ORIGINAL RESEARCH ARTICLE

Electrochemical quantification of the levels of hydrogen peroxide in cassava using glassy carbon electrode modified with chitosan/silver nano-hybrid

Akitoye A. A, Ibrahim G. O, Okiei W. O*

Affiliation
Chemistry Department, University of Lagos, Akoka, Yaba, Lagos

***For Correspondence**
Email: wokiei@unilag.edu.ng

Abstract
This study correlates the production of hydrogen peroxide in cassava with its rapid postharvest physiological deterioration (PPD). Chitosan/silver nanohybrid was synthesized and immobilized on glassy carbon electrode for improved detection of hydrogen peroxide in electrochemical studies. The cathodic peak current for the reduction of hydrogen peroxide to water and oxygen occurred at -550 mV and β-carotene contents of the cassava cultivars were quantified using UV-Vis spectroscopy at a wavelength of 480 nm. No significant amount of hydrogen peroxide was found in the root tubers on the first, second, third and fourth day. However, the production of hydrogen peroxide from the different cultivars on the fifth and sixth day after harvest was found to correlate with their respective β-carotene contents. The cultivar with the highest β-carotene content (Yellow roots- IBA070593: 0.0044 mg/g) was found to have the lowest level of hydrogen peroxide on day 5 and day 6: 0.096 mmol/g FW and 0.037 mmol/g respectively; while that with the least β-carotene level (White roots- IBA980505: 0.0000 mg/g) demonstrated the highest level of hydrogen peroxide content on day 5 and day 6: 0.177 mmol/g FW and 0.096 mmol/g respectively; and highest percentage increase from the fifth to the sixth day of the PPD process. It is seen from this study that an increase in the level of hydrogen peroxide indicates PPD, and that antioxidants with hydrogen peroxide scavenging properties can help increase shelf-life of cassava cultivars.

Keywords: Cassava, food security, postharvest physiological deterioration.

Introduction

Cassava (*Manihot esculenta* Crantz) commonly referred to as manioc or tapioca is a major tuberous root of great importance. It is the most important staple root crop in the world (De Bruijn & Fresco, 1989) and is ranked the fourth most important food crop after rice, maize and sugar cane Cock, 1985), providing the necessary food energy intake for nearly 1 billion people in 105 countries worldwide (Li et al., 2017). Cassava serves as a staple food for 800 million people mainly in sub-Saharan Africa (Nassar & Ortiz, 2010), 300 million people in the tropics (Kawano, 1980) and 50 million people in Nigeria (Oluwole et al., 2004). The roots are an excellent source of carbohydrate (Montagnac et al., 2009a), providing more than 10 percent of the daily dietary calorie intake for Africans. The roots also contain significant levels of vitamin C, riboflavin, thiamin and niacin (Simonyan, 2014).

Cassava has been said to be the cheapest source of calories among all food crops and is estimated to provide over 12% of the daily per capita calorie needs for the people of Sub-Saharan Africa (Montagnac et al., 2009b). The multifarious plant has a wide variety of uses. As the cheapest source of starch, it is employed for use in over 300 industrial products (Li et al., 2017). Its most popular product in Nigeria is garri (white or yellow). Other products from cassava include flour, fufu (or foofoo in Ghana, Nigeria and Congo), livestock feeds, textiles, glues and confectionaries. It is useful in the food processing industry for the production of monosodium glutamate and sweeteners and also has its application in the pharmaceutical industry as dextrin (Tonukari, 2004). In China, it is mainly used for industrial purposes; The Guangxi Zhuangzu autonomous region in southern China produced 139 million litres of ethanol from cassava in 2007 (Dai et al., 2006; Drapcho et al., 2008).

Despite the significance of this crop in terms of consumption, industrial utilization and potency for food security, its major limitation to massive commercialization is its rapid deterioration rate. According to an *ex-ante* estimate, extending the shelf life of cassava by several weeks would reduce financial losses by $2.9 billion in Nigeria alone over a 20-year period (Rudi et al., 2010).

Cassava undergoes several biochemical changes emanating from wound responses known as postharvest physiological deterioration (PPD). Within 48 hours after harvesting, the root tubers of cassava suffer a wound-response and undergo biochemical changes. PPD is a complex process involving enzymatic stress responses to wound healing, changes in gene expression and protein synthesis as well as accumulation of secondary metabolites (Blagbrough et al., 2010). It has been reported that PPD of cassava is influenced by environmental factors, as well as by genotypes. Other factors that have been correlated with PPD include dry matter content, harvest period, soil preparation and physiological state of the plant (Zidenga et al., 2012). PPD reduces starch quality and renders the cassava roots unmarketable and unpalatable. It starts as a physiological process which is triggered by physical damage of the roots during harvesting and followed by microbial deterioration. It is observed as a blue or brown discoloration of the vascular parenchyma that starts to appear between 24 and 72 hr of harvest (Blagbrough et al., 2010).

The wound on the roots triggers an oxidative burst of the super oxide radical (O_2-), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2). Therefore, PPD can be delayed by the exclusion of oxygen, storing the roots in polyethylene bags, in a water-bath, or by coating the tubers with wax (Rickard, 1985; Best, 1990). Although a number of secondary metabolites accumulate during the storage process, their occurrence has been linked with microbial decay, which dominates after about six days of storage (Buschmann et al., 2000; Huang et al., 2001). Reported symptoms of PPD include increased respiration (Uritani, 1998), failure in wound healing response of the plants (Booth et al., 1976), changes in lipid composition (Lalaguna & Agudo, 1989) and synthesis of chemical compounds such as ethene (Hirose et al., 1984). Other symptoms include rapid increase in uptake of calcium ion (Ca^{2+}), production and accumulation of phenolic compounds such as scopoletin, scopolin, esculin and proanthocyanidins which cause discoloration of the vascular tissues (Rickard, 1985). Predominantly, only the accumulation of hydrogen peroxide, scopoletin and scopolin occur during physiological deterioration process (Buschmann et al., 2000; Reilly et al., 2004; Iyer et al., 2010).

In view of the important role of cassava in food security, as identified and prioritized by Food and Agriculture Organization of the United Nations (FAO), the chemistry and biochemistry of PPD has been widely studied, with intent to improve its shelf life to a minimum of 14 days.

Many of these studies have placed reactive oxygen species (ROS) production as one of the earliest events in cassava's deterioration process, and hydrogen peroxide as the major ROS causing cassava PPD (Lalaguna & Agudo 1989; Buschmann et al., 2000). A study has also correlated the production of hydrogen peroxide in cassava with cyanogenesis (Zidenga et al., 2012).

Several studies have identified and quantified hydrogen peroxide in cassava roots in relation to its PPD, but this is the first study that will employ an electrochemical method for the quantification of the level of hydrogen peroxide as it relates to cassava PPD and correlate the presence of beta-carotene with the production of hydrogen peroxide in different cassava cultivars.

Materials and Methods

Reagents and chemicals

All chemical reagents employed in this research were of analytical grade, needing no further purification. Sodium hydroxide (NaOH), acetic acid, silver nitrate ($AgNO_3$), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), hydrochloric acid (HCl- 37%), acetone $(CH_3)_2CO$, hydrogen peroxide (H_2O_2), alumina Al_2O_3 (0.05 µm and1 µm))**, e**thanol, perchloric acid (70%) and methanol were purchased from BDH Chemicals Ltd.

Sample Collection & Preparation

Four different cultivars of cassava root tubers were collected from International Institute of Tropical Agriculture (IITA), Ibadan Nigeria and used in the study. The samples were identified at the herbarium of the University of Lagos and assigned sample numbers. Five root tubers of each cultivar were used in the study. The four different cassava cultivars are IBA070593- Yellow root (8 months old before harvest), IBA120008- Yellow root (15 months old before harvest), TME 419- White root (8 months old before harvest) and IBA980505- White root (8 months old before harvest). These samples were harvested on the same day.

Table I: Cassava cultivars and sample codes

Sample Name	Code
IBA070593- Yellow root (8 months old)	S3
IBA120008- Yellow root (15 months old)	S8
IBA980505- White root (8 months old)	S5
TME 419- White root (8 months old)	S9

Storage and Extraction of Roots

The root tubers were stored at ambient conditions in the laboratory at 26^o C during the six days of analysis. The sample roots were washed in running water to remove dirt before analysis. Distal and proximal ends were cut, and the body was wrapped in a cling film, using the method described in literature (Uarrota et al., 2016). Some of the fresh cassava roots were left uncut, while others were transversely cut into 1 cm slices and stored at room temperature. The roots were observed and analyzed over a period of six days.

Deacetylation of Chitin

Chitosan was obtained by the deacetylation of chitin. 40% NaOH solution was prepared in a 100 mL standard flask and 10 mL of this was taken and added into a round bottomed flask containing 15 mL of isopropanol and 3 g of chitin (queen crab 45-60 mesh). The resulting mixture was refluxed, decanted and filtered with a Whatman (125 mm) filter paper; washed several times with de-ionized water (milli-Q^R), followed by the addition of 3.3 mL of 5% w/v ethylene-diamine-tetra-acetic acid (EDTA) with stirring at room temperature for 2 additional hours for precipitation of any heavy metals (Qian & Glanville 2005). The purified chitosan obtained was then used in the synthesis of the chitosan/silver nanohybrid.

Synthesis of Chitosan/Silver NanoHybrid (Cs/AgNps) & Characterization

Chitosan silver nanohybrid (AgNP) solution was prepared following the method of Tran et al., (2010). 25 mL 0.1 M $AgNO_3$ was prepared and added to a mixture of 1% w/w chitosan/acetic acid/water composition and refluxed at 90^o C. The derived AgNPs was an orange-red colloidal solution which was centrifuged at 5,000 rpm for 1 hour to eliminate particles present. The supernatant obtained was a clear orange-yellow solution which served as the CS/AgNPs stock**.**

Characterization of the chitosan/silver nanohybrid

The chitosan/silver nanohybrid was characterized using X-ray Diffraction (XRD) technique and a High-Resolution Transmission Electron Microscope (HR-TEM).

Immobilization of Chitosan Silver nanoparticle hybrids on glassy carbon electrode

Glassy carbon electrodes used were first cleaned with double distilled water and polished sequentially using coarse and fine alumina respectively. The electrode was rinsed again with water, dipped in ethanol for 2 minutes, and then left to dry at room temperature. The immobilization of the electrode was done by casting 5 µL of the CS/AgNPs mixture as a drop onto the surface of the electrode and left to dry for1 h before electrochemical measurements were carried out.

Electrochemical determination of hydrogen peroxide

A BASI-Epsilon potentiostat/galvanostat obtained from Bioanalytical Systems InC. (West Lafayette, USA) was used in the electrochemical study, employing the conventional three-electrode configuration for the measurements. The working electrode (3 mm) was made of the CS/AgNPs immobilized on a glassy carbon electrode. Ag/AgCl served as the reference electrode, while a platinum electrode (1.6 mm) served as the auxiliary electrode.

Standard solutions of hydrogen peroxide (1.0mM-5.0 mM) were prepared in 0.1 M phosphate buffer, pH 7.0. Each solution was purged with nitrogen for 10 min before scanning the potential from 0.1 V to 1 V using a scan rate of 50 mV/s. The cathodic peak currents obtained at -550 mV was subsequently used to construct the calibration curve. Electrochemical determinations for the reduction of hydrogen peroxide in 0.1 M PBS pH 7.0 on bare glassy carbon electrode were similarly carried out.

Determination of the β- carotene contents of the Cassava cultivars

Beta-carotene contents of the cassava cultivars were determined according to the method reported by Harborne, 1973. 250 mg of the cassava root samples were blended with 25 mL of 80% acetone and filtered using a Whatmann filter paper. The supernatant was made up to the 25 mL mark with 80% acetone and the absorbance read at 480 nm on the UV-Vis spectrophotometer.

Determination of the levels of hydrogen peroxide by UV-Visible spectrophotometry

Standard solutions of hydrogen peroxide were prepared. Each solution was mixed with a fixed concentration of the CS/AgNPs, stirred and left to react for 15 minutes before reading the absorbance at 400 nm. The values obtained were used to prepare a calibration curve for the hydrogen peroxide.

Results and Discussions.

Synthesis of metal nanoparticles has gained increasing attention because of their potential applicability in electronics, chemistry, energy and medicine (Saxena et al., 2012). The silver nanoparticles have a large number of applications in nonlinear optics, spectrally selective coating for solar energy absorption, biolabeling, intercalation materials for electrical batteries as optical receptors, catalyst in chemical reactions, antibacterial materials, chemically stable materials and good electrical conductors (Sharma et al., 2009; Zargar et al., 2014).

The synthesized chitosan silver nanoparticle hybrid was characterized by UV-Visible spectrophotometry, X-ray diffraction analysis (XRD) and Transmission electron microscopy (TEM). The results are shown in figures 1-4.

The formation of the chitosan silver nanohybrid was accompanied by a colour change from a colourless solution of the silver nitrate to a golden yellow hue of the nanocomposite.

UV–Vis spectroscopy is the most important technique and the simplest way to confirm the formation of nanoparticles. The absorbance spectrum of the colloidal composite was obtained in the range of 300–700 nm (figure 1). The chitosan silver nanohybrid shows a maximum absorption at 400 nm as against 425 nm, indicating conversion of the silver ions from $AgNO_3$ to chitosan silver nanohybrid. It reflects the interphase interaction of the chitosan with the silver nanoparticle. Typically, a strong, broad peak shown around 400 nm characterizes the surface plasmon resonance bands of poly dispersed AgNPs. The bands are often influenced by factors such as shape, size, morphology, composition and dielectric environment of prepared nanoparticles (Buschmann et al., 2004; Sharma et al., 2009; Sandeep, 2017).

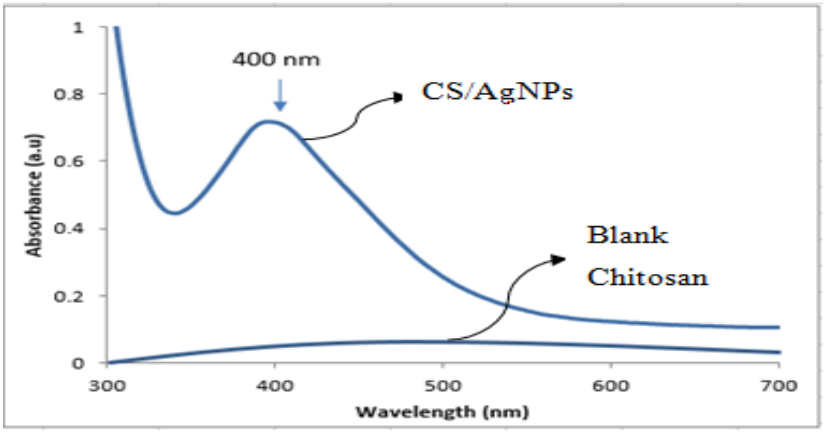

Figure 1: UV-Visible absorption of Chitosan silver nanohybrid

A subsequent characterization of the chitosan silver nanohybrid was carried out using X-Ray diffraction technique (figure 2). The diffracted intensities were recorded from 30° to 70°. Three strong Bragg reflections were observed at 38.45°, 46.35°, 64.75° corresponding to the planes of (1 1 1), (2 0 0) and (2 2 0) respectively, indexed according to the face centered cubic crystal structure of silver. These values are in agreement with the values reported in literature (Rhim et

al., 2006; Govindan et al., 2012; Olaniyan et al., 2016). The results were corroborated against the Energy Dispersive X-ray spectroscopy (EDX) (figure 3), which show strong signals indicating the crystalline property of the silver atoms. The presence of O and N peaks along with the Ag signals is indicative of the chitosan molecule used to cap the AgNPs.

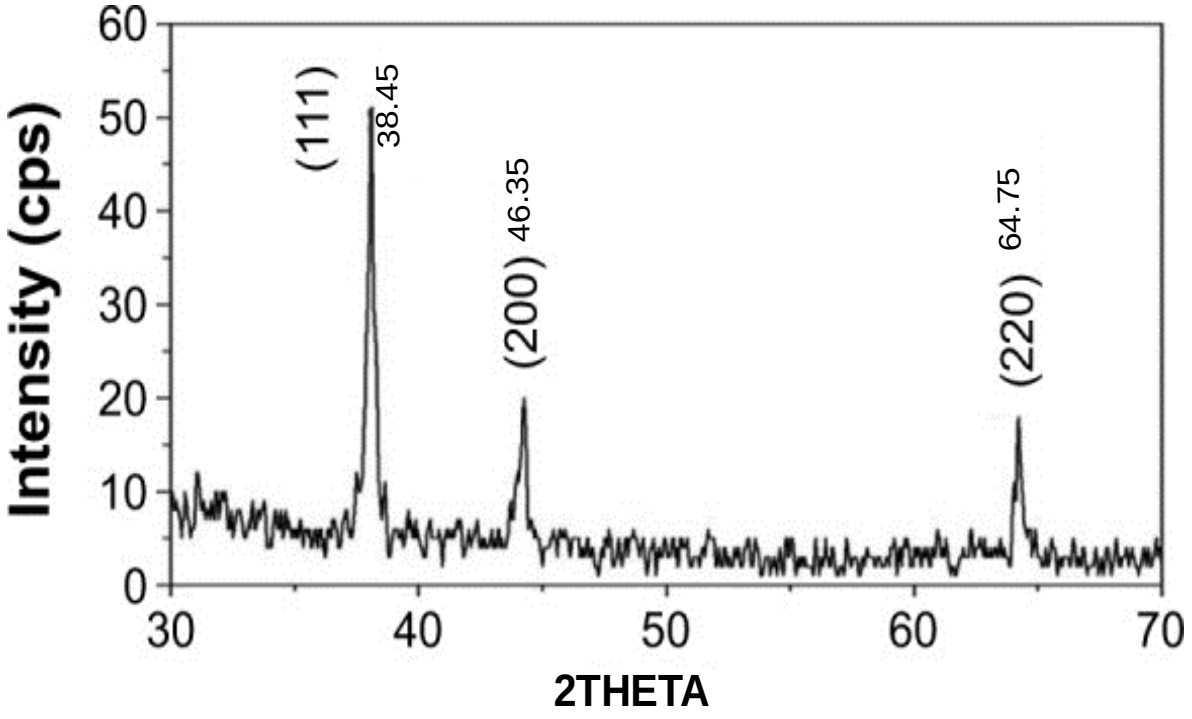

Figure 2: XRD characterization of Synthesized CS/AgNPs

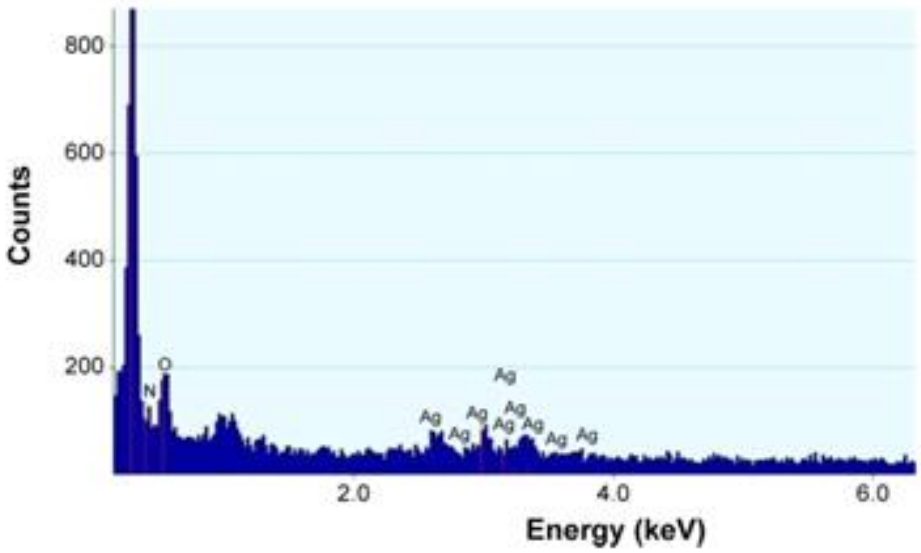

Figure 3: Energy dispersive X-ray spectrum of the CS/AgNPs

The HR-TEM images of the synthesized AgNPs (figure 4) indicates that the particles are predominantly spherical in shape with an average particle size of 24.6178 nm as determined from the TEM micrograph. This falls within the range earlier reported by Atanda et al., 2019.

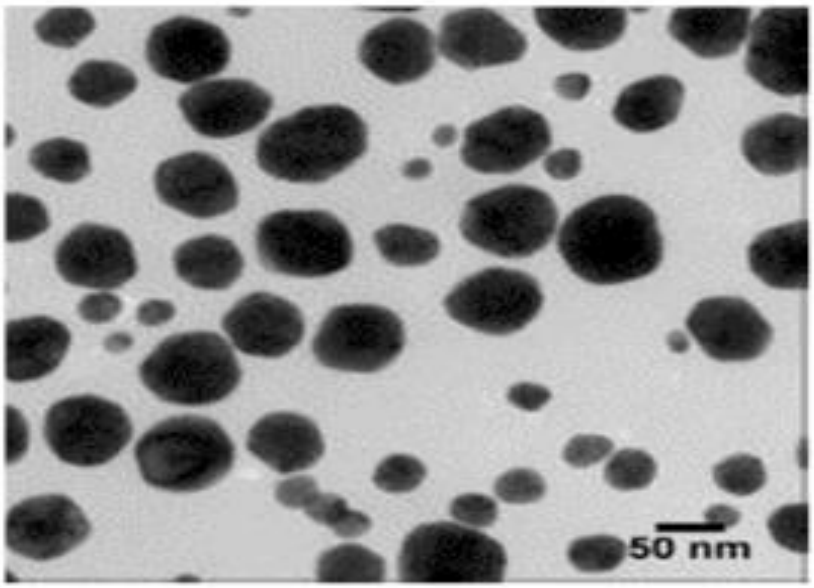

Figure 4: High Resolution Transmission Electron Microscopy (HR-TEM) of the CS/Ag nanohybrid.

The result for the determination of the β-carotene contents of the cassava cultivars are shown in Table II. The yellow root cassava samples were found to contain higher levels of β-carotene, the highest being found in sample S3. S8 has a minimal amount of β-carotene while the white root samples did not have β-carotene.

Table II: β-Carotene Content in analyzed Cassava Samples

Sample	Absorbance at 480nm	β-Carotene content/100mg cassava roots
S3	0.011	0.0044
S5	-0.008	0.0000
S8	0.001	0.0004
S9	-0.003	0.0000

Electrochemical Determination of Standard Hydrogen Peroxide Concentrations and Comparison with UV-Vis Data

As a step in the determination of the levels of hydrogen peroxide in the deteriorating cassava samples, standard hydrogen peroxide solutions were prepared and the potential scanned between 0.1V and 1 V using the modified glassy carbon electrode. The results obtained were compared with those of the UV-Vis spectrophotometry. The overlay of the voltammograms obtained in the cyclic voltammetric determination of different concentrations of hydrogen peroxide are shown in figure 5. Evidently, the cathodic peak current for the reduction of hydrogen peroxide to water and oxygen occurred at -550 mV.

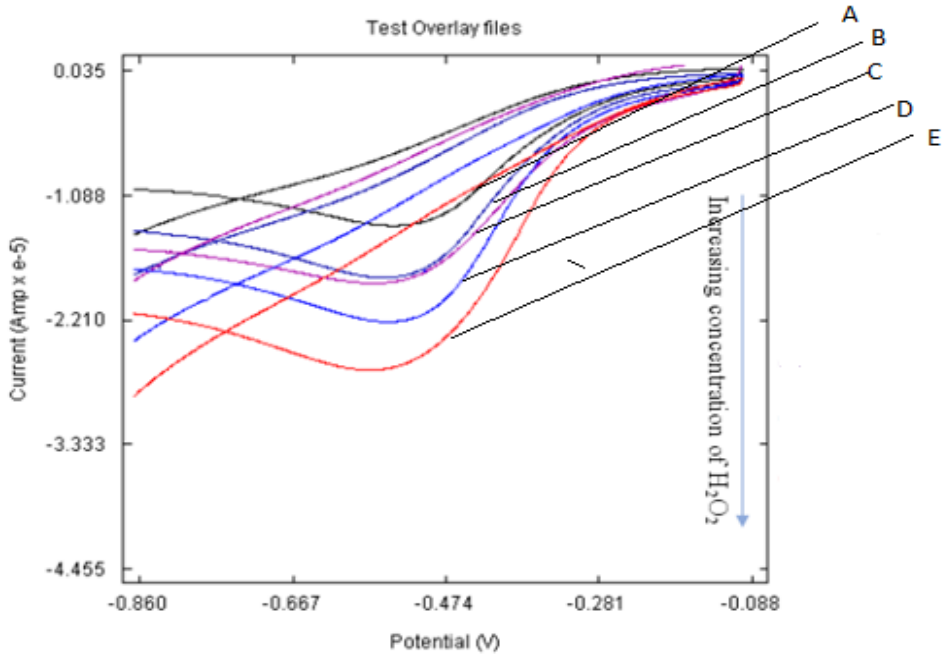

Figure 5: Overlay of voltammograms for the electrochemical reduction of standard solutions of hydrogen peroxide (A:1 mM, B: 2 mM, C: 3 mM, D: 4 mM, E: 5 mM)

It can be seen in figure 5 that the cathodic peak current at -550 mV correlated well with the electrocatalytic ability of the AgNPs, capped with chitosan to reduce H_2O_2 into water (H_2O) and oxygen (O_2) (Tran et al., 2010**)**. Figure 6 shows a schematic diagram of the catalytic ability of the AgNPs.

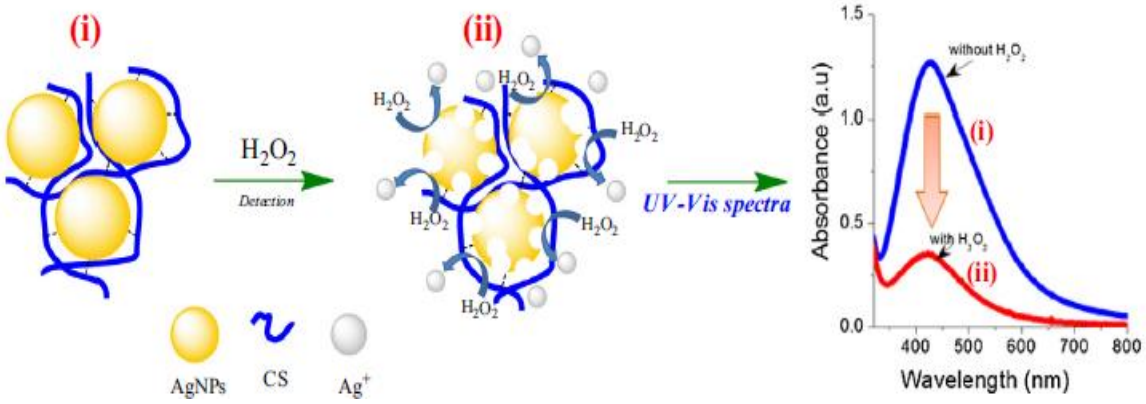

Figure 6: Reducing ability of CS/AgNPs (Tran et al., 2010)

The catalytic ability of the AgNPs capped with chitosan to reduce hydrogen peroxide to water and oxygen was also observed in the UV-vis measurements shown in figure 7.

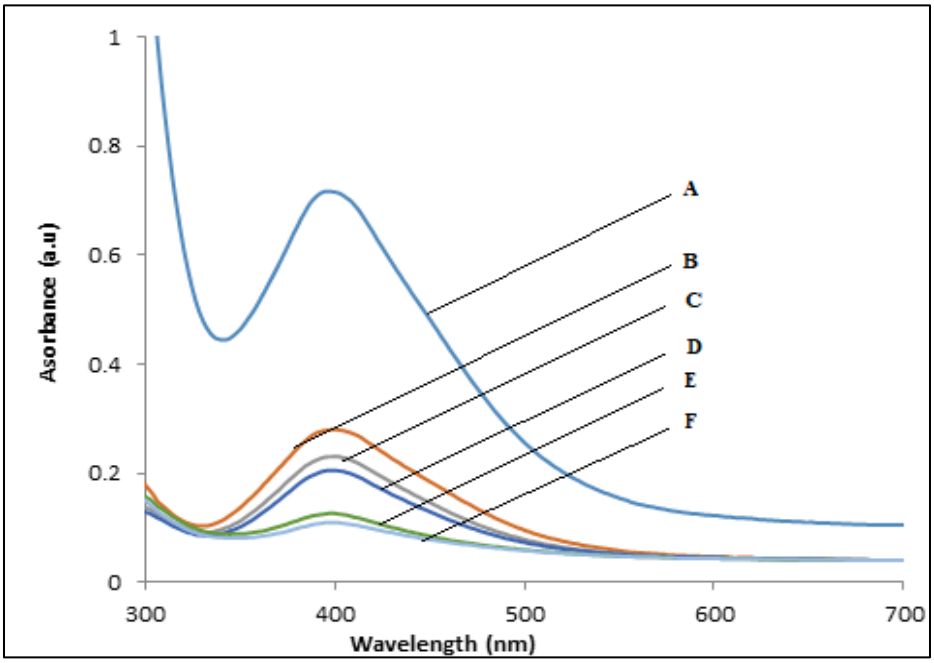

Figure 7: UV-Vis absorption spectra of CS/AgNPs hybrid solution in the presence of A- No H_2O_2, B: 1 mM, C: 2 mM, D: 3 mM, E: 4 mM and F: 5 mM H_2O_2

The results in figure 7 show a peak reduction at 400 nm in the absorbance of the Cs/AgNPs with increasing concentration of the H_2O_2. A calibration curve was obtained by plotting ($\Delta A/Ao$) * 100 versus H_2O_2 concentration ($\Delta A = A_0 - A_c$) using the data shown in Table III. The plot is shown in figure 8.

Table III: Table for absorbance values for calibration curve 1 to 5 mM H_2O_2

A	A_0	ΔA	$\Delta A/A_0$	$(\Delta A/A_0) * 100$
0.280	0.716	0.436	0.608939	60.89385475
0.230	0.716	0.486	0.678771	67.87709497
0.205	0.716	0.511	0.713687	71.36871508
0.126	0.716	0.590	0.824022	82.40223464
0.109	0.716	0.607	0.847765	84.77653631

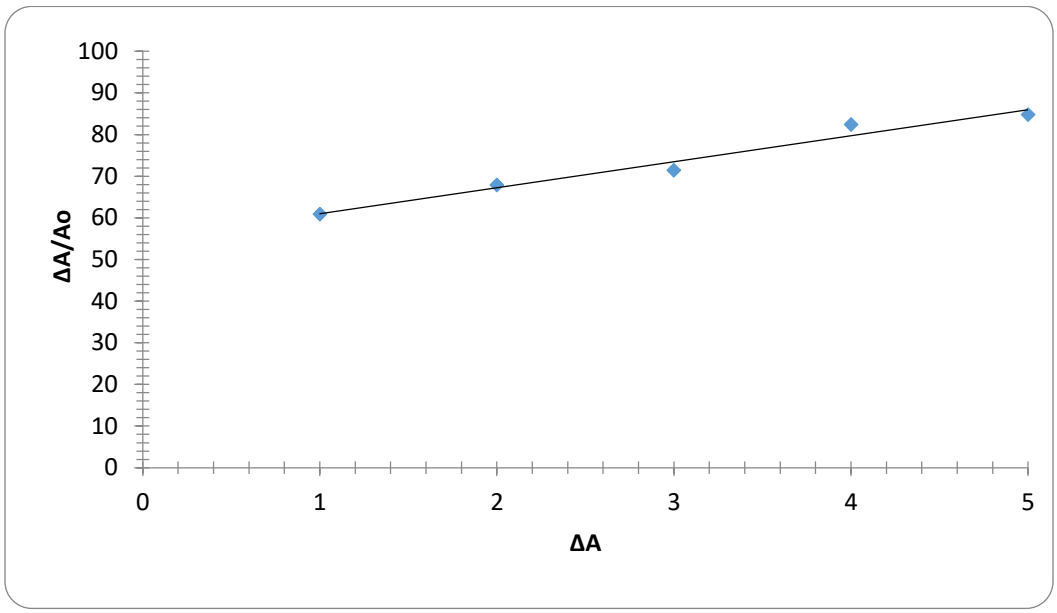

Figure 8: Calibration plot of (ΔA/Ao) against ΔA

The overlay of the voltammograms for the detection of hydrogen peroxide in the cassava cultivars are shown in figures 9-12. No significant difference was shown in the voltammograms for the detection of hydrogen peroxide in the cassava cultivars on the first and second day. This suggests that the samples did not experience rapid deterioration as expected.

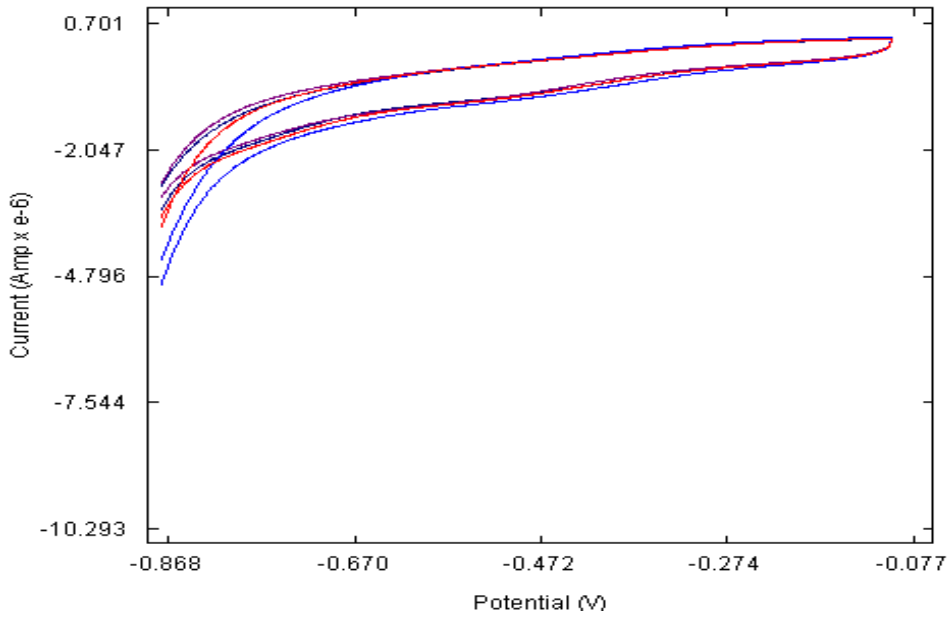

Figure 9: Overlay of the voltammograms for determination of hydrogen peroxide levels in different cassava cultivars on day one and two after harvest.

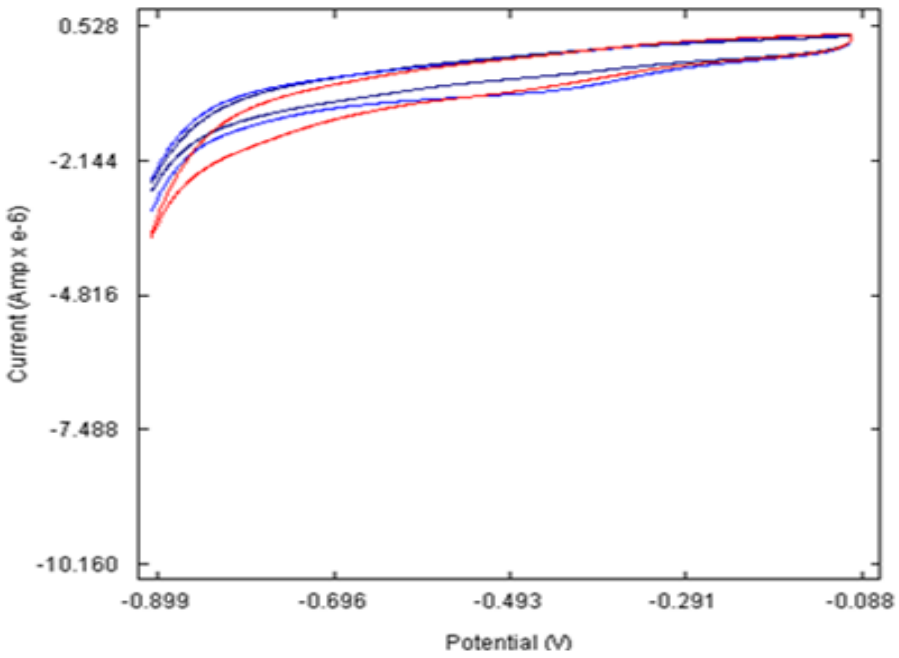

Figure 10: Overlay of the voltammograms for determination of hydrogen peroxide levels in different cassava cultivars on day three and day four after harvest.

However, the voltammograms for the reduction of hydrogen peroxide in the cassava cultivars are well separated on the fifth day based on the production of hydrogen peroxide in the tubers. A similar trend was observed in day 6, confirming the production and accumulation of hydrogen peroxide in the cassava roots.

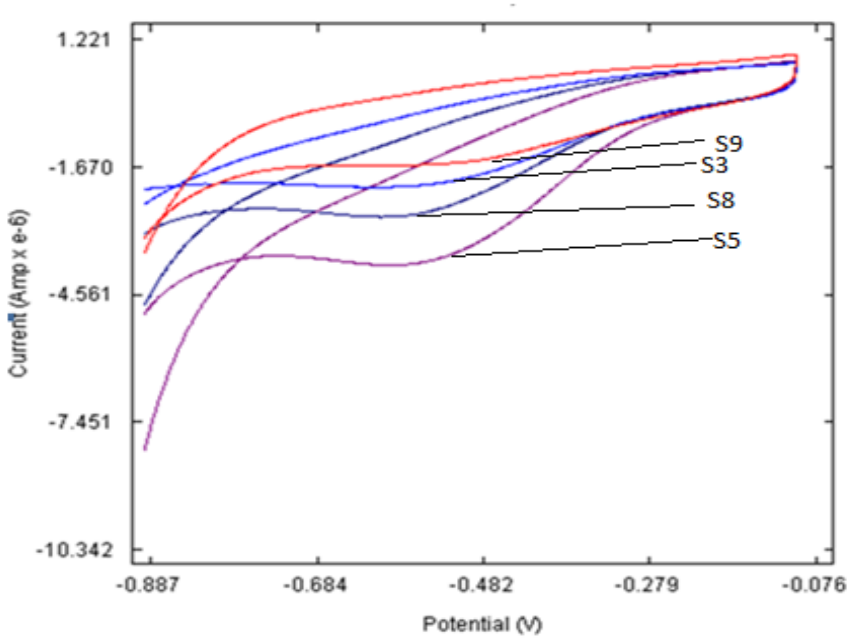

Figure 11: Overlay of the voltammograms for determination of hydrogen peroxide levels in different cassava cultivars on day five after harvest.

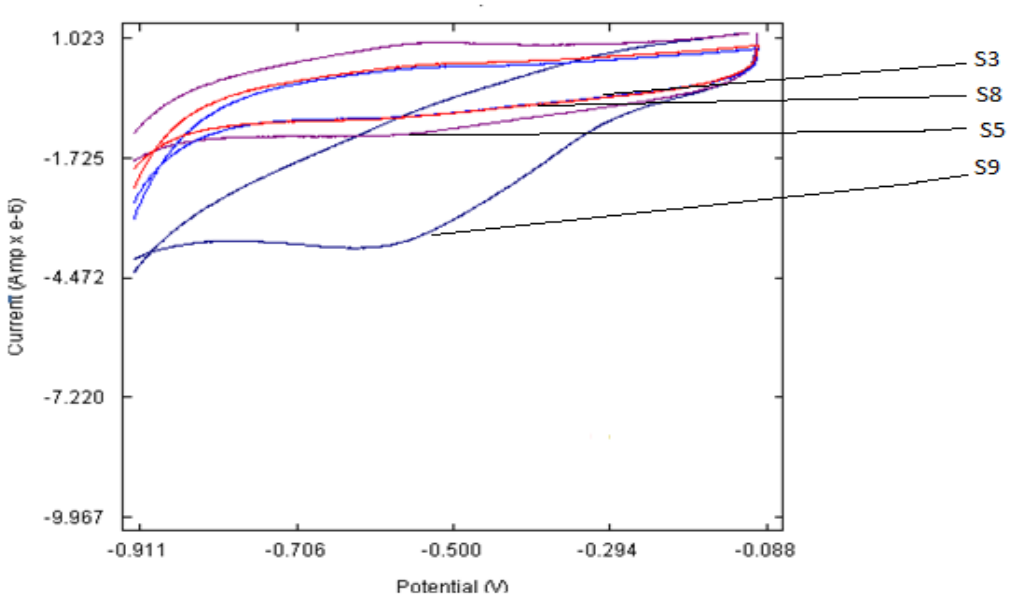

Figure 12: Overlay of the voltammograms for determination of hydrogen peroxide in different cassava cultivars on day six after harvest.

Table IV: Amount of hydrogen peroxide in cassava samples on day 5

Sample Code	Current (µA)	Concentration of H_2O_2
S9	0.496	0.071 mmol/g FW
S3	0.992	0.096 mmol/g FW
S5	1.550	0.177 mmol/g FW
S8	0.672	0.141 mmol/g FW

Table V: Concentration of H_2O_2 on Day 6

Sample Code	Current (µA)	Concentration of H_2O_2 (mM)
S9	0.936	0.133 mmol/g FW
S3	0.26	0.037 mmol/g FW
S5	0.672	0.096 mmol/g FW
S8	0.66	0.094 mmol/g FW

FW – Fresh weight

Table VI: Summary of Percentage Change in Samples

SAMPLE CODE	DAY 5	DAY 6	% CHANGE
S9	0.071 mmol/g FW	0.133 mmol/g FW	87 % increase
S3	0.096 mmol/g FW	0.037 mmol/g FW	61 % decrease
S5	0.177 mmol/g FW	0.096 mmol/g FW	33 % decrease
S8	0.141 mmol/g FW	0.094 mmol/g FW	46 % decrease

FW – Fresh weight

Conclusion

The utilization of the chitosan silver nanoparticles immobilized on glassy carbon has proven to be an efficient, fast and low-cost method for quantifying hydrogen peroxide in samples. A high level of specificity in determining the analyte of interest, compared to other electrodes, including bare glassy carbon electrode was achieved and this specificity is of interest to many analytical processes, especially when working with plant species with diverse compounds observable within close range.

The synthesis of the chitosan silver nanocomposite was validated by data from the Uv-Vis measurement. The UV-vis indicates plasmon band resonance of 400 nm. The study also showed the data obtained from TEM, XRD and EDX measurements.

The complete process of modification was also confirmed by the respective voltammograms derived from the unmodified electrode alongside with the modified one, with the latter giving an improved detection of the hydrogen peroxide in samples by exhibiting improved peaks.

The four cultivars of cassava analyzed showed variations in the concentration of hydrogen peroxide profoundly on days five & six. These variations are explainable, as rate of physiological postharvest deterioration depends on the type of cultivar as well as environmental conditions (Sanchez et al., 2006).

The yellow tubers used in the study, IBA070593 and IBA120008 were confirmed to contain β-carotene which slowed down the postharvest deterioration. The white cassava tuber did not contain β-carotene and recorded high levels of postharvest deterioration. The physiological postharvest deterioration (PPD) has been correlated with the β-carotene contents of different cultivars, showing that β-carotene plays a role in the reduction of cassava PPD. Carotenoids are a class of metabolites acting as chain breaking antioxidants, thus, protecting cells and organisms against photooxidation (Sanchez et al., 2006; Priya & Siva, 2014). The antioxidant properties of carotenoids help to extend the shelf-life of cassava storage roots (Priya & Siva, 2014).

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