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DRUG METABOLISM BY WHITE ROT FUNGI AND BROWN ROT FUNGI

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ABSTRACT

Biotransformation is the chemical modification/alteration of chemical compounds like amino acids, toxins, xenobiotic and drugs brought by an enzyme or an enzyme system of an organism to produce bioactive molecules were selected. Biotransformation of Diclofenac, Paracetamol and Aspirin (1% w/v) concentration of each by Basidiomycetes,*Gleophyllum sp* Brown rot fungi and *Agaricus sp* White rot fungi were studied. The said drugs are the most commonly used by human and have adverse effect on ecosystem when excreted. Post biotransformation metabolites of drugs were checked by TLC and HPLC. From the results of TLC and HPLC the metabolites of Paracetamol matched with Hydroquinone and Glutathione disulphide, metabolites of Diclofenac found to be 4-hydroxy diclofenac and unknown D2 and for Aspirin metabolites matches with Gentisic acid and unknown A2.Thus, Fungal biotransformation of the above mentioned drugs to less /nontoxic metabolite is an ecofriendly approach.

KEYWORDS: **Biotransformation, Basidiomycetes,Gleophyllum sp,Agaricus sp,Diclofenac, Paracetamol, Aspirin**

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INTRODUCTION

In municipal water, partially metabolised drugs are present which have increased the pollution level in water bodies whereby the compounds have been detected in ground water and even in potable water[1]Prodrugs are the inactive pharmaceutical compounds which are administered in human to increase their bioavailability, adsorption and to reduce their toxic effects in human beings. The mammalian system contains Cytochrome P450 monooxygenase enzyme system which is present in liver. This enzyme system contains Phase I(functionalization) and Phase II (conjugation) enzymes which are involved in metabolism of drugs. Metabolism of drugs brings about detoxification and sometimes drugs are partially metabolised to an intermediate compound which more toxic to the ecosystem[2]. Phase II enzymes transforms drugs to more water soluble and less toxic compound, which are easily eliminated from the body[3].

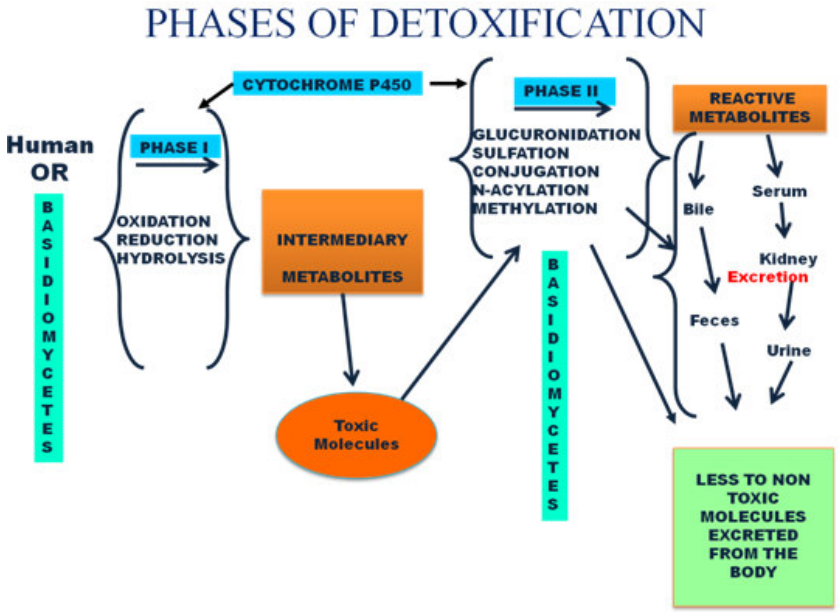


Figure1
Comparison Between Human And Basidiomycetes Detoxification Reactions

Fungi as Microbial Model

The fungal kingdom including yeast, mold, and Basidiomycetes inhabit a wide range of environments and play fundamental roles in nutrient cycling. Basidiomycetes are capable of converting recalcitrant environmental xenobiotic. Basidiomycetes can mimic the mammalian metabolism through their cytochrome P450 intra cellular enzyme system.

$$RH + NAD(P)H + H^+ + O_2 = ROH + NAD(P)^+ + H_2O$$

Fungi play promising role in drug biotransformation. They possess metabolic enzymes similar to human metabolic enzymes, therefore they are "biological chemists".[4]Fungi are more tolerant to high concentration of pollutants White rot fungi and Brown rot fungi are known to degrade wide range of xenobiotic compound and has the similar Cytochrome P450 monooxygenase enzymatic system as mammalian.[5]Higher fungi are advantageous over bacteria and other lower fungi as they possess the unique ability for complete detoxification of the toxic compounds and they mimic the human enzymatic system so it is easy to compare the metabolites which are formed after biotransformation with that of human metabolites. [6]In this study Biotransformation by *Gleophyllum* sp and *Agaricus* spof Brown rot and White rot fungi respectively[6,7]were checked for Diclofenac, Paracetamol and Aspirin because these are used extensively as over the counter non-prescription drugs. Toxic metabolite of these drugs has the adverse effect on human and when excreted out it contaminates the ground water which is harmful for the aquatic system.

MATERIALS AND METHODS

Isolation Growth Characterization

Twenty six different Basidiomycetes were collected from Sinhagad fort, one from Botanical garden (Pune) and one from Azam campus (Pune). Collected samples were maintained at room temperature.

Culture Media

For isolating fungi, samples from above sources were inoculated on Potato dextrose agar (PDB). Different culture media such as Glucose Malt Yeast Extract (GMY), Basidiomyctes Rich Medium (BRM)

a. Composition of GMY: - Glucose-10g; Malt extract-3.5g, Yeast extract-2.5g; MgSO4.7H2O-0.5g

b. Basidiomyctes Rich Medium (BRM):-L-aspargine-0.6g; KH_2PO_4-1.0g; MgSO4. $7H_2O$ -1.0g;KCL-0.5g;Yeastextract-0.5g;$FeSO_4.7H_2O$-.01g;$ZnNO_3.4H_2O$-2mg;$Ca(NO_3)_2.4H_2O$-50mg;$CaSO_4.5H_2O$-mg;1%glucoseWt./Vol);1%peptone (Wt./Vol)

c. PDB:- Potato, infusion broth-200g/litre, dextrose-20g/litre, final pH-5.1

Fungi were inoculated individually in all three medium mentioned above to find out suitable production media

for growth of fungal biomass. The inoculated media was incubated 28 ± 2ºC for 7-15 days.

Characterization
Spore print of all 28 Basidiomycetes.

Procedure for Spore Print
Remove the stem from the smaller mushroom and for larger mushroom, slice off a section of the cap, gills, or pores downwards on piece of paper. Place a cup or glass upside-down on top of your mushroom to keep air currents away. Some spore prints can appear within few hours or to wait overnight. Remove the cup and lift the mushroom cap and find print of mushroom on filter paper and spore print reflects the pattern of the mushroom's gills, since the spores fell directly downward on filter paper.

For Fungal Isolation and Growth Condition
Spores were collected from spore prints and spore suspension was prepared (10^7 spore/ml).This spore suspension was inoculated in 250 ml flasks containing 100 ml of PDB and kept on rotary shaker at R.T at 150 rpm for 5-7 days. Aliquot from this broth was spot inoculated on PDA plates and kept at RT for 4-5 days.

Morphological Characterization of Fungi
The fungal strains were taken for morphological identification by using spore print and Lacto-phenol Cotton Blue staining. The fungal spores were viewed under 40x and 100x magnification using light microscope and phase contrast microscope. *Agaricus* sp and *Gleophyllum*sp were selected for further studies[8].

Preparation of Drugs for Biotransformation
Three drugs were selected for biotransformation study i.e. Diclofenac, Paracetamol and Aspirin. Standardforms of these drugs were obtained from Sigma Aldrich. Stock solution was prepared for all the three drugs i.e. 0.5g of all 3 drugs was dissolved in 50 ml of methanol (1%).

Procedure for Biotransformation
The 1 cm^2 of PDA agar containing mycelia growth of *Gleophyllum* sp and *Agaricus* sp was inoculated in 100ml flask containing 50ml of PDB. 1 ml of each drug was added in 3 different flasks (final concentration of drug 0.5%) and incubated at room temperature for 2-7 days. For study of biotransformation of Aspirin, the brown rot fungi of *Gleophyllum* sp were used and same protocol was followed of Diclofenac and Paracetamol.

Detection of Metabolite
Thin Layer Chromatography (TLC) Analysis
The progress of biotransformation process was checked every 24 hours. Thin layer chromatography of Diclofenac, Paracetamol, and Aspirin metabolites were performed on silica gel. The metabolites of all three drugs were separated in different solvents system .The solvent system used as mobile phase for Paracetamol and Diclofenac was- n-exane: 1% CTAB in Methanol: Ethyl Acetate: Acetic Acid (13:1:4:2) and for Aspirin it was n-Hexane: Ethyl Acetate: Acetic Acid (6.5:3:0.5). The metabolites of all three drugs were spotted manually using capillary tube on precoated silica gel TLC plates(Merck).The spotted plates were put into the solvent system and the mobile phase allowed to run up to 3/4$^{th\ of}$ the plate and immediately the solvent front was recorded. The plates were air dried and the spots were visualised in iodine chamber as well as in UV. The spot were marked and Rf values were calculated by using the following formula:

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

High Performance Liquid Chromatography (HPLC) Analysis
Sample preparation
On respective days, samples were taken from all three drugs. They were filtered by using membrane filter assembly, (Millipore filter paper 0.45µ) to remove spores and fungal hyphae from the samples.

HPLC procedure
10µl sample is injected through injection port of C18 HPLC column having 25cm by 4.6cm inner diameter The run time was 10mins and mobile phase was methanol 1% (v/v) at flow rate of 1ml/min was used to separate metabolites for all three drugs. Retention time (Rt) of all three Standard drugs and that of metabolites were observed and compared with that of standard.

RESULTS

Identification of Basidiomycetes
Two Basidiomycetes were selected for studying the biotransformation of Diclofenac, Paracetamol and Aspirin.

a. White Rot Fungi **b.Brown Rot Fungi**

Figure 2
Spore print

These two species were microscopically observed by Lacto-phenol cotton blue staining (Figure.3-4)

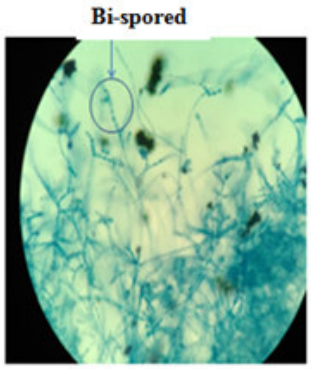


Figure 3
Microscopic View of WRF

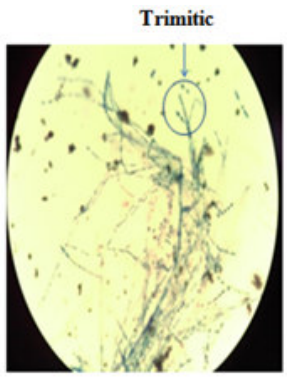
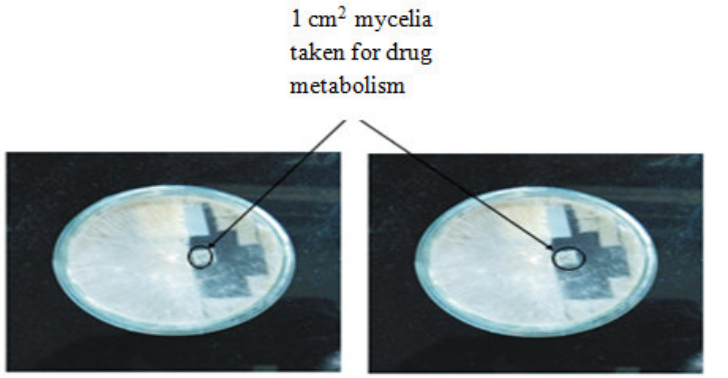


Figure 4
Microscopic View of BRF

WHITE ROT FUNGI
Spore Print- Brown.
Microscopic Features-
- Spores- 5.5-8.5 * 4-6.5µ, Elliptical, smooth.
- Basidia are bi-spored.
- Clamp connection is observed in hyphae.

BROWN ROT FUNGI
Spore Print- White.
Microscopic Features-
- Spores- 9-13 *3.5µ, smooth, cylindrical
- Basidia are elongated.
- Hyphal system is trimitic.

By the macroscopic study of spore print and microscopic study with Lacto-phenol cotton the two isolates were identified as*Agaricus* sp of WRF and *Gleophyllum* sp of BRF[8].Growth of *Agaricus* sp and *Gleophyllum* sp on Potato Dextrose Agar was seen.



a. Growth of *Agaricus* sp **b.Growth of *Gleophyllum* sp**

Figure 5
Growth of mycelia on PDA plate

Biotransformation
Growth of *Agaricus* sp was observed in all the tubes containing Diclofenac and Paracetamol except Aspirin.*Gleophyllum* sp was selected for biotransformation of Aspirin and the growth was observed.

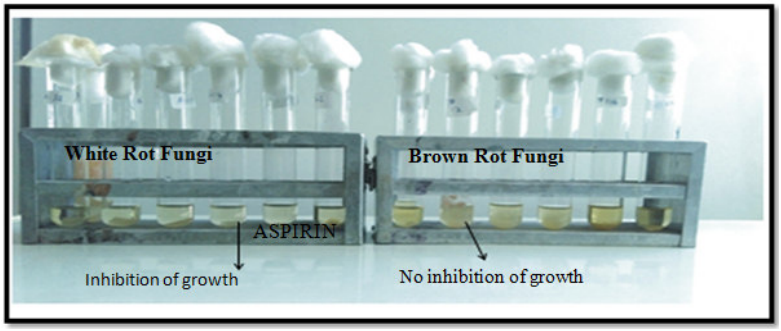


Figure 6
Biotransformation of Drugs

Detection of standard drug and its metabolites by TLC

Thin layer chromatography (TLC) was performed initially to detect the metabolites. It was found that the Diclofenac is biotransformed on 3rd day of incubation, Paracetamol on 6th day of incubation and Aspirin On 2nd and 3rd day. Rf values of Standard drugs and that of metabolites were calculated and compared with standard values (Table 1).

Table 1
Primary Detection of Metabolite by TLC

Sr.No.	Drug	Cal. Rf value	Std. Rf value	Metabolite
1.	Diclofenac	0.57	0.54	StandardDiclofenac
		0.3	0.29	4-hydroxydiclofenac
2.	Paracetamol	0.56	0.58	StandardParacetamol
		0.17	0.18	Hydroquinone
3.	Aspirin	0.81	0.89	Standard Aspirin
		0.53	0.60	Gentisic acid

Diclofenac (D) Aspirin (A) Paracetamo (P)

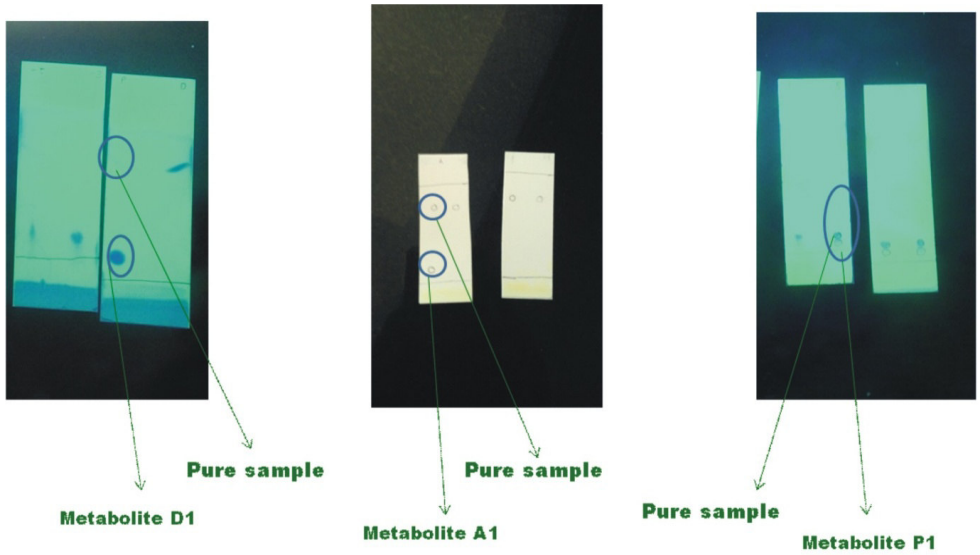


Figure 7
Primary Detection of Metabolite by TLC

Detection of standard drug and its metabolites by HPLC

To detect the metabolite and their purity High pressure liquid chromatography (HPLC) has been performed on same days of incubation as TLC. Reported retention times are compared with standard (Table 2).

Table 2
Detection of Metabolite by HPLC

Sr.No.	Drug	Cal. Rt value	Std. Rt value	Metabolite
1.	Diclofenac	3.62	3.9	StandardDiclofenac
		5.38	4.9	4 OH Diclofenac
		6.55	Unknown	Unknown
2.	Paracetamol	2.81	2.85	StandardParacetamol
		4.21	4.47	Hydroquinone
		5.50	5.0	Glutathione disulphide
3.	Aspirin	2.81	2.45	Standard Aspirin
		5.80	5.30	Gentisic Acid
		3.28	Unknown	Unknown

HPLC Reports

Aspirin-A1 metabolite of Aspirin is obtained after 48 hrs. (2 days) and A2 metabolite on incubation of 72hrs (3 days).

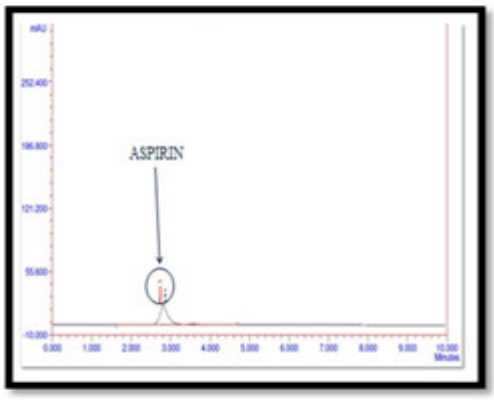


Figure 8
Retention Time of standard Aspirin=2.81

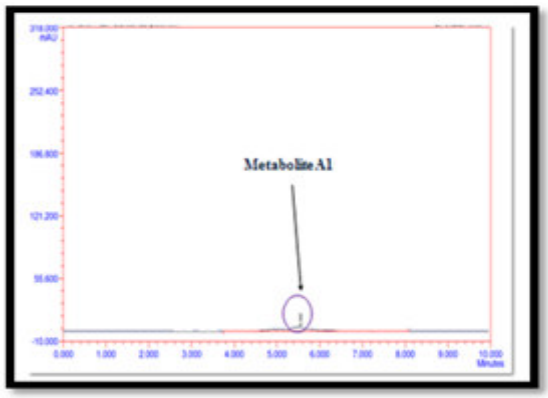


Figure 9
Retention Time of Aspirin MetaboliteA1=5.50

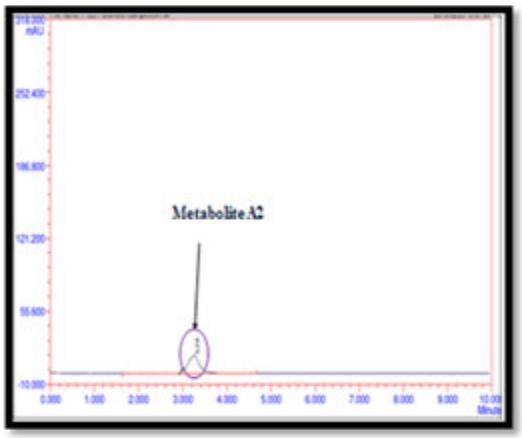


Figure10
Retention Time of Aspirin MetaboliteA2=3.28

HPLC Report Paracetamol-P1 and P2 both metabolites of Paracetamol are obtained on 6th day of incubation

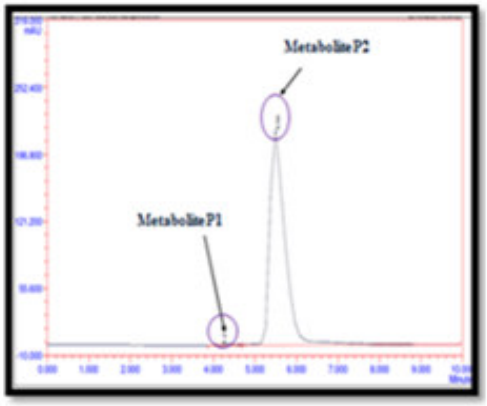


Figure 11
Retention Time of standard Paracetamol=2.81

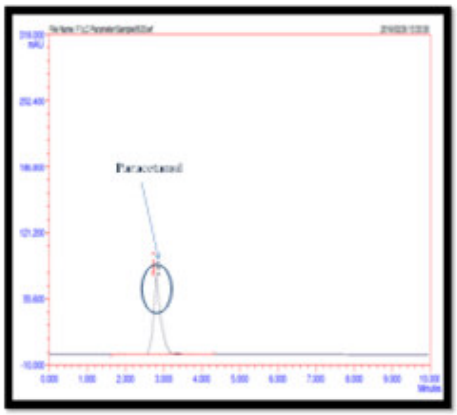


Figure 12
Retention Time of P1=4.21 and P2=5.50

HPLC Report Diclofenac-Metabolites of Diclofenac D1 and D2 are found on 3rd day of incubation.

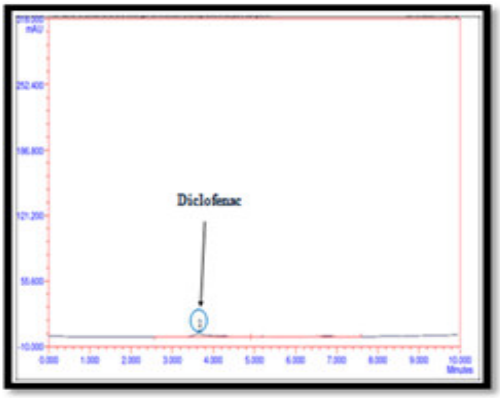


Figure 13
Retention time of Standard Diclofenac=3.62

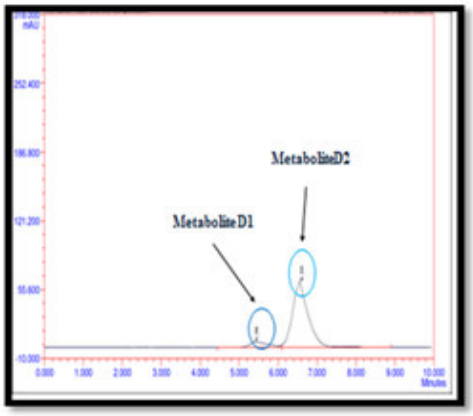
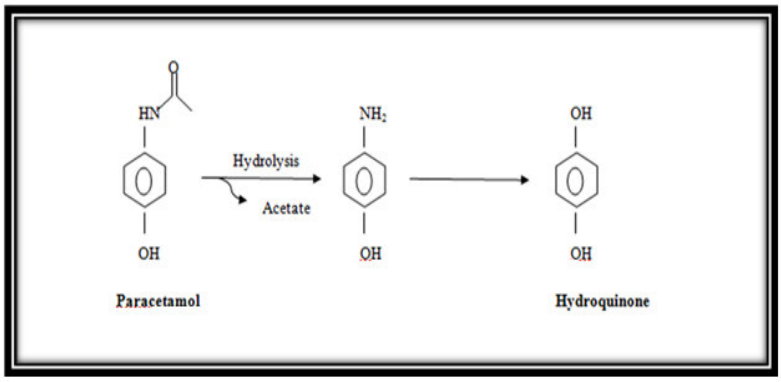


Figure 14
Retention Time of D1=5.38 and D2=6.55

DISCUSSION

In the previous study, *Cunninghamella echinulata* was shown to metabolize various drugs to their respective metabolites which are similar to mammalian metabolite.[9] *Cunninghamella echinulata* was able to metabolize Paracetamol to N-acetyl p-benzoquinone imine (NAPQI), a hepatotoxic metabolite of Paracetamol. The phase-I metabolite of Paracetamol in human beings is also NAPQI.[10,11,12] which is a toxic metabolite which has ability to bind to macromolecules of liver both in animals and human beings.[13] It was observed that *Cunninghamella* possess cytochrome P450 monooxygenase systems analogous to those in mammals and phase-II drug metabolism enzymes[14,15,16]*Penicillium species* has the potential to utilise Paracetamol as nitrogen source[17]. Primarily in animals Paracetamol is metabolized to non-toxic compounds via three metabolic routes :(1) Glucuronidation(2) Sulfation(3) Hydroxylation and rearrangement. The enzyme system cytochrome P450 metabolizes Paracetamol into a minor alkylating metabolite known asNAPQI.By conjugation NAPQI is then irreversibly join with the sulfhydryl groups of glutathione. About 30 and 55 % of administered Paracetamol is excreted in urine as conjugates Paracetamol-sulphate and Paracetamol-glucuronide, respectively[18]. A small amount of Paracetamol is probably transformed by a third metabolic pathway, that is, oxidation by the microsomal cytochrome P450 (CYP)-containing multi-functional oxidase system to NAPQI. Hydroquinone pathway is the major route in Paracetamol biodegradation[19].In our biotransformation study of Paracetamol By using *Agaricus*sp, Paracetamol is converted into Hydroquinone. The results of TLC and HPLC were compared with standard values and it was found to be matching with Hydroquinone which is consistent with previous study by *Penicillium.* In HPLC one more metabolite is detected which may be a Glutathionedisulphide.



Reaction 1
Paracetamol to Hydroquinone

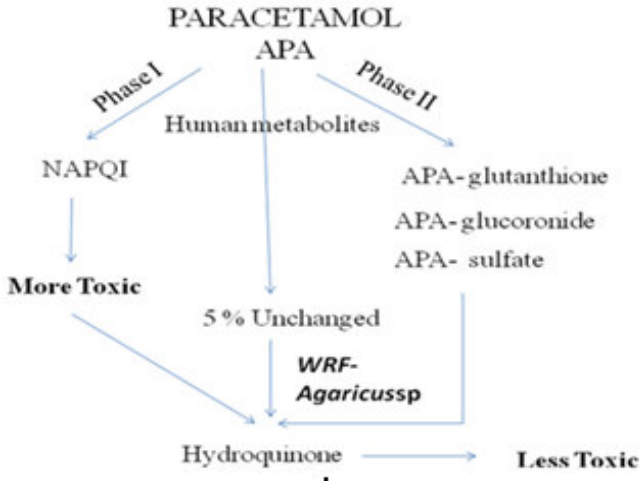
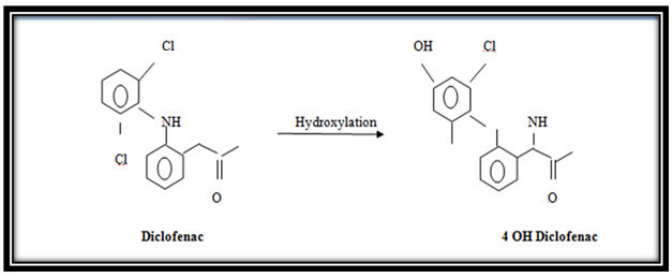


Figure 15
Degradation pathway of Paracetamol

Diclofenac is metabolized in the liver via two major pathways in both humans and animals. First, Diclofenac undergoes ring hydroxylation, major oxidative metabolite 4-OH Diclofenac is formed which is catalysed by cytochromeP450 (CYP) isoenzyme CYP2C9).[20]Other, quantitatively minor metabolite of oxidation reaction include 5- OH-Diclofenac, catalysed by CYP3A4[21,22]and other mono- or di-hydroxylated metabolites such as 4,5-dihydroxy Diclofenac.[23,24] 4'-OH-Diclofenac was shown to be the predominant metabolite, and5-OH-Diclofenac and 4', 5-diOH-Diclofenac occurred as minor metabolites by treatment of Diclofenac with the white rot fungus *P. sordida*YK-624. This suggested that *P. sordid* YK-624 possesses functions similar to those of mammalian CYP2C9 and CYP3A4[1]In our study the metabolites of Diclofenac detected by TLC and HPLC could be 4-hydroxy Diclofenac which is the major metabolite and it does not have any toxic effect on environment. In HPLC one more Peak is observed which is not matching with any of the metabolites of Diclofenac.



Reaction 2
Diclofenac to 4-OH Diclofenac

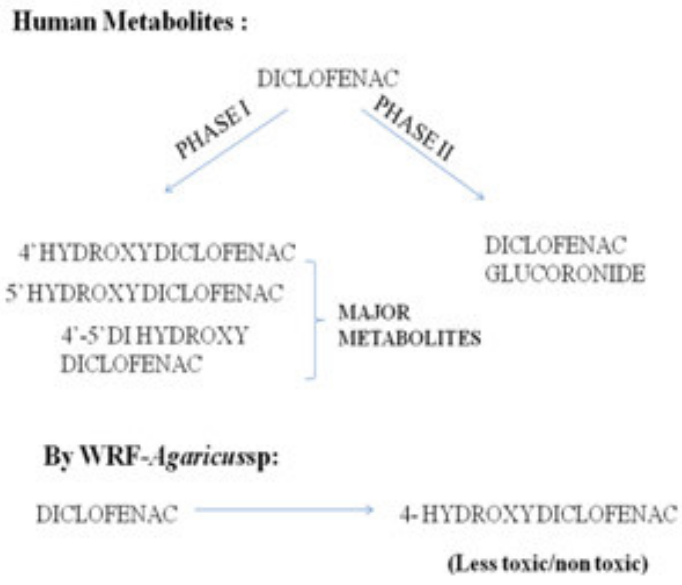
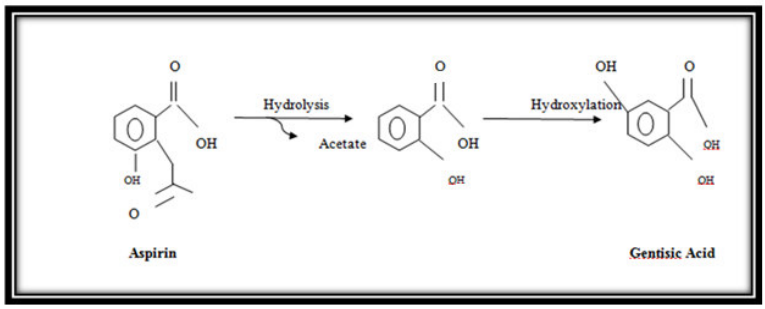


Figure 16
Degradation pathway of Diclofenac

Till date there is no report for Aspirin biotransformation by lower or higher fungi. The metabolites of Aspirin are compared with human metabolites.In our investigation we carried out Aspirin Biotransformation by *Gleophyllum* sp because it is observed that Aspirin inhibit the growth of *Agaricus.* By comparing calculated and observed Rf values and Rt it is found that Gentisic acid may be one of the metabolite, In HPLC results of Aspirin one more metabolite is observed whose Rt is not matching with any other metabolites of Aspirin.



Reaction3
Aspirin to Gentisic acid

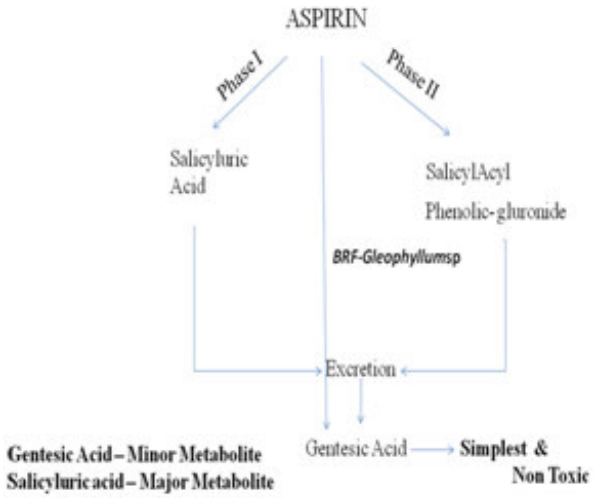


Figure 17
Degradation pathway of Aspirin

From the present study it can be concluded that by using White rot and Brown rot Fungi we can detoxify the toxic metabolites which are harmful to the ecosystem as well as to aquatic system. In further study we can separate the metabolites, purify them and its molecular mass and molecular structure can be found out by LC-MS and NMR. In future it would be of interest to study the role the P450 in biotransformation and the new metabolites revealing the possibility of producing compounds with novel structure and biological activity.

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