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Effect of Bioaugmentation on Crude Oil Contaminated Pond Water as revealed by Oxidative Stress in African Catfish (*Clarias gariepinus*)

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ABSTRACT: Contaminated water bodies arising from oil exploration and production activities pose serious threats to aquatic life. In the present study, juvenile African catfish (*Clarias gariepinus*) were exposed to lethal concentration of crude oil (0.9 %, v/v) and bioaugmentation treatment using 10% mixed culture of *Alcaligenes eutrophus* (BNS-09) and *Bacillus sphaericus* (BNS-05) for 96 h compared with control. Life sustaining indices (pH, dissolved oxygen (DO) and conductivity were monitored. Markers of oxidative stress (superoxide dismutase, catalase, reduced glutathione and lipid peroxidation (malondialdehyde) levels) were evaluated in catfish liver. Results showed significant decrease in pH and DO with increase in conductivity in crude oil polluted water (CPW) compared with control. There was no significant difference between bioaugmentation treated water (BTW) and control (C). After 96 h, lipid peroxidation increased significantly in liver of catfish in CPW group while there was no significant increase in liver of catfish in BTW group compared with control. Reduced glutathione level, superoxide dismutase and catalase activity decreased significantly in liver of catfish in CPW group with no significant difference found between BTW group and control. These findings reveal that bioaugmentation technique is effective in overcoming oxidative stress in fish exposed to crude oil even at lethal concentrations.

Keywords: *Alcaligenes eutrophus*; *Bacillus sphaericus*; bioaugmentation; catfish; *Clarias gariepinus*; crude oil

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INTRODUCTION

Crude oil is made up of thousands of complex gaseous, liquid and solid organic compounds with hydrocarbons being the most abundant (Kerambrun *et al*., 2012). The polycyclic aromatic hydrocarbons (PAHs) in crude oil exhibit high toxicity on aquatic life (Achuba *et al*., 2003, Gonzalez *et al*., 2008). PAHs are primarily metabolized and detoxified, by catalytic activities linked to cytochrome P450 system (Sturve *et al*., 2006) and its metabolism leads to the formation of reactive oxygen species (ROS) through the formation of redox labile metabolites. Oxidative stress occurs when reactive oxygen species (ROS) such as superoxide radicals (O_2), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO) are produced in excess, leading to oxidization of cellular components (Almeida *et al*., 2007). This condition has different detrimental effects on organisms such as loss of DNA integrity, induction of mutations, chromosomal aberrations and birth defects (Frenzilli *et al*., 2004).

Another harmful effect of ROS is the lipid peroxidation, process in which the cell membranes are oxidized leading to the formation of by-products such as malondialdehyde (MDA) which is a biomarker widely used to indicate injuries caused by oxidative stress (Van der Oost

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et al., 2003). In order to protect the cells against excessive ROS, the organisms have many antioxidant defenses, such as enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These enzymes act by removing ROS, thus promoting a protection against its harmful effects (Livingstone, 2001). Increased attention has therefore been paid to developing and implementing innovative technology for cleaning up oil spill in the environment.

Bioremediation is an attractive approach of cleaning up crude oil because it is simple to maintain, applicable over large areas, cost-effective and leads to the complete mineralization of the contaminant (Prince *et al*., 2010). Bioremediation functions basically on biodegradation, which involves the complete mineralization of organic contaminants into carbon dioxide, water and inorganic compounds by biological agents such as microorganisms.

Bioremediation for the use of oil spill clean-up is either by bioaugmentation or biostimulation. Bioaugmentation is the addition of microorganism capable of degrading the toxic hydrocarbons, in order to achieve a decrease of the pollutants. On the other hand, biostimulation is the addition of nutrients needed by indigenous hydrocarbon degrading microorganisms in order to achieve maximum degradation of toxic compounds present in the oil (Boufadel *et al*., 2006). However, only very limited number of field trials providing efficacy of this technology have been reported (Venosa *et al*., 2002).

Previous studies in the Enzyme and Microbial Technology (EMT) laboratory, Department of Biochemistry Federal University of Technology, Akure (FUTA), Nigeria revealed high crude oil degradation efficiency of some bacteria and actinobacteria (Olajuyigbe *et al*., 2015) Among the bacteria are *Bacillus sphaericus and Alcaligenes eutrophus*. The use of *B. sphaericus* in bioremediation of heavy metal polluted soil has been previously reported (Benton *et al*., 2005). In this study, mixed culture of *B. sphaericus* and *A. eutrophus* was used for bioremediation of crude oil polluted pond water. Juvenile catfish (*Clarias gariepinus)* was selected as the aquatic organism for this study due to their quick response and high sensitivity to toxicants (Chatla *et al*., 2012). Here, we investigated and reported the effects of bioaugmentation treatment on oxidative stress in the liver of catfish exposed to lethal concentration of crude oil.

MATERIALS AND METHODS

Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brent, a sweet light crude oil with approximately 0.37% Sulphur, was obtained from the Department of Petroleum Resources (DPR), Nigerian National Petroleum Corporation (NNPC), Port Harcourt, NE Nigeria.

Microorganisms

Alcaligenes eutrophus (BNS-09) and *Bacillus sphaericus (*BNS-05) were obtained from the culture collection of EMT laboratory, Department of Biochemistry, Federal University of Technology, Akure (FUTA), Nigeria. The two strains were seeded overnight in nutrient broth at pH 7.4 and incubated in a shaking incubator (Stuart) at 180 rpm after which, 5% inoculum of each of the bacteria was introduced into the basal medium used for this study. The two bacteria were selected for mixed culture based on previous optimization studies in the laboratory using different microbial consortia for crude oil degradation (unpublished data)

Test organisms and experimental conditions

Catfish specimens (mean body weight and length of 15.12 ± 0.141 g and 11.82± 1.5 cm) were obtained live from fish farm of FUTA, Ondo State, South West Nigeria. Both males and females were used. The fish were placed in individual plastic

tanks (15 L) with pond water at a controlled temperature (25°C). The catfish were acclimatized for 7 d prior to crude oil exposure and were fed with commercial feed once a day during acclimatization period. The catfish were starved during the study.

Acute toxicity study

Preliminary studies carried out using varied concentrations of crude oil provided the basis for the spread of test concentrations to crude oil polluted pond water (CPW). Fish plastic tanks filled with only pond water was used as positive control. Juvenile *C. gariepinus* (20) were exposed to 0.5, 0.6, 0.7, 0.8 and 0.9% (v/v) crude oil polluted pond water (CPW). Each test tank was covered with wire mesh to prevent the organisms from jumping out of the tanks. The experimental fish were taken as dead when there were no opercula and other forms of body movements even on prodding with a glass rod.

Experimental procedure

Sixty catfish (*C. gariepinus*) specimens were divided into three groups. There were two real replicates for each experimental group. The groups comprised ten catfish each exposed to different conditions in tanks containing pond water. Group 1 contained catfish in pond water without any contaminant. Group 2 comprised catfish in pond water polluted with lethal concentration of crude oil (0.9% v/v). Group 3 contained catfish under bioaugmentation treatment (catfish were in pond water polluted with lethal concentration of crude oil with added inoculum of 10% mixed culture of *A. eutrophus* (BNS-09) and *B. sphaericus (*BNS-05). The lethal concentration of crude oil used in this study was based on preliminary studies done in our laboratory (unpublished data). At the end of 96 h experimental period, the fish were anesthetized with benzocaine (90 mg/L) to remove liver. Liver samples were stored immediately at -20 °C prior to biochemical analysis.

Determination of physicochemical parameters (life sustaining indices)

Samples of pond water from the three groups were taken for the determination of physicochemical parameters such as pH, conductivity, and dissolved oxygen which are life sustaining indices. These were monitored at 24 h intervals over the 96 h study period.

Biochemical analyses

Sample preparation and protein quantification

Liver tissues were homogenized (1:4, w/v) in 50 Mm Tris-HCl buffer, (pH 7.4) containing 5 mM sucrose, 15 mM KCl and 1 mM PMSF. The homogenized samples were centrifuged at 10,000g for 15 min at 4°C. The supernatant was collected. The resulting supernatant was used for assay of superoxide dismutase (SOD) and catalase (CAT) and determination of total glutathione (GSH). Total protein concentrations in supernatants were determined using the method of Bradford (1976) with bovine serum albumin as standard.

Total glutathione (GSH)

The GSH (total glutathione) concentration in liver was measured according to Vandeputte *et al*. (1994). Ten (10 µL) of TCA deproteinized sample was mixed with phosphate buffer pH 7.4 containing 0.3 mM NADPH and 1 mM Ellman's reagent. The enzymatic reaction was monitored spectrophotometrically at 405 nm and the results were expressed in µmol of GSH/g of protein.

Superoxide dismutase (SOD)

SOD activity was measured using the assay developed by Paoletti *et al*., 1986. The assay mixture contained 200 ul of sample, 2.5 ml of sodium carbonate and 0.3 mM epinephrine, at pH 10.2. Kinetic of auto-oxidation of epinephrine was determined at 480 nm. The results were presented in U of SOD/mg of proteins.

Catalase (CAT)

CAT activity was assayed as described by Babo and Vasseur (1992). The assay mixture comprised 0.08 g protein/L diluted sample and 28 mM hydrogen peroxide. The kinetics of hydrogen peroxide degradation was determined at 280nm. Results were expressed as U of CAT/ mg of protein.

Lipid peroxidation

Lipid peroxidation levels were determined via malondialdehyde (MDA) content using the method described by Beuge and Aust (1978). One gram of liver was homogenized with ice cold 1.15% KCl/Tris-HCl pH 7.4. An aliquot of 0.5 ml of the homogenate was mixed with 2 ml of TCA-TBA reagent and heated to 80 °C for 20 min. The reaction mixture was cooled and centrifuged at 3500 rpm for 5 minutes. The absorbance of the supernatant was recorded at 532 nm.

Statistical analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA) to assess the effects of different exposure conditions. Data were expressed as the mean ± standard deviation of the mean (SD) corresponding to groups of twenty fish (n = 20). The ANOVA was used to compare the difference between the groups. Difference was considered significant at the 95% confidence level ($p < 0.05$).

RESULTS AND DISCUSSION

Mortality

No mortality was recorded in the 0.5% (v/v) CPW. However, 40% mortality was recorded in 0.6 % (v/v) CPW, while 50% mortality was recorded in the 0.7% (v/v). Seventy (70%) was recorded in the 0.8% (v/v) CPW while 100% mortality was recorded in the 0.9% (v/v) CPW (Figure 1). The 96 hour LC_{50} is known to vary for toxicants and for different concentrations of toxicants (Ayotunde *et al.*, 2010). In this study, the 96 hour LC_{50} of CPW was found to be concentration dependent after series of preliminary tests (Figure 1). This led to lethal concentration used for this study. The mortality in crude oil polluted pond water (CPW) and bioaugmentation treated pond water (BTW) were also recorded during bioremediation study. It was observed that there was total (100 %) mortality in crude oil polluted pond water (CPW) after 96 h exposure while there was no mortality recorded in bioaugmentation treated pond water (BTW). The 96 h LC_{50} for *C. gariepinus* is shown in Figure 1.

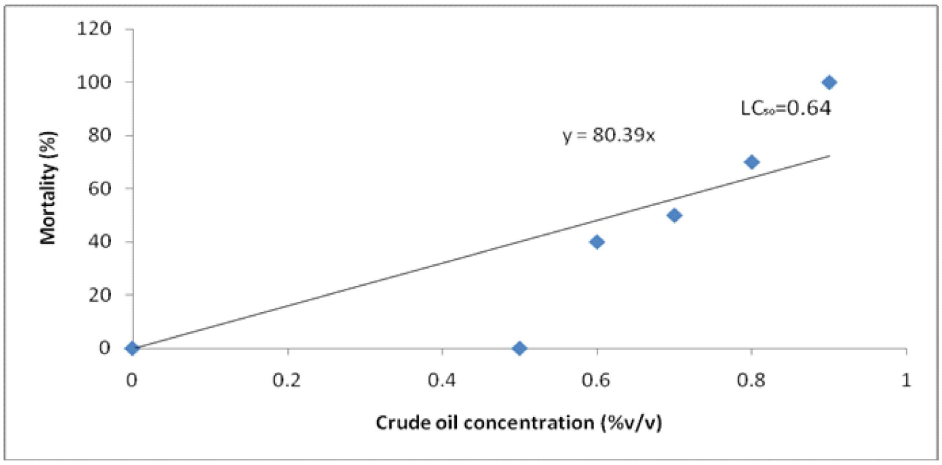

Figure 1: Effects of 96 h acute toxicity of crude oil on *C. gariepinus*

Life sustaining indices

Crude oil polluted water (CPW)

The severity of problems in inhabitants of crude oil producing areas is dependent upon the point of contact with the polluted water (Edema, 2012). This was the basis on which physicochemical properties were monitored in this study. The desirable range for pond pH is 6.5 - 9.5 (Stone and Thomforde, 2003) .There was decrease in pH of crude oil polluted pond water after 96 h from 6.96 ±0.028 to 6.38 ±0.020 (Table 1). The decrease in pH which poses a lethal effect on fish is indicated to be from the crude oil pollution (Table 1).

Similar trend was also observed in dissolved oxygen (DO) under this exposure. There was a significant decrease ($p \leq 0.05$) in dissolved oxygen from 6.10±0.028 mg/L to 3.27±0.049 mg/L (Table 1).

It has been reported that low DO causes anaerobic decomposition of organic matter in water, forming noxious and toxic substances such as hydrogen sulphide and methane which ultimately might have deleterious effect on fish (Basau, 2009). This observation could be attributed to the oil film formation that reduces the dissolution of atmospheric oxygen that comes in contact with pond water consequently reducing the dissolved oxygen in crude oil polluted pond water (CPW). There was corresponding increase in conductivity in CPW from 36 ±0.141 µS/cm to 39.1±0.070 µS/cm over a period of 96 h (Table 1). High conductivity is an indication of the non-portability of the water (Chatterjee *et al*., 2013).

Bioaugmentation treated water (BTW)

Under bioaugmentation treatment there was no significant increase in pH from 6.7±0.028 to 6.95±0.021 (Table 1) when compared with control (C) which is within the desirable range for pond water pH, 6.5 - 9.5 (Stone and Thomforde, 2003). Dissolved oxygen (DO) insignificantly ($p>0.05$) decreased from 6.10±0.028 mg/L to 5.27±0.049 mg/L when compared with control (C) which made the environment favourable for the survival of the fish. Saloom and Duncan (2005) also pointed out that the minimum dissolved oxygen should be 5 mg/l for tropical fish. Conductivity insignificantly ($p>0.05$) increased from 25.35±2.050 µS/cm to 30.3 ±2.545 µS/cm (Table 1) when compared with crude oil exposure, this may be due to the clean-up of the oil by the degradative activities of the inoculated microbes

Biochemical Analysis

Under normal physiological status, the antioxidant defense systems including Glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) can be induced by slight oxidative stress as a compensatory response, and thus the reactive oxygen species (ROS) can be removed to protect the organisms from oxidative damage (Livingstone, 2001,

Table 1: Physicochemical parameters of pond water containing African Catfish (*Clarias gariepinus*) under crude oil exposure and bioaugmentation treatment at 24 h and 96 h

Parameters	Time (h)	Control	*CPW	*BTW
pH	**24**	6.88 ± 0.007^{b}	6.96 ± 0.028^{a}	6.7 ± 0.028^{c}
	96	6.99 ± 0.056^{a}	6.38 ± 0.02^{b}	6.95 ± 0.021^{a}
Conductivity (µS/cm)	**24**	27.3 ± 0.021^{b}	36 ± 0.141^{a}	25.35 ± 2.050^{c}
	96	28 ± 0.070^{c}	39.1 ± 0.070^{a}	30.3 ± 2.545^{b}
Dissolved Oxygen (mg/L)	**24**	7.08 ± 0.042^{a}	6.10 ± 0.028^{b}	6.10 ± 0.028^{b}
	96	5.6 ± 0.141^{a}	3.27 ± 0.049^{c}	5.27 ± 0.049^{b}

Values are means ± SEM for 20 catfish. Means with different superscripts (a,b,c) are significantly different ($p < 0.05$) across the rows. *CPW: Crude oil polluted water; *BTW: Bioaugmentation Treated Water

Milinkovitcha *et al*., 2011) . The activity of antioxidant enzymes may be increased or inhibited under chemical stress depending on the intensity and duration of stress applied as well as susceptibility of exposure species. The liver relies on antioxidant enzymes to protect them from the activity of reactive oxygen species which are products of transformation of xenobiotics. Oxidative damage and decrease in antioxidant defense enzymes occurs as a result of toxic effects of pollutants (Otitoloju, 2006).

In this study, GSH activity in fish exposed to lethal concentration of crude oil significantly ($p<0.05$) decreased (3.950±0.070 U/mg) when compared to the control (5.25±0.07 U/mg). Table 2 shows the statistical analysis while the graphical representation is shown by Figure 2. GSH is an important antioxidant in fish preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Wang *et al*., 2008) but once oxidized, glutathione can be

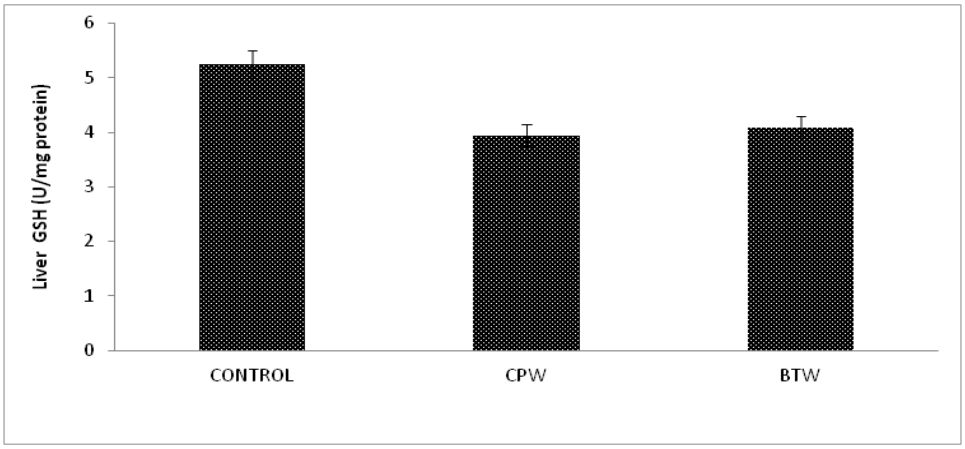

Figure 2: Reduced glutathione level in liver of African catfish under crude oil exposure (CPW) and bioaugmentation treatment (BTW).

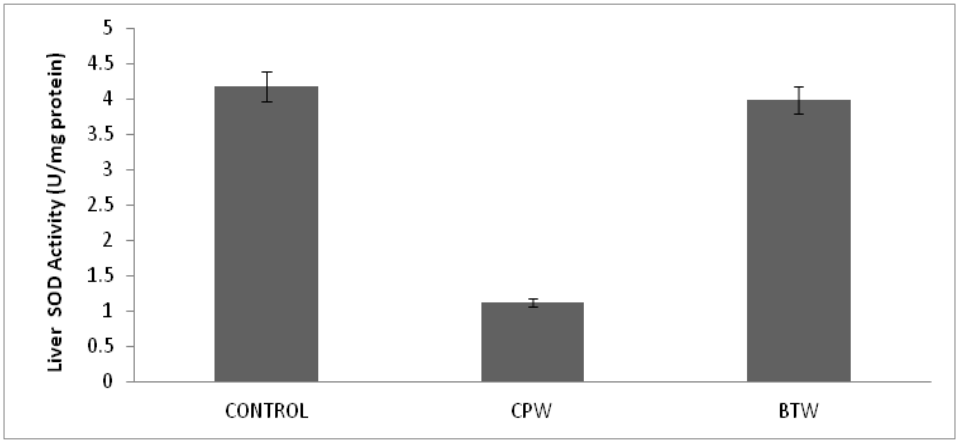

Figure 3: Superoxide dismutase activity in liver of African catfish under crude oil exposure and bioaugmentation treatment. CPW: Crude oil polluted water, BTW: Bioaugmentation treated water

Table 2: Reduced glutathione level, lipid peroxidation (LPO) and activities of superoxide dismutase (SOD) and catalase (CAT) in liver of *C. gariepinus*

Treatments	SOD (U/mg protein)	CAT (U/mg protein)	GSH (U/mg protein)	LPO (nmol MDA/g tissue)
Control	4.19±0.014[a]	4.56±0.141[a]	5.25±0.077[a]	3.99±0.007[c]
Crude Oil Exposure	1.120±0.113[c]	2.55±0.212[b]	3.950±0.070[b]	7.250±1.343[a]
Bioaugmentation	3.99±0.014[b]	4.5±0.354[a]	5.20±0.212[a]	4.1±0.141[b]

Values are means ± SEM for 20 catfish. Means with different superscripts (a,b,c) are significantly different ($p < 0.05$) across the columns.

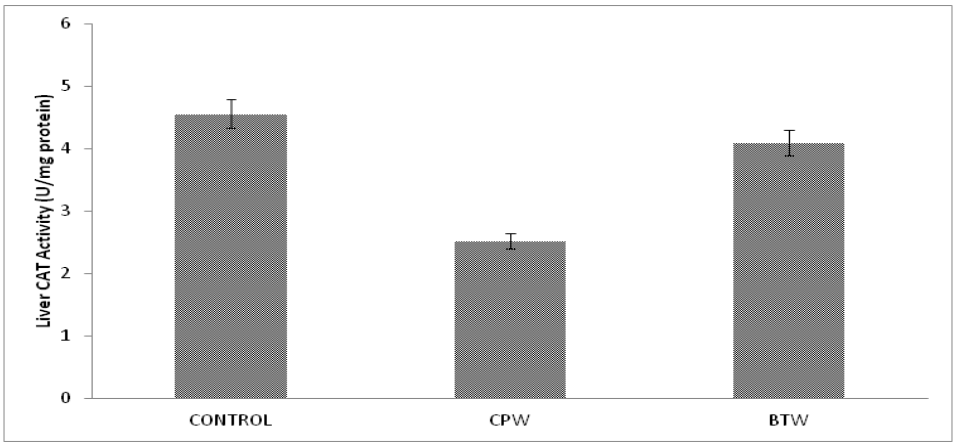

Figure 4: Catalase activity in liver African catfish under crude oil exposure and bioaugmentation treatment. CPW: Crude oil polluted water, BTW: Bioaugmentation treated water.

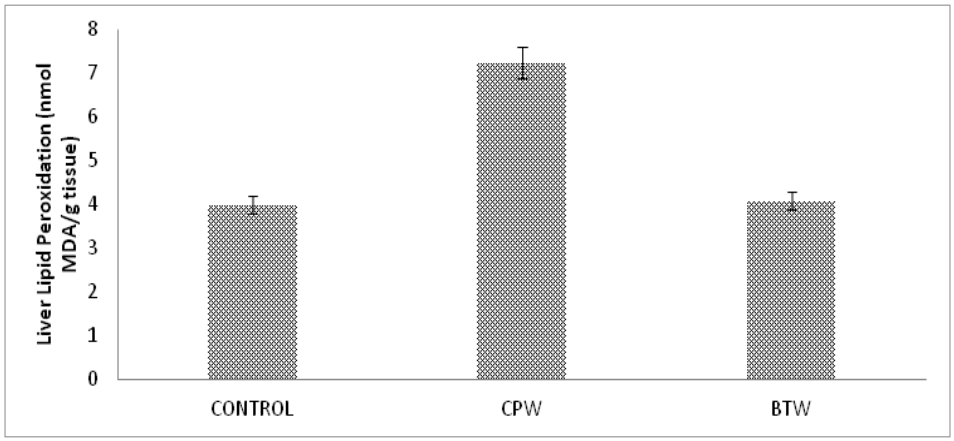

Figure 5: Lipid peroxidation in liver of African catfish under crude oil exposure and bioaugmentation treatment. CPW: Crude oil polluted water, BTW: Bioaugmentation treated water.

reduced back by glutathione reductase, using NADPH as an electron donor (Coulon *et al*., 2006).

A significant decrease in reduced glutathione and superoxide dismutase in the liver of *Clarias gariepinus* exposed to crude oil, benzene and xylene had previously been reported (Otitoloju and Olagoke, 2011). The liver SOD and CAT activities also significantly ($p<0.05$) decreased under lethal concentration of crude oil exposure (1.120±0.113 U/mg, 2.55±0.212 U/mg) when compared to the control (4.19±0.014 U/mg) (Table 2, Figures 3 and 4).

Decrease in the activities of the antioxidant enzymes was accompanied by increased formation of reactive oxygen species. Malonylaldehyde (MDA) which is one of the oxidative damage products of lipid peroxidation was found to increase significantly ($p<0.05$) under lethal concentration of crude oil exposure (7.250±1.343 nmol MDA/g tissue) when compared with control (3.99±0.007 nmol MDA/g tissue) (Table 2, Figure 5). Previous response has shown increase in lipid peroxidation (LPO) in fish gills after 48 h of exposure and in fish livers after 16 h of exposure, respectively (Ahmad *et al*., 2005; Milinkovitch *et al*., 2011). Oxidative stress due to the toxic effect of pollutants is usually indicated by increased levels MDA and subsequent decrease in defense enzymes (GST-GSH, SOD and CAT) due to overwhelming effects of pollutants (Otitoloju, 2006).

Remarkably, in the liver of fish under bioaugmentation treatment, there was no significant decrease ($p>0.05$) in the activity of GSH, SOD and CAT and (5.20±0.212 U/mg, 3.99±0.014 U/mg, 4.5±0.354 U/mg,) when compared with control (Figures 2 - 4). Similarly, there was no significant change ($p>0.05$) in MDA level (4.1±0.141 nmolMDA/g tissue) when compared with control.

Results revealed that the mixed culture of *B. sphaericus* and *A. eutrophus* used in this study cleaned up crude oil in the pond water and reduced the overwhelming effect of crude oil pollution on the anti-oxidant enzymes in the liver of fish under bioaugmentation treatment.

The mixed culture of *B. sphaericus* and *A. eutrophus* used might have released peroxidases that catalyzed the breakdown of crude oil to compounds that were less toxic to the fish which subsequently led to no significant change ($p>0.05$) in the activities of anti-oxidant enzymes and level of lipid peroxidation in the liver of fish under the bioaugmentation treatment when compared with control.

CONCLUSION

This study shows that bioaugmentation treatment is capable of cleaning up crude oil in contaminated areas and overcoming oxidative stress in fish exposed to lethal crude oil concentration. This suggests the effectiveness and practicability of bioaugmentation treatment as an environmental friendly bioremediation technique.

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