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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-Isopropyldiene-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...
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 (CuSO₄) sterile solution as laccase-inducer and incubated at 25°C for 5 days (Kiiskinen et al., 2004).
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, CaCl2.2H2O 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 28°C for 12 days at 150 rpm (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 (Å) 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⁻¹cm⁻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:

\[
\text{Laccase activities (U/mL)} = \frac{\Delta A_{470}/\text{min} \times 4 \times V_t \times \text{dilution factor}}{\epsilon \times V_s}
\]

Where, Vt = final volume of reaction mixture; Vs = sample volume; \(\epsilon\) = extinction coefficient of guaiacol = 6740 M⁻¹cm⁻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 redissolved 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 (IIIRO) Oshodi, Lagos and cultured on the plate potato dextrose agar plate (Plate 1) to
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*.

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

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.

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

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

<table>
<thead>
<tr>
<th>Peak</th>
<th>Wave number (cm(^{-1}))</th>
<th>Type of vibration</th>
<th>Functional group</th>
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<tbody>
<tr>
<td>1</td>
<td>1018.45</td>
<td>Stretching</td>
<td>C-O</td>
</tr>
<tr>
<td>2</td>
<td>1111.03</td>
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<td>C-O</td>
</tr>
<tr>
<td>3</td>
<td>1411.94</td>
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<td>S=O</td>
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<td>4</td>
<td>1450.52</td>
<td>Bending</td>
<td>C-H</td>
</tr>
<tr>
<td>5</td>
<td>1651.12</td>
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</tr>
<tr>
<td>6</td>
<td>2229.79</td>
<td>Stretching</td>
<td>C≡N</td>
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<td>C-H</td>
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<td>2924.18</td>
<td>Stretching</td>
<td>C-H</td>
</tr>
<tr>
<td>9</td>
<td>3356.25</td>
<td>Stretching</td>
<td>N-H</td>
</tr>
</tbody>
</table>

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-dihydropuran-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
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 6 mm) 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., 2016).

![Plate 3. Seed germination test.](image-url)
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 \textit{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 \textit{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 difference (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-distearate, 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-tetrahydroxanaphthalene, 14-hexadecenyl, palergone, 2-hexyl-1-decanol, 5-isopropylidene, 3,3-dimethyl-dihydrofuran-2-one, tetradec-1-ene, methylamine, N-(1-ethylpentylidene), 1-docosenoic, octadec-11-enoic acid, 1,3-diesterin, cis-13-docosenamide, palmitic amide, 3-benzilylhexahydropryrolo[1,2-alpyrazine-1,4-dione, 10-undecenyl aldehyde, 2-undecen-1-ol and diocetyl 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 hexahydropryrolo[1,2-alpyrazine-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 \textit{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.
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 biodegradation 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.

The 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 was 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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Full Length Research Paper

Screening of hydrocarbon degrading fungi in crude oil polluted soil isolated in the Niger Delta

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Bioremediation has been argued to be cheaper and more environmental friendly when compared to other remediation technology. Bioremediation make use of the ability of bacteria and fungi to biodegrade organic compound to cleanup hydrocarbon pollution. Many fungi have been shown to biodegrade simple and complex polycyclic aromatic hydrocarbon. In this study, petroleum hydrocarbon degrading fungi have been isolated and identified by microscopic and macroscopic methods in oil polluted soil. The polluted soil samples were collected from an oil polluted site within an oil bearing community in Bayelsa state of Nigeria. Eight different strains of fungi were isolated and identified and they include, Penicillium spp, Candida species, Aspergillus niger, Mucor spp, Rhodotorulla spp, Rhizopus, Trichorderma spp and Cladospermorium spp. Population of hydrocarbon degrading fungi, isolated from the soil collected in three different sampling stations are 5.3x10\(^4\), 8.3x10\(^3\) and 2.51x10\(^4\) Cfu/g for station 1, 2 and 3, respectively. While total hetetrophilic fungi are 3.6x10\(^5\), 7.1x10\(^5\), and 9.51x10\(^6\)Cfu/g, Penicillium spp , A. niger, Mucor spp, Rhodotorulla spp, Rhizopus, Phanerochaete spp, Alternaria alternate, Fusarium spp are strains of fungi that have been discovered by different scholars to degrade different components of petroleum. This study has revealed that oil polluted soil in the Niger Delta is habited by hydrocarbon degrading fungi, which can be biostimulated to enhance oil pollution cleanup in the area.

Key words: Bioremediation, biodegradation, fungi, polluted soil biostimulate, isolated and identified.

INTRODUCTION

Fungi are group of microorganisms scientifically regarded as non-green plants. They are widely distributed in the environment and mostly found in moist dark places. While some are harmful as a result of their toxic secretion, some are edible. Apart from being edible some are also beneficial to man in other ways. For instance,

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some help in the breakdown of organic waste that would have otherwise pose threat to humans and the environment.

Like some bacteria and Protists, fungi digest insoluble organic matter by secreting exoenzymes, which absorb the solubilized nutrients (Prescott et al., 2008). Yeast, molds, mushroom, mildews and some other types of fungi are wide spread in the environment and help to breakdown complex organic matter. The ability of fungi and some bacteria to breakdown complex organic and harmful matter into simple and harmless or less toxic compounds which assimilates them as food, has made them useful and find application in the science of environmental restoration (Bioremediation).

Bioremediation is a technology that utilizes the metabolic potentials of microorganisms such as fungi and bacteria to degrade petroleum hydrocarbon spilled in the environment into harmless compounds (Watannabe, 2001). These microbes either degrade, bioaccumulate, bioabsorbs or bioadsorbs the contaminant in sediment, water or soil. (Odokuma and Dickson, 2003). Of all the petroleum pollution cleanup techniques that have been developed, the use of petroleum hydrocarbon degrading microbes has received most attention (Macaulay, 2015).

Petroleum hydrocarbon pollution is hazardous to the ecosystem and public health (Illeoma, et al., 2015). It is because of toxicity of petroleum hydrocarbon that immediate and urgent attention should be given with view to cleaning up the spill. Abi and Nwosu (2009) reported that crude oil negatively affects soil agricultural productivity and soil organisms. Crude oil has also been shown to reduce plant germination and productivity (Agbogidi, et al., 2007). A study by Wokocha, Ermeodu and Ihenko (2011) also reported that crude oil pollution increase soil acidity. High soil acidity is not good for plants because it can destroy plants and impotent soil organisms.

Crude oil also kill fishes in concentration level of 4000ppm (Prasad, 1987). Benzene, a volatile compound in crude oil is also reported by Abdel-Shafy and Mansour (2016), to cause Leukemia in humans. Poly aromatic hydrocarbon another component of crude oil is also reported by Selina, (2005) to cause cancer in humans.

Bayelsa as one of the Niger Delta States where oil companies based their operation has experience many oil spillages hence, oil polluted sites are wide spread in the state. Inspite of the numerous oil pollution which have occurred in the state, much research have not been carried out to identify oil degrading fungi and bacteria, as it done in Ogoni land in Rivers State with view to harness them for bioremediation of the numerous oil polluted sites across the state. Bioremediation offers a very feasible alternative for the decontamination of oil spills (Greetha et al., 2013).

This technique is considered as an effective technology for the treatment of oil pollution (Malatova, 2005).

Bioremediation is an effective, economical and environmentally friendly treatment method in which microbes are used to degrade hydrocarbons (Greetha et al., 2013). Considering the economic viability and effectiveness of bioremediation, it has become very necessary to isolate and identify potential hydrocarbon degrading microbes such as fungi in crude oil polluted sites in Bayelsa state, within the Niger Delta region of Nigeria. For instance, in a research published by Uzoamaka et al. (2009), eight fungi isolates from petroleum polluted soil were identified to be hydrocarbon degrading which include Aspergillus Versicolor, Aspergillus niger, Aspergillus Flavus, Syncephalastrum spp. Tricoderma spp. Neurospora Sitophila, Rhizopus arrhizus and Mucor spp.

Some of these fungi have been previously reported as hydrocarbon degrading. Fungi have proven useful in bioremediation of polluted environment due to their ability to grow under stressed environment, secretion of extracellular enzymes (Chikere and Azubike, 2014). There is also specialty in the components of hydrocarbon degraded by microorganisms. Fungi can degrade certain components of hydrocarbon that bacteria cannot degrade and certain components that fungi may find difficult to degrade can be easily degraded by certain bacteria. Chikere and Azubike (2014) also reported that the ability of fungi to secrete extracellular enzyme which aid in the biodegradation of petroleum hydrocarbon makes these species of microorganisms useful in the science of bioremediation. This study is focus on the isolation and identification of hydrocarbon utilizing (degrading) fungi in oil spilled soil in the Niger Delta region of Nigeria.

MATERIALS AND METHODS

The materials used in for this research include: trowel, shovel, recyclable polythene bags and sack bags for the polluted soil sample collection apparatus which include microscope, glass slides, Petri dishes conical flasks and dropping pipette etc. Crude oil polluted soil samples were collected from three different locations in an oil spill site in Ikaramar community in the Okordia Zarama clan, within Yenagoo Local Government Area of Bayelsa State. This community is host to SPDC and Agip oil facilities within the Niger Delta. The soil samples were collected from a depth of 0 to 30cm of the top layer soil, put into clean and sterile black polythene bag and transported to the laboratory for biological analysis.

Chemicals and media

Chemicals and media include 95% ethanol lactophenol blue stain, Sabouraud Dextrose Agar, Bushnell Haas broth medium and nutrient Agar. The composition of the Sabouraud Dextrose Agar include: Dextrose (40 g/l), Peptone (10 g/l) and agar (15 g/l), Bushnell Haas broth medium include: MgSO₄ (0.2 g/l), KH₂PO₄ (1 g/l), K-HPO₄ (1 g/l), CaC₂O₄ (0.02 g/l), NH₄NO₃ (1 g/l) and FeCl₃ (0.05 g/l) with Chloramphenicol and Nutrient Agar include: Peptide digest (5 g/l), Beef extract (5 g/l), Yeast extract (1.5 g/l), NaCl (5 g/l) and Agar (1.5 g/l) PH (7.4).
Table 1. Microscopic and macroscopic morphology of Fungi isolates.

<table>
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<tr>
<th>Organisms</th>
<th>Microscopic morphology</th>
<th>Macroscopic morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>Presence of septate hyphae, black long, smooth, erect conidiophores Hyalineichoromously branched vesicle round, radiate head.</td>
<td>Brownish-black mycelium with dark pores on the surface.</td>
</tr>
<tr>
<td>Aspergillus fumigates</td>
<td>Presence of rough conidiophores, with uni/biseriate phialides whose vesicle is round with radiate head.</td>
<td>Presence of blue-green to yellow coloration from surface.</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>Presence of septate hyphae, ongsmooth, colorless and sometimes brownish conidiophores with round radiate head vesicles and biseriate phialides.</td>
<td>A yellow-green color with a creamy edge appears on the surface, which appears golden to reddish brown on the reverse side.</td>
</tr>
<tr>
<td>Candida species</td>
<td>Single clusters of blastoconidia which is round and elongate. Long branched pseudohyphae were also observed.</td>
<td>A creamy to yelowish colonies with smooth, pasty, glistening or dry, wrinkled and dull color.</td>
</tr>
<tr>
<td>Penicillium species</td>
<td>Presence of red pigment with edges surrounded by whitish margin. Also the conidiophores are branched. Septate and fruity mycelium is observed.</td>
<td>A bluish-green filament is seen which changes to powdery greenish brown. It has brush phialospheres arrangement.</td>
</tr>
<tr>
<td>Mucor species</td>
<td>Presence of visible spore and short sporangiospores with non septatehyphae.</td>
<td>Slimy colonies of texture with dark pigmented spores.</td>
</tr>
<tr>
<td>Fusarium species</td>
<td>Presence of dark pigment of micro and macro conidiophores.</td>
<td>Presence of sickle-shaped macroconidia that is yellow to purple in colour.</td>
</tr>
<tr>
<td>Rhodotorula</td>
<td>Spherical to elongate budding yeast-like cells or blastoconidia.</td>
<td>Colonies are coral red to salmon-coloured or slightly orange, smooth to wrinkled, highly glossy to semi-glossy.</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>Presence of stolon’s and pigmented rhizoids, the formation of sporangiospores, singly or in groups from nodes directly above the rhizoids and apophysate, columnellate, multisспорed, generally globose sporangia.</td>
<td>Presence of zygomycetes spores with dark pigment colonies are fast growing and cover an agar surface with a dense cottony growth that is at first white becoming grey or yellow brown with sporulation.</td>
</tr>
<tr>
<td>Trichodermatopsis</td>
<td>Presence of conidiophores repeatedly branched irregularly verticillate, bearing clusters of divergent, often irregularly bent, flask-shaped phialides.</td>
<td>Presences of green conidia filament that resembles pencilli conidia are mostly green, sometimes. Hyphae with smooth or rough walls and are formed in slimy conidia heads (glorospora) clustered at the tips of the phialides.</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>Microscopy shows ascending to erect olivaceous-green apically branched, elongate conidiophores producing branched acropetal chains of smooth-walled conidia.</td>
<td>Presence of light to grayish surface, gray to black surface blastoconidia.</td>
</tr>
</tbody>
</table>

**Isolation, enumeration and identification**

The fungi were isolated from the soil sample by culturing them under growth conditions of media (Sabouraud Dextrose Agar and nutrient agar). 1 g of homogenized and sieved oil polluted soil sample was weighed and dissolved in 9 ml of sterilized distilled water in nine replicates and properly shaken. Each of the resultant solution was made to 10⁰ and the diluents were further serially diluted up to 10⁻⁰. 1ml of each diluent was then inoculated into sterilized petri dish. The micro and sterilized Sabouraud Dextrose Agar media was poured into each of the Petri dish containing soil water inoculums. The plates were rotated in clockwise and anticlockwise direction to ensure uniform spread of the inoculums. These plates were allowed to set and then incubated upside down position at 37°C for one week. The plates were observed with magnifying hand lens for hydrocarbon degrading fungal growth after 3 to 5 days.

The plate showing between 30 to 300 colonies was recorded. From the counting, the total viable microbe cells in the sample were expressed as colony forming unit (cfu/g) of the sample.

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**Screening of Fungi hydrocarbon degradation (utility)**

A pure culture of hydrocarbon degrading fungi was further subcultured in another prepared Sabouraud Dextrose agar enriched with nutrient agar for further purification and identification.

Fragment of a well grown pure culture was immersed in 0.1 ml of 95% ethanol on a glass slide. The ethanol was allowed to evaporate and the specimen was stained with 0.1 ml lactophenol blue stain. The stained specimen was then covered with cover slide and viewed microscopically. The resultant morphology was compared using the guide provided by Pepper and Gerba (2005); Els et al. (2007); Barton and Hunter (1972); Benson (2002) (Table 1).

**Microscopic identification of fungi**

A pure culture of hydrocarbon degrading fungi was further subcultured in another prepared Sabouraud Dextrose agar enriched with nutrient agar for further purification and identification.

Fragment of a well grown pure culture was immersed in 0.1 ml of 95% ethanol on a glass slide. The ethanol was allowed to evaporate and the specimen was stained with 0.1 ml lactophenol blue stain. The stained specimen was then covered with cover slide and viewed microscopically. The resultant morphology was compared using the guide provided by Pepper and Gerba (2005); Els et al. (2007); Barton and Hunter (1972); Benson (2002) (Table 1).
### Table 2. Fungi isolates from the three biosludge samples.

<table>
<thead>
<tr>
<th>Samples locations</th>
<th>Location 1</th>
<th>Location 2</th>
<th>Location 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>Aspergillus niger</td>
<td>Aspergillus niger</td>
<td></td>
</tr>
<tr>
<td>Candida species</td>
<td>-</td>
<td>Candida species</td>
<td></td>
</tr>
<tr>
<td>Penicillium species</td>
<td>Penicillium species</td>
<td>Penicillium species</td>
<td></td>
</tr>
<tr>
<td>Mucor species</td>
<td>Mucor species</td>
<td>Mucor species</td>
<td></td>
</tr>
<tr>
<td>Rhodotorulla species</td>
<td>Rhodotorulla species</td>
<td>Rhodotorulla species</td>
<td></td>
</tr>
<tr>
<td>Rhizopus</td>
<td>Rhizopus</td>
<td>Rhizopus</td>
<td></td>
</tr>
<tr>
<td>Trichorderma species</td>
<td>Trichorderma species</td>
<td>Trichorderma species</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Cladospermorium species</td>
<td>Cladospermorium species</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Fungi density in each of the polluted soil samples.

<table>
<thead>
<tr>
<th>Soil Samples</th>
<th>Location 1</th>
<th>Location 2</th>
<th>Location 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total heterotrophic Fungi</td>
<td>3.6x10^5</td>
<td>7.1x10^5</td>
<td>9.51x10^6</td>
</tr>
<tr>
<td>Hydrocarbon utilizing Fungi</td>
<td>5.3x10^4</td>
<td>8.3x10^3</td>
<td>2.51x10^4</td>
</tr>
</tbody>
</table>

### RESULTS

#### Isolated and identified fungi in biosludge samples

Table 2 shows the isolated and identified fungi biosludge while Table 3 show fungi enumeration for each polluted soil.

### DISCUSSION

Bioremediation has been shown to be an effective, economical and environmentally friendly organic pollutant treatment method in which microbes are used to degrade hydrocarbons (Greetha et al., 2013). In this work, hydrocarbon degrading fungi were isolated and screened then identified by microscopic and macroscopic method, in crude oil polluted soil. The soil as stated in the methodology was collected from crude oil spilled site in Ikarama community, in Yenagoa Local Government Area of Bayelsa State. Eight different strains of fungi were isolated and identified. These include, *Penicillium* spp, *Candida* spp, *A. niger*, *Mucor* spp, *Rhodotorulla* spp, *Rhizopus*, *Trichorderma* spp and *Cladospermorium* spp.

The population of hydrocarbon degrading fungi, isolated from the soil which was collected from three different sampling stations is 5.3x10^4, 8.3x10^3 and 2.51x10^4 CFU/g for station 1, 2 and 3, respectively. While total heterotrophic fungi are 3.6x10^5, 7.1x10^5, and 9.51x10^6. *Penicillium* spp, *Trichorderma Candida* spp, *A. niger*, *Mucor* spp, *Rhodotorulla* spp, *Rhizopus*, *Phanerochaete* spp, *Alternaria alternate* and *Fusarium* spp are strains of fungi that have already been discovered by different scholars, to degrade different components of petroleum.

This study has further revealed that, oil polluted soil in the Niger Delta is habited by Hydrocarbon degrading fungi, which can be biostimulated to enhance oil pollution cleanup in the area. The result of this study is an indication that many of the oil spilled soil in Bayelsa may be inhabited by potential hydrocarbon degrading fungi which could be exploited for the cleanup of the polluted environment in the state.
Conclusion

In this study, eight different strains of hydrocarbon utilizing fungi have been isolated and screened in polluted soil, obtained from polluted site in Okordia/Zarama land of Bayelsa state. The result of this study has proven that apart from bacteria that are versatile in the environment and known for hydrocarbon degradation, the oil polluted soil in Okordia/Zarama area of Bayelsa state also contains fungi that can be harnessed and exploited for oil pollution cleanup.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCE


Technical evaluation of soil and water conservation measures in Maego Watershed, North Ethiopia

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Mekelle Agricultural Research Center, P. O. Box 492, Mekelle, Ethiopia.

Received 11 February, 2018; Accepted 5 April, 2018

Soil and water conservation (SWC) practices have been carried out to solve land degradation problems in Ethiopia since the last 3 decades. Technical evaluation of the implemented SWC structures is essential for effectiveness and sustainability of the measures. Therefore, the objective of this study was to identify and evaluate the technical quality of constructed physical SWC structures in Maego watershed, Ethiopia. The performance of the structures was evaluated based on technical standards set by Ethiopian Ministry of Agriculture. Parallel transect walk method was used to measure the dimensions of the structures. The major types of SWC structures constructed in the study watershed are stone bund, hillside terrace, bench terrace, stone bund/hillside terrace + trench and gabion check dams. Some of the structures, especially the old ones which are found on the uncultivated land did not meet the site specific width and height standards. Furthermore, some of the spacing of SWC structures was not set depending on the desired vertical intervals/slope. With similar biophysical features of the watershed, some of the structures were narrowly spaced and some others were spaced greater than the standard. Most of the structures, especially the old ones are filled by sediment deposition. The main reasons for the failure to achieve the technical quality based on the technical standards were knowledge and skill gaps, free grazing and more attention was given to coverage areas rather than technical quality at the beginning of the watershed treatment. Furthermore, there is no maintenance of the structures on the communal lands. Therefore, awareness creation for the local community and capacity building about layout of structures for the local technical leaders should be given. Moreover, more attention should be given to technical quality rather than coverage area of SWC structures.

Key words: Soil and water conservation, technical evaluation, Maego watershed.

INTRODUCTION

Ethiopia is one of the countries in sub-Saharan Africa most seriously affected by land degradation (FDRE, 2015). Land degradation is the major cause of low agricultural productivity and loss of soil nutrients in the country (Adimassu et al., 2014; Haregeweyn et al., 2015). Without accounting for downstream and offsite effects
such as flooding and damage to infrastructure resulting from erosion, the minimum estimated annual costs of land degradation in Ethiopia range from 2 to 3% of agricultural GDP (FDRE, 2015). Most of the soils in Ethiopia are highly degraded (ATA, 2013). The highest land degradation in the form of soil erosion has been recorded in the highlands of Ethiopia (>1500 masl) (vanmaercke et al., 2014). These high erosion rates constitute a significant threat to sustainable food production in the region (taye et al., 2013). Rapid growth in human and livestock population (over grazing) causing increased soil erosion rates, loss in plant productivity, and a generalized over-exploitation of natural resources (FDRE, 2015; galdino et al., 2016). Furthermore, erosive rainfall and rugged topography are some of the factors attributed to soil erosion. abera (2003) reported that the average annual rate of soil loss in the country is estimated to be 12 tha⁻¹ year⁻¹ and it can be even higher (300 tha⁻¹ year⁻¹) on steep slopes and on places where the vegetation cover is low. Poor land and water management practices and lack of effective planning and implementation approaches for conservation are responsible for accelerating degradation on agricultural lands. In the tigray region, northern Ethiopia, steep slopes have been cultivated for many centuries and hence they are subjected to serious soil erosion (mekuria et al., 2007).

Soil and water conservation (SWC) measures have been extensively carried out in Ethiopia by governmental and non-governmental organizations so as to reduce soil erosion (moard, 2005). The impacts of physical SWC measures can be classified into short- and long-term effects based on the time needed to become effective against soil erosion (morgan, 2005). The short-term effects of stone bunds are the reduction in slope length and the creation of small retention basins for run-off and sediment. SWC practices can considerably reduce soil loss due to water erosion if they are well planned, correctly constructed and properly maintained. Even though a lot of efforts have been carried out in SWC practices, the main problem of the country for agricultural productivity is still land degradation with a limited success due to less willingness of the local community to maintain the extensively introduced practices of SWC practices in the communal areas. ofgeha (2017) also reported that various hindering factors such as lack of capital, poverty, small size of their land and other socio-economic and physical factors were observed which obstacle to apply the SWC technologies. Failures in SWC suggest that more detailed information should be used for appropriate layout and design of site specific SWC measures. For a specific area, it is necessary to consider where, and how to start SWC practices. The most important factor that requires immediate consideration for SWC activities is that they have to be carefully designed and constructed taking into account ground realities of the required sites (gebre and weldemariam, 2013). It is also suggested that the performance of SWC structures should be better monitored over time. Hence, in order to support the country’s effort in planning and implementation of SWC measures, a study that evaluates the quality of SWC practices implemented through watershed approach is essential. The objective of this research is, therefore, to identify and evaluate the quality of the physical SWC measures implemented in maego watershed, Ethiopia; and relate their ages to their effectiveness in trapping sediments and their sustainability.

MATERIALS AND METHODS

Description of the study area

The research was conducted in maego watershed, negash village, kilite awulaelo district, tigray, ethiopia (figure 1). This watershed was selected for the study due to its representativeness with respect to intensive SWC practices such as stone bunds, hillside and bench terraces, trenches and gabion check dams have been carried out. SWC interventions have been started before 15 years ago in this watershed; but they have not been evaluated scientifically. It is dominated by rugged topography. Its elevation ranges from 2218 to 2665 m.a.s.l (figure 2). The study area is characterized by weina-dega (middle highland).

It receives an annual rainfall of 300 to 1200 mm with an average of 583.9 mm and its temperature range is between 16 and 34°C (Ethiopia National Meteorological Service Agency, unpublished). The rainfall in the study area is unimodal with erratic in variability and amount within and among seasons. The main rainy season is very short and extends from June to the first week of September. Agriculture is the main source of income in the area, where the farming system is characterized by small-scale production of mixed crops and livestock. Vegetation types and the agriculture production are influenced by seasonality in rainfall distribution. The major crops grown in the area are wheat and barley and the major livestock production are composition of cattle, sheep, goat, chickens and bee colony. The most dominant indigenous and exotic trees grown on the uncultivated part of the watershed are acacia etbaica and eucalyptus camaldulensis, respectively. The land holding size of most farmers in the study area was less than 1 ha. The major soil types of the study site were lithic leptosol, eutric leptosol, chromic luvisol and calcric cambisol (FAO, 1998).

Data collection

Primary data were collected from the performance evaluation of already implemented SWC structures in the watershed with less than and greater than 7 years old after implementation, because intensive SWC measures have been conducted before 7 years ago in the watershed. Secondary data were collected from the bureau of agriculture and rural development. The topographic transect walk method was employed for the assessment of existing SWC measures in the watershed. During the transect walk, observations in level of soil erosion before and after the SWC measures, and types of SWC structures were recorded. Slope map and land use map of the watershed were prepared using digital elevation model (DEM) with 30 m resolution and Arc-GIS software (figure 3). The area of the watershed is dominated by bush land (table 1). To evaluate the technical quality of the main soil conservation structures in the study area, a careful investigation was conducted...
along 7 parallel transect lines performed from the upper to the bottom position of the watershed (Figure 3). Geographical Position System (GPS) with an error of ±3 was used for the direction of each transect walk. Along each transect walk, the spacing between structures, the height and width of each structure as shown in Figure 4 and sediment deposition were measured with an interval of 150 m in each slope category of cultivated and uncultivated lands. A total of 50 sample plots were used in all transect lines. Five consecutive SWC structures were measured in each sample plot. The measured technical values were compared with the site specific technical standards based on the technical manual/packages (MoA, 2005).

The accumulated sediment rate (t ha\(^{-1}\) year\(^{-1}\)) behind the major SWC structures was estimated by adopting the Equations 1, 2 and 3 described by Gebremichael et al. (2005) as:

\[
AA = \frac{10MA}{(T \times D)} (1) \\
MA = BD \times VA (2) \\
VA = WA \times HA (3)
\]

where AA is the annual sediment accumulation behind SWC structures (t ha\(^{-1}\) year\(^{-1}\)); MA, mass of accumulated sediment per unit length (kg m\(^{-1}\)); T, age of SWC structure (year); D, average spacing between SWC structures (m); BD, dry bulk density of sediment accumulated behind SWC structure (kg m\(^{-3}\)); VA, the unit volume of accumulated sediment (m\(^3\) m\(^{-1}\)); HA = depth of the accumulated sediment (m); WA, width of the sediment zone; and *, multiplication symbol. The value of D was calculated as a mean of the spacing between consecutive bunds on different fields in the watershed. Furthermore, informal interview was conducted with the local farmers and development agents.

Data analysis

The data collected were organized and summarized using Microsoft Excel and analyzed using descriptive statistics such as percentage and mean. Qualitative method was also used to describe the local farmers’ perception towards the limiting factors of SWC measures.

RESULTS AND DISCUSSION

Soil and water conservation practices in the study area

The major physical SWC structures constructed on farmlands, closure areas and grazing land of the watershed include stone bunds, trenches, bench terrace, stone bund + trench, hillside terrace and gully treatment with gabion check dams. Construction of stone bunds and hillside terraces started since 1998 in the watershed, whereas deep trenches and bench terraces were introduced before seven years ago. Even though most of the SWC structures in the watershed were constructed by
food for work programmes, there was also free contribution of the local community (MoARD, unpublished). The main purpose of construction of SWC measures was for conserving and rehabilitating of degraded area and increase agricultural productivity (MoARD, 2005). Even though more attention was given to physical SWC measures, some biological measures such as rehabilitation of gullies by elephant grass, plantation of *E. camaldulensis*, *Acacia saligna* and *Sesbania sesban* trees seedlings were started in the watershed. According to the local farmers, a lot of tree seedlings have been planted as biological measures in the watershed; but their survival was low due to moisture stress and free grazing of livestock.

**Stone bund structures**

Stone bunds have been constructed where stones were readily available on or near the part of the study watershed. They have been constructed perpendicular to slopes to reduce runoff and soil erosion in sloping areas (up to 20% slope of the watershed), and excess water can pass more easily through stone terraces. With continuing soil erosion, soil has been deposited uphill of the bund which resulted in the built up of terraces. It improves water availability for crop production. Nyssen et al. (2007) stated that crops that grow better on both sides of stone bunds could be due to water conservation. However, construction does require a large amount of labor; they are not convenient for ox-plowing and can habitats for potential harmful weeds and rodents (Teshome et al., 2014), they occupy part of the land.

Sediment deposition behind stone bunds in the study watershed was 192.3 and 83.6 t ha\(^{-1}\) year\(^{-1}\) for less than and greater than 7 years old of stone bunds, respectively. This is higher than other studies such as Nyssen et al. (2007) who reported that 57.26 t ha\(^{-1}\) year\(^{-1}\) soil deposited behind stone bunds in Degua Tembien, Ethiopia. Mekonen and Tesfahunegn (2011) also revealed that about 65.3 t ha\(^{-1}\) year\(^{-1}\) sediment was deposited behind SWC structures. Similarly, 59 t ha\(^{-1}\) year\(^{-1}\) sediment deposition was estimated in the Tigray region, Ethiopia (Gebrernichael et al., 2005). The different results could have occurred due to differences in agroecology and biophysical features. If there is high and erratic rain fall and the topography is with high slope category, more sediment deposition could be recorded. Moreover, the height of the bund should be high to deposit more sediments transported by run-off. The annual sediment accumulated on the young stone bund structures was by 108.7 t ha\(^{-1}\) year\(^{-1}\) higher than the sediment deposited on the old structures after their construction. This is because young structures have the capacity (space) to retain soil, which older bunds lack and the height of younger stone bunds is higher than the height of the old ones. According to the informal interview of the local farmers, more focus was not given to technical standards during the construction of old structures. Some bund structures were silted up and damaged due to runoff overtopping the bunds.

**Hillside terraces**

These structures are the most common physical measures constructed in steep degraded slopes (up to 50%) and shallow soils (Figure 5). Its objective is to convert steep slopes into a series of steps with nearly horizontal benches. The sediment deposition behind hillside terraces was 116.7 t ha\(^{-1}\) year\(^{-1}\) with less than 7 years old, but it was 91.5 t ha\(^{-1}\) year\(^{-1}\) with greater than 7 years hillside terraces old. Large amount of sediment was deposited in the lower part of the hillside areas because its their upper side was not well treated as shown in Figure 4. Terraces and check dams were filled with soil up to 1.5 m deep in Medego watershed, Ethiopia (Mekonen and Tesfahunegn, 2011). Hillside terrace is highly labour intensive.
**Figure 3.** (a) Land use map of the watershed and (b) Slope map of the watershed.

**Figure 4.** Survey layout of SWC structures.

**Stone faced trench**

This was constructed on up to 20% slope areas in both cultivated and uncultivated lands of the watershed. The trench was constructed above side of the stone bund with the dimensions of 3 m long and 1 m width and depth. Stone faced trench is very effective in controlling runoff and soil erosion. For low and medium rainfall areas, conservation measures should allow maximum retention of rainwater (Danano, 2002). Sediment deposition behind
stone faced trench with less than 7 years old was 89 t year⁻¹. They are suitable in areas with high stoniness and stable soils.

**Gully control measures**

From the field observation, the main causes of gully in the study area were over grazing, poor design of physical SWC structures and deforestation. In the study area, several gully rehabilitation measures were implemented in the past 15 years. The measures include loose stone check dam, gabion check dam, gully reshaping, biological measures such as elephant grass, *S. sesban* and other local grasses. However, most of the check dams are filled with sediment deposition and some of them started to collapse. The failures of these check dams were poor design lay out and specifications selection. Furthermore, there is no maintenance of the structures and the biological measures are being destroyed by free grazing. However, the study indicates that the potential of gullies is huge if more attention is given to technical standards, avoiding of free grazing and maintenance is performed on time.

**Evaluation of technical quality of soil and water conservation structures**

Top width of stone bunds on the slope categories of 0 to 8 and 8 to 20% was 20 to 120 and 43 to 120 cm, respectively. Whereas, the height of the bunds on the specified slope categories was 25 to 101 and 53 to 125 cm, respectively. Similarly, the height of hillside terraces on the slope categories of 20 to 45 and >45% was 48 to 198 and 75 to 225 cm, respectively; and the top width of the bunds was 30 to 100 and 20 to 60 cm on the specified slope categories, respectively. Danano (2002) stated that farmland terraces on slopes >10% should have a dimension of 65 cm height during construction. Majority of the stone bunds and hillside terraces have smaller height than site specific technical standards based on the guideline prepared by the MoA for each agroecology (Table 1). However, most of the stone bunds have higher top width than the recommended values and the top width of most of the hillside terraces have been constructed according to the technical standards (Table 2). Most of the stone bunds and hillside terraces which are below the recommended values in their height are old aged structures and are found on uncultivated land. This means, recently constructed SWC measures are with better technical quality than the old ones. Better maintenance of SWC structures by individual farmers has been observed on the cultivated land.

**Table 1. Area of each land use.**

<table>
<thead>
<tr>
<th>Land use type</th>
<th>Area (ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rain fed cultivated</td>
<td>237.52</td>
</tr>
<tr>
<td>Irrigated land</td>
<td>7.18</td>
</tr>
<tr>
<td>Forest land</td>
<td>94.21</td>
</tr>
<tr>
<td>Grass land</td>
<td>26.29</td>
</tr>
<tr>
<td>Bush land</td>
<td>607.3</td>
</tr>
<tr>
<td>Total</td>
<td>972.5</td>
</tr>
</tbody>
</table>
Table 2. Width and height of major SWC structures compared to site specific technical standards.

<table>
<thead>
<tr>
<th>Slope category (%)</th>
<th>Type of structure</th>
<th>Top width of structure compared to the recommended values (%)</th>
<th>Height of structure (lower side) compared to the recommended values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Above the standard</td>
<td>Equal with the standard</td>
</tr>
<tr>
<td>0 - 8</td>
<td>Stone bund</td>
<td>83.3</td>
<td>-</td>
</tr>
<tr>
<td>8 - 20</td>
<td>Stone bund</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>20 - 45</td>
<td>Hillside terrace</td>
<td>4.4</td>
<td>60.8</td>
</tr>
<tr>
<td>&gt;45</td>
<td>Hillside terrace</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. The spacing between SWC structures.

<table>
<thead>
<tr>
<th>Slope category (%)</th>
<th>Type of structure</th>
<th>Spacing between structures in each land use (m)</th>
<th>Recommended spacing (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cultivated</td>
<td>Uncultivated</td>
</tr>
<tr>
<td>0 - 8</td>
<td>Stone bund, trench</td>
<td>11.9 - 17.2</td>
<td>3 - 10.2</td>
</tr>
<tr>
<td>8 - 20</td>
<td>Stone bund</td>
<td>3.1 - 25.5</td>
<td>1.5 - 16.5</td>
</tr>
<tr>
<td>20 - 45</td>
<td>Hillside terrace, hillside terrace + trench</td>
<td>7.3 - 28.5</td>
<td>2.2 - 21</td>
</tr>
<tr>
<td>&gt;45</td>
<td>Hillside terrace</td>
<td>-</td>
<td>2.9 - 15</td>
</tr>
</tbody>
</table>

Most of the hillside terraces on the communal land of very steep slope and bunds have zero height in their upper side due to sediment deposition, so maintenance is needed. As reported by other authors, the different height of SWC structures resulted in soil depth gradients across the slope of the land (Gebremichael et al., 2005; Nyssen et al., 2007). Terraces on steep slopes which have not been built by large stones and are not supported by biological measures have started to collapse. The other observed problem in the study watershed was putting of large stones on small stones during construction of SWC structures. As per discussion with the farmers, the main reason for the failure to achieve some of the SWC structures based on the technical standards were knowledge and skill gaps, more attention was given to coverage areas rather than technical quality, shortage of stones and no maintenance in the communal lands. Furthermore, according to the response of the farmers from informal interviews, free grazing of livestock is among the major limitations to sustainable management of SWC measures. Without introducing and formulating improved livestock husbandry and livestock management policies, conservation interventions would never be sustained, no matter to what extent, the techniques are efficient and effective, and the approaches are relevant and accepted (Danano, 2002).

The spacing between SWC structures in the study area depends on slope and land use types. Their spacing was also determined by the interest of individual farmers. On the slope category of 0 to 8%, the spacing of stone bunds and trenches on cultivated land is almost similar with the recommended technical standards; however, there are narrowly and widely spaced bunds and trenches on the uncultivated land on the 0 to 8% slope (Table 3). There is irregular spacing between the existing SWC structures on the slope categories of 8 to 20, 20 to 45 and >45% on both land use types (Table 3). This means, with similar slopes and land use types, some of the structures are constructed much narrowed and some of them are spaced more than the recommended standards. Depending on the site specific measurements, 83, 38 and 32% of the structures on cultivated land have been constructed according to the technical standard spacing on the slope categories of 0 to 8, 8 to 20 and 20 to 45%, respectively. Even though 75 and 60% of the structures on the uncultivated land are narrowly spaced on 0 to 8 and 8 to 20% slopes, respectively, 55% of them have been constructed with the standard spacing on the 20 to 45% slope.
Construction of SWC structures more than the recommended standards could lead to unnecessary wastage of labor and land.

On the other hand, 78% of the structures on the cultivated land are widely (greater than recommendation) spaced on the highest slope (>45%) category. The spacing of structures increased with increasing slope (Table 3). Especially, the widest spacing between hillside terraces on the 20 to 45% slope of the cultivated land is not recommended. Technically, the spacing should decrease when slope gradient increases (Gizaw et al., 2009). According to WTCER (2011), the basic principles for determining the spacing of bunds are: seepage zone below the upper bund should meet the saturation zone of lower bund, the bunds should check the surface runoff at the point where flow attains an erosive velocity and the bund should not cause inconvenience in agricultural operations. Other authors reported that closely spaced and taller structures are constructed on the steep slopes, however, such structures are limited as the slope becomes very steep (Olarieta et al., 2008; Nyssen et al., 2007). Farmland terraces on slopes >10% are to be spaced at 1 m vertical interval (VI) and terraces on slopes <13% are to be spaced at 1.5 m VI (Danano, 2002). In the study area, technical layout for spacing of SWC structures has been done by selected local farmers who can read and write. However, they have technical limitation in using vertical interval and knowing of slope to set the spacing of structures, that is why different spacing of SWC structures has been recorded on similar biophysical features of the watershed.

Table 3 shows that the spacing of structures on cultivated land is wider than the spacing on uncultivated land with similar slopes. Most farmers perceived that constructing bunds in narrow spacing may create difficulty in plowing activities and reduces farm size. In this study, the land occupied by stone bund, hillside terrace and deep trench of SWC structures ranges from 3.75 to 13.6% of the cultivated land. In Ethiopia, it was recommended that fanya juu (type of SWC structure) occupies 2 to 15% of the land area for a slope of 3 to 15%, stone bunds occupy 5 to 25% for a slope of 5 to 50% and soil bunds occupy 2 to 30% for a slope of 3 to 30% (Teshome et al., 2014). In experimental plots established in the central highlands of Ethiopia, soil bunds occupy 8.6% of cultivable land (Adimassu et al., 2012).

Most of the farmers in the bottom part of the watershed have used cut-off drain as traditional SWC to protect their cultivated land from run off and soil erosion. However, this was not supported by technical experts, so can lead to rill and gully formation as observed in the field. Cutoff drains are essential mechanical structures to dispose runoff water coming from up slopes and hillsides at safe velocities and protect cultivated lands. For laying out cutoff drains, a gradient of 0.5 to 1% is recommended (Danano, 2002). Gizaw (2010) indicated that farmers have constructed a small drainage ditch across the slope to protect the lower field from concentrated runoff during heavy storms. Wallie and Fiseha (2015) reported that the fitness of existing SWC practices with the recommended ones was low from required soil conservation structures. Stability of SWC structures depend on various factors such as slope of the land, construction quality, construction material, support of physical structures by biological measures, and appropriateness of structure to the site conditions (Olarieta et al., 2008). For example, appropriateness of SWC structures with increasing slope comes in the order of soil bund, stone-faced bund, stone terrace, bench terrace and hillside terrace, respectively (Nyssen et al., 2007).

**Effect of SWC practices on inter-terrace slope**

The highest slope reduction was recorded on stone bund structures followed by stone bund with trenches (Table 4). This could be related to their difference in ages after construction. On average, after stone bund building, slope gradient decreases by 1% every 3 years (Nyssen et al., 2007). Reduction of slope steepness after construction of SWC structures shows that bunds are changed into benches due to sediment deposition.

**Conclusions**

The major constructed physical SWC structures in the watershed are stone bunds, trenches, bench terrace, stone bund + trench, hillside terrace and gully treatment with gabion check dams. Even though more attention was given to physical SWC measures at the beginning of the watershed management, a lot of tree seedlings have
been planted as biological measures in the watershed; but their survival was low due to free grazing of livestock and other factors. The highest sediment accumulation behind SWC structures was recorded on the young SWC structures. Some old structures have been tilted up and damaged due to runoff overtopping of them. Some of the height and width of SWC structures, especially the old ones have not been constructed according to the standard technical specifications. Furthermore, there are layout limitations in spacing of the structures according to site specific biophysical features. Some of the structures are constructed more narrowed than the standards and some of them are spaced more than the recommended standards. According to the local farmers, the main reason for the failure to achieve some of the SWC structures based on the technical standards were knowledge and skill gaps, free grazing and more attention was given to coverage areas rather than technical quality. Furthermore, there is no maintenance of the structures on the communal lands. Therefore, awareness creation about the use of integration of physical and biological SWC measures following watershed approach, institutional setups and avoiding of free grazing should be given to the local community members. Education level is essential for layout of SWC measures, and intensive capacity building about the layout of SWC structures should be given to them. Moreover, more attention should be given to technical quality rather than coverage area of SWC structures.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Determination of cadmium, chromium and lead in four brands of herbal bitters preparation sold in Benin-city, Southern Nigeria

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This study evaluated the level of three toxic heavy metals (cadmium, chromium and lead) in four selected brands of herbal bitter preparations sold in Benin City, Southern Nigeria purchased from chemists' shops, using the flame atomic absorption spectrophotometric technique. The result of the atomic absorption spectroscopy (AAS) analysis showed that Chromium (Cr) was not detected in any of the four brands. Lead (Pb) was present in only two herbal bitters B (28.0 ppm) < S (75.0 ppm) while Cadmium (Cd) was detected in all the herbal bitters with a trend A (3.2 ppm) < Y (5.3 ppm) < B( 31.0 ppm) < S (45.8 ppm). This observed trend could be attributed to the use of untreated organic manure, poor waste management and disposal practices, and the use of unsafe portable water for the preparations. It signals an epidemiological timed bomb that should be prevented by the Foods and Drug regulatory bodies.

Key words: Epidemiology, heavy metal, herbal bitter preparations, waste disposal, waste management.

INTRODUCTION

Over three-quarter of the third world's population relies on traditional herbal remedies to meet their primary health care needs (WHO, 2007; Osamor and Owumi 2010). Herbal preparations are sold in major markets, motor parks and in health shops. They are in form of herbal teas and bitters, packaged liquid preparations, capsules, packaged powdered medicinal plant parts, and fresh medicinal plants part.

In most countries, these products although not licensed as drugs or pharmaceutical products by the appropriate regulatory agencies, are recognized as dietary supplements. Of the herbal preparations, the use of herbal bitters is on the increase with scarce documentation on the usage extent and pattern among any given population (Showande and Amokeodo, 2014). These poly-herbal liquid preparations which contain bitter herbs are commonly used as carminatives, aphrodisiacs, immune boosters, anti-infectives, aperitifs and to improve digestion (Showande and Amokeyodo, 2014). Most of these preparations are made from plants

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collected from the environment with water as a medium for the extraction of the bioactive constituents. This underscores the need for proper waste disposal and management practices in addition to quality assessment of water used for these preparations. Also of concern is that most of these products have not been certified safe for patient’s use. They find their way into the market with little or no data on their safety and efficacy due to poor regulation and monitoring by the appropriate agencies in the third world countries.

In Nigeria today, like most third world countries, these products are ubiquitous with acclaimed cure-all potencies despite little or no scientific documentation on their safety, potencies, stability, and relevant physico-chemical profile.

The medicinal plants used for the formulation of these herbal preparations are collected from the environment with reported high incidence of pollution due to unregulated emissions from industrial and agricultural activities. This further underscores the urgent need for quality assessment of these herbal products found in the drug markets which a large percentage of the population depend on for the treatment of illnesses. This will provide information on their safety, potency and stability that could be used to develop a monograph for their standardization thus improving on public health. Medicinal plants like every other plant are vulnerable to bioaccumulation of toxic heavy metals from the environment. Several factors like; the nature of soil and its heavy metal concentration, proximity of farmlands to dumpsites and automobile traffic, use of untreated organic manures and sewages sludge, atmospheric depositions and stage of development of the plant have been known to affect the bioaccumulation of heavy metals in plants (Lake et al., 1984; Scott et al., 1996; Voutsas et al., 1996, Sinha et al., 2005; Sharma et al., 2006, Liu et al., 2007; Nwachukwu et al., 2010).

The control of their level in medicinal plants used for medicinal herbal preparations is therefore an imperative to safeguard their quality and safety. In view of this, the objective of this study is focused on the use of atomic absorption spectrophotometry to evaluate the level of three toxic heavy metals: Pb, Cd and Cr in four selected brands of herbal bitter preparations purchased from chemist shops in the Oba market area of Benin City, Edo State, Nigeria.

MATERIALS AND METHODS

Study area and sample collection

The study area, Benin City (6.34°N and 5.63°E), is located in Edo State, and it is one of the major cities in the South-South geopolitical region of Nigeria with a population of approximately 1.2 million people. The study samples were purchased in August 2013 from the chemist shops in the Oba market that is one of the major markets in the city.

Sample collection and description

Four herbal bitters preparation (coded as A, B, S and Y) and containing extracts from various medicinal plants (Table 1) were bought from the Village Chemist shops in August 2013. The samples were selected such that they were still within the expiration limit specified by their respective manufacturers. The samples were kept in the refrigerator at 4°C prior to analysis carried out at the Springboard Research Laboratory, a private laboratory in Awka, South Eastern Nigeria.

Experimental methodology

The wet digestion method (Adrian, 1973; Allen et al., 1986) was used with modification. The concentrations of the toxic heavy metals (Pb, Cd and Cr) in the four selected brands were determined using a 240FS Agilent flame Atomic Absorption Spectrophotometer (AAS) after prior digestion (on a hot plate) of 1 ml of the sample in a fume chamber. 15 ml of a ternary mixture of the concentrated acids, HNO$_3$:H$_2$SO$_4$:HClO$_4$ (5:2:8 v/v/v) was used for the digestion. The digestion was completed when a transparent solution was obtained. The digested mixture was allowed to cool and filtered using an ashless filter paper and the filtrate diluted to 50 ml with deionised water. This resulting diluted filtrate solution of the digested sample was then analysed for the presence of the heavy metals using the AAS calibrated using the standard nitrate salt solutions of the respective heavy metals. The instrument was operated following the Manufacturer’s Instruction (Agilent, 2013).

RESULTS AND DISCUSSION

Cr was not detected in any of the four brands. Lead was present in the herbal bitters B (28.0 ppm) and S (75.0 ppm) but was not detected in the herbal bitters A and Y. Cadmium was detected in all the four herbal bitters with a trend A (3.2 ppm) < Y (5.3 ppm) < B (31.0 ppm) < S (45.8 ppm) (Figure 1). In all the selected brands, the values of the evaluated heavy metals were above approved limits in Table 2 (WHO, 1998; FAO, 2005; WHO, 2007).

Similar findings on high level of the heavy metals: Cd (0.83-10.6 ppm) and Pb (2.6-48 ppm), in herbal preparation were reported by Nwoko and Mgbeahuruike (2011) from Aba, Abakaliki, Enugu and Onitsha South Eastern Nigeria. Kalagbor et al. (2014) and Umedum et al. (2014) also reported on heavy metal pollution in medicinal plants collected from farmlands in Nigeria. Similar reports by other authors from other parts of the world are also documented (Gasser et al., 2009; Maobe et al., 2012; Lakshmi et al., 2013).

Toxic heavy metals are of no physiological use with deleterious health implication even at low exposure. Several cases of human disease, disorders, malfunction and malformation of organs due to metal toxicity have been reported (Adepoju-Bello and Alabi, 2005; Obi et al., 2006). Pb causes hyperactivity in children affecting the cognitive performance (Owen and Pikering, 1994),
Table 1. Samples description.

<table>
<thead>
<tr>
<th>Herbal bitters code</th>
<th>A</th>
<th>B</th>
<th>S</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packaging</td>
<td>Supplied as 200ml liquid content in slight greenish opaque plastic bottle</td>
<td>Supplied as 200ml liquid content in a brownish plastic bottle</td>
<td>Supplied as 330ml net volume in a brownish opaque plastic bottle</td>
<td>Supplied as 200 ml liquid content in a transparent plastic bottle</td>
</tr>
<tr>
<td>NAFDAC registration status</td>
<td>Registered</td>
<td>Registered</td>
<td>Not registered</td>
<td>Registered</td>
</tr>
<tr>
<td>Indication</td>
<td>None</td>
<td>None</td>
<td>Management of hypertension, insomnia and strengthening the immune system.</td>
<td>Anti-oxidants</td>
</tr>
</tbody>
</table>

Figure 1. Concentration of Cr, Pb and Cd in four market brands of herbal bitters preparation in Nigeria.
impaired blood synthesis and hypertension. Cd is extremely toxic causing bone porosity, inhibition of bone repairs and death. The observed trend in the level of the heavy metals evaluated in this study could be attributed to environmental pollution due to industrial activities, poor waste management and disposal practices, and the use of unsafe portable water for the preparations (Adeyeye, 2005).

This underscores the need for the provision of waste handling facilities in most developing and underdeveloped countries, use of treated organic manure and sewage sludges in farmlands, cleaning of contaminated sites and strict enforcement of relevant environment and waste management and disposal laws by the relevant authorities, strict enforcement of town/urban planning master plan, strict monitoring of quality assurance procedures and market surveillance for medicinal herbal preparations by the relevant foods and drug regulatory authorities in third world countries as it is done for orthodox preparations.

Table 2. Limits of Cd, Cr and Pb in medicinal herbals.

<table>
<thead>
<tr>
<th>Heavy Metal</th>
<th>FAOa (ppm of Food products)</th>
<th>WHOb (mg/Kg of HM)</th>
<th>Canadae (ppm of RHM)</th>
<th>Canadae (mg/day for FHP)</th>
<th>Chinae (ppm of HM)</th>
<th>Malaysiae (mg/Kg of FHP)</th>
<th>Thailandc (ppm of HM/FHP)</th>
<th>NSFc (ppm of RDS)</th>
<th>NSFc (mg/day for FDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.01</td>
<td>0.3</td>
<td>0.3</td>
<td>0.006</td>
<td>1.0</td>
<td>na</td>
<td>0.3</td>
<td>0.3</td>
<td>0.006</td>
</tr>
<tr>
<td>Cr</td>
<td>0.08</td>
<td>na</td>
<td>2.0</td>
<td>0.02</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Pb</td>
<td>0.01</td>
<td>10</td>
<td>10.0</td>
<td>0.02</td>
<td>10.0</td>
<td>10</td>
<td>10.0</td>
<td>10.0</td>
<td>0.02</td>
</tr>
</tbody>
</table>


The authors have not declared any conflict of interests.

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