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Full Length Research Paper

Biotransformation and detoxification of reactive black dye by *Ganoderma tsugae*

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In this study, the decolourization potential of the white-rot fungus *Ganoderma tsugae*, which is capable of producing laccase was investigated to degrade reactive black dye. Biodegradation of reactive black dye was analyzed by using spectrophotometer at an absorbance of 585 nm. Laccase, manganese peroxidase and pH were served as biodegradation indices. Fourier transform infrared (FTIR) and gas chromatography mass spectrometry (GCMS) were used to analyze degradation products. Seed germination study was carried out on maize and beans seeds with distilled water (control), degraded dye products and non-degraded dye. Microtoxicity assay was also performed on the test cultures *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* using the degraded dye metabolite/non-degraded dye. The non-degraded dye components inhibited the growth of *P. aeruginosa* (clear zone of 6 mm) and *E. coli* (clear zone of 3 mm). *K. pneumoniae* was resistant to the toxicity of the dye components. The following metabolites were detected; 3-Benzyl hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one, N-[(Z)-1-Ethylpentylidene] methanamine and 10-Undecenyl aldehyde with retention time of 23.317, 16.475, 16.850 and 23.500 min, respectively.

Key words: Detoxification, reactive black, phytotoxicity, white rot, Fourier transform infrared (FTIR), gas chromatography mass spectrometry (GCMS).

INTRODUCTION

Reactive black dye is a high-toxic compound containing rigid aromatic molecules with azo-based chromophores possessing different reactive groups and regarded as commonly used synthetic reactive dyes in the dyeing industry (El Bouraie and El Din, 2016; Kaneva et al., 2016). It is typically and highly recalcitrant to conventional

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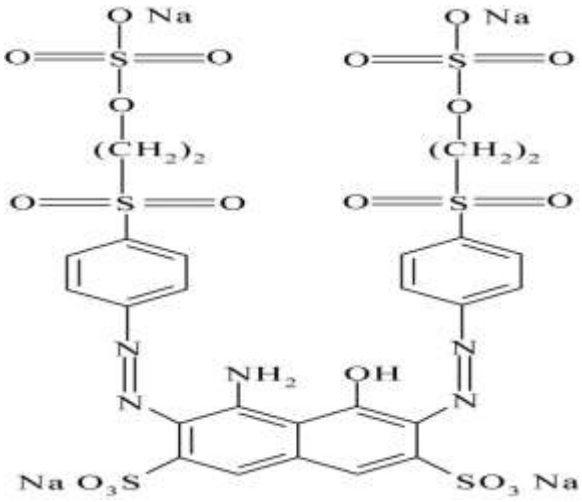


Figure 1. Chemical structure of reactive black.

Wastewater treatment processes (Lucas, 2006; Adnan et al., 2014). They are made up of one or more azo groups (R1-N=N-R2) and aromatic rings; the latter are mostly substituted by sulfonate groups thereby making them highly soluble in water and hence difficult to be removed from wastewater (Figure 1) (Saratale et al., 2011). The complex structure and xenobiotic nature makes the azo dyes and their degradation products recalcitrant to biodegradation and, in many cases, they have been reported to be mutagenic and carcinogenic (Khan and Malik, 2016; Bilal et al., 2017a, b, c, d; Chatha et al., 2017).

The release of dye-containing effluents into the aquatic environment is undesirable without proper treatment because it can remain persistent in the environment for an extended period (Irshad et al., 2012; Iqbal and Asgher, 2013; El Bouraie and El Din, 2016). Such practice of discharging untreated textile effluents into nearby water bodies and soil constitute a serious threat to human health and aquatic life (Asgher et al., 2013a, b; Shilpa and Shikha, 2015). Therefore, effective removal of azo dyes from wastewater effluents before discharge into the environment is of great concern. Various physiochemical strategies for the decolorization of textile wastewater are not promising due to different limitations associated with each of them, therefore, there is a need for a technique which is efficient and also meets the environmental regulatory requirements (Shilpa and Shikha, 2015; Bilal et al., 2017b). More than one process such as physical and chemical treatments has been used for the treatment of wastewater containing dyes due to their complicated structures and recalcitrant nature (Türgay et al., 2011) but these techniques are not cost-effective and pose operational difficulties. Some of the other treatments already used to degrade direct dyes include photocatalysis, oxidation, etc. which are again energy intensive processes (Krishnan et al., 2016).

Although some of these processes have been effective, their application is limited due to the high cost, excess usage of chemicals, and excessive sludge generation with subsequent disposal problem (Saratale et al., 2011). Biological treatment methods are eco-friendly, have been proven to be efficient and more cost-effective and hence are gaining importance in today's situation. Microorganisms such as actinomycetes, fungi, algae, yeast, aerobic and anaerobic bacteria and their enzymes have been successfully utilized to degrade a wide variety of dyes (Kaushik and Malik, 2009; Gupta et al., 2010; Srinivasan et al., 2014; Asgher et al., 2016). But the use of bacteria in the biological treatment of wastewater may result in the generation of colorless, dead-end aromatic amine which is generally more hazardous than the parent compounds and thus may have poor usage and limited application in the treatment of dye effluents (Guaratini et al., 2001; Hadibarata et al., 2013). Therefore, the use of white-rot fungi and their extracellular enzymes are currently an effective solution for removal of synthetic dye containing wastewater (Ali, 2010; Asgher et al., 2013b; Bilal et al., 2016). There are two important mechanisms for treatment of dye by white-rot fungi which are by biosorption of dye to the fungal biomass and biodegradation of dye into another compound by an extracellular enzyme (Banat et al., 1996).

Fungal treatment of dyed effluents removes several chromophoric groups and thus decreases its toxicity and aesthetic impact in the receiving water bodies. However, *Ganoderma tsugae* has been known to possess medicinal values but much less work was devoted to its decolorization ability. In this work and for the first time, a new ability exhibited by growing cultures of a *G. tsugae* was reported. Additionally, its performance during batch biodegradation of the industrially important reactive black dye as well as the decolorization mechanism is discussed.

MATERIALS AND METHODS

Microorganism and culture conditions

The white rot fungal strain of *G. tsugae* was obtained from culture of the Federal Institute of Industrial Research Oshodi, Lagos State, Nigeria. The culture mycelium was stored on malt extract agar slant at 4°C.

Screening for laccase production

The screening for the production of laccase by the test organism was done using potato dextrose agar. The potato dextrose agar (PDA) plates were prepared in duplicate maintaining the pH at 6.5 with the addition of 0.02% of guaiacol. The cultures were supplemented with 150 mM copper sulfate ($CuSO_4$) sterile solution as laccase-inducer and incubated at 25°C for 5 days (Kiiskinen et al., 2004).



Plate 1. Pure culture of *Ganoderma tsugae.*

Dye decolorization experiment

Decolorization study of Reactive Black was carried out by using 0.01 % of dye in 250ml Erlenmeyer flask containing the nutrient solution and five agar plugs 10 mm in diameter, from the edge of a 7-day-old agar culture of Ganoderma tsugae growing mycelia. This nutrient solution was autoclaved at 121°C for 30 min before being inoculated with fungal mycelia. The nutrient solution contained the following chemicals (g/L in distilled water): Glucose (10 g); KH2PO4 (2 g); MgSO4.7H2O, CaCl2.2H20 and NH4H2PO4 each 0.5 g; NH4Cl, FeSO4.5H2O and MnSO4 each 0.1 g; CoSO4, ZnSO4, CuSO4.5H2O and Na2HPO4 each 0.05g. The contents were inoculated and incubated at 150 rpm for 12 days at 28 0C (Shanmuga Priya et al., 2013). The uninoculated medium with reactive black dye served as blank. Percentage of decolorization was calculated by using the following formula (Hassan et al., 2013):

$$\text{Decolorization} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100\%$$

The decolorization potential, an extracellular protein, pH and production of lignin-degrading enzymes were monitored periodically in order to evaluate the performance of the fungal cells in decolorization. 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 2 days; centrifugation of the aliquots were carried out using the centrifuge at 4,000 rpm for 15 min, and then the supernatant were used to determine dye decolorization by monitoring the decrease in absorbance at the wavelength (λ) of 595 nm for each dye using a spectrophotometer (Visible Spectrophotometer L1-722) (Da Silva et al., 2009).

Extracellular laccase activity assay

Laccase activity was measured spectrophotometrically using guaiacol as a substrate with an absorbance coefficient value of 6800 $M^{-1}cm^{-1}$ at 470 nm (Collins and Dobson, 1997). The reaction mixture consisted of 3 mL of 100 mM of guaiacol dissolved in 10% acetone (v/v) in sodium acetate buffer (100 mM, pH 5.0), and 1 mL culture filtrate was used. The mixture was incubated for 15 min and the absorbance read at 470 nm. One unit (U) of laccase activity will be defined as the amount of enzyme catalyzing the production of one micromole of coloured product per minute per milliliter. Laccase activities was calculated using the following equation:

$$\text{Laccase activities (U/mL)} = \frac{\Delta\ \text{A470/min x 4 x Vt x dilution factor}}{€\ \text{X Vs}}$$

Where, Vt = final volume of reaction mixture; Vs = sample volume; € = extinction coefficient of guaiacol = 6740 $M^{-1}cm^{-1}$ and 4 = derived from unit definition and principle.

Assay of manganese dependent peroxidase

Enzyme activity was determined spectrophotometrically at 25°C. Manganese peroxidase (MnP) activity was assayed at 468 nm using dimethoxyphenol (DMP) as the substrate (Field et al., 1993). One unit (U) of enzyme activity was defined as the amount of enzyme required in producing one micromole of product per minute.

Determination of protein content

The protein content was determined according to the Bradford's method as reported by Singh and Abraham (2013). To 0.1 ml of culture filtrate water, Bradford reagent was added in required amount. The reaction mixture was incubated in the dark for 20 min and the absorbance was read at 595 nm. The protein was estimated taking bovine serum albumin (BSA) as standard.

Determination of residual metabolites

Biodegradation was determined by comparing the Fourier transformed infrared spectroscopy (FTIR) peak profiles of the metabolite of reactive black dye and those of its abiotic control. An attempt was also made to identify the dye metabolites using their gas chromatography-mass spectroscopy (GC-MS) spectra. The decolorized reactive black dye solution, withdrawn after 48 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 black dye 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 to 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 analyses. The identification of metabolites formed after degradation was done using a QP2010 GC-MS system (Shimadzu, Japan).

RESULTS

Enzyme activity

Ligninolytic enzymes are one of the important groups of enzymes involved in bioremediation, which are produced by various white rot fungi in greater extent. In this study, *G. tsugae* was obtained from the Federal institution of Industrial Research (FIIRO) Oshodi, Lagos and cultured on the plate potato dextrose agar plate (Plate 1) to

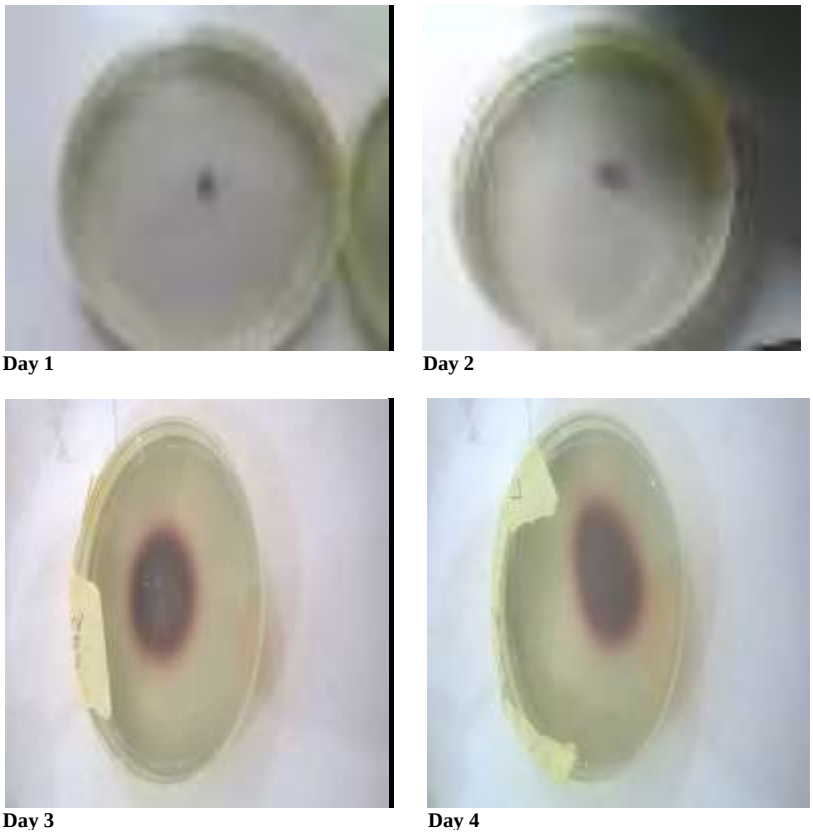


Plate 2. Screening for laccase production.

observe its colonial morphology. During screening for enzyme activity (Plate 2), the presence of a reddish brown color zone on the fungal culture plates confirmed laccase enzyme. The Laccase production found to increase as the incubation period progressed shown in Plate 2. Table 1 shows all the biodegradation indices for monitoring biodegradation process such as pH, extracellular protein content, laccasse activities, manganese peroxidase and percentage of decolourization. The pH values of the culture filtrate are shown in Table 1; ranged from 4.50±0.12 to 8.50±1.20 for the period of 288 h. The highest pH value was 4.50 after 48 h of incubation. The mean values of the pH increased progressively with increase in the period of incubation. Duncan multiple range test (DMRT) comparison at $P<0.05$ showed that the values were not significant from 96 to 144 h of incubation but significantly different after incubation for 288 h. Extra cellular protein, manganese peroxidase and laccase activities production followed the same patterns with the highest production after 48 h of incubation with mean values of 22.10 ± 2.55, 0.54 ± 0.00 and 0.66 ± 0.06, respectively and decreased progressively over a period of incubation while the lowest mean values were 3.00±0.50, 0.09±0.00 and 0.15±0.00, respectively. Duncan multiple range test (DMRT) comparison at $P<0.05$ showed that there were no significant differences from 96 to 144 h of incubation in extracellular protein, manganese peroxidase and laccase activities. The increase in pH seemed to bring about the decrease in extra cellular protein, manganese peroxidase activities and laccase activities. The lowest decolorization percentage was recorded after 48 h of incubation while the highest was after 288 h of incubation. The percentage of decolorization after 240 and 288 h were not significant.

Product characterization using analytical methods

Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infrared spectroscopy (FTIR) was used to monitor biotransformation/biodegradation of dyes. Figures 2 and 3 show peaks at 3700 cm^{-1} (O-H stretch), 3600 cm^{-1} (O-H Stretch), 3400 cm^{-1} (N-H stretch), 2350 cm^{-1} (O=C=O stretch), 1649 cm^{-1} (C=O), 1625 cm^{-1} (C=C), 1421 cm^{-1} (O-H bend) 1351 cm^{-1} (S=O stretch), 1300 cm^{-1} (S=O stretch), 1125 cm^{-1} (C-O stretch), 994 cm^{-1} (C=C bend), 962 cm^{-1} (C=C bend), 850

Table 1. Changes in pH, protein, degradation and ligninolytic enzyme activities during biodegradation of reactive Black dye by *Ganoderma tsugae.*

Time (h)	pH	Extracellular protein (Mg/mL^{-1})	Manganese peroxidase (U/ML^{-1})	Laccase activities (U/mL^{-1})	Decolorization (%)
48	4.50±0.12^{d}	22.10±2.55^{a}	0.54±0.00^{a}	0.66±0.06^{a}	25±1.51^{d}
96	5.35±0.00^{c}	18.25±0.33^{ab}	0.51±0.01^{a}	0.60±0.00^{a}	43±2.00^{c}
144	6.00±0.33^{c}	16.33±0.11^{ab}	0.38±0.00^{ab}	0.40±0.03^{ab}	55±4.81^{b}
192	6.50±0.11^{ab}	12.22±0.00^{b}	0.25±0.02^{b}	0.33±0.00^{b}	64±3.00^{ab}
240	7.50±0.00^{b}	7.86±0.20^{c}	0.12±0.00^{c}	0.22±0.02^{c}	75±500^{a}
288	8.50±1.20^{a}	3.00±0.50^{d}	0.09±0.00^{d}	0.15±0.00^{d}	82±4.33^{a}

Values are presented as mean ± SEM (n = 3). All groups are compared to each other at P < 0.05. Values with the same superscripts along the same column are not statistically different from each other.

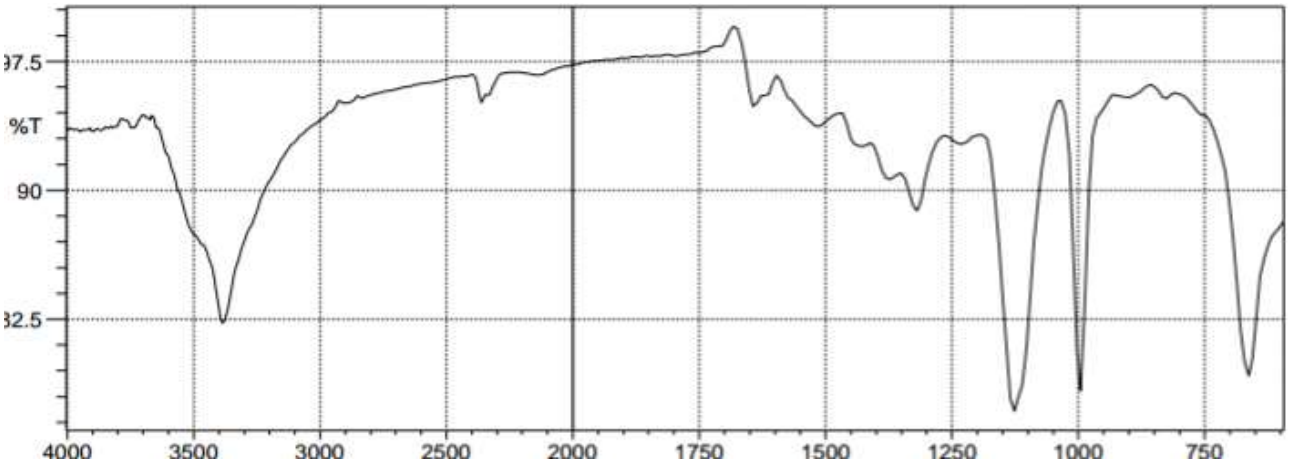


Figure 2. FTIR spectral of non-degraded reactive black dye (control).

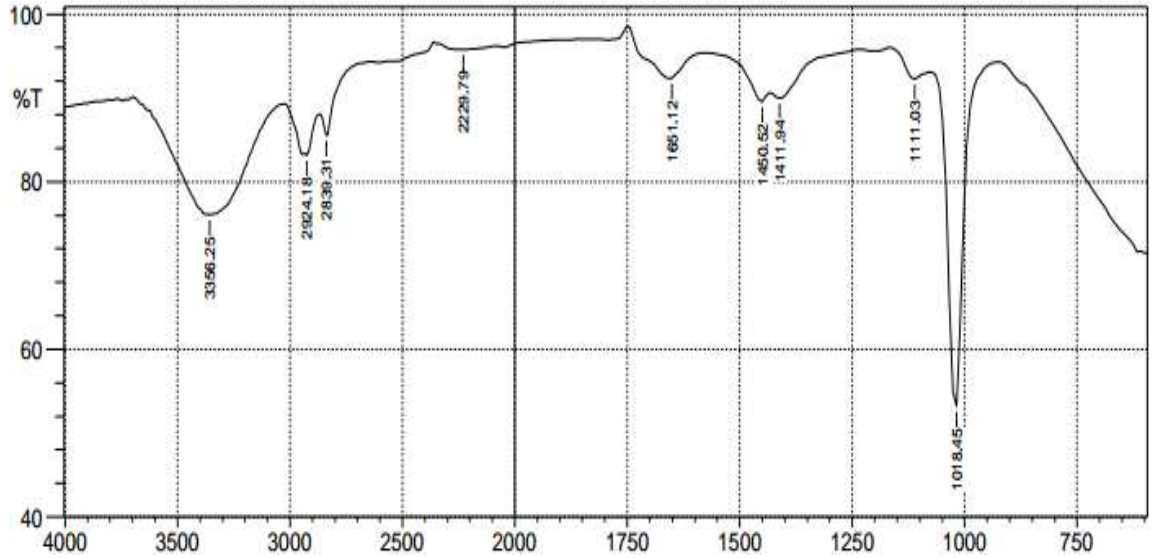


Figure 3. FTIR spectral of degraded reactive black dye.

Table 2. Interpretation of infrared spectral of the functional groups in non-degraded reactive black dye.

Peak	Wave number (cm^{-1})	Type of vibration	Functional group
1	649.13	Stretching	C-Br
2	801.25	Bending	C=C
3	850.11	Stretching	C-Cl
4	962.30	Bending	C=C
5	994.42	Bending	C=C
6	1125.73	Stretching	C-O
7	1300	Stretching	S=O
8	1351.22	Stretching	S=O
9	1421.40	Bending	O-H
10	1625.19	Stretching	C=C
11	2350.37	Stretching	O=C=O
12	3400.21	Stretching	N-H
13	3600	Stretching	O-H
14	3700	Stretching	O-H

Table 3. Interpretation of infrared spectral of the functional groups in degraded reactive black dye.

Peak	Wave number (cm^{-1})	Type of vibration	Functional group
1	1018.45	Stretching	C-O
2	1111.03	Stretching	C-O
3	1411.94	Stretching	S=O
4	1450.52	Bending	C-H
5	1651.12	Stretching	C=C
6	2229.79	Stretching	C≡N
7	2839.31	Stretching	C-H
8	2924.18	Stretching	C-H
9	3356.25	Stretching	N-H

cm^{-1} (C-Cl stretch), 801 cm^{-1} (C=C bend), and 649 cm^{-1} (C-Br stretch). It is observed in Tables 2 and 3 that C=C groups were predominant in non-reactive black dyes, and they all possess bending type of vibrations appearing at the low peak of the spectrum and are all double bonded, while some functional groups were not seen in the degraded dye. Though the remaining functional group is quite different in terms of structure, wave number and vibration, they all possess single and double bonds as a unifying factor.

Gas chromatography-mass spectrometry (GC-MS)

The degraded samples were analyzed by GC-MS. The GC degradation products of reactive black dye showed the presence of several peaks. The structures and nomenclature of the detected intermediate compounds were assigned from the fragmentation pattern and m/z values obtained from GC chromatogram and mass spectral analysis. Each of the peaks represents a particular compound on the whole; the total numbers of compounds present in the dye before and after degradation were found to be 12 and 23 in degraded and non-degraded reactive black dyes respectively. Interestingly, the following metabolites were only found in the degraded dye but not in original reactive black dyes such as 3-Benzyl hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one, N-[(Z)-1-Ethylpentylidene] methanamine and 10-Undecenyl aldehyde with retention time of 23.317, 16.475, 16.850 and 23.500 min, respectively as shown in Figure 5.

Seed germination assay

The seed germination study (plate assay) was carried out



Plate 3. Seed germination test.

using beans and maize seeds (Plate 3). Following 3 days of incubation, the germination percentage was calculated. The result of the seed germination analysis revealed inhibition of germination for each seed of maize and beans by 82.5 and 87.5%, respectively (Plate 3). However, about 77.5 and 65.7% germination was observed in both seeds irrigated with dye degradation metabolites (Figure 6).

Toxicity assay

Toxicity assay was carried out using the test cultures of *K. pneumoniae*, *E. coli* and *P. aeruginosa.* After incubation for 48 h, zones of clearance were observed and measured (Plate 3). The results indicate that the non-degraded dye components inhibited the growth of the test cultures of *P. aeruginosa* (clear zone of 6mm) and *E. coli* (clear zone of 3 mm) due to the presence of toxic compounds in the dye. *K. pneumoniae* was able to resist the toxicity of the dye components and no clear zone was found around the bore hole containing the non-degraded dye. On the other hand, *K. pneumoniae*, *E. coli* and *P. aeruginosa* were able to grow on the degraded dye.

DISCUSSION

The rapid growth of the *G. tsugae* on the plate of potato dextrose agar with fluffy white mycelium shown on the plate affirmed the assertion of Stanley and Nyenke (2011) who reported that the increase in such growth rate can be attributed to the availability of growth factor present in the nutrient medium supporting the growth of the fungus (Stanley and Nyenke, 2011). Ragunatha et al. (2003) described that *G. tsugae* has a rich white aerial with a reverse colorless mycelium on potato dextrose agar plate similar to what was observed in the current study (Plate 1). The use of dyes and coloured indicators that enable visual detection of lignolytic activities is a simple method of screening as no measurement is required. The plate-test is an efficient and simple method for bioprospecting fungi with novel lignolytic enzymes for industrial application purposes (Machado et al., 2005). Therefore Reddish brown color formation was observed when guaiacol is used as an indicator to confirm the presence of extracellular fungal laccase production which increased as the incubation progressed as it is shown in Table 2 (Alfarra et al., 2013)

One of the parameters widely used in the detection of ligninolytic enzymes is the chromogen. In the present study, guaiacol was used as a chromogen. The reddish brown zone surrounding the mycelia of the culture on the plate supplemented with guaiacol was an indication of Bevandamm's reaction (Thakur and Gupte, 2014; Sara et al., 2016). Several authors have reported the involvement of different lignolytic enzymes in biodegradation of synthetic dye by laccase and manganese peroxidase by white rot fungi (Hadibarata et al., 2013; Adnan et al.,

2014; Adnan et al., 2015); this confirmed the activities of laccase and Mnp in this current study.

In similar work, the use of guaiacol proofs to be a sensitive substrate for the screening of laccase producing organisms as produced by *G. tsugae* (Tekere et al., 2001). Chromogen is used in the detection of ligninolytic enzymes of which guaiacol is one that brings out Bevandamm's reaction (Sara et al., 2016). The mean values of pH, laccase activities and manganese peroxidase were found to be correlated with decolorization of reactive black dye (Table 1) as biodegradation progressed. The enzymatic breaking down of the reactive dye can largely be attributed to the laccase and other ligninolytic enzymes secrete by the organism. Among the several ligninolytic enzymes; laccase was found to be highly studied for its role in decolorization and detoxification of various industrial and textile dyes. It has been reported that laccase is solely accounted for the decolorization and degradation of dyes (Liu et al., 2004; Abdulla et al., 2000; Rodriguez et al., 1999; Casieri et al., 2005; Poojary et al., 2012). Azo dye is a recalcitrant and complex molecule for degradation as it consists of fused aromatic rings (Kumari et al., 2007). Despite this, *G. tsugae* still have the capability to decolorize reactive dye up to 82%. The differences in the dye decolorization capacity have been related to fungal variations, the molecular complexity of dyes as well as culture conditions (Levin et al., 2004; Machado et al., 2006). Such recalcitrance of dye degradation by the fungus may also, be attributed to higher molecular mass, structural complexity and the presence of inhibitory groups such as sulfides, chlorides, and aromatics in the dyes (Hu et al., 2001). Nutrient medium used in the current study contained a large amount of glucose meaning that *G. tsugae* did not utilize reactive black as a source of carbon and energy but via co-metabolic biodegradation. Though, it has been reported that the basidiomycetes could not utilize the dye as a sole carbon and energy source for growth and production of enzymes (Adosinda et al., 2001). Glucose or other carbon sources served as a co-metabolic substrate for dye decolorization which supplied an essential substrate for the production of enzyme and growth of the cell, in which the production of ligninolytic enzyme was performed throughout their secondary metabolism (Hadibarata et al., 2011). It has been reported that 1,2- and 2,3-dioxygenase synthesis during cell growth might be responsible for decolorization and glucose might play significant roles in this process except as carbon or energy source for fungi growth (Hadibarata et al., 2011). The white rot fungus contains various enzymes and is, therefore, able to degrade or mineralize several organic pollutants (Gao et al., 2010). The optimum pH was between pH 3 and 5 because the pH range is suitable for the growth and enzymatic production by white-rot fungi (O'Mahony et al., 2002). In a similar study, Vaithanomsat et al. (2002) reported the decolorization of reactive black as being better degraded under acidic conditions which also is in agreement with the work of Young and Yu (1997) that found an azo-based dye to be more efficiently degraded by white-rot fungi under acidic conditions. The maximum extracellular protein, laccase activities, and manganese peroxidase activities were obtained at pH 4.5 and 5.35 (Table 1). Though the growth of the fungi is ideal at low pH (Sunil et al., 2011) in similar finding, Zhixin et al. (2010) obtained the maximum activity at pH 4.4 which is approximately similar to that achieved by Khushal et al. (2010). Protein content, Lac and MnP activity detected early growth period, with maximum extracellular protein, Lac and MnP activity at 22.10±2.55, 0.54±0.00 and 0.66±0.06 after 48 h of cultivation, respectively. This maximum enzyme activity observed much earlier in the liquid cultivation could be attributed to the presence of more carbon and nitrogen sources in the medium that may have stimulated the growth and enzyme production of *G. tsugae*; the same pattern was observed in the work of Vaithanomsat et al. (2002). This also corroborated those of Buddolla et al. (2008) who obtained maximum laccase activity of 600 U/L after the 4th day of incubation using potato dextrose broth (PDB) as a culture medium. The laccase activity is predominant during dyes degradation by different fungi compared to the MnP activity (Valderrama et al., 2003). The same trend was obtained in the current study where laccase activities were higher than that of manganese peroxidase activities although, with no significant difference ($p<0.05$). Liu et al. (2004) also reported that laccase is solely responsible for the decolourization and degradation of dyes. Also, Hou et al. (2004) findings, affirmed the assertion that laccase was the only ligninolytic enzyme activity present in the supernatant when the fungus was grown in liquid culture with or without shaking. Similarly, Zouari-Mechichi et al. (2006) found that the sole ligninolytic activity detected in liquid cultures using a glucose-peptone medium was laccase in contrary to the current findings in which Lac and MnP activities were detected; this disparity may be attributed to the differences in white-rot fungi used and culture medium. The presence of laccase and MnP activities agreed with the findings obtained by other researchers that laccase-MnP combination is the most common group of extracellular enzymes in the white rot fungi (Nerud and Misurcova, 1996). It has also been proposed that the activity of laccase and/or MnP may be sufficient for lignin degradation in some fungi (Nerud and Misurcova, 1996). During the reactive black degradation, laccase activities, manganese peroxidase, and extracellular protein secretion were detected in the early period of culture and correlated with a decrease in pH and decolorization. This is in agreement with the work of Hadibarata et al. (2011) who reported that the production of MnP was a kind of response to the presence of dye by the fungi and related with the process of color removal. In this study Lac and

MnP were detected in the filtrate and presumed to be involved in the biodegradation of reactive black dye contrary to the work of Vaithanomsat et al. (2010) who reported the detection of highest Lac activity whereas there was no detection of MnP and LiP activities and concluded that the *Datronia* sp. KAPI0039 was able to degrade reactive dyes, and Lac was considered to be an only major lignin-degradation enzyme in this reaction. In similar study, Placido et al. (2007) showed that the laccase and MnP from the *F. trogii* ATCC 200800 were essential enzymes for the decolorization and that decolorization is not a single step reaction but rather, a more complex phenomenon in which more than one enzyme is involved. The highest percentage decolorization was attained after 240 and 280 h of degradation with no significant different ($p<0.05$) which may be as a result of catabolic activities of the secreted ligninolytic enzymes. In this study higher decolorization under shaking conditions, which could be due to better oxygenation of the fungus and regular contact of secreted enzymes with dye molecules to decolorize it. The removal of dye color may be attributed to the enzymatic degradation of chromophore present in dye molecules. Paszczynski et al. (1991) affirmed that the degradation of azo dyes might be attributed to the cleavage of its aromatic compounds which may be due to the substitution of its precursors with the phenolic, amino, acetamido, 2-methoxyphenol or other easily biodegradable functional groups, resulting in a greater extent of degradation. To have an insight and understanding of the mechanism of biodegradation of molecules which occurred would require the chemical identification of the breakdown of metabolites and functional groups. Fourier transform infrared and GC-MS were used to analyze residual metabolites in the experimental flask. The differences regarding chemical structure and their functional groups of textile dyes on aromatic base greatly influence their decolorization rates of textile dyes (Harshad et al., 2015; Bilal et al., 2017e). This plainly demonstrates that decolorization was breaking down of dyes into its simpler forms. FT-IR analysis was done to characterize the metabolites produced. The results of the FT-IR analysis of the dye control and the metabolite obtained after decolorization showed various peaks. The FT-IR spectra of dye control displayed peaks at 650, 1000, 1150, 1350, 1600, 2400, 3450 and 3600 cm^{-1}. The FTIR spectra of dye and dye degradation products differed with some peaks and their positions (Figures 2 and 3). A significant change in FTIR spectrum in degraded dye metabolite confirms biotransformation of dye into other compounds.

The differences in chemical structure of textile dyes that accounts for its recalcitrance are as a result of the substitution of various functional groups on aromatic base that greatly affect their decolorization rates (Harshad et al., 2015). This demonstrates that decolorization was largely due to degradation of dyes into intermediate products. After reactive black dye degradation, a significant difference in FT-IR spectrum was observed in Figures 2 and 3. Peaks at mono-substitutedand para-disubstituted benzene rings prominent in non-degraded reactive black dye completely disappeared in final degraded products; while the entirely new peaks appear, this can be as a result of cleavage of benzene rings due to biotransformation of the reactive black dye. Tables 2 and 3 are used for the interpretation of the infrared spectral of the FTIR.

The GC degradation products of reactive black 5 dye show the presence of several peaks. The nomenclatures of the detected compounds were assigned from the fragmentation patterns and m/z values obtained from the GC-MS analysis. Figure 4 shows the compounds present in the non-degraded and degraded dye; the non-degraded dye has a total number of 12 compounds present. Some complex compound like glyceryl trilaurate was broken down into glyceryl 1,3-distereate, palmitic acid into palmitic amide and methyl octanoate broke down into methyl decanoate in the degraded dye. Upon degradation, there was decrease in percentage concentration in some compounds like oleic acid amide and myristic acid both which are present in the control and degraded dye. Some new compounds formed during the degradation of the dye includes acetaldehyde, undecane, 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene, 14-hexadecenal, pelargone, 2-hexyl-1-decanol, 5-isopropylidene,3-3-dimethyl-dihydrofuran-2-one, tetradec-1-ene, methylamine, N-(1-ethylpentylidene), 1-docosene, octadec-11-enoic acid, methyl ester, methyldecanoate, lauric amide, glyceryl 1,3-diestearin, cis-13-docosenamide, palmitic amide, 3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 10-undecenyl aldehyde, 2-undecen-1-ol and dioctyl phthalate. This phenomenon shows that the dye has been degraded, that is, some compounds disappeared due to biodegradation and also some complex compounds are broken down into simpler compounds; there are increase and decrease in percentage content of some compounds. The mass spectra of the following complex compounds were detected such as 3-benzyl hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, 5-isopropylidene-3,3-dimethyl-dihydrofuran-2-one, N-[(Z)-1-ethylpentylidene] methanamine and 10-undecenyl aldehyde with a retention time of 23.317, 16.475, 16.850 and 23.500 min, respectively. This represents some of the metabolites present in the degradation products of reactive black dye by *G. tsugae*. The biodegradation process is promising in degrading dye but the toxicity is still needed to be ascertained (Bilal et al., 2016). Thus, both sulfonated and un-sulfonated aromatic amines are important groups of environmental pollutants formed during reduction of (sulfonated) azo dyes, that can be potentially pass through biological treatment system

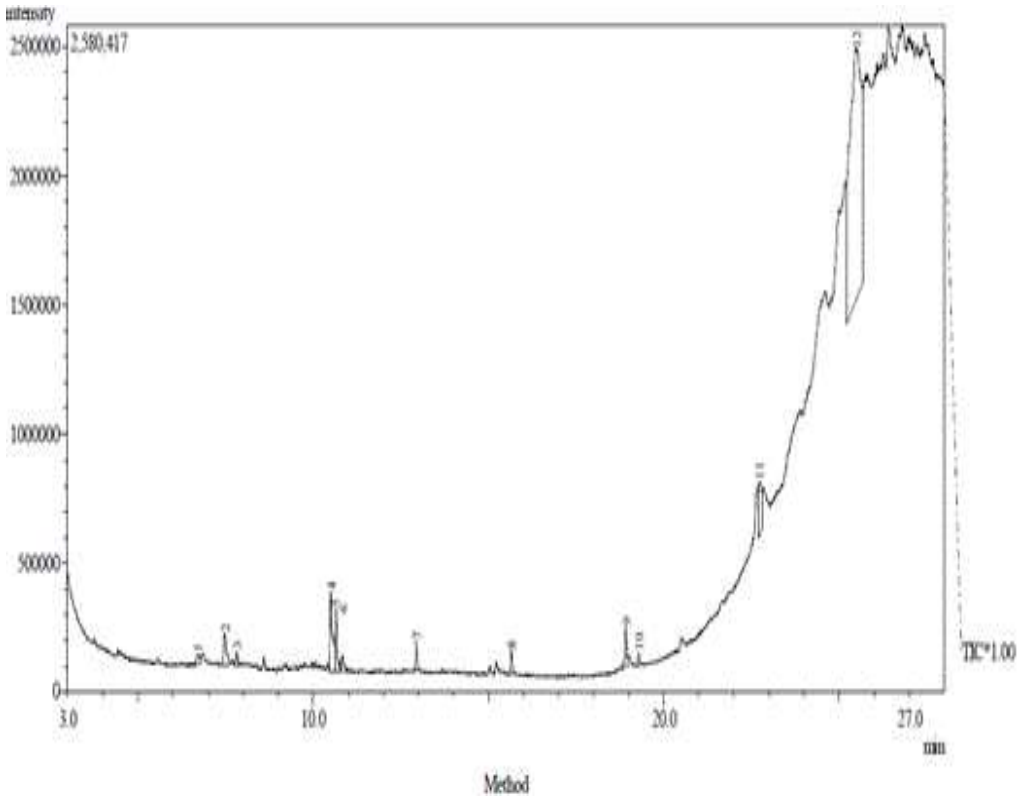


Figure 4. GC-chromatogram of degraded reactive black dye.

(Kalyani et al., 2008). Therefore, it was of public health and ecological concerns to assess the toxicity of the dye before and after degradation, because degraded by-products may exert more toxicity than parent dyes molecule. As a result, the toxicity evaluation of treated dyes using reliable and standard analytical method/ bioassays is important (Nouren et al., 2017). The results of the seed germination assay revealed strong influence of physiological characteristics in untreated dye seeds and it suggests that reactive Black dye has a toxic effect on the seeds as it inhibited germination. Since the treated effluent containing reactive dyes will eventually discharge into either receiving water or soil. Therefore, toxicity reactive black dye was evaluated on some bacterial pathogens. Non-degraded reactive black dye inhibited the growth of both *E. coli* and *P. aeruginosa* while *K. pneumoniae* was found to be resistant to the dye. Its mechanism of resistance may be attributed to the same mechanism used by the organisms to antibiotics such as efflux pumps and modification of target sites. Interestingly, degraded reactive black showed no inhibitory effect on the test bacteria. This can be inferred to be due to the detoxification and biotransformation of the reactive black dye into nontoxic form by the action of the white rot fungus *G. tsugae*. Phytotoxicity was performed using *Zea mays* and bean seed assay. Seeds were exposed to treated and not degraded reactive black dye samples. The result of the seed germination analysis revealed inhibition of germination by reactive black for each seed of maize and beans by 17.5 and 12.5%, respectively (Plate 3). However, about 77.5 and 65.7% germination was observed in both seeds irrigated with dye degradation metabolites. In comparison to control, non-degraded reactive black dye revealed toxicity signs, whereas seeds exposed to treated reactive black dye solution showed a considerable improvement in germination. Results suggest that *G. tsugae* transformed and reduced the toxicity of reactive black dye, which indicates that bio-degradation is efficient in toxicity reduction. These findings are in line with previous studies that the toxicity of dyes can be reduced as a result of biodegradation (Bilal and Asgher, 2015; Bilal et al., 2016; Iqbal, 2016; Nouren et al., 2017). This study reveals that the metabolites generated after the biodegradation of reactive black is less toxic compared to original dye. Given efficient toxicity reduction, the biodegradation is an eco-friendly method and *G. tsugae* is not only been able to decolorize the reactive black dye but also completely detoxify it. This suggests the future application of *G. tsugae* for low-cost biodegradation as well as

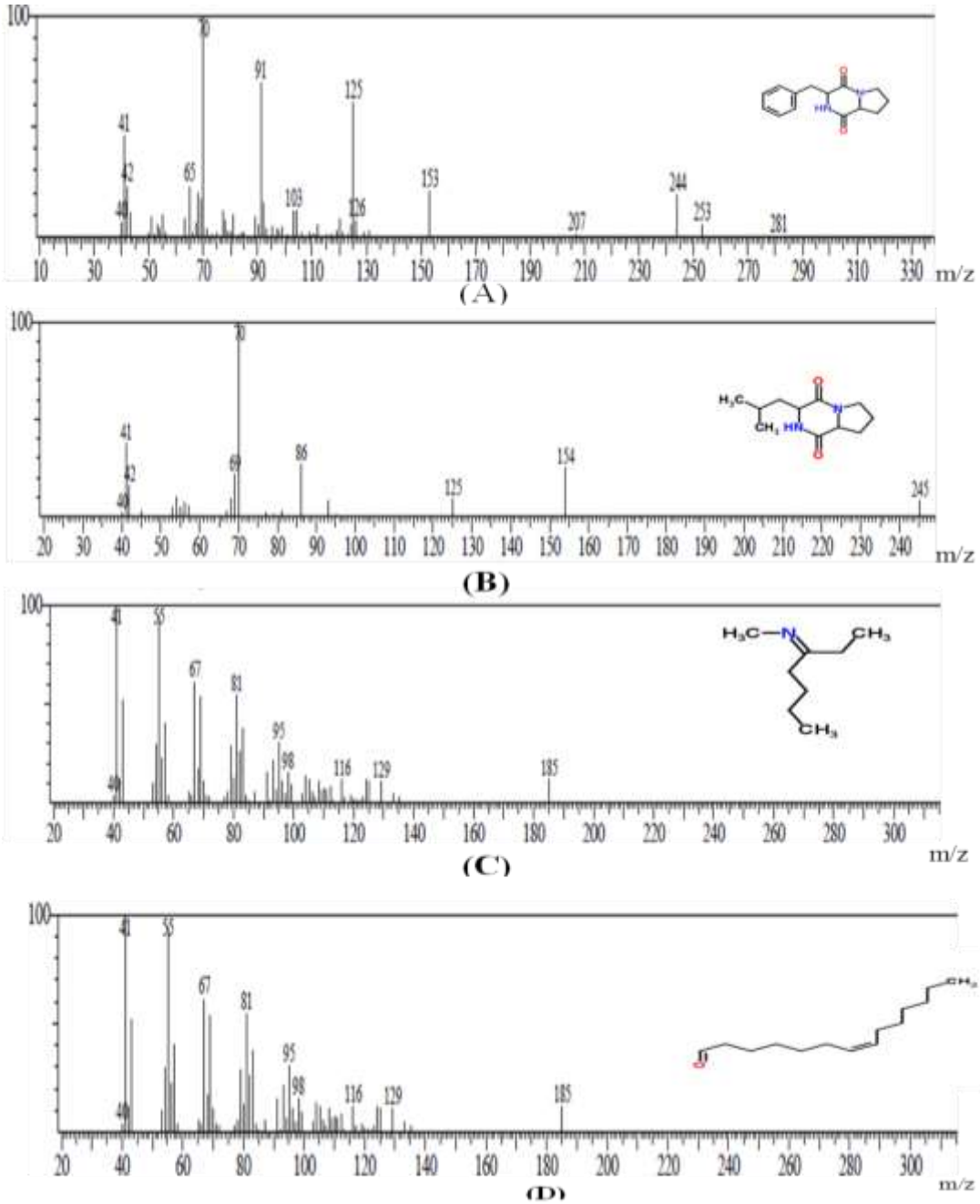


Figure 5. Identification of metabolites of reactive black by GC–MS. **(a)** 3-Benzyl hexahydropyrrolo[1,2-a]pyrazine-1,4-dione M.W 244, mass peak (m/z) 244. **(b)** 5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one M.W 154, mass peak (m/z) 154. **(c)** N-[(Z)-1-Ethylpentylidene] methanamine M.W. 127, mass peak (m/z) 127. **(d)** 10-Undecenyl aldehyde, M.W 168, mass peak (m/z) 168.

detoxification of azo dye contaminated wastewaters.

Conclusion

This work evaluated the decolorization and detoxification of reactive black dyes by *G. tsugae*. Batch culture degradation process showed that the *G. tsugae* possesses not only medicinal values but also an effective enzymatic capacity for the cleavage of reactive black dye such as laccase and manganese peroxidase that played major role in biotransformation of synthetic dyes. The

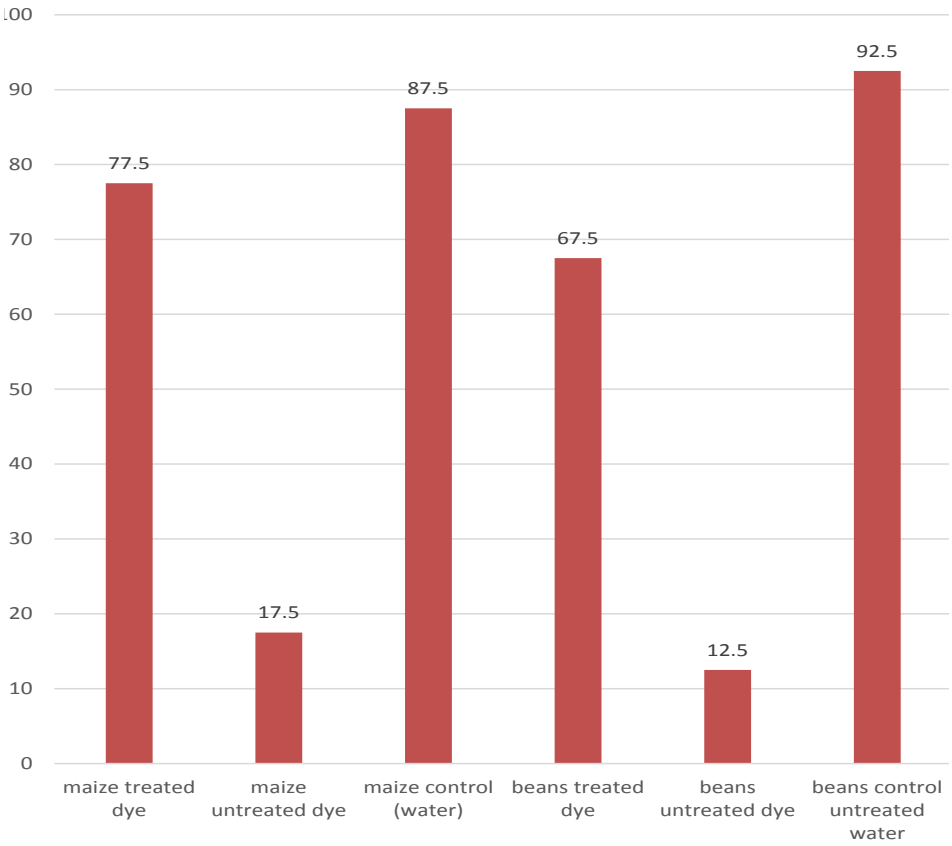


Figure 6. Percentage of seed germination of treated and untreated reactive black dye on beans and maize seeds.

mechanism of degradation might be through benzene ring cleavage and hydroxylation. FT-IR and GC-MS analysis were used to confirm the degradation products. Toxicity of the degraded products were tested on both microorganisms, bean and maize seeds which confirmed the detoxification capacity of *G. tsugae* on reactive black dye when compared with non-degraded reactive black dyes. It could successfully be employed in the treatment of textile effluent. This study shows that both laccase and manganese peroxidase enzyme from fungus *G. tsugae* play a significant role in the biodegradation of reactive dye and as such it can be applied in bioremediation of wastewater from textile industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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