



Original Research

Fungal Enzyme Hydrolysis of Agricultural Biomass Wastes: Comparative Studies of Different Lignocellulosics

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Abstract

Environmental pollution from agricultural activities remains a challenge, especially in developing economies. Due to recalcitrance to hydrolysis, such byproducts tend to persist in the environment owing to their high lignocellulose content. Alternatively, eco-friendly hydrolytic approaches are imperative to mitigate environmental pollution. Besides, hydrolysis holds promise for reducing sugar production, which could serve as a starting material for bioethanol production towards a clean energy future and sustainable energy production. Some of the abundant agro-byproduct biowastes come from sugarcane, rice husks, and groundnut. To this end, five varieties of sugarcane, namely National Cereals Sugarcane (NCS)-, NCS-003, NCS-006, NCS-007, Coimbatore 957, and a local variety, besides rice husk and groundnut shells, were studied for ease of fungal cellulase degradation and hydrolysis for reducing sugar production, using isolated wild strains of *Aspergillus niger* and *Spadicoides* spp, using established methods. Extracellular enzyme production potential was assessed by measuring the protein secretion capacity of the fungal strains in broth culture. *Spadicoides* spp produced 1.23 mg/mL and 0.38 mg/mL of extracellular protein when grown on NCS-003 (W) and rice husk, respectively. *Aspergillus niger* grown on NCS-003 (W) and NCS-007 (UW) secreted extracellular proteins of 0.98 mg/mL and 0.50 mg/mL, respectively. Co 957 and Local showed an almost linear relationship between activity and substrate concentration. Reducing sugar production was highest in Local (192.22 activity units) and least in NCS-003 (78.33 units). Therefore, Local was adjudged the SCB with the highest potential for fungal cellulase-mediated biodegradation to reduce sugar production.

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Statement of Sustainability: Anthropogenic activities such as production and consumption of fossil fuels negatively impact the environment. Industrial processing of foods such as sugar, rice, and peanuts creates byproducts as environmental pollutants. This research is premised on using byproducts to add value by converting them to useful products, such as reducing sugars, useful for bioethanol production. The outcome shows that environmental pollution can be mitigated, bioethanol can be produced, and enzymes, a commercial value product, can also be produced from this process. This will create cleaner fuel and an environment by mitigating pollution using microbial organisms and creating value-added products from industrial byproducts.

1. Introduction

Lignocellulosic biomass represents the most abundant biopolymer in the world (Silva et al., 2024; Okafoagu and Nzelibe, 2006). In the agricultural industry, it is found in stalks, stems, and husks. It is a major component of woody and non-woody plants and is the primary source of renewable organic matter (Thapa et al., 2020). It is produced mainly by photosynthesis (Das et al., 2023). Therefore, agricultural wastes (e.g., rice husks, corn stover, sugar cane bagasse), forestry wastes (e.g. sawdust, mill wastes), municipal solid waste (e.g. human excreta, paper waste are derived from similar sources), and several other industrial waste



products contain essentially lignocellulose. Lignocellulosic biomass is composed of three biopolymers: lignin, hemicellulose, and cellulose (Das et al., 2023; Kirubahar et al., 2008). The percentage composition of lignocellulose monomers varies among plant species (Das et al., 2023). Moreover, the composition within a single plant varies with age, growth stage, and apical proximity (Das et al., 2023; Hori et al., 2003), among other factors. Grabber (2005) reported that, with maturity, the ease with which enzymes degrade lignocellulosic biomass is greatly reduced due to progressive lignification of tissues, a typical feature of the secondary plant cell wall, which confers strength, stability, and support for physiological functions. The composition of lignin monomeric units varies depending on the plant species. For instance, coniferous wood lignin is derived almost exclusively from coniferyl alcohol, producing a G-rich lignin with minor p-hydroxyphenyl (H) units, with a smaller content of coumaryl alcohol and minor amounts of sinapyl alcohol. In angiosperm lignin, there is approximately equal content of coniferyl and sinapyl alcohol and minor contents of coumaryl alcohol, while hardwoods contain guaiacyl (G) and syringyl (S) units. These variations in content are adjusted in response to environmental adaptations and survival strategies. Another major component of lignocellulosics is hemicellulose. It is a complex carbohydrate polymer that constitutes 25-30% of the total dry weight and ranks second only to cellulose in abundance (Das et al., 2023; Cullen and Kersten, 1992). It is composed of assorted monosaccharides, even in modified forms and in varying proportions, of which the exact composition depends on plant species (Das et al., 2023; Howard et al., 2003; Badger, 2014). The third component is cellulose. It makes up 45% of the dry weight of wood and is the most abundant biological polymer on earth (Thapa et al., 2020). It is composed of insoluble linear chains of D-glucose linked by β -1,4-glycosidic bonds, forming cellobiose units. This cellobiose is thus considered the repeating structural unit of cellulose (Howard et al., 2003). The degree of polymerization depends on the plant species (Howard et al., 2003) as structural conformity aligns with the polymer's adaptive features and physiological roles.

The natural decomposition of lignocellulosics employs an array of microbial enzymes in its mineralization (Adejumo et al., 2015), especially those of fungal origin (Adejumo et al., 2015; Sivaramanan, 2014). This process is one of the most important events in the carbon cycle (Silva et al., 2024). Several carbon sources, both synthetic and natural, have been used in attempts to produce cellulase. Yusoff et al. (2000) reported producing cellulase from the growth on SCB. Ojumu *et al.* (2003) compared SCB, corncobs, and sawdust and reported that sawdust was the best of the three because it produced the highest cellulase activity. Nwodo-Chinedu *et al.* (2007) compared sawdust and cellulose and reported cellulose as the better substrate, producing cellulase with activities of 100.0 Units/L and 92.2 Units/L for sawdust. Muthuvelayudham & Viruthagiri (2006) compared different substrates, both singly and in combination with many of the sources. Between SCB and rice straw, SCB was better (5.66 Filter Paper Units, (FPU)/ml) than rice straw (5.26 FPU/ml). Sendelinus (2005) studied SCB degradation for bioethanol production and reported that pretreated SCB showed easier hydrolysis than untreated SCB.

Therefore, several carbon sources have been employed in both experimental and industrial production of cellulases and reducing sugars. The need to source more economically viable substrates to serve as starting materials for cheaper, more affordable cellulases remains a challenge (Das et al., 2023). This step is critical for producing reducing sugars from feedstock and is necessary to avoid disrupting food supply chains in many economies. An ideal carbon source should be readily available, inexpensive, and preferably derived from agricultural waste or industrial waste. This offers multiple advantages, such as combating environmental pollution caused by the accumulation of poorly degraded waste (Howard et al., 2003), thereby helping to tackle environmental pollution and ensure food security. Examples of such materials, agricultural byproducts and industrial wastes, include sugar cane bagasse (SCB), rice husks, and groundnut shells. For every one tonne of sugar refined, two tonnes of SCB are produced (Guillaume, 2008). Thus, it is abundant in sugar-producing countries such as Brazil and Nigeria. Its burning to generate electricity contributes to CO₂ emissions. Moreover, sugar cane is a well-known photosynthesizer that converts the potent greenhouse gas (GHG) CO₂ into biomass, thereby reducing the deleterious ozone-layer-degrading effect of the GHG and its attendant consequences on the biosphere and the atmosphere.

Several varieties of sugar cane are cultivated in Nigeria. However, no study has demonstrated the feasibility of using them as a starting material for biofuel production. On the other hand, rice milling, an agro-industrial activity undertaken across the country, produces tonnes of rice husks. Also, groundnut cultivation in Nigeria, especially in the northern part, produces tonnes of groundnut shells following processing. These are largely environmental threats that demand urgent attention to ease the burden of environmental pollution. This multi-pronged study is designed to: test the hydrolytic capacity of locally isolated fungal strains for bioresource development, hydrolyze identified agro-industrial wastes, and compare the relative ease of hydrolysis of different waste materials for the production of reducing cellulases and reducing sugars for biofuel or other industrial activities. The isolation of the bioresources and their optimization were previously reported (Yarkwan & Anyanwu, 2016).

2. Materials and Methods

2.1. Reagents

Ammonium sulphate (NH₄)₂SO₄ (British Drug House (BDH) Chemicals Ltd., Poole, England), Ammonium chloride (NH₄Cl) (Fisher Scientific Company, USA). Dipotassium hydrogen orthophosphate trihydrate (K₂HPO₄·3H₂O), Kermel, India. Sodium potassium tartrate (C₄H₄KNaO₆·4H₂O) (Rochelle salt) Merck, Germany. Sodium acetate (CH₃COONa) Vickers Laboratories Ltd,



West Yorkshire, London. Sodium hydroxide (NaOH) Merck, Germany, and Sodium carbonate (Na_2CO_3) (Sigma-Aldrich). Standard buffers pH 4.01, pH 7.01, and pH 9.0 Qualigens Fine Chemicals, a division of GlaxoSmithKline, London. 3,5-dinitrosalicylic acid (DNS) ($\text{C}_7\text{H}_4\text{N}_2\text{O}_7$) Lab. Tech. Chemicals, Avighkar India. Hydrochloric acid (HCl) conc. Reagente Puro Erba (RPE). Calcium carbonate (CaCO_3) from May and Baker Ltd., England. Copper tetraoxosulphate vi (CuSO_4). Merck, Germany (Analytical grade). Bovine Serum Albumin (BSA). D (+) Glucose (Industrial grade) M & B Ltd. Dagenham, England. All chemicals were of analytical grade. Whatman No. 1 filter paper.

2.2. Apparatus

Weighing balance Ohaus Dial-O-Gram. Ohaus Corporation, N.J., USA. Jeffco Food and Fodder Cutter – Grinder Model 365B, size 10 (Jeffress Bros Ltd., 351 Molton Road, Northingham, Brisbane, Queensland, Australia). Apex Hydraulic press, 30104 (Apex Construction Ltd.). Autoclave: UDAY BURDON'S Patent Autoclave, Made in India. Milling machine: Thomas Willey Laboratory Mill model 4, Author H (Thomas Company, Philadelphia, USA). Oven: Gallenkamp Hotbox. Made in England. Water bath: Grant Instruments (Cambridge) Ltd, Barrington, Cambridge. UV Spectrophotometer: UNICO UV 2102 PC spectrophotometer. pH meter: Ecoscan pH meter, made in Singapore. All instruments were working well and, where required, were calibrated in accordance with the manufacturer's specifications.

2.3. Collection and Crushing of Sugar Cane Samples

Four improved varieties of sugar cane, namely, National Cereals Sugar (NCS) NCS-003, NCS-006, NCS-007 and Coimbatore (Co) Co 957 (a variety imported from India), were generously donated by the Sugarcane breeding program of the National Cereals Research Institute (NCRI), Badegi ($9^\circ 05' 69''\text{N}$ $6^\circ 14' 34''\text{E}$), Niger State. A local variety was bought from farmers around the Badegi settlement and tagged as "local" in this study. The improved varieties were two seasons old, while the local variety was only one season old. The local variety grown in Fadama lands was fresher than the improved varieties, which were cultivated in uplands, because the samples were collected in the dry season, with dry soil and hence reduced soil water content. Equal weights (6.00 kg) of all samples were weighed on a top-load balance and transferred to the milling room.

The weighed sugar cane samples were crushed using the Jeffco Food and Fodder Cutter- Grinder. After crushing, the bagasse was pressed using the Apex Hydraulic Press in order to remove the brix. The bagasse was then packaged in labeled polythene bags and transported to the laboratory for further processing.

Rice husk was collected from the Adani Rice Mill ($6^\circ 73' 35''\text{N}$, $7^\circ 00' 92''\text{E}$) in the Uzo-Uwani Local Government Area of Enugu State. Freshly deposited groundnut shells were gathered from refuse dump sites around the University of Nigeria, Nsukka (UNN). Both were washed with distilled water and oven-dried, a treatment that was also applied to the washed portion of the SCB samples upon delivery to the laboratory at UNN ($6^\circ 86' 68''\text{N}$ $7^\circ 41' 34''\text{E}$).

2.4. Laboratory Treatment of the Sugar Cane Bagasse (SCB)

Each of the five sugar cane bagasse samples was divided into two equal-weight parts using a Gallenkamp S/No. 588 top-load weighing balance. One half was rinsed three times in running tap water, squeezed out by hand, and marked 'washed, W'. The other part was not washed and marked 'unwashed, UW'. Both samples were dried in the oven at 105°C until constant weight, according to Vermerris (2005). The dried SCB was milled using a Thomas Willey Laboratory Mill. The milled SCB was then sieved using a 0.5-mm mesh. The fine-sieved SCB was then packaged in polythene bags and shelf-preserved in the laboratory.

2.5. Dry Matter, Moisture Content, Ashing, and Acid Digestion for Metal Ion Analysis.

This was carried out according to established methods applicable to such materials. Briefly, ashing was performed on 2 g of the oven-dried SCB (NCS-006) at 500°C for 4 hr for mineral analysis. For the digestion for heavy metals analysis, 50 mL of 30% HCl (prepared by mixing 30 mL of concentrated HCl with 70 mL of deionized water) was used to dissolve the ash (0.027 g and 0.026 g for the W and UW samples, respectively) and was kept in a clean, dry plastic container until analysis. The instrument blank consisted solely of 30% HCl. Metal ion concentrations were determined using an atomic absorption spectrophotometer (AAS).

2.6. Procedure for Protein Determination

The standard method for protein analysis, as reported in the literature (Lowry et al., 1951), was adopted. Ten test tubes were arranged in duplicate, containing 0.0 – 1.0 mL of 0.2 mg of protein stock solution. The volume was made up to 1 mL with distilled water. 5 mL of alkaline solution E was added and then allowed to stand at room temperature for about 10 min. 0.5 mL of solution C (diluted Folin Ciocalteu reagent) was added with rapid mixing. After standing at room temperature for 30 minutes, the absorbance was taken at 750 nm. A calibration curve of absorbance against standard BSA concentration was made. The protein concentration per mL in each tube was calculated using the equation of the line derived from the calibration curve.

Components of Protein Determination Reagent:

Solution A: An alkaline sodium carbonate (Na_2CO_3) solution was prepared by dissolving 2 g of Na_2CO_3 in 100ml of 0.1 M NaOH



(0.4g of sodium hydroxide pellets were dissolved in 100ml of distilled water).

Solution B: A copper tetraoxosulphate (VI) – sodium potassium tartrate solution was prepared by dissolving 0.5g of CuSO_4 in 1g of sodium potassium tartrate, all in 100ml of distilled water. It was prepared fresh by mixing the stock solution, and this was done whenever required.

Solution C: Folin-Ciocalteu reagent was made by diluting the commercial reagent with water in a ratio of 1:1 on the day of use.

Solution D: Standard protein (Bovine Serum Albumin, BSA) solution

Solution E: Freshly prepared alkaline solution was made by mixing 50ml of solution A and 1ml of solution B.

2.7. The Fermentation Broth

The fermentation broth contained (w/v): 1% SCB, 0.1% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% of NH_4Cl , and 0.1% of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, measured out into a clean, dry 250 mL Erlenmeyer flask containing 50 mL of distilled water. This was sealed with a stopper enclosed in aluminum foil, and autoclaved at 121°C and 15 psi for 15 min. This was done to sterilize the broth. Inoculation, cultivation of the broth, and harvest of the broth were accomplished following standard procedures. Briefly, prepared broth was inoculated with three-day-old fungal cultures, and kept in the laboratory at an average temperature of $29 \pm 1^\circ\text{C}$ until the day of harvest.

2.8. Partial Purification of Harvested: Ammonium Sulphate Precipitation and Dialysis

One liter of crude enzyme filtrate was prepared. Then 75% ammonium sulphate precipitation was carried out by dissolving gently 476g of the salt in the filtrate and stirring gently till the salt was completely dissolved. The ammonium sulphate – crude enzyme solution was allowed to stand in the cold temperatures of about 4°C for 30 hr until the supernatant could be gently decanted off. The precipitate was then collected and centrifuged for 10 min at 10 000g. The protein was obtained and dissolved in 50 mM Sodium Acetate (NaOAc) buffer pH 5.50 and kept under cold conditions. Following complete centrifugation, dialysis was performed. Dialysis tubes preserved in 90% ethanol were rinsed several times with distilled water till they were clean of the ethanol. Fifty mL of NaOAc buffer pH 5.5 was used for dialysis using a magnetic stirrer with a magnetic fleece inserted into it. Dialysis was carried out for 14 hr with continuous stirring, and the buffer was changed every 6 hr. After dialysis was completed, the partially purified enzyme was stored frozen at -24°C .

2.9. Cellulase Assay

Whatman No. 1 filter paper strips measuring 1 cm x 3 cm were carefully cut out. These were used as the assay substrate. Each strip contains, by standard units, an equivalent of 25 mg of glucose.

The assay mixture containing two mL of 0.2 M NaOAc buffer, pH 5.5, was pipetted into the test tube. Then 0.5 mL of the enzyme filtrate was added. A strip of filter paper was then immersed entirely in the mixture and incubated in a water bath at 50°C for 6 hr, unless otherwise stated. (This reduction in filter paper size was to ensure a limited supply of the assay kit was maintained owing to limited supply. The extension in incubation was considered in the final activity computation results). An enzyme blank was prepared following a similar procedure, but no enzyme was added. A substrate blank was also prepared by mixing 0.5 mL of the enzyme with 2.0 mL of the buffer, with no substrate added. The tubes were removed, and the reaction was terminated by adding 0.5 mL of DNS reagent. The mixture was then placed in boiling water for 10 minutes to deactivate the enzyme. After cooling, the absorbance was read at 540 nm. The blank was used to zero the instrument before taking the readings and periodically during the reading time.

The reducing sugar formed per mL was calculated as described by Wu et al. (2006). One unit of activity was defined as the amount (in micromoles) of reducing sugar formed per minute per milligram of protein under the assay conditions.

2.10. Statistics and Data Analysis

Results obtained from the analysis performed were collected and analyzed using Microsoft Excel Version 2015 for basic chart construction and determination of means of triplicate experiments, where applicable.

3. Results and Discussion

3.1. Ash and Dry Matter

The organic matter content of the sugar cane bagasse ranged from 98.00% to 99.00% across all samples (Table 1). Dry matter was generally higher in the unwashed samples than in the washed samples. This could be attributed to residual sugar in the unwashed samples, which contributes to their carbon content. Co 957 had the highest dry matter content, i.e., 64.00% (UW) compared with 52.90% (W). NCS-003 (UW) had the highest weight loss at 56.40%, while NCS-006 had 64.80%, the highest among the washed samples. Weight loss was higher in all washed samples than in the unwashed samples. This could be explained by the fact that the unwashed samples were machine-pressed, while the same machine or a similar one was not available at UNN for pressing, and



the hand-pressing force may not match the machine pressure in squeezing out the water. Thus, this difference merely reflects the residual moisture content in the samples before oven drying. The ash content ranged from 1.00% for Co 957 (W) to 2.00% for both Local (UW) and Co 957 (UW), with the lowest and highest values, respectively. NCS-003 (W) occupied an intermediate position relative to the other samples (Table 1). Since ash is a reflection of the inorganic matter content, it is thought that the higher ash content in the washed sample of NCS-003 than in the other samples in the washed group (except for the local variety) may be a result of the variety's higher capacity for metal uptake, even while growing within a similar field. Since this was not confirmed, it is expedient to have specific further comparative studies done to confirm this presumption. Since metal uptake is a good property for phytoremediation of metal pollution, any sugar variety with strong metal bioaccumulation potential could also be employed in phytoremediation of metal-polluted sites, in addition to its known primary role in carbon dioxide uptake.

Table 1. Composition of ash, dry matter, organic matter, and weight losses in the different sugar cane bagasse samples.

Parameter (%)	NCS 003	NCS 006	NCS 007	Co 957	Local
Weight loss of W	60.00	64.80	52.60	47.10	59.90
Weight loss of UW	56.40	50.40	42.10	35.30	54.20
Organic matter of W	98.50	99.00	99.00	99.00	98.00
Organic matter of UW	98.00	99.00	99.00	98.00	98.00
Dry matter of W	40.00	35.20	47.40	52.90	40.10
Dry matter of UW	43.60	49.60	57.90	64.70	55.80
Ash of W	1.50	1.00	1.00	1.00	2.00
Ash of UW	1.00	1.00	1.00	2.00	2.00

Moisture content, determined as the percentage weight loss after the Brix was pressed out, reflected the moisture content in the SCB, not in the sugar cane stalks. The moisture content of Co 957 (W) was 47.1%, close to the 47.8% reported elsewhere (Herrera, 2000). However, it was lower than the 71.0% reported by Herrera (2000). This could be due to the harvest season, as these samples were harvested in February, a dry season in Nigeria, with very low soil water content, thereby causing plants to lose water. All other SCB samples had moisture content higher than the value reported by Pessoa et al. (1997) but lower than the value reported by Herrera (2000). Moisture content can be affected by maturity, harvest period, and the differential water-holding capabilities of species and variety(s) of sugar cane. The sugar cane samples used were very mature, having stayed for two seasons on the farm and therefore having lost freshness due to drying and depleted soil water content. These contributed to the lower moisture content and dry matter, since the total dry matter of the sugar cane bagasse (SCB) samples was high, ranging from 35.2% to 52.9% for the washed samples and 43.6% to 64.70% for the unwashed samples. These values are higher than the 29% earlier reported (Herrera, 2000).

The percentage ash content of all the SCB samples was higher than the 0.50% reported by Herrera (2000). Ash content of washed samples was 1.00% for each of Co 957 and NCS-006, and 1.50% for NCS-003. The unwashed samples of NCS-003, NCS-006, and NCS-007 each had 1.00%. These results agreed with the 1.00% earlier reported (Muthuvelayudham & Viruthagiri, 2006) but disagreed with the 2.0% reported elsewhere (Goncalves et al., 2005). The ash content of Local (W), Co 957 (UW), and Local (UW) was 2.00%, which agreed with the 2.00% reported by Goncalves et al. (2005). Ash content represents the inorganic constituents of the sample and may be affected by washing, which could either add inorganic ions from the running water to the sample or remove certain traces of inorganic substances from the sample due to the distilled water rinse (de Oriani et al., 2003).

Owing to the paucity of funds for analytical costs typical of self-funded research activities predominant in the Nigerian educational landscape, the determination of metals in the samples was restricted to one sample, NCS-006, because it was the predominant sample cultivated by the Institute. Determination of metal ions in NCS-006 shows that calcium has the highest concentration in both the W (61.49 ppm) and UW (33.64 ppm) SCB NCS-006 (Table 2). Manganese was lowest for the W (0.61 ppm), and zinc was lowest for the UW (0.33 ppm).

Table 2. Concentration (ppm) of some metal ions of sugar cane bagasse NCS-006 (W is Washed; UW is Unwashed).

Sample	Metal	Mn	Cu	Zn	Fe	Ca	Mg	K	Na
SCB sample W (ppm)		0.61	1.92	0.65	2.30	33.64	21.18	3.61	4.98
SCB sample UW (ppm)		0.86	1.65	0.33	2.08	61.49	51.18	2.97	4.33

Literature on the content of metal ions in SCB is scarce, but Fareed et al. (2024) highlighted the presence of heavy metals, including Cd, Pb, Zn, and Fe. However, the physiological roles of metal ions in plants have been well documented, as metals such as Zn, Mn, Cu, Fe, Ca, and Mg are known to activate certain enzymes, while K and Na function as electrolytes in living systems (Anosike, 2001). Mg, K, Na, and Ca are major elements of plant nutrition, while the remaining ones are minor elements. The focus here is



on the organisms' ability to degrade the SCB. At the concentrations found in the representative sample, these metals did not cause any inactivation or denaturation of the enzymes. This is evident by the appreciable cellulase activity observed (Figures 1, 2 and 3) from the cultivation of the organisms on this carbon source. The increase in biomass accumulation is reflected by the extracellular enzyme production observed in both curves. However, since only NCS-006 was studied, this conclusion may not be extrapolated to include the other varieties whose independent studies will be critical in determining the levels of heavy metals content in them. Moreover, frequent soil examination for heavy metals is important to ensure the ensuing sugar cane obtained from such soils which may become contaminated owing to increasing anthropogenic activities does not impact the quality of the sugar brix produced from such sugar cane harvest, as has been reported elsewhere (Onah et al., 2026).

3.2. pH Change in Different Substrates During Fermentation

The pH of the UW samples was in all cases higher than that of the W samples fermented by the same organism (Table 3). The final pH either increased (NCS-003W, G/nut) or decreased (NCS-006 W and NCS-007 W).

Table 3. pH Change of the broth of different substrates fermented by *Spadicoides* spp and *Aspergillus niger* for 4 days.

Substrate	Initial pH	Final pH <i>Spadicoides</i> spp	Final pH <i>Aspergillus niger</i>
Co 957 W	5.75	5.91	5.33
Co 957 UW	5.81	6.20	5.84
Local W	5.81	3.55	3.60
Local UW	5.80	6.06	5.25
NCS-003 W	4.85	4.90	5.06
NCS-003 UW	6.04	6.36	6.50
NCS-006 W	5.33	4.16	3.81
NCS-006 UW	5.55	6.26	5.72
NCS-007 W	5.25	4.18	4.32
NCS-007 UW	6.24	6.15	6.12
G/NUT	6.41	7.03	6.92
R. HUSK	6.15	6.61	5.09

The pH in all cases changed, though not with a defined pattern. The pH change towards the acidic range was observed in the Local variety cultivated with *Spadicoides* spp, while the increase towards the alkaline range was observed for groundnut shells cultivated with *Spadicoides* spp (Table III). This decrease in pH, observed in most substrates, could be due to the accumulation of organic acids such as lactic acid, citric acid, and acetic acid, as well as other acids from metabolic pathways such as glycolysis (Das et al., 2023), and byproducts of cellular respiration. Moreover, NH₄Cl was the source of nitrogen used. The breakdown of this salt to its constituents to obtain nitrogen for amino acid biosynthesis is thought to contribute towards increased concentration of the positively charged species. If this is the case, it is expected that increased biomass accumulation over time, arising partly from ammonium salt consumption, will result in a corresponding increase in pH. Dashtban et al. (2009) reported that cellulolytic fungi of soil origin, such as those employed in this study, grow better at a pH below 6.0. Therefore, this study agrees with previous reports.

3.3. Extracellular Protein Production Using Different SCB Substrates

Two cellulase-hyperproducing fungi, *Spadicoides* spp (SWA1) and *Aspergillus niger* (DLS A1), were employed in these studies (Figure 1). The extracellular protein assay showed that *Spadicoides* spp grown on NCS-003 (W) produced 1.23 mg/mL of protein. The same organism grown on rice husk produced only 0.38 mg/ml. *Aspergillus niger* grown on NCS-003 (W) produced 0.98 mg/ml and produced 0.51 mg/mL when grown on groundnut shells. These quantities of the extracellular protein produced by these organisms on different substrates are higher than the 120 µg/mL reported (Narashimha et al., 2006), but less than the 7.94 mg/mL reported by Muthuvelayudham & Viruthagiri (2006), who worked with a mutant strain of *T. reesei* grown on SCB.

The extracellular protein secreted from SCB-supported broth is several times higher than the secretion when rice husk and groundnut shells were the carbon source (Figure 1). This could be due to the relative ease of biodegradability of SCB, compared to the relatively higher recalcitrance of the other lignocellulosic agro-byproducts. This finding portrays SCB as a relatively easier source of starting material for reducing substrates, which could be employed in biofuels for clean, sustainable energy production, or other industrial applications. It also showcases the prospects of engaging these fungal strains in the bioremediation of SCB-polluted environments, either on farms where large quantities are harvested or where wastes are indiscriminately dumped. It could also be a potential bioresource for sugar-producing companies to engage in environmental remediation of SCB pollution.

The different organisms were shown to have cellulase activity. The activity was higher than that reported by previous researchers (Milala et al., 2005; Ojumu et al., 2003) but lower than that of some mutant strains of *T. reesei*. Just like protein secretion, the cellulase activity differed with the fermentation substrate (Figure 2). This indicates that the SCB samples are capable of inducing cellulase

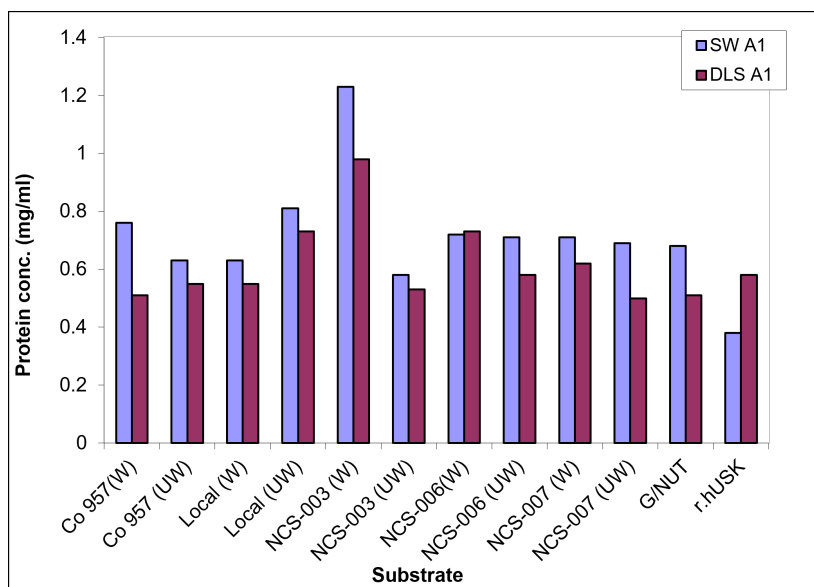


Figure 1. Protein concentration (mg/ml) of SW A1 and DLS A1 grown on different substrates for 4 days. Extracellular protein was highest in SW A1 grown on NCS-003 W (1.23 mg/ml), while DLS A1 grown on the same substrate produced 0.98 mg/ml, being the highest value for this organism. *Spadicoides* spp (SW A1) grown on rice husk produced 0.38 mg/ml, while *Aspergillus niger* (DLS A1) grown on NCS-007 UW produced 0.50 mg/ml, being the lowest protein produced by the organisms. (SWA1 = *Spadicoides* spp and DLS A1 = *Aspergillus niger*)

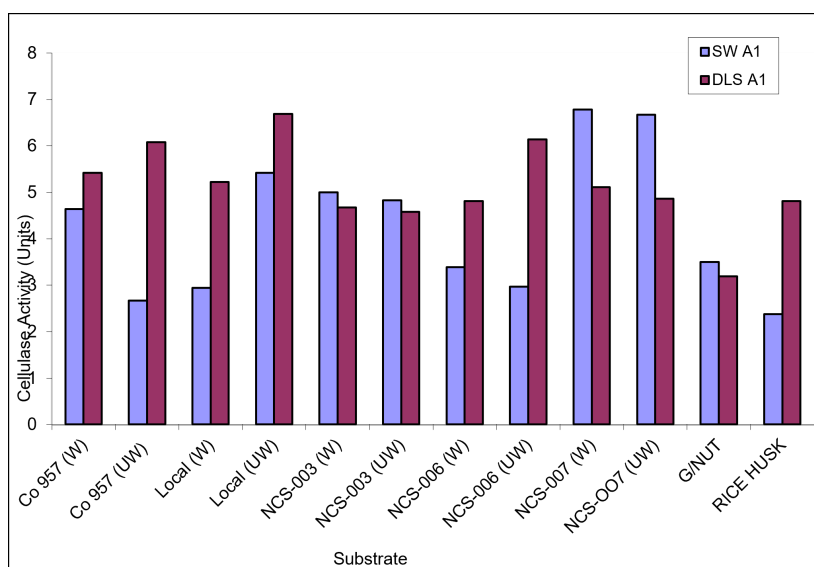


Figure 2. Cellulase activity of SW A1 and DLS A1 isolates grown on different substrates. SW A1 grown on NCS-007 W had the highest activity (6.78 units), while DLS A1 grown on Local UW had the highest activity (6.69 units) (Figure 2). SW A1 grown on rice husk had the lowest activity (2.38 units), while DLS A1 grown on groundnut shell had the lowest activity (3.19 units). Of the 12 pairs of samples, DLS A1 had the highest activity in 7 samples compared with SW A1, which had higher activity than DLS A1 in 5 samples. The substrate for the assay was filter paper. Activity is given in units (Unit = micromoles/min) (SWA1 = *Spadicoides* spp and DLS A1 = *Aspergillus niger*)

production in *Aspergillus niger* and *Spadicoides* spp. The cellulase of *Aspergillus niger* is more active against filter paper than that of *Spadicoides* spp., indicating higher exoglucanase activity in the former. Rice husk and groundnut shell were included in the

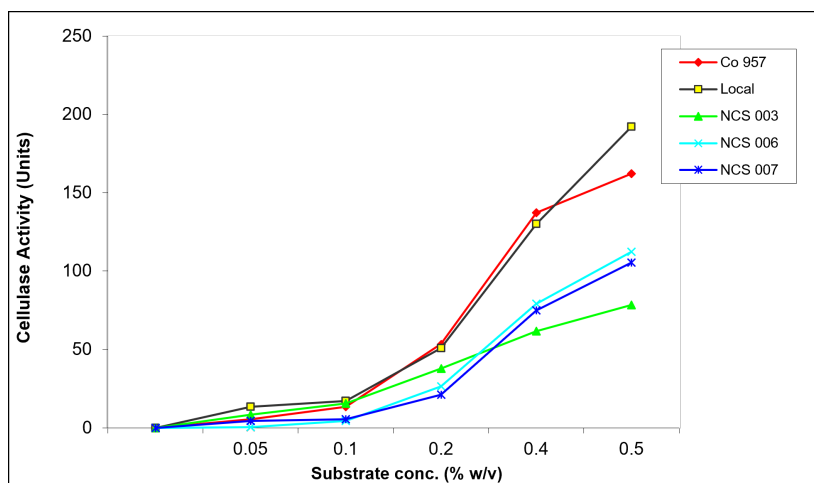


Figure 3. Hydrolysis of Different SCB Substrates by the partially purified enzyme of *Aspergillus niger* DLS A1 and SW A1. An increase in substrate concentration increased enzyme activity across the different SCB substrates. Local was degraded the most (192.20 units), while NCS-003 was degraded the lowest (78.33 units). For all substrates, activity increased most between 0.2% and 0.4% SCB concentration. Activity is given in units. (Unit = micromoles/min). (SWA1 = *Spadicoides* spp and DLS A1 = *Aspergillus niger*)

study to assess their potential to support the growth of cellulolytic organisms and, consequently, cellulase production. Rice husk showed higher cellulase activity than groundnut shells. The enzyme activity from fungal growth on rice husk is less than that reported earlier (Muthuvelayudham & Viruthagiri, 2006). Perhaps this was because of the mutant strain of *T. reesei* they used, the substrate being less recalcitrant rice straw, and pretreatment, which can open encrusted cellulose chains, exposing them to greater penetration and hydrolysis by cellulolytic enzymes. Moreover, *T. reesei* differs largely from *A. niger*, which has been studied here, thereby presenting differences in the kinetic properties of their respective enzymes. The difficulty in microbial degradation of rice husk was also reported (Sinigani, 2005) when comparing wheat, barley, and rice husks as carbon sources for growing five different cellulolytic organisms. Ordinarily, groundnut shells are hard. Since the toughness of plant tissues is due to the extent of lignification, this implies that groundnut shells have a higher lignin content than the other substrates, hence the difficulty they pose to enzymatic degradation, a view also reported by Oliveira et al. (2020). The results, therefore, indicate that SCB is a better substrate for cellulase production than both groundnut shell and rice husk. Five different washed SCB samples were tested as possible substrates for the enzymes (Figure 3). Co 957 and Local showed an almost linear relationship between activity and substrate concentration. Reducing sugar production was highest in Local (192.22 activity units). This was closely followed by Co 957 (162.22 activity units). These values are higher than those reported by several authors. For instance, Muthuvelayudham & Viruthagiri (2006) reported values of 68 U/ml, 5.66 U/ml, and 2.80 U/mL for CMCase, FPase, and β -glucosidase, respectively. Ojumu et al. (2003) reported 0.0573 IU/ml, while Yusoff et al. (2000) reported 0.55 IU/mL and 0.08 IU/mL activity for FPase and β -glucosidase, respectively. Higher reducing sugar yield is typical of low lignin content, which is inversely related to the ease of hydrolysis (Mota et al., 2020). Thus, it's plausible to suggest that the local variety of sugar has lower lignin content and is therefore more susceptible to fungal enzyme hydrolysis. Since it is not a specifically engineered sugar variety, it is thought that it may not have undergone any modifications, hence its lower lignin content. Several factors determine the lignin content of plants. These include, among others, age and species (Das et al., 2023). Since Co 957 in this work was twice as old as Local, it may be reasoned that due to its age, it may have acquired more lignification than Local, though this was not tested and may not be conclusive. One may reason that if Co 957 was the same age as Local, it might have been more easily degraded. However, since lignin content and composition were not measured in this study, this may be speculative and subject to confirmation from empirical evidence using these samples. Thus, it is cautiously stated that lignin composition might have impacted the results, based on established knowledge (Vermerris et al., 2005) not tested in this particular instance. This presents a possible area of research to follow up on. Vermerris et al. (2005) reported that the ease with which a lignocellulose material degrades is dependent upon the lignin composition and not the lignin content per se. In the hydrolysis of maize from a mutant species, it was observed that the ease of degradation was due to a lower content of synapyl and *p*-coumaryl residues in the lignin. Oliveira et al. (2020) pointed out that the ease with which microbes degrade grasses is due to the larger content of cinnamyl units in their lignin structure. Besides lignin composition, other effects on enzyme production could arise from variations in crystallinity, structural heterogeneity, and differences in the lignocellulose fractions of cellulose and hemicellulose, as well as from inorganic metal ions. Subsequent studies should use sugar cane of similar age for all samples to facilitate better comparisons between the results on ease of degradability. Also, the content and composition of lignin for these five



sugar varieties should be studied as a means of providing informed empirical evidence for the structural basis of relative ease of hydrolysis among the tested varieties.

4. Conclusion

Heavy metals were found in the SCB tested but not at concentrations to impact fungal growth. Different quantities of protein and extracellular enzymes were produced when the fungi were grown on the lignocellulosic biomass. The ease of hydrolysis, represented by the amount of reducing sugar produced per unit of lignocellulosic biomass, followed the order of Local > Co957 > NCS006 > NCS007 > NCS003 for both organisms. For the lignocellulose biomasses, the quantity of extracellular cellulase secreted in the broth for *Aspergillus niger* was in the order of: Local (UW) > Co957(UW) > NCS006(UW) > Co957(W) > Local(W) > NCS006(W) > NCS007(UW) > NCS007(UW) > Rice husk > NCS003(W) > NCS003(UW) > Groundnut Shells; while that for *Spadicoides* spp was in the order: NCS007(W) > NCS007(UW) > Local(UW) > Co957(W) > NCS003(W) > NCS003(UW) > Co957(UW) > Groundnut shells > NCS006(W) > NCS006(UW) > Local(W) > Rice Husk. This demonstrates the sequence for ease of hydrolysis of these lignocellulosics using the wild-type strain bioresources. The highest reducing sugar production was obtained when the carbon source was between 0.2% and 0.4% (w/v); beyond this range, increasing concentration did not yield a corresponding increase in the reducing sugar released.

Authors' Contributions

Baturh Yarkwan: Conceptualization, Execution of the experiments, collation of data, analysis of data, drafting the manuscript; Ferdinand C. Chilaka: Supervision, provision of guidance in experimental designs, provision of research chemicals, and oversight and vetting of the write-up; Clement Wada: Provision of sugar cane varieties, technical staff to support harvesting, transportation, fodder cutting, and all other substrate-related logistics and housing the researcher at the research station.

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Declarations

Conflicts of Interest: The author(s) declare no conflict of interest.

Institutional/Ethical Approval: This study did not involve human or animal subjects, and no institutional or ethical approval was required.

Data Availability/Sharing: The datasets used and analyzed during the current study will be made available from the corresponding author upon a reasonable request.

Supplementary Information Availability: Not applicable.

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