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Characterization of lignocellulolytic bacterial strains associated with decomposing wood residues in the Lagos lagoon, Nigeria

Olanike Maria Buraimoh*, Matthew Olusoji Ilori, and Olukayode Oladipo Amund

Department of Microbiology, University of Lagos, Akoka, Lagos, Nigeria.
Email: marianiks@yahoo.com

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ABSTRACT

Aims: The presence of lignocelluloses, especially sawdust in the Lagos lagoon and the attendant ecological problems warranted studies on their degradation. This study aimed to isolate and identify the indigenous bacterial strains capable of utilizing lignocellulosic wastes under the prevalent tropical estuarine conditions.
Methodology and results: Nine bacterial species were obtained by elective culture from decomposing wood residues in the lagoon. They were identified on the basis of morphology, biochemical characteristics and analysis of their 16S rRNA gene sequences as *Streptomyces*, *Bacillus* and *Paenibacillus* species. They were cultured on various lignin-related lignocellulosic substrates over a period of 7 to 12 days. All the isolates showed moderate to very good growth on sugarcane baggase. *Streptomyces albogriseolus* strain AOB and *Paenibacillus* sp. ROB showed good growth on grass while on sawdust, only *Streptomyces* AOB, and *Bacillus megaterium* strain NOB showed good growth. High performance liquid chromatographic analysis showed that the *Streptomyces* species completely utilized coniferyl alcohol, *B. megaterium* strain NOB utilized 90-100% of all the lignin- related aromatic compounds. All the bacterial species utilized less than 40% of sinapyl alcohol, *Bacillus* sp. OOB and *Paenibacillus* sp. strain ROB failed to utilize vanillic acid.
Conclusion, significance and impact of study: The isolates degraded lignocellulosic wastes and lignin-related compounds. The role of fungi in the breakdown of lignocellulose in the Lagos lagoon had been the subject of previous research considerations whilst the role of bacteria spp was unreported. Autochthonous bacterial species may equally play a role in the bio-rehabilitation of the sawdust-polluted water of the Lagos lagoon.

Keywords: Lignocellulose, *Streptomyces*, *Bacillus, Paenibacillus*, pollution

INTRODUCTION

Lagos, a commercial city in Nigeria is a natural destination of wood obtained from felled trees because it is close to the rain forest belt of the country. Timbers are transported along the creeks and lagoons to Lagos for various purposes. The Lagos lagoon is about 6354.708 sq km in area and 285 km in perimeter (Ajagbe *et al*., 2012). Although the lagoon provides a means of transportation and livelihood for the fishing communities, it is also a dumpsite for residential and industrial wastes (Amund and Igiri, 1990). About 104,000 cm^3 of sawdust is generated daily from various sawmills fringing the lagoon and this constitutes one of the major sources of lignocellulose in the Lagos lagoon (Abulude, 2006). Other major sources of lignocellulose include houses constructed with wood, which lie suspended in the lagoon inhabited by the native fishermen and their stilt supports as well as floating logs which are transported from the rain forest belt. Other sources of lignocellulose in the lagoon include abandoned wooden boats, seaweeds, especially the water hyacinth (*Eichornia crassipes*), grasses (*Paspalum* sp.), paper wastes, sugarcane baggase and fruit peels that are constantly being dumped into the lagoon do decompose after a long period to pollute the lagoon. The problems associated with this lignocellulose-polluted lagoon include addition of nutrients leading to algal bloom, enhancement of microbial growth leading to increased oxygen demand and development of anoxic conditions resulting in the death or migration of aquatic organisms. Furthermore, loss of aesthetic value as a result of bad odour emanating from decomposition of the sawdust under anaerobic conditions make the lagoon unsuitable for use as a tourist and relaxation centre, which could have enhanced the local economy and provide employment for the populace. The presence of lignocellulosic residues in the lagoon is also known to cause the narrowing of navigation pathways for transportation facilities such as haulage vessels for people and goods including ferries and boats. Most importantly, destruction of sawdust by dumping into the lagoon or by burning is a gross wastage of natural

*Corresponding author

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resources. Biological degradation of sawdust and other lignocellulosic wastes could be of economic value as these so called "wastes" could be converted into useful products such as biofuels, bioethanol, textile dyes, food additives and other chemicals of medical importance (Howard *et al*., 2003; Kirby, 2005; Ruiz-Duenas *et al*., 2009; Dong *et al*., 2011).

Although lignocellulose is renewable and abundant in nature, it is a major constituent of plant materials that must be degraded through the biogeochemical cycling for the ecosystem to function properly and for efficient biotechnological purposes. The dumping of sawdust into the Lagos lagoon and the attendant ecological problems had warranted studies on its biodegradation. The role of fungi in the breakdown of lignocellulose in the Lagos lagoon had been the subject of previous research considerations (Akpata, 1980, 1986; Chinedu, 2008), whilst the role of autochthonous actinomycetes and other bacteria was unreported. The intent of this paper is to bridge the information gaps by characterizing the indigenous bacterial strains associated with decomposing sawmill wastes and other woody residues dumped into the Lagos lagoon.

MATERIALS AND METHODS

Collection of samples for isolation of actinomycetes and other bacteria

For the isolation of bacterial cultures, decomposing sawdust was collected from the Lagos lagoon at Okobaba sawmill at Ebute-Metta axis of Lagos Mainland fringing the lagoon (Co-ordinates: N 6°29'21.8''; E 003°23'29.3'') in sterile sample bottles, carefully labelled and stored in the refrigerator at 4 °C , before processing within 24 h.

Isolation of microorganisms

Decomposing sawdust (1.0 g) was serially diluted. Aliquots (0.1 mL)) from 10^{-3} dilution were inoculated by spreading on sterilized (121 °C, pH 7.2) Starch-Casein agar plates containing: soluble starch (1.0 g), K_2HPO_4 (2.0 g), KNO_3 (2.0 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $CaCO_3$ (0.02 g) Casein (0.3 g), NaCl (2.0 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), agar (15.0 g), Cycloheximide (100.0 mg), deionised water (1000 mL) and aerobically incubated (28 °C) for at least 5 days. Pure isolates were stored on starch-casein agar slants at 4 °C.

Screening for lignocellulose degraders

Sterile filter papers were aseptically placed on the surface of freshly prepared sterile Starch-Casein agar plates. Each pure culture was then streaked on the surface of the filter paper. The plates were incubated (28 °C) for at least 5 days. Isolates were selected based on their abilities to break down the filter paper. Screening for utilization of aromatic acids was carried out in 250 mL conical flasks containing minimal agar medium (pH 7.2) which contained 1.0 g/L aromatic acids (vanillic or veratric acid), trace elements (1.0 mL), phosphate buffer and the pH indicator bromothymol blue as previously described by Nishimura *et al.* (2006). The catabolism of aromatic acids resulted in increased pH of the medium, which can be seen visually by a change of colour from green (pH 7.2) to blue (pH > 7.2).

Cultural and morphological characteristics

Cultural attributes of isolates were observed visually on starch-casein agar plates using a hand lens. Cellular morphology was observed by the use of an epiflourescence light microscope.

Scanning electron microscopy

The samples used for scanning electron microscopy (SEM) were prepared by the method of Bozzola and Russell (1999).The surfaces of spores and spore chains were examined by SEM using a Hitachi S-3500N model electron microscope (ThermoNaran, Hitachi technologies, America Inc.).

Biochemical characteristics

Catalase reaction, oxidase test, nitrate reduction, starch and gelatin hydrolysis were studied as described by Lanyi (1987). Pure cultures of bacterial isolates were identified according to the identification scheme of Bergey's manual of determinative bacteriology (Holt *et al*., 1994).

Amplification and identification of bacterial isolates by 16S rRNA gene analysis

Total bacterial genomic DNA from each primary isolate was extracted from 1.0 mL of 48 h culture in Luria broth using the master pure Gram positive DNA kit (www.epicentre.com).

Gene sequences (16S rRNA) of isolates were amplified by PCR using bacterial universal primers adapted from Relman (1993), based on the protocol of Schuller *et al.* (2010). The universal primers supplied by integrated DNA technologies (IDT) were: Foward primers: universal 1 (UI) 5'–ACG CGT CGA CAG AGT TTG ATC CTG GCT–3', universal 2 (U2) 5'–CGC GGA TCC GCT ACC TTG TTA CGA CTT–3', universal 3 (U3) 5'–AGT GCC AGC AGC CGC GGT AA–3', universal 4 (U4) 5'–AGG CCC GGG AAC GTA TTC AC–3', universal 5 (U5) 5'–TCA AAK GAA TTG ACG GGG GC–3' (K=G+T). Reverse primer: short universal 1 (UIR) 5'– CGA CTA CCA GGG TAT CTA AT–3'.

For culture isolates, primers were used in the following combinations to amplify the l6S rRNA gene - U1 + U2, U3 + U4, U5 + U4. The DNA amplification was performed using a T- gradient thermo cycler (Whatman Biometre Gmb H Rudof-Wissel-Street 30). Initial denaturation was at 95 °C for 15 min, followed by 30 cycles of denaturation at 96 °C for 15 sec., an annealing temperature of 55 °C for 1 min. 30 sec, elongation at 72 °C for 2 min and a final extension step at 72 °C for 10 min.

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Agarose gel electrophoresis of DNA fragments

Polymerase chain reaction-amplified DNA segments were separated by electrophoresis in a 2% agarose gel, using 100 bp DNA marker (Promega, USA) as DNA standard, *Pantoe agglomerans* DNA (positive control), Millipore water (blank) was used as negative control and ethidium bromide was used as the stain. Gel was run for 80 min at 100 V. The amplified products were observed under the Kodak fluorescent imaging equipment, model IS 4000R (Kodak image station, care stream molecular imaging health inc. Rochester, NY, USA.).

16S rRNA gene sequencing

Purification of PCR products was carried out using PCR clean-up system Wizard SV gel (Promega, Madison, W1, USA). Quantification of the nucleic acid was carried out using a Nanodrop 1000 spectrophotometer (Nanodrop technologies, Wilmington, DE, USA.). Sequencing of the 16S rRNA gene was performed using the dye terminator cycle capillary sequencing (ABI 3100x1) at the molecular and cellular imaging centre, Ohio agricultural research development centre (OARDC), Wooster, Ohio, USA. The 16S rRNA gene sequences of isolates were edited (Bio Edit), aligned (MegAlign) and analysed using a BLAST (http://www.ncbi.nim.nih.gov/BLAST). Phylogenetic trees for *Bacillus* and *Streptomyces* 16S rRNA gene sequences were constructed by the neighbour-joining method of Saitou and Nei (1987). Evolutionary distances were computed using the parameter method of Kimura (1980). Analysis involving 15 and 21 nucleotides respectively was computed using Mega 6 software version.

Substrate utilization studies

A forty-eight hour old culture (1.0 mL) of each pure isolate was grown in a 250 mL conical flask at 30 °C with agitation (150 rpm) in minimal salts medium (100 mL, pH 7.2) containing; $KHPO_4$ (2.0 g), K_2HPO_4 (2.5 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $MnCl \cdot 7H_2O$ (0.0003 g), $ZnCl \cdot 7H_2O$ (0.0003 g), yeast extract (0.5 g), deionised water (1000 mL) and 0.1 g aromatic compound as a sole carbon source. The lignin-related aromatic compounds tested were protocatechuic, 2,5-dihydroxybenzoic, 4-hydroxybenzioc, vanillic, ferulic, 3,4-dimethoxybenzoic and *trans*-cinnamic acids, as well as coniferyl, coumaryl and sinapyl alcohols. The culture fluids were sampled at an interval of 24 h and subjected to high pressure liquid chromatography (HPLC) quantitative analysis (Agilent technologies 1200 series, Santa Clara, USA) to measure the depletion of the substrates over a period of 7 days. The mobile phase was 0.005N H_2SO_4 with a flow rate of 0.6mL/min, Rezex polymer based column (Phenomenex, Bio-Rad, ROA organic part no OOH - 0138 - KO and refractive index detector. Injection volume was 20 µL, column temperature was 80 °C while the detector temperature was 55 °C). Controls (uninoculated mineral salts medium with substrate, inoculated mineral salts medium without substrates) were put in place and monitored to rule out contamination.

Similarly, each of the pure isolates were grown in different conical flask (250 mL) at 30 °C with agitation (150 rpm) in MSM (100 mL) containing 1.0 g of various lignocellulosic substrates as sole carbon sources, including sugarcane baggase, grass, cottonwool, filter paper and sawdust for a period of 12 days. Two sets of controls (the uninoculated control and the inoculated MSM without any substrate) were put in place to monitor the extent of growth of each isolate and to rule out contamination of the set up. The extent of growth was determined by the turbidity of the growth medium compared to the controls.

Optimization of bacterial cell growth conditions using crystalline cellulose as a model substrate

Biomass of *Streptomyces* strains was determined using the dry weight method as described earlier (Ball *et al.*, 1989). This is because *Streptomyces* species grow in mycelial chains; hence the turbidity method would not be suitable. For the optimization of growth conditions of each strain, the effects of pH, temperature and substrate concentrations were determined. For pH determination, fermentation was carried out in 250 mL plugged Erlenmeyer flasks, each containing 100 mL of mineral salts medium prepared in buffer in the range 4.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 with crystalline cellulose (1.0 g) as the sole carbon source. Sterilization was achieved in an autoclave at 121 °C, while each of the flasks was then appropriately inoculated with 1.0 mL of pure bacterial culture (approximately 10^6 CFU). Incubation was at 30 °C on a rotary shaker (150 rpm) for 12 days. Likewise, the effects of temperature and substrate concentrations were monitored as earlier described but the pH of the growth medium was maintained at 7.0. The temperature ranges of 28, 37, 45, 60 70 °C and substrate concentrations ranges of 0.5, 1.0, 1.5 and 2.0 mg/mL respectively were adopted for optimization study. At an interval of 24 h, flasks from each set were harvested and the contents were filtered using a pre-weighed filtered paper (Whatman, No 42). The filtered microbial mass was dried at 60 °C in an oven until the weight is constant, allowed to cool in desiccators, and then the final weight was obtained. The weight of the filter paper was deducted from total weight in order to obtain the mycelia dry weight (biomass). For the *Bacillus* strains, turbidity was monitored using a spectrophotometer at 620 nm. The cell density values were extrapolated from the standard reference curve as previously described (Chandra *et al*., 2007).

Determination of optimal culture conditions of strains using natural substrates

The effects of temperature, pH and metal ion concentration on bacterial growth using natural substrates (sawdust, grass, cotton wool, filter paper and sugarcane baggase) were determined by assaying for extracellular

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cellulase enzyme activity of selected strains. This was monitored by the amount of reducing sugar released into the medium as a measure of growth. This method was used to determine the optimal cultivation conditions because the *Streptomyces* strains grow in pellets and mycelial chains, they get mixed, interlock or tangle with the substrates hence dry weight method would not be suitable because of the separation challenge. Fermentation was carried out in 250 mL plugged Erlenmeyer flasks, each containing 100 mL of medium (pH 7.2). Strains were cultured using 1.0 g of oven- dried sterile finely ground natural substrate as a sole carbon source. Before inoculation, the set up was sterilized in an autoclave at 121 °C for 15 min. Each flask was inoculated with 1.0 mL of pure bacterial culture (approximately 10^6 CFU). Incubation was at 30 °C on a rotary shaker (150 rpm) for 12 days. When indicated, a higher temperature in the range 28, 37 and 45 °C was adopted for optimization study. Harvesting was at 2 days interval for a total period of 12 days. Crude extract (2.0) mL which served as a source of enzyme was directly taken from the culture flask and centrifuged (10,000 ×*g*, 4 °C for 5 min). One mL of the clear supernatant was mixed with 1.0 mL (1.0%) of carboxymethyl cellulose (CMC) in test tubes containing potassium phosphate buffer (pH 7.0), the set up was incubated for 6 h at different temperature ranges and the amount of reducing sugars was determined as described previously (Ogunyemi *et al*., 2010). Enzyme activity determinations represent mean values of three independent measurements performed in three replicates. In the same manner, the effects of pH (4.0, 7.0, 9.0 and concentration of metal ion (manganese and zinc, 0.003, 0.006 and 0.009 g/L were determined.

RESULTS

Twenty-five bacterial strains were isolated from the Lagos lagoon out of which nine were found to grow on both cellulose and lignin-related aromatic compounds. All the strains were Gram positive. Colonies of strains coded as AOB, BOB, COB, DOB, EOB and FOB appeared woolly on light microscope while strains NOB, OOB and ROB were Gram positive *Bacillus*-type strains. Colonies of strains AOB, BOB, and COB appeared tough, leathery, greyish with powdery whitish surface, fruity/earthy smell (geosmin) and produced black melanin-like pigments. Colonies of EOB, FOB and ROB appeared whitish and dry but without pigmentation. Colonies of strains NOB and OOB were cream-coloured, glossy and spreading without any noticeable smell or pigmentation. Epiflourescence light microscopy of strains AOB, BOB, COB, DOB, EOB and FOB showed characteristic mycelial mats, while strain NOB appeared as single rods. Scanning electron micrographs (Figure 1) showed that strains AOB, BOB, COB, DOB, EOB and FOB on the starch casein agar had fungi-like aerial hyphae and mycelia which appeared straight or curly with rodlets and spores. Strain OOB appeared as rods held together in chains, NOB appeared as single rods while the cells of strain ROB are ellipsoidal in long chains.

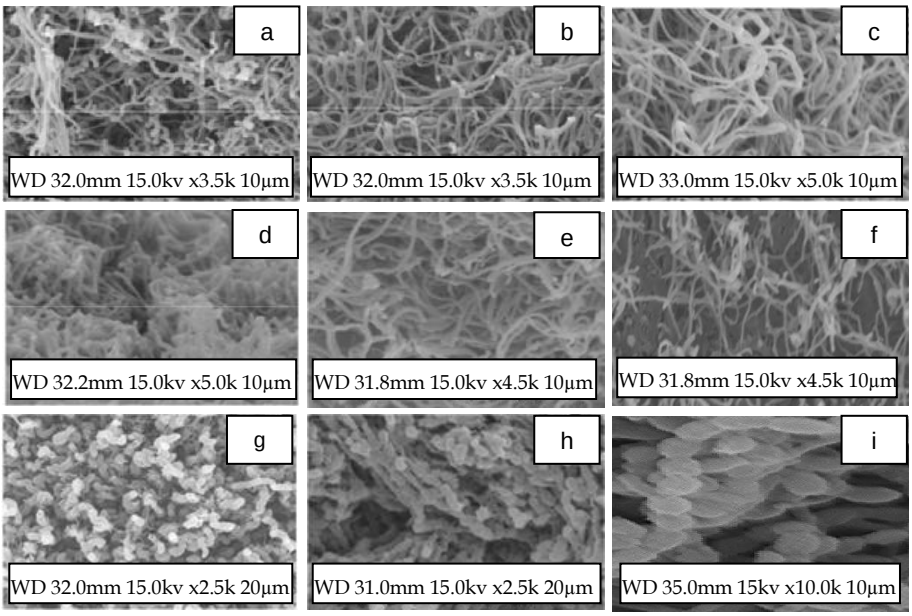

Figure 1: Scanning electron micrographs of a, *Streptomyces albogriseolus* AOB KF977548 (×3.5K); b, *S. aureus* BOB KF977549 (×3.5K); c, *S. ceolicolor* COB KF977550 (×5.0K); d, *S. albus* DOB KF977551 (×5.0K); e, *S. pseudogriseolus* EOB KF977552 (×4.5K); f, *Bacillus bataviensis* FOB KF977553 (×4.5K); g, *B. megaterium* NOB KF977554 (×2.5K); h, *Bacillus* sp. OOB KF977555 (×2.5K); i, *Paenibacillus* sp. ROB KF977556 (×10.0K).

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Biochemical characteristics

All isolates were Gram positive. They were catalase and oxidase positive with the exception of strain NOB. All isolates were able to hydrolyse starch, gelatin and casein except strains OOB and ROB which could not hydrolyse casein. All isolates were lactose fermenters except strain ROB. The isolates were able to produce gas from glucose except strain OOB.

Genotypic identities of isolates

A positive identification of isolates was established from the 16S rRNA gene sequences of the strains. The 16S rRNA encoding the genes of strains AOB, BOB, COB, DOB, EOB, FOB, NOB OOB and ROB were sequenced (approximately 1500 bp each),with GenBank accession numbers KF977548, KF977549, KF977550, KF977551 KF977552, KF977553, KF97754, KF977555 and KF977556 respectively. A homology search showed that the 16S rRNA gene sequences had a high level of identities (95-100%) with similar strains in the GenBank data base. Evidences from 16S rRNA gene sequence analyses as summarized (Table 1) showed that the 16S rRNA genes of strains AOB and COB each *had* 100% similarity to *S. albogriseolus* GQ925802 and *S. coelicolor* KF977550 respectively, while the 16S rRNA genes of strains NOB and ROB are similar to those of *B. megaterium* L2S3 EU221414 and *Paenibacillus* sp. HQ-5 by 98 and 99.8% respectively. The phylogenetic tree which was constructed for comparison of the sequences of some marine strains associated with lignocellulose degradation in the GenBank indicated that strains AOB, BOB, COB, DOB and EOB belong to the genus *Streptomyces* (Figure 2), strains FOB, OOB and NOB belong to the genus *Bacillus* while strain ROB belongs to the genus *Paenibacillus*. The phylogenetic tree showed two distinct clusters; *Streptomyces* group that are closely related and are likely to have evolved from same ancestors, and the *Paenibacillus* and *Bacillus* group that are closely related to each other, but are distant from the *Streptomyces* group.

Substrate utilization

The isolates demonstrated differential abilities to utilize lignin-related aromatic compounds (Figures 3 and 4). All isolates utilized protocatechuic acid fed into the medium completely except *Streptomyces* strain EOB, *Bacillus* strains FOB and OOB which utilized 60-90% of the substrate. All isolates utilized less than 40% of 2, 5-dihydroxybenzoic acid whilst *Bacillus* strains FOB and OOB utilized it completely. All *Streptomyces* species utilized vanillic acid to the tune of 100%, except *S. aureus* BOB. *Bacillus strains* FOB and NOB also utilized vanillic acid while *Bacillus* strain OOB and *Paenibacillus* strain ROB failed to utilize it. All isolates utilized 100% of 4-hydroxybenzoic acid except *Bacillus* sp. OOB and *Paenibacillus* sp. ROB. All *Streptomyces species* utilized 100% of coniferyl alcohol substrate in the medium. *Bacillus* strains NOB, OOB and FOB utilized 10-100% of coniferyl alcohol while *Paenibacillus* sp*.* ROB utilized 25% of the compound in culture. On coumaryl alcohol, *Streptomyces* spp. strains BOB and DOB and *Bacillus* strain NOB showed 100% utilization. *Streptomyces* strains AOB, COB and EOB utilized less than 20% while *Bacillus* strains FOB, OOB and *Paenibacillus* sp*.* strain ROB utilized less than 10% of coumaryl alcohol. *Streptomyces* spp. utilized sinapyl alcohol to a level of 22-38%. The *Bacillus* species utilized less than 30% of sinapyl alcohol while *Paenibacillus* sp*.* ROB gave 25% utilization. *Streptomyces coelicolor* COB, *Bacillus* spp. NOB, FOB and OOB utilized *trans*-Cinnamic acid completely to the level of 100%, whilst *Paenibacillus* sp. ROB failed to do so. All isolates showed visible signs of growth on lignocellulosic substrates tested (Table 2). Strains AOB, COB, FOB and ROB showed very good growth on sugarcane bagasse, whilst moderate growth was shown on grass and filter paper.

Table 1: Genotypic identities of lignocellulose-degrading bacterial isolates from amplified sequences of 16S rRNA fragment of genomic DNA.

Bacterial strain	Tentative Identity	GenBank accession number	Closest relative	% identity	GenBank accession number
AOB	*Streptomyces albogriseolus*	KF977548	*Streptomyces albogriseolus* ABRIIW EA1145	100	GQ925802.1
BOB	*Streptomyces aureus*	KF977549	*Streptomyces aureus* 3184	99	EF371429.1
COB	*Streptomyces coelicolor*	KF977550	*Streptomyces coelicolor* WBF-16	100	HQ848084.1
DOB	*Streptomyces albus*	KF977551	*Streptomyces albus* NBRC 13689	95	AB249915.1
EOB	*Streptomyces pseudogriseolus*	KF977552	*Streptomyces pseudogriseolus* S41	98	HQ850412.1
FOB	*Bacillus bataviensis*	KF977553	*Bacillus bataviensis* LMG 21832	98	AJ542507.1
NOB	*Bacillus megaterium*	KF977554	*Bacillus megaterium* L2S3	98	EU221414.1
OOB	*Bacillus* sp.	KF977555	*Bacillus* sp. LS29	97	FJ937891.1
ROB	*Paenibacillus* sp.	KF977556	*Paenibacillus* sp. HQ-5	99.8	AM162315.1

A, *S. albogriseolus* strain AOB; B, *S. aureus* strain BOB; C, *S. coelicolor* strain COB; D, *S. albus* strain DOB; E, *S. pseudogriseolus* strain EOB

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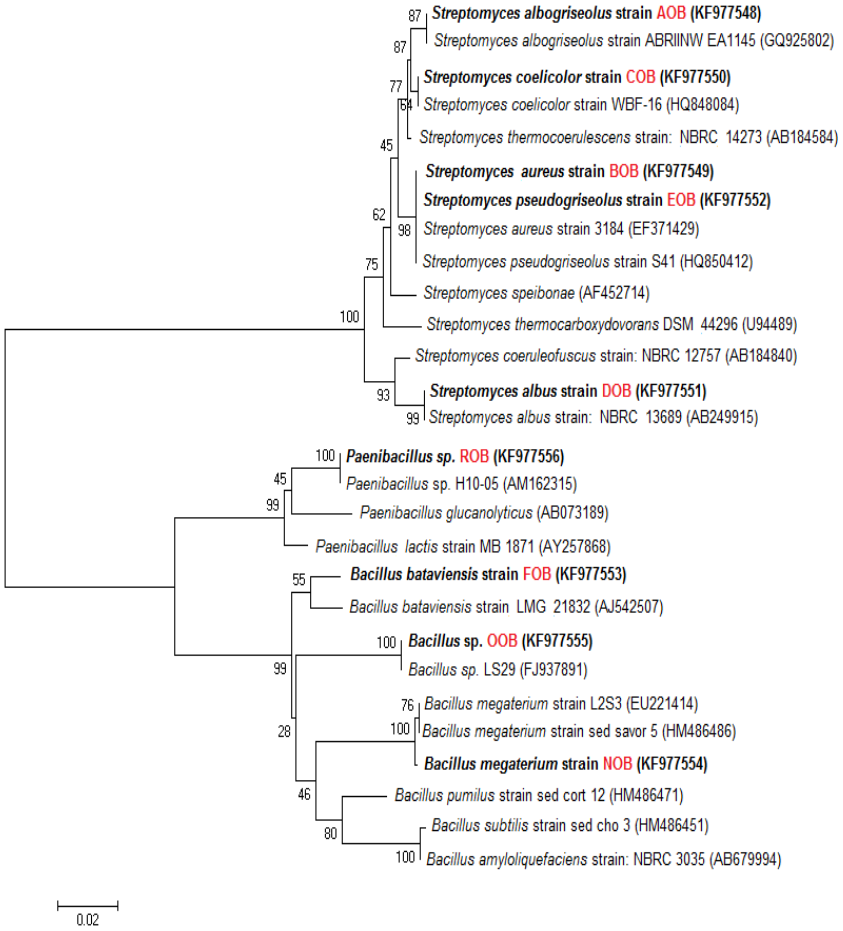

Figure 2: Phylogenetic tree of lignocellulose-degrading *Streptomyces*, *Bacillus* and *Paenibacillus* species 16S rRNA sequences using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap test = 1000 replicates. The evolutionary distances were computed using the Kimura (1980) parameter method. Analysis involving 28 nucleotide sequences was computed using Mega 6 software.

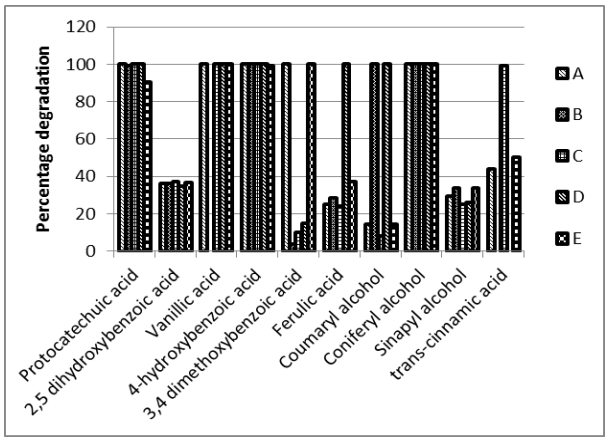

Figure 3: Summary of percentage degradation of lignin-related compounds by *Streptomyces* strains during substrate specificity testing.

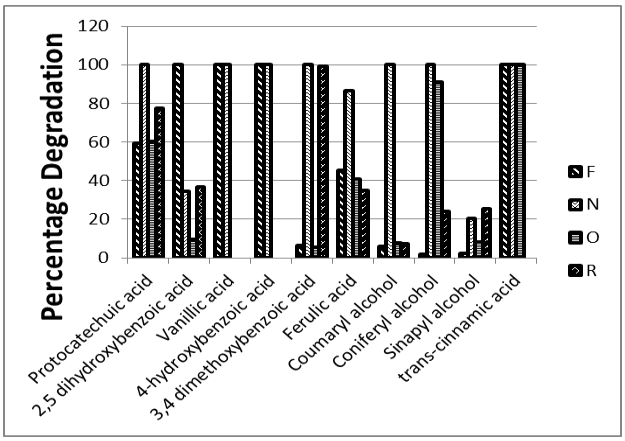

Figure 4: Summary of percentage degradation of lignin-related compounds by *Bacillus* and *Paenibacillus* strains during substrate specificity testing.

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Table 2: Substrate diversity of isolates.

Substrates	Isolates								
	AOB	BOB	COB	DOB	EOB	FOB	NOB	OOB	ROB
Sugarcane baggase	+++	++	++++	+++	+++	++++	+++	+++	++++
Grass (*Setaria* sp.)	+++	+	+++	+++	+++	+++	++	++	+++
Cotton wool	+++	+	+++	++	+++	++	+	+	++
Filter paper	+++	+	+++	+++	+++	+++	+++	+	+++
Sawdust	+++	+	++	++	+++	++	+++	+	++

++++, very good growth; +++, good growth; ++, moderate growth; +, poor growth.

Optimal conditions of bacterial growth

The summary of the bacterial cell growth optimization study (Table 3) showed that strain AOB grew at a temperature range of 28 -70 °C, but grew optimally at 60 °C. The pH range was between 4.0 and 11.0 with an optimum pH at 8.0. Even though the strain could grow at varied substrate concentrations, its growth was optimal at a concentration of 1.0 mg/mL of crystalline cellulose. Strains COB, FOB, NOB and OOB grew optimally at pH 7.0, but their optimal temperatures were 60, 37, 37, and 60 °C respectively.

The effect of temperature, pH, substrate concentration and metal ion on cellulase activities of selected strains following growth on various natural substrates was carried out using cell-free extracts as enzyme sources while carboxymethyl cellulose was the assay substrate. The results showed that under different conditions of cultivation, the strains had varied cellulase activities when different natural substrates were used as the sole carbon sources. At a temperature of 28 °C, *Paenibacillus* sp. strain ROB gave the highest cellulase activity of 0.225 mg/mL.min. At 37 °C, strain DOB gave the highest activity of 0.210 mg/mL.min while at 45 °C, *S. pseudogriseolus* strain EOB gave the highest cellulase activity (0.211 mg/mL.min),when sugarcane baggase was used as the growth substrate (supplementary Table 1).

When grass was used as the substrate, *Paenibacillus* sp. strain ROB gave the highest cellulase activity (0.216 mg/mL.min) at 28 °C. At 37 °C *S. albus* gave the highest activity (0.186 mg/mL.min), while at 45°C, *S. pseudogrieseolus* strain EOB gave the highest activity (0.196 mg/mL.min). When sugarcane baggase was used as the growth substrate, *S. albus* strain DOB gave the highest cellulase activity of 0.182 and 0.164 mg/mL.min at pH 4.0 and 7.0 respectively while *Paenibacillus* sp. strain ROB gave the highest cellulase activity at alkaline pH of 9.0 (0.124 mg/mL.min). On Grass, at pH 4.0, the highest cellulase activity was 0.162 mg/mL.min by *S. albus* strain DOB. At pH 7.0, the highest activity was by *B. megaterium* strain NOB (0.447mg/mL.min) while at pH 9.0, the highest activity was by *Paenibacillus* sp. ROB (0.133 mg/mL.min) as summarised (supplementary Table 2).

When filter paper was used as the substrate, the result showed an activity of 0.134 mg/mL.min (highest at pH 4.0), 0.134 mg/mL.min (highest at pH 7.0) and 0.105 mg/mL.min at alkaline pH of 9.0 by *S. albus* DOB, *S. pseudogriseolus* EOB and *Paenibacillus* sp. ROB respectively.

The effect of concentration of manganese on cellulase activities showed that *S. albus* strain DOB produced the highest cellulase activities (0.198, 0.181 and 0.167 mg/mL.min) at a manganese concentration of 0.009 g/L when sugarcane baggase, grass and filter paper respectively were used to cultivate the organism. When cotton wool and sawdust were used as the growth substrates, strain DOB had its highest activity at the manganese concentration of 0.006 g/L (supplementary Table 3) while the activities reduced at a concentration of 0.009 g/L. For *Streptomyces* EOB, *B. megaterium* and for *Paenibacilllus* sp. strain ROB, an increase in concentration of manganese brought about an increase in the cellulase activities, irrespective of the growth substrate.

The selected strains showed varied cellulase activities when varied natural substrates were used as the growth substrate at varied concentration of zinc metal ions. *Streptomyces albus* strain DOB showed highest cellulase activities, when the zinc concentration was 0.003 g/L on sugarcane baggase, filter paper, cotton wool and sawdust (0.155, 0.136, 0.112 and 0.101 mg/mL.min respectively), while the highest activity when grass was the growth substrate was obtained at a zinc concentration of 0.009 g/L (supplementary Table 4). *Streptomyces* strain EOB showed highest activity when the zinc concentration was 0.006 g/L in all natural substrates except grass. The cellulase activity of strain NOB varied depending on the type of growth substrate used. The highest activity (0.400 mg/mL.min occurred when filter paper was used as the growth substrate at a zinc concentration of 0.006 g/L. *Paenibacillus* sp. strain ROB had the highest cellulase activity when zinc concentration was 0.009 g/L irrespective of the substrate used.

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Table 3: Optimal growth condition of strains on carboxymethyl cellulose.

Strains	pH		Temperature (°C)		Substrate concentration (mg/mL)	
	Range	Optimum	Range	Optimum	Range	Optimum
AOB	4.0 - 11.0	8.0	28 - 70	60	0.5-2.0	1.5
BOB	6.0 - 9.0	7.0	28- 70	50	0.5 -1.5	1.0
COB	4.0 - 11.0	7.0	28 - 70	60	0.5 - 20	2.0
DOB	4.0 - 11.0	4.0	28 - 60	37	0.5 - 2.0	2.0
EOB	4.0 - 11.0	6.5	28 - 70	60	0.5- 1.5	1.0
FOB	4.0 - 11.0	7.0	28 -70	37	0.5 - 2.0	1.0
NOB	4.0 - 11.0	7.0	28 - 60	37	0.5 - 1.5	0.5
OOB	7.0 - 11.0	7.0	28 - 70	60	0.5- 2.0	0.5
ROB	4.0 -11.0	4.0	28 - 70	70	0.5 - 2.0	0.5

DISCUSSION

Previous studies carried out in the Lagos lagoon on the biodegradation of sawdust had focused mainly on the role of fungi (Akpata, 1980; 1986). However, in this study, nine lignocellulose**-**utilizing bacteria were isolated from decomposing sawdust in the lagoon, which were identified as; *S. albogriseolus* strain AOB, *S. aureus* strain BOB, *S. coelicolor* strain COB, *S. albus* strain DOB, *S. pseudogriseolus* strain EOB, *B. bataviensis* strain FOB, *B. megaterium* strain NOB, *Bacillus* sp. strain OOB and *Paenibacillus* sp. strain ROB. The morphological, cultural and the biochemical characteristics as well as the 16S rRNA gene sequences suggested that strains AOB, BOB, COB, DOB and EOB be assigned to the genus *Streptomyces*. Their characteristics were similar to those previously reported by Li (1997) and Meyers *et al.* (2003). The phylogenetic tree of *Streptomyces* species isolated in this study shows that they are closely related to one another. *Streptomyces thermoceorulescens* (AB184584) is the nearest neighbour to *S. coelicolor*, while *B. pumulus* strain sed cort12 (HM486471) is the nearest neighbour to *B. megaterium* strain NOB. Sequence analysis of 16S rRNA gene has been used by several authors for successful identification of *Streptomyces* spp. (Moran, 1995; Nishimura *et al.*, 2006). The unrooted phylogenetic tree shows that *Bacillus* and *Paenibacillus* spp. appeared to be closely related and are likely to have evolved from the same ancestor. This is in agreement with Maki *et al.* (2011). The authors showed that *Bacillus* spp. and *Paenibacillus* spp. belong to the phylum firmicutes. The observation of the authors, that *Bacillus* and *Paenibacillus* spp. are distantly related to *Streptomyces* species (actinobacteria), is in line with the observation made in this study. Although the *Bacillus* and *Paenibacillus* species are distantly related to the *Streptomyces* group, a common phenotypic attribute among them in this study is their ability to produce either conidia or endospores for vegetative propagation, which may enable them to survive under harsh environmental conditions.

Substrate utilization studies showed that the isolates had varied preferences for different substrates. The plausible reasons for this have been well established by Kirby (2005), Nishimura *et al*. (2006) and Maki *et al*. (2011). The authors suggested that most isolates will preferentially metabolize the less complex compounds within a mixture of substrates. Other reasons could also be the ability of the isolates to produce the requisite enzymes for metabolizing the compounds, the level of production of these enzymes, the incubation period and culture conditions such as pH and temperature. It has also been established previously by Ray *et al*. (2011) that the enzymatic removal of lignin from lignocellulosic biomass facilitates the availability of cellulose to degradation by cellulase**-**producing organisms. The isolates showed abilities to grow better on sugarcane baggase compared to other lignocellulosic biomass presented in this study. It is probably because of its complex composition, especially because of its high percentage content of cellulose, a major component of the plant cell wall. Natural substrates had previously been shown to support the growth of *Streptomyces* spp. (Njoku and Antai, 1987), *Bacillus* spp. (Sethi *et al.*, 2013) and *Paenibacillus* sp. (Maki *et al.*, 2011; Kumar *et al*., 2012). Physicochemical factors such as pH, temperature and substrate concentration have been reported to limit lignin and cellulose degradation by microorganisms (Pometto and Crawford, 1983; Alam *et al*., 2004). During growth optimization experiments, cellulose and various wastes (sugarcane baggase, grass, filter paper, cotton wool and sawdust) were used as substrates (sole carbon sources) for strains which were selected based on their performances on the degradation of kraft lignin and lignin related aromatic compounds (DOB,EOB,NOB and ROB) under the same environmental conditions (data not shown). It was observed that the selected strains had varied optimum growth conditions when crystalline cellulose was used as substrate. Generally speaking, the strains showed varied cellulase activities depending on the growth substrates used. This is most likely to be as a result of the constituents of each substrate. It was

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therefore not a surprise that the highest cellulase activity in this study was obtained when sugarcane baggase was used as the growth substrate. Shanker and Isiarasu (2011) had earlier suggested that the highest cellulase productivity with sugarcane baggase may be due to its very high percentage of cellulose, a major component of cell walls.

Evidence that culture conditions and nature of substrate are important factors in the production of cellulase had been demonstrated in this study. For example, *Streptomyces albus* DOB gave a cellulase activity of 0.182 mg/mL.min at pH 4.0, and 28 °C, but at 37 °C, and neutral pH, the isolate gave an activity of 0.210 mg/mL.min at the same incubation period when sugarcane baggase was used as the growth substrate. Alam *et al.* (2004) had earlier reported no growth at a temperature of 25 °C, a heavy growth at 35 and 40 °C but a moderate growth at 45 °C. It was therefore not surprising that *S. pseudogriseolus* strain EOB gave its highest cellulase activity at 45 °C at an optimum pH of 4.0 irrespective of the growth substrate.

It is evident that the *Bacillus* and *Paenibacillus* species obtained in this study produced their maximum cellulase activities at 28 °C irrespective of the natural growth substrate used as carbon source. *Bacillus megaterium* strain NOB selected for optimization in this study had it maximum cellulase activity at pH 7.0 irrespective of the growth substrate, although grass was preferred by this isolate. *Paenibacillus* sp. gave its maximum cellulase activity at pH 4.0; incidentally, this organism also reached its highest biomass level at a pH of 4.0 when crystalline cellulose was the growth substrate. Interestingly, that was the only organism among the selected ones that produced cellulase activity of up to 0.124 mg/mL.min at an alkaline pH of 9.0 irrespective of growth substrate. When crystalline cellulose was used as the growth substrate to produce biomass, the optimum temperature of growth for the *Bacillus* and *Paenibacillus* strains (FOB, NOB, OOB and ROB respectively) were higher (37, 37, 60 and 70 °C) although they grew at a temperature range of 28 to 7 °C. A similar observation was made by Kumar *et al.* (2012), who characterized carboxymethyl cellulase from *P. polymyxa* using the mango peel. From their results, this isolate exhibited maximum cellulase activity at 30 °C and pH 5.0. The purified enzyme was found by the authors to be stable over the range of 20-60 °C at pH 4.0-7.5.

The results of the optimization study showed that the concentration of manganese and zinc ions in the growth medium will either act as a repressor (inhibitor) or inducer, depending on the type of growth substrate used. This is probably due to the fact that different lignocellulosic wastes have different constituents, metal ions inclusive. Even though metal ions are essential nutrients, it is well established that their presence in excess may be detrimental to living organisms. Prema *et al.* (2005) observed an increase in manganese peroxidase activity by *S. psammoticus* from 0.25 µmol/mL to 3.06 µmol/mL when $MnSO_4$ was increased from 0.5 mM to 1.75 mM in the growth medium. They reported that the concentration of Mn^{2+} had significant effect on enzyme activity. In their reaction medium, maximum activity was observed at $MnSO_4$ concentration of 0.15 mM and beyond that, there was a reduction in the activity.

The results of enzyme optimization study were generally in agreement with the results of several authors. Pandey *et al.* (2001) is of the opinion that the type of strain, culture condition, nature of substrate and nutrients are important factors affecting growth and enzyme production. Shankar and Isaiarasu (2011) studied cellulase production by *B. pumilus* EWBCMI under varying culture conditions and concluded that various production parameters like pH, temperature, carbon source, metal ions, inoculum size and incubation time should be optimized from the economic point of view so that it can be used for cheaper and more easily available resources than expensive and refined media. During the optimization study of *B. cereus* MRKI for cellulase production and its biostoning, Kumar *et al.* (2012) found that the isolate gave its highest cellulase activity at 32 °C, pH 8.0, within 48 h. Bhat *et al.* (2000) showed that CMCase activities alter with varying pH and temperature and that characterization of produced enzymes require knowledge about optimum pH, temperature stability and substrate so as to find out the best level of performance for enhanced efficiency of biotechnological processes. This study showed that the bacterial strains isolated from the Lagos lagoon can tolerate wider pH and temperature ranges and may therefore be good candidates for biotechnological applications.

CONCLUSION

Even though much credence had been ascribed to fungi in the breakdown of lignocelluloses (especially the white rot fungi), it is worthy of note that bacteria, because of their immense environmental adaptability and biochemical versatility have the potential for the breakdown of enormous number of organic molecules. Most of the bacterial isolates in this study possessed the abilities to breakdown lignocellulosic biomass and lignin-related aromatic compounds and may therefore play a key role in the bio-rehabilitation of the sawdust-polluted environment and in biotechnological processes. With respect to the utilization of lignin-related aromatic compounds studied, strain OOB performed poorly and may not be of further utility. Although strain ROB was less dramatic in the degradation of lignin-related aromatic compounds, it had shown preference for cellulose as a substrate and may have the potential for use in the conversion of cellulosic biomass. Further research work would focus on the ability of each isolate to degrade polymeric lignin with a view to determine the degradative enzymes involved and the metabolic routes employed. The metabolic products of lignin and sawdust degradation by the selected organisms would also be determined.

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