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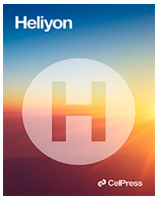

Research paper

Production of cellulases by *Xylaria* sp. and *Nemania* sp. using lignocellulose substrates for bioethanol production from maize cobs

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ABSTRACT

Two species of *Xylaria* (KM01, KM03) and *Nemania* sp.KM02 isolated from decaying plant biomass were evaluated for their ability to produce cellulases on maize cob, eucalyptus, and cypress substrates under solid-state fermentation. A total of 10 fungal samples from decaying plant biomass were collected from Karura forest based on morphological variations. The fungi isolated were screened for cellulase activity and positive isolates were selected for the study. ITS4 R and ITS86 F primers were used to identify the fungal isolates with accuracy ranging from 98 % to 100 %. The crude cellulases produced was assayed for FPase, exoglucanase, endoglucanase and β-glucosidase. Cellulases of *Xylaria* sp. KM01 produced higher FPase and exoglucanase (2.01 ± 0.13 IU/ml and 0.94 ± 0.08 IU/ml) on pretreated maize cobs with 0.1M HCl at 121°C, while that of *Xylaria* sp.KM03 produced higher β-glucosidase and endoglucanase (588.6 ± 64.2 IU/ml and 3.59 ± 0.02 IU/ml) on maize cobs pretreated with 0.1M NaOH at 121°C. However, cellulases of *Xylaria* sp. KM01 produced higher β-glucosidase and FPase (629.7 ± 20.2 IU/ml and 1.67 ± 0.03 IU/ml) on untreated maize cobs after the 9th day of incubation, whereas cellulases of *Xylaria* sp. KM03 and *Nemania* sp.KM02 produced higher endoglucanase and exoglucanase (2.80 ± 0.21 IU/ml and 0.83 ± 0.02 IU/ml) on untreated maize cobs after the 3rd and 6th day of incubation. Saccharification of maize cobs by cellulase of *Xylaria* sp.KM03 produced the highest reducing sugars at 8 % substrate loading (10.17 ± 0.37 mg/ml) after 72 h of incubation. Simultaneous hydrolysis and fermentation of maize cobs by cellulase of *Nemania* sp.KM02 and *Saccharomyces cerevisiae* yielded higher bioethanol (28.72 ± 3.82 mg/ml) after 96 h of fermentation. Maize cob is established as a suitable feedstock for cellulases and bioethanol production.

1. Introduction

As the world's population grows, there is an increasing energy demand and limited fossil fuel (oil, coal, and gas) reserves to sustainably meet this demand [1]. The projected depletion of fossil fuels and the increase in global warming have led countries to explore the need for non-conventional and natural energy sources [2,3]. Serious environmental concerns posed by fossil fuels can be overcome by producing bioethanol from agricultural wastes. Pretreatment and enzyme optimization are key aspects of improving saccharification of biomass for bioethanol production. These two methods deal with the challenge by efficiently hydrolyzing cellulose

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(saccharification) into reducing sugars, [4]. The reducing sugars produced from lignocellulose hydrolysis can be fermented by; i) separate hydrolysis and fermentation, ii) simultaneous saccharification and fermentation (SSF), iii) solid-state fermentation (SSF), iv) simultaneous saccharification and co-fermentation (SSCF), v) consolidation bioprocessing (CBP) or v) batch fermentation [5]. In this study, separate hydrolysis and fermentation were used to produce ethanol from maize cobs.

Lignocellulose primarily consists of 35 %–50 % cellulose, 20 %–35 % hemicellulose, and 5 %–30 % lignin by dry weight and forms a major structural component of plant cell walls [6–8]. Cellulose consists of glucose molecules linked together by β-(1–4) glycosidic bonds with repeating cellobiose units [9,10]. Cellulose molecules (about 30 units) are packaged into elementary fibrils, which are

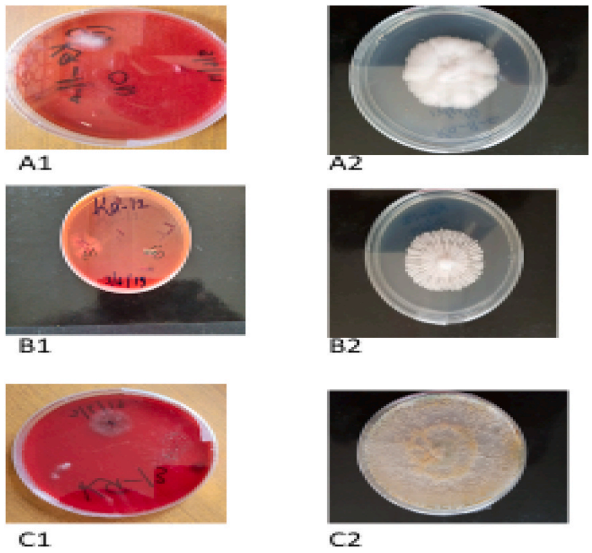


Fig. 1. Cellulase activities and colony morphologies of *Xylaria* sp. KM01(A1, A2), *Nemania* sp. KM02 (B1, B2) and *Xylaria* sp. KM03 (C1, C2) cultured on 1 % CMC-Congo red agar and sub-cultured on PDA respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

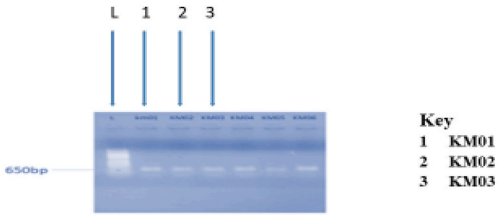


Fig. 2. An image of an agarose gel indicating the amplification of ITS target region of *Xylaria* sp. KM01, *Nemania* sp. KM02 and *Xylaria* sp. KM03.

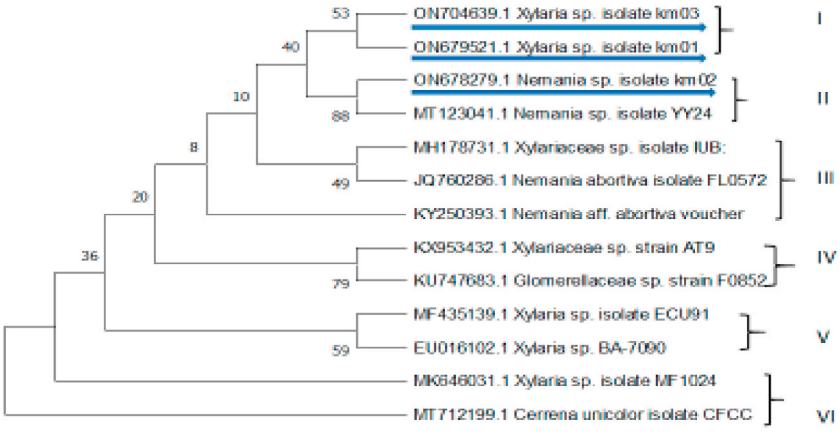
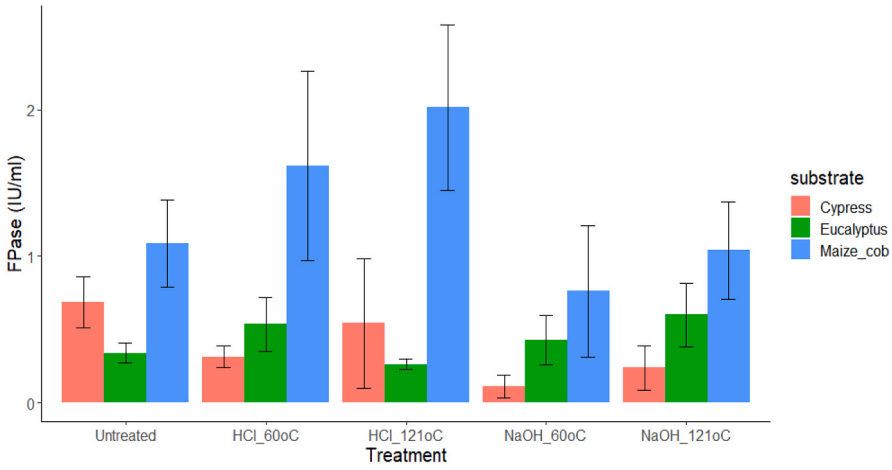


Fig. 3. Phylogenetic tree for *Xylaria* sp. KM01, *Nemania* sp. KM02 and *Xylaria* sp. KM03. *Cerrena unicolor* isolate CFCC was used as an outgroup.



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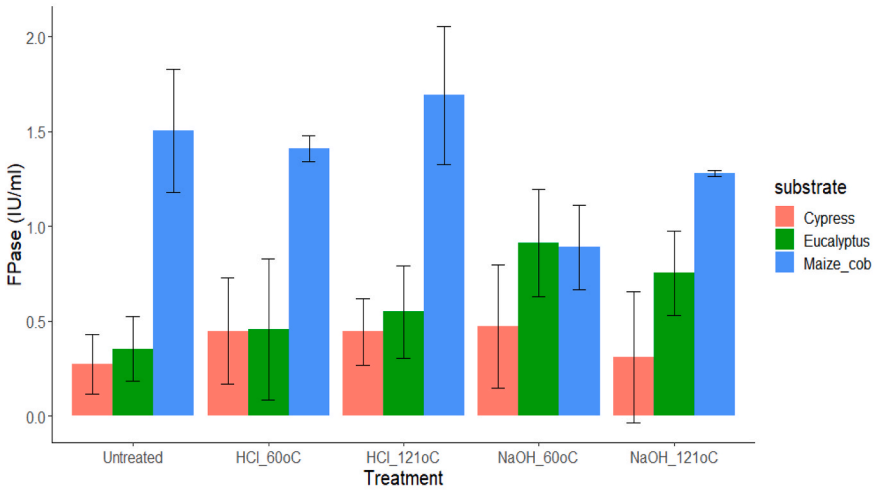

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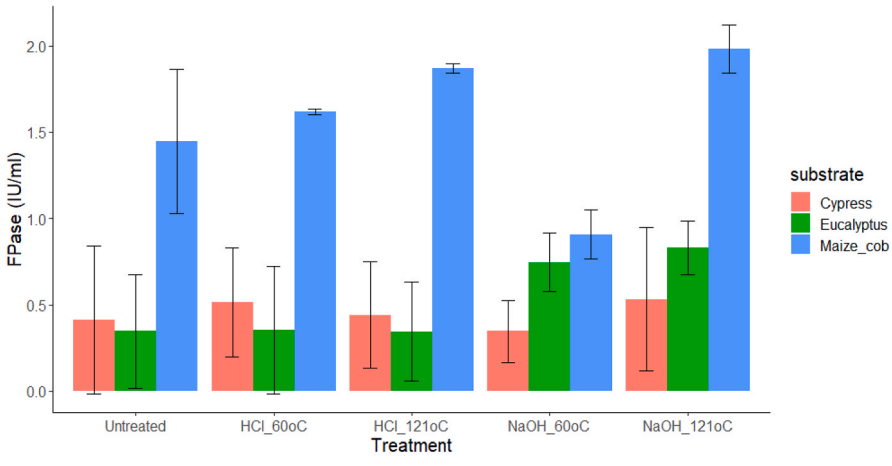

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Fig. 4. FPase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02, and (C) *Xylaria* sp. KM03 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

reassembled into microfibrils, and finally assembled into cellulose fibers [11]. Cellulose degradation synergistically is done through the action of three enzymes (endoglucanase, exoglucanase and β-glucosidase) based on their catalytic action [12]. Endo-glucanase cleaves randomly in the internal D-glycosidic linkages preferentially in the amorphous units of the cellulose, easily rendering cellulose more prone for exo-glucanase cleavage to form free ends chain [13]. Exoglucanase (cellobiohydrolase, CBH) hydrolyzes cellulose to produce cellobiose units. CBH I cleave cellulose from the reducing end while CBH II cleaves from the non-reducing end. The presence of cellobiose, a by product of substrate breakdown will however prevent the activity of cellobiohydrolase [14]. β-glucosidase catalyzes the breakdown of cellobiose and oligosaccharides into glucose monosaccharides [15]. However, the production or presence of glucose competitively inhibits β-glucosidase activity [13].

Different microorganisms secret cellulolytic enzymes in nature, which can be purified and used to break down lignocellulose into simple fermentable sugars for biofuel production. *Trichoderma* sp. and *Aspergillus* sp. are commonly utilized species to source lignocellulases [16]. Despite numerous reports of isolation of lignocellulolytic enzymes from microorganisms in other parts of the world, the biotechnological benefit of novel lignocellulolytic enzymes from Kenyan microbial communities has not been fully exploited. Plant biomass like agricultural and forestry residues are renewable natural sources of organic matter that can serve as low-cost sources of feedstock that could be utilized to produce enzymes and alternative fuel among other commodity products [6]. It is, therefore, necessary to focus on isolating and characterizing new microorganisms from the local environment as sources of lignocellulolytic enzymes that hydrolyze lignocellulose biomass to reduce the cost and process of producing biofuels, among other products in today's market [17–19]. Cellulase enzyme production can be done using chemically defined media under submerged fermentation or by the use of lignocellulose materials under solid-state fermentation. Solid state fermentation (SSF) is a technique in which the microbial cell growth preferably fungi is performed in a fermenter with a moist substrate in no free-moving water environment [20,21]. This fermentation technology has been widely used for metabolite production from plant biomass such as lignocellulases, gibberellins, and biopesticides among others [22]. Recently, this technology has been adopted in cellulase production as compared to submerged fermentation systems due to high enzyme production [23].

Bioethanol is produced from the sugar component of agro-waste through fermentation under controlled environmental conditions releasing carbon dioxide (CO_2) [24]. The fermentation process is anaerobically and follows the Embden-Meyerhoff pathway (EMP) which is catalyzed by bacterial and fungal enzymes [24]. In this process, the substrates are first hydrolyzed by chemical means or with cellulase enzymes to reduce sugars, followed by fermentation by ethanologenic microorganisms to produce bioethanol [25]. Bioethanol production from corn starch, though faces challenges due to the shortage of edible crops, but this can be substituted by using non-edible food crops and agro-waste. This would therefore facilitate proper farming for food security. Large amounts of agricultural waste are generated through human and animal activities and pose an environmental concern, a problem usually aggravated by biomass burning [26].

Several cellulosic materials widely used in SSF are sugarcane bagasse, wheat (bran and straw), rice (bran, straw, and husk) maize bran, soy hull, sawdust, and corncobs among others [16,27]. The choice of the substrate in a solid-state fermentation is dependent on many factors such as the cost of raw materials and substrate availability. In this study, we screened untreated and pretreated maize cob, cypress, and eucalyptus substrates for their suitability as carbon sources for cellulase enzyme production. The carbon source resulting in high cellulase production was used to produce enzymes for the saccharification of maize cobs for ethanol production using separate hydrolysis and fermentation technology.

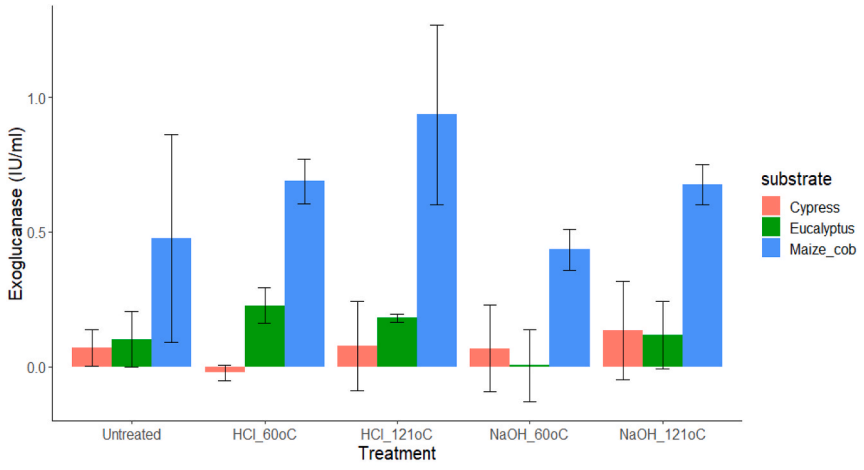
2. Materials and methods

2.1. Sampling area and technique

The sampling area for the present study was Karura forest reserve, located north of central Nairobi (−1.250916S 36.845914 E) along Kiambu road. Ten (10) fungal samples from decaying plant biomass were collected from different sites in the forest. The distribution of the sample units was carried out randomly by locating the sites with fungal species found growing on decaying plant biomass. The specimens were carefully collected, placed into sample envelopes, and labeled before transporting them to the Microbial Biotechnology Laboratory, Department of Biochemistry, Microbiology and Biotechnology at Kenyatta University.

2.2. Experimental design

To examine the performance of cypress, eucalyptus, and maize cobs substrates for the production of cellulase enzymes utilizing the three isolated fungi, a multifactorial experimental design was used in the study. The tests were done in triplicates for reproducibility. The three independent variables for this study were the three isolated fungi, three substrates, and four pretreatment methods while the dependent variables were cellulase enzyme produced and ethanol.



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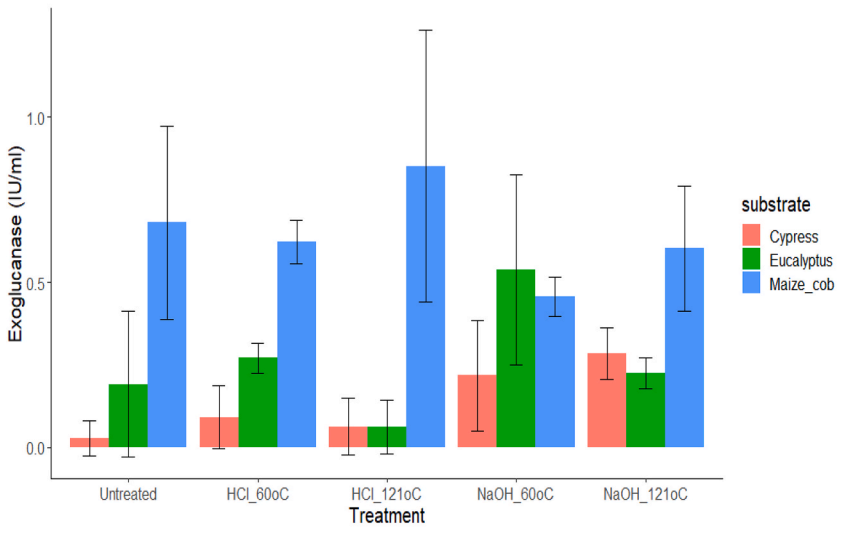

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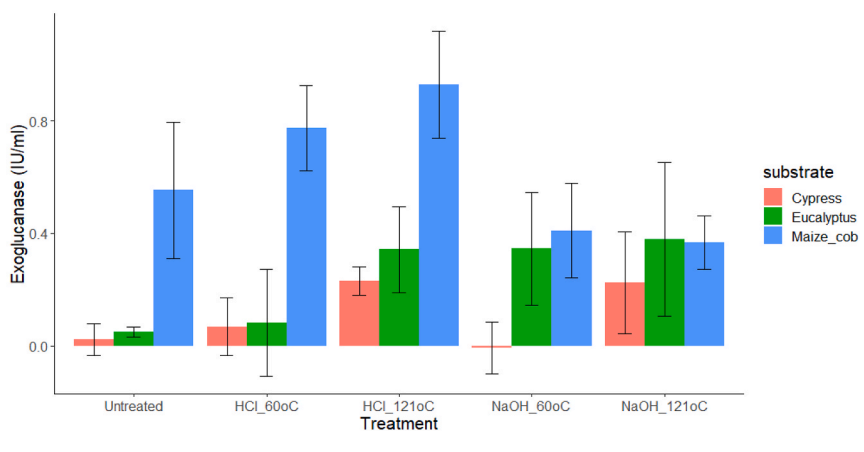

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Fig. 5. Exoglucanase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02, and (C)*Xylaria* sp. KM03 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

2.3. Isolation and screening of fungi

The fruiting body and hyphae of the mushrooms were cut into 0.5 mm by 0.5 mm in size and sterilized with 1.5 % sodium hypochlorite for 30 s, rinsed 5–7 times with sterile distilled water before culturing on screening media [28]. Fungal strains were selected on their morphological variations and were cultured on 1 % CMC-Congo red medium according to Legodi et al. [29]. The composition of the medium was 1 % Carboxymethylcellulose powder (CMC) 10(g/l), glucose 1(g/l), agar 15(g/l), and Congo red 0.1(g/l) were placed in 2 L of Erlenmeyer flask and made to a liter with distilled H_2O. Cultures were incubated at 25°C for 7 days and monitored daily to visualize the hydrolysis zones [28,30]. Three cultures with large clearing zones were selected for cellulase enzyme production. The cellulase-producing fungi were sub-cultured on potato dextrose agar (PDA). The medium was autoclaved at 121°C, 15 psi for 20 min. After cooling 2.5 ml of gentamycin antibiotic solution (100 mg/l) was added before dispensing into sterile petri dishes.

2.4. Culture maintenance

The selected fungal isolates were maintained on PDA by sub-culturing every 14 days and stored at 4 °C for future studies.

2.5. Molecular identification of fungi

Molecular characterization was based on the PCR amplification of the conserved nucleotide sequence of ribosomal internal transcribed spacer (ITS) using gene coding for the 18S, 5.8S, and 28S rRNA [31].

2.5.1. Fungal genomic DNA extraction

Mycelia of the three fungal strains were used for genomic DNA isolation by the Graham method.

[32]. Fungal mycelia were scraped with the help of a fine sterile scalpel followed by freezing in liquid nitrogen and suspended in 100 μl of extraction buffer solution [100 mM Tris-HCl, pH 8.0, 60 mM EDTA, pH8.0] in a sterile mortar and pestle and ground into fine powder. The powder was placed in 2000 μl microcentrifuge tubes and 400 μl of lysis buffer [400 mM Tris-HCl, pH 8.0, 60 mM EDTA, pH 8.0, 150 mM NaCl, 1 % sodium dodecyl sulfate] was added and the tube contents left at room temperature for 10 min. This was followed by adding 10 μl of Proteinase K [20 mg/ml], mixed gently, and incubated at 65 °C for 15 min in a water bath. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and centrifuged at 13,200 rpm for 5 min at 4 °C. The supernatant was transferred to a new tube and an equal volume of 3M Sodium acetate and isopropyl alcohol were added. The tubes were gently inverted 5 times. The tubes were centrifuged at 13,200 rpm for 10 min, and the supernatant was discarded. The resultant DNA pellet was washed with 300 μl of 70 % ethanol by spinning at 10,000 rpm for 1 min, the supernatant was discarded. The DNA pellet was air-dried and dissolved in 50 μl of 1 x Tris-EDTA, pH 8.0. The quality of DNA was checked on 1 % agarose gel.

2.5.2. PCR amplification

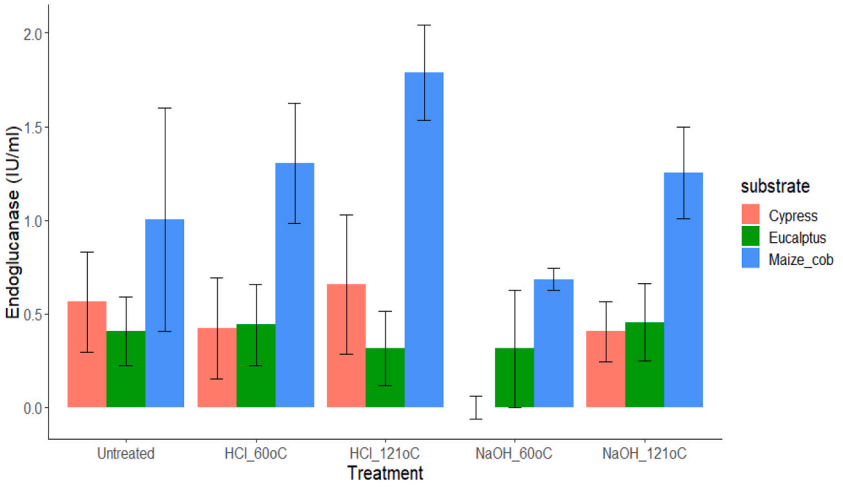
The internal transcribed spacer ITS target region coding gene for 5.8S rRNA was amplified using ITS4 (R) 5′TCCTCC GCT TAT TGA TAT GC 3′for the reverse strand and ITS86 (F)5′ GTG AAT CAT CGA ATC TTT GAA 3′ for the forward strand [31]. Amplification was done in 22 μl reaction mixture (3 μl of fungal DNA template, 0.4 μl Taq polymerase, and 4.0 μl buffer with dNTPs and MgCl2, 4 μl of both primers and 10.6 μl of PCR H_2O). The thermocycler was initiated with a denaturation process at 95°C for 3 min, followed by 32 cycles of second denaturation at 95°C for 30 s, annealing at 55°C for 2 min, and elongation at 72°C for 1 min. Final elongation was done at 72°C for 10 min before maintaining at 4°C [33]. The quality of amplicons was checked on 1.2 % agarose gel.

2.5.3. Sequencing of PCR amplicons

The amplified DNA samples were packed in 1.0 ml microcentrifuge tubes and sent to Microgen Inc. in the Netherlands for sequencing using the Sanger deoxy chain termination method [33].

2.5.4. Phylogenetic analysis

Phylogenetic analysis of the ITS region of fungal isolates with sequences of related strains obtained from NCBI BLAST was done with MEGA11 software package, [34]. The phylogenetic tree was created using a matrix of pairwise distances estimated using the Neighbor-Joining method [35]. The robustness of the tree topologies was evaluated by bootstrap analysis based on 1000 re-samplings of the sequence alignment.



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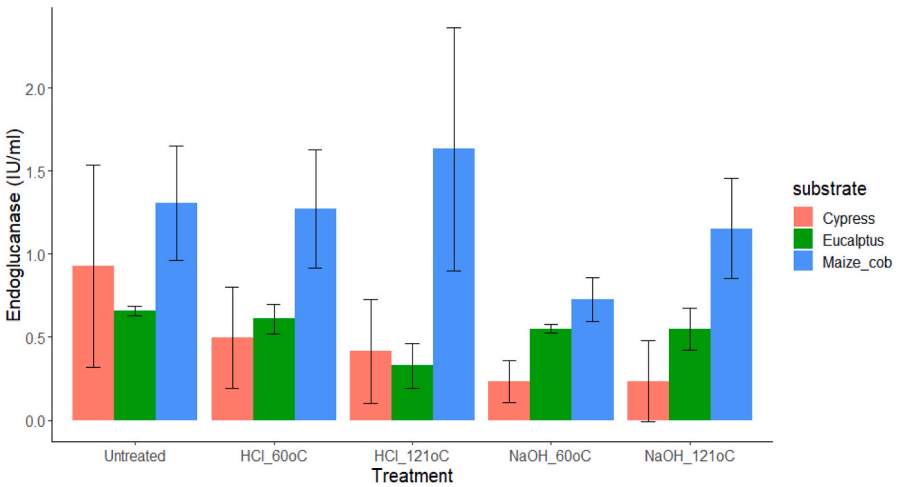

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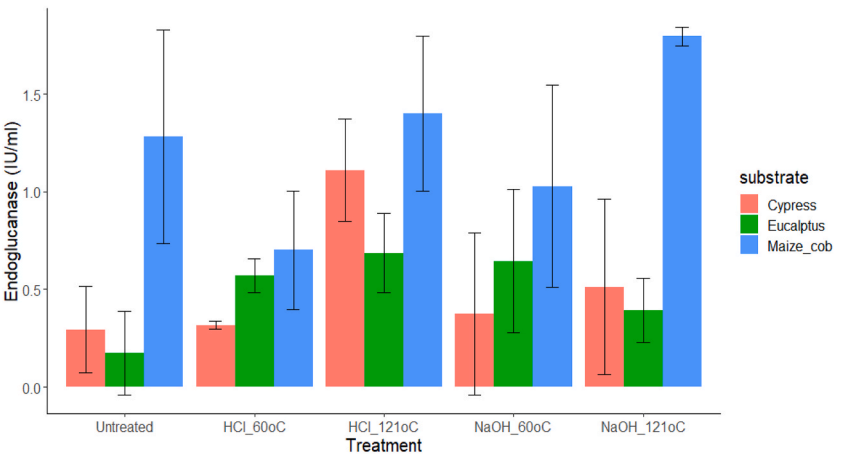

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Fig. 6. Endoglucanase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02, and (C)*Xylaria* sp. KM03 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

2.6. Solid-state fermentation (SSF)

Production of cellulases from three locally available substrates (maize cobs, eucalyptus, and cypress) as carbon sources was done in SSF. These substrates were dried, ground into fine powder by an electric mill, and sieved using a 2 mm sieve. Five (5) g of each substrate powder was weighed and dispensed into 350 ml bottles and 10 ml basal salt solution [1.25 g/l $NaNO_3$, 1.0 g/l KH_2PO_4, 0.05 g/l $CaCl_2 \bullet 6H_2O$, 0.1 g/l $MgSO_4 \bullet 7H_2O$, 0.1 g/l Nacl] was added to each bottle. The bottles were plugged with cotton wool and autoclaved at 121^o C, 15 psi for 15 min.

2.7. Inoculation of mycelium into SSF system

Inoculum was prepared from fungal cultures grown on a PDA medium after 7 days. Spores were suspended in 20 ml of 5 % sucrose solution. For fungi that did not sporulate, mycelium was scrapped from a PDA plate and blended in a sterilized blender for 30 s. The resulting mycelium extract was used as inoculum. Two (2) ml of the spore suspension or mycelia extract was transferred into each bottle in a biosafety cabinet, then thoroughly mixed with substrate and incubated at room temperature.

2.7.1. Effects of substrate pretreatment on cellulase enzyme production

Maize cob, cypress, and eucalyptus substrates were pretreated using 0.1M NaOH (w/v) and 0.1M HCl (v/v) at 60^{o}C for 2 h and at 121^{o}C (15 psi) for 15 min. After pretreatment, the substrates were washed with distilled water until neutral pH. The substrates were then dried at 70^{o}C until constant weight is achieved. The pretreated substrates were inoculated with the three cellulolytic fungi as described in inoculation section above (2.7).

2.7.2. Effects of incubation time on cellulase enzyme production

The SSF process was carried out for 15 days to determine the effect of incubation time on cellulase enzyme production. Samples were taken at 3-day intervals for cellulase enzyme determination. All assays were carried out in triplicates.

2.8. Cellulase extraction and assays

The crude enzyme was extracted by adding 50 ml of 50 mM citrate buffer pH 4.8 to all bottles. Culture bottles were vigorously shaken and left to stand at room temperature for 2 h. The mixture was sieved through muslin cloth and the filtrate collected, was centrifuged for 10 min at 10,000 rpm. The crude enzyme extract was used for cellulolytic enzyme assays, using the DNS method [36].

2.8.1. Total cellulase activity (FPase) (filter paper unit (FPU))

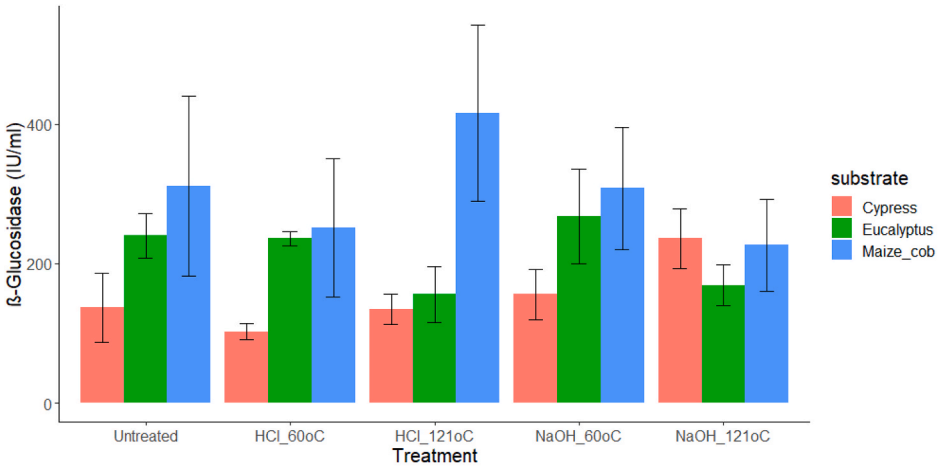
FPase activity was determined by a strip of Whatman's No.1 filter paper (1 cm × 6 cm) approximately 50 mg according to Ghose [37]. The mixture contained 500 μl of 5 mM citrate buffer pH 4.8, filter paper strip, and 500 μl of crude enzyme extract in 50 mM citrate buffer, pH 4.8, accordingly. The mixture was incubated at 50 °C for 1 h in a water bath. The released reducing sugar was estimated by the addition of 3,5-dinitrosalicylic acid (DNS) reagent (10 g of 3,5-dinitrosalicylic acid, 0.5g of sodium sulfite, 10 g of sodium hydroxide, and 2 g of phenol were placed in a 1000 ml volumetric flask and dissolved with distilled water up to 1000 ml mark) with glucose as standard. The mixture was boiled for 5 min followed by adding 300 μl sodium potassium tartrate (400 g of potassium sodium tartrate powder was placed in a 1000 ml volumetric flask and dissolved with distilled water) after cooling. Reducing sugars produced during the reaction were assayed in triplicates using a Jenway 6300 UV/Vis spectrophotometer at 540 nm. One unit of FPU was defined as the amount of enzyme required to release 1 μmol of glucose (reducing sugar equivalents) per minute from filter paper under the assay conditions.

2.8.2. Exoglucanase assay

Exoglucanase activity was determined according to the method described by Ghose [37]. The reaction mixture contained 500 μl of 1.25 % Avicel (microcrystalline) 0.1M Na acetate buffer, pH 5.0, and 500 μl of crude enzyme. The mixture was incubated at 50˚C for 30min and the released reducing sugar was estimated as indicated in the assay for FPase above. One unit of exoglucanase activity was defined as the amount of enzyme releasing 1 μmol of glucose (reducing sugar equivalents) per minute per unit from avicel under the assay conditions.

2.8.3. Endoglucanase assay

Endoglucanase activity was determined according to the method described by Ghose [37]. The reaction mixture contained 500 μl of



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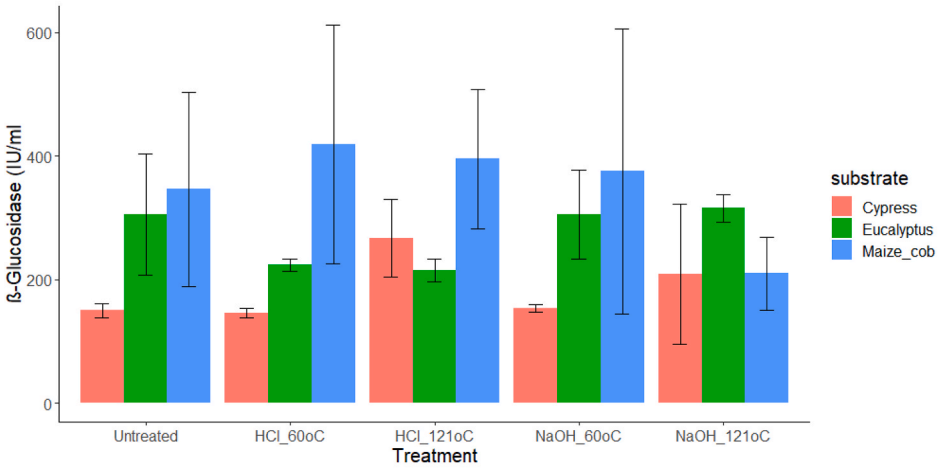

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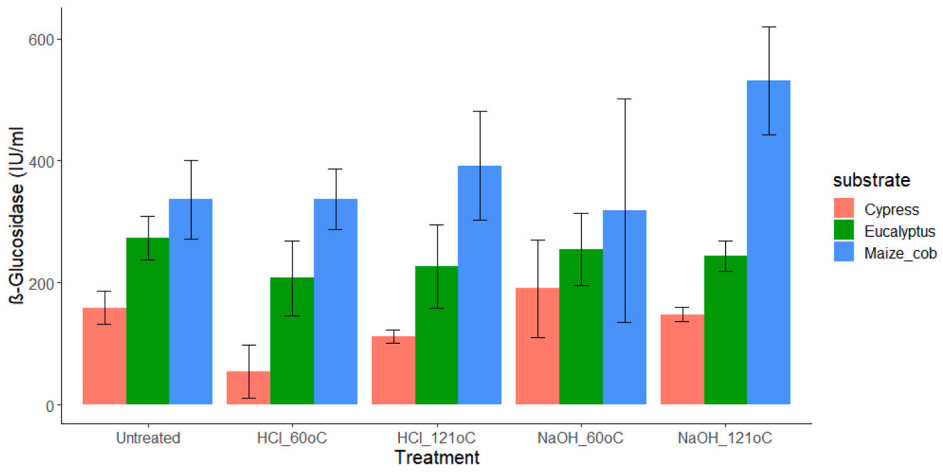

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Fig. 7. β-Glucosidase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02, and (C) *Xylaria* sp. KM03 cultured on pretreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

1 % CMC in 0.1M Na acetate buffer, pH 5.0, and 500 μl of crude enzyme. The mixture was incubated at 50˚C for 30 min and the released reducing sugar was estimated as indicated in the assay for FPase above. One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 μmol of glucose (reducing sugar equivalents) per minute per unit from CMC under the assay conditions.

2.8.4. *β glucosidase assay*

β-Glucosidase activity was determined according to the method described by Herr [38]. The reaction mixture contained 100 μl of 5 mM para-nitro phenol-β- D-glucopyranoside (pNPG) in 100 mM sodium acetate buffer, pH 4.8, 100 μl of crude enzyme solution, and 0.4 ml of 100 mM sodium acetate buffer, pH 4.8. The substrate control contained 0.2 ml of 5 mM pNPG prepared in 100 mM citrate buffer at pH 4.8 and 0.4 ml of 100 mM sodium acetate buffer, at pH 4.8. The mixture was incubated at 50 °C for 15 min in a water bath. The reaction was stopped by adding 800 μl ml of 50 mM NaOH-glycine buffer, pH 10.8. The enzyme activity indicated by the release of p-nitrophenol was determined at 420 nm using a Jenway 6300 UV/Vis spectrophotometer. One unit of β glucosidase activity was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol per minute under the same assay conditions.

2.9. *Concentration of crude enzymes for saccharification*

The crude cellulases of isolated fungal strains from maize cob fermentation of higher enzyme activity were extracted through sieving with muslin cloth and separated using a high-speed centrifuge (10,000×*g*, 20 °C, 10 min). The crude extract was concentrated five-fold by freeze-drying using a BIOBASE™ tabletop freeze dryer as described by Jasińska et al. [39],.and used for the hydrolysis process.

2.10. *Saccharomyces cerevisiae inoculum for ethanol production*

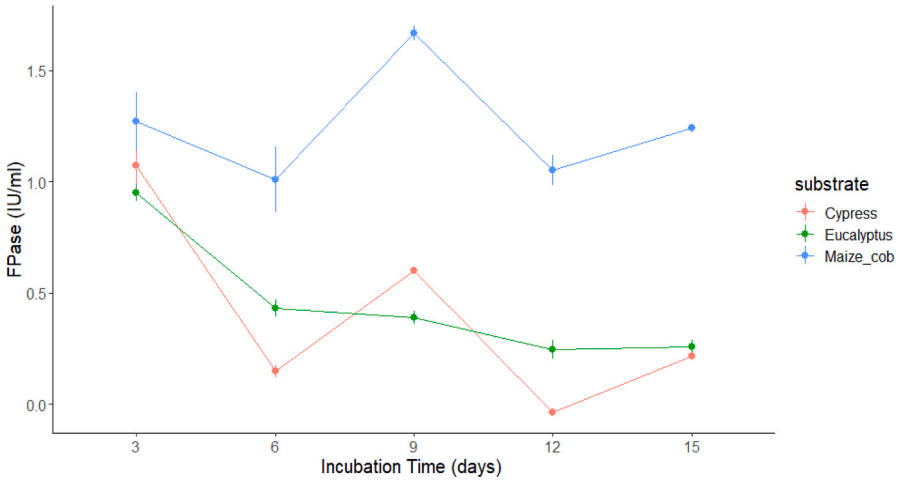
Baker's yeast, *Saccharomyces cerevisiae* was used in the fermentation process for ethanol production. Dried yeast (1 g) was inoculated into a 5 % glucose solution in a sterile 50 ml Erlenmeyer flask and incubated in a shaker (150 rpm) at 25^{o}C for 24 h. A loopful of the yeast colony was transferred on the agar plate and incubated at 25^{o}C for 24 h to check for viability and contamination, [40]. The total viable yeast cells were then determined using a Neubauer chamber with 0.4 % trypan blue stain [41]. The yeast cells loading concentration used in the maize cobs hydrolysate (20 ml) was adjusted to 1.0×10^{6} cells/ml.

2.11. *Saccharification of maize cobs with cellulases of isolated fungi*

Hydrolysis of maize cobs with crude cellulases of isolated fungi was done according to Mandels et al. [42]. Saccharification mixture comprised of untreated maize cobs, (4 %, 8 %, 18 %, & 20 % w/v) in 18 ml of citrate buffer at pH 4.8 and autoclaved at 121^{o}C for 15 min. After cooling, 1 ml of crude enzyme extract (5 FPU), 0.5 ml of gentamycin (200 mg/l), and 0.5 ml of griseofulvin (200 mg/l) to inhibit microbial growth were added to make a total volume of 20 ml in 50 ml falcon tubes. The saccharification mixture was incubated at 25 °C for 72 h with agitation at 150 rpm. Five (5) FPU (Catalog #C1184 Sigma-Aldrich) cellulase and 100 IU/ml β-glucosidase (Catalog # 49290 Sigma-Aldrich) enzymes were used as positive control while citrate buffer at pH 4.8 was used as negative control. Samples (1 ml) were withdrawn aseptically from each tube at 12-h intervals and analyzed for reducing sugar by the DNS method described by Miller [36], to determine the best time for the maximum reducing sugar production.

2.12. *Simultaneous saccharification and fermentation*

Simultaneous saccharification and fermentation were used for ethanol production in 50 ml falcon tubes. The fermentation mixture comprised untreated maize cobs (4 %, 8 %, 18 %, & 20 % w/v) and 18 ml of citrate buffer at pH 4.8 and autoclaved at 121^{o}C for 15 min. After cooling, 1 ml of crude enzyme extract (5 FPU, Catalog #C1184 Sigma-Aldrich), 0.5 ml of gentamycin (200 mg/l) to inhibit microbial growth, and 1 ml of *Saccharomyces cerevisiae* (1.0×10^{6} cells/ml) were added and incubated at 37 °C for 96 h with agitation at 150 rpm. Five (5) FPU (Catalog #C1184 Sigma-Aldrich) cellulase and 100 IU/ml β-glucosidase (Catalog # 49290 Sigma-Aldrich) enzymes were used as positive control while citrate buffer at pH 4.8 was used as negative control. Samples (0.5 ml) were collected aseptically at 24-h intervals, and the amount of ethanol produced was determined by the acidified potassium dichromate method described by Ref. [43]. Ethanol sample (500 μl) was collected from fermentation mixture in a 2 ml micro centrifuge tube and 500 μl acidified potassium dichromate reagent (20 g of potassium dichromate (VI) dissolved in 1000 ml volumetric flask) was added. Concentrated sulfuric acid (100 ml) was slowly added to the solution at 60^{o}C in a water bath. The mixture was diluted with 200 μl of sterile distilled water. Standard mixture was prepared from stock solution (20 mg/ml) at different concentrations (0.0, 0.2, 0.4, 0.8,



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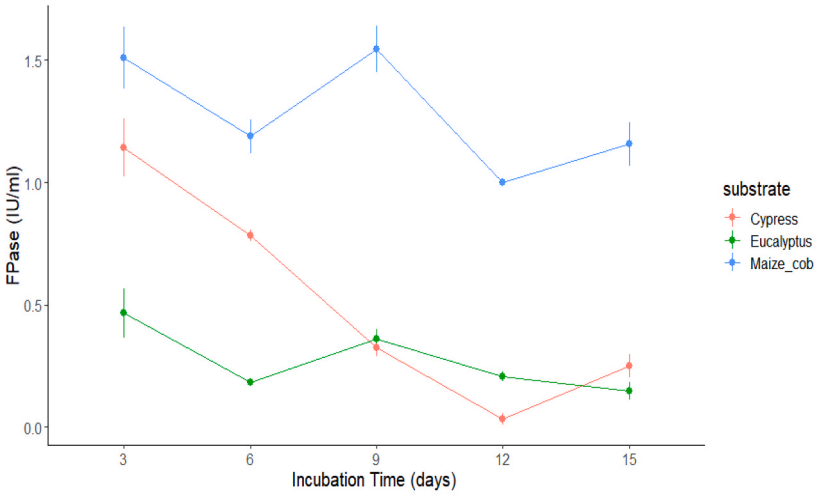

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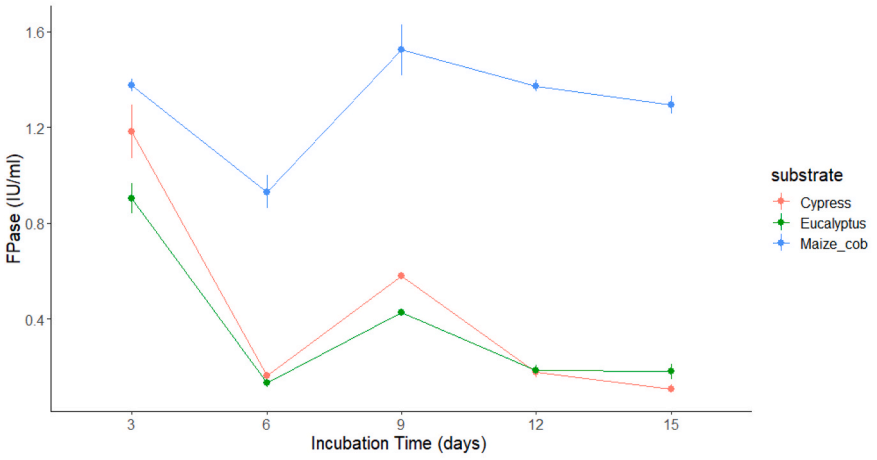

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Fig. 8. FPase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02 and (C) *Xylaria* sp. KM03 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

1.2, 1.6, and 2.0). The absorbance was measured at 600 nm using a Jenway 6300 UV/Vis spectrophotometer in triplicates and used in calculating the amount of ethanol (mg/ml) produced.

2.13. Data management and analysis

Enzyme activity absorbance data was converted into international units (IU/ml) using glucose and para-nitrophenol standard curves as described by Rahnama et al. [44]. Enzyme activity was calculated based on the following equations.

i) FPase, Exoglucanase and Endoglucanase (IU/ml)

$$= \frac{final\ abs - c}{m} \times \frac{df}{sample\ volume} \times \frac{1}{time} \times \frac{1000\ \mu g}{1\ mg} \times \frac{1 \mu mole}{180.16 \mu g}$$

ii) β-glucosidase (IU/ml)

$$= \frac{final\ abs - c}{m} \times \frac{df}{sample\ volume} \times \frac{1}{time} \times \frac{1000\ \mu g}{1\ mg} \times \frac{1 \mu mole}{139.1 \mu g}$$

Where c = intercept, m = slope obtained from glucose and para-nitro phenol standard curves, ab is the absorbance, and df is the dilution factor. Time is the reaction time and is expressed in minutes.

Ethanol yield obtained in triplicates was entered into an Excel spreadsheet and converted into mg/ml of ethanol. The data was imported in R software version 3.5.1 [45] and checked for normality using Shapiro-Wilk and equality of variance using Levene's test. Data in triplicates were statistically analyzed using one-way ANOVA at $P \leq 0.05$ significant level. Any significant differences in the factors affecting enzyme and ethanol production were determined by Tukey's HSD Post Hoc test. For molecular data analysis sequences were compared with the sequences in the nucleotide database (NCBI) using the BLAST technique. The MEGA software version X program was used for phylogenetic analysis and multiple alignments using CLUSTAL W [46]. Tables and figures were used to present the data.

3. Results

3.1. Isolation of cellulolytic fungi

The cellulolytic activities of the fungi were demonstrated on an agar medium containing 1 % carboxyl methyl cellulose and Congo red (Fig. 1). The ability to grow on a medium containing carboxyl methyl cellulose was taken as a measure of the cellulase activity of the fungi. This method has been used to measure the initial hydrolysis rate for cellulases using soluble cellulose derivatives [47,48], and is strongly recommended along with the determination of reducing sugar. The isolated fungi represent microorganisms obtained from decaying plant biomass from the Karura forest able to utilize and grow on lignocellulose [48]. These reports of their ability to utilize cellulose as a carbon source were confirmed in this project by their ability to exhibit growth on cellulolytic agrowastes such as sugarcane, cypress, eucalyptus, and maize cobs.

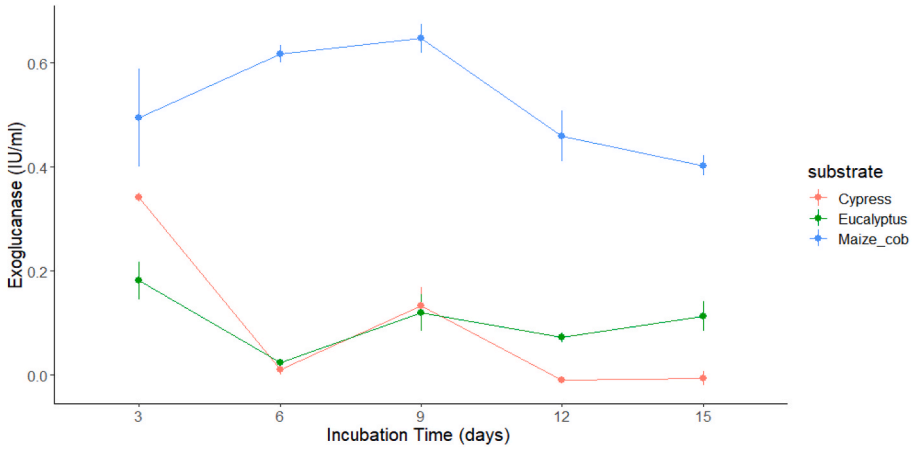
3.2. Molecular identification of the cellulolytic fungi

The fungal isolates were identified based on their molecular characteristics using ITS primers.

Fungal genomic DNA from the three fungi was isolated, amplified and amplicons sizes of 650 bp were obtained (Fig. 2). The fungal isolates were identified as; KM01, KM02 and KM03.

The sequence data of the isolated fungal strains were submitted to the NCBI Gene Bank database for comparison with available sequences using BLAST (Fig. 3).

The evolutionary history of *Xylaria* sp. KM01, *Xylaria* sp. KM03 and *Nemania* sp. KM02 were inferred using the Neighbor-Joining method [35]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches Felsenstein [49]. The evolutionary distances in the units of the number of base substitutions per site were computed using the Kimura 2-parameter method (Kimura M [50]. There was inclusion of 1st+2nd+3rd + Noncoding codon



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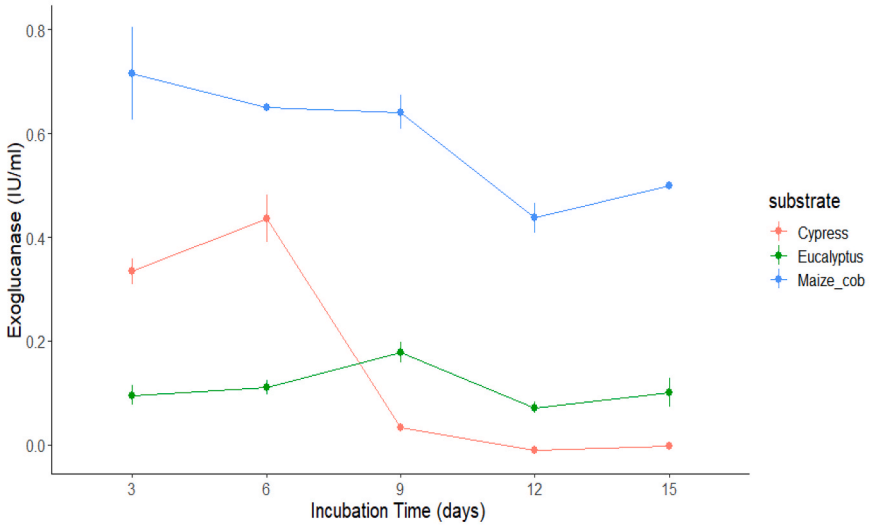

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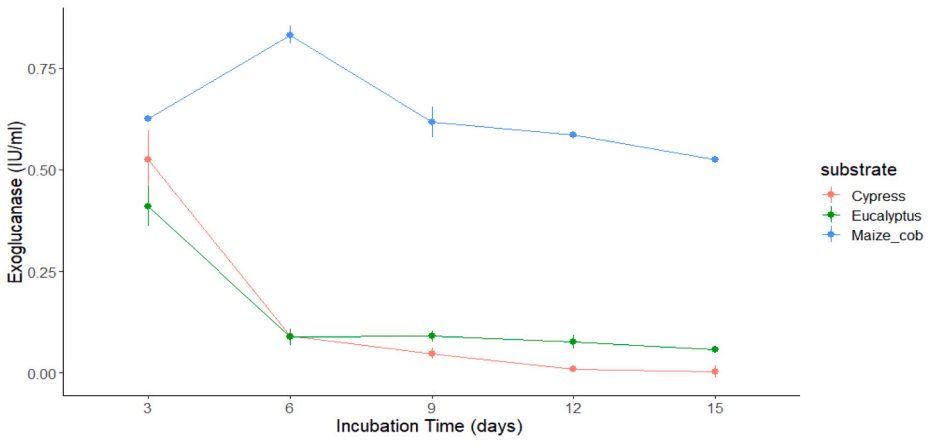

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Fig. 9. Exoglucanase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02 and (C) *Xylaria* sp. KM03 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

positions. The bootstrap values are represented by figures at the nodes [34,35,49]. MEGA11 was used for evolutionary analyses (Tamura et al., 2021).

3.3. Effect of substrate pretreatment on cellulase production by Xylaria sp. KM01, Nemania sp. KM02 and Xylaria sp. KM03

The effect of pretreatment on FPase, exoglucanase, endoglucanase and β-glucosidase was investigated for the three fungal strains. The pretreatment was done at 60°C for 1 h and 121°C for 15 min with 0.1M HCl and 0.1M NaOH on cypress, eucalyptus and maize cob substrates under the SSF process. The crude enzyme was extracted on the 5th day of fermentation. A quantitative evaluation of cellulase production by the selected fungi was assayed using filter paper, avicel, CMC and pNPG as commercial substrates to assess the effect of pretreatment on FPase, exoglucanase, endoglucanase and β-glucosidase for the three fungal strains.

3.3.1. Effect of substrates pretreatment on total cellulase activity (FPase)

Maximum FPase was observed after 5 days for all the fungal strains (Fig. 4). On maize cob, *Xylaria* sp. KM01 and *Nemania* sp. KM02 produced 2.01 ± 0.131 IU/ml and 1.69 ± 0.085 IU/ml when pretreated with 0.1MHCl at 121°C. *Xylaria* sp. KM03 produced 1.98 ± 0.033 IU/ml when pretreated with 0.1M NaOH at 121°C ($P < 0.05$). *Xylaria* sp. KM01 produced 6.84 ± 0.040 IU/ml with untreated cypress while *Xylaria* sp. KM03 produced 0.6 ± 0.050 IU/ml followed by *Nemania* sp. KM02 with 0.53 ± 0.096 IU/ml when cypress pretreated with 0.1M NaOH at 121°C.on eucalyptus, *Nemania* sp. KM02 produced 0.91 ± 0.065 IU/ml when pretreated with 0.1M NaOH at 60°C. *Xylaria* sp. KM03 produced 0.83 ± 0.036 IU/ml followed by *Xylaria* sp. KM01 with 0.6 ± 0.050 IU/ml when pretreated with 0.1M NaOH at 121°C.

3.3.2. Effect of substrate pretreatment on exoglucanase activity

The highest exoglucanase activity was recorded on maize cob for all the fungal strains (Fig. 5). *Xylaria* sp. KM01 and KM03 produced activities of 0.94 ± 0.077 IU/ml and 0.93 ± 0.044 IU/ml respectively, whereas *Nemania* sp. KM02 recorded 0.85 ± 0.096 IU/ml when pretreated with 0.1M HCl at 121°C. This was followed by eucalyptus with the highest recorded by *Nemania* sp. KM02 with 0.54 ± 0.6IU/ml when pretreated with 0.1M NaOH at 60°C, *Xylaria* sp. KM03 produced an amount of 0.38 ± 0.063 IU/ml when pretreated with 0.1M NaOH at 121°C and *Xylaria* sp. KM01 0.23 ± 0.015IU/ml when pretreated with 0.1M HCl at 60°C. The lowest activity noted on cypress of 0.28 ± 0.018 IU/ml produced by *Nemania* sp. KM02 when pretreated with 0.1M NaOH at 121°C, *Xylaria* sp. KM03 produced an amount of 0.23 ± 0.018 IU/ml when pretreated with 0.1M NaOH at 121°C, while *Xylaria* sp. KM01 produced 0.14 ± 0.042 IU/ml when pretreated with 0.1M NaOH at 121°C.

3.3.3. Effect of substrate pretreatment on endoglucanase activity

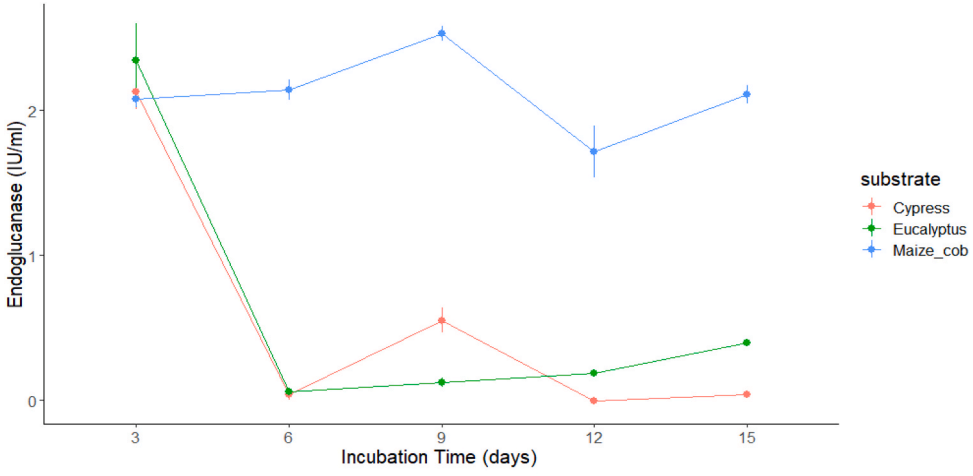
The production of endoglucanase activity was higher on maize cob for all the fungal strains (Fig. 6). *Xylaria* sp. KM01 and *Nemania* sp. KM02 produced activities of 3.58 ± 0.12 IU/ml and 3.26 ± 0.34 IU/ml respectively, whereas *Xylaria* sp. KM03 recorded 2.80 ± 0.19 IU/ml when pretreated with 0.1M HCl at 121°C. On cypress, *Xylaria* sp. KM03 recorded 2.22 ± 0.12 IU/ml when pretreated with 0.1M HCl at 121°C, whereas *Nemania* sp. KM02 produced 1.85 ± 0.28 IU/ml with untreated cypress. The lowest activity of 1.31 ± 0.17 IU/ml was noted for *Xylaria* sp. KM03 when pretreated with 0.1M HCl at 121°C.

3.3.4. Effect of substrate pretreatment on β-glucosidase activity

The levels of β-glucosidase produced vary among the fungal strains (Fig. 7). On maize cob, *Xylaria* sp. KM03, *Nemania* sp. KM02 and *Xylaria* sp. KM01 produced significantly higher activities of 588.6 ± 64.2 IU/ml when pretreated with 0.1M NaOH at 121°C, 418.7 ± 44.9 IU/ml when pretreated with 0.1M HCL at 60°C, 415.7 ± 29.4 IU/ml when pretreated with 0.1M HCL at 121°C respectively, compared to the other substrates tested ($P \leq 0.05$). On eucalyptus, *Xylaria* sp. KM03, *Nemania* sp. KM02 and Xylaria sp. KM01 produced activities of 298.5 ± 57.9 IU/ml when pretreated with 0.1M NaOH at 121°C, 295.2 ± 30.0 IU/ml when untreated and 267.9 ± 15.9 IU/ml when pretreated with 0.1M NaOH at 60°C. On cypress, *Nemania* sp. KM02, *Xylaria* sp. KM01 and KM03 produced activities of 244.4 ± 32.3 IU/ml when pretreated with 0.1M HCL at 121°C, 236.0 ± 10.0 IU/ml when pretreated with 0.1M NaOH at 121°C, 190.9 ± 18.6 IU/ml when pretreated with 0.1M NaOH at 60°C.

3.4. Effect of incubation time on cellulase production by Xylaria sp.KM01, Nemania sp. KM02 and Xylaria sp. KM03

The production of cellulase with changes in incubation time was also investigated in this study (Figs. 8–11). The fermentation was carried out at room temperature using cypress, eucalyptus and maize cob substrates under the SSF process. The crude enzyme was collected at 3-day intervals during fermentation. Quantitative estimation of cellulase produced by the selected fungi was assayed using filter paper, avicel, CMC and pNPG as commercial substrates to assess the effect of incubation time on FPase, exoglucanase,



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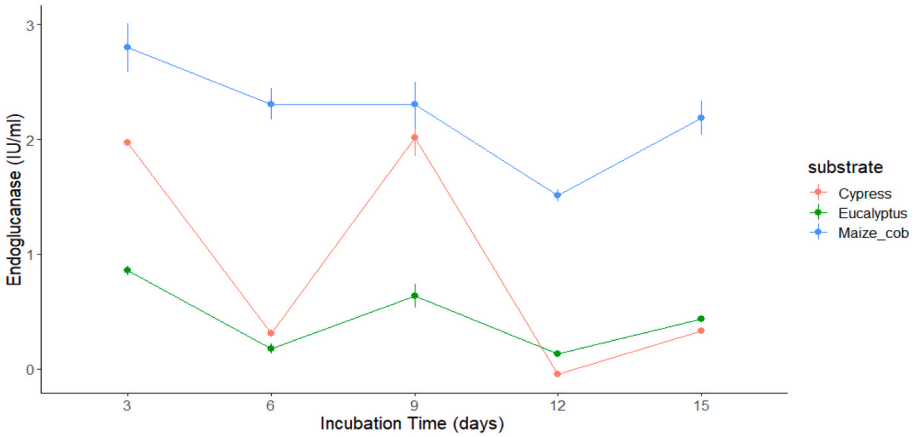

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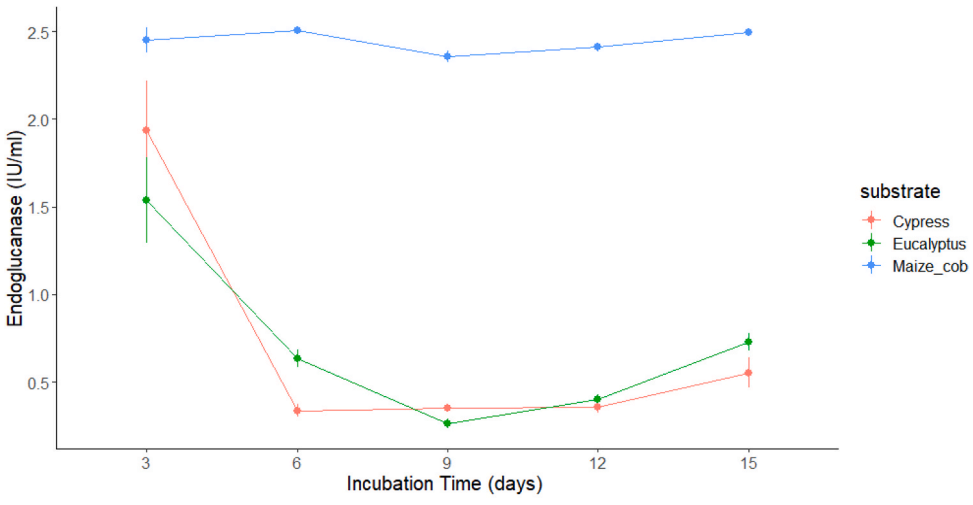

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Fig. 10. Endoglucanase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02 and (C) *Xylaria* sp. KM03 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

endoglucanase and β-glucosidase for the three fungal strains.

3.4.1. Effect of incubation time on total cellulase activity (FPase)

Maximum FPase activities of 1.67 ± 0.03 IU/ml by *Xylaria* sp. KM01, 1.54 ± 0.1 IU/ml by *Nemania* sp. KM02 and 1.52 ± 0.11 IU/ml by *Xylaria* sp. KM03 were obtained when cultured on maize cob on the 9th day of incubation ($P \leq 0.05$), compared to cypress with production amounts of 1.14 ± 0.12 IU/ml by *Nemania* sp. KM02, 1.18 ± 0.11 IU/ml by *Xylaria* sp. KM03 and 1.07 ± 0.09 IU/ml by *Xylaria* sp.KM01 and eucalyptus with production of 0.95 ± 0.04 IU/ml by *Xylaria* sp. KM01, 0.90 ± 0.06 IU/ml by *Xylaria* sp. KM03, 0.73 ± 0.14 IU/ml by *Nemania* sp. KM02 on the 3rd day of incubation respectively (Fig. 8).

3.4.2. Effect of incubation time on exoglucanase activity

The production of exoglucanase was higher on maize cob for all the fungal strains (Fig. 9). *Xylaria* sp. KM03 and KM01 produced higher activities of 0.83 ± 0.02 IU/ml and 0.62 ± 0.02 IU/ml on the 6th day of incubation respectively, whereas *Nemania* sp. KM02 produced activity of 0.72 ± 0.09 IU/ml on the 3rd day of incubation. On cypress, *Xylaria* sp. KM03 and *Nemania* sp. KM02 recorded 0.52 ± 0.073 IU/ml and 0.44 ± 0.05 IU/ml on the 6th day of incubation whereas *Xylaria* sp. KM01 recorded 0.34 ± 0.009 IU/ml on the 3rd day of incubation respectively. On eucalyptus, *Xylaria* sp. KM03 and KM03 recorded 0.41 ± 0.05 IU/ml and 0.18 ± 0.04 IU/ml on the 3rd day of incubation whereas *Nemania* sp. KM02 recorded 0.18 ± 0.00 IU/ml on the 9th day of incubation. *Xylaria* sp. KM03 recorded a steady decrease in activity after 3rd day of incubation on cypress and eucalyptus substrates.

3.4.3. Effect of incubation time on endoglucanase activity

The highest production of endoglucanase was also favored by the time of incubation for the three fungal strains (Fig. 10). On maize cob, *Nemania* sp. KM02 produced 2.80 ± 0.212 IU/ml on the 3rd day of incubation followed by *Xylaria* sp. KM01 with amounts of 2.53 ± 0.052 IU/ml on the 9th day of incubation and *Xylaria* sp. KM03 produced 2.51 ± 0.01 IU/ml on the 9th day of incubation respectively. On cypress, *Xylaria* sp. KM01 and KM03 produced 2.13 ± 0.12 IU/ml and 1.94 ± 0.28 IU/ml on the 3rd day of incubation respectively, whereas *Nemania* sp. KM02 produced 2.1 ± 0.16 IU/ml on the 9th day of incubation. On eucalyptus, *Xylaria* sp. KM01 and KM03 produced 2.4 ± 0.34 IU/ml and 1.54 ± 0.24 IU/ml, while *Nemania* sp. KM02 produced 0.7.28 ± 0.158 IU/ml on the 3rd day of incubation respectively. Both *Xylaria* sp. KM01 and KM03 recorded a steady decrease in activity after 3rd day of incubation on cypress and eucalyptus substrates.

3.4.4. Effect of incubation time on β-glucosidase activity

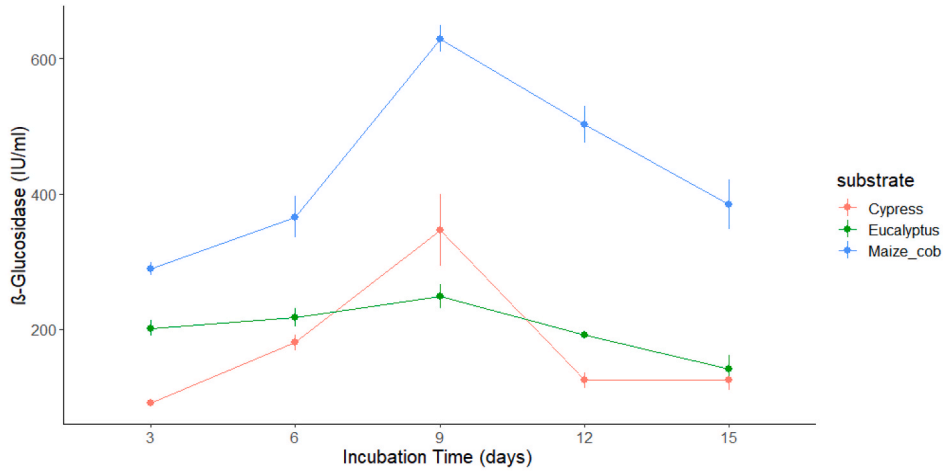
The levels of β-glucosidase produced vary among the fungal strains (Fig. 11). Generally, β-glucosidase activity was the highest on maize cob ($P \leq 0.05$). *Xylaria* sp. KM01 produced higher activity of 629.7 ± 20.2 IU/ml on the 9th day of incubation followed by *Xylaria* sp. KM03 and *Nemania* sp. KM02 with amounts of 587.2 ± 29.7 IU/ml and 455.9 ± 28.9 IU/ml on the 12th day of incubation respectively. On cypress, *Xylaria* sp. KM01 and *Nemania* sp. KM02 produced 346.3 ± 53.2 IU/ml and 222.5 ± 19.6 IU/ml on the 9th day of incubation respectively. *Xylaria* sp. KM01 produced 243.1 ± 43.3 IU/ml on the 12th day of incubation. On eucalyptus, *Xylaria* sp. KM03 produced 396.0 ± 19.1 IU/ml on the 12th day of incubation, *Nemania* sp. KM02 produced 284.5 ± 17.4 IU/ml on the 6th day of incubation and *Xylaria* sp. KM01 produced 248.7 ± 17.3 IU/ml on the 9th day of incubation respectively.

3.5. Saccharification of maize cobs with cellulases of isolated fungi

The results show the amount of sugars during the fermentation period (Table 1). Maize cob hydrolysis by crude cellulases of isolated fungi produced higher reducing sugars. At 8 % concentration, cellulases of *Xylaria* sp. KM03 produced the highest amounts of sugar at 10.17 ± 0.37 mg/ml, *Xylaria* sp. KM01 produced yields of 9.48 ± 0.13 mg/ml, whereas *Nemania* sp. KM02 produced 9.41 ± 0.27 mg/ml after 72 h of fermentation period. It was observed that the sugar yield increased steadily reaching the peak after 72 h of fermentation at all the concentrations.

3.6. Ethanol production from maize cob using simultaneous hydrolysis and fermentation

Table 2 shows the amount of ethanol yield (mg/ml) produced from untreated maize cobs hydrolysate of different concentrations fermented with saccharomyces cerevisiae during the fermentation period (Table 2). Hydrolysis of maize cobs by cellulases of *Xylaria* sp. KM01, *Nemania* sp. KM02 and *Xylaria* sp. KM03 recorded higher amounts of ethanol after 96 h of fermentation. At 8 % concentration, cellulases of Xylaria sp. KM03 produced the highest amounts of ethanol at 28.59 ± 1.58 mg/ml, Nemania sp. KM01 produced yields of 28.72 ± 3.82 mg/ml, whereas *Xylaria* sp. KM02 produced 25.75 ± 0.21 mg/ml after 96 h of fermentation period. It was



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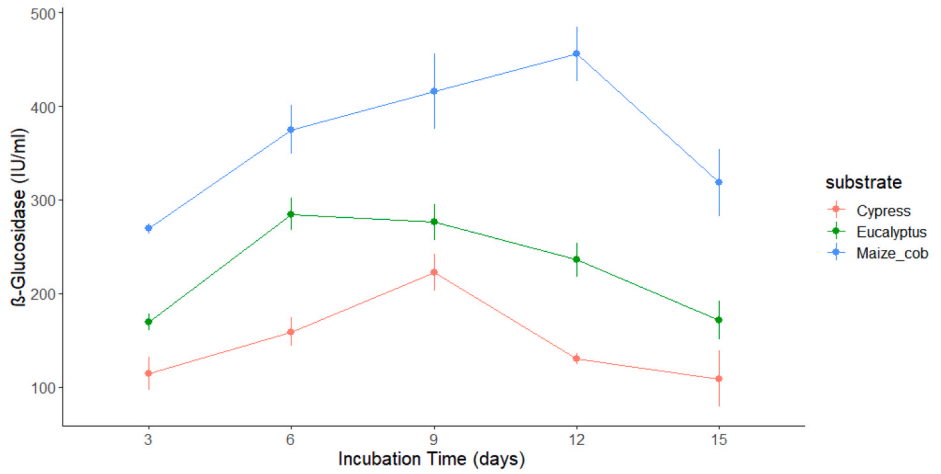

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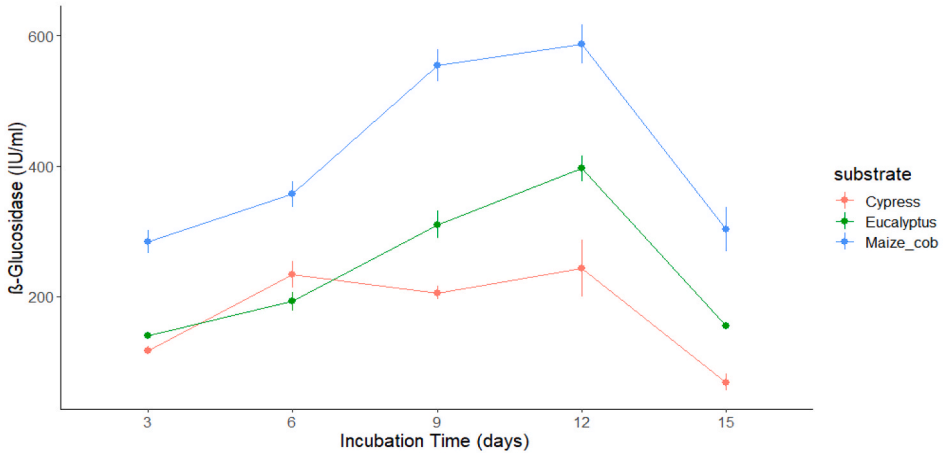

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Fig. 11. β-Glucosidase Exoglucanase activities of (A) *Xylaria* sp. KM01, (B) *Nemania* sp. KM02 and (C) *Xylaria* sp. KM03 cultured on untreated cypress, eucalyptus and maize cob substrates. Values are means of three replicates ± SEM.

Table 1
Sugar yield (mg/ml) from hydrolysis of untreated maize cobs of different concentration by cellulases of isolated fungi.

		Total reducing sugar (mg/ml)					
		(Maize cob concentration %)					
		4	8	18	20	Control (+)	Control (−)
Fungi	Time (Hrs)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Xylaria sp. KM01	12	$2.35 \pm 0.11b^{cC}$	4.12 ± 1.21^{aB}	3.58 ± 0.40^{abC}	4.03 ± 0.48^{aD}	4.53 ± 0.035^{aD}	1.59 ± 0.05^{cD}
	24	3.17 ± 0.73^{cC}	8.21 ± 0.36^{aA}	6.84 ± 0.13^{bB}	6.42 ± 1.37^{bC}	7.32 ± 0.45^{abC}	1.85 ± 0.04^{dC}
	36	$7.54 \pm 0.36b^{cB}$	8.78 ± 0.13^{aA}	7.19 ± 0.2^{bcB}	6.76 ± 0.03^{cBC}	7.79 ± 0.55^{bBC}	1.88 ± 0.00^{dBC}
	48	$8.37 \pm 0a^{AB}$	8.89 ± 0.00^{aA}	7.55 ± 0.08^{abB}	6.89 ± 0.06^{bBC}	8.25 ± 0.86^{aBC}	1.90 ± 0.00^{cBC}
	60	8.51 ± 0.16^{abA}	9.17 ± 0.04^{aA}	8.37 ± 0.53^{bA}	7.14 ± 0.23^{cAB}	8.84 ± 0.38^{abB}	1.95 ± 0.00^{dB}
	72	8.83 ± 0.07^{cA}	9.48 ± 0.13^{bA}	8.84 ± 0.08^{cA}	7.73 ± 0.28^{dA}	11.21 ± 0.29^{aA}	2.09 ± 0.00^{eA}
Nemania sp. KM02	12	2.99 ± 0.11^{cD}	5.51 ± 0.30^{aD}	4.63 ± 0.45^{bC}	4.40 ± 0.05^{bD}	2.10 ± 0.07^{dD}	1.63 ± 0.00^{dD}
	24	3.50 ± 0.38^{bcD}	6.40 ± 0.21^{aC}	5.82 ± 0.03^{aBC}	4.63 ± 0.01^{bCD}	3.40 ± 0.91^{cD}	1.90 ± 0.00^{dC}
	36	5.12 ± 0.73^{bcC}	7.10 ± 0.37^{aC}	6.00 ± 0.14^{bBC}	4.76 ± 0.06^{cC}	5.84 ± 0.23^{bC}	1.90 ± 0.00^{dC}
	48	6.41 ± 0.43^{bB}	8.52 ± 0.12^{aB}	6.20 ± 0.00^{bcBC}	5.19 ± 0.04^{cB}	6.70 ± 0.37^{bC}	1.90 ± 0.00^{dC}
	60	7.73 ± 0.62^{abA}	9.13 ± 0.44^{aAB}	6.88 ± 0.70^{bcB}	5.50 ± 0.20^{cB}	9.18 ± 1.40^{aB}	2.14 ± 0.00^{dB}
	72	8.73 ± 0.12^{bA}	9.41 ± 0.27^{bA}	8.67 ± 0.29^{bA}	6.07 ± 0.21^{cA}	11.69 ± 0.56^{aA}	2.23 ± 0.00^{dA}
Xylaria sp. KM03	12	3.39 ± 0.35^{dE}	7.15 ± 0.15^{aE}	5.25 ± 0.27^{bD}	4.31 ± 0.02^{cC}	2.53 ± 0.62^{dE}	1.63 ± 0.00^{eE}
	24	4.27 ± 0.47^{cDE}	7.51 ± 0.13^{aDE}	6.13 ± 0.20^{bC}	4.41 ± 0.08^{cC}	4.76 ± 0.12^{cD}	1.88 ± 0.00^{dD}
	36	5.39 ± 0.57^{cCD}	7.83 ± 0.15^{aD}	6.55 ± 0.09^{bcBC}	5.66 ± 0.91^{cB}	7.38 ± 0.12^{abC}	1.90 ± 0.00^{dC}
	48	6.44 ± 0.32^{cBC}	8.56 ± 0.28^{bC}	6.67 ± 0.01^{cBC}	6.45 ± 0.12^{cAB}	9.75 ± 0.14^{aB}	1.90 ± 0.00^{dC}
	60	6.92 ± 0.28^{cAB}	9.19 ± 0.12^{bB}	6.94 ± 0.19^{cB}	6.57 ± 0.00^{cAB}	10.21 ± 0.40^{aB}	1.93 ± 0.00^{dB}
	72	8.13 ± 0.64^{cA}	10.17 ± 0.37^{bA}	9.07 ± 0.50^{cA}	6.82 ± 0.17^{dA}	12.81 ± 0.24^{aA}	2.14 ± 0.00^{eA}

Table 1 shows amount of Sugar yield (mg/ml) from hydrolysis of untreated maize cobs of different concentration with partially purified cellulases of fungal isolates. Values are means of three replicates ± SEM and are expressed as mg/ml. Means expressed with different superscript capital letters in the same column are significantly different. Means expressed with different superscript small letters in the same row are significantly different at $P < 0.05$.

Table 2
Ethanol yield (mg/ml) produced from untreated maize cob hydrolysate fermented with *saccharomyces cerevisiae*.

		Ethanol yield (mg/ml)					
		(Maize cob concentration %)					
		4	8	18	20	Control (+)	Control (−)
Fungi	Time(Hrs)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Xylaria sp. KM01	12	13.22 ± 1.66^{bF}	13.35 ± 1.15^{bF}	14.62 ± 0.46^{bD}	9.03 ± 2.14^{cF}	18.62 ± 0.46^{aE}	8.25 ± 0.00^{cG}
	24	17.20 ± 1.31^{bE}	16.32 ± 1.74^{bE}	15.93 ± 0.50^{bCD}	14.33 ± 0.69^{bE}	24.82 ± 3.66^{aD}	8.59 ± 0.04^{cF}
	36	18.71 ± 0.12^{bDE}	18.75 ± 0.61^{bDE}	16.76 ± 0.07^{bcC}	15.89 ± 0.55^{cDE}	29.65 ± 0.62^{aC}	8.59 ± 0.00^{dF}
	48	20.52 ± 0.50^{bCD}	21.00 ± 0.42^{bCD}	17.04 ± 0.09^{cC}	17.07 ± 1.03^{cCD}	30.90 ± 0.64^{aC}	8.93 ± 0.00^{dE}
	60	21.42 ± 0.17^{bBC}	22.11 ± 0.17^{bC}	19.17 ± 0.79^{cB}	19.17 ± 0.69^{cBC}	33.31 ± 1.36^{aC}	9.61 ± 0.00^{dD}
	72	22.05 ± 0.33^{bBC}	23.15 ± 0.69^{bBC}	20.23 ± 0.39^{cB}	20.23 ± 0.17^{cAB}	37.67 ± 0.55^{aB}	9.73 ± 0.00^{dC}
	84	23.20 ± 0.74^{bAB}	24.29 ± 0.66^{bAB}	21.06 ± 0.46^{cB}	21.12 ± 0.48^{cAB}	38.66 ± 0.54^{aB}	9.90 ± 0.00^{dB}
	96	24.61 ± 0.72^{bA}	25.75 ± 0.21^{bA}	24.40 ± 1.47^{bA}	22.54 ± 0.41^{bA}	43.09 ± 1.12^{aA}	10.24 ± 0.00^{cA}
Nemania sp. KM02	12	13.06 ± 1.43^{cG}	16.32 ± 1.15^{bE}	14.47 ± 0.23^{bcF}	10.39 ± 0.07^{dF}	23.81 ± 1.18^{aE}	7.51 ± 0.00^{eE}
	24	14.96 ± 0.51^{cF}	17.88 ± 1.74^{bDE}	16.82 ± 0.14^{bEF}	11.13 ± 0.69^{dF}	25.65 ± 0.59^{aE}	7.51 ± 0.04^{eE}
	36	16.40 ± 0.31^{cEF}	18.62 ± 0.26^{bCDE}	17.65 ± 0.23^{bDE}	12.10 ± 0.58^{dEF}	28.57 ± 0.72^{aD}	7.79 ± 0.00^{eD}
	48	17.39 ± 0.26^{dDE}	20.38 ± 0.82^{bBCDE}	18.62 ± 0.14^{cCDE}	13.37 ± 0.21^{eDE}	29.54 ± 0.53^{aD}	8.25 ± 0.00^{fC}
	60	18.47 ± 0.63^{bCD}	21.75 ± 0.40^{bBCD}	19.26 ± 0.39^{cCD}	14.49 ± 0.32^{dCD}	30.75 ± 0.17^{aCD}	8.53 ± 0.00^{eB}
	72	19.77 ± 0.16^{cBC}	22.69 ± 0.46^{bBC}	20.67 ± 0.76^{cBC}	15.74 ± 0.89^{cBC}	32.32 ± 0.96^{aC}	8.70 ± 0.00^{dA}
	84	20.40 ± 0.43^{cAB}	24.10 ± 0.43^{bB}	22.90 ± 1.31^{bcB}	17.44 ± 0.71^{dB}	35.27 ± 1.86^{aB}	8.70 ± 0.00^{eA}
	96	21.65 ± 1.79^{cA}	28.72 ± 3.82^{bA}	28.66 ± 1.79^{bA}	20.02 ± 1.14^{cA}	43.08 ± 0.41^{aA}	8.70 ± 0.00^{dA}
Xylaria sp. KM03	12	11.09 ± 0.55^{bE}	15.24 ± 0.69^{aF}	7.85 ± 1.08^{cF}	9.86 ± 1.11^{bcE}	17.04 ± 1.20^{aD}	5.23 ± 0.00^{dG}
	24	13.06 ± 0.16^{cD}	18.37 ± 0.16^{bE}	11.00 ± 2.09^{cdE}	11.83 ± 0.64^{cdDE}	23.23 ± 1.82^{aC}	8.59 ± 0.00^{dF}
	36	13.60 ± 0.16^{cD}	20.06 ± 0.17^{bDE}	13.80 ± 0.32^{cDE}	$13{,}73 \pm 0.33^{cCDE}$	26.13 ± 0.60^{aBC}	8.70 ± 0.00^{dE}
	48	14.28 ± 0.12^{dCD}	20.91 ± 0.48^{bCD}	15.93 ± 1.10^{cCD}	14.60 ± 0.43^{cdCD}	27.07 ± 0.20^{aBC}	8.99 ± 0.00^{eD}
	60	15.45 ± 0.52^{dC}	22.13 ± 0.41^{bBCD}	18.34 ± 1.24^{cBC}	15.24 ± 0.10^{dCD}	28.21 ± 0.68^{aBC}	9.73 ± 0.00^{eC}
	72	16.72 ± 0.64^{dB}	22.79 ± 0.17^{bBC}	20.04 ± 0.09^{cAB}	16.46 ± 0.42^{dBC}	31.95 ± 1.06^{aAB}	9.73 ± 0.00^{eC}
	84	18.17 ± 0.27^{cA}	24.04 ± 0.71^{bB}	20.69 ± 0.39^{cAB}	20.82 ± 1.53^{bcB}	35.72 ± 2.38^{aA}	10.18 ± 0.00^{dB}
	96	19.19 ± 0.66^{dA}	28.59 ± 1.58^{bA}	22.49 ± 1.15^{cdA}	27.02 ± 4.04^{bcA}	38.54 ± 1.26^{aA}	10.30 ± 0.00^{eA}

Table 2 shows the amount of ethanol yield (mg/ml) produced from untreated maize cob hydrolysate of different concentration fermented with saccharomyces cerevisiae. Values are means of three replicates ± SEM and are expressed as mg/ml. Means expressed with different superscript capital letters in the same column are significantly different at $P < 0.05$. Means expressed with different superscript small letters in the same row are significantly different at $P < 0.05$.

Table 3
Ethanol yield (%) produced from glucose (synthetic medium) and corn cob (complex medium) hydrolysate by cellulase of *A niger* fermented with *saccharomyces cerevisiae*.

Fermentation time (Hrs)	Ethanol yield (%) (Glucose)		(Corn cobs)	
	1 %	10 %	1 %	10 %
24	1.76	3.47	2.21	2.77
48	2.38	5.21	3.44	4.80
72	3.45	6.23	4.17	6.17
96	2.85	6.01	3.89	5.11
120	2.67	5.62	3.57	4.89

(adapted from Ref. [51]).

observed that there was a steady increase in ethanol yield with the maximum produced after 96 h of fermentation at all the concentrations. Table 3, adapted from Ref. [51] shows a comparison with a similar trend where at all concentrations of substrates, the ethanol yield increased steadily and recorded maximum yield after 72 h of fermentation. This shows that corn cobs can be one of the cheaper substrates for ethanol production.

4. Discussion

Fungal species are mostly used in cellulase production because of their capacity to provide large amounts of extracellular cellulases using cheap lignocellulose substrates. Cellulase enzyme production occurs through submerged fermentation or solid-state fermentation. The cost of cellulase enzymes prevents the economical application of ethanol production from lignocellulose. The cost could be reduced by culturing cellulase-producing fungi isolated from the local environment on cheap substrates. Our research was aimed at isolating and characterizing local fungi from the Karura forest for the production of cellulase enzymes using locally available substrates such as maize cobs, cypress and eucalyptus and subsequently used for saccharification of maize cobs for ethanol production using Saccharomyces cerevisiae. Considering the advantages of the solid-state fermentation process, we investigated several parameters that influence cellulase production, such as (i) time of incubation, (ii) pretreatment and (iii) substrate loading on maize cobs saccharification for ethanol production.

4.1. Isolation and screening of fungi

Ten (10) fungal samples from decaying plant biomass were collected from different sites in the forest. Growth on 1 % CMC Congo red agar was used for the isolation of cellulolytic fungal strains in this study. Three fungal strains were selected based on fast and abundant growth on CMC agar plates. This method provided a relatively easy tool for the specific detection of fungi that produce cellulase activity. A similar result was obtained by Ref. [29,48] where the isolated fungi were able to grow and secrete endoglucanase which hydrolyzed CMC bound to CR dye. This was revealed by appearance of pale yellow “halo zone” around the fungal growth, an indication of CMC hydrolysis.

4.2. Molecular identification of cellulase producing fungi

Molecular identification was based on the detection of conserved sequences in the 5.8S rRNA region and the amplification of the ITS2 region [31]. ITS4 R and ITS86 F primers were used to identify these fungal isolates by comparing sequences to those in NCBI databases using BLAST with accuracy ranging from 98 % to 100 %. The three fungal isolates were identified as Xylaria sp. KM01 (Genebank accession number: ON679521), Nemania sp. KM02 (Genebank accession number: ON678279), and Xylaria sp. KM03 (Genebank accession number: ON704639), (ncbi.nlm.nih.gov/blast)

4.3. Effect of pretreatment on cellulase production

Production of cellulase enzymes from complex lignocellulose is not significantly achieved by disrupting the recalcitrant structure through ordinary microbial attack. Therefore, the selection of a simple and effective method for treating lignocellulose materials should be considered. Several types of treatments were investigated for cellulose hydrolysis including acid and alkali pretreatments [52–56]. The proper mechanism is to attack hemicellulose for dissolution to initiate the loosening of the complex structure of lignocellulose.

A higher yield of FPase activity was produced by the three fungi on pretreated maize cob, *Xylaria* sp. KM01 produced the highest enzyme activity (2.11 ± 0.13 IU/ml) when pretreated with HCl at 121 °C. *Xylaria* sp. KM03 (1.98 ± 0.033 IU/ml) when pretreated with NaOH at 121°C *Nemania* sp. KM02 (16.9 ± 0.85 IU/ml) when pretreated with HCl at 121°C. Kakde et al. [57] reported similar findings when corn cob was pretreated with 5 % (w/v) HCl autoclaved at 121 °C for 60 min recorded maximum cellulase production with 3.46 FPU/ml. This was contrary to *Xylaria* sp. KM03 which recorded the maximum activity when pretreated with 0.1M NaOH at 121°C. A similar finding was reported by Ojumu et al. [58] where cellulase activity by *Penicillium decumbens* was recorded significantly

higher on corn cob pre-treated with dilute alkali (NaOH).

Higher production of exoglucanase activities were produced by the three fungi on pretreated maize cob when pretreated with HCl at 121°C. *Xylaria* sp. KM01 produced the highest activity (0.94 ± 0.077 IU/ml), *Xylaria* sp. KM03 (9.28 ± 0.44 IU/ml) and *Nemania* sp. KM02 (0.85 ± 0.1 IU/ml), respectively [57]. reported similar findings when corn cob was pretreated with 5 % (w/v) HCl autoclaved at 121 °C for 60 min recorded maximum cellulase production with 3.46 FPU/ml.

Higher amounts of endoglucanase activity were produced by the three fungi on pretreated maize cob, *Xylaria* sp. KM03 produced the highest enzyme activity (3.59 ± 0.022 IU/ml) when pretreated with NaOH at 121°C. *Xylaria* sp. KM01 (3.58 ± 0.19 IU/ml) and *Nemania* sp. KM02 (3.26 ± 0.34 IU/ml) when pretreated with HCl at 121°C respectively. Kakde et al. [57] reported similar findings when corn cob was pretreated with 5 % (w/v) HCl autoclaved at 121 °C for 60 min recorded maximum cellulase production with 3.46 FPU/ml This was contrary to *Xylaria* sp. KM03 which recorded the maximum activity when pretreated with 0.1M NaOH at 121°C. A similar finding was reported by Ojumu et al. [58] where cellulase activity by *Penicillium decumbens* was recorded significantly higher on corn cob pre-treated with dilute alkali (NaOH).

Higher levels of β-glucosidase activity were produced by the three fungi on pretreated maize cobs, *Xylaria* sp. KM03 produced the highest enzyme activity (588.6 ± 64.2 IU/ml) when pretreated with NaOH at 121°C. *Nemania* sp. KM02 (418.7 ± 44.9 IU/ml) when pretreated with HCl at 60°C, and *Xylaria* sp. KM01 (415.7 ± 29.4 IU/ml) when pretreated with HCl at 121°C. Kakde et al. [57] reported similar findings when corn cob was pretreated with 5 % (w/v) HCl autoclaved at 121 °C for 60 min recorded maximum cellulase production with 3.46 FPU/ml. This was contrary to *Xylaria* sp. KM03 which recorded the maximum activity when pretreated with 0.1M NaOH at 121°C for 15 min. A similar finding was reported by Ojumu et al. [58] where cellulase activity by *Penicillium decumbens* was recorded significantly higher on corn cob pre-treated with dilute alkali (NaOH). In this study acid pretreatment was the most effective method for enzyme production as compared to alkali pretreatment. The possible mechanism involves the dissolution of hemicellulose causing loosening of the structure of raw material, which makes acid pretreatment an important method for the production of enzymes ([53,59]; Pandey et al. [60].

4.4. Effect of time of incubation on cellulase production

The optimal time of incubation for cellulase enzyme production depends on the type of substrate used and the fungal species involved [61]. The time of incubation significantly influenced cellulase enzyme activities from the 3rd day of incubation. The highest FPase activity for all three fungal strains were recorded on maize cob. *Xylaria* sp. KM01 produced the highest activity (1.67 ± 0.034 IU/ml), *Nemania* sp. KM02 (1.54 ± 0.01 IU/ml) and *Xylaria* sp KM03 (1.52 ± 0.11 IU/ml) on the 9th day of incubation respectively. These results were similar to Gautam et al., 2011 findings on *Aspergillus niger* which showed the highest cellulase activities were recorded after 5 days of incubation period cultured on a corn cob. However, contrary when they recorded a trend in which cellulase activities by *Trichoderma viride* cultured on corn cob peaked on the 3rd day of incubation and decreased afterward.

Exoglucanase activity produced by the three studied fungi recorded higher yields on maize cob. *Xylaria* sp. KM03 produced the highest activity (0.83 ± 0.023 IU/ml) on the 6th day of incubation. *Nemania* sp. KM02 (0.716 ± 0.089 IU/ml) on the 3rd day of incubation. *Xylaria* sp. KM01 (0.647 ± 0.028 IU/ml) on the 9th day of incubation. These results were similar to Ref. [62] findings on *Aspergillus niger* which showed the highest exoglucanase activity was recorded after 5 days of incubation and a similar trend in which cellulase activities by *Trichoderma viride* peaked on the 3rd day of incubation when cultured on a corn cob. Exoglucanase activity by *Xylaria* sp. KM03 recorded a steady decrease after 3rd day of incubation on cypress and eucalyptus substrates. This could be attributed to fungal species (genetic make-up) and substrates used for growth [63].

Endoglucanase produced by the three fungal strains recorded higher activity on maize cob. *Nemania* sp. KM02 (2.80 ± 0.212 IU/ml) on the 3rd day of incubation. *Xylaria* sp. KM01 (2.53 ± 0.052 IU/ml) on the 9th day of incubation. *Xylaria* sp. KM03 (2.51 ± 0.01 IU/ml) on the 6th day of incubation. These results were similar to Ref. [62] findings on *Aspergillus niger* which showed the highest cellulase activities were recorded after 5 days of incubation period cultured on a corn cob. However, endoglucanase activities by *Xylaria* sp. KM01 and KM03 recorded a steady decrease after 3rd day of incubation on cypress and eucalyptus substrates. This could be attributed to fungal species (genetic make-up) and substrates used for growth [63]. High viscosity of the substrate, decreases the oxygen supply to the organisms. It could also be inhibitory effects of accumulated cellobiose generated by the action of cellulases [14].

β-glucosidase activity produced by the three studied fungi was the highest on maize cobs. *Xylaria* sp. KM01 (629.7 ± 20.2 IU/ml) on the 9th day of incubation. *Xylaria* sp. KM03 (597.2 ± 29.7 IU/ml) on the 12th day of incubation and *Nemania* sp. KM02 (455.9 ± 28.7 IU/ml) on the 12th day of incubation [62]. recorded similar findings in which cellulase activities by *Trichoderma viride* cultured on corn cob peaked after the 5th day of incubation and decreased afterward. These differences could be due to the difference in the type and composition of substrates [64]. It could be also attributed to the maximum metabolic activity of the fungus attributed to the genetic make-up of the fungal strains as a result of adaptations to different habitats, and the concentration of soluble sugar in the substrate [65]. The low cellulase activities could be a result of an inhibitory effect of accumulated cellobiose [14].

4.5. Saccharification of maize cobs for sugar production

In the presence of a given amount of enzyme, the rate of an enzymatic reaction increases as the

substrate loading concentration increases until a limiting rate is reached, after which a further increase in the substrate concentration produces no significant change in the reaction rate. A higher yield of reducing sugars was optimized on substrate loading with 8 % at 72 h of saccharification by three fungi with the highest produced by *Xylaria* sp. KM03 (10.17 ± 0.37 mg/ml), *Xylaria* sp. KM01 (9.48 ± 0.13 mg/ml) and *Nemania* sp. KM02 (9.41 ± 0.27 mg/ml) respectively. A varying yield of sugars from corn cob has also been

reported in other studies. Saliu and Sani [66]. recorded a yield of 7.63 mg/ml of reducing sugar produced on hydrolysis of corn cob with cellulases of *A. niger* within 48 h. Jaafaru and Fagade [67] recorded a similar trend when corn cob was pretreated with alkali (5.55 mg/ml) of reducing sugar on hydrolysis with culture filtrates of *A. niger* compared to 4.25 mg/ml from untreated corn cob of between 3 % and 5 % substrate concentration.

4.6. Ethanol production from maize cob in simultaneous hydrolysis and fermentation processes

Fermentation of maize cob hydrolysate by *Saccharomyces cerevisiae* yielded varying ethanol concentrations. A higher yield of ethanol was optimized on substrate loading with 8 % at 96 h of fermentation. The highest ethanol concentration was produced by cellulase of *Nemania* sp. KM02 (28.72 ± 3.82 mg/ml), followed by *Xylaria* sp. KM03 (28.59 ± 1.58 mg/ml) and Xylaria sp. KM01 (25.75 ± 0.21 mg/ml) respectively. Similar findings were recorded by Ref. [51] and Saliu and Sani [66] when corn cob was hydrolyzed by cellulases of *A. niger*, recording ethanol yield (6.17 % and 2.67 %) respectively after 72 h of fermentation by *Saccharomyces cerevisiae*. The presence of fermentable sugar in negative control also recorded low ethanol yield due to the presence of free soluble sugar in the substrate. Yeast, however, consumes sugar for the growth and production of other metabolic products. Moreover, since growth commences during the aerobic phase, some amount of sugar gets used up before the anaerobic stage which is characterized by ethanol production, [66].

Bioethanol yield generally increases during the fermentation period with maximum bioethanol production at 96 h of incubation. As ethanol level increases it reduces the pH that favors *Saccharomyces cerevisiae* which converts the sugar present in the medium to ethanol and also provides an acidic condition that prevents bacterial contamination during fermentation. Nester et al. [68] recorded similar results, however, the fermentation did not proceed after 96 h, and the projection was a decrease afterward as the yeast consumes these nutrients from the medium and depletes the amount of sugar as the fermentation progresses.

4.7. Conclusions

As a conventional plant, maize cob proved its feasibility as a viable feedstock and as a cheap substrate for cellulase and bioethanol production. In the study, the supremacy of the cellulase of *Xylaria* sp. KM03 in hydrolyzing maize cobs to fermentable sugars recorded 10.17 ± 0.37 mg/ml reducing sugars, while hydrolyzed by cellulase of *Nemania* sp. KM02 recorded 28.72 ± 3.82 mg/ml ethanol yield. From this study, it can be concluded that, *Xylaria* sp. KM01, *Nemania* sp.KM02 and *Xylaria* sp. KM03 isolated from decaying plant biomass are cellulase-producing fungi. Time of incubation significantly affected cellulase enzyme production by the three fungal isolates using cypress eucalyptus and maize cobs substrates. Pretreatment of substrates affected cellulase enzyme production by three isolated fungi. The crude enzymes produced by the three fungal isolates saccharified maize cobs to produce fermentable sugars. Hydrolysate from saccharified maize cobs was fermented to ethanol using *Saccharomyces cerevisiae*.

Data availability statement

The experimental data used to support the findings of this study are available from the corresponding author upon request and have not been deposited into a publicly available repository.

CRediT authorship contribution statement

Stephen M. Kamande: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **George I. Omwenga:** Supervision, Project administration. **Mathew P. Ngugi:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36802.

References

[1] World Bank, World Development Report 2010: Development and Climate Change, 2010. © Washington, DC, http://hdl.handle.net/10986/4387License:CCBY3.0IGO.
[2] H. Tuo, Energy and exergy-based working fluid selection for organic Rankine cycle recovering waste heat from high temperature solid oxide fuel cell and gas turbine hybrid systems, International Journal of Energy Resources 37 (14) (2013) 1831–1841.
[3] C. Zhao, Z. Ma, Q. Shao, B. Li, J. Ye, H. Peng, Enzymatic hydrolysis and physiochemical characterization of corn leaf after H-AFEX pretreatment, Energy Fuels 28 (7) (2016) 4288–4293.
[4] R.C. Kuhad, R. Gupta, A. Singh, Microbial Cellulases and Their Industrial Application, vol. 2011, Enzyme Research, 2011 280696.
[5] S.H.M. Azhar, R. Abdulla, S.A. Jambo, H. Marbawi, J.A. Gansau, A.A.M. Faik, K.F. Rodrigues, Yeasts in sustainable bioethanol production: a review, Biochemistry and Biophysics Report 10 (2017) 52–61.
[6] B.K. Acharya, S. Mohana, R. Jog, J. Divecha, D. Madamwar, Utilization of anaerobically treated distillery spent wash for production of cellulases under solid state fermentation, J. Environ. Manag. 91 (2010) 2019–2027.
[7] D. Deswal, Y.P. Khasa, R.C. Kuhad, Optimization of cellulose production by a brown rot fungus Fomitopsis sp. RCK under solid state fermentation, Bioresour. Technol. 102 (2011) 6065–6072.
[8] S. Montoya, C.E. Orrego, L. Levin, Growth, fruiting and lignocellulolytic enzyme production by the edible mushroom Grifola frondosa (maitake), World J. Microbiol. Biotechnol. 28 (2011) 1533–1541.
[9] H. Li, Y. Pu, R. Kumar, A.J. Ragauskas, C.E. Wyman, Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect of cellulose hydrolysis, and underlying mechanisms, Biotechnol. Bioeng. 111 (3) (2014) 485–492.
[10] B.T. Wang, S. Hu, X.Y. Yu, L. Jin, Y.J. Zhu, F.J. Jin, Studies of cellulose and starch utilization and the regulatory mechanisms of related enzymes in fungi, Polymers 12 (3) (2020) 530.
[11] R.J. Moon, A. Martini, J. Nairn, J. Simonsen, Cellulose nanomaterials review: structure, properties and nanocomposites, Chem. Soc. Rev. 40 (2011) 3941–3994.
[12] C. Sawatdeenarunat, K.C. Surendra, D. Takara, H. Oechsner, S.K. Khanal, s. Anaerobic Digestion of Lignocellulosic Biomass: Challenges and Opportunitie, Bioresource Technology, 2015, pp. 178–186.
[13] L.H. Guimarães, Carbohydrates from biomass: sources and transformation by microbial enzymes, in: C. Changed (Ed.), Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology. Carbohydrates Technologies, 2012, pp. 441–458 (Chapter 20).
[14] Z. Yue, W. Bin, Y. Baixu, G. Peiji, Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase, Sci. China C Life Sci. 47 (1) (2004) 18–24.
[15] B. Ouyang, G. Wang, N. Zhang, J. Zuo, Y. Huang, X. Zhao, Recent advances in β-glucosidase sequence and structure engineering: a brief review, Molecules 25 (13) (2023) 28.
[16] A. Pandey, P. Nigam, P. Selvakumar, C.R. Soccol, Solid state fermentation for the production of industrial enzymes, Curr. Sci. 77 (1999) 149–162.
[17] A.L.T. Benatti, M. Polizeli, Lignocellulolytic biocatalysts: the main players involved in multiple biotechnological processes for biomass valorization, Microorganisms 11 (1) (2023) 162.
[18] O.B. Chukwuma, M. Rafatullah, H.A. Tajarudin, N. Ismail, Lignocellulolytic enzymes in biotechnological and industrial processes: a review, Sustainability 12 (2020) 7282, 2020.
[19] N. Ilić, M. Milić, S. Beluhan, S.D. Branković, Cellulases: From Lignocellulosic Biomass to Improved Production, Energies 16 (8) (2023) 3598.
[20] A. Archana, T. Satyanarayana, Xylanase production by *thermophilic Bacillus licheniformis* A 99 in solid state fermentation, Enzym. Microb. Technol. 21 (1997) 12–17.
[21] A. Pandey, F. Francis, C.R. Soccol, General aspects in solid state fermentation, in: Ashok Pandey (Ed.), Concise Encyclopedia of Bioresource Technology, Haworth Press, New York, 2004, pp. 702–718.
[22] M. Raimbault, General and microbiological aspects of solid substrate fermentation, Electron. J. Biotechnol. 1 (3) (1998) 174–189.
[23] D.K. Arora, D. Bhatnagar, Handbook of Fungal Biotechnology, CRC press, 2003, pp. 471–493.
[24] Y. Sun, J. Cheng, Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresour. Technol. 83 (2) (2002) 1–11.
[25] C. Martín, M. Galbe, C.F. Wahlbom, B. Hahn-Hägerdal, Jönsson, L.J. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose utilizing *Saccharomyces cerevisiae*, Enzym. Microb. Technol. 31 (2002) 274–282.
[26] H.M.N. Iqbal, G. Kyazze, T. Keshavarz, Advances in the valorization of lignocellulosic materials by biotechnology: an overview, Bioresources (2) (2013) 3157–3176.
[27] N. Jacob, P. Prema, Influence of mode of fermentation on production of polygalacturonase by a novel strain of *Streptomyces lydicus*, Food Biotechnol. 44 (2) (2006) 263–267.
[28] A. Tilay, U. Annapure, Novel simplified and rapid method for screening and isolation of polyunsaturated fatty acids producing marine bacteria, Biotechnology Research International 542721 (2012) 1–8.
[29] M.L. Legodi, D. La Grange, E.L. Jansen van Rensburg, L. Ncube, Isolation of Cellulose Degrading Fungi from Decaying Banana Pseudostem and Strelitzia Alba, vol. 2019, Enzyme Research, 2019 1390890.
[30] K. Sukesh, An Introduction to Industrial Microbiology. *S*, Chand Publishing, New Delhi, 2010, p. 22.
[31] T.J. White, T. Bruns, S. Lee, J.W. Taylor, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: M.A. Innis, D.H. Gelfand, J. J. Sninsky, T.J. White (Eds.), PCR Protocols, Academic Press, New York, NY, 1990, pp. 315–322.
[32] G.C. Graham, P. Meyers, R.J.A. Henry, Simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis, Biotechniques 16 (1994) 48–50.
[33] L. Delgado-Serrano, S. Restrepo, J.R. Bustos, M.M. Zambrano, J.M. Anzola, Mycofier: a new machine learning-based classifier for fungal ITS sequences, BioMed Central Research Notes 9 (2016) 402.
[34] K. Tamura, G. Stecher, S. Kumar, MEGA11: molecular evolutionary genetics analysis version 11, Mol. Biol. Evol. 38 (7) (2021) 3022–3027.
[35] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[36] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, J. Anal. Chem. 31 (1959) 426–428.
[37] T.K. Ghose, Measurement of cellulase activities, Pure Appl. Chem. 59 (1987) 257–268, 1987.
[38] D. Herr, Secretion of cellulase and beta-glucosidase by Trichoderma viride ITCC-1433 in submerged culture on different substrates, Biotechnol. Bioeng. 21 (8) (1979) 1361–1371, 1979 Aug.
[39] K. Jasińska, B. Zieniuk, D. Nowak, A. Fabiszewska, Studies on the catalytic properties of crude freeze-dried preparations of yarrowia lipolytica extracellular lipases for geranyl ester derivative synthesis, Biomolecules 11 (6) (2021) 839.
[40] S. Suh, N. Zhang, N. Nguyen, S. Gross, M. Blackwell, Lab Manual for Yeast Study. Mycology Lab, Louisiana state university. Statistical assistance, 2007, pp. 200–208.
[41] S. Alfenore, C. Molina-Jouve, S. Guillouet, J.L. Uribelarrea, G. Goma, L. Benbadis, Improving ethanol production and viability of Saccharomyces cerevisiae by a vitamin feeding strategy during fed-batch process, Appl. Microbiol. Biotechnol. 60 (1) (2002) 67–72.
[42] M. Mandels, L. Hontz, J. Nystrom, Enzymatic Hydrolysis of Waste Cellulose. Biotechnology and Bioengineering, xvi, U.S. Army Natick Laboratories, 1974, pp. 1471–1493. Natick Massachusetts 01760.
[43] A. Caputi, M.T. Ueda, T. Brown, Spectrophotometric determination of ethanol in wine, AJEV (Am. J. Enol. Vitic.) 19 (1968) L160–L165.
[44] N. Rahnama, H.L. Foo, N.A. Abdul Rahman, et al., Saccharification of rice straw by cellulose from a local *Trichoderma harzianum* SNRS3 for biobutanol production, BMC Biotechnol. 14 (2014) 103.
[45] R Core Team. R: A Language and Environment for Statistical Computing_, R Foundation for Statistical Computing, Vienna, Austria, 2018. https://www.R-project.org/.

[46] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, Mega X: molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. 35 (6) (2018) 1547.
[47] Y.H.P. Zhang, M.E. Himmel, J.R. Mielenz, Outlook for cellulase improvement: screening and selection strategies, Biotechnol. Adv. 24 (2006) 452–481.
[48] H.D. Zakpaa, E.E. Mak-Mensah, F.S. Johnson, Production of bio-ethanol from corn cobs using Aspergillus Niger and saccharomyces cerevisae in simultaneous saccharification and fermentation, Afr. J. Biotechnol. 8 (2009) 3018–3022.
[49] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution 39 (1985) 783–791.
[50] M. Kimura, A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences, J. Mol. Evol. 16 (1980) 111–120.
[51] S.A. Ado, G.U. Kachalla, M.B. Tijjani, M.S. Aliyu, Ethanol production from corn cobs by co-cultures of saccharomyces cerevisiae and aspergillus Niger Bayero, J. Pure Appl. Sci. (Ankara) 2 (2) (2009) 99–101.
[52] A.K. Forrest, J. Hernandez, M.T. Holtzapple, Effects of temperature and pretreatment conditions on mixed-acid fermentation of water hyacinths using a mixed culture of thermophilic microorganisms, Bioresour. Technol. 101 (2010) 7510–7515.
[53] F. Ma, N. Yang, C. Xu, H. Yu, J. Wu, X. Zhang, Combination of biological pretreatment with mild acid pretreatment for enzymatic hydrolysis and ethanol production from water hyacinth, Bioresour. Technol. 101 (2010) 9600–9604.
[54] J. Gao, L. Chen, Z. Yan, L. Wang, Effect of ionic liquid pretreatment on the composition, structure and biogas production of water hyacinth (Eichhornia crassipes), Bioresour. Technol. 132 (2013) 361–364.
[55] R. Timung, M. Mohan, B. Chilukoti, S. Sasmal, T. Banerjee, V.V. Goud, Optimization of dilute acid and hot water pretreatment of different lignocellulosic biomass: a comparative study, Biomass Bioenergy 81 (2015) 9–18.
[56] J. Yan, Z. Wei, Q. Wang, M. He, S. Li, C. Irbis, Bioethanol production from sodium hydroxide/hydrogen peroxide pretreated water hyacinth via simultaneous saccharification and fermentation with a newly isolated thermo tolerant Kluyveromyces marxianu strain, Bioresour. Technol. 193 (2015) 103–109.
[57] P.R. Kakde, S.C. Aithal, Effect of pretreatment on agro-residues for cellulase production by solid state fermentation using fungi, International Journal of Recent Scientific Research 11 (1) (2020) 36903–36906, 2020.
[58] T.V. Ojumu, B.O. Solomon, E. Betiku, S.K. Layokun, B. Amigun, Cellulase Production by Aspergillus flavus Linn isolate NSPR 101 fermented in sawdust, bagasse and corn cob, Afr. J. Biotechnol. 2 (2003) 150–152.
[59] A. Xia, J. Cheng, W. Song, C. Yu, J. Zhou, K. Cen, Enhancing enzymatic saccharification of water hyacinth through microwave heating with dilute acid pretreatment for biomass energy utilization, Energy 61 (2013) 158–166.
[60] S. Pandey, M. Shrivastava, M. Shahid, V. Kumar, A. Singh, S. Trivedi, Y.K. Srivastava, *Trichoderma species* cellulases produced by solid state fermentation, J. Data Min. Genom. Proteonomics 6 (2015) 170.
[61] U. Ilyas, S. Ahmed, A. Majeed, M. Nadeem, Biohydrolysis of Saccharum spontaneum for cellulase production by Aspergillus terreus, Afr. J. Biotechnol. (21) (2012) 4914–4920.
[62] S.P. Gautam, P.S. Bundela, A.K. Pandey, J. Khan, M.K. Awasthi, S. Sarsaiya, Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi, Biotechnology Resources International 81 (2011) 4–25.
[63] P.R. Yadav, P.B. Chauhan, M. Gahlout, Isolation, screening and optimization of process parameters for enhanced production of cellulase by solid state fermentation, Int. J. Adv. Res. Biol. Sci. 3 (2016) 21–27.
[64] B.A. Bhat, G.R. Khan, K. Asokan, Role of substrate effects on the morphological, structural, electrical and thermoelectrical properties of V2O5 thin films, Resour. Adv. 5 (2015) 552–602.
[65] L.W. Yoon, T.N. Ang, G.C. Ngoh, A.S.M. Chua, Fungal solid-state fermentation and various methods of enhancement in cellulase production, Biomass Bioenergy 67 (2014) 319–338.
[66] B.K. Saliu, A. Sani, Bioethanol potentials of corn cob hydrolysed using cellulases of Aspergillus Niger and Penicillium decumbens, EXCLI J 11 (2012) 468–479, 2012 Aug 15.
[67] M.I. Jaafaru, O.E. Fagade, Cellulase production and enzymatic hydrolysis of some selected local lignocellulosic substrates by a strain of Aspergillus Niger, Res. J. Biol. Sci. 2 (2007) 13–16.
[68] E.W. Nester, D.G. Anderson, C.E. Roberts, N.N. Pearsall, M.T. Nester, Dynamics of Prokaryotic Growth in Microbiology*: A Human perspective*.3 Rd, McGraw-Hill, New York, 2001, pp. 87–108.