremediation & synthetic dye removal, especially in immobilized form.

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