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Broad-range pH/Temperature-stable Cellulase from a Novel Hydrocarbon Contaminated Mangrove soil Bacterium, *Bacillus licheniformis* VVA21

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Authors' contributions

This work was carried out in collaboration between all authors. Author VGA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VGA, AAI, CNA and VE managed the analyses of the study. Authors VGA, AAI and VE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study reports the production of cellulase by *Bacillus licheniformis* VVA21 isolated from hydrocarbon contaminated Kegbara-Dere mangrove in Ogoniland, Nigeria.
Methodology: Baseline physicochemical characteristics of the hydrocarbon contaminated soil were established. Twenty-two bacterial isolates were screened for cellulolytic activity on carboxymethyl cellulose (CMC) agar using the spread plate technique. The isolate with the highest zone of clearance was selected and assayed further. Crude cellulase was extracted and partial purification achieved by ammonium sulphate precipitation, followed by dialysis, and final purification

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by Sephadex G-100 chromatography. The best cellulase-producing bacterium was identified by analytical profile index (API) and 16S rRNA gene analyses.
Results: The contaminated soil revealed sulphate, nitrate, phosphate, polycyclic aromatic hydrocarbon (PAH), benzene, toluene, ethylbenzene, and xylene (BTEX) and total petroleum hydrocarbon (TPH) contents of 30.23, 24.77, 17.56, 11.66, 100.22 and 11560 mg/kg, respectively. Out of the 22 bacterial isolates screened for cellulolytic activity, isolate VVA21 gave the highest zone of clearance (16 mm) with maximum cellulase activity of 871.6 U/mL after 48 h of incubation. Specific cellulase activity increased across the different purification steps (from crude fraction to Sephadex G-100 fraction), ranging between 16.8 and 68.9 U/ /mg/mL. The purified cellulase was stable over a pH range of 4 to 10 and temperature range of 30 to 100°C. The best stability for pH (102.46 U/mg) was observed at pH of 8.5 whereas the best stability for temperature (99.96 U/mg) was observed at 30°C. API and 16S rRNA gene product analyses revealed the best cellulase-producing strain as *Bacillus licheniformis* strain VVA21. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number MF581271.1.
Conclusion: The *Bacillus licheniformis* VVA21, isolated from hydrocarbon contaminated mangrove soil, showed high potentials for cellulase production with high stability over broad temperature and pH ranges.

Keywords: Bacillus licheniformis; mangrove; hydrocarbon contaminated soil; cellulase; Ogoniland.

1. INTRODUCTION

Mangroves are salt-tolerant (halophytic) plant communities found in sheltered coastline areas in tropical and sub-tropical intertidal regions of the world, such as bays, creeks, delta, estuaries, lagoons, mashes, and mudflats [1]. The term 'mangrove' describes both the ecosystem and the plant families that have developed specialized adaptation to live in the tidal environment [2]. Biologically, mangroves are highly productive marine ecosystem where microorganisms actively take part in biomineralization and biotransformation [3]. The distribution of microbial activities in estuarine systems is clearly variable. Mangrove-derived detritus constitute a large reservoir of carbon and energy, potentially available to estuarine food web and bacteria, the major participants in the carbon, sulphur, nitrogen and phosphorus cycles within the mangrove ecosystem [4].

Hydrocarbon contamination of mangrove soils has been widely reported. Proffitt [5] noted that mangroves are highly susceptible to oil pollution. Oil slicks enter the swamps during high tidal conditions and become deposited on aerial roots and surface sediments. Conventionally, mangrove swamps may be adversely affected by crude oil production because of known high persistence of oil in low-energy muddy environments [6,7]. Mangroves are killed by heavy or viscous oil that covers the trees' breathing pores, leading to loss of oxygen available to the roots. The impact of oil on mangrove vegetation in some part of the Niger Delta region of Nigeria especially, Ogoniland, has been disastrous [8]. Impacts vary from extreme stress to total destruction. In the most impacted areas, only the roots of the mangrove remain, with no stem or leaves; the roots are completely coated in oil [8,9], authors' field experience]. Toxic substances such as petroleum hydrocarbons (PHs) can knock-off mangroves; their aerial root systems and their intertidal muddy nature make them susceptible to crude oil pollution. This susceptibility to oiling ensures that crude oil in mangrove swamps persists [10,11]. Crude oil pollution has not only reduced productive outputs of the Niger Delta mangrove but has also led to bio-accumulation of total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs) in edible seafoods from mangrove [12,13,14]. The mangroves are principal places that are impacted by oil exploration and associated activities [15]. Factors responsible for oil spillage in the zone include corrosion of oil pipes, tanks sabotage, port operations, inadequate care in oil production operations, and engineering drills [16]. The consequence is massive oiling of the environment and destruction of vulnerable ecological units [17,18].

Industrial enzymes generate massive revenue and are applied in bio-pharmaceutical, medical, food, cosmetic, and agricultural industries. Sudha [19] isolated halophilic bacteria from mangrove soil, which produced asparaginase. Arylsulfatase, an important enzyme that participates in the metabolism of sulphuric acid esters produced predominantly by *Bacillus* spp.

followed by *Vibiro* spp., has been widely isolated from mangroves. Behera et al. [20] isolated and identified cellulose-degrading bacteria: *Bacillus* spp., *Brucella* spp., and *Pseudomonas* spp., all from mangrove soils. Cellulase importance as an industrial enzyme is well known. Cellulase hydrolyses β-1, 4-glycosidic bonds in cellulose polymer to generate glucose subunits. It is among the industrially important hydrolytic enzymes and is of great significance in present-day biotechnology. Bacterial cellulases have gained significant attention due to their wide applicability in various industrial processes including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture. In addition to these major industrial applications of cellulases, they are used in animal feeds for improving nutritional quality and digestibility. This study investigated the production of cellulase by *Bacillus licheniformis* isolated from hydrocarbon contaminated Kegbara-Dere mangrove in Ogoniland, Nigeria.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were obtained from mangrove swamp located at Kegbara-Dere (K-Dere) in Gokana Local Government Area of Rivers State, Nigeria. Geographically, Kegbara-Dere is located between N4^{o} 45$^{'}$ Latitudes and E6^{o} 59$^{'}$ Longitudes. Soil auger was used to collect soil samples randomly (at depths between 0 and 15 cm) and the samples were afterwards bulked to make a composite sample. The sample was transferred into a non-reactive glass container and transported immediately to the laboratory for physicochemical and microbiological analyses.

2.2 Physicochemical Analyses

Physicochemical parameters analysed for the hydrocarbon contaminated mangrove soil included pH, temperature, sulphate, nitrate and phosphate contents, TPH, PAH, BTEX and heavy metals (Ni, Fe, Cd, Cr, V, Cu, Zn, and Pb).

The pH of the soil sample was measured in 1:1 soil to water suspension with a glass electrode pH meter (Mettler Toledo, Switzerland) (sensitivity ± 0.01). Nitrate and sulphate contents were determined by the methods described by APHA [21]. Heavy metals concentrations were estimated after nitric acid digestion by means of an atomic absorption spectrophotometer. The phosphate content was estimated using colorimetric method described by UNEP [22].

2.3 Total Petroleum Hydrocarbon (TPH)

The method of APHA [21] was adopted for the estimation of the TPH concentration. Soil sample (10 g) was weighed into extraction bottle and 20 mL of extraction mixture (dichloromethane), in the ratio 2:1, was added. The mixture was subjected to sonication for 1 h and the organic-aqueous layer decanted. Extracted organic phase was dried using anhydrous sodium sulphate salt and concentrated using vacuum rotary evaporator gas to about 1.0 mL. Round bottom flask was rinsed to make the final volume of the extract 1.0 mL. One millilitre of the final extract was injected into an already calibrated Gas Chromatograph (GC) (HP 5890, USA) equipped with capillary column. The peak areas were used in the quantifications. Commercially available TPH Standard, C8-C40 (Hydrocarbon window defining standards, Accu Standard DRH 0085) was used for the GC calibration.

2.4 Polycyclic Aromatic Hydrocarbons (PAHs) and Benzene, Toluene, Ethylbenzene, Xylene (BTEX)

PAHs and BTEX were analysed using the method described by APHA [21]. A known weight of the soil was poured into an extraction bottle and dried with sodium sulphate. The sample was then extracted with a total volume of 1:1 dichloromethane:hexane mixture in 25 mL portion. The extract was reduced to about 2 mL in an atmosphere of nitrogen and fractionated in a silica gel column using hexane. This fraction was stored in a refrigerator prior to the analysis. Each sample was spiked with naphthalene and scalene for quality control purpose. The same sample was further fractionated with a chromatographic grade dichloromethane for the analysis of polycyclic aromatic hydrocarbon. Both samples for BTEX and PAH were analysed with a programmed gas chromatograph (HP, 5890).

2.5 Isolation and Screening of Cellulase-Producing Mangrove Bacteria

Composite soil sample (25 g), obtained from Kegbara-Dere mangrove was homogenized in 250 mL of Ringers solution and serially diluted. The third-fold dilution was used for analyses [23]. Cellulolytic bacterial strains were isolated using

the 10^{-3} diluent and spread plate technique adopted according to the method of Jalal et al. [23]. Carboxymethyl cellulose (CMC) agar with components described by Ezebuiro et al. [24] was prepared and used for the isolation of cellulolytic bacteria. The inoculated CMC agar plates were incubated (Gaint Binder Incubator, USA) at 35°C for 48 h.

After 48 h of incubation, each of the duplicate plates was screened for cellulase activity by flooding the plate with 0.1% Congo red (Sigma Aldrich, Germany) solution. The plates were left undisturbed for 15 min and then destained with 1 M NaCl (Oxoid, UK) [20]. Halo zones around the growing cellulolytic bacteria confirmed positive isolates. The ratio of the clear zone diameter to colony diameter was measured and the highest cellulase producer selected. The largest ratio was assumed to contain the highest activity [24]. The selected isolate was transferred into minimal CMC agar slants and the slants maintained at 4°C for further analysis.

2.6 Inoculum Development

A loopful of 24 h pure culture of the representative bacterium was inoculated in 100 mL of cellulase broth medium (containing: $MgS0_4$, 0.01%; K_2HP0_4, 0.2%; KH_2P0_4, 0.7%; Sodium citrate, 0.05%; yeast extract, 0.1%; glucose, 0.1%; and 100 mL distilled H_2O), sterilized in an autoclave at 121^{o}C for 15 min at 1 atm and incubated in a shaker incubator at 35^{o}C for 24 h. After 24 h, vegetative cells were obtained and used as inoculum source for cellulase assay in a submerged fermentation process according to the methods described by Ray et al. [25]; Irfan et al. [26] and Ire et al. [27].

2.7 Cellulase Production

The fermentation medium (containing in 100 mL of distilled water: $MgSO_4$, 0.01%; K_2HPO_4, 0.7%; KH_2PO_4, 0.2%; sodium citrate, 0.05%; yeast extract, 0.1%; CMC, 0.1%) was transferred into a 250 mL cotton-plugged conical flask, sterilized in an autoclave at 121°C for 15 min at 1 atm and allowed to cool before use under ultra-violet sterilizer to minimize contamination [28,29].

The flasks were then each inoculated with 1 mL of 0.5 McFarland standardised inoculum and incubated in a shaker incubator at 35°C for 24 h at 259 x g, after which, 10 mL of culture was taken into sterile test tube and centrifuged in a centrifuge (Uniscope, England) at 6467 x g for 15 min. The clear supernatant (cell free extract) obtained after centrifugation, served as the crude enzyme source and was subjected to enzyme assay [30,31].

2.8 Determination of Cellulase Activity/ Enzyme Assay

Cellulase activity was assayed using dinitrosalicylic acid (DNS) reagent (Lab M, India) by estimating the reducing sugars released from CMC solubilised in 0.05 M phosphate buffer at pH 8 [32]. Crude enzyme was added to 0.5 mL of 1% CMC in 0.05 M phosphate buffer and incubated at 50°C for 30 min. After incubation, the reaction was stopped by the addition of 3 mL of DNS reagent and boiled at 100ºC in water bath for 5 min. Development of colour was observed after boiling and sugars liberated were estimated by measuring absorbance at 540 nm with a spectrophotometer (Perkin Elmer, USA). Cellulase production was estimated using glucose calibration curves. One unit (U) of cellulase activity was expressed as the quantity of enzyme, required to release 1 µmole of glucose per min per mL under standard assay conditions.

2.9 Ammonium Sulphate Precipitation of Cellulase

The method of De-moraes et al. [33] with minor modifications was adopted for the purification of the cellulase from *Bacillus licheniformis*. In brief, crude extract, obtained from submerged fermentation, was centrifuged at 6467 x g (4 ^{o}C) for 15 min to increase clarity. Solid crystals of ammonium sulphate were added to the crude enzyme extract until it was 70% saturated. The mixture was kept for 6 h at 4°C. The resulting precipitate was collected by centrifugation at 6467 x g for 15 min. The obtained pellets of precipitated proteins were kept aside and more crystals of ammonium sulphate were added to the supernatant to attain 85% saturation. This was again kept for 6 h at 4°C and centrifuged as described previously. After centrifugation, the supernatant separated and sediments were dissolved in small amount (about 10 mL) of 0.2 M phosphate buffer (pH 5.5) and 0.2 M Tris-HCl buffer (pH 8.0) and assayed accordingly [34,35, 36,37].

2.10 Desalting by Dialysis for Cellulase

The solution obtained from ammonium sulphate precipitation was subjected to a process of

dialysis. It was desalted using a Float-A-Lyzer dialysis bag (Spectrum Lab) with 10kDa MWCO by rinsing against the same buffer. One end of the dialysis bag was tied tightly and the solution was poured into the bag and the other end of the dialysis bag sealed to prevent any leakage. Thereafter, the dialysis bag was suspended in beakers and dialyzed against the same buffer with 4 regular changes of buffer after every 6 h to remove low molecular weight substances, ions, and other impurities that interfere with the enzyme activity. Total protein and activity of partially purified cellulase were determined before and after dialysis of the ammonium sulphate fraction. This process ensured further purification of the partially purified cellulase [36, 37,38].

2.11 Sephadex G-100 on Gel Filtration Chromatography for Cellulase

The dialyzed cellulase fraction was subjected to gel filtration chromatography using Sephadex G-100 (Sigma, USA) column for further purification. The column was packed to the height of 120 cm in a glass column with internal diameter of 2.0 cm. The sample was poured onto the column packing and eluted with phosphate buffer pH 5.5. The flow rate was maintained at 0.5 mL/min; up to 20 fractions of 1 mL each were collected and both the enzyme activity and protein content were determined for each separate fraction via spectrophotometer following standard assay protocols. The corresponding fraction with the highest activity was pooled and kept in water ice for further studies [37].

2.12 Determination of Protein Content

Determination of protein was performed according to the method of Lowry et al. [39]. Protein extract (0.2 mL) was measured into a tube and 0.8 mL distilled water added to it. About 1 mL of distilled water was used as blank; while bovine serum albumin (BSA) standard curve was equally set up. Five millilitres (5.0 mL) of alkaline solution prepared accordingly, was added to the tube, mixed thoroughly and allowed to stand for 10 min. Thereafter, 0.5 mL of Folin Ciocalteau solution was added to the test tube and left for 30 min after which the optical density was read at 650 nm wavelength using a spectrophotometer. The protein concentration was estimated using values extrapolated from standard graph.

2.13 Effect of pH on Activity and Stability of Purified Cellulase

The optimum pH of the purified cellulase was determined by replacing 0.2 M phosphate buffer (pH 7.5) in the cellulase assay with various buffers: sodium acetate buffer 0.2 M (pH 4.0-5.5), phosphate buffer 0.2 M (pH 6.0-8.0) and glycine-NaOH buffer (pH 9-12). The cellulase assay was carried out as previously described by Muhammad and Hafiz [40] and Olajuyigbe and Falade [41]. The effect of pH on stability of the pure cellulase was measured by incubating 2 mL of the cellulase with 2.0 mL of relevant buffers of varying pH (4-12); 0.2 M sodium acetate buffer (pH 4-5.5), 0.2 M phosphate buffer (pH 6-8) and 0.2 M glycine-NaOH buffer (pH 9-12) without a substrate for 30 min and assayed accordingly. Thereafter, the residual cellulolytic activity was measured using standard cellulase assay method.

2.14 Effect of Temperature on Activity and Stability of Purified Cellulase

Optimum temperature for the purified cellulase was determined by incubating the reaction mixture of the cellulase assay at different temperatures: 30, 35, 37, 40, 45, 50, 55, and 60^{o}C and monitoring for cellulolytic activities. The study was carried out using the method described by Olajuyigbe and Falade [41]. Thermal stability of the purified cellulase was measured by incubating 2 mL of pure cellulase at pH 7.5 without a substrate at the various temperatures of 30 to 100°C for 30 min. Thereafter, standard cellulase assay as described by Ire et al. [27] was performed to determine the residual activities.

2.15 Phenotypic and Biochemical Characterisation

The cellulase-producing bacterium was subjected to various biochemical tests using the methods described by Holts et al. [42]; MacFaddin [43] and Madigan et al. [44]. The bacterial colonial growth characteristics on agar plates were observed and recorded. Macroscopic examination was based on shape of colony, colony size, elevation, margin, colour on the reverse side of plate, texture, surface appearance, and opacity. The different biochemical test carried out on the isolate included motility, endospore formation, oxidase, citrate, catalase, indole, urease, methyl red,

Voges-Proskauer, starch production and sugar fermentation tests (glucose, lactose, mannitol, maltose, fructose, and sucrose).

2.16 Analytical Profile Index (API)

The isolate was identified using API Kit [45]. The test was performed according to the manufacturer's instructions and is summarised briefly. Culture of the bacterium on mineral salt agar after about 24 h incubation and purity check was harvested from the solid media using sterile swab stick. The inoculum was prepared in an API 50 CHB medium. The test was carried out and the reaction of each test recorded on the record sheet provided. The isolate was identified with the help of the API web identification software. All positive tests were indicated by a colour change from pink to yellow due to acid formation while the negative test had no colour change.

2.17 Molecular Identification

Extraction of the cellulase-producing bacterial DNA, PCR amplification of 16S rRNA gene and gel electrophoresis of the isolate were carried out at the Molecular Biology Laboratory of National Institute for Medical Research (NIMR) Yaba, Lagos, Nigeria. The amplicon obtained from the gel electrophoresis was sent to GATC Biotech AG (European Genome and Diagnostics Centre - Jakob-Stadier-Platz 7, 78467 Constance, Germany) for Sanger Sequencing.

2.17.1 DNA extraction and polymerase chain reaction (PCR)

The genomic DNA of the isolate was extracted directly from freshly grown culture of the isolate using a Qiagen QiaAMP DNA extraction kit. PCR amplification of the 16S rRNA gene accompanied the extraction process with the primer set 27F-5'- AGA GTT TGA TGC TGG CTC AG -3', and 515R 5'- TTA CCG CGG CKG CTG GCA C-3' according to the method described by Yamada et al. [46] and Katsura et al. [47]. In brief, 20 µL reaction mixture containing 1X PCR buffer (Solis Biodyne, Estonia), 1.5 mM $MgCl_2$ (Solis Biodyne, Estonia), 0.2 mM of each dNTP (Solis Biodyne, Estonia), 2 U Taq DNA Polymerase (Solis Biodyne, Estonia), 20 pMol of each primer and sterile water was used to make up the reaction mixture. PCR was carried out in an Eppendorf Nexus Thermal Cycler with the following cycling parameters: an initial denaturation step at 95°C for 5 min., followed by 30 consecutive cycles of denaturation at 95°C for 30 sec., annealing at 55°C for 45 sec. and extension at 72°C for 1 min. Finally, extension stage was carried out at 72°C for 10 min.

After the PCR reaction, the product was separated and purified on a 1.5% agarose gel (Solis Biodyne, Estonia). One hundred base pair (100 bp) DNA ladder (Solis Biodyne, Estonia) was used as DNA molecular weight marker. Electrophoresis was done at 80 V for 1 h 30 min. and the gel was viewed under UV light after staining with ethidium bromide (Solis Biodyne, Estonia).

2.17.2 Sequence analysis and phylogenetic tree construction

The sequence generated by ABI 3130 automated sequencer (Hitachi, Japan) was visualized using Chromas Lite (version 2.1.1, Technelysium Pty Ltd). BioEdit Sequence Alignment Editor was used for sequence editing, before performing a basic local alignment search tool (BLAST) using NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with the highest similarities were downloaded and aligned with ClustalW. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.89615307 was constructed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown (Fig. 4) next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and were in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 655 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [48].

2.18 Statistical Analysis

All data generated from this study were subjected to statistical analyses to determine any significant difference between experimental mean.

3. RESULTS

3.1 Physicochemical Characteristics of the Hydrocarbon Contaminated Soil

Table 1 presents the physicochemical characteristics of hydrocarbon contaminated Kegbara-Dere mangrove soil from which the cellulase-producing bacterial strain was isolated. The data show that the environment was sufficiently contaminated with petroleum hydrocarbons. The TPH value was 11560 mg/kg (Fig. 1). The pH was however near neutral (6.79). The soil sample also had traces of heavy metals (Cu, Zn, Ni, Fe, and Pd) (Table 1).

3.2 Characteristics of the Different Purification Stages of the Cellulase

Table 2 shows results (protein content, cellulase activity, and total yield) obtained with crude enzyme fraction and the other various fractions at different purification stages. The results reveal inverse relationship between cellulase activity and specific activity across the different purification stages. Cellulase activity decreased from crude fraction (871.65 U/mL) to sephadex G-100 fraction (116.45 U/mL) while specific activity (U/mg) increased from 16.63 for crude fraction to 67.37 for sephadex G-100 fraction.

Table 1. Physicochemical and microbiological characteristics of hydrocarbon contaminated Kegbara-Dere mangrove soil

Parameters	Concentration	DPR Target	DPR Intervention
Physicochemical			
pH	6.79	6.5	8.5
Sulphate (mg/kg)	30.23	NA	NA
Nitrate (mg/kg)	24.77	NA	NA
Phosphate (mg/kg)	17.56	NA	NA
Copper (mg/kg)	32.45	36	190
Zinc (mg/kg)	97.22	140	720
Nickel (mg/kg)	25.48	35	210
Iron (mg/kg)	1256	NA	NA
Lead (mg/kg)	15.22	85	530
THC (mg/kg)	21320	NA	NA
TPHs (mg/kg)	11560	50	5000
PAHs (mg/kg)	11.66	1.0	40
BTEX (mg/kg)	100.22	0.05	206
Microbiological			
TCHB	$2.73x10^6$	NA	NA
TCHUB	$1.55x10^4$	NA	NA

NA: not available; BTEX: Benzene, Toluene, Ethylbenzene, and Xylene; DPR: department of petroleum resources; TPH: total petroleum hydrocarbons; PAHs: polycyclic aromatic hydrocarbons; THC: total hydrocarbon content; TCHB: total culturable heterotrophic bacteria; TCHUB: total culturable hydrocarbon utilizing bacteria

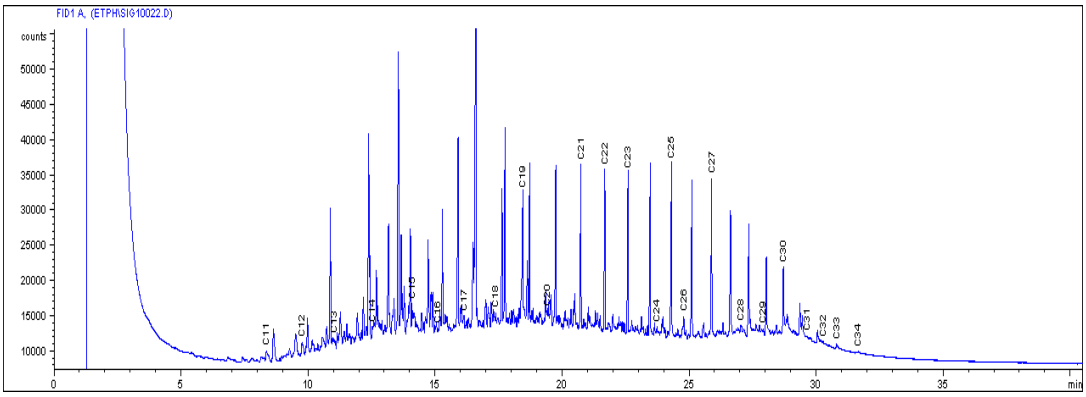

Fig. 1. TPH Chromatogram for Kegbara-Dere mangrove soil sample

The respective protein contents for crude, ammonium sulphate, and sephadex G-100 fractions were 52.41, 24.48, and 1.73 mg/mL. The changes in cellulase/specific activity at the different purification steps are presented clearly in Fig. 2.

3.3 Effects of pH and Temperature on the Activity and Stability of the Sephadex G-100 Purified Cellulase

Fig. 3 shows the effect of temperature and pH, on the purified cellulase activity and stability. The results revealed that maximum activity occurred within pH range of 4 to 6.5 with peak activity at pH of 5.5. The temperature range was between 30 and 60°C, with maximum activity at 50°C. The cellulase was stable over a pH range of 4 to 10 and temperature range of 30 to 100°C. The best stability for pH (102.46 U/mg) was observed at pH of 8.5 while the best stability for temperature (99.96 U/mg) was observed at 30°C.

3.4 Phenotypic, Biochemical and Molecular Characterisation of the Cellulase-Producing Bacterium

Basic local alignment search tool (BLAST) of the sequence obtained from the DNA of the isolate using NCBI (National Centre for Biotechnology Information) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed 99% similarity with the following GenBank isolates: *Bacillus licheniformis* strain BCRC 11702, *B. licheniformis* strain ATCC 14580, *B. sonorensis* strain NRRL B-23154, *B. licheniformis* strain NBRC 12200, *B. licheniformis* strain DSM 13, *B. paralicheniformis* strain KJ-16, *B. sonorensis* strain NBRC 101234, *B. subtilis* strain JCM 1465, and *B. shackletonii* strain LMG 18435. Phylogenetic analysis of these similar sequences revealed *Bacillus licheniformis* strain BCRC 11702 as the closest match. The phylogenetic tree construction showing the relationship of the cellulase producer used in this study and other GenBank closest matches is given in Fig. 4.

Table 2. Characteristics of the different steps of cellulase obtained from *Bacillus licheniformis* VVA21

Parameters	Fractions from various purification stages		
	Crude enzyme	Ammonium sulphate	Sephadex G-100
Protein Contents (mg/mL)	52.41	24.48	1.73
Cellulase activity (U/mL)	871.65	717.96	116.45
Protein volume (mL)	100	69.0	33.5
Total protein (mg)	5241.0	1689.1	57.9
Total activities (U)	87165.0	49539.24	3901.07
Specific activity (U/mg)	16.63	29.32	67.37
Yield (%)	100.0	56.83	4.47
Fold	1.0	1.76	4.05

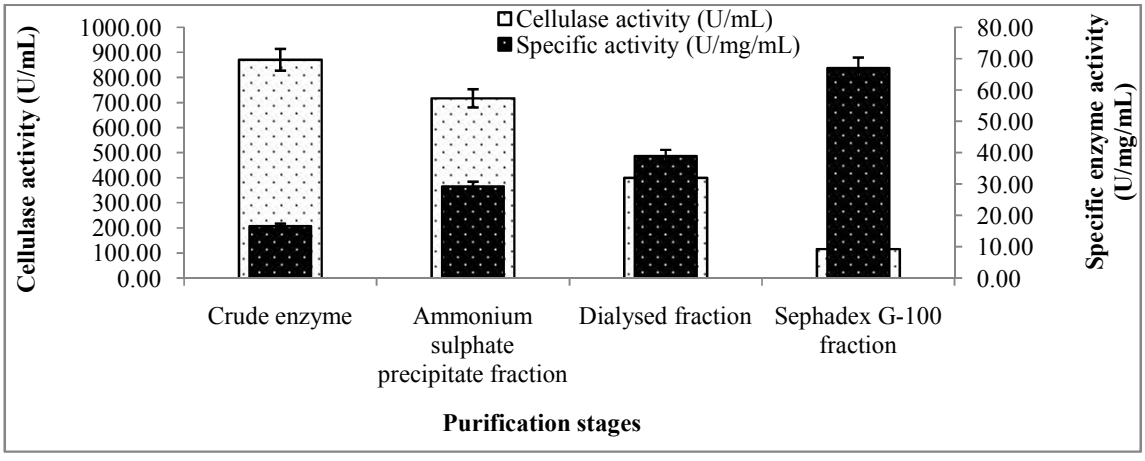

Fig. 2. Changes in cellulase/specific activity at different purification steps

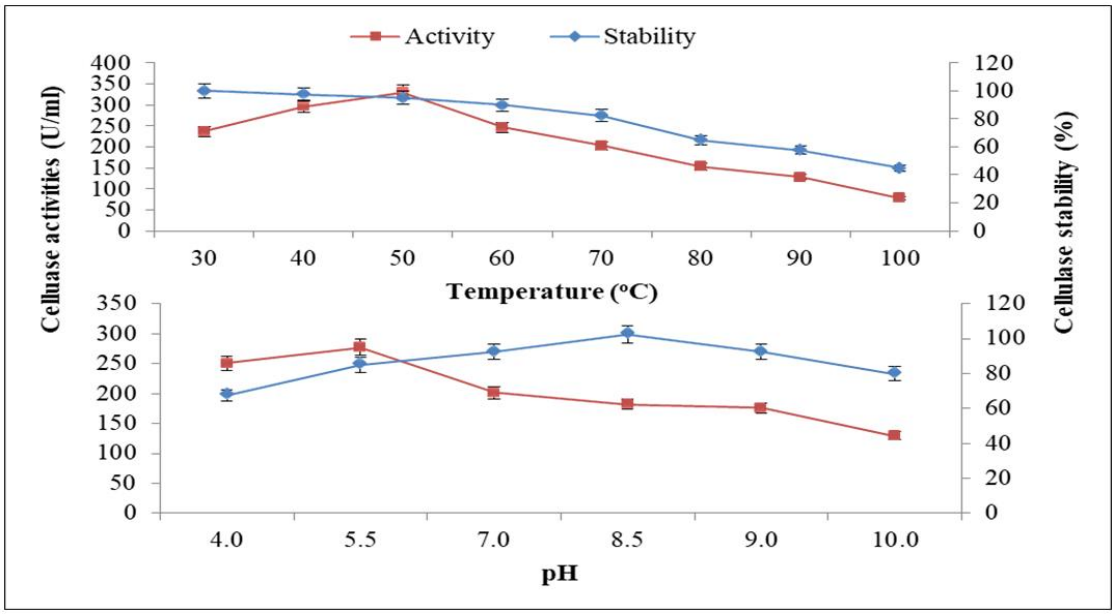

Fig. 3. Effect of pH and temperature on the activity and stability of Sephadex G-100 purified cellulase from *Bacillus licheniformis* VVA21

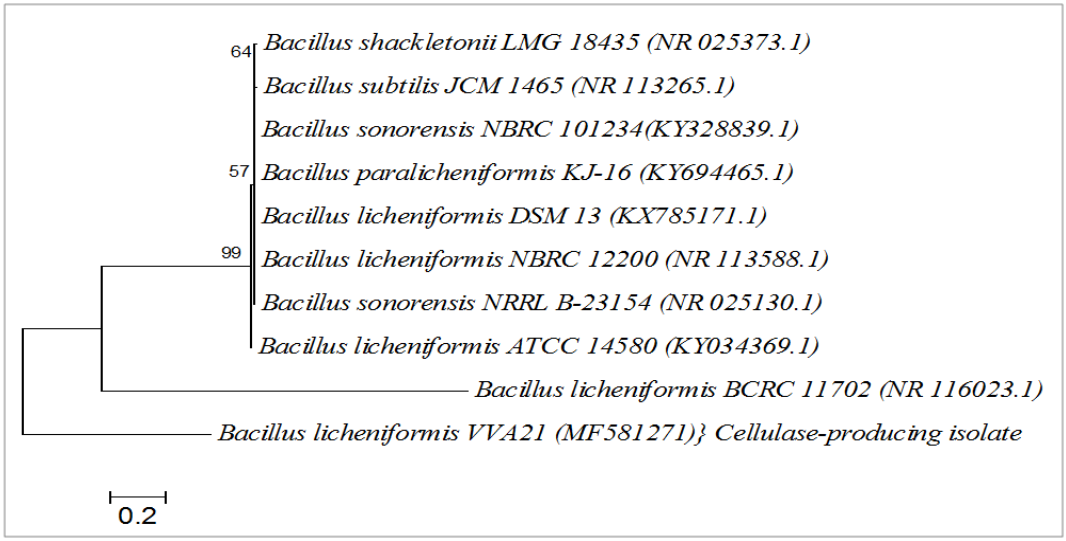

Fig. 4. Estimated phylogenetic relationship of the cellulase-producing *Bacillus licheniformis* VVA21 used in this study with closest GenBank isolates. The tree was rooted by designating strain VVA21 as the out-group

4. DISCUSSION

This study recovered highly stable-cellulase (an industrial enzyme) from a crude oil contaminated mangrove soil, which could be scaled up for industrial production. The result obtained from physicochemical analyses of the mangrove soil showed high crude oil contamination. Respective TPHs, PAHs, and BTEX values of 11560, 11.66, 100.22 mg/kg were above local (Department of Petroleum Resources - DPR) regulatory target values with TPH exceeding even the DPR intervention limit of 5000 mg/kg. Analyses of heavy metals in the soil sample revealed contamination by heavy metals although they were still within DPR target and intervention

limits. Similar high heavy metal concentrations of hydrocarbon polluted soil samples in Bodo, a community in Ogoniland, has been reported by Chikere and Ekwuabu [49]. The high TPH and PAH values reported in this study are similar to other reports. UNEP [8] reported TPH values of 52,000 mg/kg in mangrove soils in Gokana, a community in Ogoniland, Nigeria. Similarly, PAH value of 53.25 mg/kg was reported by Chikere and Ekwuabu [49] on assessment of hydrocarbon contaminated soils in Bodo, Ogoniland. Mangroves are highly productive ecosystems where bacteria actively take part in biomineralisation and biotransformation [20]. They present vital resources that serve the inhabitants of the Niger Delta, areas of active oil exploration in Nigeria. In the Niger Delta, mangroves remain very important to the indigenous people and cover large areas approximately 70,000 km^2 of land [50]. Nevertheless, the human impact of poor upstream management or economic activities around the mangrove areas coupled with constant pollution by oil has caused 5 to 10% of these mangroves to disappear, thus affecting their values [9]. According to Robertson et al. [51], Chikere et al. [52], and Sahoo and Dhal [53] mangrove is a home of "bio-industry", which harbours diversity of bacteria with high specificities for various applications, a claim this study has further validated. However, this study reveals that these contaminated mangroves could still offer potentials for the recovery of industrially relevant substances.

Soil sample obtained from the mangrove soil was used for the isolation of cellulase-producing bacteria. *Bacillus licheniformis* strain VVA21 with a zone of clearance of 16.0 mm was the preferred cellulase producer among the various other bacterial isolates screened for cellulase activity. The implications of *Bacillus* spp. in cellulase production have been widely reported. Behera et al. [20] obtained bacteria isolates including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, and *Bacillus brevis*, from mangrove soil of Mahanadi River Delta, India, with maximum zone of clearance ranging between 4 and 21 mm. Shaikh et al. [54] screened 20 cellulolytic bacterial isolates out of 34 isolates from different environments and identified the isolates as members of the genera *Pseudomonas* and *Bacillus* with zone of clearance ranging between 7 and 14 mm. Ezebuiro et al. [24] isolated cellulase-producing *Bacillus cereus* GBPS9 from agro-waste-impacted soil. Thus, the zone of clearance obtained from this study compares well with those obtained from other *Bacillus* spp from previous studies.

The crude cellulase was purified through various steps namely: partial purification using ammonium sulphate precipitation, dialysis of the partially purified fraction, and the final purification with sephadex G-100. The protein content of each fraction obtained from the various purification steps was equally determined. It was observed that cellulase activity decreased across the purification steps, whereas, specific activity increased across the purification steps. This trend could be as a result of the removal of protein concentrations, ions and other impurities across the purification steps that may be interfering with cellulase activity [55,56]. Igbal et al. [37] observed cellulase activities of 79,600 U/mL with specific activities of 45 U/mg/mL by a cellulase producing-bacterium purified by sephadex G-100 gel filtration column. In another study conducted by Begium and Absar [57] cellulase purified by DEAE-cellulose chromatography recorded the specific activity of 43.9 U/mg/mL. Furthermore, Sultana [58] observed an increase in specific activity of cellulase across the purification steps and recorded specific activity of 13.71 U/mg/mL by *Bacillus* sp. Therefore, the specific cellulase activity obtained in this study after sephadex G-100 purification (68.9 U/mL/mg) compares well with the results from other studies.

The cellulase showed stability over pH range of 4 to 10 and temperature range of 30 to 100°C. Such high stability over wide ranges of pH and temperature may be due to the adaptation of the cellulase-producing isolate to the stress in the environment from which it was obtained. Hydrocarbon contamination has been known to modify the physical, chemical, and ecological characteristics of any given ecosystem [13,49]. The findings of this study are similar with other reports. Lin et al. [59] reported that purified cellulase (JqCel5A), from *Jonesia quinghaiensis* displayed maximum activity (21.7 U/mg) at 55°C and pH 7.0 towards the substrate carboxymethyl cellulose. They also reported that JqCel5A exhibited high pH stability over a broad pH range of pH 3 to 11. In another study [60], cellulase activity over a broad range of temperatures (40 to 100^{o}C) and pH (3.5 to 10) was reported. Mawadza et al. [61] reported that the key characteristic feature of cellulase secreted by *Bacillus* spp is its activity over broad pH range.

The cellulase-producing isolate was identified as *Bacillus licheniformis* using API kit at 99% confidence interval. The 16S rRNA gene analyses further characterized the isolates as *Bacillus licheniformis* strain VVA21. The gene sequences have since been deposited at the GenBank with accession number (MF581271) assigned to it. *Bacillus licheniformis* is taxonomically classified as: Bacteria (domain), Firmicutes (phylum), Bacilli (class), Bacillales (order), Bacillaceae (family), and Bacillus (genus). *Bacillus licheniformis* VVA21 is a rod-shaped gram positive motile bacterium and is closely related to the widely studied *B. subtilis* and *B. sonorensis*. The phylogenetic tree analyses showed that it was closely related to other *Bacillus licheniformis* strains deposited at the GenBank. Rey et al. [62] reported that the ability of *B. licheniformis* to form endospores allows it to survive in the harsh environments (such as used in this study: hydrocarbon contaminated mangrove soil) required to manufacture industrial enzymes, chemicals, and antibiotics.

5. CONCLUSION

Bacillus licheniformis strain VVA21, isolated from hydrocarbon contaminated mangrove soil, used in this study showed high potentials for cellulase production; this could be scaled up for various industrial applications. The cellulase was stable over pH range 4 to 10 and temperature range of 30 to 100°C. This study reveals the potentials of highly stressed mangrove ecosystem in the production of bioactive compounds. It therefore shows, that stressed environments may offer solution to the production of highly stable bioactive substances.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Tomlison PB, Ashton PS, Hubbel SP, Janzen DH, Raven PH. Traditional and medicinal uses of mangroves biogeography In: The botany of mangroves, Cambridge University Press, New York. 1986;133-148.
2. Omogoriola HO, Williams AB, Ukaonu SC, Adegbile OM, Olakolu FC, Mbawuike BC, Akinnigbagbe AE, Ajulo AA. Survey, biodiversity and impacts of economic activities on mangroves ecosystem in Eastern Part of Lagos. Nature Sci. 2012; 10(10):30-34.
3. Gonzalez-Acosta B, Bashan Y, Hernandez-Sa-avedra NY, Ascenaio F, Cruz-Guero G. Seasonal sea water as a major determinant for population of culturable bacteria in soils of in-tact mangrove in an Arid region. Microbiol. Ecol. 2006;55(2):311-321.
4. Rojas A, Holguin G, Glick BR, Bashan Y. Synergism between *Phyllobacterium* sp. (N_2-Fixer) and *Bacillus licheniformis* (P-Solubilizer), both from a semi-arid mangrove rhizosphere. Microbiol. Ecol. 2001;35:181-187.
5. Proffitt CE. Managing oil spills in mangrove ecosystems: Effects, remediation, restoration, and modelling. OCS Study MMS 97-0003. New Orleans: US Department of the Interior, Minerals Management Service, Gulf of Mexico; 1996.
6. Duke NC, Burns KA, Swannell RPJ, Dalhans O, Rubb RJ. Dispersant use and a bioremediation strategy as alternate means of reducing the impacts of large oil spills on mangroves: The Gladstone field trials. Marine Poll. Bull. 2000;41:403-412.
7. Odokuma LO, Dickson AA. Bioremediation for crude oil polluted tropical mangrove environmental. J Appl Sci Environ Manage. 2003;7(2):23-29.
8. UNEP. Environmental Assessment of Ogoniland, 1st ed. United Nations Environment Programme. Nairobi, Kenya; 2011.
9. Orji FA, Ibiene AA, Ugbogu OC. Petroleum hydrocarbon pollution of mangrove swamps: The promises of remediation by enhanced natural attenuation. Am. J. Agric. Biol. Sci. 2012;7(2):207-216.
10. Vandermelen JH, Gillfilan ES. Petroleum pollution corals and mangroves. Marine Technol. Soc. J. 1985;18:11-16.
11. IPIECA. Metcalf & Eddy, wastewater engineering: Treatment and reuse. International Petroleum Industry Environmental Conservation Association, McGraw-Hill Inc., New York; 2003.
12. Suni S, Koskinen K, Kauppi S, Hnnula E, Ryynanen T. Removal by sorption and *in-situ* biodegradation of oil spills limits damage to marine biota: Laboratory simulation. Ambiology. 2007;36:173-179.
13. Ibiene AA, Orji FA, Orji-Nwosu EC. Microbial population dynamics in crude oil-

polluted soils in the Niger Delta. N. J. Agric. Food Environ. 2011;7:8-13.

14. Ibiene AA, Orji FA, Ezidi CO, Ngwobia CL. Bioremediation of hydrocarbon contaminated soil using spent mushroom compost and other organic wastes. N. J. Agric. Food Environ. 2011;7:1-7.
15. Shigenaka G. Oil spill in mangrove: Oil toxicity. Office of Response and Restoration Service, National Oceanic and Atmospheric Administration (NOAA) Washington; 2002.
16. Wegwu MO, Uwakwe AA, Anabi MA. Efficacy of enhanced natural attenuation (land farming) technique in the remediation of crude oil-polluted agricultural land. Arch. Appl. Sci. Res. 2010;2:431-442.
17. Ebuehi OAT, Abibo IB, Shekwolo PD, Sigismund KI, Adoki A. Remediation of Crude Oil Contaminated Soil by Enhanced Natural Attenuation Technique. J. Appl. Sci. Environ. Manage. 2005;9:103-106.
18. Ajibola-James O. Assessment of mangrove vegetation recovery from the of crude oil spillage in the Niger Delta (1986-2008). Department of Geography, Obafemi Awolowo University (O.A.U), Ile-Ife, Nigeria; 2011.
19. Sudha V. Studies on halophilic bacteria associated with mangrove sediments: (a) *Biovalvemollussc anadararhombea* (Arcidae) and (b) *L. asparaginase* (Anti-leukamic agent) from an extremely halophilic bacterium. Ph. D. Thesis. Annamalai University, Parangipettai, India; 1981.
20. Behera BC, Parida S, Dutta SK, Thatoi HN. Isolation and identification of cellulose degrading bacteria from mangrove soil of Mahanadi River Delta and their cellulase production ability. Am J Microbiol. Res. 2014;2:41-46.
21. APHA. Standard Methods for the Examination of Water and Wastewater (21st ed.). American Public Health Association. Washington, DC; 2005.
22. UNEP. Mangroves of Western and Central African Regional Seas Programme, United Nations Environment Programme (UNEP)-World Conservation Monitoring Centre (WCMC). Data analysis, Cambridge, UK; 2007.
23. Jalal KCA, Fatin UTN, Mardiana MA, John BA, Kamaruzzama YB, Shahbudin S, Omar MN. Antibiotics resistance microbes in tropical mangrove sediments, East Coast Pennisular Malaysia. Afr. J. Microbiol. Res. 2010;4:640-645.
24. Ezebuiro V, Ogugbue CJ, Oruwari B, Ire FS. Bioethanol production by an ethanol-tolerant *Bacillus cereus* strain GBPS9 using sugarcane bagasse and cassava peels as feedstocks. J. Biotechnol. Biomater. 2015;5:213.
25. Ray AK, Bairagi A, Ghosh KS, Sen SK. Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acta. Ichtyol. Pisc. 2007; 37(1):47-53.
26. Irfan M, Safdar A, Syed Q, Nadeem M. Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. Turk. J. Biochem. 2012;37(3):287-293.
27. Ire FS, Ezebuiro V, Ogugbue CJ. Production of bioethanol by bacterial co-culture from agro-wastes impacted soil through simultaneous saccharification and co-fermentation of steam exploded bagasse. Bioresour. Bioprocess. 2016; 3:26.
DOI: 10.1186/s40643-016-0104-x.
28. Nascimento WCA, Martins MLL. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. Braz. J. Microbiol. 2004;35:1-2.
29. Oyeleke SB, Egwim EC, Oyewole OA, John EE. Production of cellulase and protease from microorganisms isolated from gut of *Archachatina marginata* (giant African snail). J. Sci. Technol. 2012;2(1): 15-20.
30. Saraswati BM, Ravikuma DJ, Mukesh-kumar P, Balashanmugam MD, Bala K, Kalaichelvan PT. Cellulase production by *Bacillus subtilis* isolated from Cow Dung. Arch. Appl. Sci. Res. 2012;4(1):269–279.
31. Srinavas L, Aruna K, Venkata C. Isolation and biochemical characterization of protease isolated from a *Bacillus* species. Int. J. Res. Pure Appl. Microbiol. 2013; 3(3):94-101.
32. Bailey JE, Ollis DF. Applications of enzymes in the pulp and paper industry. Biotechnol. Prog. 1997;15:147-157.
33. De-Moraes LMP, Filhos SA, Ulhaa CJ. Purification and some properties of an alpha amylase and glucoamylase fusion protein from *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 1999;15: 561-564.

34. Beg KB, Gupta R. Purification and characterization of an oxidation-stable thiol-dependent serine alkaline protease from *Bacillus mycoides*. *Enzyme* Microb. Technol. 2003;32:294-304.
35. Alem MZ, Manchur MA, Anwar MN. Isolation, purification, characterization of cellulolytic enzymes produced by the isolate *Streptomyces omiyaensis*. Pak. J. Bio. Sci. 2004;10:1647-1653.
36. Sharma J. Partial purification of an alkaline protease from a new strain of *Aspergillus oryzae* AWT 20 and its enhanced stabilization in entrapped ca-Alginate beads. Biotechnol. Adv. 2006;23(2):53-69.
37. Igbal N, Muhammad H, Ahmed I, Muhammed I. Purification and Characterization of the kinetic parameters of cellulose produced from wheat straw by *Trichoderna viride* under SSF and its detergent compatibility. Adv. Biosci. Biotechnol. 2011;2:149-156.
38. Pokhrel B, Aghaya B, Magor RT. Production Purification and characterization of cellulose from *Bacillus subtilis* isolated from soil. European J. Biotechnol. 2014;2(5):31-37.
39. Lowry OH, Rosebrough NJ, Farr AL, Randall JR. Protein measurement with folin phenol reagent. J. Biochem. 1951; 242:265-275.
40. Muhammad A, Hafiz M. Purification and kinetic parameter characterization of an alkaline protease produced from *Bacillus subtilis* through submerged fermentation technique. *World* Appl. Sci. J. 2011;12(6): 751-757.
41. Olajuyigbe FM, Falade AM. Purification and partial characterization of serine alkaline protease from *Bacillus brevis.* Bioresour. Bioprocess. 2014;1(8):1-10.
42. Holt JG, Krieg NR, Sneath PHA. Bergey's Manual of Determinative Bacteriology. Lippincott Williams & Wilkins, USA; 1994.
43. MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. Lippincott Williams and Wilkins, Philadelphia; 2000.
44. Madigan MT, Martinko JM, Stahl DA, Clark DP. Brock biology of microorganisms. Benjamin Cummings California, USA; 2012.
45. Awong-Taylor J, Crven KS, Griffiths L, Bass C, Muscarella M. Comparison of biochemical and molecular methods for the identification of bacterial isolates associated with failed loggerhead sea turtle eggs. J Appl Microbiol. 2008;104(5): 1244-1251.
46. Yamada R, Tanaka T, Ohnishi Y, Suematsu K, Minami M, Seki T, Yukioka M, Maeda A, Murata N, Saiki O, Teshima R, Kudo O, Ishikawa K, Ueyosi A, Tateishi H, Inaba M, Goto H, Nishizawa Y, Tohma S, Ochi T, Yamamoto K, Nakamura Y. Identification of 142 single nucleotide polymorphisms in 41 candidate genes for rheumatoid arthritis in the Japanese population. Hum. Genet. 2000;106:293–297.
47. Katsura K, Kawasaki H, Potacharoen W, Saono S, Seki T, Yamada Y, Uchimura T, Komagata K. *Asaia siamensis* sp. nov., an acetic acid bacterium in the α-proteobacteria. Int. J. Syst. Evol. Micr. 2001;51:559–563.
48. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 2013;30:2725–2729.
49. Chikere CB, Ekwuabu CB. Culture-dependent characterization of hydrocarbon utilizing bacteria in selected crude oil-impacted sites in Bodo, Ogoniland, Nigeria. Afr. J. Environ. Sci. Technol. 2014; 8(6):401-406.
50. Mmom PC, Arokoyu SB. Mangrove forest depletion, biodiversity, loss and traditional resources management practices in the Niger Delta, Nigeria. Res. J. Appl. Sci. Engineer. Technol. 2010;2(1): 28-34.
51. Robertson AI, Alongi DM, Boto KG. Food chains and carbon fluxes: Tropical mangrove ecosystems, Washington, DC. American Geophysical Union; 1992.
52. Chikere CB, Okpokwasili GC, Chikere BO. Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation. Afr. J. Biotechnol. 2006;8(11):2535-2540.
53. Sahoo R, Dhal NK. Potential microbial diversity in mangrove ecosystem: A review. Indian J. Mar. Sci. 2009;38(2):249-256.
54. Shaikh NM, Patel AA, Melita SA. Isolation and Screening of celluloytic bacteria inhabiting different environment and optimization of cellulase production. J. Environ. Res. Technol. 2013;3(1):39-49.
55. Fujwara N, Masui A, Imanaka T. Purification and properties of highly themostable alkaline protease from an alkliphilic and thermophilic *Bacillus* sp. J. Biotechnol. 1993;30:245-256.

56. Hoondal G, Tiwari R, Tewari R, Dahiya N, Beg Q. Microbial alkaline pectinases and their industrial applications: A review. Appl. Microbiol. Biotechnol. 2002;59:409-418.
57. Begium F, Abser N. Purification and characterization of intracellular cellulase from *Aspergillus orybae*. Microbiology 2009;37(2):122-212.
58. Sultana S. Isolation of cellulositic microoganisms and their activities M.Phil. Thesis Biochemistry Department Rajshashi University Bangladesh. 1999;32.
59. Lin L, Xiaozhou L, Yating Z, Linyan G, Jiajia H, Weiqian H**.** A novel pH-stable, endoglucanase (JqCel5A) isolated from a salt-lake microorganism. Jonesia quinghaiensis. Electron J Biotechnol. 2016; 19(6):56-62.
Available:http://dx.doi.org/10.1016/j.ejbt.2016.09.004
60. Kazeem MO, Shah UK, Baharuddin AS, Abdul Rahman NA. Enhanced cellulase production by a novel thermophilic *Bacillus licheniformis* 2D55: Characterisation and application in lignocellulosic saccharification. Bioresources. 2016; 11(2):5404-5423.
61. Mawadza C, Hatti-Kaul R, Zvauya R, Mattiasson B. Purification and characterisation of cellulases produced by two *Bacillus* strains. J. Biotechnol. 2000; 83:177-187.
62. Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, Zaretsky EJ, Tang M, Lopez de Leon A, Xiang H, Gusti V, Clausen IG, Olsen PB, Rasmussen MD, Andersen JT, Jorgensen PL, Larsen TS, Sorokin A, Bolotin A, Lapidus A, Galleron N, Ehrlich SD, Berka RM. Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related Bacillus species. Genome Biol. 2004;5(10):77.

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