BIODEGRADATION AND ELUCIDATION OF BYE-METABOLITES OF REACTIVE YELLOW DYE BY *PLEUROTUS PULMONARIUS KP826832*

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Abstract

The current work is aimed to evaluate the degradation of Reactive Yellow Dye by Pleurotus pulmonarius (a whit rot fungus). The induction of the lignolytic enzyme activities of the organism during the decolorization of reactive yellow suggests their probable role in the biodegradation process. Metabolites obtained after decolorization were analyzed with GC-MS and FTIR studies which confirmed the biodegradation of the dye. Metabolites produced after biodegradation of reactive yellow were identified by GC-MS analysis. Predicted pathway of biodegradation of reactive yellow by Pleurotus pulmonarius was elucidated for first time. The results showed efficient biodegradation of reactive yellow to low molecular weight compounds, namely, 2-methyl 3-hydroxypyrone; 1-hydroxy 2,4dichloro benzene; 4-hepten-3-one, 3-chloro-4-methyl-2-pentanol; 4,hydroxyphenethyl alcohol; 1,6-anhydro-beta d-glucopyranose and fatty acids. Pleurotus pulmonarius was found to be versatile and could be used for textile effluent biodegradation and consequently environmental bioremediation.

Keywords: Biodegradation, Metabolites, *Pleurotus pulmonarius*, Reactive yellow, FTIR, GCMS

Introduction

Rapid industrialization has necessitated the manufacture and use of different chemicals in day to day life (Sathiya *et al.*, 2007; Shah *et al.*, 2013). The textile industry is one of them, which extensively uses synthetic chemicals as dyes. Wastewaters from textile industries pose a threat to the environment, as large amount of chemically different dyes are used. A significant proportion

of these dyes enter the environment via wastewater. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide (Rafi *et al.,* 1990; Mathur and Kumar, 2013; Pankaj *et al*., 2017). Among all textile dyestuff used, the azo dyes constitute about 70 % and are being used worldwide (Saratale *et al.,* 2011). The discharge of azo dyes containing wastewaters into the environment may lead to bioaccumulation which causes toxic effect on aquatic life and even carcinogenic and mutagenic effect on humans because of the conversion of azo group into aromatic amines (Modi *et al.,* 2010; Zhang *et al.,* 2010).

Aside from the human toxicity, colour of dyes interrupts the aquatic environment by reducing light penetration, gas solubility, and interference of phytoplankton's photosynthesis (Sharma, 2009; Lade *et al*., 2015; Bose and Anitha, 2016). Therefore, treatment of textile wastewater becomes essential before discharging into the water streams. Additionally, limited supply and increasing cost of water for industrial sector have made the treatment and reuse of dyeing effluent mandatory to avoid the environmental pollution as well as reduce the production cost (Lade *et al*., 2015).

Textile dyes are recalcitrant to degrade by the conventional wastewater treatment systems (Naik *et al.,* 2008). Several physicochemical methodologies such as coagulation and flocculation which are most commonly used worldwide for treatment of textile effluent (Meric *et al.,* 2005 and Verma *et al.,* 2012) have many disadvantages in application, including high energy costs, high-sludge production and formation of the secondary toxic by-products (Sarioglu *et al.,* 2007) as well as excessive use of chemicals, low efficacies, and high operational cost all these discourage the employment of these methods (Davies *et al.,* 2005; Lade *et al*., 2015).

However, the use of biological methods is a promising alternative to the harsh physicochemical methods usually employed for many of the industrial purposes, including pollution abatement. Current study is aimed at selecting white rot fungus with the ability to produce large quantities of laccases for the degradation and characterization of by-products of a popular textile dye- Reactive Yellow Dye.

Materials and Methods

Procurement of test Microorganism and Dye: The white rot fungal strain of *Pleurotus pulmonarius KP826832* was obtained from culture from Federal Institute of Industrial Research Oshodi (FIIRO) Collection Center in Lagos State, Nigeria. The culture mycelium was stored on malt extract agar slant at 4 ^{0}C. The reactive yellow dye used for this study was obtained from a retailer shop.

Decolorization and Detoxification Experiment: For decolorization experiments, inoculation was carried out directly in Erlenmeyer flasks. Six plugs (diameter 3 mm) from 5-day-old fungal cultures of *Pleurotus pulmonarius KP826832* on three agar plugs (5 mm^{2}) of active mycelium from PDA plates were transferred aseptically into 250 ml Erlenmeyer flasks containing 100 ml of PDB (Potato Dextrose Broth) medium with 100 mg/ml of dyes reactive black dye. After fungal growth, the cultures were supplemented with 150 mMCuSO4 sterile solution as laccase-inducer. The Erlenmeyer flasks were incubated statically for 12 days at 28 °C (Shanmuga Priya *et al.*, 2013). Decolorizing activity was observed for the period of days and the preparation was done in duplicate. Aliquots of the fungal culture incubation were collected at an interval of 3 days; centrifugation of the aliquots was carried out using the at 4,000 rpm for 15 minutes, and then the supernatants were used to determine dye decolorization by monitoring the decrease in absorbance at the wavelength of maximum absorption for each dye using a spectrophotometer (Visible Spectrophotometer L1-722) (Da Silva *et al.*, 2009).

Analysis of Reactive Yellow Degradation by GC-MS and FTIR: Biodegradation was determined by comparing the Fourier transformed infrared spectroscopy (FTIR) peak profiles of the metabolite of Reactive Yellow and those of its abiotic control. Attempt was also made to identify the dye metabolites using their Gas chromatography-mass spectroscopy (GC-MS) spectra. The decolorized Reactive yellow solution, withdrawn after 24 h and centrifuged at 8,944 ×g for 10 min, was extracted using ethyl acetate. The extract was dried in a rotary evaporator and re-dissolved in high-performance liquid chromatography grade methanol for GC-MS analyses. FTIR analysis of biodegraded Reactive yellow was carried out using a Shimadzu 800 spectrophotometer and compared with that of the control dye. The FTIR analysis was done in the IR region of 400-4,000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr for pellets formation and the pellets were used for the analysis. The identification of metabolites

formed after degradation was done using a QP2010 GC-MS system (Shimadzu, Japan). Gas chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial column temperature was 80°C for 2 min, after which it increased linearly at 10°C/min to 280°C, which was held for 7 min. The ionization voltage was 70 eV. The temperature of the injection port was 280 °C and the GC-MS interface was maintained at 290°C. The helium carrier gas flow rate was 1.0 ml/min. Degradation products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta; Shimadzu) of the GC-MS.

Biodegradation Assay via FTIR spectrum: The FTIR spectrum of non-degraded dye (control) shows ten peaks (Figure 1) while Table 1 shows functional groups represented on each peak thus: 624.50cm/1 (C-Br stretch), 698.41 cm^{-1} (C ≡ C- H bend), 750.10 cm^{-1} (C-Cl stretch), 834.49cm^{-1} (C-Cl stretch), 1043.54 cm^{-1} (C-N stretch), 1196.93 cm^{-1} (C-N stretch), 1504.46 cm/1 (N-O stretch),1652.83 cm^{-1} (C=C stretch), 2432. 64 cm^{-1} (COOH stretch) and 3496.72 (O-H bend). After degradation of Reactive yellow, significant peaks are (12) in FT-IR spectrum was observed in. The peaks are 632.67 cm^{-1} (C ≡ C- H bend), 1026.16 cm^{-1} (C-N stretch), 1111.03 cm^{-1} (O-H stretch), 1211.34 cm^{-1}(CH_2X),1411.94 cm^{-1} (C-C stretch), 1481.38 (N-O stretch), 1666.55 (C=C stretch), 2044. 61 (N=C=S stretch), 2831.60 (N-H stretch), 2885. 60 (C-H stretch), 2985.91 (C-H stretch) and 3348. 54 (N-H stretch).

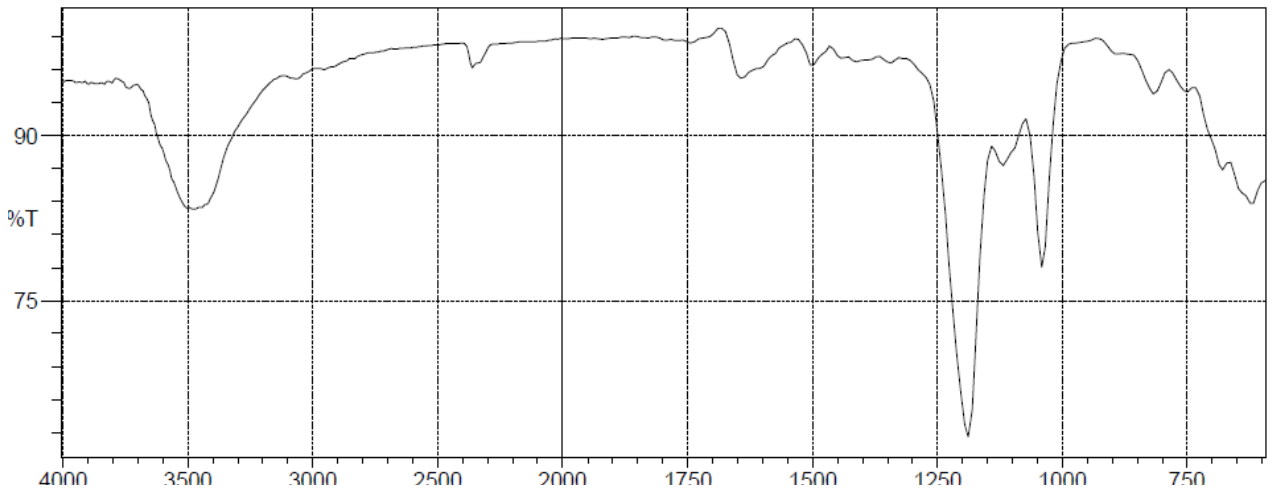


Figure 1: FTIR finger print of non-degraded the dye (control)

Table 1 shows the FT-IR profiling of non-degraded Reactive Yellow with the ten wave numbers and eight functional groups comprising of 3 alkyl halides, 2 aliphatic amines, and 1 each for alkyne, nitro, alkene, carboxylic acid and alcohol compounds with alkyne and alcohol having bend vibration while others showed the stretch type of vibration.

Table 1: FT-IR profiling of non-degraded (control) Reactive Yellow

Peak no	Wave no	Type of vibration	Functional group	Chemical formula
1	624. 50	Stretch	Alkyl halide	C-Br
2	698.41	Bend	Alkyne	C ≡ C- H
3	750.10	Stretch	Alkyl halide	C-Cl
4	834.49	Stretch	Alkyl halide	C-Cl
5	1043. 54	Stretch	Aliphatic amine	C-N
6	1196.93	Stretch	Aliphatic amine	C-N
7	1504.93	Stretch	Nitro compound	N-O
8	1652.83	Stretch	Alkene	C=C
9	2432. 64	Stretch	Carboxylic acid	COOH
10	3494.72	Bend	Alcohol	O-H

On the other hand, after degradation of reactive yellow dye by *P. Pulmonarius*, component compounds formed are in twelve different functional groups as shown in Figure 2 and table 2. New compounds like alkanes, and thiocyanate appeared while carboxylic acid compounds disappeared and aliphatic primary amines replaced the aliphatic amines in the fragmented dye.

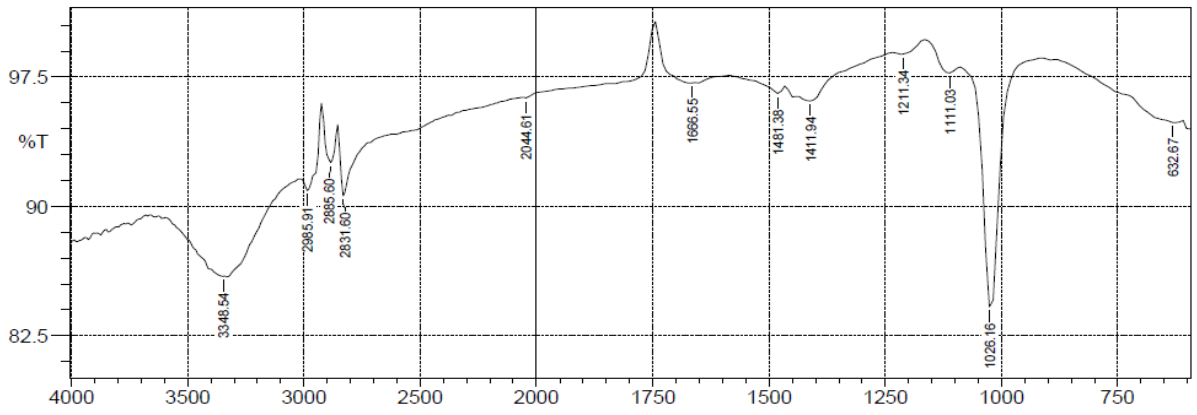


Figure 2: FTIR spectrum of reactive yellow dye degraded by *Pleurotus pulmonarius*

The only alkyne compound occurred with bend type of vibration while others were stretch type (Table 2). The table also shows 3 alkanes, 3 aliphatic amines (2 being primary amines)and 1 each for alkyne, alcohol, alkyl halide, nitro, alkene and thiocyanate compounds.

Table 2: FT-IR profiling of Reactive Yellow Dye degraded by *Pleurotus pulmonarius*

Peak no	Wave no	Type of vibration	Functional group	Chemical formula
1	632. 67	bend	Alkyne	C ≡ C - H
2	1026.16	Stretch	Aliphatic amine	C - N
3	1111.03	Stretch	Alcohol	O - H
4	1211.34	Stretch	Alkyl halide	CH_2X
5	1411.94	Stretch	Alkane	C - C
6	1481.38	Stretch	Nitro compound	N - O
7	1666. 55	Stretch	Alkene	C = C
8	2044. 61	Stretch	Thiocyanate	N=C=S
9	2831. 60	Stretch	Aliphatic primary amine	N - H
10	2885. 60	Stretch	Alkane	C - H
11	2985.91	Stretch	Alkane	C - H
12	3348. 54	Stretch	Aliphatic primary amine	N - H

The fingerprints from GC-MS for raw and fragmented reactive yellow dye are shown in Figures 3 and 4 respectively. The chromatographs show 20 peaks for non-degraded dye while there were only fourteen for the degraded dye.

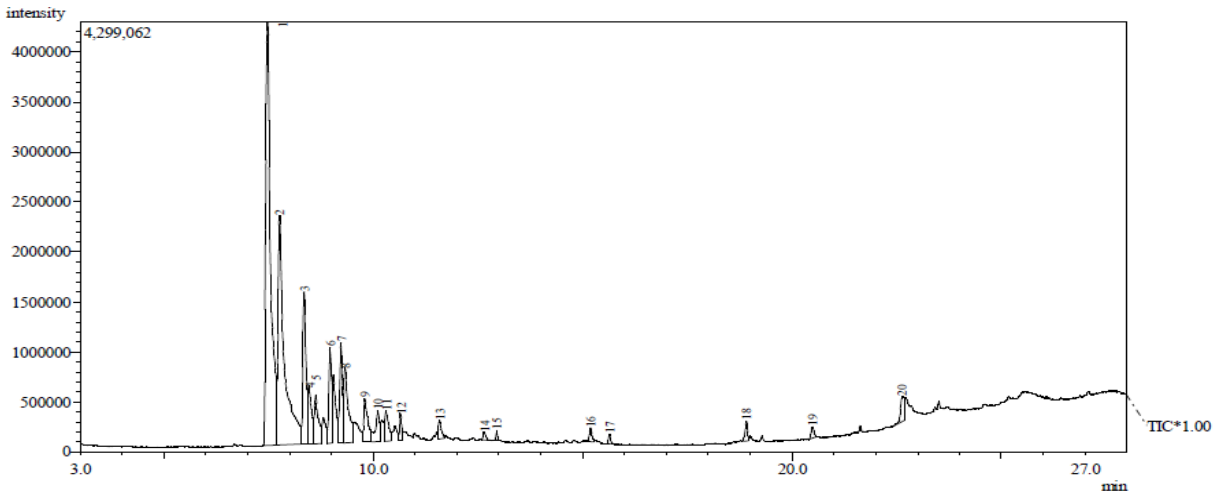


Figure 3: GC MS fingerprint of non-degraded reactive yellow dye (control)

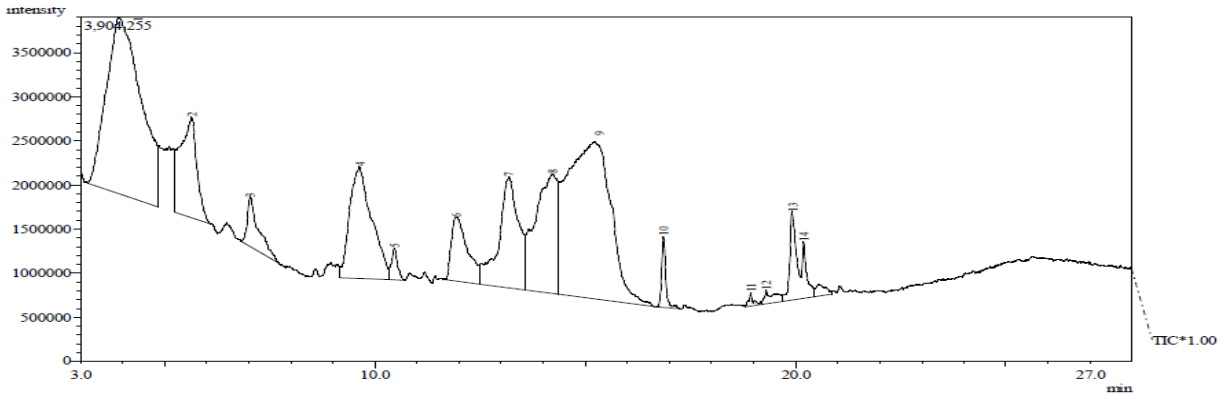
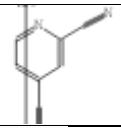
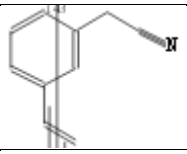
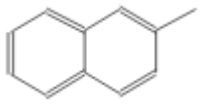
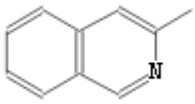
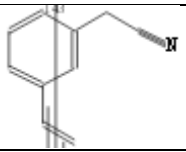


Figure 4: GC MS fingerprint of degraded reactive yellow dye

The profiling results identified the actual compounds with their predicted structures, molecular weights and retention time for each of them (Tables 3 and 4). The molecular weight varied from 129 to 296 for the non-degraded while it varied from 126 to 340 in the degraded dye. However, about 60 % of component compounds in the degraded dye were of low molecular weights and also, the fragments after degradation survived for a shorter period than components of the raw dye.

Table 3: GC MS Profiling of non degraded reactive yellow dye

Peak no	Name of compound	Retention Time	Molecular weight	Chemical formular	Structure
1	2,4 pyridine dicarbonitrile	7.479	129	$C_7H_3N_3$	
2	Phenylpropiolaldehyde	7.763	130	C_6H_6O	
3	3-vinylphenyl acetonitrile	8.347	143	$C_{10}H_9N$	
4	2-methylnaphthalene	8.461	142	$C_{11}H_{10}$	
5	3-methylisoquinoline	8.624	143	$C_{10}H_9N$	
6	3-vinylphenyl acetonitrile	8.966	143	$C_{10}H_9N$	

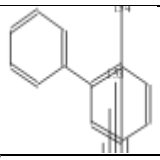
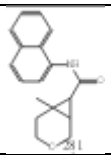
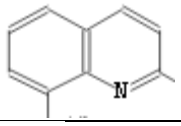
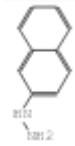
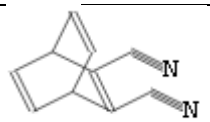
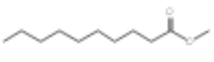
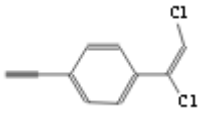
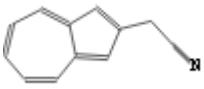
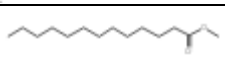
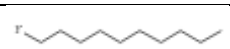
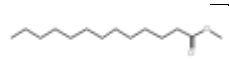
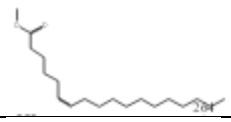
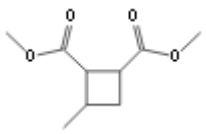
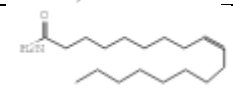
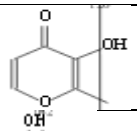
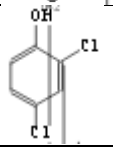
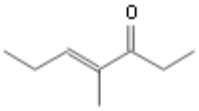
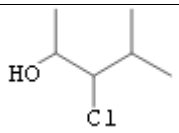
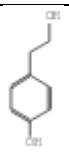
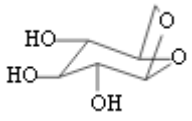
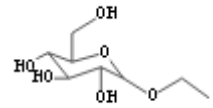
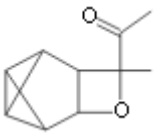
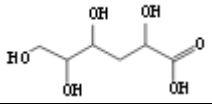
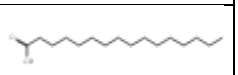
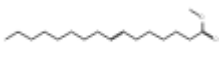
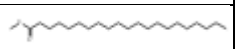
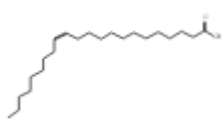
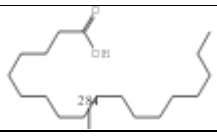
7	1,1 diphenyl	9.226	154	$C_{12}H_{10}$	
8	6-methyl-N-(1-naphthyl)	9.327	281	$C_{18}H_{19}NO_2$	
9	2,8 dimethylquinolonine	9.802	157	$C_{11}H_{11}N$	
10	1-(2-napthyl)hydrazine	10.107	158	$C_{10}H_{10}N_2$	
11	7-triene2,3 dicarbonitrile	10.303	154	$C_{10}H_6N_2$	
12	Methyldecanoate	10.653	186	$C_{11}H_{22}O_2$	
13	1(1,2-dichloroethenyl)4-ethynyl	11.578	196	$C_{10}H_6Cl_2$	
14	2-azulenyl acetonitrile	12.649	167	$C_{12}H_9N$	
15	Methyltridecanoate	12.945	228	$C_{14}H_{28}O_2$	
16	n-decyl fluoride	15.190	160	$C_{10}H_{21}F$	
17	Tridecanoic methyl ester	15.643	228	$C_{14}H_{28}O_2$	
18	Methyl cis 6-octadecanoate	18.910	296	$C_{19}H_{36}O_2$	
19	1,2 cyclobutanedicarboxylic acid	20.488	186	$C_9H_{14}O_4$	
20	Oleic acid amide	22.620	281	$C_{18}H_{25}NO$	

Table 4: GC MS Profiling of reactive yellow dye degraded by *Pleurotus pulmonarius*

Peak no	Retention time	Name of Compound	Molecular weight	Chemical Formula	Structure of Compound
1	3.922	2-methyl 3-hydroxypyrone	126	$C_6H_6O_3$	
2	5.642	1-hydroxy 2,4dichloro benzene	162	$C_6H_4Cl_2O$	
3	7.033	4-hepten-3-one	126	$C_8H_{14}O$	
4	9.636	3-chloro-4-methyl-2-pentanol	136	$C_6H_{13}ClO$	
5	10.468	4,hydroxyphenethyl alcohol	138	$C_8H_{10}O_2$	
6	11.935	1,6-anhydro-beta d-glucopyranose	162	$C_6H_{10}O_5$	
7	13.196	Ethyl alpha-d-glucopyronoside	208	$C_8H_{16}O_6$	
8	14.226	7-Oxatetracyclo[4.2.0.0(2,4).0(3,5)]octane	164	$C_{10}H_{12}O_2$	
9	15.226	3-deoxy-d-mannonic acid	180	$C_6H_{12}O_6$	
10	16.884	Pentadecane carboxylic acid	256	$C_{16}H_{32}O_2$	
11	18.948	7-hexadecanoic acid	268	$C_{17}H_{32}O_2$	
12	19.550	Methyl-henicosanoate	340	$C_{22}H_{44}O_2$	
13	19.953	Cis-13-docosenoic acid	338	$C_{22}H_{42}O_2$	

14	20.600	Octadecanoic acid	284	$C_{18}H_{36}O_2$	

Discussion of Results

The chemical structural differences in textile dyes due to the substitution of various functional groups on aromatic base greatly influence their decolorization rates (Harshad *et al*., 2015). This clearly indicates that decolorization was due to degradation of dyes into intermediate products.

The FT-IR spectra of control dye display peaks at 624.50, 698.41, 750.10, 834.49, 1043.54, 1196.93, 1504.46, 1652.83, 2432.64, and 3494.72. The FT-IR spectra of degradation product display peak for N–H stretching. The peak characteristic of azo bond at 1411.94 of dye was absent in the decolorized sample, indicating degradation of dye to aromatic amines as intermediate products which are subjected to oxidation giving rise to simpler compounds. The FTIR spectra of dye and dye degradation products differed with number of peaks and their positions. The appearance of peak at 1481.38 cm^{-1} confirmed the presence of aromatic nitro compound and azo group in dye whereas peaks at 2885.60 and 2985.91 cm^{-1} were alkane (aliphatic) compounds. The N–H stretching is indicated by peaks at 2831.60 and 3348.54 cm-1. The peaks at 2044.61 cm^{-1} is due to the presence of the sulphonated dye compound. The peak at 1111.03 cm^{-1} indicates primary alcoholic group and peak at 632. 67 cm^{-1} indicates C–H deformation of benzene ring. The sharp peak at 1026.16 cm-1 in azo compounds absent in the FTIR spectral analysis of degraded products confirms that the amine is now aliphatic. The peak at 1211.34 due to C–Br stretching indicates halogenation of the product. A significant change in FTIR spectrum in degraded dye sample which displayed peaks at 2432. 64 cm^{-1} for –COOH stretching, which has been degraded totally. Thus, the FTIR analysis confirms biotransformation of dye into other compounds. The strong absorption at 1632$cm^{-1}$and 1402 cm^{-1} is because of presence of Azo bond (-N=N-). Absence of these peaks after decolorization indicates cleavage of azo bond. In IR spectra of treated sample new peaks at 2985.92cm-1,1411.94 cm^{-1} and 1481.38 cm^{-1} were observed which indicates that there may be formation of new compounds originating from the fragmentation of parent dye molecule.

The control IR spectrum of RY shows the following observation. The peaks in between 3700-3100 cm^{-1} shows the presence of –OH,-NH-, =C-H (amides and amines). The peaks in between 2700-2000cm^{-1} responsible for nitriles, azide compounds in the sample and 800-400cm^{-1} peaks are

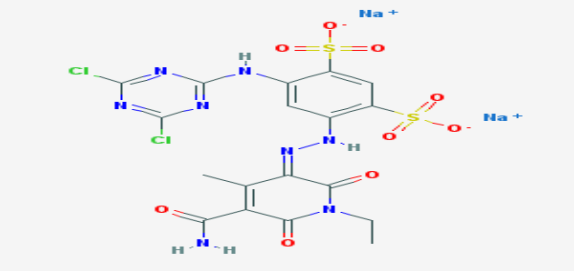
associated with aromatic compounds in the sample. The IR spectra obtained from treated sample shows the several variations in the region at 3700 - 3100 cm^{-1}, 2700-2000cm^{-1} and 800-400 cm^{-1} as it is compared with control IR spectra of reactive yellow. It may be the because of drastic destruction of dye by *Pleurotus pulmonarius*. The presence of aromatic amine in the degraded sample indicates the effect of laccase activity. It has been reported that the azo compounds with hydroxyl or amino groups were more likely to be degraded than those with methyl, methoxy, sulfo or nitro groups. Usually, the presence of sulphonates in reactive dye structures results in low levels of colour removal. However, this is not applicable to direct dyes that usually exhibit high levels of colour removal independent of the number of sulphonate groups in the dye structure, reinforcing the idea that steric hindrance and the number of azo bonds are responsible for the different degradation times (Whiteley, 2007).

In this study, the metabolites of reactive yellow dye fractions were identified by comparing the mass spectra with data in the NIST98 library and independently by interpreting the fragmentation patterns. The GC-MS Fingerprint of degraded and non-degraded dye showed the following components such as 2,4 pyridine dicarbonitrile with the retention time of 7.48min and a mass peak of 129; Phenylpropiolaldehyde with the retention time of 7.77min and a mass peak of 130; 2-methylnaphthalene with the retention time 8.46 min and a mass peak of 142. The metabolites with retention times of 8.35, 8. 62 and 8.97min, identified as 3-vinylphenyl acetonitrile with same mass peak of 143 ;1,1 Diphenyl with the retention time 9.23min and mass peak of 154; 6 methyl N (1 naphthyl) with retention time of 9.33min and mass peak of 281; 2,8 dimetylquinoline with retention time of 9.80min and mass peak of 157; 1-(2-napthyl)hydrazine with retention time of 10.10min and mass peak of 158; 7-triene2,3 dicarbonitrile with retention time of10.30min and mass peak of 154; Methyldecanoate with retention time of 10. 66 and mass peak of 186; 1(1,2-dichloroethenyl)4-ethynyl with retention time of 11.58 min and mass peak of 196; 2-azulenyl acetonitrile with retention time of 12. 65min and mass peak of 167; Methyltridecanoate with retention time 12.95min and mass peak of 228; n-decyl fluoride with retention time of 15.19min and mass peak of 160; Tridecanoic methyl ester with retention time of 15. 64min and mass peak of 228; Methyl cis 6-octadecanoate with retention time of 18.91min and mass peak of 296; 1,2 cyclobutanedicarboxylic acid with retention time of 20.49min and mass peak of 186,Oleic acid amide with retention time of 22. 62mins and mass peak of 281 The metabolite with retention time of 8.35, 8. 62 and 8.97mins, identified as 3-vinylphenyl acetonitrile.

Reactive yellow dye is degraded into various intermediates such as 2-methyl 3-hydroxypyrone and 4-hepten-3-one with mass of 126; 1-hydroxy 2,4dichloro benzene with mass of 162; 3-chloro-4-methyl-2-pentanol with mass of 136; 4,hydroxyphenethyl alcohol with mass of 138; 1,6-anhydro-beta d-glucopyranose with mass of 162; Ethyl alpha-d-glucopyronoside with mass of 208; 7-Oxatetracyclo[4.2.0.0(2,4).0(3,5)]octane with mass of 164; 3-deoxy-d-mannonic acid with mass of 180; Pentadecane carboxylic acid with mass of 256; 7-hexadecanoic acid with mass of 268; Methyl-henicosanoate with mass of 340; Cis-13-docosenoic acid with of 338 and Octadecanoic acid with mass of 284.

It is evident from the mass analyses report that reactive yellow dye, in the presence of laccase, undergoes degradation phenomenon and result in various intermediate metabolites which is in corroboration with the findings of Pandey et al. (2007).

The mechanism of degradation that could be proposed for the degradation reactive yellow dye is shown in Figure 5. Some azo dyes are more resistant to removal by microorganisms and this may be attributed to their structural differences. These isolates probably have acquired natural adaptation to survive in the presence of the dyes (Khadijah *et al*. 2009). The complete degradation of dye could be achieved during study which was confirmed by FT-IR analysis. This suggests that microbial isolates may have an efficient enzymatic system for the cleavage of parent dye. The hypothesis that could explain mechanism of degradation is *ara operon* through which certain metabolites produced from co-substrate that can induce synthesis of various enzymes involved in biodegradation of dye. It could successfully be employed in the treatment of textile effluent. However, further work is needed to identify the genes responsible for this kind of textile azo dyes decolorization. Gomare and Govindwar (2009), Yuan *et al.* (2016) reported that the metabolites of dyes can induce or inhibit biotransformation, so the metabolites generated during the degradation process of dye might cause different side effects on enzymes.



REACTIVE YELLOW DYE

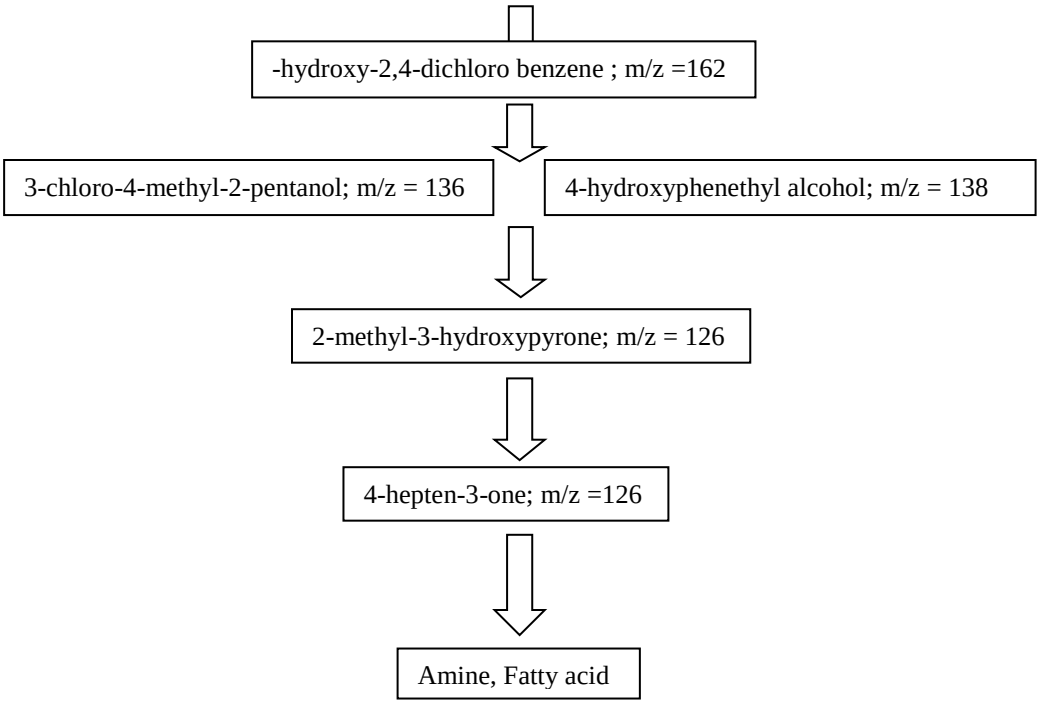


Figure 5: Proposed mechanism of degradation of reactive yellow dye

Conclusion

In conclusion, a strain of *Pleurotus pulminorius KP826832* possesses degradative and enzymatic capability to degrade reactive yellow dye. A pathway for the biodegradation of reactive yellow dye was proposed based on metabolites identified using GC-MS and FTIR. The research also suggested that *Pleurotus pulmonarius* might be more important than its consumption as food and medicinal values.

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