Original Research Article

Enzymatic Activities of Halotolerant and Halophilic Fungi isolated from Iko River Estuary, South-South Nigeria.

ABSTRACT

Aim: Fungi that can tolerate unfavorable conditions are a potential source of stable bioactive metabolites that can be used for various industrial processes. Therefore this study aimed to isolate halotolerant and halophilic fungi from the Iko river estuary and screen them for three industrially important enzymes: amylase, cellulase, and mannanase.

Place and Duration of Study: Department of Microbiology, Akwa Ibom State University, Nigeria, between September 2021 and August 2022.

Methodology: Five sediment-dwelling fungi isolated from three locations along the Iko river estuary were assayed for their halotolerance on PDA containing different NaCl concentrations while their amylolytic, cellulolytic, and mannolytic activities were evaluated on agar plates containing 10% NaCl. The highest producer of the screened enzymes was identified using molecular techniques and further subjected to various submerged fermentation conditions to optimize the production of salt tolerant amylase, cellulase, and mannanase.

Results: All isolates were halotolerant, best adapted to a salinity of 5% NaCl (w/v), and capable of secreting at least one of the three tested enzymes in 10% NaCl (w/v). *Aspergillus niger* isolate L2S1 had the highest enzymatic index among other isolates thus requiring optimization. The optimum production of amylase, cellulase, and mannanase was obtained at an incubation temperature of 30^{o}C, initial pH of 6.0, and NaCl concentration of 5% (w/v). Out of the tested agro-wastes, wheat bran was most suited for the production of amylase, corncob was optimum for cellulase production, and maximum mannanase yield was obtained using copra meal. Amylase and cellulase production was optimal at 96 hours of incubation, whereas mannanase required 144 hours of fermentation to reach its maximum activity.

Conclusion: *Aspergillus niger* isolate L2S1 was capable of utilizing different carbon sources including cheap agro-residues as substrates for the production of halostable amylase, cellulase, and mannanase.

Keywords: Estuary, Sediments, Fungi, *Aspergillus, Penicillium,* Enzymes.

1 INTRODUCTION

Microorganisms found in saline environments are equipped with the capacity to balance the osmotic pressure of their surroundings and resist the denaturing effects of salt [1]. These organisms are spread across the three domains of life and are classified according to their salt requirements: non-halophiles grow optimally at NaCl concentration lesser than 0.2 M, slight halophiles show optimum growth at 0.2 – 0.5 M NaCl, moderate halophiles grow best at 0.5 – 2.5 M NaCl, borderline extreme halophiles grow at NaCl concentration of 2.5 – 4.0 M NaCl, and extreme halophiles can tolerate NaCl concentrations above 4.0 M [2]. Halotolerant microorganisms can adjust to different saline conditions but they do not completely require salt for growth while those that can tolerate NaCl concentrations above 2.5 M are classified as extremely halotolerant [2, 3].

Estuaries are characterized by constantly fluctuating salinity due to the influx of marine and freshwater, with noticeably increased salinity during the dry season due to evaporation and drought [4]. Therefore, the fungal diversity of these estuarine ecosystems has devised different adaptive strategies to cope with unstable salinity. One such mechanism used to prevent plasmolysis caused by osmotic and ionic stress is the increased production and accumulation of compatible solutes such as polyols, sugars, and amino acids in the cytoplasm of these fungi [5]. Several fungal species populating saline environments are also capable of producing enzymes, antibiotics, antioxidants, anti-cancer compounds, hydrophobins, biosurfactants, pigments, polysaccharides, and nanoparticles that have found potential applications in various industries such as textile, detergent, pharmaceuticals, food, cosmetics, paper, biofuel, and waste management [6, 7, 8, 9]. The enzymes secreted by the above-mentioned fungi are characterized by an excessive amount of acidic amino acids on their surface and decreased hydrophobic amino acids making them active over a broad range of salinity which could be challenging for regular enzymes [10]. Since these bioactive catalysts are mostly extracellular, they are easily extracted and are more effective in biotechnology [6].

Agwu and Oluwagunke [11], hypothesized that the varying salinity observed across Nigerian coastal waters suggests the possible existence of interesting halotolerant and halophilic organisms with different biotechnological and industrial importance. Several studies conducted on the mycobiota of the Iko River estuary have focused on their diversity and ecological importance, however, their salt tolerance and enzymatic potential remain undocumented [12, 13, 14]. Therefore, this study aimed at investigating the halotolerance of fungal species inhabiting the Iko River estuary alongside their ability to produce three halostable enzymes (cellulase, amylase, and mannanase) with the objectives of identifying high enzyme-producing isolates and also determining the optimal conditions required for the production of these enzymes.

2. MATERIALS & METHODS

2.1. Study Area

The Iko River is located within the petroleum belt of the Niger Delta region in Nigeria (7°30'N 7°45'N, and longitude 7°30' E/7°40'E). The river takes its course from the Qua Iboe River catchments and drains directly into the Atlantic Ocean at the Bight of Bonny [15, 16]. The river is more than 30km long with a shadow depth ranging from 1.0 meters to 7.0 meters at flood and ebb tide and an average width of 16metres [17]. As part of the tropics, this area experiences two seasons, the dry season from October to May and the wet season from April to October, with annual rainfall averaging about 2500 mm [18, 19]. The Iko River is bordered by a diversity of flora such as red mangrove (*Rhizophora mangle*), *Avicennia africana, Lancungularia, Raphia hookeri,* nipa palm (*Nypa fruiticans*), and *Sargassum* sp that is normally found during the wet season. Oil palm (*Elaeis guineensis*) and coconut palm (*Cocoa nucifera*) are also widely distributed in the villages [19]. Sediments in the Iko River are well sorted, composed of mainly coarse quartz sand, shell debris, fecal pellets, silt, and clay [17].

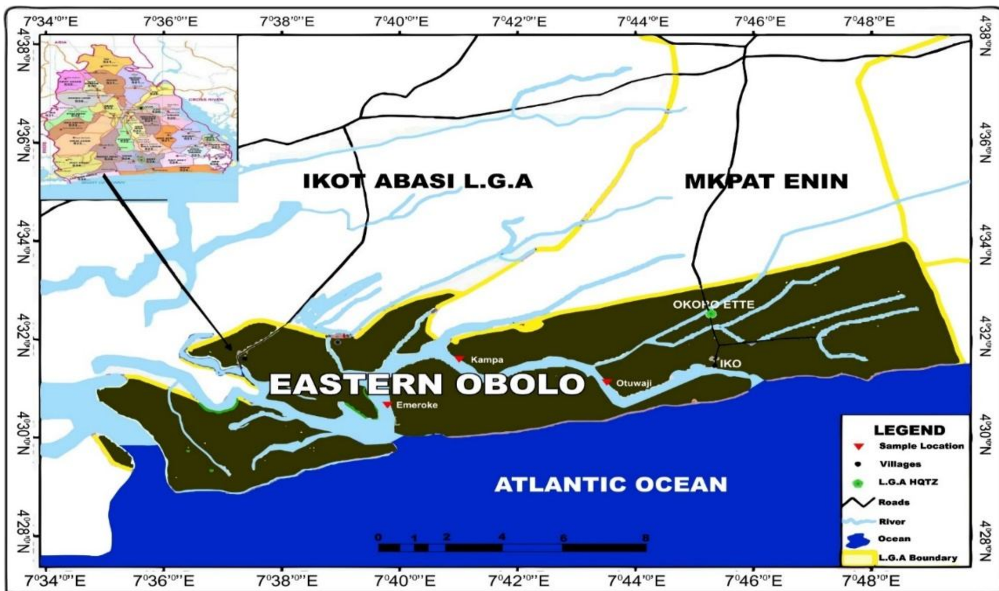

Fig 1. Iko estuary **Map of river**

showing sampling locations.

2.2. Sample collection

Three locations along the Iko River Estuary (Otuwaji, Kampa, and Emeroke) were selected for sampling. Sediment samples were collected using an Ekman Grab sampler in sterile bags and transported to the laboratory for microbial and physicochemical analysis.

2.3. Physicochemical analysis of Iko River sediment

Sediment samples were prepared for physicochemical analysis by air-drying for 24 h and sieved using a 2mm sieve**.** Electrical conductivity and pH were measured using the 1:5 soil/water method described by FAO [20] and FAO [21]. Organic carbon was determined using the Walkley-Black oxidation method described by FAO [22]. Available phosphorus was determined using the Bray-1 method described by FAO [23] and the total nitrogen in the sediment samples was estimated using the Kjeldahl method as described by FAO [24].

2.4. Isolation of Halophilic and Halotolerant Fungi

Wet sediment samples were serially diluted according to the methods of Cheesbrough [25]. One milliliter (1 mL) of each decimal dilution (10^{-3} and 10^{-4}) was inoculated on potato dextrose agar (PDA) supplemented with 10% NaCl using the pour-plating method described by Sanders [26]. The plates were incubated at 30^{o}C and observed regularly for fungal growth after which they were subcultured on fresh PDA plates supplemented with 10% NaCl to obtain pure cultures.

2.5. Morphological Characterization of Isolates

The morphological characterization of the isolates was carried out using the Lacto-phenol cotton blue staining method (X40). The identification keys of Pitt and Hocking [27], the pictorial atlas of soil and seed fungi by Watanabe [28], and the methods of Campbell *et al*. [29] were used to classify isolates into different genera.

2.6. Halotolerance Test

To estimate the salt requirement and tolerance of the fungal isolates, a halotolerance test was carried out by culturing 5 mm of each fungal isolate on a fresh PDA medium supplemented with different concentrations of NaCl (0%, 5%, 10%, 15%, 20%, and 25%) and incubated at 27^oC for 7 days.

2.7. Screening of Isolates for Enzyme Activity

2.7.1 Primary Screening of Enzyme Activity

The ability of halotolerant fungi to produce amylase, cellulase, and mannanase was initially assayed on agar plates containing the necessary substrates for each enzyme and supplemented with 10% NaCl. Inoculation was made by drilling a depth of 1 cm into each plate using a cork borer and filling it with 5 mm of each fungal isolate. Amylase activity was evaluated on basal medium containing: 3 g.L^{-1} $NaNO_3$, 0.5 g.L^{-1} $MgSO_4.7H_2O$, 5 g.L^{-1} KCl, 1 g.L^{-1} KH_2PO_4, 0.01 g.L^{-1} $FeSO_4.7H_2O$, 0.1 g.L^{-1} $CaCl_2$, 10 g.L^{-1} Starch and 15 g.L^{-1} agar. After inoculation and incubation, the cultures were flooded with a solution of Gram's iodine and the appearance of a clear zone around the colony revealed the presence of amylase. Cellulase activity was tested on medium containing: 7.0 g.L^{-1} KH_2PO_4, 2.0 g.L^{-1} K_2HPO_4, 0.1 g.L^{-1} $MgSO_47.H_2O$, 1.0 g.L^{-1} $(NH_4)_2SO_4$, 0.6 g.L^{-1} yeast extract, 10 g.L^{-1} CMC and 15 g.L^{-1} agar. After inoculation and incubation, the plates were then flooded with 5 ml of 0.1% Congo red solution followed by 1 M NaCl for 15 to 20 minutes to visualize the hydrolysis zone as described by Kasana *et al*. [30]. Mannanase activity was detected on Dox-medium containing: 10 g.L^{-1} locust bean gum, 2 g.L^{-1} $NaNO_3$, 1 g.L^{-1} K_2HPO_4, 0.5 g.L^{-1} $MgSO_4.7H_2O$, 0.5 g.L^{-1} KCl, 0.01 g.L^{-1} $FeSO_4.7H_2O$, 20 g.L^{-1} agar. After inoculation and incubation at room temperature for 72hrs, the zones of clearance were spotted using Congo red. Enzyme activity was determined following the method described by Chamekh *et al*. [31] where enzymatic index (EI) = R/r (R is the diameter of the halo zone and r is the diameter of the colony). Isolates with EI values greater than or equal to 2 millimeters were considered good producers of the screened enzyme.

2.7.2. Secondary Screening of Enzyme Activity

The secondary screening was carried out under submerged fermentation conditions by preparing liquid media with the required substrates (Starch, CMC & LBG) and supplemented with 10% NaCl.

2.7.2.1. Inoculum Preparation

The isolate with the highest enzymatic index from the plate assay was selected for secondary screening. The inoculum was prepared by culturing the isolate in a McCartney bottle containing 10 ml of PDA. After 72 h of incubation at 27 ^{o} C, the agar slant was flooded with 5 ml of distilled water and 1ml (1.0 x 10^6 spores ml $^{-1}$) of this suspension served as the inoculum.

2.7.2.2. Crude Enzyme Production

Amylase activity was determined in basal medium comprising (3 g.L^{-1} $NaNO_3$, 0.5 g.L^{-1} $MgSO_4{\cdot}7\ H_2O$, 5 g.L^{-1} KCl, 1 g.L^{-1} KH_2PO_4, 0.01 g.L^{-1} $FeSO_4{\cdot}7\ H_2O$, 0.1 g.L^{-1} $CaCl_2$), and 1% starch as carbon source. Cellulase activity was estimated in basal medium containing (3 g.L^{-1} $NaNO_3$, 0.5 g.L^{-1} KCl, 0.5 g.L^{-1} $MgSO_4.7H_2O$, 1.0 g.L^{-1} KH_2PO_4, 0.01 g.L^{-1} $FeSO_4.7H_2O$) and 1.0% CMC. Mannanase activity was measured in basal medium consisting of, (2 g.L^{-1} $NaNO_3$, 1 g.L^{-1} K_2HPO_4, 0.5 g.L^{-1} $MgSO_4.7H_2O$, 0.5 g.L^{-1} KCl, 0.01 g.L^{-1} $FeSO_4.7H_2O$) and 10 g.L^{-1} locust bean gum. One hundred milliliters (100ml) of each media was prepared in three separate 250ml Erlenmeyer flasks and supplemented with 10% NaCl respectively. The culture media were autoclaved for 15 min at 121^oC. After sterilization, the flasks were inoculated and incubated at 30ºC for 7 days.

2.7.2.3. Enzyme Extraction

The fermentation broths were filtered using Whatman no.1 filter paper after which the filtrates were centrifuged at 8000x g for 10 minutes at 10 ^{o} C to obtain the supernatants that were used for enzyme assay.

2.7.2.4. Enzyme assay

As described by Miller [32], enzyme activity was quantified using the 3,5-dinitrosalicyclic acid (DNS) reducing sugar method. The assay was carried out by mixing 1 ml of each enzyme filtrate with 1 ml of 50mM phosphate buffer (pH 7) and 1ml of the substrate (Starch, CMC, and LBG). The mixture was incubated for 10 min at 30^{o}C, thereafter, 1 ml of DNS was added to stop the reaction. The final mixture was incubated in a water bath at 100^{o}C for 10 min and then quickly cooled to room temperature. The degree of enzymatic hydrolysis of the substrates was determined spectrophotometrically by measuring the optical density at 540 nm. The concentration of each of the released reducing sugar was obtained through their respective standard curves. Enzyme activity is expressed as the amount of the enzyme that released 1 µmole of the reducing sugar from the substrate per minute per ml at pH 7 and a temperature of 30^{o}C.

2.8. Molecular Identification

2.8.1. Purification of fungal isolate

This was performed by culturing the desired isolate in a McCartney bottle containing 20 ml of sterilized Potatoes Dextrose Agar (PDA) supplemented with 10% NaCl and 50 µg ml^{-1} of chloramphenicol. The isolate was left to grow for 5 days after which it was analyzed using molecular techniques for accurate identification to species level.

2.8.2. DNA Extraction and Quantification

The genomic DNA of the fungal isolate was extracted following the protocol of the Quick-DNA Fungal/Bacterial MiniPrep Kit (Zymo Research Group, California, USA) as described by the manufacturer. The eluted DNA purity, concentration, UV/ Visible absorbance, and absorbance ratio were quantified using the NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

2.8.3. Polymerase Chain Reaction (PCR)

Polymerase chain reaction amplification was performed using the ABI GeneAmp 9700 PCR System (Thermo Fisher Scientific, Waltham, MA). The 25 µl reaction mixture contained 12.5 µl master mix (Taq polymerase, buffer, dNTPs, $MgCl_2$), 2 µl genomic DNA, 0.5 µl ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), 0.5 µl ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and 9.5 µl of nuclease-free water. The PCR reaction was performed under the following conditions; initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 52°C for 30 s, and 72°C for 1 min with a final extension step of 72 °C for 7 min. The quality of the obtained amplicon was checked on 1.2% agarose gel electrophoresis using ethidium bromide as the staining agent and purified using the DNA Clean and Concentration^{TM} Kit (Zymo Research Group, California, USA).

2.8.4. DNA Sequencing & Phylogenetic Analysis

Sanger sequencing was carried out using ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). The sequenced data was analyzed and exported to FASTA format using BioEdit. A BLAST search of the obtained ITS sequence against the nucleotide collection database (GenBank) of the National Center for Biotechnology Information (NCBI) was carried out to determine the closest matching sequences. The resulting sequences were added to a sequence alignment on MEGA 11 and multiple sequence alignment was achieved using the MUSCLE program. The phylogenetic tree was constructed using the Neighbor-Joining method and the evolutionary distances were computed using the Maximum Composite Likelihood method.

2.9. Optimization of fermentation conditions for enzyme production

The optimization of amylase, cellulase, and mannanase production was evaluated using the OVAT (one-variable-at-a-time) system which involves changing one independent variable while keeping other factors constant.

2.9.1. Effect of initial pH

The effect of initial pH on the production of amylase, cellulase, and mannanase enzyme was evaluated on culture broth supplemented with 10% NaCl. The culture broths were adjusted across an initial pH of 5, 6, 7, 8, 9, and 10 using acetate buffer (5 - 6), phosphate buffer (7 – 8), and ammonia buffer (9 - 10). After sterilization and inoculation, the cultures were incubated at 30°C for 7 days and enzyme activities were measured using the DNS method.

2.9.2. Effect of incubation temperature

The medium broth for amylase, cellulase, and mannanase production was supplemented with 10% NaCl, sterilized, and inoculated with the fungal isolate. Following inoculation, the cultures were incubated at different temperatures (20, 25, 30, 35, 40, 45, and 50°C) for 7 days after which enzyme activities were measured using the DNS method.

2.9.3. Effect of salinity

The effect of salinity on the production of amylase, cellulase, and mannanase enzymes was assessed in different flasks containing culture broth supplemented with 5, 10, 15, 20 & 25% NaCl, sterilized, inoculated, and incubated at 30 °C. The enzyme activities were measured after 7 days using the DNS method.

2.9.4. Effect of agro-waste materials on enzyme production

The effect of various plant-based biomasses such as cassava bagasse (CB), wheat bran (WB), sawdust (SD), palm kernel cake (PKC), and copra meal (CM) at a concentration of 1% was examined for optimum enzyme production in the fermentation medium. The medium was amended with 10% NaCl, sterilized for 15 mins at 121°C, inoculated with the fungal isolate, and incubated at 30°C. The enzyme activities were measured after 7 days using the DNS method.

2.9.5. Effect of incubation time on enzyme activity

The production of amylase, cellulase, and mannanase enzymes was evaluated by measuring the enzymatic activity at different incubation times. The fungal isolate was inoculated in medium broth supplemented with 10% of NaCl and cultivated at 30°C. After 24, 48, 72, 96, 120, 144, and 168 h of incubation, the enzyme activities were determined using the DNS method.

3. Physicochemical analysis of sediment samples

The analysis revealed that the sediments were slightly acidic with a mean pH value of 6.7±0.081. The conductivity value ranged from 50.31 - 56.62 ms cm^{-1} and the mean salinity value was 3.46±0.169%. The mean organic carbon was 4.2±0.163%, available phosphorus was 6.16±0.016 mg kg^{-1} and the total nitrogen ranged from (0.52 - 0.54%). The results of the physicochemical analysis are presented in Table 1.

3.2. Halotolerance Assay

The salt tolerance test result displayed in Table 2 revealed that all isolates were halotolerant and none were halophilic since they could grow on PDA media devoid of NaCl as well as media supplemented with

10% NaCl. All five isolates displayed optimum growth at 5% NaCl concentration, but none could tolerate NaCl concentrations above 10%.

3.3. Primary Screening of Enzyme Activity

Amylase and cellulase were produced by the five isolates, , however, only three isolates belonging to the genus *Aspergillus* were able to produce mannanase. Isolate L_2S_1 (*Aspergillus* sp.) showed the highest production of amylase with an EI value of 2.06 followed by Isolate L_1S_2 (*Aspergillus* sp.) with an EI of 1.66. Cellulase production was also observed in all isolates with Isolate L_2S_1 (*Aspergillus* sp.) having the highest EI value of 1.90. Mannanase was detected in isolates L_1S_1 (*Aspergillus* sp.), L_1S_2 (*Aspergillus* sp.), and L_2S_1 (*Aspergillus* sp.) with an EI value of 1.01, 1.20, and 1.62 respectively. Isolate L_2S_1 (*Aspergillus* sp.) was found to be the most promising isolate showing the presence of all extracellular enzymes screened in this study. The results of the primary screening are presented in Table 3.

Table 1. Mean and Standard Deviation of Physicochemical Analysis of Iko River Estuary

Parameters	L1 (Otuwaji)	L2 (Kampa)	L3 (Emeroke)	Mean	Standard deviation
pH	6.6	6.7	6.8	6.7	0.081
Salinity (%)	3.3	3.4	3.7	3.46	0.169
Electrical Conductivity (ms cm $^{-1}$)	50.31	52.57	56.62	53.16	2.61
Organic Carbon (%)	4.2	4.4	4.0	4.2	0.163
Available Phosphorus (mg kg^{-1})	6.14	6.18	6.16	6.16	0.016
Total Nitrogen (%)	0.54	0.53	0.52	0.53	0.008

Sediments

Table 2. Results Showing the Salt tolerance level of the isolates.

Isolate	Salt tolerance (NaCl %)		Classification
	Growth interval (%)	Optimal growth (%)	
L_1S_1 (*Aspergillus* sp.)	0-10%	5%	Halotolerant
L_1S_2 (*Aspergillus* sp.)	0-10%	5%	Halotolerant
L_2S_1 (*Aspergillus* sp.)	0-10%	5%	Halotolerant
L_3S_1 (*Penicillium* sp.)	0-10%	5%	Halotolerant
L_3S_2 (*Penicillium* sp.)	0-10%	5%	Halotolerant

Table 3: The enzyme activities of the isolates for the three enzymes studied

Isolate	Enzymatic Index (mm)		
	Amylase	**Cellulase**	**Mannanase**
L_1S_1 (*Aspergillus* sp.)	**1.49**	**1.42**	**1.01**
L_1S_2 (*Aspergillus* sp.)	**1.66**	**1.27**	**1.20**
L_2S_1 (*Aspergillus* sp.)	**2.06**	**1.90**	**1.62**
L_3S_1 (*Penicillium* sp.)	**1.52**	**1.36**	**-**
L_3S_2 (*Penicillium* sp.)	**1.20**	**1.04**	**-**

EI = R/r (diameter of the halo zone/ diameter of the colony) −: No enzyme activity: (-) Low enzyme activity: EI < 1, Moderate enzyme activity: EI ≥ 1 < 2, High enzyme activity: EI ≥ 2 [30].

3.4. Secondary Screening of Enzyme Activity

Isolate L_2S_1 (*Aspergillus* sp.) was further screened for its ability to produce amylase, cellulase, and mannanase using submerged fermentation (SmF). Isolate L_2S_1 demonstrated the ability to produce amylase, cellulase, and mannanase with enzyme activities of 0.216, 0.184, and 0.290 U/ml, respectively.

3.5. Molecular Identification

The nucleotide length of the ITS sequence obtained from isolate L_2S_1 was revealed to be 560 base pairs using Sangers sequencing. The BLAST result for isolate L_2S_1 obtained from the NCBI GenBank database showed that it was 99.64% similar to *Aspergillus niger* strain N (HQ891666.1) after which an accession number (OP242200) was assigned to the isolate. The phylogenetic tree presented in Fig 2 comprised 17 nucleotide sequences and the out-group used in this analysis was *Aspergillus flavus* strain S2. All positions containing gaps and missing data were eliminated (complete deletion option) and there was a total of 490 positions in the final dataset.

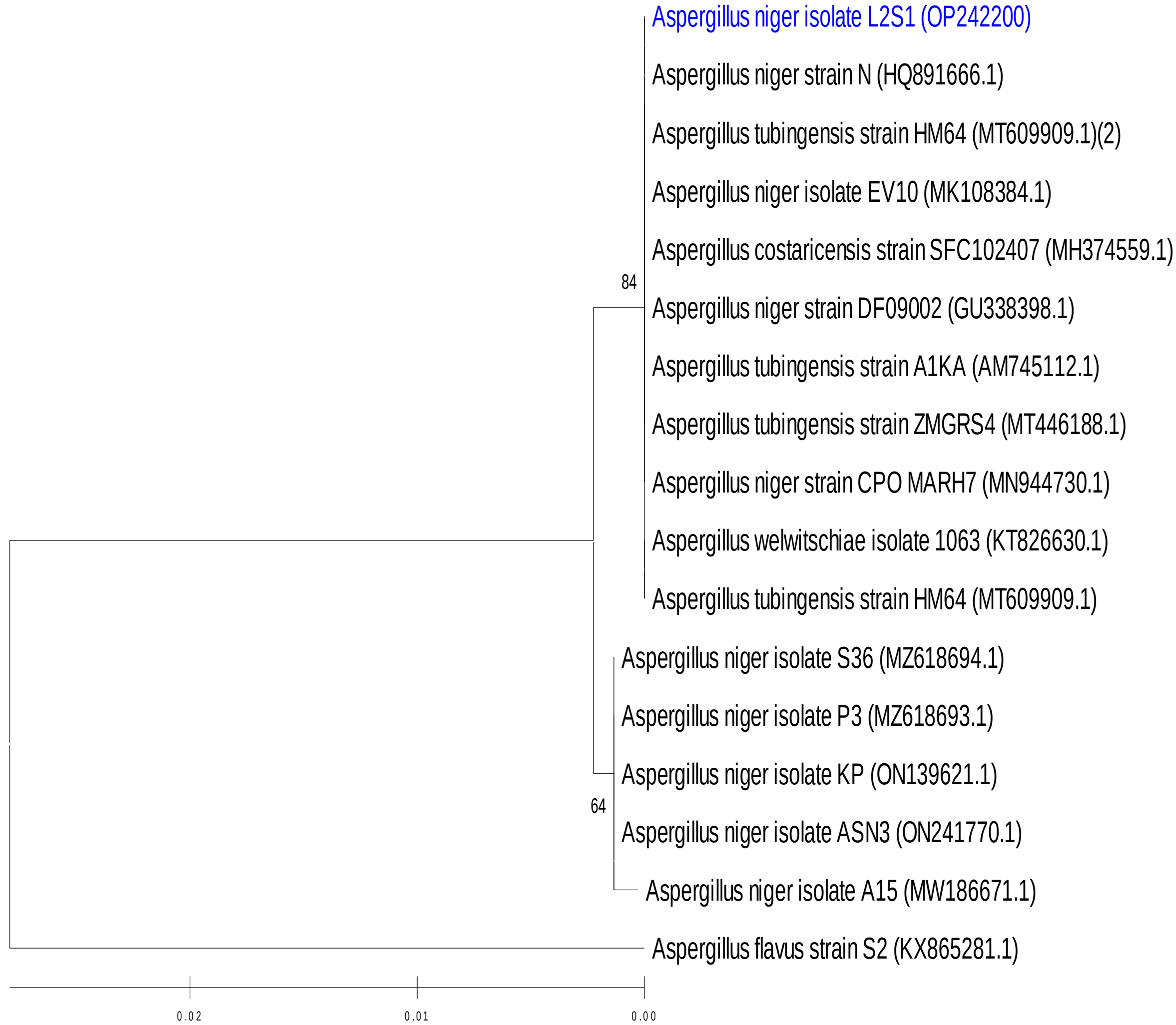

Fig 2. Phylogenetic evolutionary relationship between Isolate L_2S_1 and other closely related species extracted from GenBank using MEGA Version 11. The scale bar represents genetic distance, and the nodes' numbers indicate the percentages with which the given branch was supported by bootstrap analysis.

3.7. Optimization of fermentation conditions for enzyme production

3.7.1. Effect of initial pH on enzyme production by *Aspergillus niger* isolate L2S1

The result of the levels of amylase, cellulase, and mannanase activities of *Aspergillus niger* isolate L2S1 at different initial pH are presented in Fig 3. The maximum enzyme activity of amylase (0.284 U/ml), cellulase (0.246 U/mL), and mannanase (0.312 U/mL) was reached at pH 6. After pH 7, a gradual decrease in enzyme yield was observed and zero activity was detected at pH 10.

3.7.2. Effect of incubation temperature on enzyme production by *Aspergillus niger* isolate L2S1

The enzyme activities obtained at different incubation temperatures from *Aspergillus niger* isolate L2S1 are reported in Fig 4. The maximum amylase (0.216 U/ml), cellulase (0.184 U/ml), and mannanase activity (0.290 U/ml) were achieved at an incubation temperature of 30 °C. At temperatures greater than 35 °C, a sharp decline in enzyme activity was observed until it reached a stop at 50 °C. This shows clearly that the production of amylase, cellulase, and mannanase by *Aspergillus niger* isolate L2S1 is significantly affected by incubation temperature.

3.7.3. Effect of salinity on enzyme production by *Aspergillus niger* isolate L2S1

The measurement of enzyme activities in fermentation media supplemented with NaCl concentrations ranging from 2% to 10% (w/v) revealed that the production of amylase, cellulase, and mannanase by *Aspergillus niger* isolate L2S1 was optimum at 5% NaCl. At salt concentrations greater than 5%, a constant decline in enzyme yield was observed. The maximum amylase (0.368 U/ml), cellulase (0.321 U/ml), and mannanase activity (0.404 U/ml) are presented in Fig 5.

3.7.4. Effect of agro-waste materials on enzyme production by *Aspergillus niger* isolate L2S1

The effect of various complex plant-based biomasses as an alternative substrate for amylase, cellulase, and mannanase production under submerged fermentation was studied. Wheat bran proved to be the best agro-residue for amylase production with an activity of 0.203 U/ml. Corncob yielded the highest cellulase activity of 0.173 U/ml and copra meal was the best substitute for mannanase production yielding 0.226 U/ml. The results are presented in Fig 6.

3.7.5. Effect of incubation time on enzyme production by *Aspergillus niger* isolate L2S1

The amylase and cellulase activity of *Aspergillus niger* isolate L2S1 was optimum at 96 h of fermentation while mannanase activity reached its peak at 164 h of fermentation. The maximum activities of amylase (0.216 U/ml), cellulase (0.184 U/ml) and mannanase (0.335 U/mL) are shown in Fig 7.

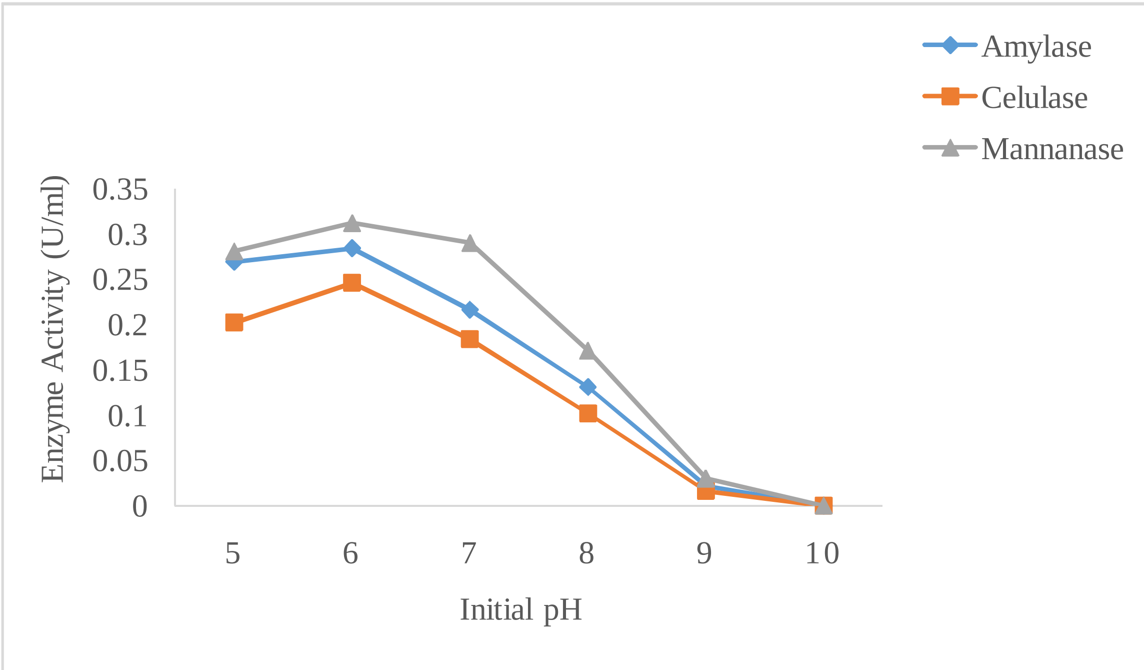

Fig 3. Effect of Initial pH on enzyme production by *Aspergillus niger* isolate L2S1

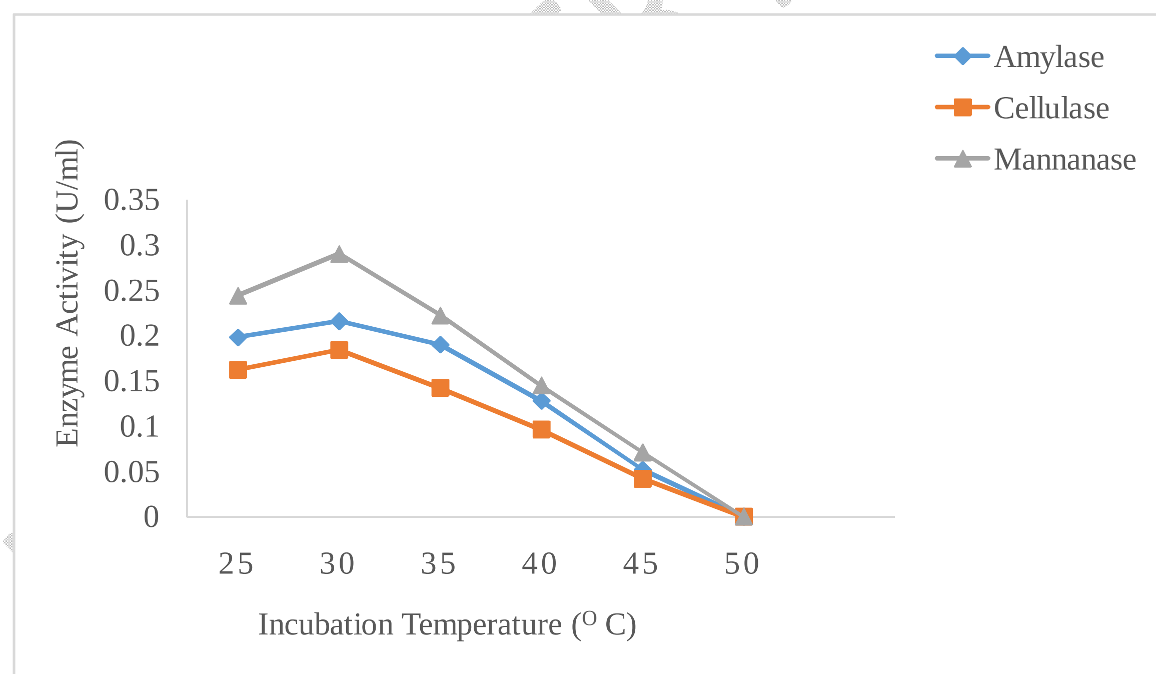

Fig 4. Effect of Incubation temperature on enzyme production by *Aspergillus niger* isolate L2S1

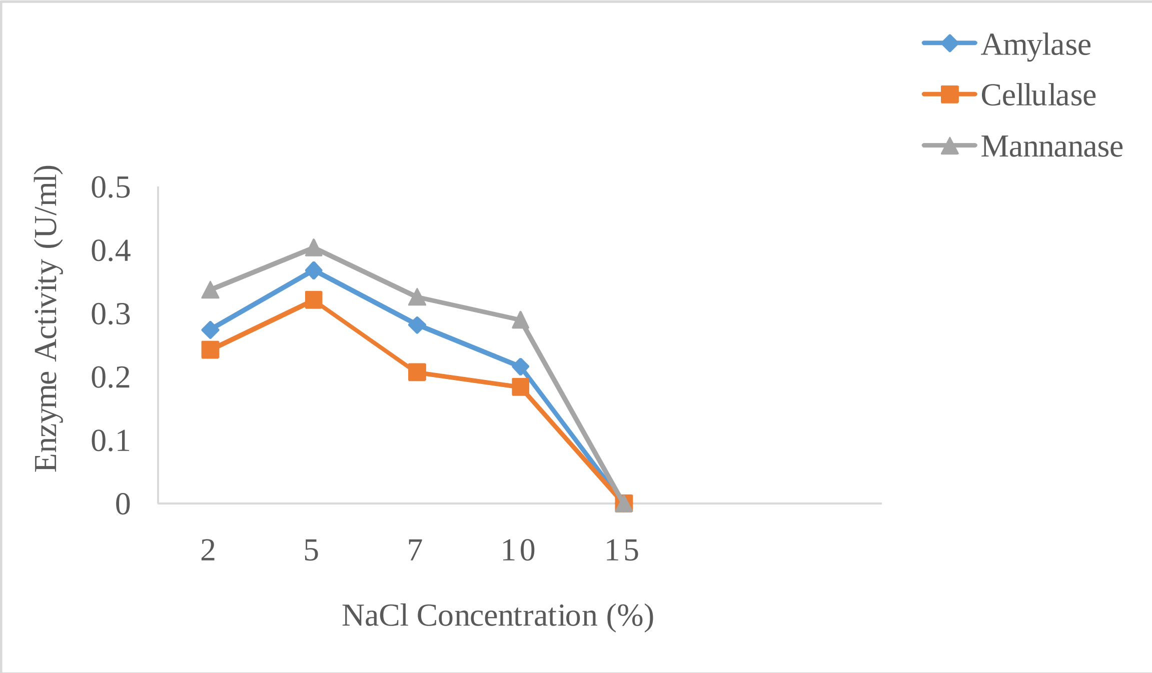

Fig 5. Effect of salinity on enzyme production by *Aspergillus niger* isolate L2S1

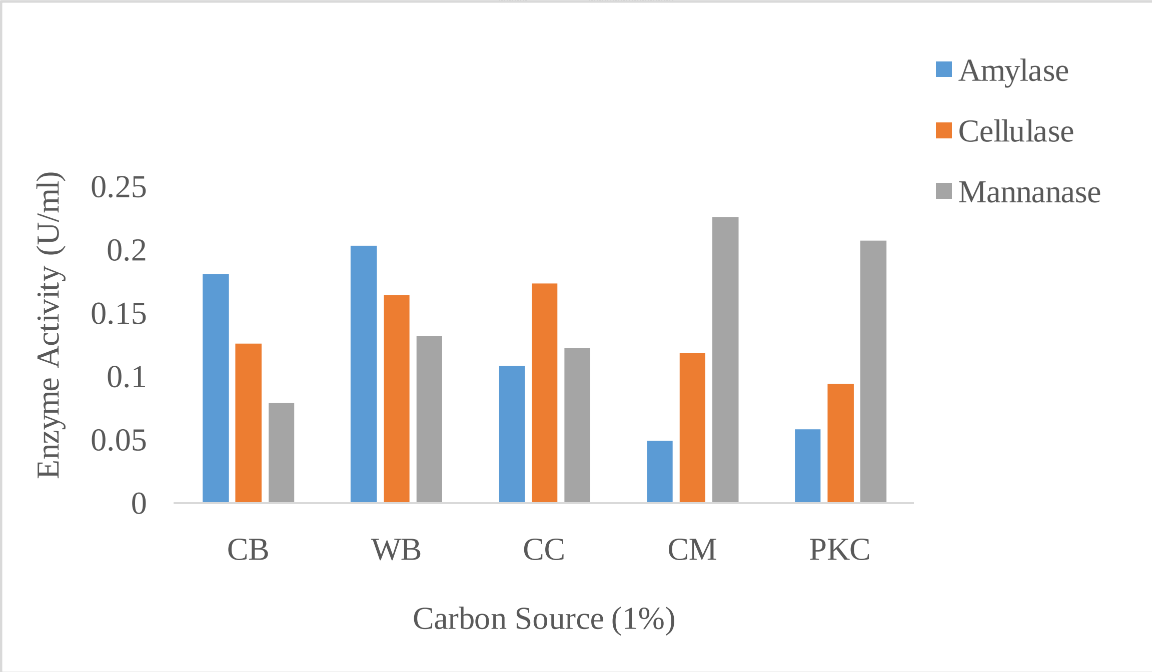

Fig 6. Effect of agro-waste materials on enzyme production by *Aspergillus niger* isolate L2S1

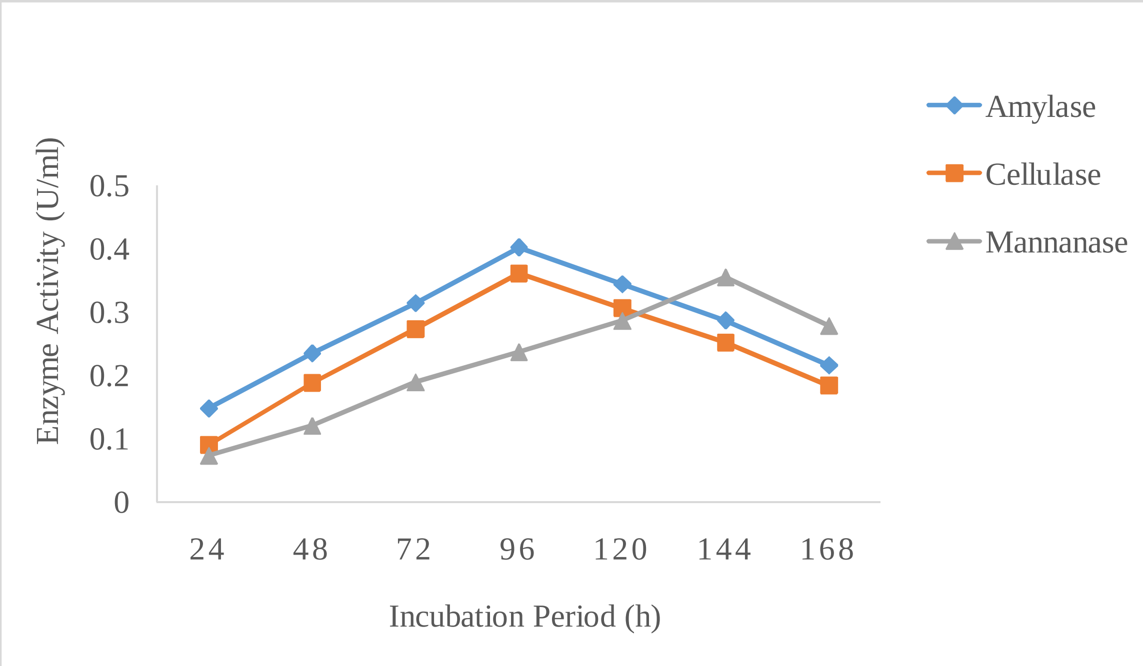

Fig 7. Effect of incubation time on enzyme production by *Aspergillus niger* isolate L2S1

4. RESULT AND DISCUSSION

This study evaluated the physicochemical characteristics of the Iko River estuary sediments. The slightly acidic mean pH of 6.7±0.081 obtained in this study is comparable to the mean pH of the Cross River estuary (6.81), Imo River estuary (6.67), and Qua Iboe River estuary (6.58) sediments reported by Ebong and John [33]. The obtained pH values are within the acceptable range of 6.5 – 8.5 specified by WHO [34], therefore, the pH levels of the Iko River estuary sediments may not harm the aquatic life inhabiting the river. The mean electrical conductivity of the sampled sediments was 53.16±2.61 mS cm^{-1}. This value is lower than 66.9±0.047 mS cm^{-1} reported by Udosen and Umana [12] and higher than 12.73 mS cm^{-1} recorded by Etesin *et al.* [17]. The variation between these studies could be a result of seasonal changes, inputs from freshwater sources, or the choice of sampling locations. The total organic carbon in the studied sediments ranged from 4.0 – 4.2%. This range is higher than the mean range of 0.87±0.15 – 2.69±0.17 reported by Wokoma and Friday [35] from the Sombreiro River, and 2.32 – 2.44% obtained from the sediments of the Silver River by Simeon *et al*. [36]. The reason for organic values greater than 1% could be attributed to the decomposition of marine organisms, terrestrial inputs from surface discharge/runoff, and anthropogenic activities such as oil spillages from bunkering activities [17, 12]. The mean concentrations of total nitrogen and available phosphorus observed in the sediment samples were 0.53±0.008% and 6.16±0.016 mg kg^{-1}. These obtained values could be attributed to the improper disposal of sewages or fertilizers containing nitrogenous compounds into the Iko River while increased phosphorus content could be linked to surface runoff from nearby land containing decayed organic matter [37, 38]. In this study, 3 species of halotolerant *Aspergillus* and 2 species of halotolerant *Penicillium* were isolated from the Iko river estuary. Members of these genera have been reported by previous studies to be part of the mycobiota dominating the Iko River estuary [12, 13, 14], other estuarine ecosystems [39, 40], and several saline environments [41, 42, 43, 44, 45] thus suggesting that species of *Aspergillus* and *Penicillium* are euryhaline in nature, tolerating wide ranges of salinity. These halotolerant fungi are able to detect high salt concentrations in the environment using the high-osmolarity glycerol (HOG) signaling pathway [5]. Enzymes secreted by fungi are characterized by their high production potency, catalytic efficiency, easy purification and separation requirements, safety, and stability during harsh industrial conditions such as low water activity and high osmotic pressure [46]. The isolated fungi in this study were screened for the production of 3 industrially important enzymes (amylase, cellulase, and mannanase) under 10% salinity. All five isolates in this study produced amylase and cellulase within an enzymatic index (EI) range of 1.20 - 2.06 for amylase and 1.04 – 1.90 for cellulase. These results were corroborated by the study of Ali *et al*. [47], Chamekh *et al*. [30], and Dendouga and Belharma [45] who also reported the production of amylase and cellulase from different halotolerant *Aspergillus* and *Penicillium* species at 10% NaCl concentration on solid medium. Although mannanase activity was not observed in the *Penicillium* species isolated in this study, the production of non-halotolerant mannanase by *Penicillium* has been reported in several studies [48, 49, 50]. The isolate with the highest enzymatic index after the primary enzyme screening was accurately identified as *Aspergillus niger* isolate L2S1 (Accession number: OP242200) using molecular identification techniques. Molecular Evolutionary Genetics Analysis (MEGA) software, version 11 (MEGA 11) is an integrated tool for automatic and manual sequence alignment that infers phylogenetic trees, mines web-based databases, estimates rates of molecular evolution, and tests evolutionary hypotheses [51]. For this reason, the evolutionary relationship between *Aspergillus niger* isolate L2S1 isolated from the Iko River estuary and other similar sequences stored in the GenBank was established using MEGA 11. Submerged fermentation (SmF) was used for secondary screening due to easy measurement and control of fermentation parameters, reduced fermentation time, and convenient harvesting of enzymes [52]. The DNS method used to quantify enzyme activity in this study measures the reducing sugar released after a substrate has been hydrolyzed by an enzyme under specified assay conditions. The reducing sugar produced by the enzyme reacts with DNS (3,5-Dinitrosalicyclic acid) to produce ANS (3-amino-5-nitrosalicyclic acid), an aromatic compound that absorbs light strongly at 540nm [53]. The results obtained from this study showed that *Aspergillus niger* isolate L2S1 was also able to produce the screened enzymes in liquid media containing 10% salt. Similar outcomes were reached by Ali *et al*. [54] who reported the production of salt-tolerant amylase from obligate halophilic *Aspergillus gracilii* strain TISTR 3638 at a salt concentration of 10%. Ali *et al*. [55] also described how *Aspergillus penicillioides* strain TISTR 3639 produced halostable amylase in a fermentation medium supplemented with 10% NaCl. Gunny *et al*. [56] recorded the production of halotolerant cellulase from halophilic *Aspergillus terreus* strain UniMAP AA-6 under 10% salt concentration also, *Aspergillus flavus* strain TISTR 3637 was used by Bano *et al*. [57] to produce salt-tolerant cellulase in liquid medium containing 10% NaCl. According to Niyonzima *et al*. [58], enzymes with desired properties can be produced efficiently by optimizing their fermentation conditions. Incubation temperature played an important role in the production of amylase, cellulase, and mannanase enzymes. The optimum temperature for growth and production of salt tolerant enzymes by *Aspergillus niger* isolate L2S1 in this study was found to be 30°C. Bellaouchi *et al.* [59] inferred that the best enzyme production in *Aspergillus* species is obtained at 30 °C, however, Zhao *et al*. [60] reported the optimum production of salt tolerant cellulase by obligate halophilic *Aspergillus flavus* strain 6830 at 35°C using solid-state fermentation (SSF). The assayed enzyme activity in this study was considerably affected by pH. The initial pH of 6.0 was the most favorable for the activity of amylase, cellulase, and mannanase. It's possible that *Aspergillus niger* isolate L2S1 was best suited to a pH of 6, which is similar to the pH of the Iko river estuary, the source of the fungal isolate. Microorganisms require sodium chloride for growth and physiological activities, however, high concentrations of sodium chloride can cause osmotic and ionic stress even for halotolerant species. In this study, increased concentration of sodium chloride in the fermentation medium led to a reduction in water activity, this limited fungal growth and inhibited enzyme production. Salt concentration above 10% inhibited the ability of *Aspergillus niger* isolate L2S1 to produce the screened enzymes while 5% NaCl was the optimum salt concentration required for enzyme production. Niknejad *et al*. [61] also reported optimum production of salt tolerant amylase from halotolerant *Fusarium incarnatum* isolate U2, *Penicillium polonicum* isolate U3*,* and *Penicillium polonicum* isolate U4 at 5% NaCl concentration. Agricultural wastes are a rich carbon source that can produce microbial biomass and metabolites, most especially as cheaper fermentation substrates for enzyme production [62, 63]. Wheat bran yielded an amylase activity of 0.203 U/ml, while cassava bagasse which yielded an amylase activity of 0.181 U/ml can also be used as a starch substitute for optimum amylase production. Corncob was the best agro-based substrate for cellulase production, yielding an activity of 0.173 U/ml. Harini and Kumaresan [64] also reached a similar conclusion using corncob although their strain of *Aspergillus niger* was non-halotolerant. Copra meal (CM) was the best by-product for mannanase production with an enzyme activity of 0.226 U/ml followed closely by palm kernel cake (PKC) at 0.207 U/ml. The proximate composition analysis of copra meal carried out by Antia *et al*. [65] revealed that it has higher protein and moisture content than palm kernel cake (PKC) thus making it ideal for both fungal growth and mannanase production. Incubation time affects enzyme production significantly and it can vary from 24 h to 168 h depending on the microorganism and other culture conditions [66]. In this study, an incubation time of 4 days (96 h) was best for amylase and cellulase production while 144 h was found to be optimum for mannanase production. Correspondingly, Gunny *et al*. [67] reported 96 h to be optimum for the production of halotolerant cellulase by *Aspergillus terreus* strain UniMAP AA-6. The decreased enzyme activity noticed after the optimum period in this study was probably due to the depletion of nutrients in the fermentation flasks over time.

5. CONCLUSION AND RECOMMENDATIONS

In conclusion, this study revealed the presence of halotolerant *Aspergillus* and *Penicillium* fungi inhabiting the Iko River estuary. The increasing demand for cheaper and natural sources of enzymes capable of withstanding several harsh industrial conditions necessitated this study. The enzymatic potentials of the screened isolates were determined and *Aspergillus niger* isolate L2S1 displayed the highest enzyme activity requiring optimization. The production of amylase and cellulase was optimized at pH of 6, 5% NaCl, and incubation temperature of 30 °C at 96 h of incubation and mannanase after 6 days (144 h) incubation period. Cheap and economical agro-waste substrates were used as alternative carbon sources to produce the salt-tolerant hydrolases and optimum enzyme activity was obtained using wheat bran for amylase, corncob for cellulase, and copra meal for mannanase. Due to the affordability of these agro-waste substrates, salt-tolerant amylase, cellulase, and mannanase could be produced commercially utilizing *Aspergillus niger* isolate L2S1.

RECOMMENDATIONS

i. The hydrolases produced by *Aspergillus niger* isolate L2S1 are capable of degrading different agro-wastes. Therefore, additional studies may be required to assess its ability to produce biofuels.
ii. Further investigations could also be directed particularly towards the purification and characterization of these enzymes to determine their molecular weight, specific activity, and optimum conditions.
iii. Lastly, genetic manipulation of the genes encoding these enzymes could be considered for the production of enzymes that can tolerate higher salt concentrations.

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