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Saccharomyces cerevisiae Strain – Growth Kinetics, Extracellular Enzymes and Production of Fermentable Sugars from a Range of Lignocellulose Residues

Egong J. Egong, Anene N. Moneke, Onyetugo C. Amadi*, Tochukwu N. Nwagu, Chukwudi I. Nnamchi, Idiunnu P. Awodirin, Reginald C. Agu, Bartholomew N. Okolo

Department of Microbiology, Faculty of Biological Sciences, University of Nigeria Nsukka, Nigeria

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ABSTRACT

Lignocellulosic materials are abundant in Nigeria and can be exploited for the production of industrially important enzymes by microorganisms. In this study, capacity of isolated *Saccharomyces cerevisiae* in producing crude enzymes and hydrolysis of selected renewable substrates – corn husks (CH) and Opete bagasse (OB) for sugar release was investigated. Fourier Transform Infrared Spectrophotometer (FTIR) was used to study the changes in the structures of the lignocellulosic substrates (CH and OB). High-Performance Liquid Chromatography (HPLC) was employed to study the effect of pretreatment on sugar release from CH and OB substrates. *S. cerevisiae* gave the highest cellulase (18.61 ± 2.35 U/gds), xylanase (54.79 ± 3.58 U/gds) and ligninase (110.79 ± 6.19 U/gds) activities after 6-day fermentation (SSF) at 28 ± 2^{o}C. Maximum glucose production (44.83 ± 1.24 mg/gds) and (49.73 ± 1.88 mg/gds) respectively from pretreatment of opete bagasse (ENZTOB) and corn husk (ENZTCH) were obtained. Maximum glucose of 35.90 ± 1.24 mg/gds and 37.87 ± 2.49 mg/gds were obtained from untreated opete bagasse (ENZOB), and untreated corn husk (ENZCH), respectively. Inoculum/cell suspension released lower glucose levels – 28.887 ± 2.03 mg/gds and 38.362 ± 1.36 mg/gds for opete bagasse (IOB) and for corn husk (ICH) respectively. The xylose sugars obtained from pre-treated opete bagasse (ENZTOB), and corn husk (ENZTCH) were 55.91 ± 3.02 mg/gds and 42.38 ± 2.44 mg/gds, respectively and are higher than 24.364 ± 1.75 mg/gds and 29.349 ± 1.74 mg/gds obtained for untreated IOB and ICH, respectively. The yeast strain may find application in consolidated bioprocessing of cellulosic mass.

***Keyword*:** Multienzymes, *Saccharomyces cerevisiae*, Waste biomass, Growth kinetics, Fermentable sugars.

Introduction

The *Saccharomyces cerevisiae* is a well-established microorganism widely used in ethanol production from sugar feedstock. They have been efficiently used in the expression of different enzymes in the bioconversion of lignocellulose biomass for bioethanol production.[1-3] However, a more recent addition to their potential is the ability to produce various enzymes.[4-6] using lignocellulosic biomass, since most agro- wastes can be utilized as substrates for enzyme production.[7,8] Earlier Shariq *et al*.,[4] reported a promising strain MK157 identified as *S. cerevisiae* in the production of endoglucanase. Whilst Qadir *et al*.,[5] reported co-culture of two yeast strains (*Saccharomyces cerevisiae* and *Candida tropicalis*) on sugarcane bagasse by SSF in the production of multienzyme preparation comprising of endoglucanase (EG), β-glucosidase (BGL), and xylanase (XYL). Similarly, Amadi *et al*.,[6] reported simultaneous production of cellulase xylanase and ligninase by an *S. cerevisiae* strain. Non-conventional yeast strains have mostly been reported for the production of lignocellulose enzymes. The prospect of having yeast strains to produce lignocellulose enzymes offers some advantages. They have a shorter generation time when compared to moulds that are commonly used. Moreover, their growth will not pose any rheological challenges during fermentation when considered for industrial application. Again, in the conversion of lignocellulose to ethanol yeast can act as sources of cellulase and xylanases as cellulose and D-xylose fermenting agents. They can conventionally be co-cultured as ethanologenic yeast by substantially reducing the cost of biofuel production. Lignocellulose biomass (cellulose and hemicelluloses) is an abundant and cheap source of fermentable carbohydrates raw materials for the fermentation industries. The study on lignocellulose biomass materials is ever-increasing and their abundance as domestic, agro-industrial, and forestry wastes is an added advantage.[8,9] Effective conversion of such wastes materials to useful products would require some pre-treatment procedures. These pre-treatments could take the form of physicochemical or biological methods. Biological methods of pre-treatment are mainly based on the application of microbial enzymes, cellulase, and xylanases. The hydrolytic enzymes (cellulase, xylanase) are important enzymes in industrial biotechnological applications for the conversion of bio-waste materials into useful products. The cellulases catalyse the degradation of cellulose by the actions of various component enzymes acting synergistically to degrade cellulosic substrates.[10,11] On the other hand, xylanases (EC 3.2.1.8) are a class of hydrolytic enzymes that can hydrolyse the polysaccharide 1,4-xylan found in hemicelluloses.[12] The enzymatic conversion of lignocellulosic material to sugars can provide a carbon source for the production of energy (fuels) and a wide range of renewable products. In the present study, the robustness of *Saccharomyces cerevisiae* in producing crude enzymes and hydrolysis capacity of selected renewable substrates in either pre-treated or untreated form for sugar release was studied. Cornhusk (CH) and '*Opete'* bagasse (OB) were the substrates used to study the

*Corresponding author. E mail: chioma.amadi@unn.edu.ng
Tel: +234813419069

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effectiveness of the multienzyme from *Saccharomyces cerevisiae* in hydrolysing these substrates to fermentable sugars and useful products. The renewable lignocellulosic raw materials are abundant in Nigeria [6,13-17] and the *Saccharomyces cerevisiae* used in this study are of local origin.

Materials and Methods

Microorganisms
The *S. cerevisae* strain studied was from the collection centre – Microbiology Department, University of Nigeria. The *S. cerevisae* strain was previously reported for the production of cellulose, xylanase and ligninase. [6] The culture was maintained on Yeast extract, Peptone, Dextrose, Agar (YPDA).

Material
The corncob was obtained from local farmers in Nsukka, Enugu State, Nigeria. Corncob (CB) was chopped into small pieces and washed with a huge amount of tap water to eliminate contaminants. After solar drying for approximately 3 h, it was oven-dried at 50℃ to constant weight. The sample was ground and sieved to obtain a particle size of 350 µm.

Solid-State fermentation (SSF) and enzyme extraction
Solid-state fermentation (SSF) for producing the enzyme complex, namely – cellulose, xylanase and ligninase enzymes was carried out as reported previously.[6,18] Extraction of crude enzyme was performed as described in previous study by Amadi *et al*.[6] whilst cellulase and xylanase activities were carried out by 3,5-dinitrosalicylic acid (DNS) method (Miller).[19] Ligninase activity was carried out using the method described by Tien and Kirk.[20]

Enzyme assay
Glucose release from the activities of cellulase and xylanase enzymes were measured using the substrates carboxymethyl cellulose (for cellulase enzyme) and xylan (for xylanase enzyme)[6,21] Ligninase peroxide (LiP) assay was as previously described.[6] One unit (U) of LiP activity was defined as the amount of enzyme which converted 1 µmole of veratryl alcohol to veratraldehyde under standard assay conditions.

Pretreatment of lignocellulosic residues
The corn husk and opete bagasse (*Costus afer*) were locally sourced and oven-dried at 50^{o}C to constant weight. Dried substrates were finely ground to pass through 710 µm screen size (to allow increased interaction of enzyme/substrate during processing). The ground samples were stored in airtight containers until further use. Exactly 3.5 g of the corn husk or opete bagasse was mixed with 15% aqueous ammonia (NH_4OH) solution and then autoclaved. The solid residue was collected, washed severally with distilled water. The solid residue was dried in an oven at 70^{o}C for 24 h before use.[22]

Enzymatic and cell suspension hydrolysis of lignocellulose substrate
The dried lignocellulose substrate corn husk (CH) and opete bagasse (OB) (1 g each) were weighed into conical flasks and sterilized at 121℃ and allowed to cool. Thereafter, 20 mL of the crude enzyme was added to the sterilized corn husk (CH) and opete bagasse (OB). This was done for both the treated and untreated substrates. A 20 mL of the inoculum (cell suspension) was also used to hydrolyze the substrates. The flasks were incubated at 25℃ for 8 days after which, the supernatants were collected and analyzed for the release of sugars. Hence the different hydrolysis treatment conditions analyzed for sugar release from selected LC biomass (CH and OB) were (i) incubation in the inoculum/cell suspension (ii) treatment with crude enzyme only (iii) incubation in a combination of ammonium pre-treated and crude enzyme (iv) and control which is substrates in sterile distilled water.

Reducing sugar estimation
The dinitrosalicylic acid (DNSA) method[19] was used to determine the concentration of glucose and xylose produced.

HPLC analysis of hydrolysed products
The hydrolysate obtained from the enzymatic hydrolysis of both untreated and pretreated lignocellulosic substrate (*opete* bagasse and corn husk) was subjected to sugar analysis using High-Performance Liquid Chromatography (HPLC). A mixture of the sample and distilled water at a ratio of 1:8 was used for the analysis. Sugar release was detected at a flow rate of 800 µL/min, using 10 mM H_2SO_4 as the mobile phase, at a maximum heating temperature 75^{o}C for 20 min. The standards used were glucose, fructose, and maltose at concentrations of 20 mg/mL.

FT-IR spectra of untreated and ammonia-treated corn cob
Changes in the treated and untreated corn husk substrates were assessed using the Fourier Transfer Infra-red spectrophotometer (Shimadzu FTIR spectrophotometer (Model IR Affinity-1) Analytical results for untreated and ammonia-treated substrates were recorded in the wavelength between 500 and 4000 cm^{-1} at room temperature (28 ± 2^{o}C). The analysis was done at the Energy Centre, University of Nigeria, Nsukka. Corn husk or opete bagasse was treated with ammonia to reduce lignin content in the substrates and also increase its accessibility toward enzyme production. Sample preparation for FTIR determination was by weighing 400 mg of potassium bromide KBr into a crucible and ground to powder. The disc was prepared by mixing 1.0 mg dried sample with the 400 mg KBr and then pressed using a mechanical pressure and moulded to form a tablet. The disc was inserted into the compartment segment of the Shimadzu FTIR spectrophotometer to generate the IR spectrum.

Results and Discussion

Table 1 shows the extracellular enzyme activity of *S. cerevisiae*. The organism expressed maximum enzyme activity at 144 h, with cellulase (18.61 ± 2.35), xylanase (54.79 ± 3.58) and ligninase (110.79 ± 6.19). Similar reports of enzyme production from the yeast *Saccharomyces cerevisiae* strains have been documented.[4-6]

Kinetic of cell growth, optimum growth conditions and enzymes production by S. cerevisae strain in SSF
Kinetic parameters of cell growth are shown in Table 2a. The enzyme kinetic study used was the Leudeking-piret model, which defines the logistic growth model and Monod model. Here, the specific growth rate (µ), relates to the velocity of biomass production at a particular time interval of biomass concentration, and was expressed as (h^{-1}).[23] *Saccharomyces* culture studied gave a maximum specific growth rate (µmax) of 0.459 h^{-1} during the exponential growth phase with a generation time of approximately 82.77 min under optimal batch growth conditions. Microorganisms possess diverse maximum specific growth rates. Results showed that the enzyme complex production increased with the specific growth rate, approaching the maximum values at the highest growth rates. Fermentation conditions are very relevant to maximize product yield, medium pH 6.0 (see Table 2b), played important role in the successful production of these enzymes as also observed in previous studies.[6,24-26] Regarding the relationship between the pattern of cell growth and enzyme complex (cellulase, xylanase and ligninase) production Figure 2c, enzyme purity is a key factor during enzymes extraction. This was determined by specific enzyme activity used to measure the enzyme kinetics. In this study, specific cellulase activity of 53.408 U/mg, xylanase activity of 182.019 U/mg and ligninase 225.728 U/mg were obtained. Based on these results, *S. cerevisae* produced purer ligninase per mg, resulting in a relatively high maximum ligninase coefficient of 148.116 U/mg glucose compared to xylanase coefficient of 43.813 U/mg glucose and cellulase coefficient of 14.653 U/mg glucose.

Profile of total reducing sugars release under varying hydrolysis treatment conditions
The profile of reducing sugars (glucose and xylose) production from various hydrolysis conditions of selected lignocellulosic materials (OB and CH) is described in Figures 1 for glucose production and Figure 2 for xylose production. It is obvious from the results in Figures 1 and 2, that treatment of the lignocellulose using ammonium hydroxide at the

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Table 1: Extracellular enzyme activity (cellulase, xylanase and ligninase) by *S. cerevisiae*

Incubation time (h)	cellulase activity (U/gds)	xylanase activity (U/gds)	ligninase activity (U/gds)	Total protein (mg/gds)
48	6.52 ± 1.19	22.88 ± 1.81	55.70 ± 5.21	15.94 ± 1.22
96	13.70 ± 3.25	36.54 ± 2.31	82.73 ± 4.33	25.55 ± 1.94
144	18.61 ± 2.35	54.79 ± 3.58	110.79 ± 6.19	39.01 ± 1.99
192	8.41 ± 0.79	40.73 ± 0.79	81.03 ± 1.90	25.15 ± 1.19

Data are presented as mean ± SD of three replicates.

lower concentration reported had a significant ($p < 0.05$) effect on the level of reducing sugars produced. Peak production of reducing sugar was obtained after four (4) days of incubation at 28 ± 2°C.

The highest glucose level (44.83 ± 1.24 mg/gds) obtained from pre-treated opete bagasse (ENZTOB) was much higher compared to 35.90 ± 1.24 mg/gds that obtained from the untreated opete bagasse (ENZOB). These results are shown in Figure 1a. Similarly, the highest glucose level was obtained from treated corn husk (ENZTCH) 49.73 ± 1.88 mg/gds compared to 37.87 ± 2.49 mg/gds obtained untreated corn husk (ENZCH) Figure 1b. Production of xylose followed a similar pattern (Figure 2a). Here, the highest xylose production (55.91 ± 3.02 mg/gds) obtained from pre-treated opete bagasse (ENZTOB) was much higher compared to 34.46 ± 1.23 mg/gds obtained from the untreated opete bagasse (ENZOB) Figure 2a. Again, highest xylose level obtained from treated corn husk (ENZTCH) was 42.38 ± 2.44 mg/gds compared to 27.25 ± 0.86 mg/gds obtained untreated corn husk (ENZCH) Figure 2b. High titres of sugar yields (glucose and xylose) from lignocellulose substrates using cocktail enzyme preparations have been reported.[27,28] However, the process of using cocktail enzyme preparations is likely to be more expensive when compared to our process where a multienzyme complex was used to achieve a similar goal. Again, our choice of using ammonium hydroxide rather than more expensive sodium hydroxide will further reduce costs. On the other hand treatment of the substrates using the inoculum/cell suspension revealed a glucose level of 28.887 ± 2.03 mg/gds for opete bagasse (IOB) and 38.362 ± 1.36 mg/gds for corn husk (ICH) by day four (4) of incubation Figure 1a and b. Whilst the release of 24.364 ± 1.75 mg/gds xylose was observed for opete bagasse (IOB) and 29.349 ± 1.74 mg/gds for corn husk (ICH) respectively by day three (3). It is worthy of note that all hydrolysis treatment conditions analysed released varying degrees of sugars.

HPLC analysis for the release of sugars

HPLC analysis results for the release of sugars from enzymatic degradation of the substrates (OB and CH) are shown in Table 3. The saccharification process and release of sugars was enhanced when alkaline pretreatment was employed. Pretreated OB substrates yielded higher sugar (1.735 g/L of glucose) compared to the untreated in the case of OB (1.386 g/L of glucose). This represents approximately 20% increase in glucose production. The mechanism that increased reducing sugars was not investigated. However, it has been reported that pretreatments of macro-substrates using dilute alkali or dilute acids results in modification of the substrates making it easier and better access for enzyme hydrolysis to occur.[29-31] Although, other pretreatments such as acid, physical and chemical pretreatments are available, alkali pretreatment is more effective for the production of different fermented products using various microbial cultures.[29,32-34] The choice of using ammonia solution, which was very effective as shown in the results, is likely to reduce cost compared to using sodium hydroxide which is more expensive.

Fourier Transform Infrared Spectroscopy (FT-IR) study of the treated and untreated opete bagasse and corn husk residues

Fourier transfer infrared (FT-IR) analysis is the analytical tool used to study the structural components as well as the chemical modifications that occurred during the transformation of lignocellulose pretreatment. Any transformation that occurs during the pretreatment process will reveal alterations in polymer inter-linkages within the structures of CH and OB. The results obtained from the (FT-IR) analyses are shown in Figure 3 for (CH) substrate and Figure 4 for (OB) and clearly, it can be seen that structural changes occurred when both substrates were pretreated compared to the untreated substrates. Pretreatment appeared to have more effect on the (OB) than on (CH) substrate (Figures 3 and 4). The bands at 3420 cm^{-1} represent the hydroxyl group stretching vibrational bands (both intra-molecular and inter-molecular) present in cellulose, hemicellulose and lignin. Hydroxyl group bond stretch was different for the pretreated (OB) and (CH) samples. Again, the bending vibration of C-H in cellulose and hemicellulose peak at 1790 cm^{-1} is different for the pretreated (OB) and (CH) substrates.

However, similar observations were found for the carboxylic peak stretching vibration of the aromatic ring at 1000 cm^{-1}.

Table 2 (a) Kinetic of cell growth (*S. cerevisiae*)

Cell growth parameter	Cell growth
Maximum cell concentration, X_{max} (CFU/gds)	2.52×10^{7}
Maximum specific growth rate μ_{max} (h^{-1})	0.459
Growth coefficient (cells/mg glucose)	2.0672×10^{7}
Generation time, Td (min)	82.766

(b) Optimum growth conditions during fermentation

Optimum conditions	Cell growth
Optimum medium pH	6.0
Fermentation time at the maximum cell concentration (days)	6.0
Moisture content (%)	80
Nitrogen concentration (g/L)	3.0
Particle size (µm)	350

(c) Enzymes production by *S. cerevisae* strain in solid state fermentation (SSF)

Enzyme production parameter	Enzyme		
	Cellulase	Xylanase	Ligninase
Fermentation time at the maximum enzyme activity (days)	6.0	6.0	6.0
Maximum enzyme production P_{max} (U/gds)	18.586	46.779	167.855
Specific enzyme activity (U/mg protein)	53.408	182.019	225.728
Enzyme coefficient (U/mg glucose)	14.653	43.813	148.116
Enzyme productivity (U/gds/h)	1112.372	10743.671	9903.613
Optimum medium pH	6.0	6.0	5.0

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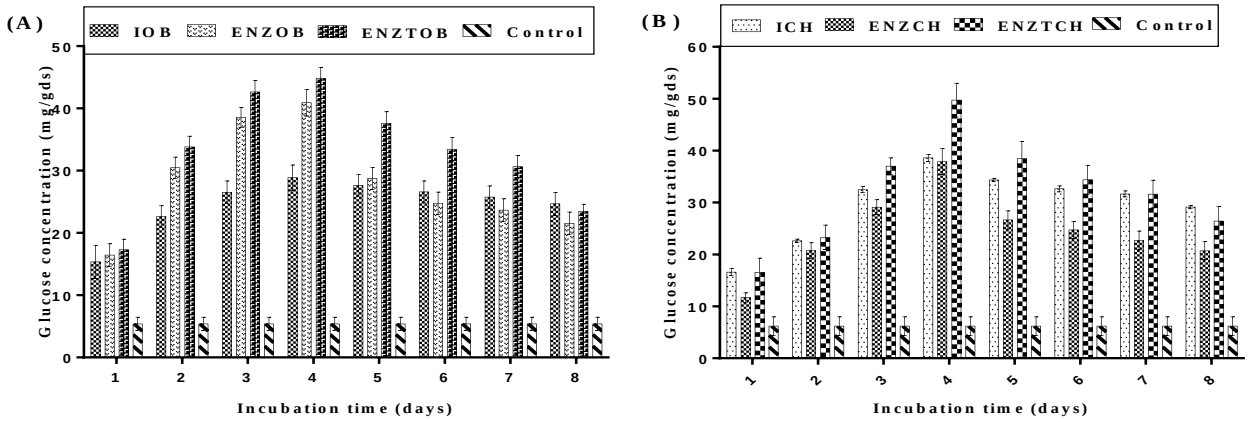

Figure 1: Glucose profile from saccharification of treated and non-treated (A) opete bagasse and (B) corn husk by crude enzyme (Enz) and culture of *Saccharomyces cerevisiae* SCPW 17 (I). Data are presented as mean ± SD of three replicates.
Inoculum incubated untreated opete bagasse (IOB); Enzyme incubated untreated opete bagasse (ENZOB); Enzyme plus ammonium pre-treated opete bagasse (ENTOB). Inoculum incubated untreated corn husk (ICH-); Enzyme incubated untreated corn husk (ENZCH); Enzyme plus ammonium pre-treated corn husk (ENTCH).

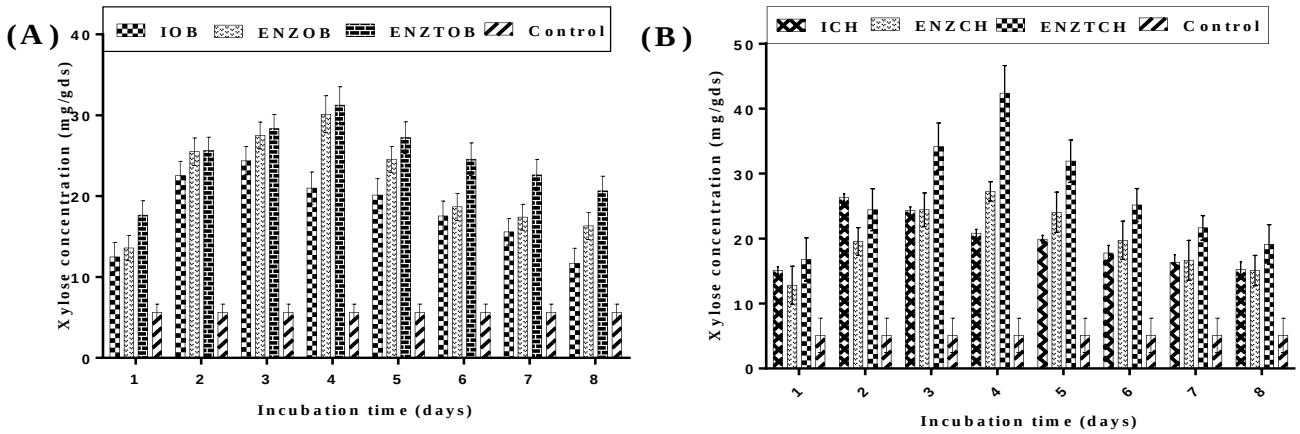

Figure 2: Xylose profile from saccharification of treated and non-treated (A) opete bagasse and (B) corn husk by crude enzyme (Enz) and culture of Saccharomyces cerevisiae SCPW 17 (I). Data are presented as mean ± SD of three replicates.
Inoculum incubated untreated opete bagasse (IOB); Enzyme incubated untreated opete bagasse (ENZOB); Enzyme treated opete bagasse (ENTOB). Inoculum incubated untreated corn husk (ICH); Enzyme incubated untreated corn husk (ENZCH); Enzyme treated corn husk (ENTCH).

Notwithstanding, these observations showed the existence of lignin.[30,35] The peaks at 2900 cm^{-1} represent the $–CH_2$ valence vibration from C_6 in cellulose. The same trend was observed for the C-H stretching vibrational bands (2900 cm^{-1}) and the C-O stretching vibrational bands (1050 cm^{-1}) in the corn husk. Also, the intensity of the bands at 950 cm^{-1} shows an increase in amorphous cellulose in the pretreated corn husk. From Figure 4, the presence of cellulose produced a characteristic peak at 1700 cm^{-1} for treated opete bagasse which is very obvious when compared with the untreated substrate. The spectra for both the ammonium hydroxide-treated and the untreated substrates were remarkably different in intensities due to changes in molecular structure in treated substrates (stretching and bending of bonds) which suggested that the microstructure of these substrates changed during alkali treatment. Therefore, the FTIR analysis showed the structural changes and delignification in the samples as a result of the ammonium hydroxide pretreatment, and it may therefore be inferred that the alkali treatment of the corn husk, as well as the opete bagasse, enabled further crude enzyme degradation (see also the results in Figures 1 and 2.

Table 3: Reducing sugars release after six days incubation analysis using High Performance Liquid Chromatography (HPLC).

	% Sugar concentration					
	Glucose		Fructose		Xylose	
Substrate	**CH**	**OB**	**CH**	**OB**	**CH**	**OB**
Inoculum	1.348	1.436	0.115	0.337	0.120	ND
Untreated	0.702	1.386	0.295	ND	0.113	0.132
Treated	1.409	1.735	0.305	0.376	0.149	0.157
Control	0.257	0.909	0.070	0.090	0.021	0.036

Untreated - untreated substrates incubated with only crude enzyme
Treated - ammonium treated substrates incubated with crude enzyme
Inoculum - substrate incubated with only cell suspension
Control - substrate incubated in distilled water
CH - corn husk OB - opete bagasse

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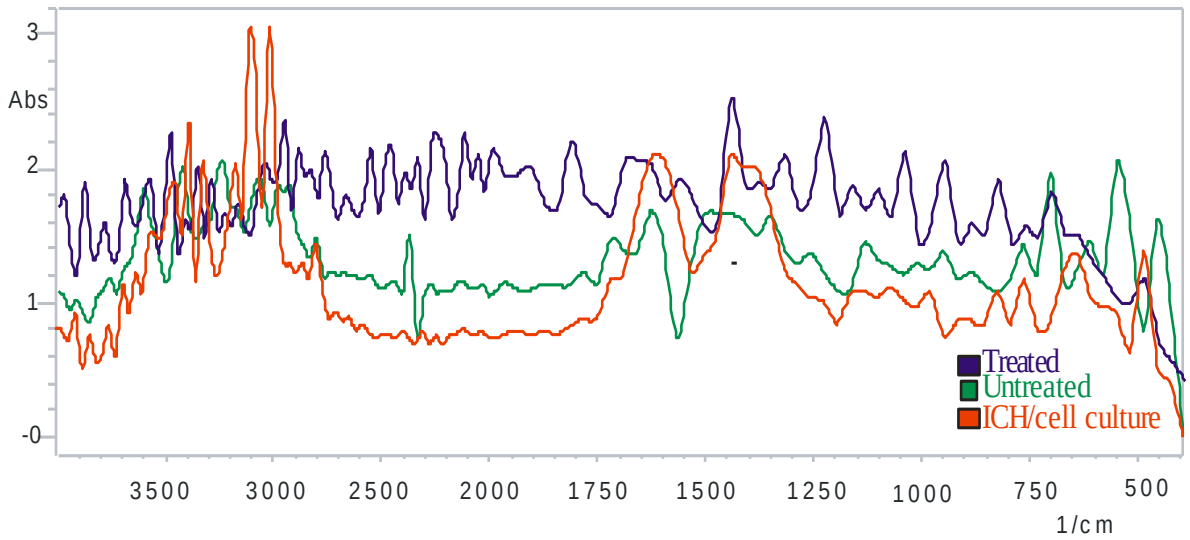

Figure 3: FT-IR spectrum for ammonium treated corn husk incubated with crude enzyme (ENZTCH, blue) untreated corn husk incubated with crude enzyme (ENZCH, green), untreated corn husk incubated with inoculum/cell suspension of *Saccharomyces cerevisiae* SCPW-17 (ICH, red).

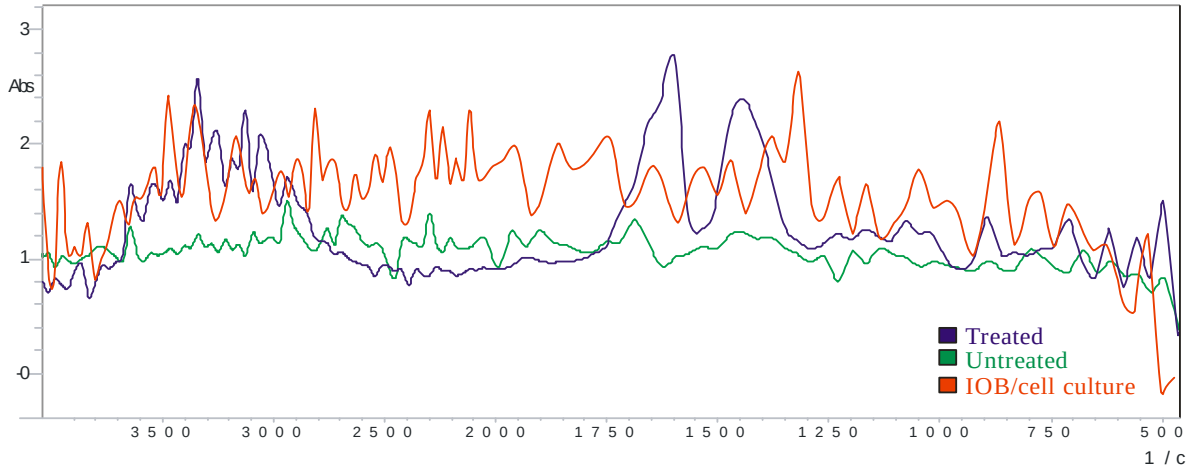

Figure 4: FT-IR spectrum for ammonium treated Opete bagasse incubated with crude enzyme (ENZTOB, blue), untreated Opete bagasse incubated with crude enzyme (ENZOB, green), and untreated Opete bagasse incubated with inoculum /cell suspension of *Saccharomyces cerevisiae* SCPW-17 (IOB, red).

Conclusion

The devastating impact of climate change on our environment has led researchers to intensify efforts on how to secure the environment. In this study, we described the kinetics and enzyme activity profile of *Saccharomyces cerevisiae* in solid-state fermentation using locally available renewable lignocellulosic raw materials corn husk and opete bagasse (CH and OB). The study also highlighted a natural (local) strain of *S. cerevisiae* that produced an enzyme complex system that can hydrolyse selected lignocellulose residues (OB) and (CH) which are cheap and abundant in Nigeria. The study further showed that a low-cost nitrogen source (Bambara meal) gave the best enzyme yield. Also, the use of cheaper ammonium hydroxide to study the influence of pretreatment on the saccharifying process, if required, will further reduce costs during industrial production. Use of these renewable substrates in bioethanol production will help in the reduction of CO_2 emission when used in commuting. The information provided from this study can also be extended to other locally available substrates such as sugar cane bagasse (SCB), corn cob (CC), "Ugu" pod (UP) and rice husk (RH) that are abundant in Nigeria, for which limited studies have been done.

Conflict of Interest

The authors declare no conflict of interest.

Authors' 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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