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Effect of Cassava Processing Effluents on Antioxidant Enzyme Activities in *Allium Cepa* L.

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ABSTRACT: The underlying principle of the biomarker approach is to analyse the physiological and/or biochemical response of an organism exposed to an environmental pollutant. Onion bulbs were exposed directly in 0%, 0.2%, 0.4%, 0.8% 1%, 2%, 3%, 4% and 5% concentrations (v/v) to each effluent of three popular Nigerian cassava meals: *garri, lafun and akpu* for 96 hours. The root growth of the onion bulb and superoxide dismutase (SOD), and catalase (CAT), activities were measured. At 96 h, the root tips showed concentration-dependent growth retardation in all the effluents with EC_{50} values of 1.5%, 2.5% and 3.5% for *garri, lafun and akpu* effluents respectively while total phytotoxic effects was induced at higher effluent concentration. The physico-chemical analyses showed that the effluents were highly acidic and contained significant ($p<0.05$) amounts of cyanide and heavy metals compared to the control. The effluents induced increased malondialdehyde (MDA) levels and accelerated SOD activity at low concentrations but decreased after attaining maximum activity in *garri* effluent at 1%. Catalase was inconsistent; however, a drastic decrease in activity was recorded at the same effluent concentration. *Lafun* and *akpu* induced similar increases in antioxidant enzymic activity up till 2% effluent concentration after which steep decline set in.

Key words: Processed cassava effluents; Cyanide; Onion bulbs; *Allium cepa*; Antioxidative enzymes.

Introduction

Biochemical markers have continued to attract a lot of interest as attempts are continually made to define and measure the effect of pollutants on the environment. They usually respond to the mechanism of toxicity rather than the presence of a specific toxicant and are increasingly being used as valid indicators of aquatic pollution (Fernandes *et al*., 2002). This concept is derived from the idea that a toxic effect manifests itself at the sub-cellular level before it becomes apparent at higher levels of biological organization. When compared to chemical residue analysis, biomarkers have the advantage of being more relevant biologically (Rees, 1993). The measurement of biochemical responses to chemical contaminants may serve to improve the assessment of biologically significant exposures to toxic chemicals and enhance the ability to assess the risk of effects on the health and survival of toxicant exposed populations. Perhaps the most studied chemical pollutants with biomarkers are heavy metal stress which leads to sharp changes in the activities of certain antioxidative enzymes notably SOD, GR, and CAT, as they show profound changes in activity upon toxic exposure (Fatima and Ahmad, 2005).

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Allium cepa L. is the common onion and is widely used in all parts of the world as flavouring vegetable. The use of the *A. cepa* root length inhibition bioassay as a sensitive, cost effective and valid indicator of toxicity test for the routine monitoring of water pollution is well documented (Fiskesjo, 1985, Monarca *et al*., 2003; Bolle *et al*., 2004, Babatunde and Bakare, 2006, Olorunfemi *et al*., 2010).

A number of investigators have utilised antioxidant enzymes as biomarkers of metallic (Luna *et al*., 1994; Cuyers *et al*., 2000, Ahmad *et al*., 2000; Geret *et al*., 2002, 2003), and recently, the efficacy of *Allium* root length inhibition bioassay was reinforced with antioxidant enzymatic studies carried out in the onion bulbs exposed to toxic heavy metals in industrial wastewater (Fatima and Ahmad, 2005). The primary aim of this study was to evaluate the potential of the antioxidant enzymes of the common onion as biomarkers of agricultural effluents and wastewaters. In this study, estimation of peroxidation and activities of two antioxidant enzymes of *Allium cepa* are measured as toxicity monitors of wastewaters obtained from the processing of cassava tubers into three popular Nigerian cassava meals; *garri, lafun and akpu*. The study would greatly increase the efficacy of the *A. cepa* root growth inhibition assay already carried out to determine the toxicity of the cassava processing effluents in a previous study.

Materials and Methods

Test Materials: The biological materials were equal-sized onion *(Allium cepa*, 2n=16*)* bulbs of the purple variety (average size 15-22 mm diameter) purchased locally in Benin City, Edo State in Nigeria (latitudes 6^o 06' N, 6^o 30' N and longitudes 5^o 30' E, 5^o 45' E). They were sun-dried for six weeks before use.
Fresh effluents from the processing of cassava into *garri*, *lafun* and *akpu* were obtained from small-scale cassava processing mills in Uselu Quarters, Benin City. The effluents were scooped from ten different positions into 10 litre plastic containers from moulds in the mills in order to form a homogenous mixture, where grated cassava packed into sacs are kept before putting them into the manual pressing machines. The effluents obtained from the processing of the cassava roots were collected at three different times (morning, afternoon and evening) and stored at 4°C until analysed for physico-chemical properties and the bioassay. Before each *Allium* test was carried out, the effluent was equilibrated to room temperature (27±2°C) and diluted with tap water to produce the series of dilutions investigated.

Analysis of Effluents for Physico-chemical Parameters: The effluents were analyzed for a number of standard physico-chemical properties, including total hardness, total solids, and chlorides, according to methods described by APHA (1998). Nine metals (including eight heavy metals) namely aluminum (Al), cadmium (Cd), copper (Cu), chromium (Cr), iron (Fe), mercury (Hg), zinc (Zn), nickel (Ni) and manganese (Mn) were analyzed in the effluents sample according to standard analytical methods (USEPA, 1996; APHA, 1998). Briefly, 100 ml of the effluents were digested by heating with concentrated HNO_3, and the volume reduced to 3-5 ml. This volume was made up to 10 ml with 0.1N HNO_3. Concentrations of the metals were estimated by using an Atomic Absorption Spectrophotometer (Perkin Eelmer E. Analyst, 2000, USA).

Enzymatic Determination of Cyanogenic Potential (CNP) in the Effluents: The procedure of enzymatic hydrolysis (Cooke, 1978) allows the determination of total cyanide, free cyanide and HCN. The method was further improved by O'Brien *et al.,* (1991) by introducing an ethanol/acid extraction step, which simplified extraction of cyanide from cooked cassava products containing gelatinised starch. The photometric procedure used in this work was developed by Essers *et al.*, (1993), who has replaced the toxic pyridine/pyrazolone colour reagent used by Cooke (1978) with a less toxic isonicotinic/chloramin T reagent. The linamarase used in the assay was purchased from VWR International AG.

The *Allium* Test: The test procedure for macroscopic evaluation was as described by Fiskesjö (1997) and modified by Bakare and Wale-Adeyemo (2004). The outer scales of the onion bulbs and brownish bottom plate were removed, leaving the ring of root primordia intact. The peeled bulbs were put into fresh tap water during the cleaning procedure to protect the primordia from drying. Thereafter, the bulbs were exposed directly in 0%, 0.2%, 0.4%, 0.8% 1%, 2%, 3%, 4% and 5% concentrations to each of the test sample (*garri*, *lafun* and *akpu* effluents). Higher concentrations above 10% at which there were no growths for any of the test samples were discarded.

Seven onion bulbs were set up in each series for each sample, out of which the best five with good root growth were selected for analysis of root growth inhibition. The experiment was set up in the dark at 25±1°C. Test liquids were changed daily. At 96 h, the onion with the poorest growth from each series was removed and the experiment was terminated by measuring the length of the roots. The total length of the bundle of roots for each bulb was determined with a meter rule giving one value for each bulb. Photographs of test materials were taken with *Nikon* Digital Camera D80 (Nikon Corp., Japan) and special note was taken of change of colour of root tip. The growth inhibition value, EC_{50} (the effective concentration at which 50% root growth of control is inhibited) was interpolated for each test material from the plot of root lengths, as percent of control, against the log of effluent concentrations. Ordinary tap water was used as growth medium for control and for the dilution of the test effluents (Fiskesjö, 1997). The tap water is ascertained to be of good quality with pH around 7 and with Ca + Mg content of about 50-70 mg/l and free from any chlorine compounds and toxic ions.

Estimation of Peroxidation and Antioxidant Enzyme Activities: The outer scales of the onion bulbs were carefully removed and the brownish bottoms were scraped away without destroying the root primordia. The peeled bulbs, fifteen for each effluent concentration, placed in tap water during the cleansing procedure to protect the primordia from drying, were randomly placed on beakers (4.5 cm diameter, 5 cm length) filled with the test liquids such that the bases were constantly moistened and observed for 48 hours. The experiments were performed at room temperature (27 ± 2°C) and in the dark. The test solutions were replaced daily. The bulbs with the poorest growth were discarded in each group. On day two when mitotic activity is presumed optimal (Fiskesjö, 1985), the root tips were excised from each bulb and prepared for total protein and peroxidation determination and enzymatic assays. The concentrations used were 0% (control), 0.2%, 0.4%, 0.8% 1%, 2%, 3%, 4% and 5% of each of the test sample (*garri*, *lafun* and *akpu* effluents)

***Estimation of Peroxidation*:** The level of lipid peroxidation products in samples was expressed as 2-thiobarbituric acid reactive materials, aldehydes, mainly malondialdehyde (MDA) and endoperoxides (Buege and Aust, 1978). 2-Thiobarbituric acid-reactive materials in samples were assayed according to the modified method of Heath and Packer (1968).
***Estimation of Total Protein*:** Total protein estimated by a modified method of Lowry *et al*., (1951).

***Estimation of Superoxide Dismutase Activity*:** SOD activity was determined by measuring its ability to inhibit the auto-oxidation of adrenaline in aqueous solution to adrenochrome in the presence of the superoxide anions (Misra and Fridovich, 1989). The amount of enzyme producing 50% inhibition is defined as one unit of the enzyme activity.

Estimation of Catalase Activity: Catalase determination is based on the method of Cohen *et al*., (1970). It hinges on the measurement of the rate of decomposition of hydrogen peroxide (H_2O_2) after the addition of the material containing the enzyme by reacting it with excess potassium tetraoxomanganate (VII), ($KMnO_4$) and then measuring the residual $KMnO_4$ spectrophotometrically at 480 nm.

Statistical Analysis: The results of the root inhibition and chromosome aberrations are presented as mean ± standard error for five onion bulbs per concentration and One-Way ANOVA was used for testing significance. Statistical significant differences between control and the different concentrations of the effluents were determined using Tukey post-hoc test. All statistical analyses were carried out using SPSS®14.0 statistical package.

Results

The physical and chemical characteristics of the effluents and tap water used in this study are presented in Table 1. The characteristics of the tap water showed that it was of good quality with a pH of 7.22 and considerable amount of calcium and magnesium without any toxic ions. The undiluted effluents were turbid with offensive odour and highly acidic with pH values of 3.96, 4.11, and 4.61 for *garri, akpu* and *lafun* effluents respectively. The effluents were characterised by appreciable amounts of cyanide and heavy metals although the concentration of these substances were relatively higher in *garri* than *akpu* and *lafun*

effluents. The amount of lead in *garri*, *akpu* and *lafun* were 1.82, 0.21 and 0.10 mg/l respectively. In *garri* effluent, the concentration of Ag, Zn, Ni, Hg and Cd, were 8.20, 5.90, 4.40, 1.05 and 0.11 mg/l respectively. Comparatively, these values were less in *akpu* and *lafun* effluents; for instance, the concentration of Ag in *akpu* and *lafun* effluents was 2.40 and 2.60 mg/l respectively.

Table 1: Physical and chemical characteristics of processed cassava effluents

Parameters	Garri	Lafun	Akpu	Tap water	FEPA[a]	USEPA[b]
pH	3.96±0.08	4.11±0.06	4.61±0.08	7.22±0.04	6 – 9	6.5 – 8.5
Appearance	Turbid	Turbid	Turbid	Colourless	NS	NS
Odour	Unpleasant	Unpleasant	Unpleasant	Odourless	NS	NS
Total Hardness	75.00±3.45	55.00±2.98	47.00±4.22	22.00±2.20	–	0 – 75
Total solids	5,600.0±120.50	4,630.0±98.80	4,780.0±68.34	20.03±5.35	NS	NS
Chlorides	516.30±44.20	149.0±32.70	113.60±24.76	13.60±4.40	600	250
Calcium	94.30±6.30	55.90±4.20	81.10±5.60	62.50±7.20	NS	NS
Aluminium	71.50±4.60	69.00±4.50	80.00±6.00	70.00±5.00	NS	NS
Magnesium	110.90±9.43	100.50±10.25	90.50±9.60	8.10±0.04	NS	NS
Lead	1.82±0.20	0.21±0.04	0.10±0.01	0.01±0.01	<1	0.003
Iron	30.9±4.90	17.0±3.02	12.0±2.01	0.20±0.01	20	0.30
Copper	2.60±0.30	1.10±0.04	1.00±0.02	0.10±0.01	<1	0.009
Manganese	7.10±0.04	5.10±0.02	3.00±0.02	0.01±0.01	5	0.05
Cadmium	0.11±0.02	0.04±0.02	0.04±0.01	0.01±0.01	0.05	0.05
Mercury	1.05±0.02	1.00±0.02	0.95±0.02	0.01±0.01	NS	NS
Chromium	1.14±0.02	109.0±0.02	1.06±0.01	0.01±0.01	<1	0.002
Zinc	5.90±0.62	1.10±0.04	1.10±0.02	0.50±0.01	<1	0.12
Silver	8.20±0.44	2.40±0.08	2.60±0.06	0.02±0.01	<1	NS
Nickel	4.40±0.78	4.00±0.04	3.60±0.04	0.02±0.01	<1	NS
Cyanide	685.0±14.50	346.0±20.80	159.0±18.20	0.00±0.00	NS	NS

Values are means of 3 replicates ±SEM
*All values are in mg/l except pH with no unit. Cyanogenic potential is expressed as µgHCl/ml (ppm)
TDS: Total dissolved solids
[a]Federal Environmental Protection Agency (1991) Permissible limits for drinking water
[b]United States Environmental Protection Agency (1999).National recommended water quality criteria – correction
NS: Not stated

Table 2 shows the mean root lengths of the *Allium* bulbs exposed to the different effluents. Effluents concentrations at 0%, 0.2%, 0.4%, 0.8% 1%, 2%, 3%, 4% and 5% induced slow growth of roots, however, strong growth retardation was observed in onion roots growing at high concentrations while total inhibition in root growth was observed at 20% effluent concentration. For instance, mean root length of 2.0 cm was obtained for onion bulb grown in 0.2 % *garri* effluent while at 1% the root length was 0.70 cm. Similar trends were observed with *akpu* and *lafun* effluents. The root growth inhibition is concentration-dependent with EC_{50} values of 1.5%, 2.5% and 3.5% for *garri*, *akpu* and *lafun* effluents respectively. Restricted root growth was observed in all effluents however, root growth inhibition was most severe in *garri* effluent, closely followed by *akpu* while *lafun* effluents were least inhibitory.

There was no change to the colour and morphology of *Allium* roots exposed to the effluents in the control and at 0.2% - 0.8% but at higher concentrations of 1% and 10%, the types of root malformations include twists, root tips bent upwards resembling hooks ('crochet hooks') and c-tumors (abnormalities appearing as swellings of the root tips). The roots were pale and at 20%, the roots were dark brown/black in colour.

Onion grown on cassava effluents resulted in a significant increase in malondialdehyde (MDA) level (Fig. 1). There was a five-fold increase of MDA level in 10% *garri* effluent compared to control. Similarly, MDA values in *akpu* and *lafun* at the same effluent concentration were more than 5 times to that of control value.

Total protein increased up till at 1% at which the increase was approximately 4 fold (compared to control) all effluents, leading to progressive decrease thereafter (Fig. 2).

SOD was most consistent in that its activity decreased after attaining maximum activity at 1% *garri* effluent concentration. Catalase was inconsistent; however, a drastic decrease in activity was recorded at the same effluent concentration. *Lafun* and *akpu* effluents had similar increases in the antioxidant enzymes up till 2% effluent concentration after which decrease in enzyme activity was observed.

Fig. 3 and 4 present the changes in superoxide dismutase and catalase activities respectively. The effluents (*garri*, *akpu* and *lafun*) at a concentration of 1% caused maximum induction of SOD activity (approximately 3 times that of control value) while higher concentrations of the effluents (3%, 4%, and 5%), SOD activity showed sharp decline (Fig. 3). CAT induction (Fig. 4) was not as consistent as that of SOD. CAT showed enhanced activity in *garri* up to a effluent concentration of 0.8%, followed by a sharp decrease at 1% after which it displayed a gradual increase up till 5% effluent concentration. Similar patterns were observed in CAT activities in *akpu* and *lafun* effluents.

Discussion

The concentrations of Cd, Cu, Cr, Pb, Hg and Zn were found to be very high. Metal-induced lipid peroxidation is mostly attributed to increased production of free radicals (Halliwell and Gutteridge, 1984., Aust *et al*., 1985). Results obtained from onion bulbs grown on the cassava effluents resulted in a significant increase in malondialdehyde (MDA) level, an indication of lipid peroxidation, a finding which is consistent with earlier reports (Chen *et al*., 2000).

Table 2: Root lengths of *Allium cepa* after cultivation in different concentrations of cassava effluents

Conc (%)	***Garri***			***Akpu***			***Lafun***		
	Mean Root length±S.E.	RG (%) of control	95% CL	Mean Root length±S.E.	RG (%) of control	95% CL	Mean Root length±S.E.	RG (%) of control	95% CL
0	4.9±0.14	-	0.7	4.9±0.14	-	0.7	4.9±0.14	-	0.7
0.2	2.0±0.11	40.8	0.9	3.2±0.40	65.3	1.8	3.3±0.27	68.4	1.1
0.4	0.9±0.11	18.4	0.4	2.2±0.17	47.0	0.6	2.4±0.08	56.1	0.3
0.8	0.8±0.10	16.3	0.4	2.0±0.13	40.8	0.4	2.1±0.13	44.2	0.5
1.0	0.7±0.10	14.2	0.6	1.6±0.29	36.4	1.2	1.8±0.04	32.5	0.6
2.0	0.7±0.09	14.2	0.5	1.4±0.21	31.2	0.8	1.5±0.21	30.6	0.8
3.0	0.5±0.04	10.2	0.3	1.2±0.12	24.5	0.3	1.2±0.09	24.5	0.2
4.0	0.4±0.04	8.2	0.2	0.8±0.09	16.3	0.4	1.0±0.11	20.9	0.2
5.0	0.2±0.01	4.1	0.4	0.6±0.10	12.3	0.5	0.7±0.05	14.2	0.4
20	Not measurable	-	-	Not measurable	-	-	Not measurable	-	-
EC_{50}	1.50%			2.50%			3.50%		

Values are Mean ± SEM
RG (%) of control expressed as % root growth of the control.
95% CL: 95% confidence limit.
$P < 0.05$, level of significance of root growth inhibition compared with the untreated control.

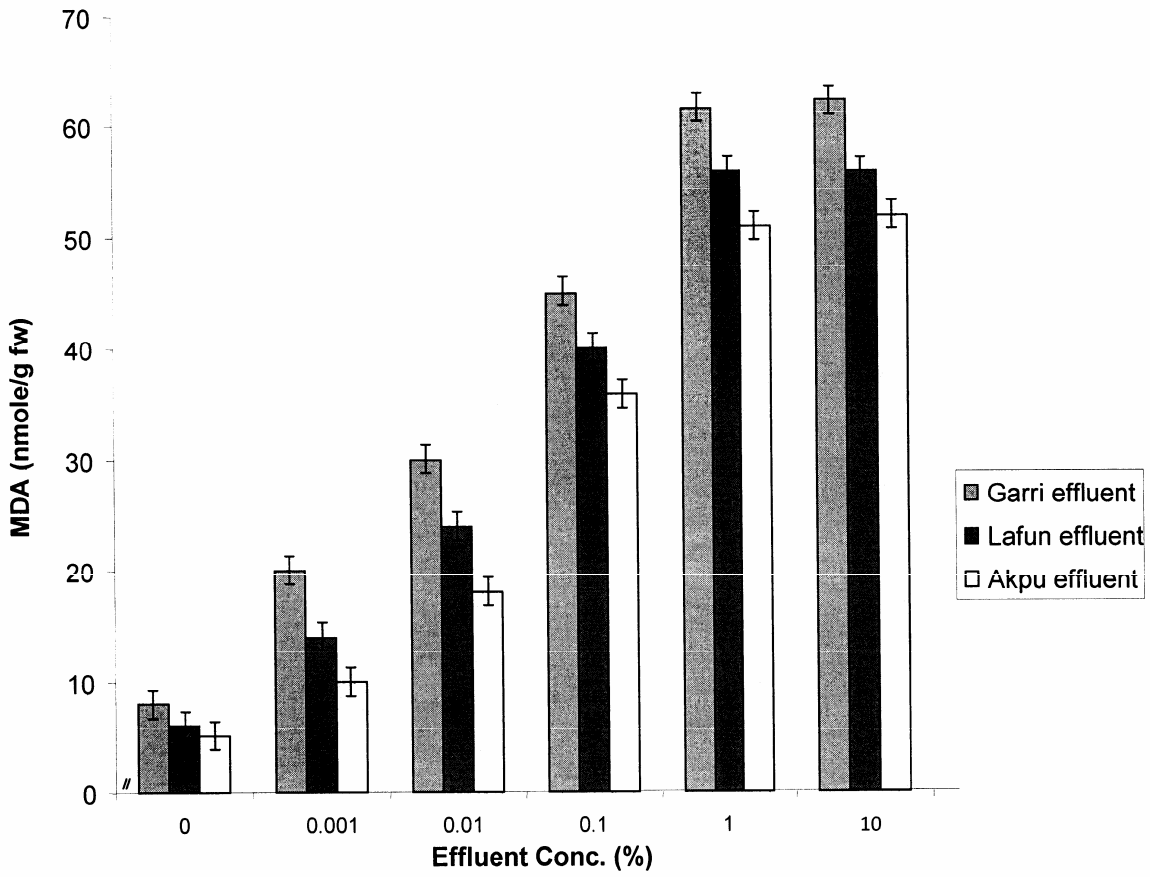

Figure 1: Effect of cassava effluents on levels of MDA in *Allium* roots

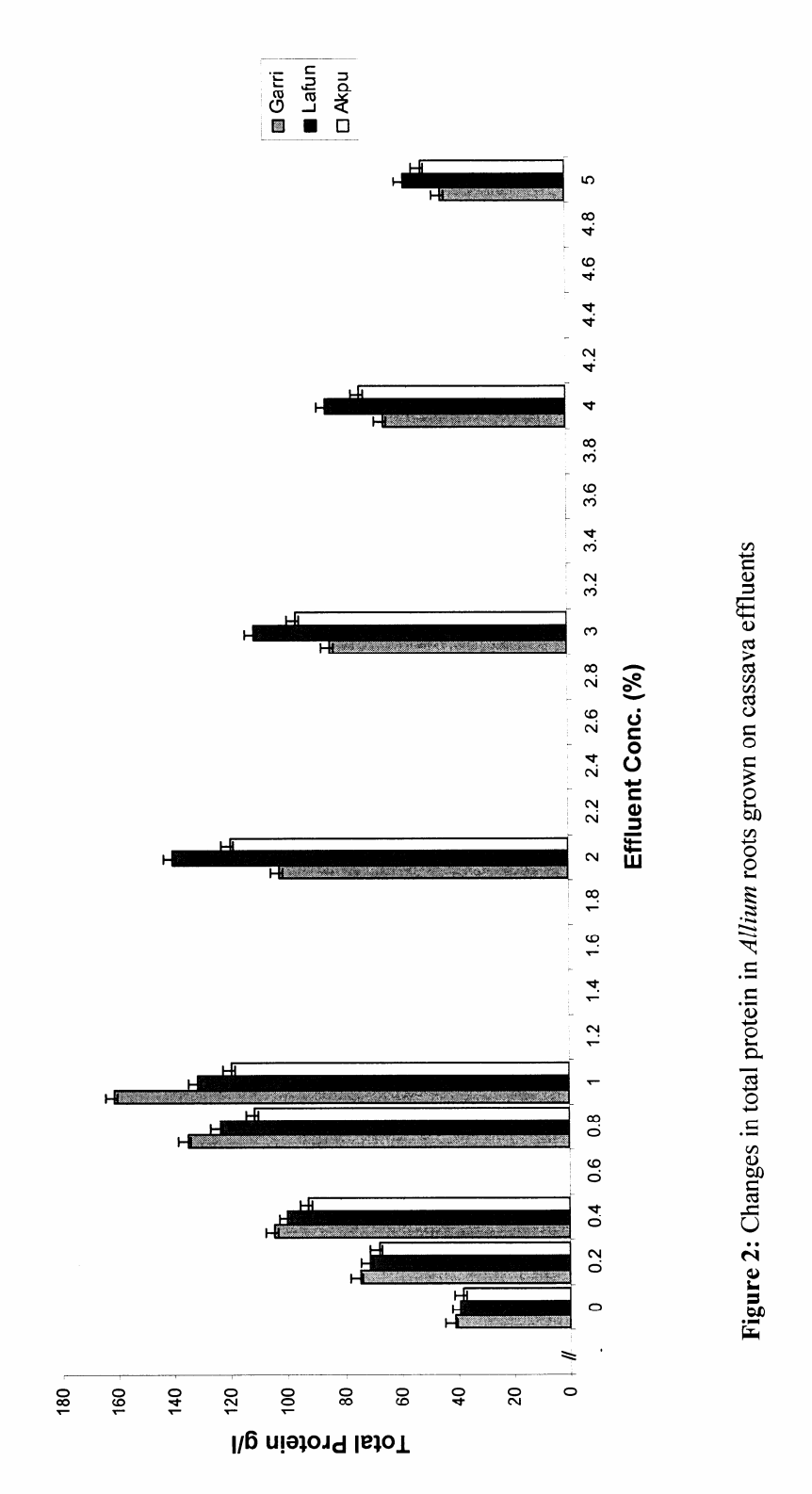

Figure 2: Changes in total protein in *Allium* roots grown on cassava effluents

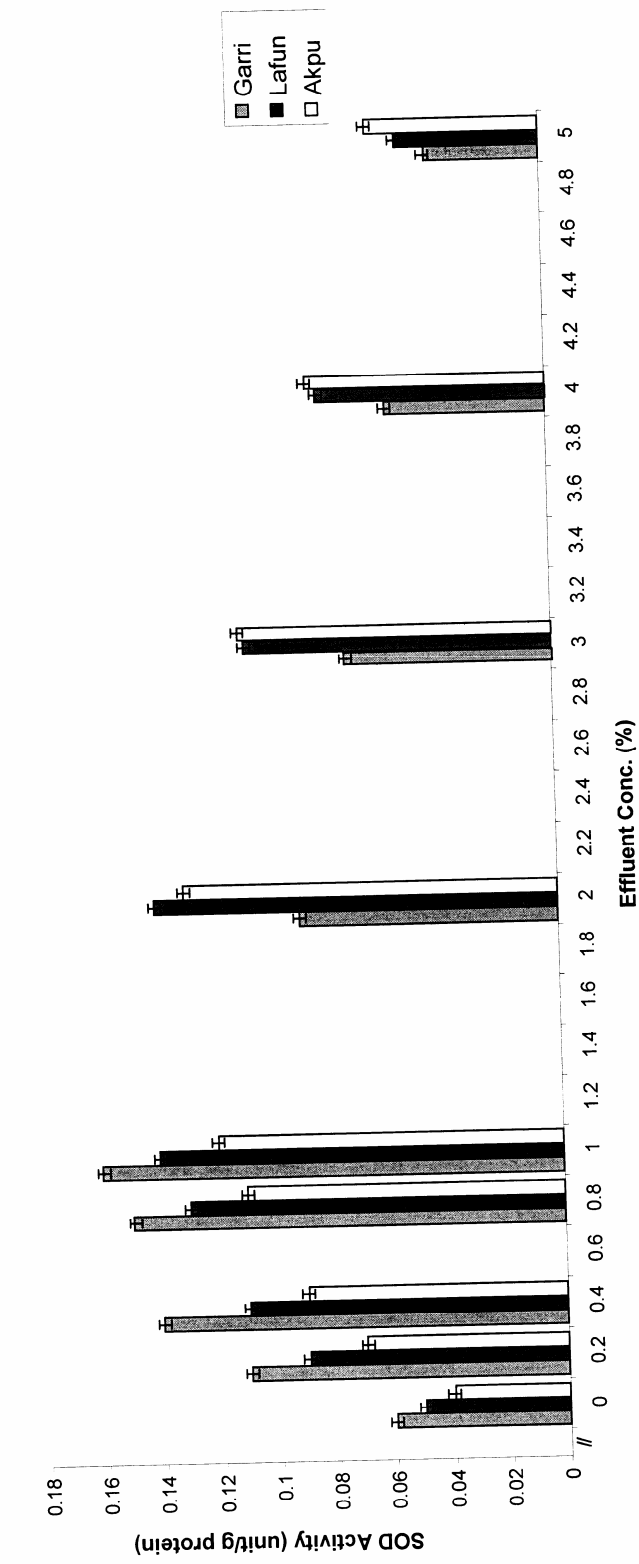

Figure 3: Changes in SOD activity in *Allium* roots grown on cassava effluents (1 unit of SOD activity is taken as the amount of SOD required to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome per minute.)

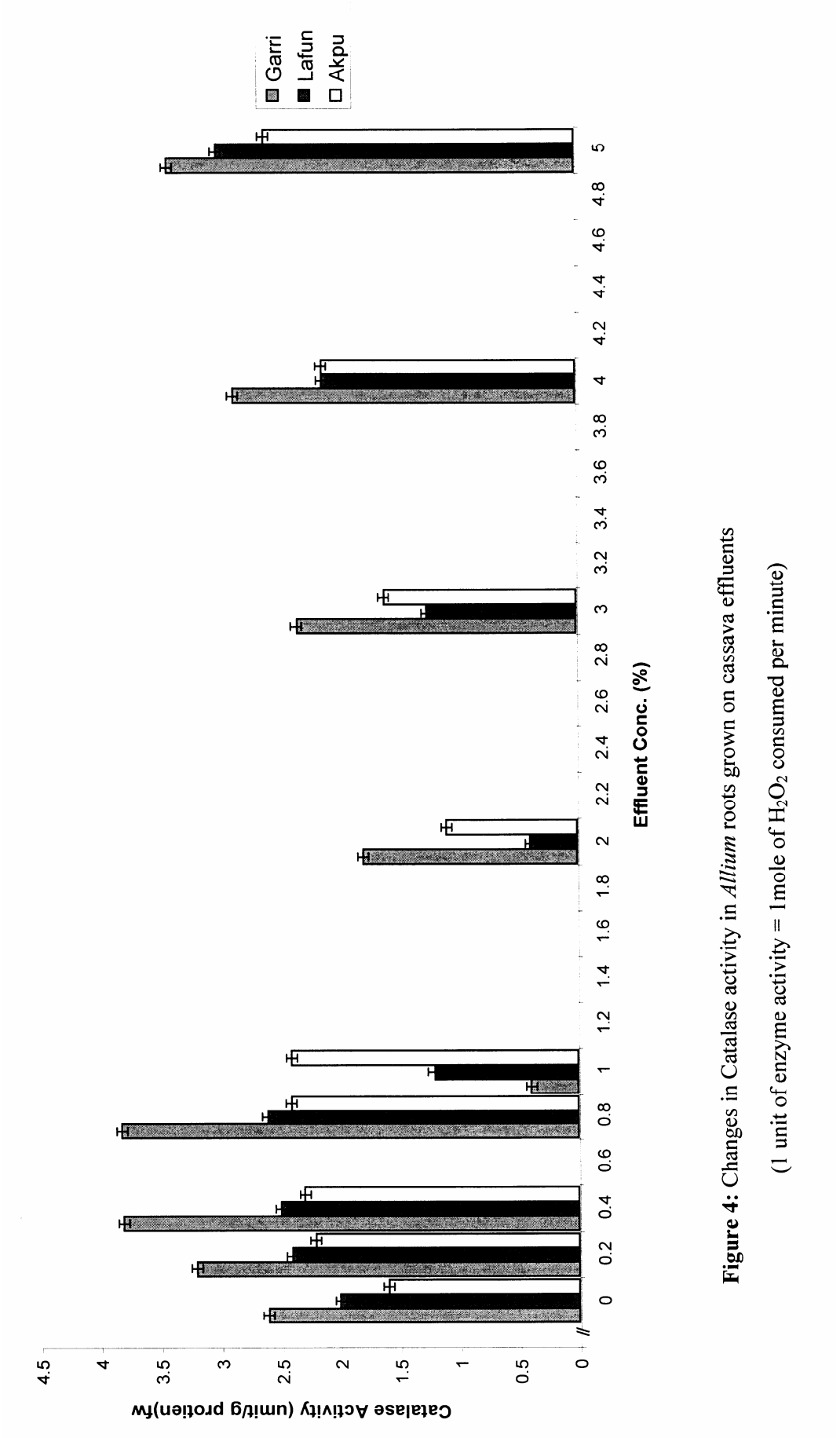

Figure 4: Changes in Catalase activity in *Allium* roots grown on cassava effluents

(1 unit of enzyme activity = 1mole of H_2O_2 consumed per minute)

The protective mechanisms adapted by plants to scavenge free radicals and peroxides involve two major antioxidative enzymes: SOD and catalase. The antioxidative enzymes are important in preventing the oxidative stress in plants. The activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions (Allen, 1995, Stevens et al., 1997, Schutzendubel et al., 2001). SOD has 3 major isoforms in higher plants, the Cu,Zn-SOD in thylakoid membranes as well as in the cytosol, Mn-SOD in the mitochondria and the Fe-SOD in the chloroplast. SOD is induced by its own substrate, the superoxide radical (Colepicolo et al., 1992; Allen and Tresini, 2000), and thus activation of cellular SOD may be an indication that the cell is experiencing pollutant induced superoxide radical stress.

The changes in SOD presented in this study reflect the changes in total SOD and not a particular isoform. Increase in SOD and catalase has been reported with environmental stresses (Tsang *et al.,* 1991; Chen and Kao, 1999). Cassava effluent treatment of the onion bulbs in this study resulted in significant increase in the activities of SOD and catalase at lower effluent concentrations while higher concentrations led to enzyme inhibition. These findings are consistent with earlier reports with heavy metal treatment in the same plant (Fatima and Ahmad, 2005).

The growth curve of the onion roots exposed to different concentrations of the effluents indicated a positive dose-response effect. Low effluent concentrations induced slow growth of roots, however, strong growth retardation was observed in the onion roots growing at high concentrations while total inhibition in root growth was observed at 20% effluent concentration; furthermore, the toxic compounds in the cassava wastes can be reduced by water dilution. The effluents induced root malformations which earlier investigation (Olorunfemi *et al*., 2010) have shown to be useful signs of toxicity.

The findings in this study clearly show that variations in the antioxidant enzymes of *A. cepa* can also serve as useful biomarkers for the detection of pollutants in the aquatic ecosystem. Catalase and the peroxidases are the major enzymes involved in H_2O_2 detoxification; CAT did not exhibit consistent change upon effluent exposure and it is known that glutathione peroxidase (GPX) has higher affinity for H_2O_2 than CAT; thus it is more effective in decomposing H_2O_2 (Halliwell, 1974). Work is on-going to determine activities of ascorbate peroxidase (APX), glutathione peroxidase (GPX) in *A. cepa* root tips exposed to cassava processing effluents.

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