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(RESEARCH ARTICLE)

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Extraction of peptide from white rot fungi, its microencapsulation and activity against aflatoxin producing *Aspergillus* spp

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Abstract

The present research work focused on the detoxification of aflatoxin by using white rot fungi, secondary metabolites and to produce the secondary metabolites using white rot fungi (*Pleurotus spp.)*, extraction of compound in the aflatoxin, checked the compound by UV-Visible spectroscopy, TLC and FTIR studies and to Evaluate the detoxification study using the toxin, and also to find out the antifungal activity using *Aspergillus spp.* later by using sodium alginate microencapsulated product produced.

Keywords: *Pleurotus spp; Aspergillus spp*; Sodium alginate; Aflatoxin etc

1. Introduction

Mycotoxins are a large group of secondary metabolic products which pose serious risks for human and animal health. Fungal growth and mycotoxin production may occur during storage, under suitable temperature and humidity conditions (Bryden, 2012). Mycotoxin contamination occurs widely in feedstuffs of plant origin, especially in cereals, fruits, vegetables, hazelnuts, almonds, seeds, fodder, and other agricultural feed or food intended for animal or human consumption (Guan *et.al* 2011;Wu *et.al* 2013, 2014, 2015a,b). The transfer of mycotoxins and their metabolites occurs in animal products, such as animal tissues, milk and eggs (CAST, 2002).

Aflatoxins, produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* (Wogan and Pong, 1970), are determined as the most hazardous mycotoxins. The major aflatoxins are called B1, B2, G1, and G2 (based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography) M1 and M2 (produced in milk and dairy products) (D'Mello and MacDonald, 1997). Aflatoxin B1 is the major aflatoxin produced by toxigenic strains and is usually the most potent natural carcinogen known (Squire, 1981). The liver is the target organ for Aflatoxin. Long-term intake of feeds contaminated with the AF results in negative effects on the liver, such as hepatic cell and tissue injury, as well as gross and microscopic abnormalities (Williams *et.al* 2011; Gholami-Ahangaran *et.al* 2016).

Aflatoxin are mainly targeting the liver (Abdel-Wahhab *et.al* 2007). Early symptoms of hepatotoxicity of liver caused by aflatoxins contains fever, malaise and anorexia followed with abdominal pain, vomiting, and hepatitis; however, cases of acute poisoning are exceptional and rare (Etzel, 2002). Chronic toxicity by aflatoxins involves immunosuppressive and carcinogenic effects. Evaluation of the effects of AFT-B1 on splenic lymphocyte phenotypes and inflammatory cytokine expression in male F344 rats have been examined (Qian *et.al* 2014). AFT-B1 decreased anti-inflammatory cytokine IL-4 expression, but increased the pro-inflammatory cytokine IFN-γ and TNF-α expression by NK cells. These findings shows that frequent AFT-B1 exposure accelerates inflammatory responses via regulation of cytokine gene

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expression. Furthermore, Mehrzad *et.al* (2014) observed that AFT-B1 interrupts the process of antigen-presenting capacity of porcine dendritic cells, recommended this perhaps one of mechanism of immunotoxicity by AFT-B1.

White-rot fungi have the ability to destroy lignin to the level of CO2 (Kirk & Farrell, 1987). The major extracellular ligninolytic enzymes of white-rot fungi involved in lignin biodegradation are Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Kirk & Farrell, 1987). There is an important role in lignin-degrading white-rot fungi and their ligninolytic enzymes because of their potential to degrade recalcitrant environmental pollutants, such as polychlorinated dibenzodioxin (Kamei *et.al* 2005), lindene (Bumpus *et.al* 1985), chlorophenols (Joshi & Gold, 1993), and polycyclic aromatic carbons (Bezalel *et.al* 1996; Collins *et.al* 1996). Recently, ligninolytic enzymes such as MnP and laccase were proved to be effective in degrading methoxychlor (Hirai *et.al* 2004) and Irgarol 1051 (Ogawa *et.al* 2004) and in eliminating the estrogenic activities of bisphenol A, nonylphenol (Tsutsumi *et.al* 2001), 4-tert-octylphenol (Tamagawa *et.al* 2007), butylparabens (Mizuno *et.al* 2009), genistein (Tamagawa *et.al* 2005), and steroidal hormones (Suzuki *et.al* 2003; Tamagawa *et.al* 2006). More recently, fungal laccases has been reported as the degradation of AFB1 (Alberts *et.al* 2009). From white rot fungi, laccase is an extracellular enzyme that can be extracted and contains four copper ions (Liu, Y. *et.al* 2021). As white rot fungi have the capability to degrade lignin, as well as a wide range of polycyclic aromatic hydrocarbons (Arora and Sharma, 2009; Mayer and Staples, 2002), their role in the degradation of other carcinogens, such as AFB1 is not known at present. Enzymes such as peroxidases (lignin- and Mg-peroxidases) and laccases of the white rot fungi have the unique mechanisms that cause lignin degradation.

In this study degradation of AFB1 by laccase enzymes was examined. With white rot fungal culture fractions, pure fungal laccase enzyme, as well as recombinant laccase enzyme fractions AFB1 was treated. The effect on the mutagenic potency of the mycotoxin and possible degradation products was also monitored to examine the biological relevance of the degradation process.

2. Materials and methods

2.1. Collection and sub culturing of fungi

Aspergillus sp. were collected from two different samples. One from onion sample and another from White rot fungi were collected from MTCC (Microbial Type Culture Collection). Malt Extract Agar and Czapek Dox Broth were used as the media for the inoculation of fungi sp.

Preparation of medium- 50ml of Malt Extract Agar and 30ml of Czapek Dok Broth were weighed and sterilized at 121°C for 15 minutes. The Czapek Dok Broth were allowed to cool and poured in 2 conical flasks and Malt extract agar were poured on 3 petri plates consecutively and allowed to solidify. One plate were inoculated with *white rot fungi*, and then one set of plates and broth in the conical flasks were inoculated each with *Aspergillus sp.* from MTCC, and *Aspergillus sp.* from onion samples. The plate and broth were incubated at room temperature for 4-5 days.

2.2. Microscopic identification of fungi

To two grease free cleaned glass slides, added 1-2 drops of Lactophenol-cottonblue stain at the center of each slides and transferred both the specimens, covered with a coverslip, without forming air bubble and observed under the microscope.

2.3. Extraction of compounds

Czapek Dox Broth were prepared. The broth were sterilized at 121°C for 15 minutes, they were allowed to cooled and inoculated with *Aspergillus sp*. from both onion sample (Os) and MTCC (Cs) and then later Incubated at room temperature for 4-5 days.

2.4. Thin layer chromatography

Thin layer chromatography is a technique used to isolate non-volatile mixtures. Aflatoxin from onion sample were treated with white rot fungi and laccase enzyme in separate tubes, same as Aflatoxin from MTCC. From these samples, TLC were done by using TLC plate. Using pencil, mark a horizontal line 1cm from one end of the plate. One TLC plate each

were required for both aflatoxin from onion sample and MTCC. Mark 3 points in the line with some distance for each, in the TLC plate. One point were spotted with aflatoxin (Os1), second point were spotted with aflatoxin treated with white

rot fungi (Os2) and third point were spotted with aflatoxin treated with laccase enzyme (Os3) for onion sample and Cs1, Cs2, Cs3 for MTCC respectively. By using fine glass capillary tube sample were spotted and wait to dry. Repeat it for 20-25 times and dry it. Then the iodine solution were poured onto a petri plate, by using eppendoff tube TLC plate were placed without touching the iodine solution. Then closed the plate for sometimes and observed the results and calculated using the formula:

RF = Distance travelled by solute

Distance travelled by solvent

2.5. UV visible spectrophotometry

The secondary metabolites were confirmed by measuring the wave length of reaction mixture in the UV-Visible spectrum. Phosphate buffer were used as blank and the aflatoxin produced from onion sample and MTCC were treated with white rot fungi and lactase enzyme used as sample.

2.6. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a technique based on the vibrations of the atoms within a molecule. An infrared (IR) spectrum is obtained by passing IR radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in the absorption spectrum appears corresponds to the frequency of vibration of a part of a sample. FTIR analysis method uses infrared light to scan test samples and observe chemical properties. It is used to identify and characterize unknown materials. Aflatoxin (AC) and aflatoxin from onion sample (OET) and MTCC (AET) were treated with laccase enzyme were used as sample.

2.7. Preparation of production media

MGYP were the production media prepared by using malt extract agar, glucose, yeast extract, peptone and distilled water. The media were sterilized at 121°C for 15 minutes. Then allowed to cool and inoculated with *white rot fungi* and incubated at room temperature for 4-5 days.

2.8. Applications

2.8.1. Preparation of Encapsulation

Czapek Dox Broth were prepared and sterilized at 121 °C for 15 minutes, they were allowed to cooled and inoculated with *white rot fungi* for 4-5 days. Then the broth were filtered, and the filtrate were added with 4% sodium alginate. Then kept it in the water bath and allowed to dissolve sodium alginate. 2% calcium chloride were prepared, to that sodium alginate sample were added drop by drop using micropipette. This were incubated for 4 hours and filtered through filter paper, then the beads were air dried. After air drying the dried capsules were weighed and transferred to empty capsules.

2.8.2. Antifungal activity of:

- *Aspergillus niger,*
- *Aspergillus flavus and*
- *Aspergillus terreus*

Prepared Beads were crushed by soaking it in 500µl distilled water and added 500µl DMSO and incubated overnight. Malt extract agar were prepared and sterilized at 121°C for 15 minutes. After sterilization, media were poured in 3 petri plates. After solidifying the media, *Aspergillus niger, Aspergillus flavus* and *Aspergillus terreus* were swabbed in each plate. Then 4 well were prepared in each plate. Two well were filled with 50µl and 100µl crushed beads, another two well with 100µl standard solution and distilled water respectively. Incubate these 3 plates at room temperature for 4-5 days.

3. Results

3.1. Collection and sub culturing of fungi

After incubation, *Aspergillus spp.* and *white rot fungi* were growed in Malt extract agar and czapek dox broth.

3.2. Microscopic identification of fungi

Stained fungi were observed under microscope.

3.3. Extraction of compound

After incubation, *Aspergillus sp*. from czapek dox broth were filtered and the filtrate were added with 10ml chloroform and kept in the shaker for 24 hours. Then the filtrate were separated and toxin produced. Then OD value were calculated.

Table 1 OD value of *Aspergillus spp.* extracted compound

	As	**Os**
260	0.049	0.076
280	0.395	0.446
310	0.407	0.547
410	0.450	0.343
540	0.314	0.256
640	0.080	0.099
700	0.036	0.069

3.4. Thin layer chromatography

The TLC were performed for the treated and non-treated *Aspergillus spp.* from onion sample and the spot were noted. The Rf value were calculated.

$$\text{Rf (Os1)} = \frac{3}{4}$$

$$= 0.75$$

$$\text{RF(Os2)} = \frac{2.5}{4}$$

$$=0.625$$

$$\text{Rf(Os2)} = \frac{3}{3.5}$$

$$= 0.86$$

Formation of single spot in (Os1) followed by two spots and no spots were observed for Os2 and Os3 respectively.

The TLC were performed for the treated and non-treated *Aspergillus spp.* from MTCC and the spot were noted. The Rf values were calculated.

$$\text{Rf(Cs1)} = \frac{3.5}{4}$$

$$= 0.875$$

$$\text{Rf(Cs2)} = \frac{3.6}{4}$$

$$= 0.9$$

$$\text{Rf(Cs2)} = \frac{3.2}{4}$$

$$= 0.8$$

$$Rf(Cs3) = \frac{3.7}{4.1}$$

$$= 0.90$$

$$Rf(Cs3) = \frac{3.5}{4}$$

$$= 0.8$$

Formation of single spot in Cs1 and two spots each for Cs2 and Cs3 respectively.

3.5. UV-Visible spectrophotometer

The characterization of protein from Os2, Os3, Cs2 and Cs3. It were monitored by UV-Visible spectrophotometer analysis. The dilute supernatant were analysed on 365nm wavelengths.

Table 2 UV-Visible spectrophotometer analysis of *Aspergillus spp.*

	2	**3**
Os	0.246	0.313
As	0.347	0.336

3.6. FTIR Analysis

The compound were characterized by using fourier transformed infrared spectroscopy.

X-axis = Wavenumber

Y-axis = Percent Transmittance

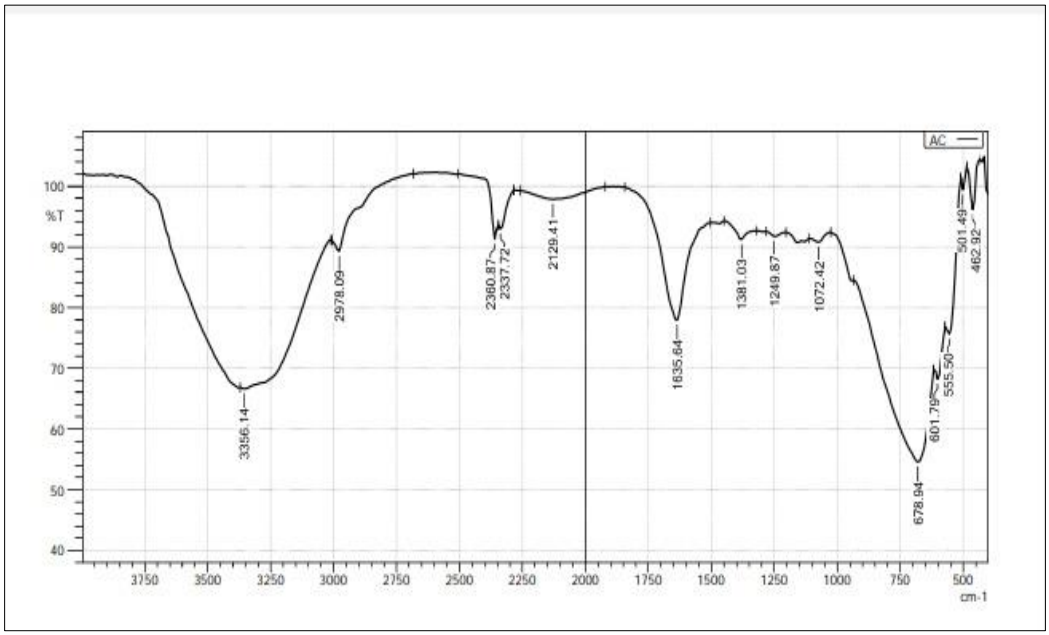

Figure 1 FTIR Analysis of aflatoxin

Table 3 Frequency table of FTIR Analysis of aflatoxin

Frequency	Functional group
3356.14	C–H stretch
1635.64	C=C alkene
1381.03	CH3 bend
1249.87	C–O–C stretch
1072.42	C–OH stretch
678.94	C–Cl
601.79	C–Cl
555.50	C–Br

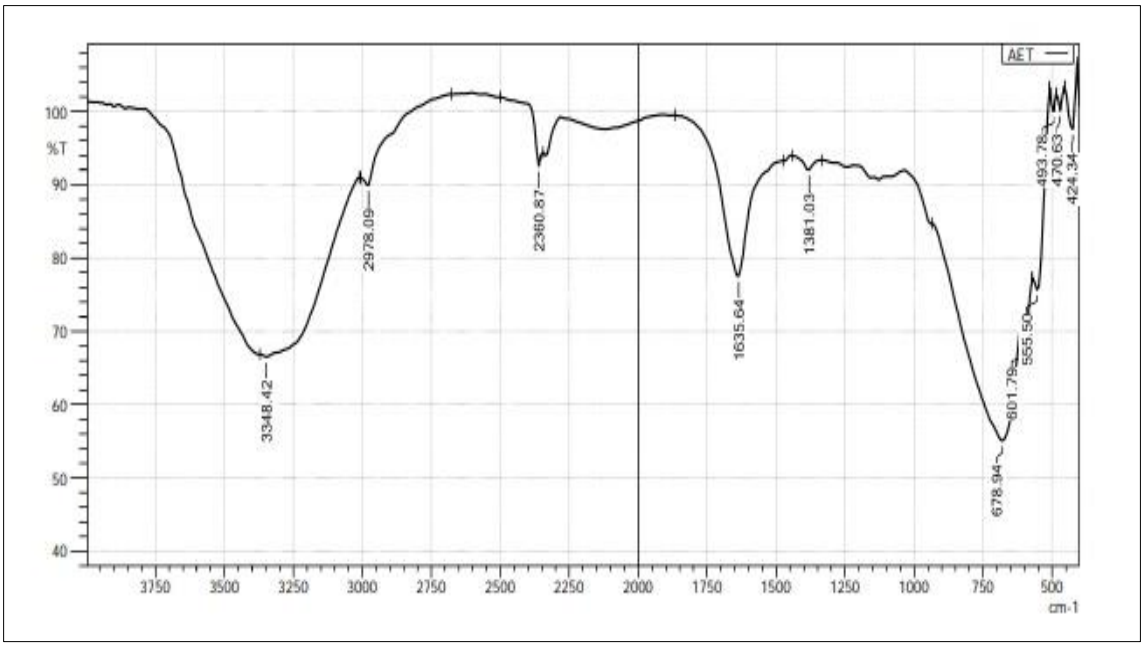

Figure 2 FTIR Analysis of enzyme treated aflatoxin

Table 4 Frequency table of FTIR Analysis of enzyme treated aflatoxin

Frequency	Functional group
1635.64	C=C alkene
1381.03	CH3 bend
678.94	C–Cl
601.79	C–Cl
555.50	C–Br

3.7. Preparation of production media

After the incubation of white rot fungi, white rot fungi in the production media were filtered. Then the filtrate were scanned at 200-800 nm and peak were observed.

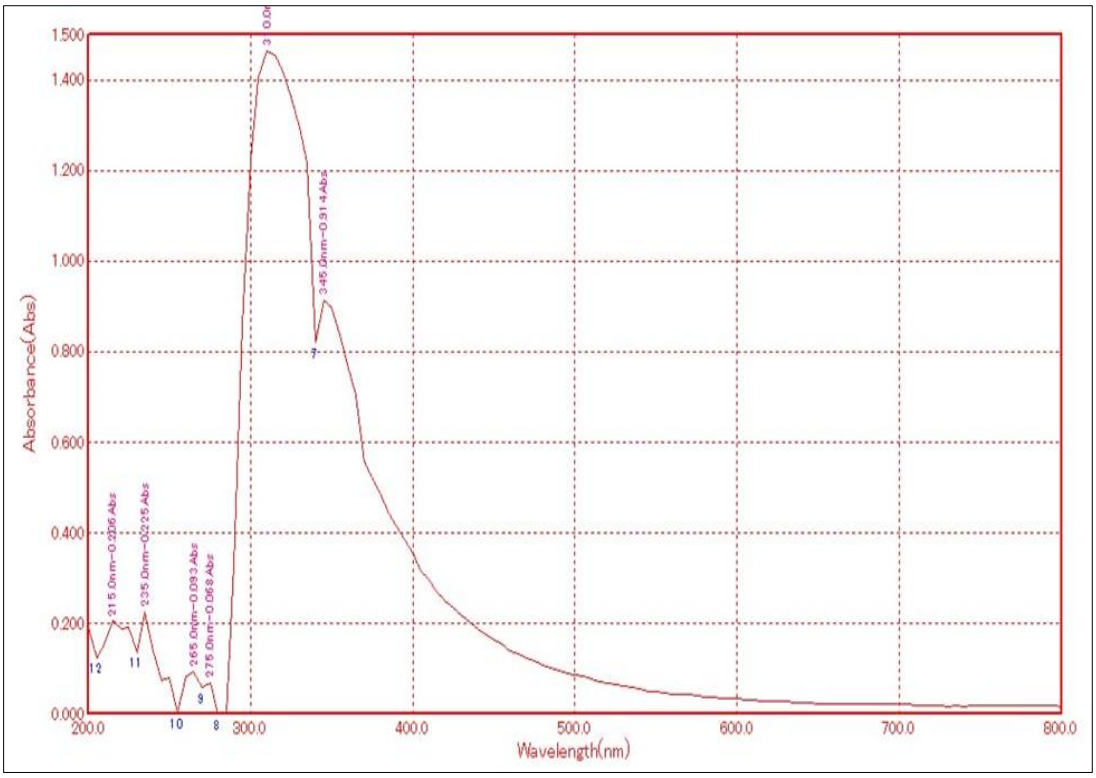

Figure 3 *white rot fungi* in MGYP

3.8. Application

Preparation of encapsulation

From the extraction of white rot fungi, capsule were prepared.

3.9. Antifungal activity

After incubation, zone were observed and their measurement is tabulated. (Aparna Ravi et.al. 2024).

Table 5 Antifungal activity

Volume	***A.niger***	***A.flavus***	***A.terreus***
Standard solution	4 mm	4 mm	2 mm
50µl	1 mm	2 mm	1 mm
100µl	3 mm	3 mm	1 mm
Distilled water	nil	nil	nil

- *Aspergillus flavus* shows more antifungal activity against aflatoxin than others.

Figure 4 *Aspergillus spp*. in Czapek agar

Figure 5 *white rot fungi* in Malt Extract Agar

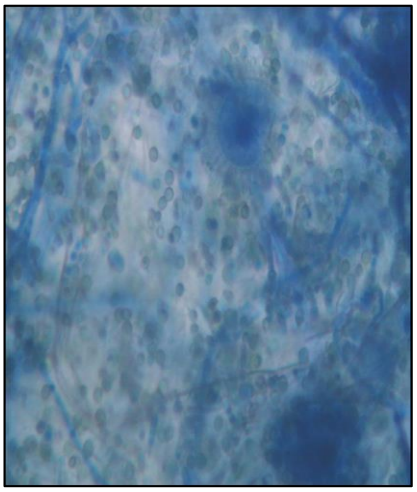

Figure 6 Extracted compound from *Aspergillus spp*

Figure 7 Fungal staining

Figure 8 *white rot fungi* in MGYP

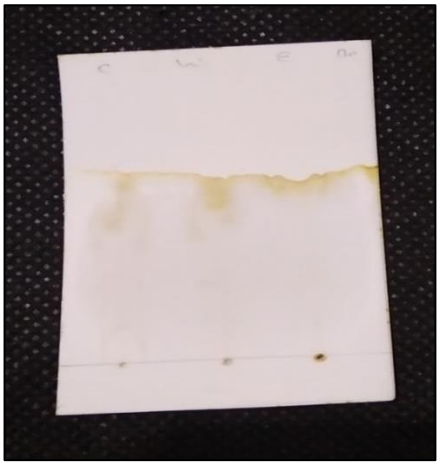

Figure 9 TLC plate of Aspergillus spp. From onion sample

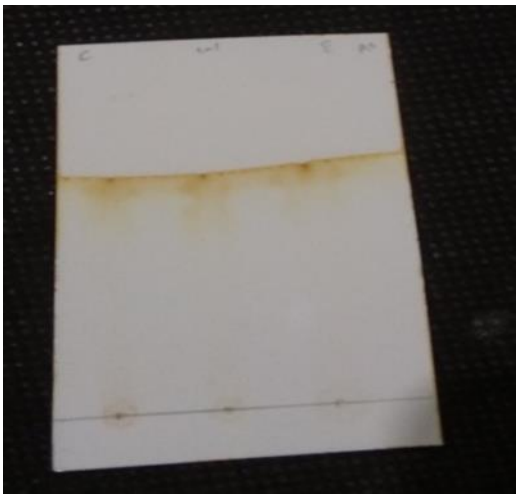

Figure 10 TLC plate of *Aspergillus spp.* From MTCC

Figure 11 Capsule prepared from *white rot fungi*

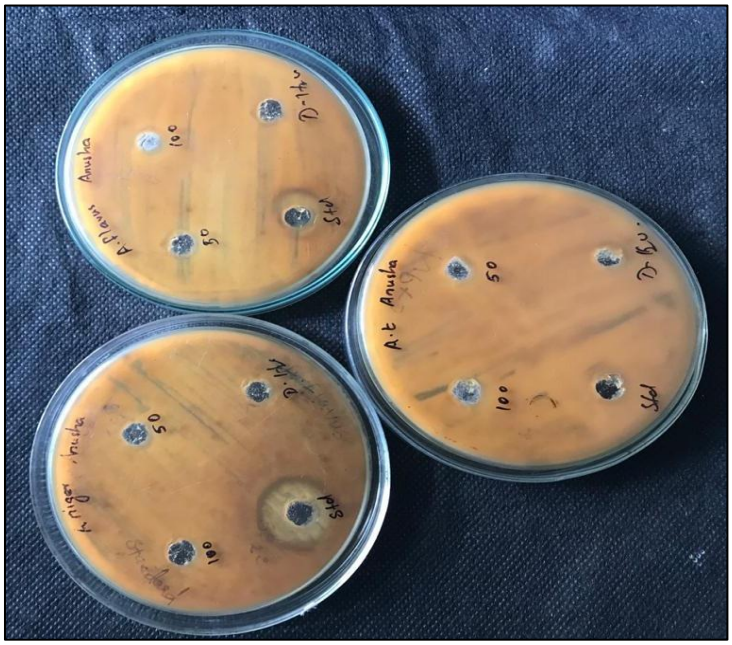

Figure 12 Antifungal activity

4. Discussion

The present research work were focused on degradation of aflatoxin using white rot fungi (Pleurotus florida). The toxin were produced using chemically defined medium and also using natural contaminated onion. By using methanol toxin were extracted and estimated. To find out the toxin UV-Visible study were carried out. TLC and FTIR were conducted to find out the degradation study of aflatoxin by using white rot fungi. In future, white rot fungi product will be used to detoxify the aflatoxin instead of chemical method.

J.F. Alberts et.al 2009 study shows that, Treatment of AFB1 with laccase enzyme produced by white rot fungi in unconcentrated culture filtrates, pure fungal laccase as well as with recombinant laccase enzymes, decreases the fluorescence properties of the AFB1 molecule as determined with HPLC. Furthermore, a considerable loss in mutagenicity were observed when treated with pure fungal laccase enzyme from T. versicolor as evaluated with the S. typhimurium mutagenicity assay. The results suggested that the treatment of AFB1 with fungal laccase enzymes targets and changes the double bond of the furofuran ring of the AFB1 molecule and as a result influences its fluorescence and

mutagenicity properties (Liu et al., 1998a,b,c). The current findings could contribute to the development of preventative strategies to reduce AFB1 contamination of food and beverages by involving enzymes and genetically engineered microbial strains with multifunctional technological properties. Treatments with enzyme preparations and application of genetically engineered microbial strains could be an important bio- control measure while significantly improving the quality, safety and acceptability of traditional fermented food and beverages.

Jianqiao Wang1 et.al 2010 showed that AFB1, which is a nonphenolic, difuranocoumarin derivate, can be oxidized by MnP from P. sordida YK-624. MnP removed approximately 70% of AFB1 after 24 h and were capable of removing AFB1 even in the absence of Tween 80. Although the complete elimination of AFB1 were not observed in the present study, it is thought that AFB1 is completely eliminated by the multitreatment with MnP. Mn(III), which is produced by MnP, could not oxidize AFB1 directly.

Co-cultivation of P. ostreatus strains on rice straw already containing A. flavus culture revealed the ability of P. ostreatus strains to rapidly colonize the substrate. Following incubation, the tubes inoculated with P. ostreatus MTCC 142 showed moderate hyphal proliferation, whereas, the tubes inoculated with P. ostreatus GHBBF10 demonstrated profuse mycelial growth. This basidiomycete strain might have synthesized higher titres of extracellular enzymes, which would have resulted in better utilization of the lignocellulosic substrate. Arijit Das et.al 2014

Co-cultivation of A. flavus and P. ostreatus were carried out on various substrates. On wheat straw, corn cobs and millet, A. flavus produced aflatoxin after 3 weeks of cultivation. A subsequent cultivation of P. ostreatus on A. flavus-contaminated straw led to detoxification of the straw and corn cobs. It were found that P. ostreatus could liquidate colonies of A. flavus. However, cultivation of P. ostreatus in the presence of 40–100 lg of AFB1/g of substrate did not result in complete detoxification of the material even after 34 days of co-cultivation, but AFB1 concentration decreased to about one-fourth of the added amount. Ginterova´ et.al 1980

5. Conclusion

The present research work were focused on degradation of aflatoxin using white rot fungi (Pleurotus florida). The toxin were produced using chemically defined medium and also using natural contaminated onion. The toxin were extracted using methanol and estimated. The UV-Visible study were carried out to find out the toxin. TLC also performed and Rf value were calculated (0.75, 0.875). The biodegradation of aflatoxin were performed using the fungi under optimized condition. The TLC and FTIR were conducted to find out the degradation study. The Rf value of control 0.75 followed by treated toxin 0.625, 0.9. The functional group in the control and treated are as follows. C=C alkene, CH3 bend, C-Cl, C-Br.

The compound were microencapsulated as product for ions storage. In future the white rot fungal product will be used to detoxify the aflatoxin instead of chemical method.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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Authors short Biography

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