The Effects of Intrasound Therapy on the Healing of Tendon Exposed to Alcohol

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ABSTRACT

Background: Functional abnormalities in wound structure has been identified as a potential complication if a patient consumes alcohol prior to injury and studies suggest that even a single incidence of acute ethanol exposure can have adverse effects on the tissue response to trauma. Ethanol ingestion has been shown to result in delayed and abnormal tendon healing 3 weeks after injury.

Objective: This study investigated the effects of low intensity intrasound therapy (LITR) given twice daily on the morphology and antioxidant parameters in the healing tendon following an acute injury in rats exposed to prior ethanol consumption.

Methods: Fifteen male rats, randomized into three groups, all had induced crush injury to the left Achilles tendon. Groups 2 and 3 had prior oral administration of 30% ethanol for six days while Group 1 (control) had no alcohol and no follow-up intervention. Group 2 received no treatment while Group 3 had LITR twice daily. LITR was commenced immediately post-injury and was given twice daily over the first 6 days. The animals were sacrificed on day 20 post-injury and the tendons were excised, and processed for histology, antioxidant and malondialdehyde (MDA) assay.

Results: The tendons in Group 2 showed disordered and haphazard collagen formation with neutrophilic infiltrates and high tenoblast population at 20 days post-injury while the LITR treated tendon demonstrated dense, organized, parallel collagen deposits with fewer tenoblasts. LITR also significantly improved the antioxidant parameters and lowered the MDA when compared with the tendon of rats that were fed alcohol but received no treatment ($p<0.05$).

Conclusion: LIRT reversed the deleterious effect of ethanol on the healing tendon and resulted in near-normal morphology of the healing tendon 20 days post-injury.

Keywords: Alcohol, intrasound therapy, tenoblasts, tendon healing, acute tendon injury.

INTRODUCTION

A significant number of patients presenting with traumatic injuries have been found to have prior acute ethanol exposure with a resultant severe impact on morbidity and mortality (1, 2). Alcohol consumption prior to injury has been implicated by some studies as being responsible for functional abnormalities in wound structure with alterations in the extracellular matrix (ECM) production and matrix proteolytic activity (3). This has been attributed to the breakdown of ethanol which generates NADH, acetaldehyde and acetate metabolites that induce tissue damage through the production of reactive oxygen species (ROS), lipid peroxidation, and alterations in signal transduction (4–6).

Overproduction or an inadequate removal of ROS in traumatic injuries normally results in oxidative stress and this, coupled with the production of ROS from the breakdown of ethanol, leads to delayed wound healing (7). Tendon healing is not exempted from these effects as prior studies have shown that tenocyte proliferation and/or viability may be affected by ROS. For instance, equine tenocytes show a decrease in proliferation when subjected to bolus addition of 10–100μM of H_2O_2 (8). However, the enzymatic antioxidant defence mechanisms against free radical-induced oxidative stress are available and these include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (9). The role of nitric oxide (NO) in tendon healing has also been investigated and studies have shown that levels of nitric oxide synthase peak after 7 days and return to baseline 14 days after tenotomy of rat Achilles tendons while inhibition of nitric oxide synthase impaired healing (10).

It has been demonstrated that acute ethanol exposure directly inhibits fibroblast function during wound healing and inhibit fibroblast proliferation (11). Tenocytes and tenoblasts are specialized tendon cells and their relative population

following an injury is an indicator of the tissue response to the treatment given (12) with most of the tenoblasts being terminally differentiated to tenocytes by the 4th week post-injury (13, 14). Hapa and colleagues (15) investigated the histological and biomechanical effects of ethanol intake on tendon healing in a rat tendon injury model and found that ethanol ingestion resulted in abnormal tenocyte morphology, disorganized collagen bundles with a tendency toward increased tenocyte number, and neovascularization 3 weeks after the tendon injury indicating delayed and abnormal healing. In addition, the study by Stephens and his co-researchers (16) demonstrated that a 1% v/v (~800 mg/dL) dose of ethanol inhibited collagen synthesis significantly by fibroblasts *in-vitro*. In view of these findings, coupled with the normal poor healing response of tendons, it is important to investigate therapeutics aimed at reversing the ethanol impairment and counteract the effects of ethanol on the repair process (11).

Intrasound therapy (ITR), which has been acclaimed to be effective in improving healing in acute inflammatory injuries (17), produces mix frequency acoustic waves in a range of 16–20,000 Hz that can penetrate up to the level of bone when applied on the skin (18). The findings from some of our previous studies suggested that ITR is effective in the healing of acute tendon injuries at any given intensity when applied twice daily (19-21). The aim of this study was to determine if low intensity intrasound therapy would reverse the effects of ethanol on the healing tendon and thus promote healing.

MATERIALS AND METHODS

MATERIALS

Animals

Fifteen 8-week old male Sprague-Dawley albino rats weighing 180–220 g were used for the study. The animals were kept in the animal room of the Department of Anatomy, College of Medicine of the University of Lagos, Lagos, Nigeria where the study was carried out. They were kept under standard conditions of 12-hour light and 12-hour darkness photoperiodicity. Ethical approval of the study was obtained from Ethical Committee of the College of Medicine, University of Lagos, Lagos, Nigeria. The rats were fed on commercial rat chow and water *ad libitum.*

Instrumentation

Novasonic- Novafon™ Intrasonic device manufactured by ™NOVASONIC MEDICAL House of Novasonic-Novafon, Denmark.

METHODS

Groupings

The animals were randomly assigned into three groups of five rats per group. The animals were assigned as follows:

Group 1: Crush injury, no alcohol and no treatment given (Experimental Control).

Group 2: Alcohol administration and a crush injury with no treatment given.

Group 3: Alcohol administration with a crush injury and treatment with LITR twice daily.

Experimental Protocol

Ethanol Administration

All the animals in Groups 2 and 3 were fed with 30% alcohol at about mid-day daily for six days by gavage through an oro-pharyngeal metal cannula (22) at a dosage of 7 mL/kg body weight (23).

Injury Procedure

After the administration of the alcohol on the 7th day, all the rats in the three groups were made to undergo an induced crush injury to the left Achilles tendon. The pre-injury circumference of the left hind limbs for each rat was taken just above the calcaneal insertion of the Achilles tendon by means of a flexible inextensible marked cord. The Achilles tendon of the left limb of each rat in all the groups was clamped transcutaneously with Number 1 artery forceps to the forceps maximum closure for 60 seconds to induce a crush injury (20, 24).

Intrasound Therapy

The Achilles tendon of the left hind limb of each of the rats in Group 3 was treated twice daily with the lowest intensity of intrasound therapy using the small treatment head applied over the Achilles tendon for 5 minutes with sterile K-Y jelly as a coupling medium (25). Treatment was given consecutively over the first six days post-injury (21, 26).

Animal Sacrifice and Tendon Harvest Process

On the 20th day post-injury, all the animals were sacrificed by cervical dislocation and the Achilles tendons excised. The tendons were processed for histology using the technique of stereology described by Young and Dyson (27).

Stereological Analysis

The slides were observed under the light microscope fitted with an ocular test grid at a magnification of x 200 and x 00 using the method of Cruz-orive and Weibel (28). The tenoblast and tenocyte profiles identified were the nuclei. Fifty random values (10 per animal) were obtained for each group. The numerical density (NA) is the number of tenoblast/tenocyte profiles per unit area of field (29). This is estimated as the profile number of tenocytes (N) within the frame of the test grid (A). N was determined by counting all the tenocyte profiles partially or totally within the frame area that did not intersect the forbidden lines, which are the top and left margins of the test grid (28). The tenoblasts were further differentiated from the typical tenocytes by their shapes. The elongated cells were identified as tenocytes while the ovoid cells were identified as tenoblasts (26). One hundred and fifty random (30 per animal) values were obtained for each group.

The total number of cells was estimated using fractionator method as shown below:

$$N = \Sigma Q \times \frac{1 \times 1 \times 1}{ssf \quad asf \quad tsf}$$

Where ΣQ is the total number of profile counted, ssf is the section-sampling fraction, asf is the area sampling fraction and tsf is the thickness sampling fraction.

Tendon MDA Assay

Tendon Malondialdehyde (MDA) levels were determined using the modified Thiobarbituric acid (TBA) method of Buege and Aust (30).

Total Superoxide Dismutase (SOD) and Catalase (CAT) Activities

In order to determine CAT activity, tissue portions were sonicated in 50 mM phosphate buffer and the resulting suspension was centrifuged at 3000*g* for 10 min. The supernatant was used for enzyme assay. SOD activity was determined by measuring the inhibition of adrenaline self-oxidation absorbance at 480 nm (31). CAT activity was measured by the rate of decrease in hydrogen peroxide (10 mM) absorbance at 240 nm (32).

Nitric Oxide (NO) Expression

The procedure was based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavenging of nitric oxide competes with oxygen leading to reduced production of nitrite ions. For this experiment, sodium nitroprusside (10 mM) in phosphate buffer saline was mixed with the sample and incubated at room temperature for 150 min. After the incubation period, 0.5 ml of Griess reagent was added and the absorbance of the chromophore formed was read at 546 nm (33).

Statistical Analysis

The statistical package used was EPI-INFO 3.5.1 2008 version. The data obtained from the stereological evaluation of the tenocytes and tenoblasts population, as well as the biochemical evaluation, were subjected to statistical analysis and expressed as mean ± SD (standard deviation). Differences between the groups were compared using Student's *t*-test for analysis of paired samples. Spearman's correlation was used to determine the relationship between the biochemical parametres and the population of the tendon cells. Chi square (χ^2) analysis was used to compare the ratio of the population of tenoblasts and tenocytes. The null hypothesis was set at a significant level of 0.05 or 5%.

RESULTS

The results obtained in this study indicate that at 20 days post-injury, the population of tenoblasts in the alcohol-fed untreated tendon was about three times that of the tenocytes in contrast to the control and intrasound treated tendons. Chi square (χ^2) analysis showed there was a significant difference in the population of the tenocytes (p=0.03) but not the tenoblasts (Table 1). The relationship between the level of NO and the tendon cells indicate a positive correlation for the tenoblasts and a negative correlation for the tenocytes in the control and intrasound treated tendons while the reverse is the case in the alcohol-fed untreated tendon (Table 2).

There was also a negative correlation between SOD/CAT and MDA in the control and intrasound treated groups while it was positive for alcohol-fed untreated tendons (Table 3). There was no significant difference in the biochemical parameters between the groups.

At 20 days post-injury, the histomorphology of the tendons in the control group show moderately aligned collagen deposits and a mixture of inactive tenocytes and more of plump round collagen forming tenoblasts (Figure 1A). In the alcohol-fed untreated tendons, there was disordered and haphazard collagen formation with neutrophilic infiltrates and mild oedema (Figure 1B) while the intrasound treated tendons had parallel well-organized collagen deposits and fewer tenoblasts than the control (Figure 1C).

Table 1: Comparative Analysis of the Population of Tenoblasts and Tenocytes and their Association with Chi Square

Group	TB (N) $\bar{X} \pm SD$	%	TC (N) $\bar{X} \pm SD$	%	Ratio to TB to TC	P value
1	710 ± 353	68.03	334 ± 120	31.97	2:1	0.12
2	693 ± 206	76.77	210 ± 52	23.23	3:1	0.02*
3	572 ± 385	56.27	445 ± 76	43.73	1:1	0.59
χ^2 (p)		1.21		0.03*		

**Significant at p<0.05. TB, Tenoblasts; TC, Tenocytes; N, Total Number of Cells/Unit Area.*

Table 2: Correlation of Tendon Cells with Nitric Oxide

Group	Spearman's Correlation (r)	
	Tenoblasts	Tenocytes
1	0.66	–0.41
2	–0.27	0.65
3	0.98	–0.98

Table 3: Correlation of Antioxidants with MDA

Group	Spearman's Correlation (r)	
	SOD	CAT
1	–0.63	–0.63
2	0.37	0.37
3	–0.98	–0.97

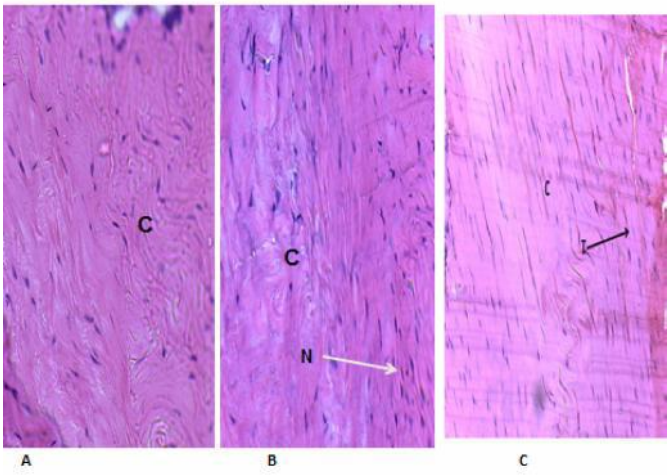

Figure 1: Longitudinal section (L/S) of tendon of rat. A: control, B: alcohol fed with no treatment and C: alcohol fed and treated with LITR (H&E) × 200. A shows moderately aligned collagen fibres (C), B shows disorganized collagen fibres (C) with neutrophilic infiltrates (N) while C shows orderly and parallel collagen deposits (C) and elongated tenocytes (T).

DISCUSSION

This study was undertaken to determine the role of intrasound therapy in ameliorating the negative effects of acute alcohol exposure on the healing of acute tendon injury. This study is pertinent because studies have shown that about 25% of alcohol related trauma victims had acute alcohol exposure (2). At this stage in the repair process, it is expected that there will be a tendency towards a terminal differentiation of most of the tenoblasts to tenocytes (16, 17). The results of this study however showed that at 20 days post-injury (Table 1), the tenoblast population in the alcohol fed untreated tendons was about three times the population of the tenocytes with a significant difference in their population ($p<0.05$). This suggests a delay in the healing process whereby cell proliferation was still on-going at a time when most of the tenoblasts should have been terminally differentiated to tenocytes (14). In the intrasound treated tendons, the ratio of both cell types was 1:1 while in the tendon that healed naturally, the proportion of the tenoblasts was double that of the tenocytes. The Chi square analysis in Table 1 showed a significant difference in the population of the tenocytes but not the tenoblasts. This could be due to the fact that at this stage in the healing process, the tenocytes are expected to be dominant (14).

Studies by Davidson and his co-researchers had suggested that the proportion of both cell types following injury is an indicator of the tissue responses to the treatment given (12). When an injured tendon heals naturally, the remodelling phase which is characterized by decreased cellularity is expected to commence after approximately three weeks (34, 35). This is corroborated by the results in Table 2 in which there was a negative correlation between NO, a signalling molecule that induces cell proliferation and the tenoblasts in the alcohol-fed untreated tendons unlike in the control and ITR groups where there was a positive correlation. The results suggest that NO had a positive effect on the proliferation of tenoblasts both in the tendons that healed normally and the intrasound treated tendons.

The deleterious effects of alcohol have been attributed partly to alcohol induced oxidative injury that is indicated by increased expression of oxidant radicals and alterations in oxidant/antioxidant balance (36). In this study as depicted in Table 3, there was a weak positive correlation between MDA and SOD/CAT in the ethanol fed untreated tendons ($r = 0.37$) which suggests that increase in oxidative stress in this tendon resulted in a weak increase in antioxidant enzymes. In the control and intrasound treated tendons, the correlations were negative ($r = 0.63$ and $0.98/0.97$ respectively) suggesting that the antioxidants were able to overcome the oxidative stress. It is interesting to observe the strong negative correlation in the intrasound treated tendons which may be a pointer to the fact that treatment with intrasound therapy resulted in significant expression of antioxidant enzymes which were able to overcome the oxidative stress in this tendon.

These results suggest that intrasound therapy had a positive impact on the healing response of the alcohol-fed tendons by reversing the deleterious effects of alcohol on the healing tendon. This effect could be seen further in the histological sections of the tendons. The alcohol fed tendons showed disorganized collagen fibres and neutrophilic infiltrates at 20 days post-injury (Figure 1b) while the intrasound treated tendons had parallel, well aligned collagen fibers and more spindle shaped tenocytes (Figure 1c). On the other hand, the tendon of the non-alcohol fed untreated rats showed moderately aligned collagen fibres (Figure 1a). These findings are in consonance with the study by Hapa et al. (15) which found that ethanol ingestion resulted in abnormal tenocyte morphology, disorganized collagen bundles with a tendency toward increased tenocyte number three weeks after the tendon injury indicating delayed and abnormal healing. In their own study, no differentiation was made between the tenocytes and tenoblasts so the entire population of the tendon cells was collectively referred to as tenocytes. These findings may be attributed to the fact that ethanol exposure alters the early inflammatory phase (37) thereby affecting the proliferative response in healing wounds (38) and rendering the cells incapable of normal, physiologic function (39).

CONCLUSION

This study showed that intrasound therapy effectively reversed the deleterious effects of alcohol on tendon healing and enhanced the healing process. However, this being a preliminary study, the sample sizes were small. Further studies could be carried out with larger sample sizes and investigations into the biomechanical and molecular effects of intrasound on the healing tendon. Also, the investigations may provide an insight into the mechanism of action of this simple non-invasive mechanical device.

DECLARATION

The authors declare that no funding was received for this study. The authors also declare no conflicting interest.

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Synthesis and Antibacterial Screening of 2-Hydroxybenzylidene-Aminophenols and its Cu(II) Complexes

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ABSTRACT

Background: The design of metal containing antimicrobial agents is currently of interest due to the growing reports of drug resistance reported for known antimicrobial agents. Structure activity relationship is crucial in the investigation of potential candidates in drug therapy.

Objective: In line with structure activity relationship, a new series of copper(II) complexes of Schiff base 2-hydroxybenzylidene-2-aminophenol (L^1), 2-hydroxybenzylidene-3-aminophenol (L^2), and 2-hydroxybenzylidene-4-aminophenol (L^3) have been synthesised. The goal of this study was to investigate the effect of position of substituent on the antibacterial activities of the ligands and its metal complexes.

Methods: The compounds were prepared using condensation method. Structural and spectroscopic properties were studied by elemental analysis, spectral (FT-IR, ^{1}H NMR, UV-Vis) and magnetic susceptibility measurements. The Schiff base ligands and its complexes were screened *in-vitro* against *E. coli*, *S. aureus*, *P. aeruginosa*, *B. cereus*, *E. faecalis* and *K. pneumonia* bacteria.

Results: The metal complexes exhibited higher antibacterial activities than the parent ligand. This can be attributed to the greater lipophilic nature of the complexes.

Conclusion: The results show that the investigated metal complexes can be employed as broad spectrum antibacterial agents.

Keywords: Antimicrobial agents, copper (II), metal complex, Schiff base, aminophenol.

INTRODUCTION

Salicylaldehyde Schiff bases and metal complexes show a wide spectrum of antimicrobial properties (1–3). Antimicrobial activity is a property of both inorganic and organic substances, and the exploitation of such activity is a matter of considerable practical importance in the development of antiseptics, sanitizers, germicides, bactericides, sporicides, virucides and disinfectants (4). Schiff bases have played a marvellous role in the development of coordination chemistry as they readily form stable complexes with most of the transition metals (5). Metal complexes of Schiff bases are specifically of interest in bioinorganic chemistry because many of these complexes provide biological models in understanding the structure of biomolecules and biological processes (6). These complexes may statistically mimic the spectroscopic or other physical properties of the enzyme (7). Many researchers have studied the synthesis, characterisation and structure activity relationship (SAR) of Schiff base metal complexes (8-11), but much work has not been reported on the effect of position of substituents on the Schiff base metal complexes of aminophenol derivatives. In line with this, we report the synthesis and characterisation of a new series of copper (II) complexes with the Schiff bases 2-hydroxybenzylidene-2-aminophenol (L^1), 2-hydroxybenzylidene-3-aminophenol (L^2), and 2-hydroxybenzylidene-4-aminophenol (L^3). The effect of position of substituents on the antimicrobial activity was studied to determine the derivative that exhibit better activity. This study reports the stereochemistry of the metal complexes and provides baseline data for further study of structure activity relationship of elated complexes.

MATERIALS AND METHODS

Materials

All reagents and solvents were of analytical/spectroscopic grade and used without further purification. Ethanol, chloroform, dimethylsulfoxide, salicylaldehyde, 2-aminophenol, 3-aminophenol, 4-aminophenol and copper (II) chloride were purchased from Aldrich-Sigma Company Limited (Missouri, USA).

Physical Measurements

Microanalytical data were obtained on a Perkin Elmer model 2400 series II CHNS/O elemental analyzer. Infrared (IR) spectra of the compounds were recorded on a Bruker FT-IR (ATR) tensor

27 spectrophotometer directly on small samples of the compounds in the range 400 to 4000 cm^{-1}. ^{1}H-NMR spectra in DMSO-d_6 solution of the ligands were recorded on a Bruker Avance III 400 MHz. Chemical shifts were reported as δ relative to TMS as internal standard. Electronic absorption spectra of the compounds were recorded from 200 to 800 nm on a freshly prepared $CHCl_3$ solution using a Cary Model 50 spectrophotometer. Melting points (°C) were determined on a Reichert Thermovar melting-point apparatus and are uncorrected. Magnetic susceptibility measurements were made on powdered samples using a Sherwood Scientific magnetic susceptibility balance. $Hg[Co(SCN)_4]$ was used as the calibrant and corrections for diamagnetism were calculated from Pascal's constants.

Synthesis of Schiff Base

Equimolar quantities (10 mmol) of salicylaldehyde and corresponding amine were dissolved in ethanol (50 ml) and stirred under reflux at 70 °C for 6 h. The precipitate formed was separated by filtration, re-crystallised from ethanol, dried and stored in a desiccator.

Synthesis of Metal Complexes

An ethanolic solution (40 ml) of Schiff base (4 mmol) was mixed with an ethanolic solution (20 ml) of Cu(II) chloride (2 mmol). The solution was made slightly alkaline with triethlyamine. The mixture was refluxed for 4 h. The solid product obtained was filtered hot, washed in ethanol and dried in vacuum.

Biological Studies

The Schiff bases and metal complexes were individually tested against a panel of standard microorganisms namely *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 19582), *Bacillus cereus* (10702), *Enterococcus faecalis* (ATCC 29212) and *Klibsella pneumoniae* (ATCC 10031).

Disc Diffusion Assay

Antibacterial activity of Schiff bases and metal complexes were carried out in triplicate using the disc diffusion method (12). Molten Mueller-Hinton agar was inoculated with the bacteria suspension which had been adjusted to the 0.5 McFarland standard and poured into sterile 90 mm Petri dishes. Schiff bases and metal complexes were dissolved in DMSO to obtain a final concentration of 10 mg/ml. Sterile Whatman No. 1 (6 mm) discs were separately impregnated with each sample to be tested and placed on the inoculated agar. The plates were incubated at 37°C for 24 h and the zones of inhibition measured at the end of the incubation period. Ampicillin was used as reference compound.

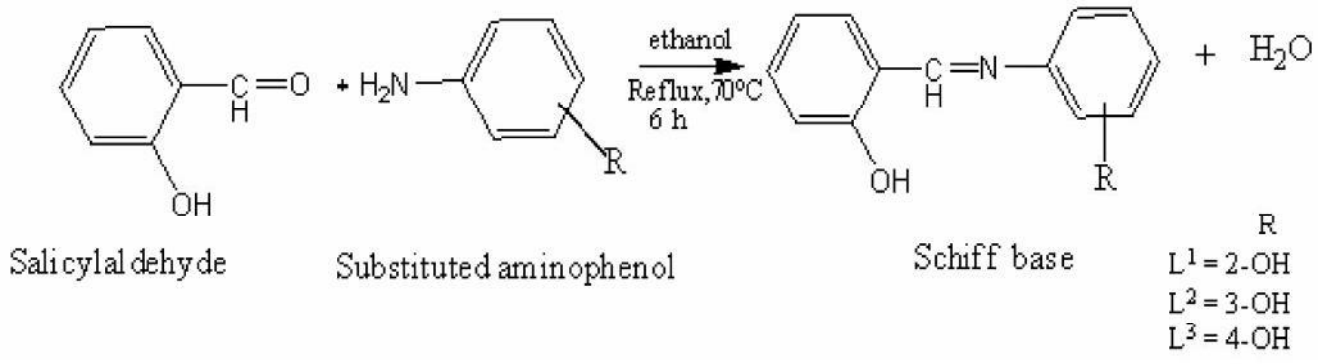
Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the Schiff bases and metal complexes were determined using the 96-well micro-plate dilution method (13). Serial plate concentrations of 5.0, 2.5, 1.25, 0.625, 0.312, 0.157, 0.078 and 0.039 $mgmL^{-1}$ were prepared for each compound. Each was inoculated with 1.5 x 10^8 CFU/mL of 0.5 McFarland standard bacteria suspension and incubated for 24 h at 37 °C. As an indicator of bacterial growth, 20 µL of 0.2 $mgmL^{-1}$ *p*-iodonitrotetrazolium solution (a colourless tetrazolium) was added to each well and incubated at 37 °C for 30 min. Growing bacteria metabolise this salt to give a red product (formazan). Inhibition prevents this conversion resulting in a clear well. MIC values were recorded as the lowest concentration of compound preventing bacterial growth.

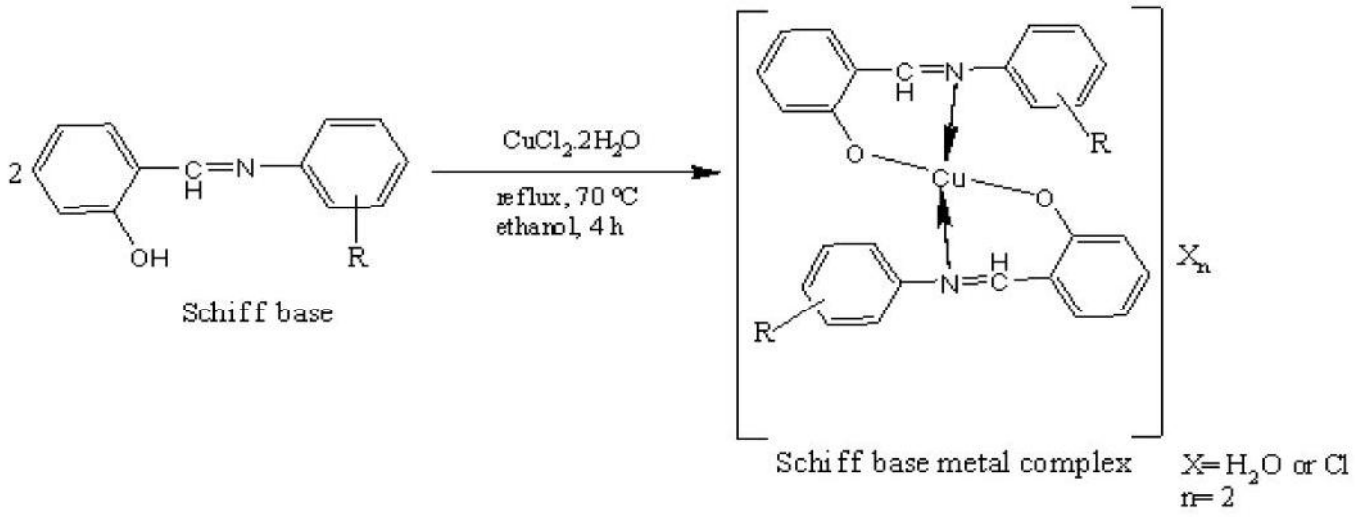
RESULTS AND DISCUSSION

Synthesis

The Schiff base ligands were prepared by the reaction of salicylaldehyde and aminophenol in a 1:1 stoichiometric ratio as illustrated in Scheme 1 below. Analytical and physical data is represented in Table 1. The compounds were isolated in good yield. ^{1}H NMR data revealed that the desired Schiff bases were isolated while microanalysis confirmed the purity of the ligand. Treatment of the ligands L^1, L^2 and L^3 with Cu(II) chloride afforded complexes (Scheme 2) corresponding to the general formula $[CuL_2]X_n$ where X = H_2O or Cl and n = 2. The analytical data show that the metal to ligand ratio is 1:2. The complexes were soluble in common organic solvents; chloroform, DMF, DMSO.



Scheme 1: Synthetic Route to Schiff Bases L^1 – L^3



Scheme 2: Synthetic Route to Schiff Base Metal Complexes Cu L^1 – Cu L^3

Table 1: Physical Properties and Analytical Data of Prepared Compounds

Compound	Formula Weight	Yield %	Colour	M.pt°C	Micro-analysis Data Found (cald)		
					%C	%H	%N
L^1	$C_{13}H_{11}NO_2$	74	Red	146 – 147	73.43(73.23)	6.44(6.57)	5.10(5.20)
CuL^1	$C_{26}H_{20}Cl_2CuN_2O_4$	57	Green	Decomp>293	56.43(55.87)	2.96(3.61)	4.89(5.01)
L^2	$C_{13}H_{11}NO_2$	71	Orange	82 – 83	73.01(73.23)	5.16(5.20)	6.57 (6.57)
CuL^2	$C_{26}H_{22}Cl_2CuN_2O_4$	61	Brown	Decomp>250	55.32(55.67)	3.97(3.95)	5.54(4.99)
L^3	$C_{13}H_{11}NO_2$	65	Orange	98 – 99	73.23(73.23)	5.11 (5.20)	6.61(6.57)
CuL^3	$C_{26}H_{24}CuN_2O_6$	79	Brown	Decomp>273	60.32(59.59)	4.95(4.62)	5.71(5.35)

Infra-red Spectroscopy

In order to study the binding mode of Schiff base to metal in the complexes, IR spectrum of the free ligand was compared with the spectra of the metal complexes. IR spectra bands of the ligands and their metal complexes are presented in Figures 1–3 and diagnostic bands reported in Table 2.

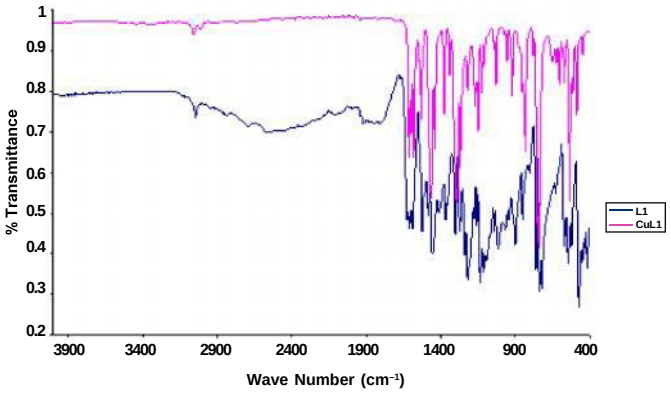


Fig. 1: IR Spectra of Schiff Base L^1 with Copper Metal Ion.

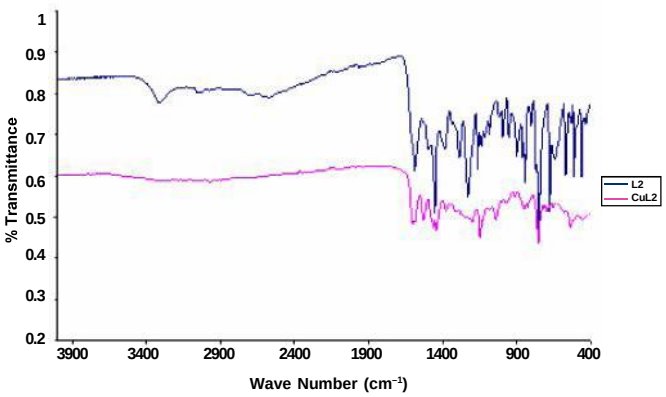


Fig. 2: IR Spectra of Schiff Base L^2 with Copper Metal Ion

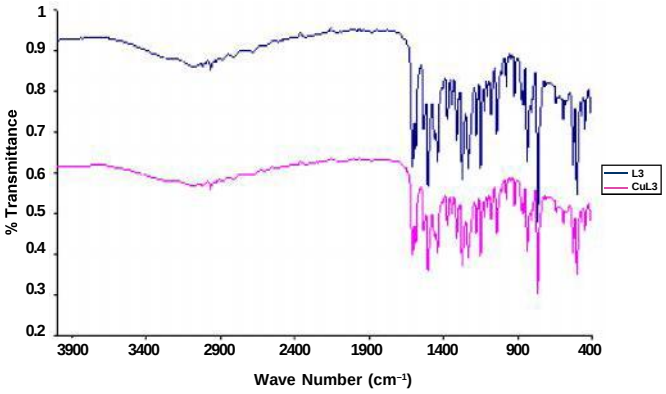


Figure 3: IR Spectra of Schiff Base L^3 With Copper Metal Ion

Magnetic Susceptibility Measurements and Electronic Absorption Spectra

The electronic spectra and magnetic moments of Schiff bases and their metal complexes are presented in Table 3. The electronic spectra of the free ligands and its metal complexes were studied in chloroform ($CHCl_3$) solution. In all the spectra of the ligand, three absorption bands appear in the region 240–268 nm, 270–303 nm and 343–355 nm assigned to $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$. Electronic spectra of Cu(II) complexes showed absorption bands in the region 306-328 nm assigned to charge transfer (CT) and 391 – 446 nm due to $d\rightarrow d$ transitions attributed to $^2B_ig\rightarrow{}^2A_ig$ transition of four coordinate, square-planar geometry. This *d–d* transition is in the region of that observed for structurally well characterized complexes of copper (II) N-alkylsalicyladiminates with square planar geometry (17). This was further corroborated with the magnetic moments values of

Table 2: IR and ^{1}H-NMR spectra data of prepared compounds

Compound	ν(C=N)	ν(C-O)	ν(O-H)	ν(Cu-N)	ν(Cu-O)	Chemical shift δ (ppm)	
						HC=N	OH
L^1	1616	1287	3040	–	–	8.69	9.11
CuL^1	1612	1268	3050	487	536	–	–
L^2	1584	1225	3294	–	–	8.38	8.77
CuL^2	1581	1190	–	442	532	–	–
L^3	1607	1272	3104	–	–	8.36	8.78
CuL^3	1601	1266	3204	513	540	–	–

Infrared spectra of the Schiff base ligands, L^1, L^2 and L^3 shows characteristics absorption bands at 1616, 1584 and1607 cm^{-1}, assignable to C=N . These bands due to the azomethine nitrogens of the Schiff base underwent a shift to lower frequencies 1612, 1581 and 1601 cm^{-1} upon complexation with Cu(II), indicating the involvement of nitrogen of the azomethine group in coordination (14). The band for C-O stretching which occurs at 1287, 1225 cm^{-1} for the ligand L^1, L^2 was moved to lower frequencies 1268, 1190 cm^{-1} (CuL^1 and CuL^2) after complexation which indicates that the shifts are due to the coordination of the phenolic oxygen of the ligand to the metal ion (15). It can be concluded that the Schiff base acts as a bidentate ligands coordinating via the azomethine *N* and the phenolic *O*. The broad band at 3204 cm^{-1} for CuL^3 complex indicates the presence of coordinated water molecule. The new bands observed in the complexes in the region 442–513 and 532–536 cm^{-1} were assigned to Cu–N and Cu–O bonds respectively (16).

^{1}H NMR Spectra

The ^{1}H NMR spectrum of the ligands (L^1, L^2 and L^3) in DMSO show signal at 8.36, 8.38, 8.69 ppm (s, IH, N=C-H) assigned to the azomethine protons. The peaks at 8.77, 8.78, 9.11 ppm are attributed to phenolic protons (s, IH, phenolic-OH) respectively.

1.76–1.88 B.M which falls within the range normally observed for one unpaired electron of Cu^{2+} complexes with square-planar geometry (18).

Table 3: Magnetic Moments and Electronic Spectra Data (nm) of Schiff Bases and their Metal Complexes

Compound	$CHCl_3$ λmax (nm)	Assignment	Magnetic Moments μ_{eff}	Geometry
L^1	240, 270 355	$\pi\rightarrow\pi^*$ $n\rightarrow\pi^*$	–	–
CuL^1	306 420, 446	CT d – d	1.79	Square-Planar
L^2	242, 270 343	$\pi\rightarrow\pi^*$ $n\rightarrow\pi^*$	–	–
CuL^2	329 422	CT d – d	1.88	Square-Planar
L^3	268, 303 346	$\pi\rightarrow\pi^*$ $n\rightarrow\pi^*$	–	–
CuL^3	308 391	CT d – d	1.76	Square-Planar

Antibacterial Activity

Antibacterial activity of the ligands and complexes were tested *in-vitro* against six human pathogenic bacteria. The activities were compared with that of ampicillin. The compounds were tested at a concentration of 10 mg/ml in DMSO using the paper disc diffusion method. The diameters of growth inhibitory zones were measured and the results are presented in Figure 4. The susceptibility zones measured were clear zones around the disc killing the bacteria; this is represented in Table 4. The complexes showed comparable activity with the reference compound ampicillin.

Table 4: Diameter of Zones of Inhibition of Bacteria in different Compounds (mm)

Bacterial Strain	L^1	CuL^1	L^2	CuL^2	L^3	CuL^3	Ampicillin
S. aureus (ATCC 6538)	10±0.70	13±0.00	10±0.00	10±0.70	10±0.35	10±0.00	13±0.00
E. faecalis (ATCC 29212)	7±0.35	11±1.41	0±0.00	10±0.00	0±0.00	9±1.41	11±0.20
B. cereus (ATCC 10702)	10±1.41	10±0.70	10±0.70	10±0.70	0±0.00	10±0.00	14±0.20
E. coli (ATCC 8739)	11±0.70	13±0.70	0±0.00	11±0.10	0±0.00	11±0.70	10±0.00
P. aureginosa (ATCC 19582)	0±0.00	12±0.00	0±0.00	10±0.28	0±0.00	8±0.28	9±0.10
K. pneumonia (ATCC 10031)	0±0.00	11±0.00	0±0.00	10±0.35	0±0.00	10±0.70	11±0.10

Diameter of zones of inhibition $\geq$ 7 = active, diameter of zones of inhibition $\leq$ 6 = not recorded (low activity = 0).

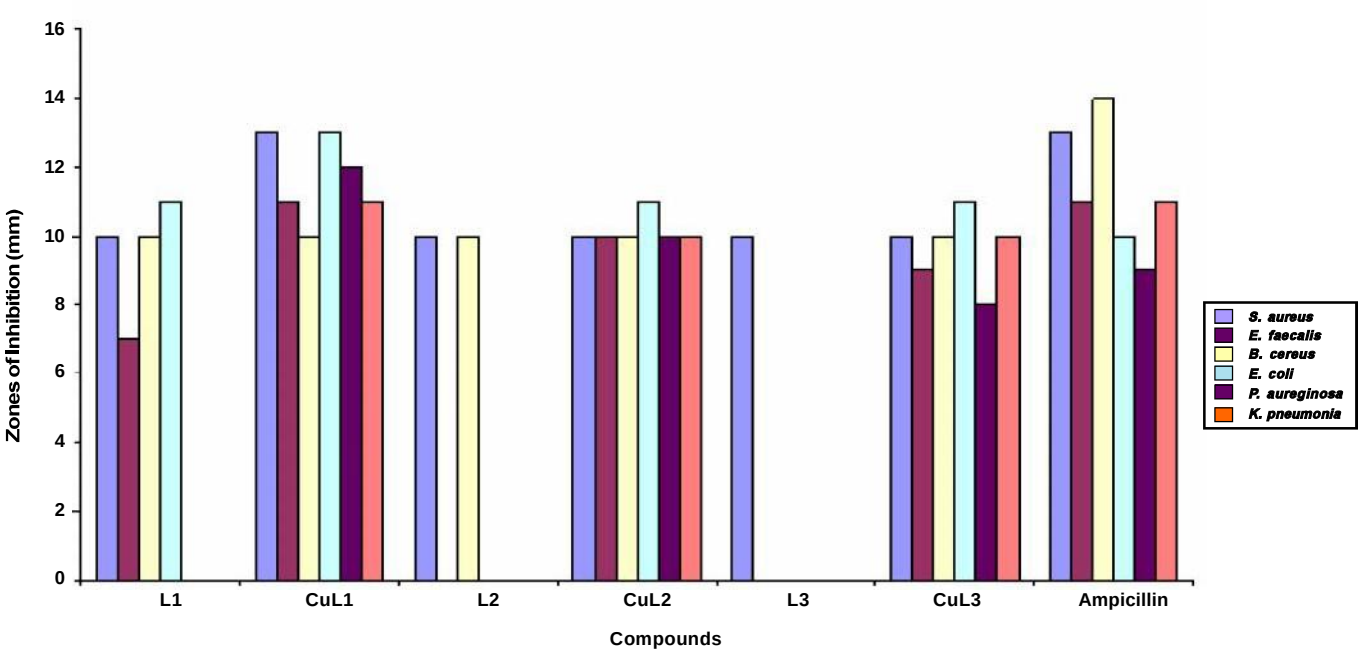


Fig. 4: Histogram showing the Comparative Activities of the Compounds

Table 5: Minimum Inhibitory Concentration (mg/ml)

Bacterial Strain	L^1	CuL^1	L^2	CuL^2	L^3	CuL^3	Ampicillin
S. aureus(ATCC 6538)	2.50	0.15	2.50	2.50	5.00	2.50	2.50
E. faecalis(ATCC 29212)	5.00	1.25	2.50	2.50	5.00	2.50	5.00
B. cereus(ATCC 10702)	5.00	0.62	5.00	5.00	5.00	2.50	5.00
E. coli(ATCC 8739)	2.50	2.50	2.50	2.50	2.50	2.50	1.25
P. aureginosa(ATCC 19582)	5.00	0.62	2.50	2.50	2.50	2.50	5.00
K. pneumonia(ATCC 10031)	2.50	0.62	2.50	2.50	2.50	2.50	2.50

The Schiff base ligands and their metal complexes all have the capacity of inhibiting the metabolic growth of the investigated bacteria to different extent. MIC values of the all the compounds against six bacteria strains used in the study are presented in Table 5. A minimum inhibitory concentration value of 0.28–1.27 mg/ml has been attributed with extremely strong activity while MIC values of 1.81–8.85 mg/ml are attributed with weak activities (19). The MIC result showed that the CuL^1 (copper(II) complex of Schiff base 2-hydroxybenzylidene-2-aminophenol) exhibited an extremely strong activity against the tested bacteria except against *E. coli* in respect of which it exhibited weak activity (19). It is known that chelation tends to make ligands act as more powerful and potent bactericidal agent and this was confirmed by the fact that the metal complexes showed enhanced antimicrobial activity against one or more strains with CuL^1 remarkably showing good activity. This can be attributed to the position of substituents, CuL^1 having the OH group at the *ortho* position (20–21). Increase in activity of this complex can be attributed to the ease of chelate formation.

CONCLUSION

Copper(II) complexes involving Schiff bases derived from 2-hydroxybenzaldehyde and aminophenols have been synthesised and characterised. Comparison of the IR spectra of the Schiff bases and their metal complexes indicate that the Schiff bases act as bidentate ligands coordinating via the azomethine *N* and the phenolic *O*. The electronic spectra of the complexes indicate square-planar geometry. The antibacterial (MIC) results show that the Cu(II) complexes were more active than the Schiff bases, with CuL^1 complex exhibiting a very low MIC compared to the other complexes. This can be attributed to the position of the substituent (OH) at the *ortho* position. The reported Cu(II) complexes particularly the 2-aminophenol complex, CuL^1 complex can be used in development of broad spectrum antimicrobial agents.

ACKNOWLEDGEMENT

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Optimization of Chemical Pre-Treatment of Cassava Bagasse for Reducing Sugar and Bioethanol Production

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ABSTRACT

Background: The indiscriminate litter of agricultural wastes which cause environmental menace and the increased interest in alternative sources of energy have led to the exploration of agro-waste as potential low cost and efficient bioproduct raw material.
Aims: This study investigated the effect of different pre-treatment solvents in the use of cassava bagasse as feedstock for reducing sugar and ethanol production in saccharification and fermentation (SSF) processes.
Methods: Cassava tubers were peeled and processed to extract starch, with the resultant waste dried to obtain cassava bagasse (CB). The CB was subjected to various chemical treatments which include hydrothermal, H_2O_2, H_2SO_4, NaOH, combined C_2H_5OH with H_2SO_4, and combined C_2H_5OH with NaOH. Pre-treated CB that gave the high cellulose yield were further subjected to enzymatic saccharification using crude enzyme extract of *Tricoderma viride* and *Aspergillus niger* prior to anaerobic production of ethanol by fermentation in the presence of commercial yeast.
Results: Pre-treatment resulted in significant ($p<0.05$) CB weight loss and increased cellulose yield compared to the unpretreated bagasse. Amongst the pre-treatment groups, H_2SO_4 pre-treatment gave the highest cellulose yield (92.30±0.27%). Saccharification with crude enzyme of *T. viride* and *A. niger* co-culture released significant ($p<0.05$) reducing sugar from the pre-treated CB slurries after 24 hours. However, fermentation of the pre-treated CB gave significant ($p<0.05$) lower ethanol yield compare to the unpretreated.
Conclusion: The present study indicates the potential use of tropical isolates and local agro-waste as raw materials for bio-production of reducing sugar and ethanol. Findings of this research suggest that the production of bio-products of interest determine the pre-treatment condition even with the same raw material base.

Keywords: Agro-industrial wastes, bioethanol, reducing sugar, renewable energy, cassava bagasse, fermentation, saccharification.

INTRODUCTION

Agricultural wastes consist of lignocelluloses (1) which are renewable sources of energy (2,3) that can be reduced, reused, recycled and upgraded to value-added products by chemical or biological procedures (4–7). These agro-wastes, which include straw, stem, stalk, husk, shell, peel, leaves, corncobs, bagasse, and palm oil effluent, are ineffectively used in Africa, particularly in Nigeria where they are mainly employed for animal feeding (8-9). A great amount of these wastes are left on the farm and are thereby decomposed by microorganisms such as fungi and bacteria. Ineffective use and/or disposal of these wastes indiscriminately cause environmental pollution (10-11).

Glasser *et al.* (1) stated that lignocellulose is compact, partly crystalline in structure, consisting of linear and crystalline polysaccharides cellulose, branched non-cellulosic and non-crystalline heteropolysaccharides (hemicelluloses), and branched (non-crystalline) lignin. These chemical properties of lignocellulosic biomass render them as substrates of huge biotechnological importance (12).

Cassava stalk, peel, leaves, and bagasse (pulp) has been considered a major source of lignocellulosic agro biomass waste especially in Nigeria where the crop is grown on both local and commercial scales (13). The bagasse is made up of fibrous root material and contains starch that may not be totally extracted (14). Ali *et al*. (15) reported the physicochemical composition of cassava bagasse (g/100 g Dry Weight) to contain 65.6% carbohydrate, 20.1% fibre, 0.2% lipids, 5.7% ash, 3.1% protein, 8.1% cellulose, 2.8% hemicellulose and 2.2% lignin. However, its poor nitrogen content (2.3%) makes it unattractive as an animal feed. Consequently, the poor nutrient content save for its carbohydrate content renders this waste majorly for use as land fill, thereby creating environmental pollution. Furthermore, the diminishing fossil reserve, increase in demand and price of fossil fuels has necessitated research into alternative renewable source of fuel such as bioethanol (16–17). In addition, meeting demands for future supply of raw materials for ethanol production has been worrisome; hence there has been a growing research to widen its raw materials base by including lignocellulosic materials or feedstocks. This technology is

termed second generation ethanol with inherent potential to decrease emission of greenhouse gas (GHG) (18). Therefore, application of cassava bagasse as substrate could solve pollution problem, as well as provide promising alternative substrates for bioprocesses.

Current researches are focusing on how to efficiently utilize the rich and abundant carbon resources in cell wall polysaccharides of agro-wastes for bioprocesses (3,8,9,12). However, the nature of plant cell wall which creates a solid structure serving as defence mechanism against pathogens, insect and hydrolytic enzyme posed much problem in utilization of this biomass for bio-product production. Consequently, optimizing the use of lignocellulosic biomass may oblige pre-treatment conditioning (19) which is a process that enhances biomass delignification and accessibility of hydrolytic enzymes to the polysaccharides in the cell wall (20). Several studies in ethanol production from lignocelluloses have involved the use of simultaneous saccharification and fermentation process (21,22). Simultaneous saccharification and fermentation is a process whereby polysaccharides are hydrolyzed into monomeric simple sugars which are bio-converted into ethanol and other value-added products such as lactic acid and citric acid (23,24).

Zayed and Meyer (25) reported bioconversion of wheat straw to ethanol using *Trichoderma viride* and *Pachysolen tannophilus*. Amutha and Gunasekaran (26) reported production of ethanol by fermentationn of liquefied cassava starch using yeast (*Saccharomyces diastaticus*) monoculture and mixed culture of *Zymomonas mobilis* and *Saccharomyces diastaticus*. Hari *et al.* (27) carried out simultaneous saccharification and fermentation (SSF) to produce ethanol from sugarcane leaves using *Trichoderma reesei* cellulase and yeast cells. Couto and Sanromàn, (28) reported the production of lactic acid from sugarcane bagasse by employing *Rhizopus oryzae.* Pandey *et al.* (29) also reported production of citric acid from cassava bagasse by using *Aspergillus niger.* Baig *et al.* (30) investigated the saccharification of banana agro-waste by cellulases of *Trichoderma lignorum.* Singhania *et al.* (31) studied cellulase production from *Trichoderma reesei* using lignocellulosic residues.

In this present study, we report the optimization of chemical pre-treatment condition of cassava bagasse and its utilization as feedstock for reducing sugar and second generation ethanol production by saccharification and fermentation (SSF) using tropical isolates.

MATERIALS AND METHODS

Sample Collection

Mature cassava (*Manihot esculenta Cranz*) tubers were obtained freshly harvested from a farm in Ogun State, Nigeria. The plant was identified and authenticated at the Department of Botany, Faculty of Science, University of Lagos, Lagos, Nigeria (Voucher specimen No LUH6478).

Fungi Isolates

Tricoderma viride was obtained from Federal Institute of Industrial Research, Oshodi, Lagos, Nigeria. *Aspergillus niger* was supplied by Dr. Musa Babalola of Biotechnology Laboratory, Department of Biochemistry, College of Medicine, University of Lagos, Nigeria. Commercial baker's yeast (*Saccharomyces cerevisiae*) was supplied by Guangxi Danbaoli Yeast Co. Ltd., Guangxi, China. Pure culture of *Trichoderma viride* and *Aspergillus niger* were maintained as stock culture grown on potato dextrose agar (PDA).

Culture Media

The culture medium as described by Mandels and Weber (32) was utilized for fermentation. The composition per liter included urea, 0.3 g; Ammonium sulfate ($(NH_4)_2SO_4$), 1.4 g; Potassium dihydrogen phosphate (KH_2PO_4), 2.0 g; Calcium chloride ($CaCl_2$), 0.3 g; Magnesium sulfate heptahydrate ($MgSO_4 7H_2O$), 0.3 g; Peptone, 0.75 g; and Yeast extract, 0.25 g. Trace elements were also added using: 1 % (v/v) solution of salts: Iron (II) sulfate heptahydrate ($FeSO_4 7H_2O$), 0.5 g; Manganese (II) sulfate ($MnSO_4$) 0.16 g; Zinc sulfate ($ZnSO_4$), 0.14 g; and Cobalt (II) chloride ($CoCl_2$), 2.0 g. The pH was adjusted by adding standardized NaOH and HCl respectively to 5.5–6.0 before sterilization.

10XYP medium was composed of Yeast extract (100 g/l) and Peptone (200 g/l). The pH of the medium was adjusted to 5.0 using 1 M sulfuric acid. The medium was sterilized by autoclaving at 121°C for 30 min.

The commercial baker's yeast (*S. cerevisiae*) culture media were prepared with slight modification according to laboratory analytical procedure (LAP) as given by the National Renewable Energy Laboratory (NREL) (24). NaCl (0.9 %) was used for yeast stock culture preparation instead of glycerol. The pH of the medium was adjusted to 5.0 using 1M sulfuric acid. This medium was autoclaved at 121 °C for 30 minutes. Baker's yeast (20 g) was added to the sterilized medium and incubated in a rotary shaker at 38 °C for 24 hours. This yeast inoculum medium was specifically prepared to support the growth of yeast cells aerobically.

Cassava Bagasse Preparation

Unpeeled cassava tubers were initially weighed, properly peeled, washed with distilled water, cut into pieces, macerated for 72 hours and starched extracted with distilled water severally. The extract was filtered to remove chaff which is the cassava bagasse. The resultant cassava bagasse was dried to a constant weight at 45 ± 3 °C according to the method of Hames *et al*. (33). The dried bagasse was co-minuted with laboratory mill (Thomas Wiley® Mill USA) of mesh size 0.5 mm to form fine bagasse powder. This was thereafter stored in an air tight container at 4 °C and severed as the stock bagasse. The percentage bagasse powder yield was calculated using the formula:

$$\text{Percentage bagasse powder yield (\%)} = \frac{\text{Weight of ground cassava bagasse}}{\text{Weight of unpeeled cassava tuber}} \times 100$$

Proximate Analysis of Cassava Bagasse

The proximate composition of the stock cassava bagasse was determined. Total carbohydrate, crude protein, crude fat,

crude fibre, total ash, and moisture content were determined according to the standard methods by the Association of Official Analytical Chemists (34).

Cellulase Enzyme Production by Submerged Fermentation

Cassava bagasse powder (10 g) was poured into two conical flasks each containing 200 ml of Mandel's medium. The conical flasks were plugged with cotton and sterilized by autoclaving at 121 °C for 15 min. The sterilized culture media were inoculated with 5 discs of *T. viride* and *A. niger* respectively freshly grown on PDA. The flasks were incubated aerobically at room temperature for 5 days on a Griffin Flask Shaker (Griffin & George Ltd UK®). After 5 days of incubation and shaking, mycelia were separated by filtration and centrifugation at 2000 *x*g for 15 min. The supernatants were stored at 4°C and subsequently used as the crude extracellular cellulase enzyme (32). In order to confirm cellulase activity of the crude extracellular extract, filter paper activity (FPase) assay was determined as described by the National Renewable Energy Laboratory (35). Whattman filter paper No. 1 (50 mg) was suspended in a mixture containing 0.5 ml of various crude enzyme dilutions (0.0125, 0.0250, 0.0375, and 0.5000 ml) and 1.0 ml of 50 mM Sodium acetate buffer (pH 4.8) in different tubes. The mixture in the tubes were incubated for 1 hour at 50°C, thereafter 1 ml were aseptically withdrawn using sterile pipettes into plain bottles, placed in boiling water bath for 5 min., then chilled on ice, centrifuged at 2000 *x*g for 15 min. and the amount of reducing sugar present in each supernatant sample were determined by 3, 5-dinitrosalicylic acid (DNS) method (36).
A graph of enzyme concentration against glucose concentration liberated was plotted. The concentration of enzyme which released exactly 2.0 mg of glucose was extrapolated from the graph. Filter paper activity (FPU) was calculated using the formula below:

$$\text{FPU} = \frac{0.37}{\text{Enzyme concentration to release 2 mg glucose}} \text{ units·ml}^{-1}$$

Crude Enzyme Hydrolysis of Standard Cellulolytic Substrates

The ability of the crude enzyme to hydrolyse standard lignocellulolytic substrates was investigated according to the method described by Ali *et al*. (15), however with slight modification. Whatman filter paper (50 mg), carboxymethyl-cellulose (1 g), and starch (1 g) were respectively placed in different test tubes and subjected to enzymatic hydrolysis by adding 5 ml of crude extracellular cellulase enzyme of *T. viride* and 5 ml of 50 mM sodium acetate buffer (pH 5.0). The tubes were incubated on a Griffin Flask Shaker (Griffin & George Ltd UK®) at agitation rate of 130 *x*g at 38 °C for 1 hour. The hydrolysate (1 ml) from each culture medium was aseptically withdrawn using sterile pipettes into plain bottles, placed on boiling water bath for 5 min., then chilled on ice, centrifuged at 2000 *x*g for 15 min. and the amount of reducing sugar present in each supernatant sample was determined by 3, 5-dinitrosalicylic acid (DNS) method (36). The same protocol was repeated with crude extracellular cellulase enzyme of *A. niger*.

Cassava Bagasse Pre-treatment

The stock cassava bagasse powder was divided into seven groups and subjected to various pre-treatment conditions in order to enhance biomass delignification and accessibility of the polysaccharides in the cell wall by hydrolytic enzymes. Groups I-VI were the chemical pre-treated groups while Group VII was the control untreated treatments. All media were autoclaved at 121°C for 15 min.

Group I: Hydrothermal (Hot-H_2O) pre-treatment: distilled water (50 ml) was added into Erlenmeyer flasks containing stock cassava bagasse powder (20 g).

Group II: Hydrogen peroxide (H_2O_2) pre-treatment: various concentrations of 6 % w/v H_2O_2 (1.5 %, 3.0 %, 4.5 %, and 6.0 % v/v; 50 ml) were respectively placed in flask containing 20 g of cassava bagasse powder.

Group III: Sulphuric acid (H_2SO_4) pre-treatment: cassava bagasse powder (20 g) was treated with 50 ml of varying concentrations of 0.1M H_2SO_4 (25 %, 50 %, 75 %, and 100 % v/v).

Group IV: Sodium hydroxide (NaOH) pre-treatment: cassava bagasse powder (20 g) was treated with 50 ml of varying concentrations of 1 % w/v NaOH (0.2 %, 0.4 %, 0.6 %, and 0.8 % v/v).

Group V: Combined Ethanol-Sulphuric acid (C_2H_5OH-H_2SO_4) pre-treatment: Cassava bagasse powder (20 g) was treated with 50 ml of varying concentrations of equal volume of 96% ethanol with varying concentrations of 0.1 M H_2SO_4 (25 %, 50 %, 75 %, and 100 % v/v).

Group VI: Combined Ethanol-Sodium hydroxide (C_2H_5OH-NaOH) pre-treatment: Cassava bagasse powder (20 g) was treated with 50 ml of varying concentrations of equal volume of 96% ethanol with varying concentrations of 1 % w/v NaOH (25 %, 50 %, 75 %, and 100 % v/v).

Group VII: This served as the control unpretreated cassava bagasse.

The cassava bagasse (CB) substrates which gave the highest cellulose yield after pre-treatment were selected and further subjected to saccharification and fermentation.

Determination of Cassava Bagasse Weight Loss and Cellulose Content after Pre-treatment

Cassava bagasse weight loss was recorded after each pre-treatment period. The resultant cassava bagasse after pre-treatment was washed in distilled water and the residue was dried to a constant weight at 45 ± 3 °C. The percentage cassava bagasse weight loss after pre-treatment was calculated using the formula:

$$\text{Percentage cassava bagasse weight loss after pre-treatment (\%)} = \frac{(W1 - W2)\,(g)}{W1\,(g)} \times 100$$

Where, W1 = the initial weight of cassava bagasse before pre-treatment
W2 = the final weight of cassava bagasse after pre-treatment
Cellulose content was estimated according to the method of van Soest and Wine (40). This is based on the fact that cellulose

undergoes acetolysis with acetic/nitric reagent forming acetylated cello-dextrins which is dissolved and hydrolyzed to form glucose molecules on treatment with 67 % H_2SO_4. Acetic acid (80 %; 15 ml) and 1.5 ml of concentrated nitric acid were added to 1g of dried pre-treated cassava bagasse (CB), and reflux for 20 min. It was filtered; the residue was washed with ethanol, dried in oven at 100-105 °C and weighed. Then it was incinerated in a muffle furnace at 540 °C. The percentage cellulose content was calculated using the formula:

$$\text{Percentage cellulose (\%)} = \frac{(W_A - W_B)\,(g)}{\text{CB weight (g)}} \times 100$$

Where, W_A = weight after drying in oven
W_B = weight after drying in muffle furnace
CB = initial weight of cassava bagasse

Saccharification of Cassava Bagasse by *T. viride* and *A. niger* Co-culture

To evaluate tropical fungi isolates release of reducing sugar from the pre-treated cassava bagasse, saccharification was carried out according to NREL method (28), however with slight modification. Pre-treated cassava bagasses which had the highest cellulose content were used for this analysis. Each saccharification fermenter bottle was loaded with 5 ml (equivalent to 5 g) of cassava bagasse substrate slurry respectively from the selected pre-treated groups, 5 ml of crude extracellular cellulase enzyme of *T. viride* and *A. niger* respectively were added, followed by 10 ml of 50 mM sodium acetate (pH 5.0) buffer and then 5 ml of 10 XYP solution was further added into each fermenter bottle. The fermenter bottles were placed on a Griffin Flask Shaker (Griffin & George Ltd UK®) at agitation rate of 130 rpm and incubated at room temperature for 96 hours. Samples (3 ml) were aseptically withdrawn at 24 hours interval using sterile pipettes into plain bottles and used to determine the amount of reducing sugar present (36).

Simultaneous Saccharification and Fermentation (SSF)

SSF was carried out according to NREL method (24), however with slight modification. Each SSF fermenter bottle was loaded with 5 ml (equivalent to 5 g) of pre-treated cassava bagasse substrate slurry, 5 ml of crude extracellular cellulase enzyme of *T. viride* and *A. niger* respectively, 10 ml of 50 mM sodium acetate (pH 5.0) buffer, 5 ml of 10 XYP solution and 5 ml of commercial yeast (2.35×10^6 cells/ml) in 0.9 % sodium chloride medium. The fermenter bottles were placed on a Griffin Flask Shaker (Griffin & George Ltd UK®) at agitation rate of 130 rpm and incubated at room temperature for 72 hours. Samples (3 ml) were aseptically withdrawn at 24 hours interval using sterile pipettes into plain bottles and used to determine the amount of reducing sugar present (36). Similarly, the amount of ethanol produced was determined using Gas chromatography (GC). The GC system used an Agilent HP-5 column (30 m × 0.32 mm × 0.25 µm), flame ionizing detector at temperature (400 °C), with inlet temperature (300 °C), injection volume (10 µl), split ratio (10:1) and nitrogen as the gas carrier.

Statistical Analysis

All experiments were carried out in triplicates. The results obtained were statistically computed using SPSS 15.0 and Microsoft Excel; and values are expressed as mean ± standard error of mean (SEM). Data were further analyzed by Tukey's Honest Significant Difference (Tukey's HSD) test.

RESULTS

Percentage Yield and Proximate Composition of Cassava Bagasse

The cassava bagasse powder yield was 11.00 %. Proximate analysis of the stock cassava bagasse showed that it was composed of carbohydrate (64.83 %), protein (2.25 %), crude fat (0.34 %), fibre (21.51 %), total ash (0.97 %) and cellulose (8.00±0.12 %). The moisture holding capacity was estimated as 66.67 %.

Cellulase Enzyme Activity (FPase) of *Tricoderma viride* and *Aspergillus niger*

T. viride and *A. niger* possessed cellulase activity as observed by the hydrolysis of Whatman filter paper by crude extracellular enzyme extract of *T. viride* and *A. niger* to yield reducing sugar. It was observed that there was increasing yield of reducing sugar with increasing amount of enzyme. Extrapolation of the amount of enzyme that released 2.0 mg of reducing sugar as glucose from 50 mg of filter paper in 60 minutes was 0.3475 and 0.295 respectively for *T. viride* and *A. niger*. Therefore, Filter Paper Activity of *T. viride* and *A. niger* was calculated as 1.065 and 1.254 (FPU/ml) respectively.

Crude Enzyme Hydrolysis of Standard Cellulolytic Substrates

T. viride and *A. niger* were able to utilize standard cellulolytic substrates to produce reducing sugar. *T. viride* released 42.37±0.116, 27.95±0.187 and 49.97±0.041 mg reducing sugar per gram of carboxymethylcellulose, starch and filter paper respectively. While *A. niger* released 75.99±0.116, 61.38±0.261 and 75.15±0.087 mg reducing sugar per gram carboxymethylcellulose, starch and filter paper respectively (Figure 1). There was significant ($p<0.05$) difference in the amount of sugar released between the two isolates.

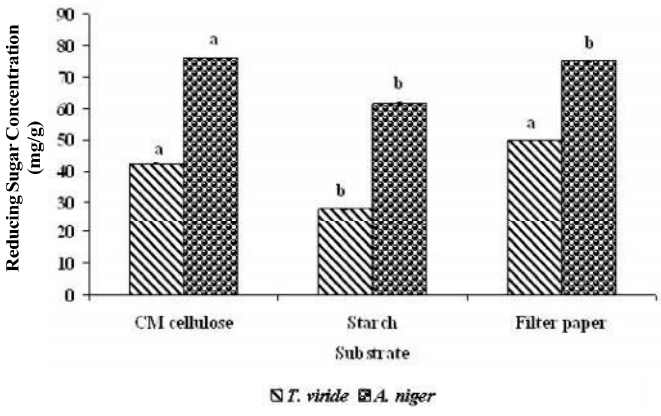

Fig. 1: Hydrolysis of Standard Cellulytic Mateirals using Crude Enzyme Extract of *Tricoderma viride* and *Aspergillus niger*

Pre-treatment Effect on Weight Loss and Cellulose Content of Cassava Bagasse

Pre-treatment methods (hydrothermal, hydrogen peroxide, sulphuric acid, sodium hydroxide, combined ethanol-sulphuric acid and combined ethanol-sodium hydroxide) employed in this study significantly ($p<0.05$) altered the percentage weight loss and cellulose content of the cassava bagasse compared to untreated bagasse. Hydrothermal pre-treatment resulted in 30.42±0.10 % weight loss and gave cellulose content of 32.47±0.31 % compared to the percentage weight (0.00±0.00 %) and cellulose content (8.00±0.28 %) of unpretreated cassava bagasse (Figure 2A). In hydrogen peroxide pre-treatment group, it was observed that increasing H_2O_2 concentration increased percentage weight loss, 4.5 % and 6.0 % H_2O_2 both gave the highest weight loss while 3.0 % H_2O_2 concentration gave the highest cellulose yield (Figure 2B).

Similarly, increasing H_2SO_4 concentration increased percentage weight loss and cellulose content (Figure 2C). There was significant ($p<0.05$) difference in the percentage weight loss amongst the various concentrations studied except with 50 and 75 % H_2SO_4 treatment. Pre-treatment with 100 % H_2SO_4 concentration gave the highest cellulose yield. Similarly, with NaOH pre-treatment, there was no significant ($p<0.05$) difference in the percentage weight loss of cassava bagasse pre-treated with the various concentrations except with 0.8 % NaOH which gave the lowest value whereas there was significant ($p<0.05$) difference in cellulose content of the cassava bagasse pre-treated with all the various NaOH concentration with 0.2% NaOH resulting to the highest cellulose yield (Figure 2D).

Combined ethanol (C_2H_5OH) and sulphuric acid (H_2SO4) pre-treatment resulted in significant ($p<0.05$) weight loss and cellulose content of cassava bagasse. Ethanol with 100 % H_2SO4 concentration gave the highest weight loss and cellulose yield (Figure 2E). Similarly, combined ethanol (C_2H_5OH) and sodium hydroxide (NaOH) pre-treatment significantly ($p<0.05$) resulted in cassava bagasse weight loss. Ethanol with 100 % NaOH concentration yielded the highest weight loss and cellulose content (Figure 2F). There was no significant ($p<0.05$) difference in the weight loss amongst the other pre-treatment concentrations. However, within the various treated groups, Hot-H_2O, 3.0 % H_2O_2, 100% H_2SO_4, 0.2 % NaOH, combined C_2H_5OH with 100 % H_2SO_4 and combined C_2H_5OH with 100 % NaOH gave the highest cellulose content.

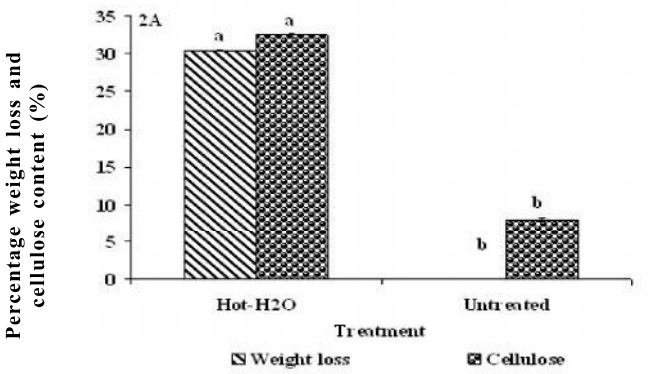

Figure 2A: Cassava Bagasse Weight Loss and Cellulose Content after Hydrothermal Pre-treatment.

Hydrothermal pre-treatment significantly ($p<0.05$) altered the percentage weight and cellulose content of cassava bagasse compared to the unpretreated cassava bagasse. Different superscript on bars with the same legend are significantly different at $p<0.05$.

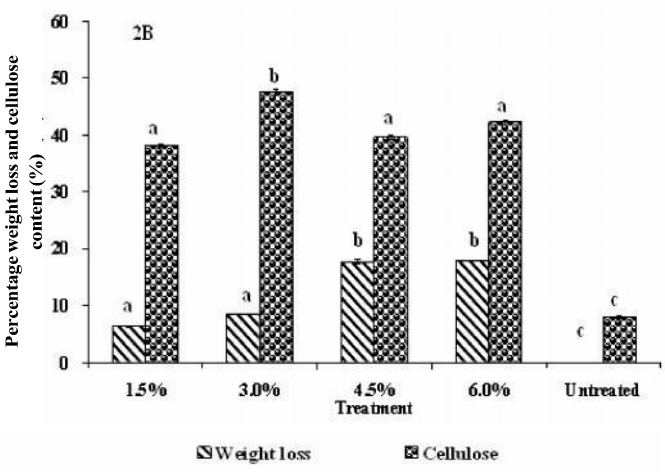

Figure 2B: Cassava bagasse weight loss and cellulose content after H_2O_2 Pre-treatment.

Hydrogen peroxide pre-treatment significantly ($p<0.05$) altered the percentage weight and cellulose content of cassava bagasse compared to the unpretreated cassava bagasse. Different superscript on bars with the same legend are significantly different at $p<0.05$.

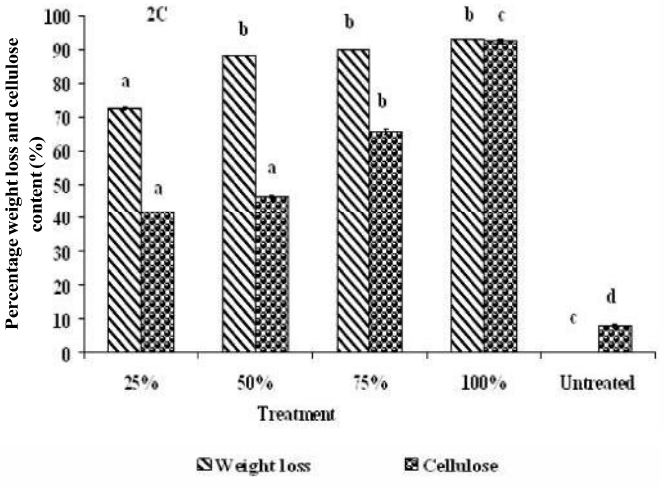

Figure 2C: Cassava Bagasse Weight Loss and Cellulose Content after H_2O_2 Pre-treatment.

Tetraoxosulphate VI acid (H_2SO_4) pre-treatment significantly ($p<0.05$) altered the percentage weight loss and cellulose content of cassava bagasse compared to the unpretreated cassava. Different superscript on bars with the same legend are significantly different at $p<0.05$.

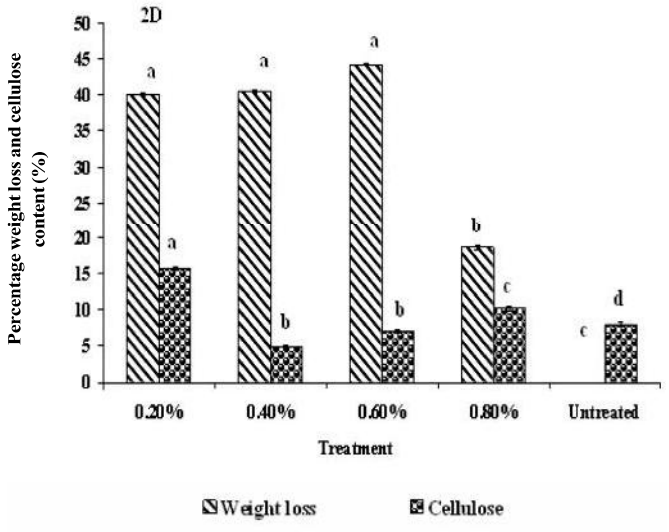

Fig. 2D: Cassava Bagasse Weight Loss and Cellulose Content after NaOH Pre-treatment.

Sodium hydroxide (NaOH) pre-treatment significantly ($p<0.05$) altered the percentage weight and cellulose content of cassava bagasse compared to the unpretreated cassava bagasse. Different superscript on bars with the same legend are significantly different at $p<0.05$.

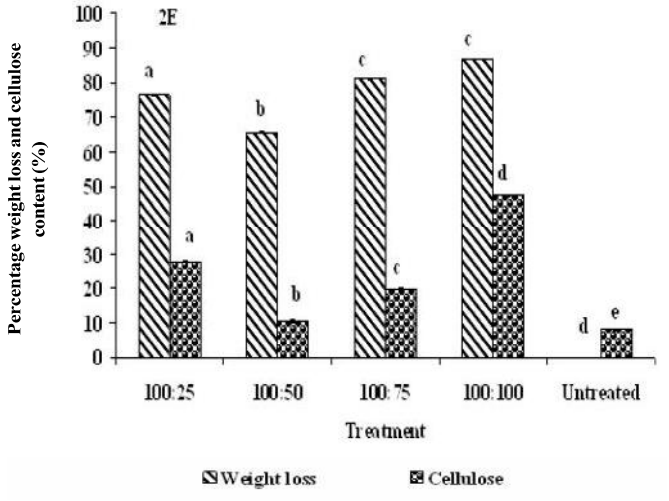

Fig. 2E: Cassava bagasse weight loss and cellulose content after combined ethanol, tetraoxosulphate VI acid and water pretreatment.

Combined ethanol, tetraoxosulphate VI acid and water pre-treatment significantly ($p<0.05$) altered the percentage weight loss and cellulose content of cassava bagasse compared to the unpretreated cassava bagasse. Different superscript on bars with the same legend are significantly different at $p<0.05$.

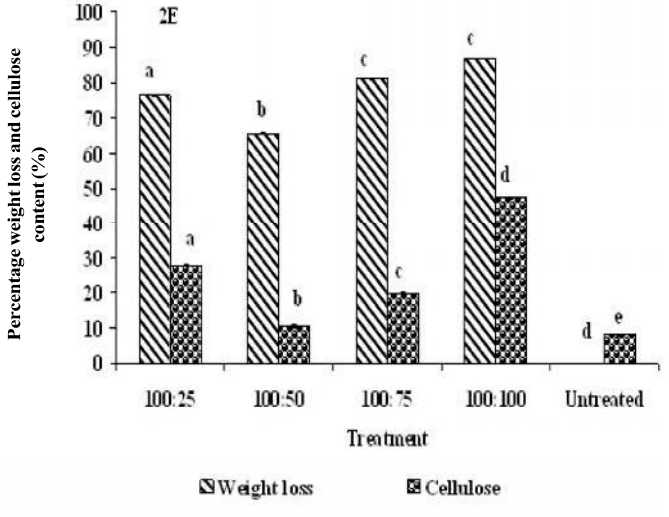

Fig. 2F: Cassava bagasse weight loss and cellulose content after combined ethanol, sodium hydroxide and water pretreatment.

Combined ethanol, sodium hydroxide and water pre-treatment significantly ($p<0.05$) altered the percentage weight and cellulose content of cassava bagasse compared to the unpretreated cassava bagasse. Different superscript on bars with the same legend are significantly different at $p<0.05$.

Saccharification by Crude Enzyme of *Tricoderma viride* and *Aspergillus niger* co-culture

Cassava bagasse pretreated with Hot-H_2O, 3.0 % H_2O_2, 100 % H_2SO_4, 0.2 % NaOH, combined C_2H_5OH with 100 % H_2SO_4, combined C_2H_5OH with 100 % NaOH and the unpretreated respectively which gave the highest cellulose yield after pretreated and subsequently subjected to saccharification with crude enzyme extract of *T. viride* and *A. niger* co-culture released significant ($p<0.05$) reducing sugar from the pre-treatment cassava bagasse slurries after 24 hours of saccharification. However, the reducing sugar released significantly ($p<0.05$) decreased after 96 hours of saccharification. H_2SO_4 (100 %, 0.1M) pre-treated cassava bagasse yielded the highest reducing sugar of 38.09±1.04 mg/g cassava bagasse compared to untreated substrate which gave 9.70 mg/g (Figure 3).

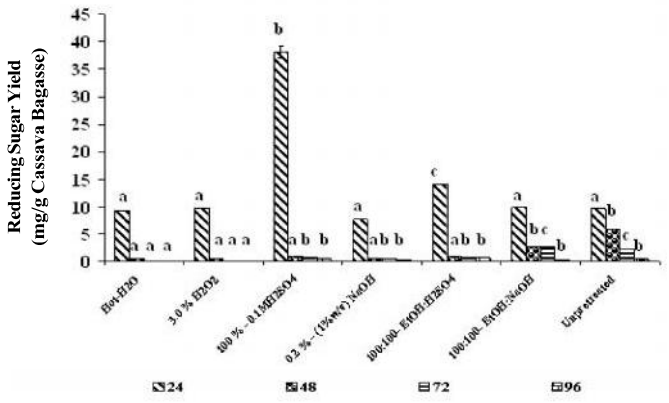

Fig. 3: Reducing sugar yield from pretreated cassava bagasse

T. viride and *A. niger* co-culture released significant ($p<0.05$) reducing sugar from the pre-treatment cassava bagasse slurries after 24 hours of saccharification. Different superscript on bars with the same legend are significantly different at $p<0.05$.

Simultaneous Saccharification and Fermentation (SSF)

Ethanol yield per gram unpretreated cassava bagasse (16.00±0.01 g/g) was significantly ($p<0.05$) higher than the ethanol yield from all the pre-treated cassava bagasse after 24 hours of fermentation. Tukey's Honest Significant Difference test showed that there was no significant ($p<0.05$) difference in the ethanol yield amongst the various cassava bagasse pre-treated groups after 24 hours of fermentation. However, ethanol yield from 100 % H_2SO_4, combined C_2H_5OH with 100 % H_2SO_4 (100:100 C_2H_5OH-H_2SO_4) pre-treated and the unpretreated cassava bagasse decreased through 72 hours of fermentation (Figure 4).

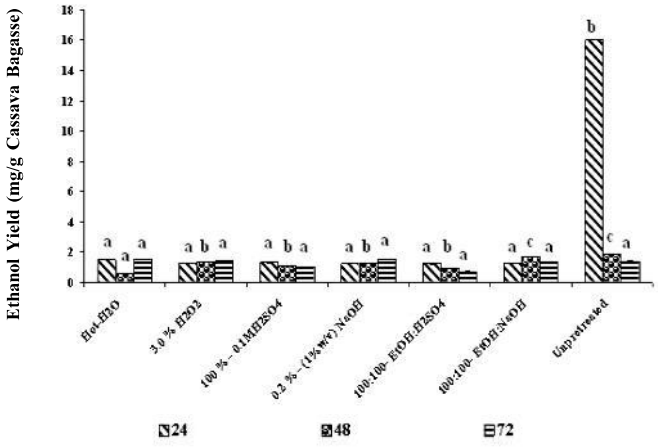

Fig. 4: Ethanol Yield from Pre-treated Cassava Bagasse.

Ethanol yield was significantly ($p<0.05$) higher using the unpretreated cassava bagasse compared to all the pre-treated cassava bagasse after 24 hours of fermentation. Different superscript on bars with the same legend are significantly different at $p<0.05$.

Furthermore, there was also significant ($p<0.05$) negative correlation (R values of - 0.132, - 0.165 and - 0.444 respectively) between reducing sugar released after 24 hours saccharification and ethanol production through 72 hours of fermentation in 100 % H_2SO_4, combined C_2H_5OH with 100 % H_2SO_4 (100:100 C_2H_5OH-H_2SO_4) pre-treated and the unpretreated cassava bagasse. However, there was significant ($p<0.05$) positive correlation between reducing sugar utilization after 48–96 hours saccharification and ethanol production through 72 hours of fermentation.

DISCUSSION

Research has shown that unsuitable handling of agricultural waste biomass such as lignocellulosics can contribute towards overall environmental pollution (11). This has led to investigations into useful alternative uses of these materials such as conversion to bioproducts such as sugars, bioethanol and enzymes. Bioethanol generation from lignocellulosic biomass has been given worldwide concentration in modern era (38). Cassava bagasse, a fibrous by-product of starch processing adds to the raw material pool for second generation ethanol (14). There have been reports on the production of some value-added products such as amylases (39), glutamate (40), and xanthan gum (41) by solid/submerged fermentation of cassava fibrous residue (cassava bagasse).

In this study, the stock cassava bagasse which composed of 64.83 % carbohydrate corroborates with the findings of Ali *et al*. (15), they observed a 65.6 % carbohydrate content while 2.25 % protein and 0.34% fat obtained in the present study is in agreement with 2.49 % protein and 0.20 % fat reported by Kosugi *et al*. (46) and Ali *et al*. (15) respectively. These confirm that cassava bagasse is a rich source of starch but of low grade for animal feed due to its poor nutrient content (13,15), thus justifying its utilization as substrates for bioprocesses as a means of contributing to its disposal and use for bio-product generation.

The observed hydrolysis of standard substrates (carboxymethylcellulose, starch and filter paper) to reducing sugars by both *Tricoderma viride* and *Aspergillus niger* could be attributed to their possession of amylolytic and cellulolytic functions. This is in agreement with Shazia *et al*. (43) who reported cellulase activity as Filter Paper Activity.

In addition, the percentage weight loss of the cassava bagasse after pre-treatment corroborates previous studies on agro-waste treatment. Curreli *et al*. (44) reported significant percentage weight loss of wheat straw powder (45.30 %) after pre-treatment with 2.0 % tetraoxosulphate IV acid at 90 °C. Weight loss could be as a result of loss of some carbon compounds (44) such as lignin, hemicelluloses, starch, and cellulose, which make up the cassava bagasse biomass. Reduced particle size of the cassava bagasse due to milling may increase its surface area to volume ratio which would have further exposed the cassava bagasse to the various chemical pre-treatment and hence increase accessibility to the inherent cellulose content embedded in the matrix of the cassava bagasse native structure. Studies have shown that pre-treatment enhances biomass delignification and accessibility of hydrolytic enzymes to the polysaccharides in the cell wall (20).

Furthermore, the significant increase in the percentage cellulose content relative to the unpretreated cassava bagasse cellulose content is in agreement with Bjerre *et al.* (45) where 70 % (w/w) cellulose content was obtained after pre-treatment of wheat straw to wet oxidation at 150 - 170 °C for 10 min. whereas the unpretreated wheat straw cellulose content was 32 %. However, present observation that cellulose content was maximal with H_2SO_4 pre-treatment compared to other pre-treatment was contrary to the findings of Curreli *et al.* (44) where cellulose content of wheat straw powder was maximal (80.2 %) with alkaline pre-treatment (1% NaOH 6-24 hours) followed by alkaline/oxidative treatment (1% NaOH, 0.3 % H_2O_2

for 6–24 hours). These differences could be as a result of variation in cell wall composition of the raw materials and duration of pre-treatment. This implies that susceptibility of substrates to pre-treatment may be dependent on substrate composition, concentration of pre-treatment chemical and time. The release of reducing sugar from pre-treated cassava bagasse using crude enzyme of *T. viride* and *A. niger* co-culture after 24 hours of saccharification corroborate the findings of Baig *et al.* (30) who investigated the saccharification of banana agro-waste by cellulases of *Trichoderma lignorum* and showed that steam treated agro-waste yielded 1.34 mg/ml of reducing sugars after 24 hours. Likewise, Olanbiwoninu and Odunfa (46) reported that reducing sugar release declines after one hour incubation period using *Pseudomonas flourescens* and *Aspergillus terreus* respectively. Although the reducing sugar release was higher in this study, this could be due to the amylolytic and cellulolytic functions of the co-culture of *T. viride* and *A. niger* which could have hydrolyzed molecules of starch remnant after pre-treatment in addition to the primarily hydrolyzed celluloses, thereby increasing reducing sugar yield.

Cellulase is a multi-enzyme complex comprised of endo-(1, 4)-β-D-glucanase, exo-(1, 4)-β-D-glucanase, and β-glucosidase (47). The decrease in reducing sugar released after 96 hours of saccharification could be due to the synergistic action of the multi-enzyme activities of cellulases of *Tricoderma viride* and *Aspergillus niger* co-culture which may have contributed to the quick and complementing enzymatic breakdown of celluloses in the cassava bagasse substrates (48).

The observed high yield of reducing sugar in H_2SO_4 pre-treated cassava bagasse is in agreement with the report of Olanbiwoninu and Odunfa (46), who investigated the optimal condition for pre-treating cassava peel with dilute sulphuric acid, methanol with catalyst (organosolv) and alkali prior to microbial enzymatic hydrolysis for the production of fermentable sugars. They observed that pre-treatment of cassava peels using 0.1 M H_2SO_4 at 120 °C for 30 min. prior to enzymatic hydrolysis yielded maximum percentage of reducing sugar of 88 % and 98 % using *Pseudomonas fluorescens* and *Aspergillus terreus* respectively, followed closely by methanol treated peels (78 and 98 %) while alkali pre-treated peels produced the least (66 and 88%).

The ethanol yield from the cassava bagasse pre-treated with H_2O_2, Hot-H_2O (hydrthermal) and NaOH after 72 hours of saccharification and fermentation is in agreement with findings of Sindhu *et al*. (49); they reported maximum ethanol concentration after 72 hours of fermentation of acetone pretreated and enzymatically saccharified rice straw. The increased significant ethanol yield from unpretreated cassava bagasse relative to the ethanol yield from the pre-treated cassava bagasse substrates could be attributed to the high starch content in the unpretreated cassava baggasse which may have been reduced in the pre-treated due to the chemical treatments. The amylolytic and cellulolytic function of the crude enzymes from *T. viride* and *A. niger* co-culture may also have concertedly degraded the starch and cellulose, and in part released ample reducing sugars for ethanol production.

In addition, due to the fact that unpretreated cassava bagasse gave the highest ethanol yield, chemical pre-treatment may not be necessary in the case of starchy agro-wastes use as raw materials for second generation ethanol technology.

In the quest to protect and clean up the environment from the menace of greenhouse effect which arises chiefly by fossil fuel use, oil spillage and unsuitable handling of agricultural waste biomass, there is therefore need to embrace the green technology to recycle, reprocess and reuse agro-waste as source of renewable fuel among other value-added products.

CONCLUSION

Given the percentage cellulose yield of 32.47±0.28, 47.50±0.50, 92.30±0.47, 15.60±0.20, 47.33±0.23 and 9.27±0.32 using Hot-H_2O, 3.0 % H_2O_2, 100% H_2SO_4, 0.2 % NaOH, combined C_2H_5OH with 100 % H_2SO_4 and combined C_2H_5OH with 100 % NaOH in pre-treatments respectively compared to 8.00±0.28 cellulose yield of unpretreated cassava bagasse, chemical pre-treatments were found to be optimum for the release of reducing sugar. The rate of release of reducing sugar over 24 hours suggests that concomitant application of crude enzyme extract of *T. viride* and *A. niger* co-culture could save time and cost of saccharification.

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