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Assessment of Extract from Glucose Oxidase-Cellulase Treated Jute Sticks and Green Amaranth Sticks for the Production of Lignocellulose-Based Bioethanol

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ABSTRACT

The possibility that some carbohydrate oxidases are capable of catalytically cleaving glycosidic bonds offers the opportunity for glucose oxidase to achieve the depolymerization of agro wastes required in the production of second-generation bioethanol. The present study aimed to ascertain the effect of glucose oxidase and cellulase isolated from *Aspergillus sp.* on locally sourced jute sticks and green amaranth sticks for the production of bioethanol. The Box Behnken design was employed to assess the effect of the different concentrations of sucrose, waste extracts and pH on fermentation efficiency, ethanol percent yield, and reducing sugar yield. The selected agro wastes were subjected to fiber detergent analysis, ATR-FTIR, XRD, and SEM. The fermentation broth was subjected to ATR-FTIR analysis. Compared to oven-dried jute extract, the maximum ethanol yield was achieved at 72 hours for 50% broth containing oven-dried green amaranth extract by a difference of 65.6%. Optimization using the Box Behnken design resulted in an increased yield of ethanol (198%), fermentation efficiency (3.86%) and reducing sugar yield (27.97%) at the combination of factor levels of 5% (sucrose concentration), 2.5% (oven-dried green amaranth extract concentration) and pH 4.5. The cleaving of glycosidic bonds in the waste samples was revealed by ATR-FTIR and further confirmed by SEM. With the evidence of the characteristic bands associated with the presence of ethanol in the fermentation broth, it was concluded that the inclusion of glucose oxidase at low concentrations in the presence of cellulase supported the release of reducing sugars required for the production of lignocellulose – based bioethanol.

Keywords: Bioethanol, glucose oxidase, cellulase, oven-dried jute sticks, oven-dried green sticks

Introduction

Africa is blessed with natural resources, including fossil fuels, which have been considered the core of the modern economy, significantly driving its contribution to prosperity.[1-3] Despite the contribution fossil fuels offer, they are associated with drawbacks that contribute significantly to global threats, including air pollution and climate change.[3-9] Irrespective of the threats from greenhouse gas emissions, their demand is still increasing.[3-5] These unsustainable impacts may still be minimized if efforts are geared towards systems that reduce/eliminate these emissions.[3-9]

One of the 17 sustainable development goals that is crucial in most countries in Africa is sustainable development goal (SDGs) 7, which functions to place value on human lives and the nation's economy.[8,9] However, in accordance with SDG 7, it has been reported that achieving clean fuel technologies is a challenge that is yet to be solved in most developing nations in Africa, as fossil fuels are still in abundance.[3,5,8]

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The susceptibility and vulnerability of the Sub-Saharan countries to the impact of the negative climate change feedback makes it important to pursue an alternative that can meet the sustainable development goal.[5,8] Bioethanol is one of the biofuel resources that has been recognized to have the capacity to serve as an alternative to well-established fossil fuels, and it is attracting a great deal of attention as its application spans almost all sectors of life.[4,5,10] However, the demand for bioethanol has placed pressure on first-generation feedstocks with subsequent increases in competition with existing food resources.[4,5,10] Unfortunately, this competition significantly impacts food availability, food accessibility and food prices, thus motivating the search for alternative resources with no food value.[4,5,10]

Despite the abundance and renewability properties of wastes, their suitability has been reported to be associated with modern perspectives on waste management, where agro-wastes are perceived to contain valuable resources within.[11] This trapped resource has been reported to serve as a medium for cultivating microorganisms or producing biochemicals such as enzymes and biofuel.[11] However, the reported complexity of the embedded resource makes its depolymerization a significant necessity in achieving a higher bio-product yield in bioethanol production.[10-12] As a large percentage of vegetable sticks in South-Western Nigeria have been identified to have no commercial importance and are consistently available, green amaranth sticks or green sticks from *Amaranthus hybridus* and jute sticks from *Corchorus olitorius* emerged as a potential fit for food waste valorization. This, in turn, helps to prevent the consequences of accumulation and waste burning.[11] The combination of waste recycling and the generation of biochemical that can function as processing aids from the recycling process will not only allow integration of one of the critical SDG 12.5 but also allow the conversion of waste to wealth.[11,13] Unfortunately,

harvesting resources from agro wastes for bioethanol production is still a continuous bottleneck and a global challenge that has raised interest in the discovery of more lignocellulosic enzymes.[4,12] With the complex nature of agro wastes and the reported application of hydrolytic enzymes to harness this complexity, a general, suitable technology that ensures efficient depolymerization of agro wastes with the purpose of increasing the productivity of second-generation bioethanol is yet to be defined.[4,12]

Despite the fact that lignocellulose biomass is thought to be depolymerized through glycoside hydrolases only, the discovery of lytic polysaccharide monooxygenase advances a perspective that involves the application of oxidoreductase as a lignocellulosic enzyme.[12,14] During depolymerization of lignocelluloses, studies have reported the release of different end products ranging from glucose to aldonic acids from the action of microbial lytic polysaccharide monooxygenase and cellobiose dehydrogenase.[12,14-16] These end products have been reported as a potential carbon source for biofuel production.[12,14-17] Despite the reported fermentation with the inclusion of oxidoreductase as an auxiliary enzyme,[4,12,14] the role of glucose oxidase as an oxidoreductase and its synergy with other hydrolytic lignocellulosic enzymes in enhancing fermentation processes is yet to be sufficiently explored in a way that projects them as biocatalysts that would be required in bioethanol production. Hence, this has created an interest that underscores the need to assess the role of fungal glucose oxidases as a potential tool in lignocellulose-based bioethanol production. It is against this background that cellulase was obtained from isolated cellulase-producing *Aspergillus sp*. The indigestible components and reducing sugar concentration present in the oven-dried green sticks (OGSW) and oven-dried jute sticks (OJSW) were evaluated. The effect of glucose oxidase and cellulase on oven-dried green sticks and oven-dried jute sticks was ascertained. The effect of glucose oxidase-cellulase treated green sticks and glucose oxidase-cellulase treated jute sticks extract as co-substrate in the production of bioethanol was assessed using Box-Behnken design (BBD) and *Zymomonas sp*. as a fermentation strain.

The assessment of these selected wastes for the production of bioethanol involves several procedures that include solid-state fermentation as a suitable approach to produce cellulase using *Aspergillus sp.* MAR 10^{-6}. The catalytic effect of glucose oxidase was measured using the o-dianisidine-horseradish peroxidase reaction system, which measures the glucose oxidase activity, and the activity of the enzyme was confirmed by the decline in the reducing sugar concentration. On the basis of the Association of Official Agricultural Chemists (AOAC) official method 2000, a fiber detergent analysis was conducted to determine acid detergent fiber (ADF) and lignin (ADL) in the selected wastes. This analysis involves the separation of plant cells into less digestible cell walls and digestible cell contents using 2 types of detergents (neutral detergent solution (NDS) and acid detergent solution (ADS)). The method allows for a sequential fractionation of the lignocellulosic components into neutral detergent fiber (the residue which is comprised of the acid detergent fiber (ADF) fraction with hemicellulose), ADF (cell wall residue made up of cellulose and lignin) and acid detergent lignin (ADL) (The lignin fraction of ADF). The nature of the wastes was determined by X-ray diffraction (XRD) analysis. The influence of citrate buffer as an extraction solution for reducing sugars was evaluated. The approximate number of bacterial cells in the culture broth was determined by turbidity. The Box-Behnken design, which focuses on the treatment combination (three levels of factors), was employed to optimize the bioethanol production process. The catalytic effects of cellulase and glucose oxidase on the wastes were analyzed by attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR) and confirmed using scanning electron microscopy technique combined with energy dispersive x-ray (SEM/EDX). The evidence of the presence of ethanol in the fermentation broth was revealed by ATR-FTIR.

Materials and methods

Materials

Collection of samples

Rice bran was obtained from the Enzyme Technology Division, Department of Biotechnology, Federal Institute of Industrial Research Oshodi, Lagos, Nigeria. The reagents for the study were of high purity and analytical grade.

The soil sample for isolating cellulase-producing fungi was obtained from the sugarcane composite, Mile 12 (GPS coordinates: Lat 6.612164, Long 3.401897), Lagos state, Nigeria. The soil sample was collected at about 1 cm –5 cm depth, labelled according to its site location, and transported to the laboratory, where the fungi were isolated.

Microorganisms

The pure culture of *Zymomonas spp.* (*palmZ-6*) was isolated from palm wine and preserved in the Department of Biotechnology, Federal Institute of Industrial Research Oshodi (GPS coordinates: Lat 6.5467, Long 3.3478), Lagos, Nigeria. PalmZ-6 was maintained on nutrient agar (HiMedia, India) and grown on nutrient broth at 37 ºC for 24 hours.

Enzyme

Glucose oxidase (1.221 U/ml, 16.9 mg/ml protein, 0.072 U/mg/ml) isolated from *Aspergillus niger* MT550028.1 was employed for the study. The enzyme was produced and purified at the Federal Institute of Industrial Research Oshodi, Lagos, Nigeria.

Glucose oxidase assay

The glucose oxidase activity was determined using the coupled o-dianisidine-peroxidase reaction.[18,19] For o-dianisidine-peroxidase assay, 1.2 ml of 1mg/ml o-dianisidine (Alfa Aesar, USA), 50 µl of 2KU horseradish peroxidase solution (Type VI salt-free lyophilized powder) (Sigma-Aldrich, USA), and 0.5 ml of 1M glucose (Loba Chemie, India) were dispensed into a test tube and allowed to equilibrate at 28 ºC. Then, 0.5 mL of sample extract was added to the cuvette and allowed to incubate for 5 minutes. Dilutions were made when necessary. The absorbance was measured at 436 nm spectrophotometrically (Visible Spectrophotometer 721, China). One unit of glucose oxidase activity was defined as the amount of enzyme oxidizing solution 1 µmol β-D-glucose to D-glucono-δ-lactone and hydrogen peroxide per minute under the assay condition.

Estimation of reducing sugars

The glucose content was determined using the dinitrosalicylic acid method.[20] A working stock solution of anhydrous glucose (1 mg/mL) (Loba Chemie, India) was prepared. Aliquots of this working stock were tightly sealed and stored frozen. Dilutions are made from the working stock (0.1 mg/ml-1 mg/ml) to produce a standard curve. Twenty milliliters of 2N sodium hydroxide (Loba Chemie, India) was transferred into 1 g dinitrosalicylic acid (SCP, Canada) in 10 ml distilled water. This mixture was dissolved in 30 g of sodium potassium tartrate (Loba Chemie, India) in 60 ml of distilled water. The prepared sample was stored in an amber bottle and kept in a dark place to prevent oxidation. An aliquot of substrate solution (0.2 ml), 0.25 ml of glucose oxidase and 0.3 ml of 0.2M citrate buffer (pH 6) sample mixture was dispensed in a test tube. he enzyme-substrate mix was incubated for 20 minutes at 25 ºC. After incubation, 0.5 ml of 3,5- dinitrosalicylic acid (DNS) reagent was added and transferred into the water bath (DK-420, England) to boil at 100 ºC for 10 minutes. This mixture was allowed to cool, and absorbance was read at 540 nm spectrophotometrically (Visible Spectrophotometer 721, China). A standard curve was prepared from glucose solution (1 mg/ml). It was subjected to the same conditions as the samples.

Cellulase assay

The total cellulase activity in the culture filtrate was determined using microcellulose powder (Alfa Aesar, USA) as substrate.[21,22] An aliquot of the sample (1 ml) was measured into the test tube containing the weighed microcrystalline cellulose (Alfa Aesar, USA) and 1 ml of 0.05M citrate buffer (pH 4.8). This mixture was subjected to incubation for 60 minutes at 50 ºC ± 2. After incubation, 1 ml of DNS reagent was added and boiled for 10 minutes. The absorbance was measured at 540

nm when the temperature was reduced to 28 ºC. Total enzyme activity was represented as U/ml. A standard curve was prepared from glucose solution (1 mg/ml). It was subjected to the same conditions as the samples.

Bioethanol assay

The estimation of ethanol content was conducted using the potassium dichromate oxidation method.[23,24] Potassium dichromate (Loba Chemie, India) solution was weighed into a 1L flask and transferred onto a previously prepared ice pack. It was diluted with 100 ml distilled water. Concentrated sulphuric acid (163 ml) (Fisher Scientific, USA) was measured into the solution and made up to 500 ml. The dichromate solution was placed on ice and allowed to cool before adding the acid. One milliliter of potassium dichromate solution was dispensed into test tubes containing an equal amount of fermented sample. Ethanol oxidizes to acetic acid in the presence of sulphuric acid and excess potassium chromate to give off a blue colour whose absorbance was measured spectrophotometrically at 570 nm. Ethanol (BDH Analar, England) standard was also subjected to the same treatment. The fermentation efficiency, ethanol percent yield and ethanol productivity were calculated from the data obtained.[24]

Turbidity measurement

The turbidity is measured at an optical density of 600 nm.[25] Fermentation broth (1ml) was transferred into the cuvette, and the optical density was measured spectrophotometrically at 600 nm.

Analysis of glucose oxidase by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

FTIR analysis was carried out to identify changes in the composition of the waste samples (treated and untreated). The waste samples and fermented broth were analyzed using an ATR-FTIR spectrophotometer (Agilent Technologies Cary 630, USA). The dried wastes were placed directly onto the ATR crystal while the broth was applied to the crystal using a dropper. The ATR crystal alignment was achieved and the baseline correction was performed to remove interference from the background. The spectrum was collected by shining an infrared beam onto the crystal and measuring the reflected light as a function of wavelength. The spectra obtained were processed to remove any baseline drift. The processed spectra were analyzed and interpreted to provide information on the changes in the wastes and composition of the broth sample. The infrared spectrum was indexed in the range of 4000 cm^{-1} to 650 cm^{-1}. The resolution was kept at 8 cm^{-1}, and the sample scan was at 32 scans were carried out on each sample.

X-ray diffraction analysis

X-ray diffractometer (Thermo Fisher Scientific ARL'XTRA, Switzerland) was used to determine the nature of the crystallinity of the agro wastes. The relative intensity was recorded as the ratio of peak intensity to the most intense peak.

Scanning electron microscopy (SEM)-EDX analysis

The morphological changes in the untreated and pre-treated OJSW and OGSW were observed by scanning electron microscope (Phenom world PRO: X:800-07334, Switzerland) with an acceleration voltage of 15 Kv. Images of all the samples were taken at a magnification of 1000X.

Isolation and screening of cellulase-producing isolate

Fungus was isolated via serial dilution technique (10^{-6}) and transferred onto potato dextrose agar (PDA) (Oxoid, United Kingdom) medium supplemented with microcrystalline cellulose powder and 0.05 g of streptomycin. Incubation was conducted at 25 ºC ± 2 for 7 days. Isolates depicting features of *Aspergillus sp.* were selected and identified using its morphological and microscopic (at a magnification of 40X) characteristics. It was further subjected to screening using the Congo red test with 1M sodium chloride (Loba Chemie, India) solution for counterstaining. [21,22] The fungal colonies showing a zone of decolourization in the Congo red test were selected for cellulase production by solid-state fermentation. [21,22]

Effect of citrate buffer on the selected wastes

Each waste substrate was measured in a tube, and the potential of citrate buffer to extract reducing sugar at different pH levels (pH 5, pH 6, pH 7) was evaluated. The extraction process was conducted at 28 ºC, 50ºC and 100ºC. The reducing sugar concentration of the extracted sample was analyzed.[20]

Solid-state fermentation for cellulase production

Rice bran was employed as a substrate for solid-state fermentation for cellulase production.[22] Fresh media containing nutrients, as shown in Table 1, were measured and dissolved in distilled water. The pH was maintained at pH 4.8 using 1M hydrochloric acid (HCL) (Fisher Scientific, USA) / sodium hydroxide (NaOH) (Loba Chemie, India). The measured rice bran (1 g) was moistened with the fresh media (1:10), and this mixture was subjected to sterilization at 121 ºC for 15 minutes. After sterilization, the sample was allowed to cool to room temperature. The moistened rice bran was aseptically inoculated with the selected *Aspergillus isolate* from fresh plates. It was incubated till ramification was observed. The ramified sample was submerged with 0.2 M citrate phosphate buffer at pH 4.8 and kept in the refrigerator for 18 hours. The total cellulase activity and reducing sugar concentration were determined using the supernatant extract obtained.

Estimation of lignocellulosic components of waste for bioethanol production

This detergent fiber analysis (neutral detergent fiber analysis and acid detergent fiber analysis) was conducted with slight modifications.[26]

Treatment of the selected wastes with glucose oxidase and cellulase

The selected wastes were moistened with citrate-phosphate buffer (0.2M, pH 6) for 30 minutes to 60 minutes. They were treated with glucose oxidase, citrate buffer and cellulase in a ratio of 0.1:10:1 at 50 ºC for 60 minutes. The enzyme mixture was kept for 2 hours in the refrigerator at 4ºC. The reducing sugar content/degree of saccharification was ascertained. The residuals of the untreated samples and treated samples were subjected to ATR -FTIR and XRD.

Production of bioethanol by static submerged fermentation

Freshly cultured *Zymomonas sp*. (*palmZ-6*) was cultivated at 150 rpm in an orbital shaker incubator (1585VWR, USA) for 24 hours in 250 mL containing 50 ml nutrient broth. *Isolate palmZ-6* was inoculated into fresh medium containing yeast extract (Biolife, United Kingdom), 0.25g; bacteriological peptone (Oxoid, United Kingdom), 0.25g; ammonium sulphate (Loba Chemie, India), 0.01g; magnesium sulfate (Loba Chemie, India), 0.01g; potassium diphosphate phosphate (Loba Chemie, India), 0.01g; Sucrose (Loba Chemie, India), 10g.[25] All components were dissolved in 100 ml of distilled water, and pH was adjusted to pH 5 using 1M HCL/NaOH.
The fermentation medium and treated waste extracts were prepared, dispensed and further subjected to autoclave (NB116, England) sterilization for 15 minutes at 121 °C, 15 psi pressure. The sterilized medium was allowed to cool and inoculated aseptically with the isolate *palmZ-6*. Under static submerged fermentation, the inoculated broth was transferred to the incubator at 37 ºC. An aliquot amount of the sample was collected at the 24-hour intervals for 72 hours. The optical density, reducing sugar concentration, fermentation efficiency, and ethanol content were estimated.[23,24]

Statistical optimization for the production of bioethanol using Box-Behnken design

The Box-Behnken design was used to optimize the effects of sucrose concentration, waste extract concentration and pH on fermentation efficiency, ethanol percent yield and reducing sugar yield. Sucrose concentration, waste extract concentration and pH were the independent variables, and fermentation efficiency, ethanol percent yield and reducing sugar yield were the dependent variables. At three different levels (-1, 0, +1), each variable in the design was studied, with all

variables taken at a central coded value of zero, as shown in Table 2. Seventeen experiments were generated, and the analysis was carried out by using the Design-Expert software package. Each run required subjection to fermentation conditions as described previously. The best-fitting mathematical model, statistical validation of the model and the appropriateness of the model to navigate the design were based on statistical parameters by the design expert software. [27]

A second-order polynomial equation was generated by the software package to describe/model the interaction/ relationships between the variables and the response.

$Y = \beta_0+\beta_1A+\beta_2B+\beta_3C + \beta_{11}A^2+ \beta_{22}B^2+ \beta_{33}C^2+ \beta_{12}AB+\beta_{13}AC+ \beta_{23}BC$ ……. (1)

The dependent variable (Y), the intercept (βo), the regression coefficients (β_1, β_2, β_3), the independent /predictor variables (A, B, and C), the interactions between variables (AB, AC, and BC), and the quadratic effect determined by A^2, B^2, C^2, are expressed in equation (1). The sign of each coefficient estimate indicates the interacting effect or main effect between a predictor variable and the response.

To validate the statistical model for optimizing production, the experiment was conducted under the optimal conditions predicted by the model. This validation experiment served to confirm the accuracy of the model in predicting the optimum concentration of factor for maximizing glucose oxidase production. Response plots generated were used to visualize the relationship between responses and the levels of each independent variable.

Methods of statistical analysis

All experiments were conducted in duplicates, with the results presented as mean ± standard deviation (SD) using Microsoft Excel 365. The data obtained were further analyzed with Design Expert software (Stat-Ease 23.1.0.0 (analysis) and Stat-Ease 23.1.8.0 (verification)) (Stat Ease, Inc.; Minneapolis, USA). Statistical significance was defined when $p<0.005$.

Results and discussion

The unsustainability of fossil fuels and the vulnerability of the Sub-Saharan countries to the impact of the negative climate change feedback makes it important to pursue an alternative that can meet the sustainable development goal.[5,8] This has been encouraged, most especially in developing nations.[5,8] In order to prevent the implications associated with the use of first-generation feedstocks, second-generation feedstocks with non-significant impact on food production have been receiving attention.[4,6,10] Despite the availability and accessibility of vegetables as a cheap source of vital nourishment, vegetable waste, which is comprised mostly of sticks and chunky stems, also accounts for a significant percentage of food waste in South-West Nigeria. The need to find utilization is essential to avert subsequent negative impacts of accumulation. As highly sought nutrient-rich vegetables, green amaranth (Figure 1A) and jute (Figure 1B) are highly consumed in South-West Nigeria. Most consumption requires just the leaves and the tender stem, while the chunky stem is usually discarded. While the resulting waste from jute and green amaranth has been utilized locally as animal feed, a large percentage of these wastes have been identified to have no commercial importance in Nigeria, thus consigned to the bin as a useless entity. As agro wastes have been recognized as a source of biochemical, further access to the trapped resources requires treatments that allow the matrix of cellulose and lignin bound by hemicellulose to be dissolved/ broken to increase the possibility of generating fermentable sugars.[4,11,12]

Fiber detergent analysis and reducing sugar concentration of selected wastes

Pretreatment, saccharification, and fermentation are identified as the critical steps in the depolymerization of waste into bioethanol.[4,6,10,12] As the suitability of wastes for bioethanol production is predicated on the relative polysaccharide composition, mechanical pretreatment was applied to the wastes to break down the coarse components into smaller structures.[4] This further makes the determination of waste components easier. In this study, the stem of the sticks was separated from ribbon-like fiber by short-term water retting.[28] Since cellulose is reported stable against temperatures up to 200-300 °C,[29] the stem was further subjected to drying in a hot air oven (New Brunswick Scientific, USA) at 100 °C for 24 hours. The significance of higher cellulose content for the production of biofuels like bioethanol has been reported. [4,30,31] The result of the fiber analysis of the wastes (Table 3) showed a higher cellulose content and low acid detergent lignin, which is indicative of the suitability of the wastes for bioethanol production. Due to its potential to support the stability of enzymes, create a suitable environment for microbial metabolic activities, function as a green

A

B

Figure 1: A) Green amaranth sticks B) Jute sticks.

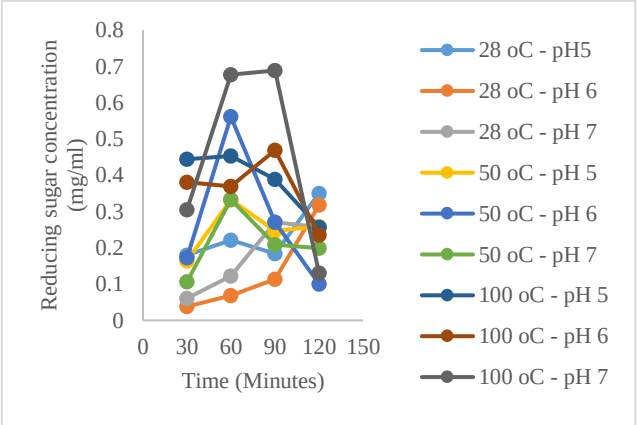

Figure 2: Effect of citrate buffer, temperature and time on reducing sugar concentration obtained from OJSW.

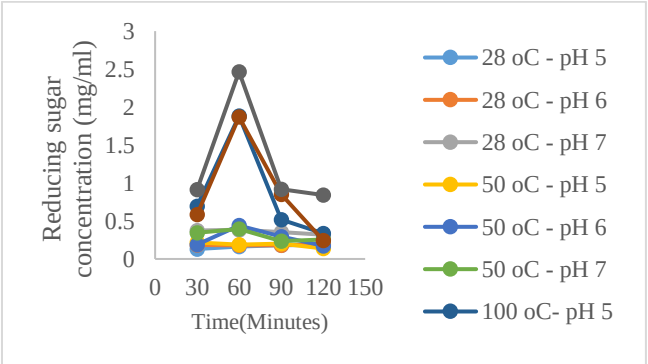

Figure 3: Effect of citrate buffer temperature and time on reducing sugar concentration obtained from OGSW.

solvent with less need for further purification process and no reported negative impact on the environment, citrate buffer was selected as moistening solvent for the wastes at temperature and pH suitable for the glucose oxidase, cellulase and isolate *palmZ-6.* The effect citrate buffer might have on the wastes at pH and temperature suitable for the enzymes' action was evaluated. It was shown that reducing sugars were released from wastes at a high temperature of 100 ºC and pH 7 as shown in Figure 2 and Figure 3. OGSW (2.465 mg/ml ± 0.247) released higher reducing sugars at 60 minutes compared to OJSW (0.689 mg/ml ± 0.0655) at 90 minutes. Lower sugar release was observed at lower temperatures. This indicates the possibility of the citrate buffer to create an environment that might be suitable for the enzymes' catalysis as well as for the fermentation process.

Catalytic effect of cellulase sample treated with purified glucose oxidase

Microbial cellulase has been identified as an essential hydrolytic and lignocellulosic enzyme required in bioethanol production.[4,12] As a colloidal crystalline portion of cellulose fibers, it was reported that microcrystalline cellulose was considered an efficient inducer for cellulase production; hence, it was used for its production.[32,33] Microcrystalline cellulose was hydrolyzed by the synergistic action of three enzymes secreted into the medium; they include endoglucanases (EC3.2.1.4), which act randomly on the cellulose chains within the crystal structure to expose two new chain ends.[4,12,21] These new ends serve as a substrate for exoglucanase (EC3.2.1.91) to produce an end product that serves as a substrate for β –β-glucosidase (EC3.2.1.21), which finally releases fermentable sugars. [4,12,21] In Nigeria, a major fallout during rice processing to meet the needs of the rising high-consumption potential population is the generation of rice bran.[11,34] In order to advert the consequence of its accumulation, rice bran was selected as a substrate due to its embedded rich source of nutrients that can support the growth of fungi. [11,34] In the presence of microcrystalline cellulose, the possibility of generating reducing sugars was high in addition to the cellulase enzyme that would be released due to the fungi's metabolic activity. In order to prevent the likelihood of end-product inhibition, the inclusion of glucose oxidase was suggested to remove the residual reducing sugars generated from cellulase action on microcrystalline cellulose. It was shown that in the absence of glucose oxidase (Figure 4), reducing sugar concentration was higher with medium A (2.267 mg/ml ± 0.032) and medium D (2.263 mg/ml ± 0.009). In contrast to medium A, at 10 minutes of incubation with glucose oxidase, medium D gave a better cellulase activity (0.481 U/ml ± 0.005) and reducing sugar concentration (1.192 mg/ml ± 0.005), which increased at 20 minutes of incubation (activity: 0.524 U/ml ± 0.083) and reducing sugar concentration (1.298 mg/ml ± 0.093). Medium D was selected for cellulase production with 20 minutes of treatment with glucose oxidase.

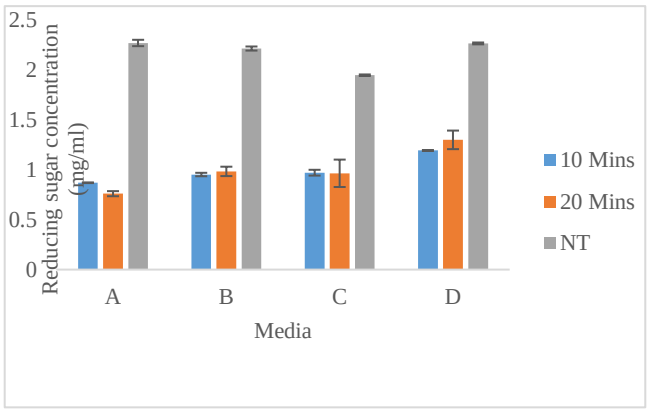

Figure 4: Effect of cellulase treated with glucose oxidase.
(A: Medium A; B: Medium B; C: Medium C; D: Medium D; min: minutes; NT: not treated with glucose oxidase)

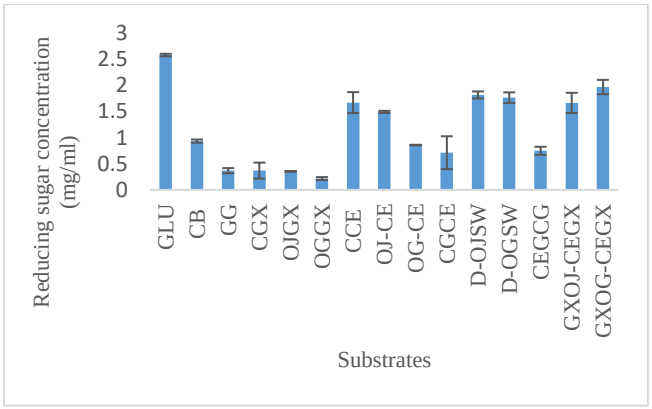

Figure 5: Effect of cellulase and glucose oxidase on selected waste resources.

OJ: Oven dried jute stick waste; OG: Oven dried green stick waste; GLU: Glucose; CB: Microcrystalline cellulose –buffer; GG: Glucose-GOX; CGX: Microcrystalline cellulose-GOX; OJGX: OJSW-GOX; OGGX:OGSW-GOX; CCE: Microcrystalline cellulose -cellulase; OJ-CE: OJSW-cellulase; OG-CE: OGSW-cellulase; CGCE: Treated cellulose-untreated cellulase; D-OJSW: Treated OJSW-untreated cellulase; D-OGSW: Treated OGSW-untreated cellulase; CEGCG; Treated microcrystalline cellulose -treated cellulase; GXOJ-CEGX; Treated OJSW-treated cellulase; GXOG-CEGX; Treated OGSW-treated cellulase; GOX: Glucose oxidase

With the potential of glucose oxidase to reduce residual sugars released during the production of cellulase, more detailed studies are required to provide insight into the role played by glucose oxidase during the extraction of cellulase.
Despite the significance of wastes in second-generation bioethanol production and the high cost of cellulase, depolymerization of lignocellulose waste components is still a bottleneck that has not been successfully resolved. [17] In addition to hydrolytic enzymes, recent studies have considered the inclusion of other carbohydrate-active enzymes after the discovery of the potential of oxidoreductase in harnessing lignocellulosic biomass. [4,14] On this note, this study introduced the processing of wastes through the oxidative route using glucose oxidase as a new approach to the separate hydrolysis and fermentation steps.
In the absence of cellulase, all the sugars were oxidized in the presence of glucose oxidase (Figure 5). In the presence of cellulase, it was observed that the time of incubation, and enzyme concentration of glucose oxidase significantly affected the release of reducing sugars. Hence, a minute amount of glucose oxidase was introduced. In comparison to glucose oxidase-treated OGSW, cellulase activity was more favored for glucose oxidase-treated OJSW by a difference of 2.936%. The treatment of OJSW with glucose oxidase displayed higher reducing sugar concentration (1.821 mg/ml ± 0.067) compared with when both cellulase and OJSW were treated (1.667 mg/ml ± 0.194). The treatment of OGSW and cellulase with glucose oxidase displayed higher reducing sugar concentration (1.972 mg/ml ± 0.138) compared with when the OGSW sample was only treated with glucose oxidase (1.768 mg/ml ± 0.102). By a difference of 16.76%, higher cellulase activity was observed when both cellulase and OGSW were treated with glucose oxidase compared to glucose oxidase treatment of OJSW and cellulase. It was suggested that glucose oxidase, through the oxidative route, cleaved the glycosidic bonds within the lignocellulose polymers, assisting the cellulase in its synergistic hydrolytic activities to yield more reducing sugars.[4,14] It was suggested that the use of citrate buffer and the application of lower temperatures may also support the action of the enzymes by enhancing their accessibility to achieve a good yield.

ATR-FTIR absorption spectra for treated oven-dried stick wastes

The ATR-FTIR was employed to analyze the effect of enzyme treatment on the selected wastes. In this study, the FTIR spectrum

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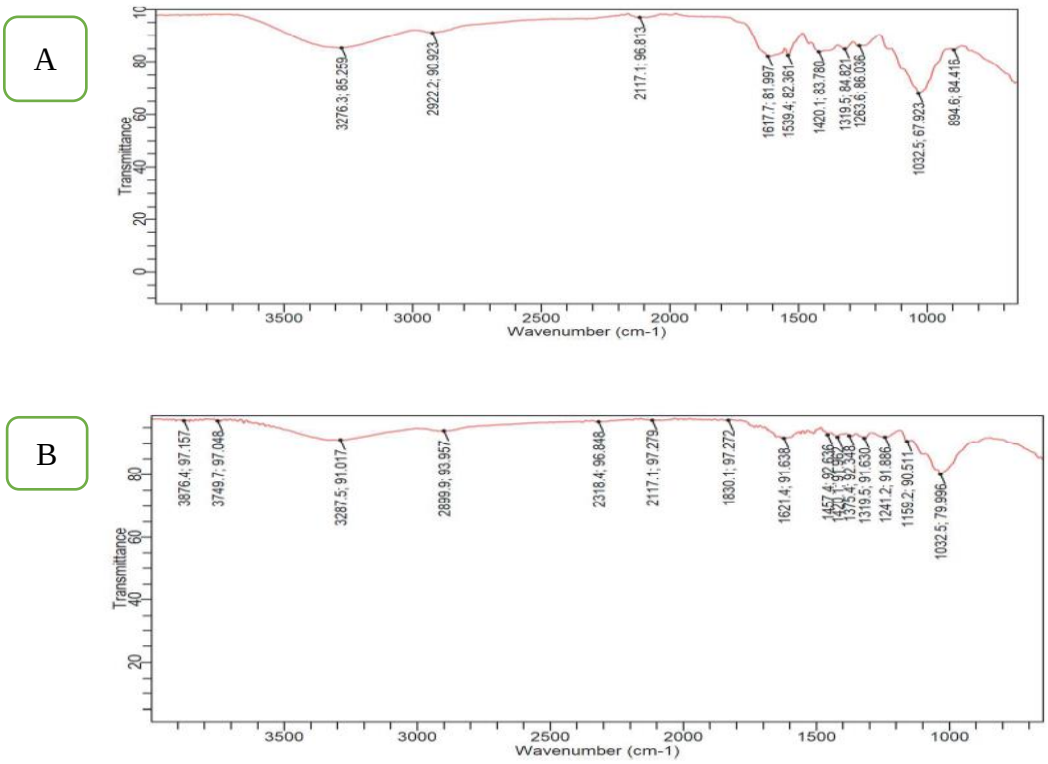

Figure 6: FTIR absorption spectra for (A) untreated OJSW and B) glucose oxidase-cellulase treated OJSW

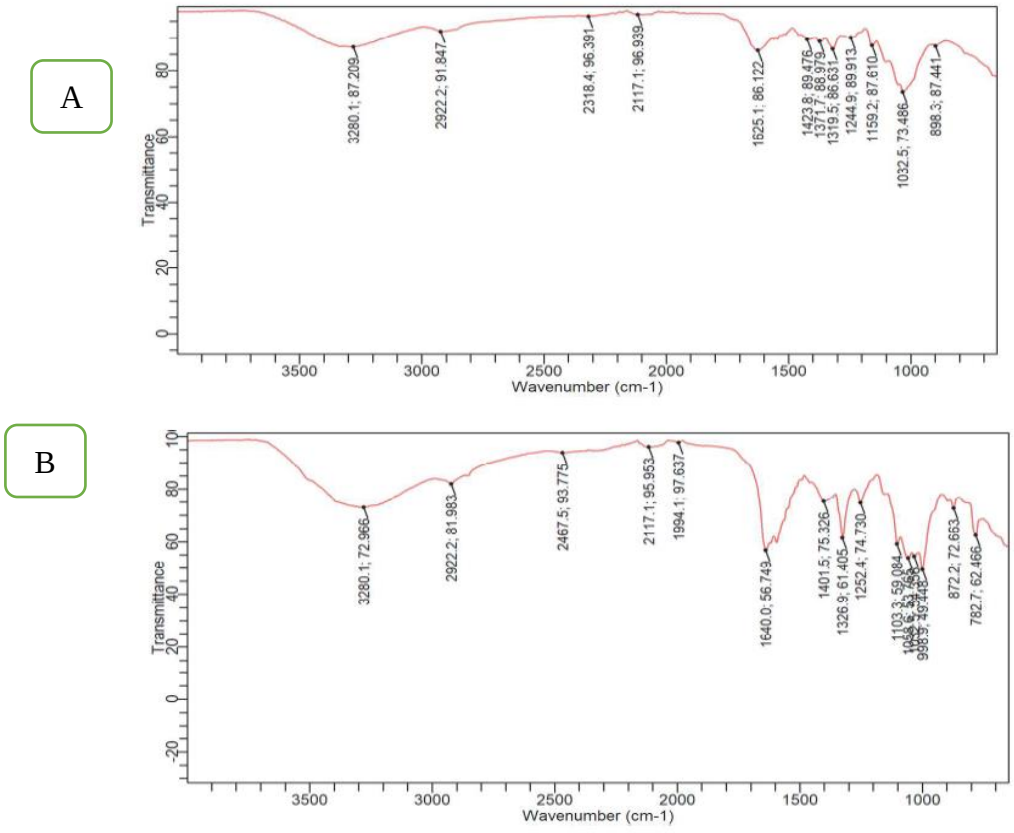

Figure 7: FTIR absorption spectra for A) untreated OGSW and B) glucose oxidase-cellulase treated OGSW

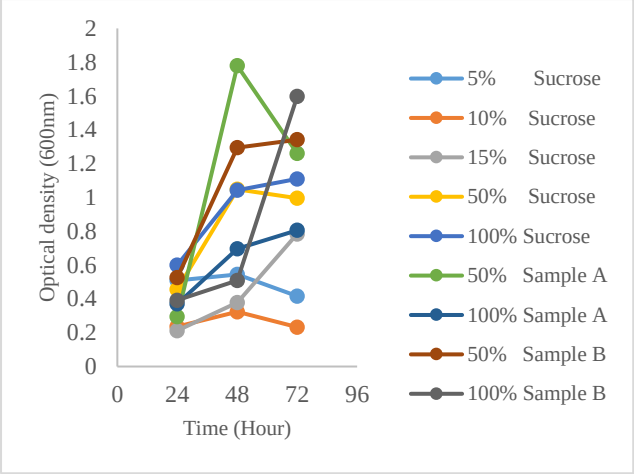

Figure 8: Growth rate of *Zymomonas sp.* in the presence of OJSW (sample A) and OGSW (sample B).

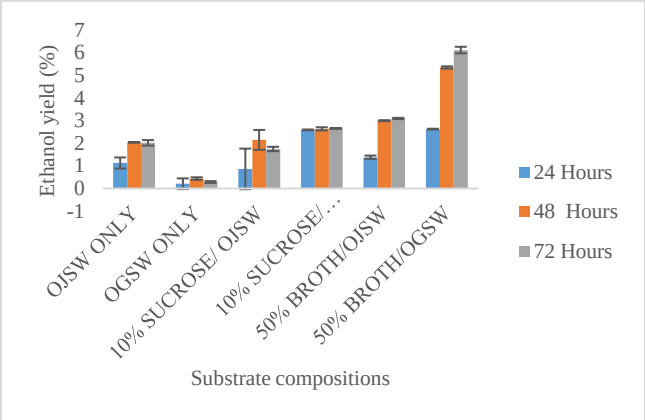

Figure 9: Bioethanol yield from 24 hours to 72 hours using treated OGSW and treated OJSW. (50% broth comprises of production medium)

showed 10 peaks generated from untreated OJSW samples (Figure 6A) and 15 peaks from treated OJSW samples (Figure 6B). OGSW samples generated 15 peaks from untreated samples (Figure 7A) and 12 peaks from treated samples (Figure 7B). This indicate the complexity of molecules within the samples.[35] In contrast to OJSW, more peaks at lower transmittance intensity were observed with the untreated OGSW. Higher transmittance was observed with treated OJSW/OGSW. Since sugars are of interest, the characteristic bands were associated mainly with functional groups present in a monosaccharide and polysaccharides, including hydroxyl groups (OH), glycosidic bonds (C-O-C), carbon-oxygen (C-O) bonds, carbon-carbon (C-C) bonds, and carbon-hydrogen (C-H) bonds. The key characteristic bands identified with untreated OJSW and untreated OGSW include a broad band around 3200 cm^{-1} and 3570 cm^{-1}, C-H stretching band, which appears around 2800 cm^{-1}-3000 cm^{-1}, bands around the fingerprint region (900 cm^{-1}-1200 cm^{-1}) representing stretching of the C-O-C bond and C-O bond, and CH2 stretch around 1415 cm^{-1}-1462 cm^{-1}.[31,35,36] These features are associated with the basic structure of sugars.[31,35,36] The bands centered at 3276.3 cm^{-1} (untreated OJSW) and 3280.1 cm^{-1} (untreated OGSW) are attributed to the strong hydrogen bond from OH stretching.[31,35] The presence of a high-frequency band at 3749.7 cm^{-1} and 3876.4 cm^{-1} at higher transmittance for treated OJSW is associated with stretching vibration of non-bonded hydroxyl group or some amine group-specific stretches.[35] While peaks around 1539 cm^{-1}-1640 cm^{-1} might be indicative of simple hetero-oxy compounds, including nitrogen oxy compounds, bands at 1319.5 cm^{-1} are both common in both waste spectra; which might be attributed to OH bend or C-H in-plane bending of cellulose.[31,35]

The fingerprint region is more complex most especially for OGSW indicative of different functional groups present in wastes. It is found useful in assessing molecular linkages and bond vibration, most especially the stretching of the C-O-C bond and C-O bond (900 cm^{-1} - 1200 cm^{-1}), which constitute a glycosidic bond present within cellulosic materials in biomass.[35,36] The presence of a band around 894 cm^{-1} - 898 cm^{-1} has been reported to correspond to C-O-C stretching vibration of β glycosidic linkage[36], a key feature for identifying cellulose (extensive network of glucose joined by β-glycosidic bond). FTIR spectra of untreated OGSW peaks around 700 cm^{-1} -1300 cm^{-1} show the presence of this bond and could be attributed to skeletal C-C vibration. Their absence in treated OJSW/OGSW samples indicates the cleaving action of glucose oxidase and cellulase. The lower intensity of the peak with the slightly wider band at 1640 cm^{-1} is indicative of simple hetero-oxy compounds, including nitrogen oxy compounds or aromatic combination bands around 1660 cm^{-1} -2000 cm^{-1}.[35] Despite the possibility of assigning the wavenumber 898 cm^{-1} in treated OGSW sample to glycosidic bond, the possibility of C-O-O- stretch from peroxide (820 cm^{-1} –890 cm^{-1}) as well as carboxylate around the region of 1550 cm^{-1} –1610 cm^{-1} followed by region of 1300 cm^{-1} –1420 cm^{-1} was not ruled out. [35,37] The broader peaks might be evidence of the amorphous nature of cellulose, which is attributed to the loss of ordered structure. Based on the above interpretation, it can be suggested that the untreated and treated OJSW/ OGSW contains complex organic compounds. There was evidence of enzyme action in their treated samples, with the possibility of the analyzed material generating free hydroxyl groups from cleaved molecules.

Production of bioethanol using the Box Behnken technique

The proficiency of an ethanologenic organism (isolate *palmZ-6)* to coordinate ethanol production using oxidative treated inexpensive wastes as co-substrate was considered. Their desirability for the commercial production of ethanol stems from fermentation efficiency toward high ethanol productivity and high osmo-tolerance.[38] Their ability to rapidly metabolize high sugars to maintain significant growth rates was associated with high expression of pyruvate decarboxylase and alcohol dehydrogenases, high-velocity facilitated diffusion glucose uptake system and high levels of the Entner–Doudoroff pathway comprising of the incomplete non-oxidative-branch of the pentose phosphate pathway and an incomplete Krebs cycle.[38] It is expected that a favorable fermentation outcome should result in the decline of reducing sugar yield with subsequent higher ethanol yield.[14] As *isolate palmZ-6* was shown to be a gram-negative, short, plump rod, and catalase-positive, maximum growth was observed at 48 hours (Figure 8) in the OJSW sample containing 50% broth. At 72 hours, 100% OGSW supported maximum growth, followed by the OGSW sample containing 50% broth. The presence of broth significantly influences the growth of *isolate palmZ-6* and the production of ethanol, as the broth contains more nutrients that could support the microbial activities of *isolate palmZ-6*. In the absence of sucrose, low ethanol was produced, as shown by the slight colour change in the presence of potassium / sulphuric solution. From 24 hours to 48 hours, in the presence of sucrose, the ethanol percent yield for glucose oxidase-cellulase treated OJSW (treated OJSW) was achieved with a 1.48-fold increase. Compared to treated OJSW, the ethanol percent yield of glucose oxidase-cellulase treated OGSW (treated OGSW) containing 50% broth (the broth enriched with micronutrients and 10% sucrose) increased by a fold of 1.33 at 72 hours. As fermentation yield is dependent on the ability of isolate palmZ-6 to utilize substrate, it is expected that the higher the fermentation efficiency, the higher the ethanol that will be produced.

In the absence of broth and sucrose, maximum fermentation efficiency was observed with OGSW at 72 hours and 24 hours for OJSW. The efficiency of fermentation and ethanol yield increased from 24 hours to 72 hours in the presence of 50% broth (Figure 9). At 72 hours, it was shown that OGSW-containing 50% broth (6.1%) gave the highest yield compared to OJSW-containing broth (3.1%).

Optimization of pH, sucrose, and treated OJSW using Box Behnken design from 24 hours to 72 hours

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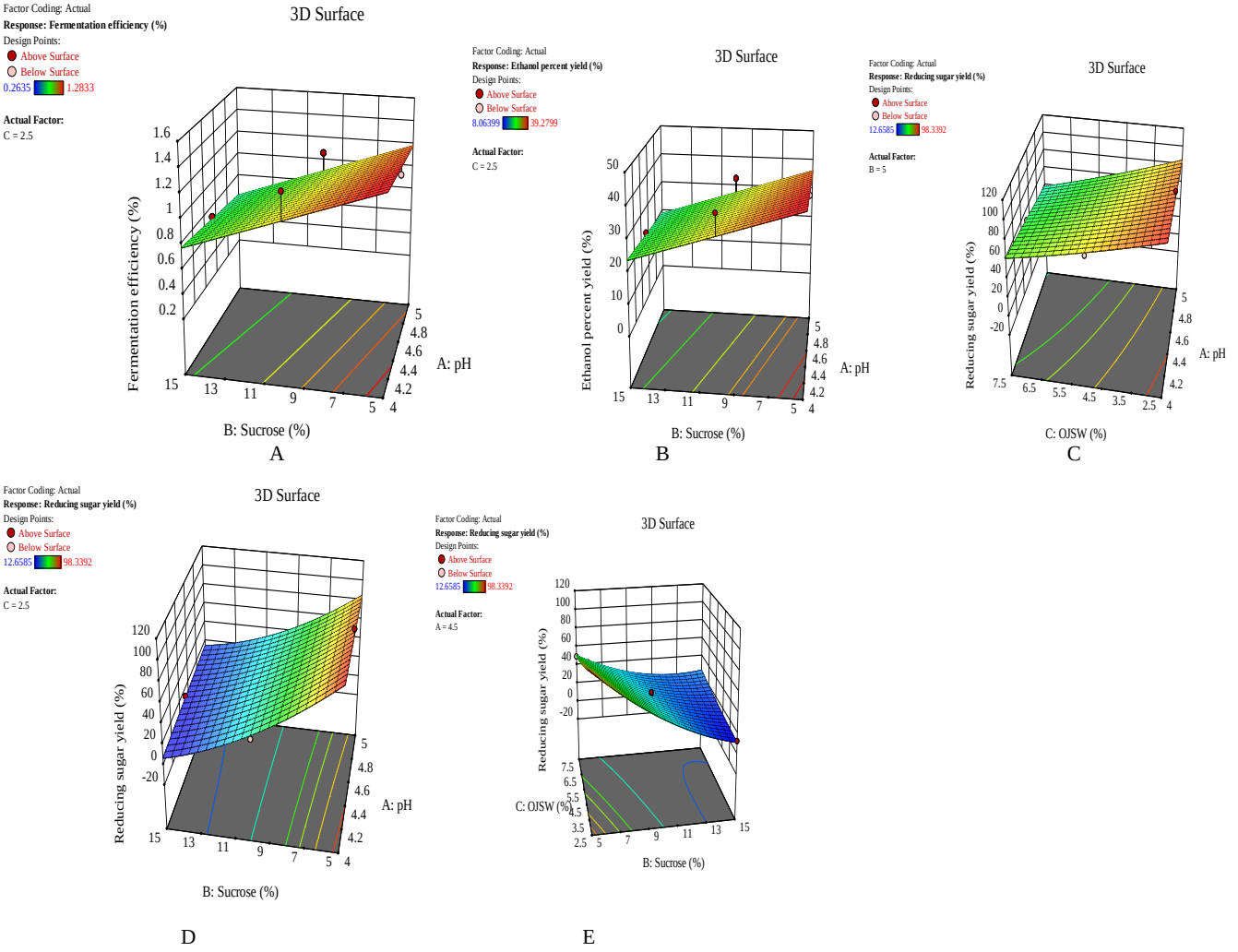
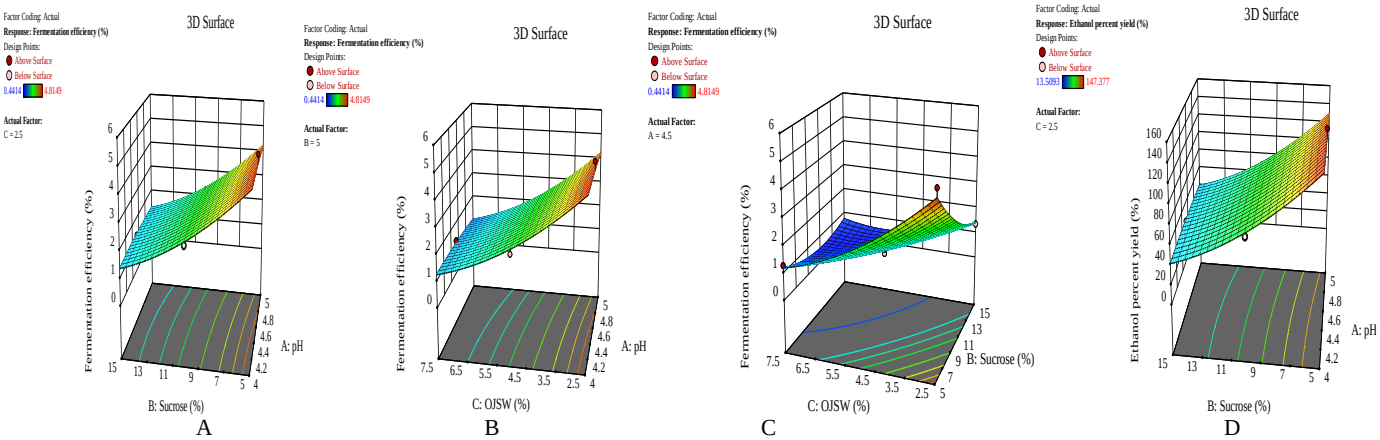


Figure 10: Response surface plot showing the effect of variables on fermentation efficiency (A/B), ethanol percent yield (C/D), and reducing sugar yield (E) at 24 hours. (Variables: OJSW, sucrose, pH)



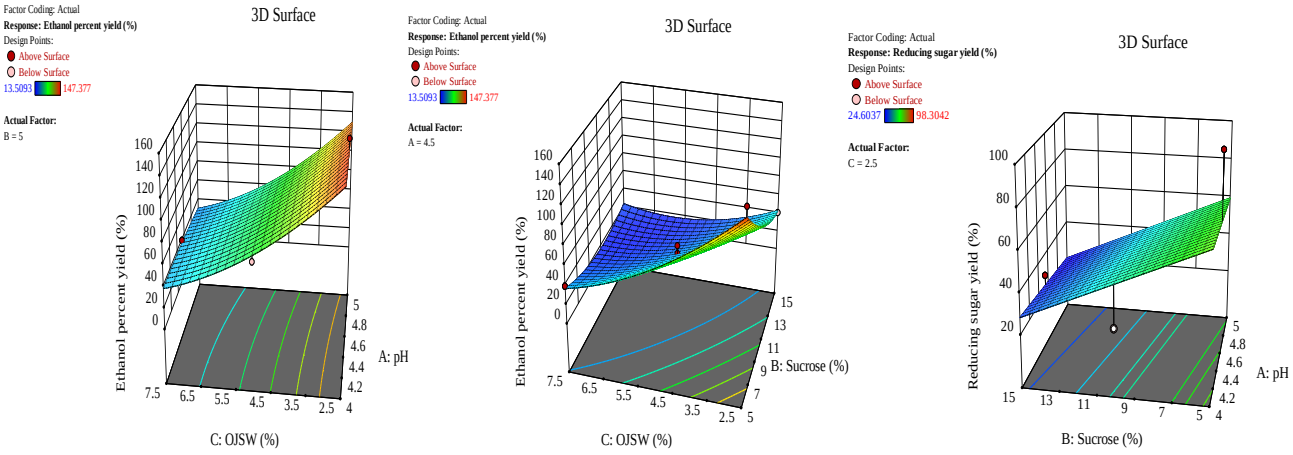


Figure 11: Response surface plot showing the effect of variables on fermentation efficiency (A-C), ethanol percent yield (D-F) and reducing sugar yield (G) at 48 hours. (Variables: OJSW, sucrose, pH)

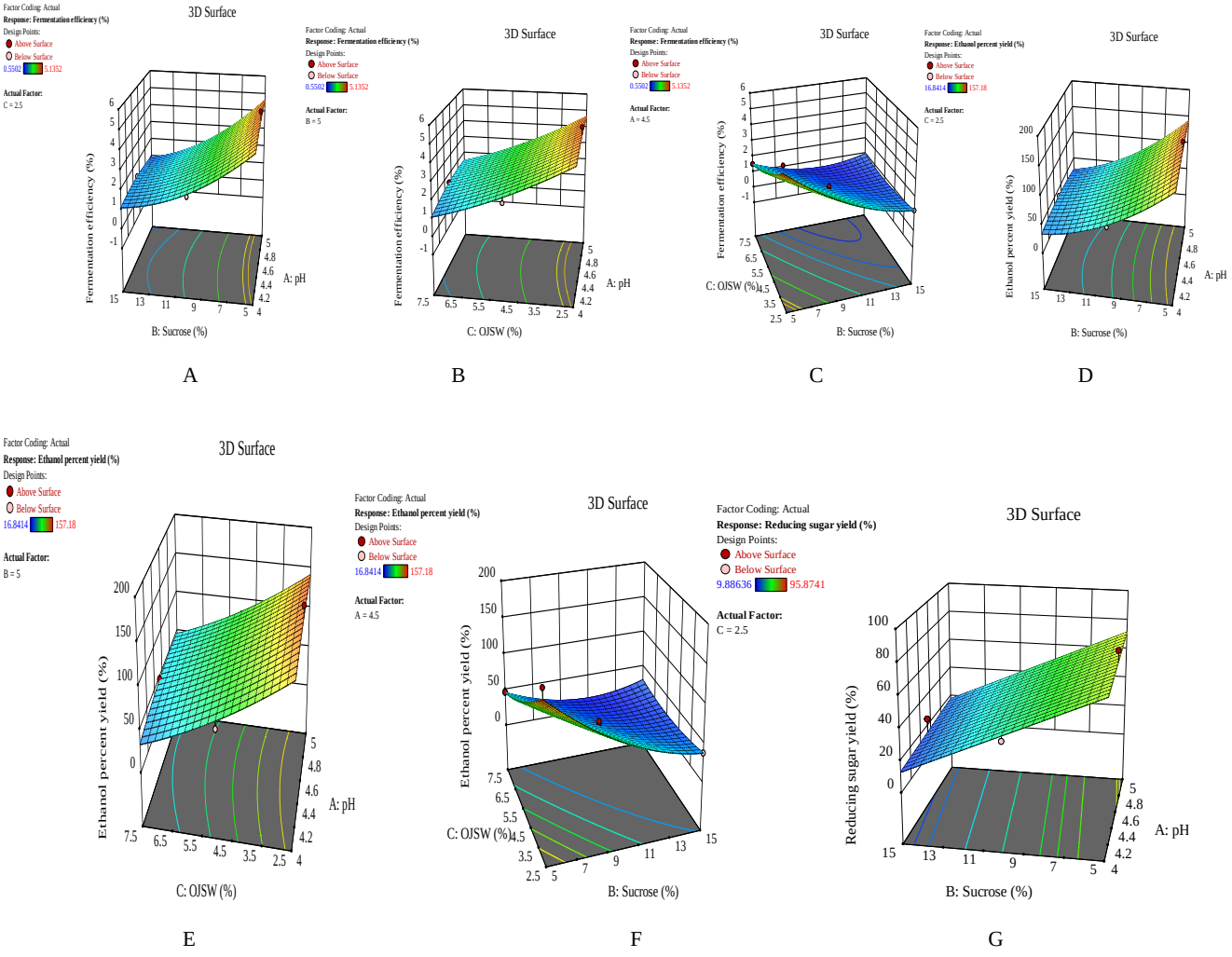


Figure 12: Response surface plot showing the effect of variables on fermentation efficiency (A-C), ethanol percent yield (D-F) and reducing sugar yield (G) at72 hours. (Variables: OJSW, sucrose, pH)

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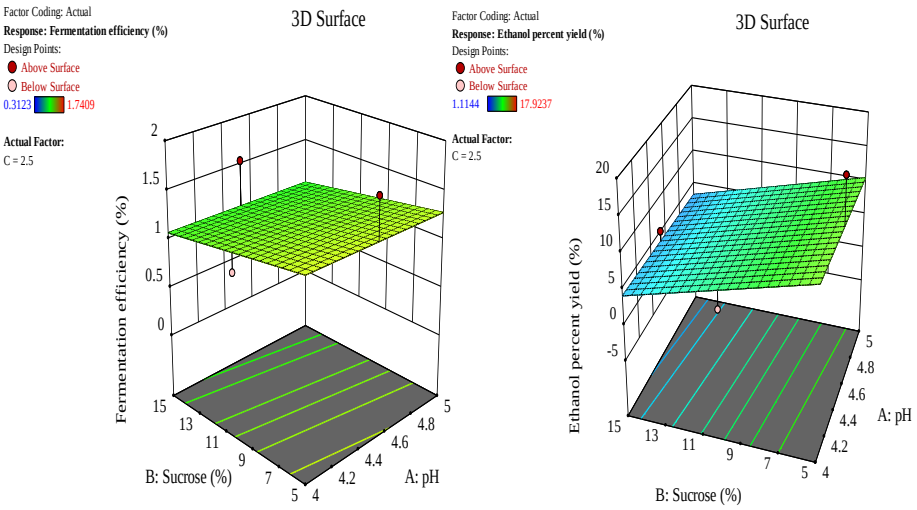

Figure 13: Response surface plot showing the effect of variables on fermentation efficiency, ethanol percent yield. (Variables: OGSW, sucrose, pH)

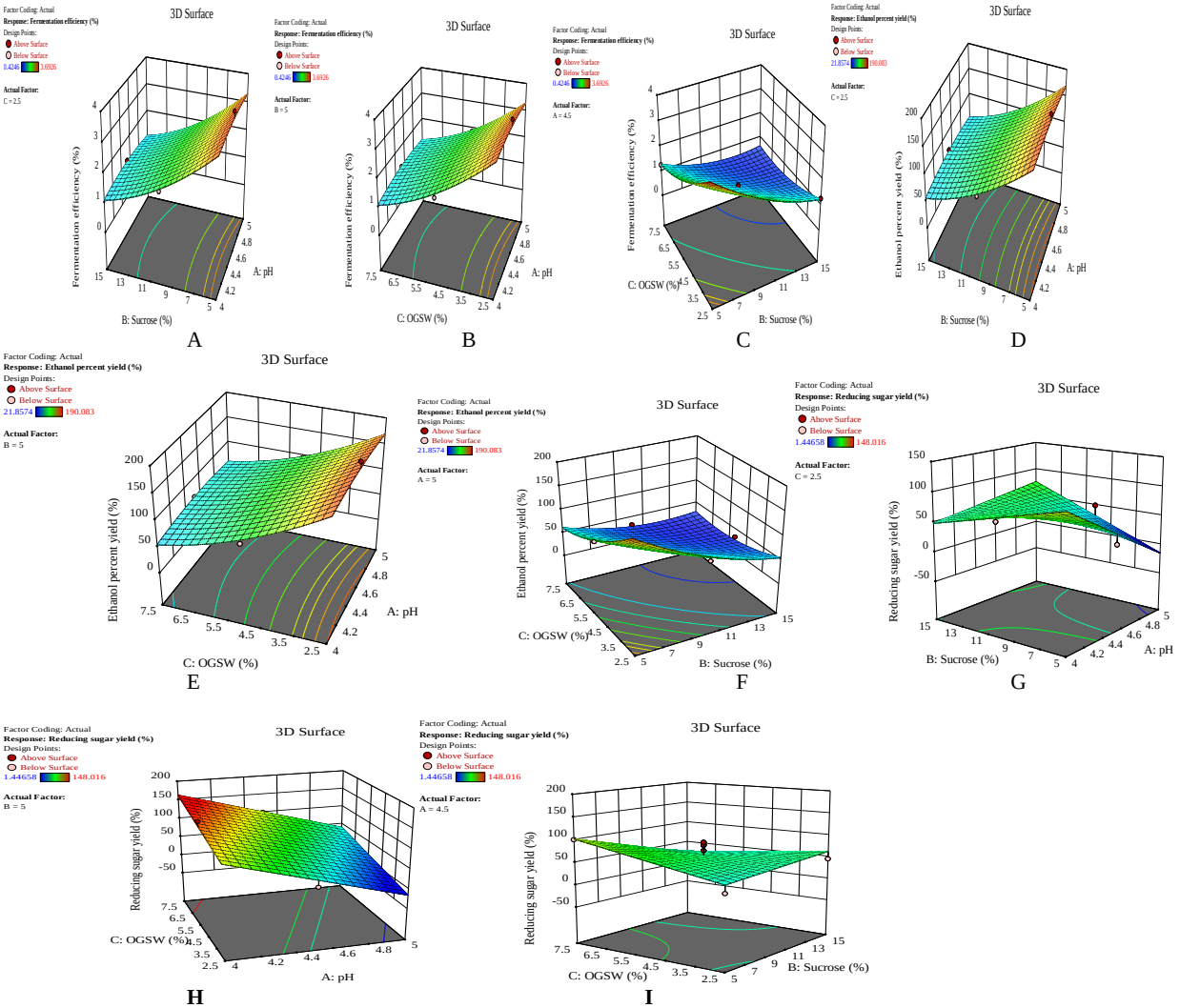

Figure 14: Response surface plot showing the effect of variables on fermentation efficiency(A-C), ethanol percent yield (D-F) and reducing sugar yield (G-I) at 48 hours (Variables: OGSW, sucrose, pH)

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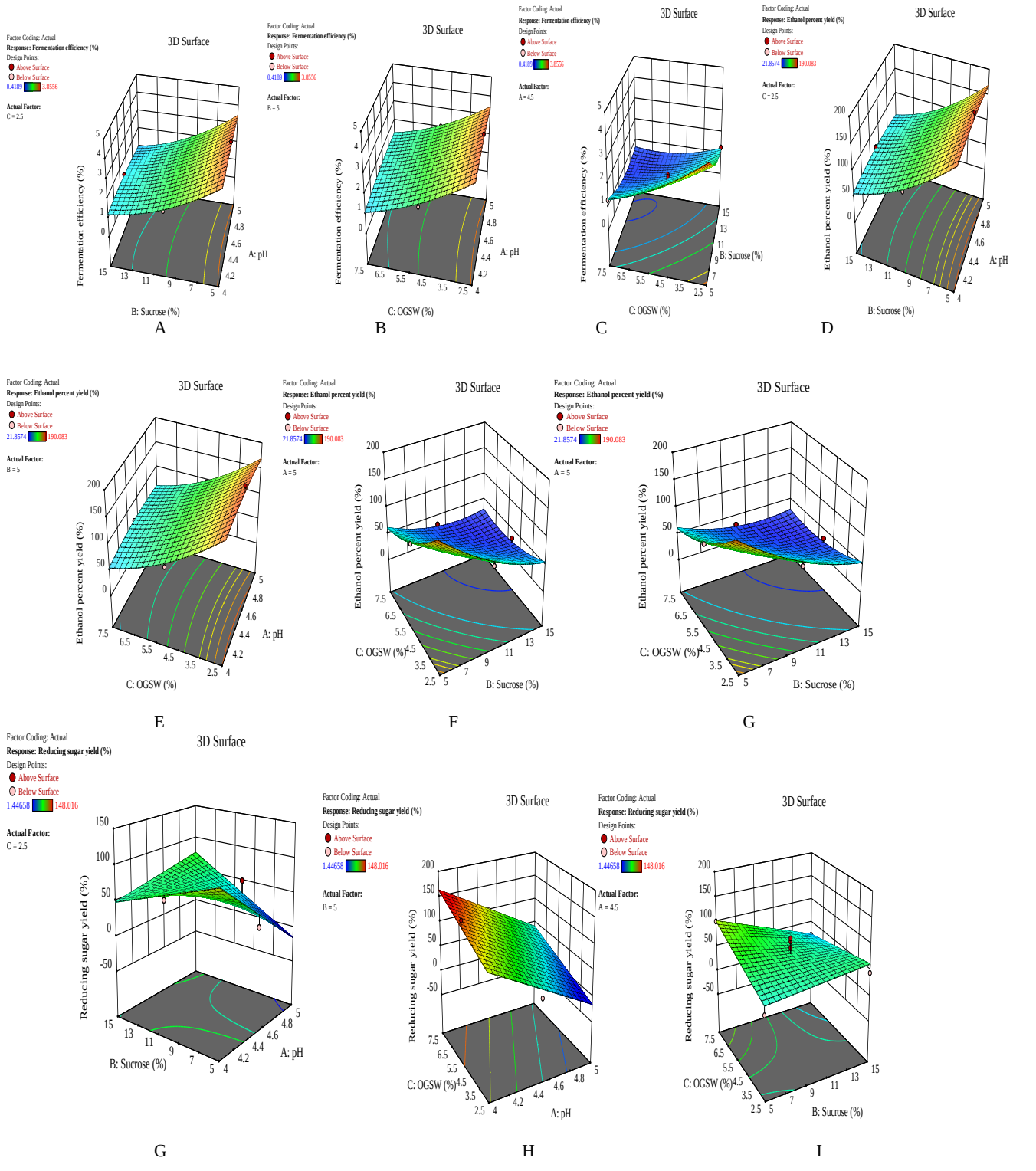

Figure 15: Response surface plot showing the effect of variables on fermentation efficiency(A-C), ethanol percent yield (D-F) and reducing sugar yield (G-I) at 72 hours. (Variables: OGSW, sucrose, pH)

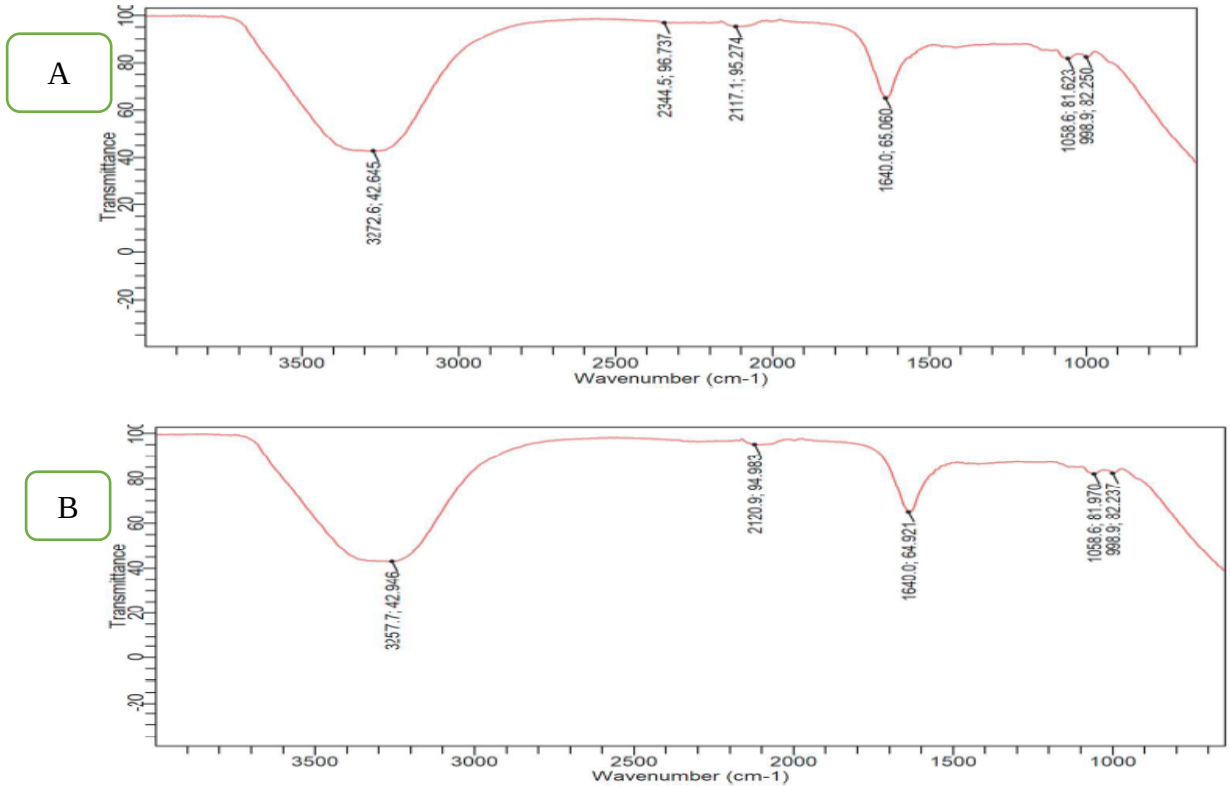

Figure 16: FTIR spectrum of A) OJSW-based fermented broth; B) OGSW based fermented broth

A Box-Behnken design was employed to identify and optimize key factors influencing bioethanol production. The response value for the fermentation efficiency, ethanol yield, and reducing sugar yield from 24 hours to 72 hours (Table S1-Table S3) and the confirmation that the model was able to predict the outcome are presented in supplementary material (Table S1.1-Table S2.1). The confirmation for 72 hours was not achievable because the factor value was outside the design space. At 24 hours, the maximum ethanol yield and the fermentation efficiency achieved were at 2.5% OJSW concentration, 10% sucrose concentration and pH 4. From 48 hours to 72 hours, maximum ethanol yield and fermentation efficiency were achieved at factor levels of 2.5% OJSW concentration, 5% sucrose concentration and pH 4.5. The saccharification decreases from 88.5% at 24 hours to 68.76% at 72 hours. The statistical validation of the model and the suitability of the model to navigate the design was based on the significant model p-value, an insignificant lack of fit F-value, and a signal-to-noise ratio greater than 4. The model's coefficient of determination for fermentation efficiency and ethanol percent yield at 78.05% (24 hours), 96.87% (48 hours) and 94.99% (72 hours) indicate strong agreement between the observed responses and the predicted outcomes. The surface response plot (Figure 10 - Figure 12) depicts the interaction effect of pH, sucrose, and treated OGSW on fermentation efficiency, ethanol per cent yield and reducing sugar yield.

The equations below describe the interaction between the variables and the responses at 24 hours:

Final equation in term of actual factors at 24 hours: Fermentation efficiency =2.5445-0.1518 *pH-0.0562* Sucrose-0.1271* OJSW, [p-value: < 0.0001, F-value: 15.41, R^2:0.7805, Adjusted R^2:0.7298, Predicted R^2:0.6339, Adeq Precision: 13.7711] Final equation in term of actual factors at 24 hours: Ethanol percent yield=77.8837-4.6475* pH-1.7212* Sucrose-3.8894* OJSW [p-value:< 0.0001, F-value: 15.41, R^2:0.7805, Adjusted R^2:0.7298, Predicted R^2:0.6339, Adeq Precision: 13.7710] Final equation in term of actual factors at 24 hours: Reducing sugar yield=267.9778-10.9102*pH-29.3214 *Sucrose-6.4437* OJSW+2.0191* pH * Sucrose-2.2360* pH * OJSW+1.0732*Sucrose * OJSW-0.5960 *pH^2+0.4771* $Sucrose^2$+0.2985 *OJSW [p-value: 0.0004, F-value: 18.40, R^2:0.9594, Adjusted R^2:0.9073, Predicted R^2: 0.5938, Adeq Precision: 15.3657]

The equations below describe the interaction between the variables and the responses at 48 hours:

Final equation in term of actual factors at 48 hours: Fermentation efficiency =7.8986+1.9579* pH-0.9063* Sucrose-1.7925*OJSW+0.0288*pH * Sucrose+0.0217* pH * OJSW+0.0528* Sucrose * OJSW-0.2796* pH^2+0.0168* $Sucrose^2$+0.0774* $OJSW^2$ [p-value: < 0.0002, F-value: 24.04, R^2:0.9687, Adjusted R^2:0.9284, Predicted R^2:0.6015, Adeq Precision: 18.5279] Final equation in term of actual factors at 48 hours: Ethanol percent yield=241.729+59.9437*pH - 27.7400*Sucrose - 54.8656 OJSW+0.8827*pH * Sucrose+0.6632 *pH * OJSW+1.6154 *Sucrose * OJSW-8.5586* pH^2+0.5148 *$Sucrose^2$+2.3695* $OJSW^2$ [p-value:< 0.0002, F-value: 24.04, R^2:0.9687, Adjusted R^2:0.9284, Predicted R^2:0.6014, Adeq Precision: 18.5262] Final equation in term of actual factors at 48 hours: Reducing sugar yield=121.8542-8.2172* pH-3.9395* Sucrose-0.5936* OJSW [p-value: 0.0147, F-value: 5.14, R^2:0.5423, Adjusted R^2:0.4367, Predicted R^2: 0.0991, Adeq Precision: 6.7510]

The equations below describe the interaction between the variables and the responses at 72 hours:

Final equation in term of actual factors at 72 hours: Fermentation efficiency =-8.7458+8.4092* pH-0.7663*Sucrose-1.4331*OJSW-0.054900*pH * Sucrose+0.0149* pH * OJSW+0.0582* Sucrose * OJSW-0.8684 *pH^2+0.0262*$Sucrose^2$+0.0444* $OJSW^2$ [p-value: 0.0009, F-value: 14.74, R^2:0.9499, Adjusted R^2:0.8854, Predicted R^2:0.2729, Adeq Precision: 15.3680] Final equation in term of actual factors at 72 hours:

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Ethanol percent yield=-267.7155+257.4026* pH-23.4565* Sucrose-43.8646* OJSW-1.6803*pH * Sucrose+0.4522* pH * OJSW+1.7812* Sucrose * OJSW-26.5805* pH^2+0.8013* $Sucrose^2$+1.3606* $OJSW^2$

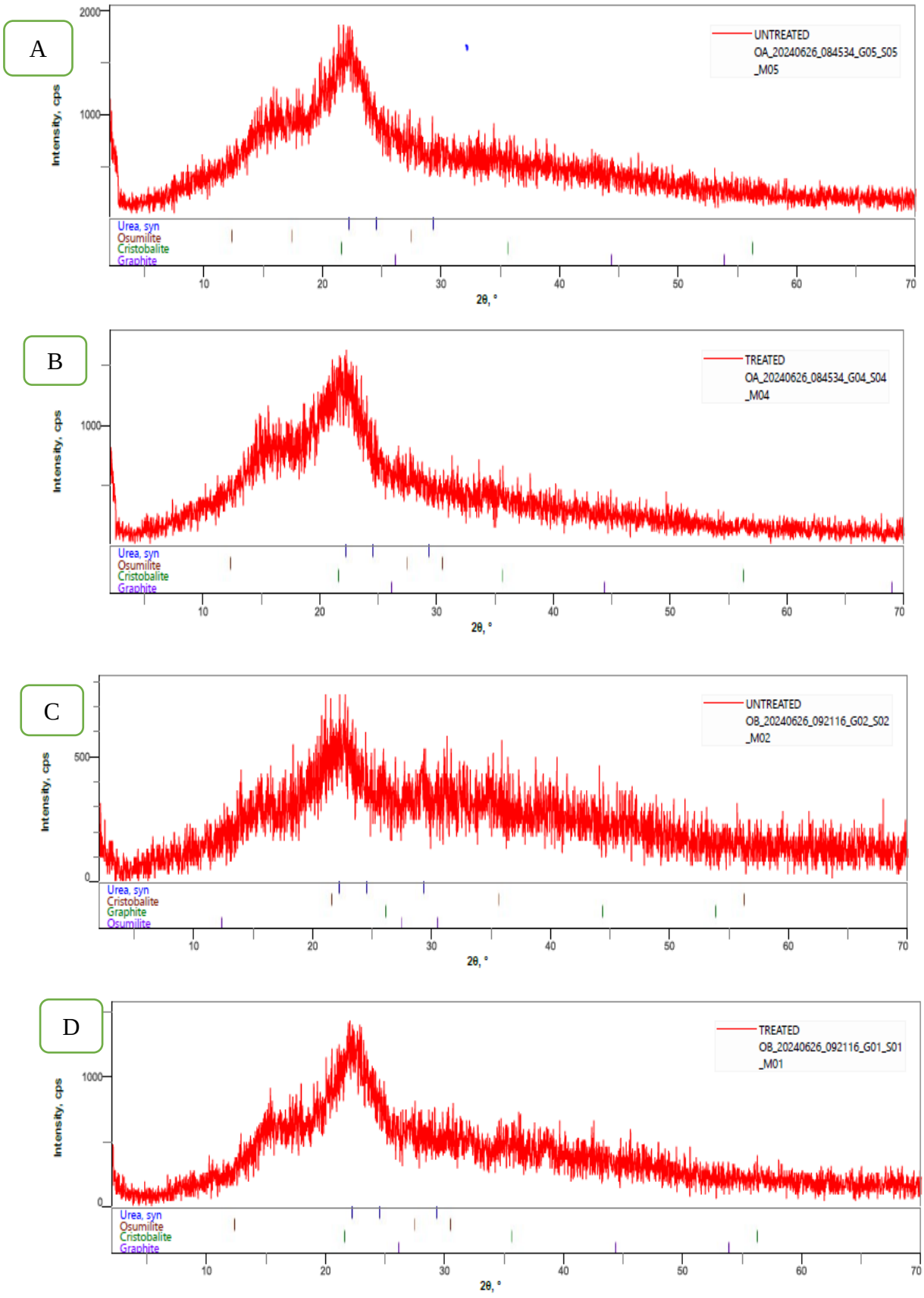

Figure 17: XRD pattern of (A): untreated OJSW (OA); B): treated OJSW (OA); C): untreated OGSW (OB); D): treated OGSW (OB))

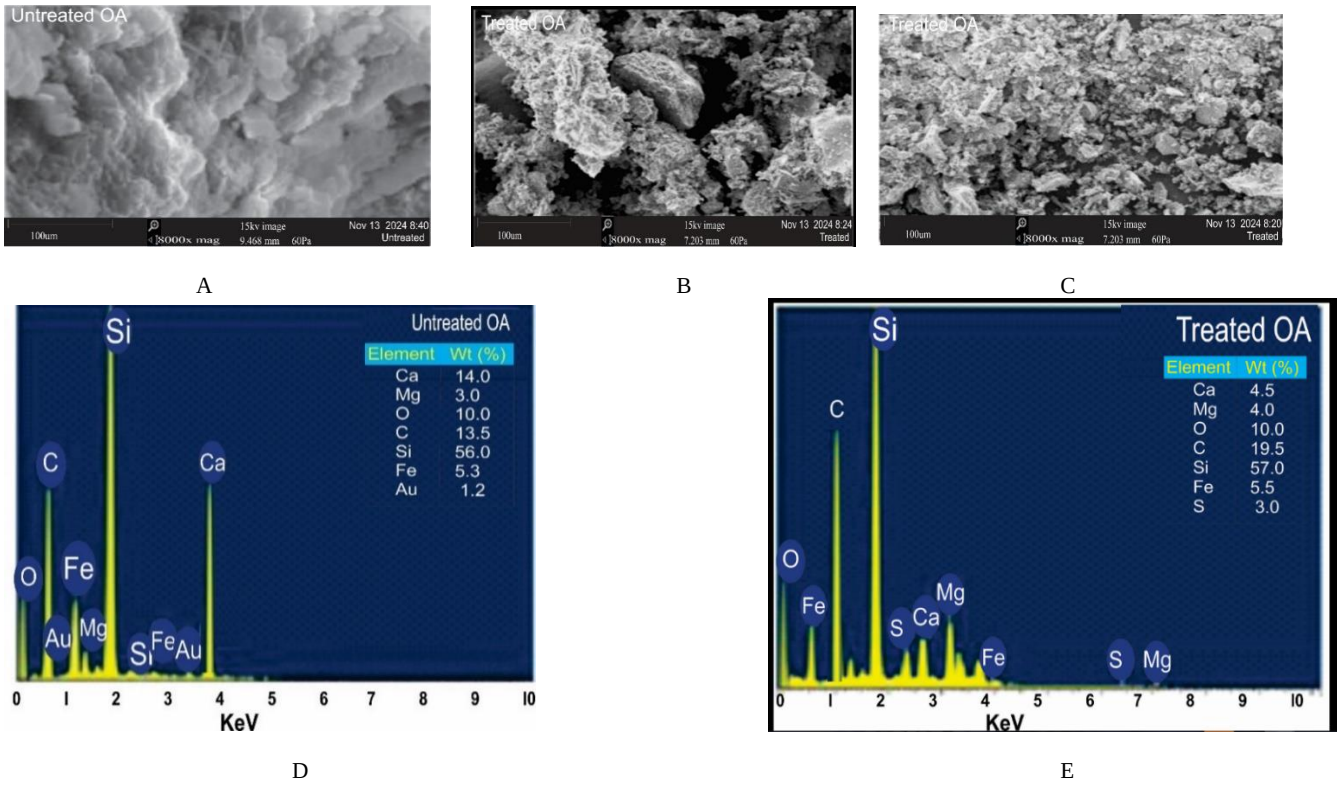

Figure 18: SEM/EDX images of A) untreated OJSW (OA); B) treated OJSW (OA); C) treated OJSW (OA) 100µM; D) EDX of the untreated treated OJSW (OA); E) EDX of the treated OJSW(OA).

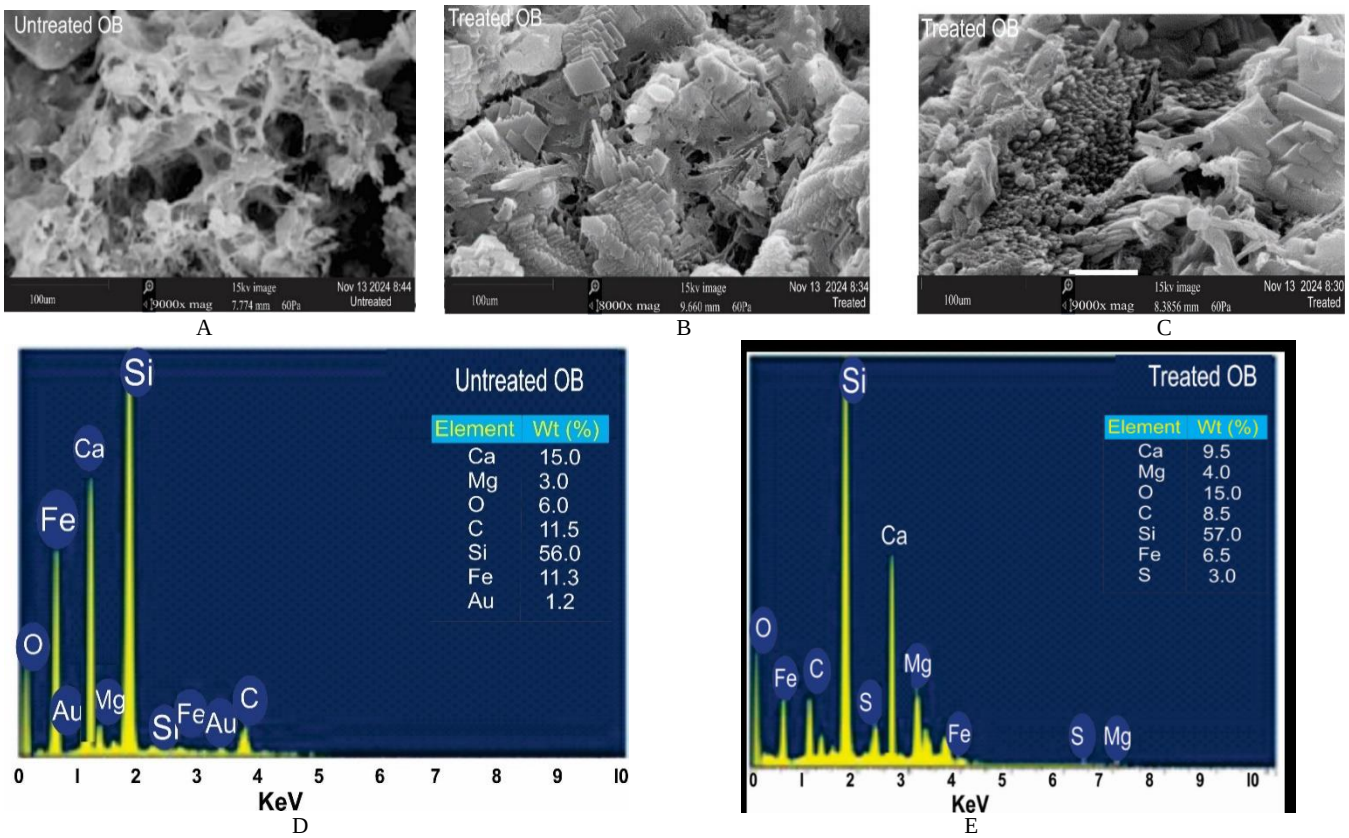

Figure 19: SEM/EDX images of A) untreated OGSW (OB); B) treated OGSW (OB); C) treated OGSW (OB) 100µM; D) EDX of the untreated treated OGSW (OB); E) EDX of the treated OGSW(OB

Table 1: Media composition for cellulase production

Medium code	Media composition	Medium code	Media composition
Medium A	Rice bran, 1g Cellulose powder, 1g Yeast extract, 0.1g Cobalt chloride 0.0185g	Medium C	Rice bran, 2g Cellulose powder, 1g Mycological peptone, 0.1g
Medium B	Rice bran, 1g Cellulose powder 0,5g Mycological peptone, 0.1g Yeast extract 0.1g Cobalt chloride 0.0185g	Medium D	Rice bran, 2g Cellulose powder, 1g Yeast extract, 0.2g Cobalt chloride 0.0236g

Table 2: Summary of the Box Behnken Design for pH, sucrose, OSW extract

Factor	Name	Units	Minimum	Maximum	Coded Low	Coded High
A	pH		4.00	5.00	-1 ↔ 4.00	+1 ↔ 5.00
B	SUCROSE	%	5.00	15.00	-1 ↔ 5.00	+1 ↔ 15.00
C	OSW extract	%	2.50	7.50	-1 ↔ 2.50	+1 ↔ 7.50

OSW: Oven-dried stick wastes

Table 3: Fiber detergent analysis

Components	OJSW	OGSW
NDF%	52.2 ± 0.028	48.72 ± 0.057
ADF%	36.695 ± 0.007	34.76 ± 0.085
ADL%	12.3 ± 0.085	10.33 ± 0.071
Cellulose	21.385 ± 0.049	22.95 ± 0.071
Hemicellulose	15.505 ± 0.035	13.96 ± 0.028

Mean ± Standard deviation; neutral detergent fiber: NDF; acid detergent fiber: ADF; acid detergent lignin: ADL

[p-value:0.0009, F-value: 14.74, R^2:0.9499, Adjusted R^2:0.8854, Predicted R^2:0.2729, Adeq Precision: 15.3677]. Final equation in term of actual factors at 72 hours: Reducing sugar yield=73.1649+4.9101* pH-5.3880 *Sucrose+0.4022* OJSW
[p-value: 0.0010, F-value: 10.20, R^2:0.7017, Adjusted R^2:0.6329, Predicted R^2: 0.5161, Adeq Precision: 8.7543]

Optimization of pH, sucrose, and treated OGSW using Box Behnken design from 24 hours to 48 hours

Maximum ethanol yield (quadratic model) with the fermentation efficiency (quadratic model) and reducing sugar yield (linear model) were achieved at factor levels of 2.5% OJSW concentration, 5% sucrose concentration and pH 4.5, as shown in the supplementary material at 24 hours (Table S4-Table S6). The analysis of the model for ethanol yield and fermentation efficiency reveals a significant p-value, an insignificant lack of fit F-value, and a signal-to-noise ratio greater than 4. These findings indicate an adequate signal that allows this model to navigate the design space. Unfortunately, at 24 hours, analysis of reducing sugar yield (quadratic model) reveals the insignificant model p-value (p=0.2332) with a value of greater than 0.05, a negative predicted R^2 (-0.3788), and the ratio of the signal to noise, which is less than 4 (3.9975). This, in turn, makes the model unsuitable. The surface response plot depicts the interaction effect of pH, sucrose, and treated OGSW on fermentation efficiency, ethanol percent yield and reducing sugar yield (Figure 13-Figure 15). The model's coefficient of determination for fermentation efficiency and ethanol percent yield at 24 hours (97.7%), 48 hours (96.87%) and 72 hours (97.08%) indicate strong agreement between the observed responses and the predicted outcomes.

The equations below model the interaction between the variables and the responses at 24 hours:

Final equation in term of actual factors at 24 hours:
Fermentation efficiency=2.0419-0.0433* pH-0.0251* Sucrose-0.1669* OGSW [p-value: 0.0286, F-value: 4.16, R^2:0.4896, Adjusted R^2:0.1104, Predicted R^2:0.2729, Adeq Precision: 6.4033] Final equation in term of actual factors at 24 hours:
Ethanol percent yield=105.1082-2.2259* pH-1.2907* Sucrose-8.5930* OGSW [p-value:0.0286, F-value: 4.16, R^2:0.4896, Adjusted R^2:0.1105, Predicted R^2:0.2729, Adeq Precision: 6.4036]

The equations below model the interaction between the variables and the responses at 48 hours:

Final equation in term of actual factors at 48 hours:
Fermentationefficiency=-2.75585+5.00842*pH-0.592406*Sucrose-1.29011 OGSW+0.00552*0 pH * Sucrose+0.041560* pH * OGSW+0.031606 *Sucrose * OGSW-0.580030* pH^2+0.013239 *$Sucrose^2$+0.050659* $OGSW^2$
[p-value: < 0.0001, F-value: 32.99, R^2:0.9770, Adjusted R^2:0.9474, Predicted R^2:0.6891, Adeq Precision: 22.1256], Final equation in term of actual factors 48 hours:
Ethanol percent yield=-141.86812+257.82156* pH-30.49676 *Sucrose-66.40984 *OGSW+0.284398 *pH * Sucrose+2.13912* pH * OGSW+1.62689 *Sucrose * OGSW-29.85860 *pH^2+0.681531 *$Sucrose^2$+2.60783 *$OGSW^2$
[p-value: < 0.0001, F-value: 33.00, R^2:0.9770, Adjusted R^2:0.9474, Predicted R^2: 0.6892, Adeq Precision: 22.1267] , Final equation in term of actual factors 48 hours:
Reducing sugar yield = 857.78546-194.1767*pH -60.3477*Sucrose + 22.4447*OGSW +14.8652* pH* Sucrose -0.3156* pH *OGSW -1.9192* Sucrose *OGSW
[p-value: 0.0073, F-value: 5.88, R^2:0.7792, Adjusted R^2:0.6467, Predicted R^2: 0.2810, Adeq Precision: 9.4029]

The equations below model the interaction between the variables and the responses at 72 hours:

Final equation in term of actual factors at 72hours:
Fermentation efficiency =-1.4390+4.4410*pH-0.5570 Sucrose-1.3066 OGSW-0.0217 pH * Sucrose+0.0479 pH * OGSW+0.0340*Sucrose * OGSW-0.4967* H^2+0.0166*$Sucrose^2$+0.0466*$OGSW^2$
[p-value: 0.0001, F-value: 25.82, R^2:0.9708, Adjusted R^2:0.9332, Predicted R^2:0.6119, Adeq Precision: 19.528], Final equation in term of actual factors at 72 hours:
Ethanol percent yield==-74.0700+228.6018* pH-28.6697* Sucrose-67.2594* OGSW-1.1168* pH * Sucrose+2.4679* pH * OGSW+1.7495* Sucrose * OGSW-25.5656 *pH^2+0.8521 *$Sucrose^2$+2.3985 *$OGSW^2$
[p-value: 0.0001, F-value: 25.82, R^2:0.9708, Adjusted R^2:0.9332, Predicted R^2:0.689, Adeq Precision: 19.527], Final equation in term of actual factors at 72 hours:
Reducing sugar yield=857.785-194.1767*pH-60.3477*Sucrose +22.4447*OGSW + 14.8652*pH*Sucrose -0.3156*pH *OGSW -1.9192*Sucrose *OGSW
[p-value: 0.0073, F-value: 5.88, R^2:0.7792, Adjusted R^2:0.6467, Predicted R^2: 0.2810, Adeq Precision: 9.4029]
From 24 hours to 72 hours, the ethanol percent yield significantly rose from 89.62% to 198.47% for OGSW treated with glucose oxidase-cellulase with an increase in fermentation efficiency (1.74% to 3.86%). At the same factor concentration level with OGSW treated with glucose oxidase-cellulase, OJSW treated with glucose oxidase-cellulase rose from 36.63% to 157.2% with higher fermentation efficiency (1.20 % to 5.14%). Reducing sugar concentration for OJSW decreased from 30.12 mg/ml to 23.4 mg/ml, and OGSW (5.466mg/ml) increased by 1.12 fold at 72 hours.

In the presence of sucrose only, OGSW increased by 5.04-fold at 72 hours compared to its maximum yield in its absence at 48 hours (0.44%). OJSW increased by 0.05-fold at 48 hours, compared to its maximum yield in its absence. Hence, optimization using Box Behnken significantly increased the yield of ethanol from 3.1% to 157% for treated OJSW and 6.12% to 198.47% for treated OGSW.

ATR-FTIR spectra analysis for fermented broth

Figure 15A and Figure 15B show the FTIR spectra of OJSW-based broth containing bioethanol and OGSW-based broth containing bioethanol. The characteristic broadband centered at 3272.6 cm^{-1} (Figure 16A) and 3257.7 cm^{-1} (Figure 16B) with very low transmittance intensity, and the bands attributed to C-O stretching (900 cm^{-1}-1200 cm^{-1}) were identified. This study agrees with FTIR results from other studies where the evidence of ethanol is based on the presence of the strong broadband (3200 cm^{-1} and 3500 cm^{-1}) associated with hydroxyl groups, C–O stretch vibration attributed to C-O bonds and C–C stretch vibration which reflect the presence of ethanol or the backbone of residual sugars.[39,40] The bands around the wavenumber 1640 cm^{-1} and between the wavenumber 2000 cm^{-1}-2200 cm^{-1} have been attributed to the bending mode of liquid water, which tends to shift towards a higher wave number as a consequence of the formation of hydrogen bonds.[41] Based on the interpretation, ethanol was evident in the broth samples containing both oven-dried stick extracts.

X-ray diffraction analysis

The insolubility of cellulose is linked to its arrangement. The absence of sharpness of these peaks reflects the amorphous region of cellulose in biomass. As XRD data revealed an amorphous nature of the agro wastes, untreated OJSW (Figure 17A) revealed two diffraction peaks at 15.20º and 22.04º, with the second peak displaying higher intensity in counts per second. Two diffraction peaks were also shown for untreated OGSW (Figure 15C) at 15.7º and 22.3º, with the second peak displaying higher intensity in counts per second. Treated OJSW and treated OGSW (Figure 17B and Figure 17D) displayed 22.56º and 22.24º, with the treated OJSW displaying the highest peak intensity with the lowest size 9.10(Å). As the crystallinity of agro wastes depends on the amount of cellulose, the type of cellulose and hemicellulose contents, the low cellulose content revealed by fiber detergent analysis with amorphous nature shown by XRD pattern indicates an overall low crystallinity of OJSW and OGSW.

Scanning electron microscopy (SEM) analysis

The morphological changes in the untreated and pre-treated OJSW (Figure 18) and OGSW (Figure 19) were visualized by scanning electron microscope combined with the energy dispersive x-ray (EDX). Comparison of the morphological structure of the untreated and treated samples of OJSW/ OGSW by scanning electron microscopy reveals evidence of catalysis. EDX shows an increase in carbon components in both treated samples, which is indicative of the enzymatic cleaving of the embedded polysaccharides to release more monomeric sugars. Compared to treated OJSW, the carbon content was lower in treated OGSW.

Conclusion

The fermentation outcome of Isolate palmZ-6 in the production of bioethanol implies the combination of cellulase and the glucose oxidase increased the reducing sugar yield when oven-dried jute stick and oven-dried green stick extracts were used as co-substrate with sucrose. With the evidence of the characteristic bands associated with the presence of ethanol in the fermentation broth, this study, therefore, has introduced the possibility of including glucose oxidase at low concentrations in the presence of cellulase to support the release of reducing sugars required for the production of lignocellulose-based bioethanol. Nevertheless, studies are required to fully demystify the biochemical mechanism of interaction between glucose oxidase and cellulase in the release of reducing sugars from lignocellulose wastes and exploit it to increase the scale of production. The success of this study lies in the availability and catalytic efficiency of required enzymes. Unfortunately, the absence of key enzyme players in most developing nations, such as Nigeria, implies that the need for enzymes is mostly met by importation. The prices of glucose oxidase and cellulase, among other enzymes, are still going on an upward trend. Investing in these enzymes will not only bridge the price and make enzyme-based analysis cheaper but will make these catalytic tools readily available for R&D required for transitioning from fossil fuel-based technology to a greener economy. Nevertheless, the role of implementing appropriate policies that encourage funding of green technology research and capacity building in Sub-Saharan Africa cannot be overemphasized in the drive toward a green future.

Conflicts of interest

The authors declare no conflicts of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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List of abbreviations

AOAC:	Association of Official Agricultural Chemists
ADF:	Acid detergent fiber
ADL:	Acid detergent lignin
ADS:	Acid detergent solution
NDS:	Neutral detergent solution
NDF	Neutral detergent fiber ADF
ANOVA:	Analysis of Variance
ATR-FTIR	Attenuated total reflectance- Fourier Transform Infrared Spectroscopy
BBD:	Box-Behnken design
CCD:	Central Composite Design
DNS:	Dinitro salicylic acid reagent
H_2O_2:	Hydrogen peroxide
HCL:	Hydrochloric acid
GMC:	Glucose methanol choline
NaOH:	Sodium hydroxide
OJSW:	Oven-dried jute stick waste
OGSW:	Oven-dried green stick waste
PDA:	Potato dextrose agar
SDG:	Sustainable development goals
SEM/EDX:	Scanning electron microscopy/energy dispersive x-ray
XRD:	X-ray diffraction

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