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Production and Characterization of Crude 1, 4 - β - endoglucanase by *Pseudomonas aeruginosa* Using Corn (*Zea mays*) Cobs and Pawpaw (*Carica papaya*) Fibres as Substrates

Francis Sopuruchukwu Ire[1*] and Dinebari Philip Berebon[1]

[1]Department of Microbiology, Faculty of Science, University of Port Harcourt, P.M.B. 5323, Choba, Port Harcourt, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author FSI designed the study and performed the statistical analysis. Author DPB wrote the protocol, managed the analyses of the study and wrote the first draft of the manuscript. Both Authors managed the literature searches. Author FSI proofread the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Lignocellulose is one of the most abundant sources of organic material in the world and it is a polysaccharide which consisted of cellulose, hemicelluloses and lignin. Cellulases are group of hydrolytic enzymes capable of degrading cellulose to smaller sugar components such as glucose units. This study aimed to isolate, screen a suitable 1,4-β-endoglucanase producing bacteria using inexpensive lignocellulosic substrates and optimize conditions for its production as well as characterize the crude enzyme.
Study Design: One-Factor-at-a-Time Methodology (OFAT) was used for culture medium optimization and characterization of crude 1,4-β-endoglucanase from *Pseudomonas aeruginosa*.
Place and Duration of Study: Department of Microbiology, Faculty of Science, University of Port Harcourt, Nigeria, between April 2014 and October 2015.
Methodology: Soil samples were collected from refuse dump site, cow dung composite soil and

**Corresponding author: E-mail: frankire4@yahoo.com, francis.ire@uniport.edu.ng;*

forest soil and screened for cellulase production on Bushnell Haas agar medium supplemented with 0.1% (w/v) carboxymethylcellulose (CMC) using 1.0% congo red dye. Isolates were identified using standard cultural, morphological and biochemical methods. Corncobs and pawpaw fibres were evaluated for the enzyme production and optimization of medium and culture conditions in submerged fermentation was investigated for maximum 1,4-β-endoglucanase production. The crude enzyme produced was characterized using standard physiochemical parameters.

Results: Nine (9) out of 21 bacterial strains isolated from soil samples showed positive potential for endoglucanase secretion but only one (1) isolate (CDB4) showing largest clear zone was selected after screening and used for further study. This isolate was identified as *Pseudomonas aeruginosa* using morphological and biochemical characteristics. Result revealed that the organism was able to secrete the enzyme when corncobs and pawpaw fibres were separately used as carbon sources. The optimal pH and temperature for 1,4-β-endoglucanase production using corncob (3%) was found to be 5.5 and 45°C after 3rd day of cultivation, while maximum enzyme yield was at 45°C in pawpaw fibre having pH 6.0 and substrate concentration of 4% after 3rd day of fermentation. The optimum temperature and pH for crude 1,4-β-endoglucanase activity were at 40°C and 6.5. The crude enzyme was most stable at pH 6.5 and 40°C, uninfluenced across a pH range of 5.5-6.5 and retained over 75% activity at 80°C after 2 h incubation. About 80.84% activity was lost when enzyme was incubated at pH 7. The enzyme was strongly activated by Co^{2+} and only slightly by Fe^{2+} and EDTA, while Zn^{2+}, Ca^{2+}, Hg^{2+} and Mg^{2+} elicited significant inhibition of the crude enzyme activity with highest repression exhibited by Zn^{2+}.

Conclusion: Results of this study revealed the ability of *Pseudomonas aeruginosa* to efficiently utilize both corncobs and pawpaw fibres without requirement for expensive pretreatment normally given to lignocellulosic residues. Therefore, this bacterium and its intrinsic metabolic capacity for endoglucanase production using inexpensive substrates at low concentration as well as the high pH stability and thermostability presented by the crude enzyme makes it useful in industrial processes.

Keywords: Pseudomonas aeruginosa; 1,4-β-endoglucanase; lignocellulosic substrates; corn cobs; pawpaw fibres; optimization and characterization.

1. INTRODUCTION

Cellulases are very different from most enzymes, as they degrade an insoluble substrate [1]. Cellulase is a generic name for the group of enzymes which catalyze the hydrolysis of cellulose and related cellu-oligosaccharide derivatives [2]. The crude cellulase enzyme complex constitutes a complete enzymatic system and usually contains three enzymes that act synergistically in the hydrolysis of cellulose: endoglucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91) and cellobiase (β-glucosidase, EC 3.2.1.21) [3]. The variable structural complexity of pure cellulose and the difficulty of working with insoluble substrates have led to the wide use of the highly soluble cellulose ether, carboxymethyl cellulose (CMC), as a substrate for studies of endoglucanase production [4,5]. Cellulose is the most abundant biopolymer in the world and can be found in plant matter and most agricultural wastes in the environment and consists mainly of long polymers of *β*-1-4, linked glucose units and forms a crystalline structure [6,7]. Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials [8]. Bacterial cellulases are constitutively produced, whereas fungal cellulase is produced only in the presence of cellulose [9]. Cellulases contribute to 8% of the worldwide industrial enzyme needs and the demand is expected to increase by 100% within 2014 [10]. In Nigeria, the microbial conversion of lignocellulolytic biowaste into cellulase and its intermediate enzymes can be a source of fund, create employment to her teeming population and serves as green waste management scheme for rural-urban agro-industrial wastes. Preliminary studies indicated that the production of cellulases can be affected by the growth media [11] or substrates used for production [12].

The application of bacteria in the production of enzymes have drawn special attention from researchers because of their high growth rate and speed of fermentation in minutes compared to hours in fungi and yeast. Generally, bacterial cellulases are constitutively secreted, whereas fungal cellulases are inducible in nature. However, the use of bacteria in cellulose production cellulase is not widely reported [13,14]. According to Sadhu [15] bacteria may also serve as a novel source of cellulases due to their higher growth rate, more complex glycoside hydrolases which provide synergy with higher

potency because of organismal diversity of extreme niches. Cellulases produced by bacteria are often more effective catalysts and may be less affected by feedback inhibition. Abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products. Ironically, much of the cellulosic waste is often disposed by biomass burning both in developing and developed countries [16]. With the help of cellulolytic system, cellulose can be converted to glucose which is a multi-utility product, in a much cheaper and biologically favorable process [16]. The high cost incurred in procurement of various purified celluloses such as Carboxymethylcellulose as carbon sources contributed adversely to endoglucanase production cost; hence the need to explore for zero cost agro-based lignocellulosic substrates which are inexpensive sources of carbon. Some of these agrowaste include: sawdust, bagasse and corncobs [17, 18], palm oil mill effluent [19], plantain peels [20], grapefruit peel waste [21], palm fruit husk [22], banana wastes and beet wastes [23]. Although most researchers have used pawpaw fruits and leaves because of its nutritional values and papain content less attention has been given to their stems (fibres) which are considered as agrowaste products. Effect of different growth parameters on endoglucanase enzyme activity by bacteria such as *Cellulomonas, Bacillus* and *Micrococcus* spp has been reported [24]. The aim of this study was to isolate a suitable 1,4-β-endoglucanase producing bacteria using inexpensive lignocellulosic substrates (corncobs and pawpaw fibres) and optimize conditions for its production as well as characterize the crude enzyme.

2. MATERIALS AND METHODS

2.1 Substrates used

The Corncobs and Pawpaw (*Carica papaya*) fibres were collected from Bodo Community in Gokana Local Government Area of Rivers State, Nigeria in polythene bags. Carboxymethyl cellulose (CMC) (Kermel, India) was used as a standard substrate for 1,4-β- endoglucanase production.

2.1.1 Processing of substrate

The corncobs and pawpaw (*Carica papaya*) fibres were chopped into smaller pieces, washed with clean water and sun-dried to reduce the moisture content. The substrate was crushed into fine powder bits using mortar and pestle previously rinsed with cotton wool soaked in ethanol .The substrate was repeatedly crushed and sun-dried between grinding. The crushed and air-dried substrate was further pulverized using hand grinding machine. Finally, the substrate was sieved through a 0.2 cm (2.0 mm) sieve size to remove larger particles and fiber. The processed substrate was then stored in sterile and air-tight container at 4°C for further use.

2.1.2 Pretreatment of substrate

Five percent (5% w/v) of substrate was added to 100ml of sterile distilled water in a ratio of 1:20 (substrate: distilled water) in a 250 ml beaker and boiled for 5 min. The beaker was covered with aluminum foil. The treated substrate was soaked overnight to possibly release some sugars. Both the filtrate and residue were preserved until use.

2.2 Sources of Organism/Sampling Site

Soil samples were collected from Bodo New Market refuse dump site located in Gokana Local Government Area, Rivers State, Nigeria. Its geographical coordinates are 4° 37′ 0″ North, 7° 16′ 0″ East. Cowdung composite soil was collected from Omokiri in Aluu LGA, Rivers State, Nigeria, located at 4°55″32.1′N, 6°55′27.3′E. Forest soil was collected from forest located opposite United Bank for Africa (UBA), Post Graduate Hostel, University of Port Harcourt, Rivers State, Nigeria, located at latitude 4.7848 and longitude 7.0055. All the soil samples were collected from a depth of 50-100 mm using of sterilized spatulas into sterile polythene bags. The soil samples were dried under shade and sieved through a 2.0 mm width mesh to remove stones and plant debris [25]. The collected soil samples were labeled, transported and stored in the refrigerator at 4^{o}C for further use.

2.2.1 Isolation of organisms

One gram of each soil sample was suspended in 9ml sterile physiological saline as the diluent to give the 10^{-1} dilution. The suspension (10^{-1} dilution) was shaken gently for 5 min and serially diluted up to 10^{-5}. Then 100 µL of the dilutions were aseptically plated out by pour plate technique in each triplicates plates of Bushnell Haas agar medium supplemented with 0.1% (w/v) of pawpaw fiber as the sole sources of carbon and energy. The inoculated plates were incubated for 24-48 h or more at 37^{o}C and observed daily for colony development. Colonies

which developed were randomly picked based on cultural characteristics (shape, size, elevation, consistency, transparency and colour). A total of twenty one isolates were obtained (visual examination) and potentially regarded as Cellulose Degrading Bacteria (CDB). Each isolate was given a code ranging from CDB1-CDB21.The selected colonies were then re-inoculated into 10 ml of sterile nutrient broth contained in test tubes and incubated for 24-48 h at 37^{o}C.The process was repeated and the isolated colonies were Gram stained to assess their degree of purity.

2.2.2 Screening of isolates

The cultures were streaked on Bushnell Haas agar medium supplemented with 0.1% (w/v) CMC contained in Petri dishes and incubated for 24-48 h. After incubation, each isolate was spot inoculated onto fresh Bushnell Haas agar medium supplemented with 0.1 % (w/v) CMC using a sterile inoculating loop. The cultures were incubated for 24-48 h for colony development. Individual spot of the colony which developed was flooded with 1.0% Congo red dye for 15-20 min and counterstained with 1M NaCl for another 15-20 min. The formation of halo or clear zone around each isolate indicated cellulose degradation [7,26].

2.2.3 Screening and estimation of cellulose degradation by isolates

The cellulose degradation potential (CDP) of the isolates screened from above was measured with a meter rule and their hydrolysis capacity (HC) estimated [16,27]. A total of twenty one isolates were screened and their cellulose degradation potential estimated as shown in Table 1. After assessing the cellulose degradation, one out of twenty one isolates was chosen for further studies based on its largest clear zone during screening on 0.1% (w/v) CMC basal agar medium. The largest clear zone was assumed to contain the highest activity [7,26]. The isolate with the highest activity was coded as CDB4 and partially purified by repeated plating as described previously and used for the preparation of stock cultures for further studies.

2.2.4 Identification of isolate

The isolate - CDB4 was presumptively identified based on cultural, morphological and some biochemical characteristics. The parameters investigated included colony morphology, Gram reactions, endospore formation, oxidase production, urease production, catalase production, VP reaction, indole production, motility, starch hydrolysis, citrate utilization, TSIA test, MR-VP test and sugar fermentation. The results were compared to Bergey's Manual of Determinative Bacteriology [28].

2.2.5 Standard inoculum preparation

The cellulolytic isolate was (CDB4) inoculated into 10 ml sterile nutrient broth contained in test tubes. The culture was incubated for 24 h at 37^{o}C.This was used as the standard inoculum for further experiments.

2.3 Fermentation Medium

Two types of media were used: Bushnell Haas medium (Bushnell and Haas, 1941) as cellulose basal medium; Nutrient broth (NB) manufactured by LAB M, UK. The Bushnell-Haas (BH) contains: $MgSO_4$ 0.2 g/L,$CaCl_2.H_2O$ 0.02 g/L, KH_2PO 1.0 g/L, NH_4NO_3 1.0 g/L, $FeCl_3$ 0.05 g/L, corncob and pawpaw fibre (separately used and compared as a sole source of carbon) 1.0 g/L, deionized water 1L,final pH 7±0.2.

2.3.1 Determination of endoglucanase activity

Enzyme assay in the crude extract was measured following the method of Miller [29]. The assay mixture contained 1.0 ml of crude enzyme solution plus 1.0 ml of 1% (w/v) Carboxymethyl cellulose (CMC, medium viscosity, India) diluted in 1.0 ml Sodium acetate buffer (pH6.5) and incubated at 50^{o}C in a shaker water bath (HH-W420 Thermostatic water bath, England) for 30 min. The assay was performed under standard reaction conditions and the reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) reagent and shake thoroughly. The colour was developed by boiling the reaction mixture for 5 min and allowed to cool for color stabilization. The optical density (OD) was read at 546 nm using spectrophotometer (Spectrumlab 752s spectrophotometer, England) and the amount of enzyme released was determined by comparing the absorbance reading of the test enzyme at 546 nm with the standard graph plotted by reacting the known concentration of glucose ranging from 0.1–100 µg/ml. One unit (U) of endoglucanase activity was defined as the amount of enzyme which catalyzed the formation of 1µmole of glucose per minute at pH 6.5 under the above standard assay condition.

2.3.2 Protein estimation

Total proteins were estimated by the Bradford method using bovine serum albumin (BSA) as standard [30].

2.4 Effect of pH on Enzyme Production

The effect of initial pH on glucanase production was investigated varying the pH from 3.0 to 9.0. The basal cellulose medium containing 0.1% (w/v) pretreated substrates (corncob and paw paw fibre) was adjusted to the desired pH using adjusting 1.0N HCl and 1.0 N NaOH prior to inoculation. The cultures were incubated at 37^{o}C for 48 h. After incubation, 10 ml of samples were aseptically withdrawn using sterile pipettes after 48 h for measurement of optical density at 546 nm for determination of reducing sugar as previously described.

2.5 Effect of Temperature on Enzyme Production

The effect of temperature on 1,4-β-endoglucanase production was studied by varying the temperature from 30^{o}C to 70^{o}C. Twenty five milliliters (25 ml) of basal cellulose medium containing 0.1% (w/v) pretreated substrates was inoculated with one milliliter of the culture from the standard inoculum and incubated at desired temperatures at the optimum pH obtained for corn cob (6.0) and pawpaw fibre (6.5) for 48 h in an orbital shaker incubator (Staurt orbital incubator, S150,OSA, UK) at 150 rpm. Samples (10 ml) were aseptically withdrawn using sterile pipettes after 48 h for measurement of optical density at 546nm for determination of reducing sugar as previously described.

2.6 Effect of Substrate Concentration on Enzyme Production

Into each of duplicate set of 100ml Erlenmeyer flasks of basal cellulose medium was separately added various concentrations of each pretreated substrates (1-5%, w/v): corncobs and pawpaw fibres. The medium was sterilized at 121^{o}C for 15 min at 15 psi and inoculated with one milliliter of standard inoculum. Cultures were incubated at optimal temperature of 45^{o}C in a shaker incubator for 96 h. Samples were withdrawn after 96 h for reducing sugar determination using calibration curve.

2.7 Effect of Incubation Time on Enzyme Production

The influence of time course on the production of endo (1,4) β-glucanase was evaluated by incubating *P. aeruginosa* at pH 5.5, 45^{o}C using corn cobs and pawpaw fibres separately for different time periods (6, 24, 48, 72, 96 and 120 h). After each hour of incubation the cell free filtrate was examined for enzyme production.

2.7.1 Crude enzyme production for characterization

Fermentation was carried out using the optimal conditions obtained when corn cobs was utilized as carbon substrates for crude endo(1,4)β-glucanase production by *P. aeruginosa*. The medium comprised: $MgSO_4$ 0.2g/L,$CaCl_2.H_2O$ 0.02 g/L, KH_2PO 1.0 g/L, NH_4NO_3 1.0 g/L, $FeCl_3$ 0.05 g/L, corncob 3%. Fermentation was performed with at 45^{o}C and pH for 3 days and thereafter crude enzyme was recovered by filtration through Whatman No. 1 filter paper. The cell free filtrate was used as crude enzyme for all the enzyme characterization.

2.8 Effect of Metal Ions and EDTA on Enzyme activity

The effect of various metal ions and Ethylene diaminotetraacetic acid (EDTA) on the enzyme activity was determined by using salts of respective cations: $ZnSO_4$, $FeSO_4.7H_2O$, $MgSO_4$, $CaCl_2$, $HgCl_2$, $Co(NO_3)_2.6H_2O$ and EDTA at 10mM concentration each. Aliquot (0.5 ml) of crude enzyme extract was dispensed into each of duplicate test tubes. Each reaction mixture contained 1.0 ml of 1% (w/v) in 1.0 ml of 0.05 M citrate buffer (pH 6.5) and 1.0 ml salt of the respective cations or EDTA. Each reaction mixture was equilibrated to room temperature for 45 min and inoculated with 0.5 ml of appropriate crude enzyme. The inoculated enzyme-substrate mixture was incubated at 50^{o}C in a water bath (HH-W420 Thermostatic water bath). The enzyme activity was assayed after incubation for 30 min. After incubation, the enzyme mixture was immediately cooled in cool water for 10-30 min. Residual enzyme activity was then determined.

2.9 Effect of pH on Enzyme Activity

The effect of pH on enzyme activity was investigated in 0.05 M citrate buffer solution adjusted to various pH values: 4.0, 5.0, 5.5, 6.0,

6.5, 7.0, 8.0 and 9.0 by addition of either 1N NaOH or 1N HCl. Each reaction mixture contained 1.0 ml of 1% (w/v) CMC in 1.0 ml of 0.05M citrate buffer of each pH value. Each reaction-mixture was inoculated with 0.5 ml of appropriate crude enzyme and incubated at 50^{o}C in a water bath (HH-W420 Thermostatic water bath). The enzyme activity was assayed after incubation for 30 min. After incubation, the enzyme mixture was immediately cooled in cool water for 10-30 min and enzyme activity assayed. The relative activity was measured by considering the highest activity to be (=100%).

2.10 Effect of pH on Enzyme Stability

The pH stability was performed as described elsewhere [31,32]. According to the procedure, 0.5ml of appropriate crude enzyme was pre-incubated (without substrate) in 1.0 ml of 0.05 M citrate buffer adjusted to appropriate pH values (4.0,5.0,5.5,6.0,6.5,7.0,8.0 and 9.0) at 50^{o}C for 2 h prior to incubation with substrate (1.0 ml of 1% (w/v) CMC). Thereafter, residual enzyme activity was assayed. The relative activity was measured by considering the highest activity to be (=100%).

2.11 Effect of Temperature on Enzyme Activity

In order to determine the effect of temperature on enzyme activity, 0.5 ml of appropriate crude enzyme solution was incubated with reaction mixture containing 1.0 ml of 1% (w/v) CMC in 1.0 ml of 0.05M citrate buffer (pH 6.5) and incubated at different temperatures ranging from 30 to 80^{o}C for 30 min. After incubation, the enzyme mixture was immediately cooled with water for 10-30 min. The residual enzyme activity was thereafter determined. The relative activity was measured by considering the highest activity to be (=100%).

2.12 Effect of Temperature on Enzyme Stability

Thermo-stability can be defined as the retention of activity after heating an enzyme extract at a selected temperature for a prolong period of time [33]. In order to determine thermal stability, 0.5ml of crude enzyme was incubated (without substrate) at increasing temperature ranging from: 30,35,40,45,50,60,70 and 80^{o}C for 2 h. The residual activity was then evaluated for 30 min and was measured by considering the highest activity to be (=100%).

2.13 Statistical Analyses

The data were subjected to one way analysis of variance (one way ANOVA) using Statistical Package for the Social Sciences (SPSS) for the determination of significant differences within different parameters at 95% confidence interval.

3. RESULTS AND DISCUSSION

3.1 Screening and Identification of Cellulose Degrading Bacteria (CDB)

A total of 21 bacterial strains were isolated from the various soil samples. Nine (9) out of the 21 isolates showed positive potential for endoglucanase secretion (Table 1). Isolate CDB4 displayed the largest zone of inhibition (20.0 mm) with enzymatic index of 4.0, followed by isolate CDB7 (18.0 mm) and enzymatic index of 3.0 while isolate CDB1 had the lowest clear zone of inhibition (3.0 mm) with enzymatic index of 1.5. Isolate CDB4 was selected for further study after the screening process and identified as *Pseudomonas aeruginosa* based on cultural, morphological and biochemical properties. The organism was used for endoglucanase production in submerged fermentation process. Among the screened cellulose degrading bacteria, *Pseudomonas aeruginosa* exhibited highest enzymatic index of 4 as shown in Table 1.

The cultural, morphological and biochemical properties of the isolate is shown in Table 2. The cultural, morphological and biochemical tests of the selected colony identified it as *Pseudomonas aeruginosa* following the standard keys of Bergey's Manual of Determinative Bacteriology.

3.2 Optimization of Some Culture Parameters

The two pretreated agricultural cellulosic residues (corncob and pawpaw fibre) were found to be good sources of carbon for the production of endoglucanase by *P. aeruginosa*. However, preliminary study showed that higher endoglucanase was achieved when corn cob was used compared to pawpaw fibres. There have been some reports on the use of corncob as substrate for efficient production of the

Table 1. Enzymatic indices of screened cellulose degrading bacteria (CDB) isolates

Isolate code	Colony size, C (mm)	Clearance zone or halo, H(mm)	Enzymatic index, EI (EI=H/C)
CDB1	2.0	3.0	1.5
CDB2	3.5	6.0	1.7
CDB3	5.0	9.0	1.8
CDB4	5.0	20.0	4.0
CDB5	4.0	8.0	2.0
CDB6	4.1	12.0	2.9
CDB7	6.0	18.0	3.0
CDB8	3.1	5.9	1.8
CDB9	3.0	7.0	2.3
*CDB10-21	-	-	-

**Isolates with EI values less than one (EI<1) or no visible halo*

enzyme [34-36]. However, several carbon sources have been reported by researchers for the production of endoglucanase. Some workers have observed higher enzyme production when wheat bran was used as carbon source [37-39]. CMC has equally been reported as the best carbon source for the production of endoglucanase by other workers [40,41]. Seo et al. [42] reported the production of endoglucanase by *B. licheniformis* using copra meal. Wheat bran and orange peel were equally found to be effective carbon sources for production of endoglucanase by *B. licheniformis* [41]. To the best of our knowledge, pawpaw fibre has been rarely used as a carbon substrate for endoglucanase production.

3.2.1 Effect of pH on enzyme production

The results obtained on effect of various pH on enzyme production by *P. aeruginosa* using pretreated corncob and pawpaw fibres as carbon source shown in Fig. 1. The result indicated that the enzyme was secreted by the bacterium in all pH ranges investigated in the study. Maximum 1,4-β-endoglucanase production (6.492 U/ml) by *P. aeruginosa* was observed at pH 5.5 and least enzyme production was observed at pH 3.0 (2.22 U/ml) when corncob was used as carbon source. When pretreated pawpaw fibres was used as carbon source, the maximum 1,4-β-endoglucanase production by *P. aeruginosa* was observed at pH 6.0 (6.448 U/ml) while the lowest enzyme production was noticed at pH 3.0 (2.12 U/ml). Further increase in initial pH of the medium beyond the ph optima for the two carbon sources resulted to decrease in enzyme production. The analysis of variance result revealed that the different pH values did not significantly affect (P>0.05) 1,4-β-endoglucanase production by *P. aeruginosa*. The optimum pH for 1,4-β-endoglucanase production from *P. aeruginosa* on corncob and pawpaw fibres ranged from pH 5.5 – pH 6.0, respectively. These results indicated that a pH value near neutrality was more favorable to cellulase activity of the organism. This pH range seemed to play a significant role in cellulose digestion by most bacterial strains for optimum endoglucanase production [23]. This organism has been reported to degrade cellulose [33,43-46]. It has been previously reported that the pH range over which the cellulases were highly active is fairly broad (pH 5.0 – 7.0) [47,48]. Our observation is similar to those obtained for endoglucanase from other sources. In a study by Nafiseh et al. [49], pH optimum ranged from 4-7 for endoglucanase of *Aspergillus terreus*. The optimum pH of 5.5 for the production of the enzyme has been reported [43,50].

3.2.2 Effect of temperature on enzyme production

The effect of temperature (30 to 70^{o}C) on 1,4-β–endoglucanase production by *P. aeruginosa* using pretreated corncob and pawpaw fibres as carbon source is shown in Fig. 2. The result obtained indicated that enzyme activity differed with variation in temperature. Optimum production of 1,4-β-endoglucanase (5.082 U/ml) in the presence of pretreated corn cobs was achieved at 45°C and further increase in temperature upto 70^{o}C, resulted to a reduction in enzyme production. The effect of temperature on 1,4-β–endoglucanase production by *Pseudomonas aeruginosa* when pretreated pawpaw fibres showed that maximum production of 1,4-β-endoglucanase by *Pseudomonas aeruginosa* occurred at 45°C (5.235 U/ml) followed by 35°C (2.923 U/ml), 30°C (2.879 U/ml) while the least 1.13 U/ml was at 70°C. The inability of the organism to elaborate the enzyme at high temperatures could be due to

denaturation of some medium components and inactivation of the enzyme by heat. One way analyses of variance (ANOVA) shows that there is significant difference ($P<0.05$) on the effect of temperature on 1,4-β-endoglucanase by *Pseudomonas aeruginosa*. Our result is consistent with previous reports on optimum temperature of 45^{o}C for cellulase production by other microorganisms: *Bacillus* sp. [51] and *Trichoderma viride* [52]. However, optimal temperature of 35^{o}C [53]; 40^{o}C [54] and 30^{o}C [53] has been reported for cellulase of *Pseudomonas fluorescens*; *Pseudomonas* sp and *P. aeruginosa* MTCC 4643, respectively. This ability of *Pseudomonas aeruginosa* endoglucanase to function beyond ambient temperature without denaturation and inactivation indicates that they can function under various culture conditions found in the industries.

Table 2. Cultural, morphological and biochemical properties of the isolate (CBD 4)

Test	Result
Size	5 mm
Shape	Round
Elevation	Raised
Colour	Blue-green
Gram's reaction	Negative
Motility	Positive
Indole	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Catalase	Positive
Citrate	Positive
Oxidase	Positive
Urease	Positive
Starch hydrolysis	Positive
Sugar fermentation	
Glucose	A/G
Dextrose	A/G
Fructose	A/G
Sucrose	A/G
Lactose	A/G
Maltose	A/G
Mannitol	A/G
TSIA	
Butt	A
Slant	B
H_2S production	Positive
Gas	Positive

Key: A = acid; B = black; G = Gas; TSIA = Triple sugar Iron agar

3.2.3 Effect of substrate concentration on enzyme production

The effect of substrate concentrations on 1,4-β-endoglucanase production using pretreated corncob and pawpaw fibre as substrates is shown is Fig. 3. Maximum enzyme production (6.227 U/ml) was obtained in cultures of *P. aeruginosa* that contained 3% (w/v), followed by 5% (w/v) and least enzyme production was observed at concentration of 2% (w/v). One way analysis of variance (ANOVA) shows that there was no significant difference ($P >.05$) in 1,4-β-endoglucanase enzyme production by *P. aeruginosa* with respect to substrate concentrations. The results obtained on effect of various concentrations on enzyme production of *P. aeruginosa* using pretreated pawpaw fibre showed that 1,4-β-endoglucanase production fairly increase with increased in pawpaw fibre concentration. Maximum enzyme production was achieved with 4% (w/v) of pawpaw fibre after which enzyme activity declined. There was no significant difference ($P>0.05$) in 1,4-β-endoglucanase production by *Pseudomonas aeruginosa* when different concentrations of the substrate was used.

The highest level of endogucanse by *Aspergillus awamori* strain VTCC-F099 and *Aspergillus fumigatus* were obtained using 3% corncob and 1% sugarcane bagasse [55,34]. Asad et al. [41] have reported maximum endoglucanase production using CMC at 5.0 g/L for *Bacillus licheniformis* KIBGE-IB2. Other researchers have reported various concentration optimal levels for cellulase production by other microorganisms: for example, 0.75% for cellulase of *C. globosum* [56], 5% level for *Pseudomonas aeruginosa* MTCC 4643 using saw dust as substrate [46], 0.75% and 0.5% for K_{22} (*B. licheniformis*) and K_{23} (*B.* sps) [57], respectively. The discrepancies observed may be attributed to the source of lignocellulosic substrate, species of cellulolytic isolate and pretreatment strategy adopted. The ANOVA result indicated that the variation in substrate concentrations did not significantly affect ($P>.05$) 1,4-β-endoglucanase enzyme production of *P. aeruginosa*.

3.2.4 Effect of incubation time on 1,4-β glucanase

The effect of time course on 1,4-β-glucanase production was studied by incubating the *P. aeruginosa* at different time intervals using the two agricultural residues (corncobs and pawpaw fibres) as substrate (Fig. 4). The endoglucanase production by the bacterium increased gradually from 0.45 U/ml at 6 h of cultivation to the maximum of 6.52 U/ml at 72 h of incubation when corncob was used as sole carbon source.

The same pattern of enzyme production was observed when pawpaw fibre (maximum enzyme activity (6.21 U/ml) at 72 h of cultivation) was used as carbon source. The result revealed that both agricultural wastes are good for the production of the enzyme by the bacterium although higher productivity was obtained when corn cob was used. Further increase in fermentation time beyond 72 h resulted to a decrease in endoglucanase production for both substrates. The fermentation time is longer compared to the results obtained by Seo et al. [42] who obtained maximum endoglucanase production by *B. licheniformis* after 48 h of incubation. Our result is in contrast with the report of other researchers who observed longer period of fermentation for maximum production of endoglucanase [34-37,39,58]. Asad et al. [41] reported maximum production of (1,4) β-glucanase by *B. licheniformis* KIBGE-IB2 at 48 h of cultivation. The decline in endoglucase production after 72 h of incubation may be as a result of change in pH, production of byproducts and depletion of nutrients in the cultivation medium as earlier opined by Asad et al. [41].

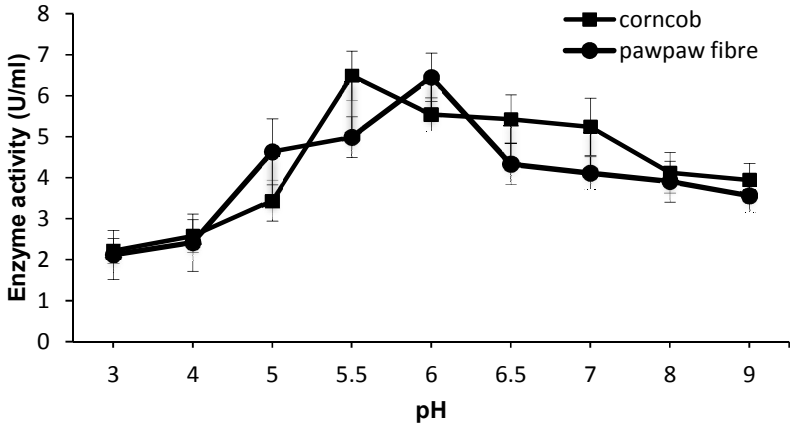

Fig. 1. Effect of pH on enzyme production of *Pseudomonas aeruginosa* using pretreated corncob and pawpaw fiibre as carbon source

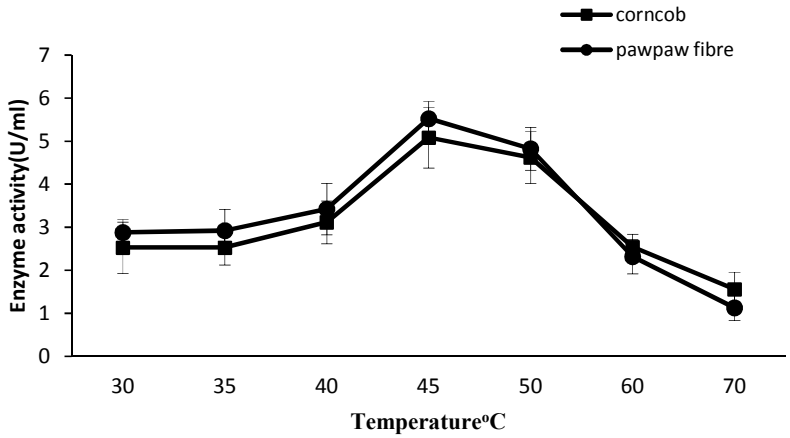

Fig. 2. Effect of temperature on enzyme production by *Pseudomonas aeruginosa* using pretreated corncob and pawpaw fibre as carbon sources

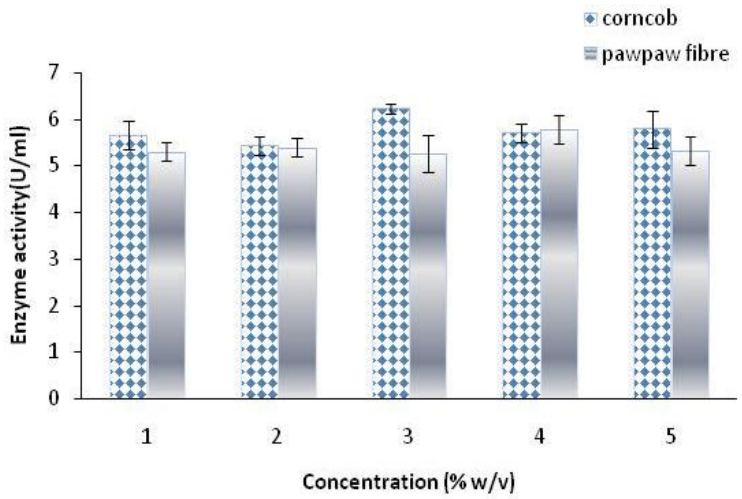

Fig. 3. Effect of substrate concentration on enzyme production by *Pseudomonas aeruginosa* using pretreated corncob and pawpaw fibre as carbon sources

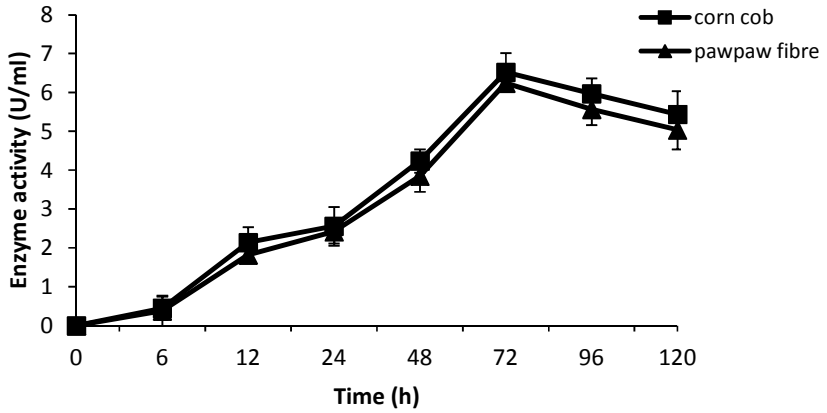

Fig. 4. Effect of time course on enzyme production

3.3 Effect of Various Metal Ions and EDTA on Crude Enzyme Activity

The effect of some metal ions and EDTA on the crude endoglucanase activity was examined, each at the concentration of 10 mM (Fig. 5). Among the metal ions tested, Co^{2+} acted as stronger stimulator of the crude enzyme activity (100%). Fe^{2+} and EDTA had moderate stimulatory effect on the crude enzyme activity. The addition of Zn^{2+}, Ca^{2+}, Hg^{2+} and Mg^{2+} showed varied levels of inhibition with the highest inhibition observed with Zn^{2+}. The finding that Co^{2+} stimulated the endoglucanase of *P. aeruginosa* is in contrast with Ni^{2+} stimulation of endo-beta-1,4-glucanase from *Peniophora* sp. NDVNOI and *L. sulphureus* var. *miniatus* [59,60]. However, our result on the stimulation of crude endoglucanase activity by Co^{2+} is in agreement with the report of Carolina et al. [39]. The activities of the crude endoglucanase enzymes of *Pseudomonas aeruginosa* were affected by low concentrations (10 mM) of various metal ions and EDTA was found to inhibit enzyme activity at all concentrations. In this study, $CaCl_2$ and $HgCl_2$ were found to inhibit enzyme activity. In general, Hg (II) behaved as a fatal cellulase inhibitor, likely because of its interaction with key sulfur-containing amino acid residues (as it does for other proteins) [61]. This is perhaps due to binding of Hg^{2+} with thiol groups and interaction with carboxyl or imidazol groups of amino acids [62]. Several studies have reported the reduction in activity of different metal ions and EDTA on

endoglucanase from other sources: *Bacillus* strain M-9 [63], *Cellulomonas* sp. ASN2 [64] and *Pseudomonas* fluorescens [54]. Results of the present study are in agreement with the earlier reports wherein Co^{2+} have stimulatory effect on cellulase of *Cellulomonas* sp. ASN2 [64] and in the case of alkaline cellulases in *Bacillus sphaericus* JS 1 [65]. Statistically, the activity of 1,4-β-endoglucanase enzyme in the presence of various metals and EDTA were not significantly different (P>.05) when corncob was used as substrate. However, there was significant difference (P<.05) in 1,4-β-endoglucanase activity when pawpaw fibre was used as substrate.

3.4 Effect of Various pH on Crude Enzyme Activity

The influence of pH on crude enzyme activity was investigated in the pH range 4.0 to 9 as presented in Fig. 6. The enzyme activity increased gradually from 28.26% at pH 4 to the maximum of 100% at pH 6.5 when incubated at 30 min. Thereafter, the activity of the enzyme decreased dramatically to 29.94% at pH 9. The optimum pH of 6.5 for endoglucanase of *P. aeruginosa* agreed with other reports [39,66,67]. Trinh et al. [60] reported an optimum pH 3.0 for endoglucanase produced by *Pheniophora* sp. NDVN01. Several researchers have reported optimum pH for endoglucanase from various organisms e.g. Bijender et al. [63] reported pH range of pH (3-10) with optimum at pH 5 for endoglucanase from *Bacillus* strain M-9. Jaradat et al. [68] reported pH range of 4-7 with maximum activity at pH 6 for *Streptomyces* sp. (Strain J2). The statistical results indicated that the pH did not significantly (P>0.05) affect 1,4-β-endoglucanase activity of *Pseudomonas aeruginosa*.

3.5 Effect of Various pH on Crude Enzyme Stability

The effect of pH on the stability of the crude enzyme is presented in Fig. 6. The crude enzyme was stable at a narrow pH range of 5.5.0 to 6.5, exhibiting optimum stability (100%) at pH 6.5 after incubation for 2 h. The crude enzyme was very unstable beyond the optimum stability pH 6.5 and about 80.84% activity was lost when the enzyme was incubated at pH 7 for 2 h. There was no significant difference (P>0.05) in the activity of 1,4-β-endoglucanase enzyme of *Pseudomonas aeruginosa* with respect to pH variation when corncob was used as substrate. Ponnuswamy and Vincent [32] reported that the enzyme was stable in the pH range of 5.0 to 7.0 and its stability was maintained for 30 min at 50°C. Chen et al. [69] reported stability for carboxymethylcellulase over the pH range of 6.0 to 9.0 with maximum stability at pH 7.0. The occurrence of more than one pH optima for endoglucanase from various organisms have been equally reported in some previous studies which suggested an isoenzyme form of endoglucanase enzyme [31].

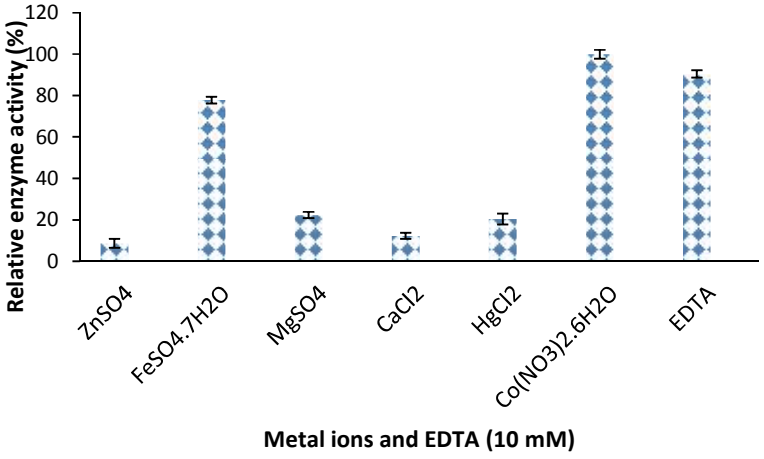

Fig. 5. Effect of metal ions and EDTA on crude enzyme activity. Relative activity is expressed as a percentage of the optimum

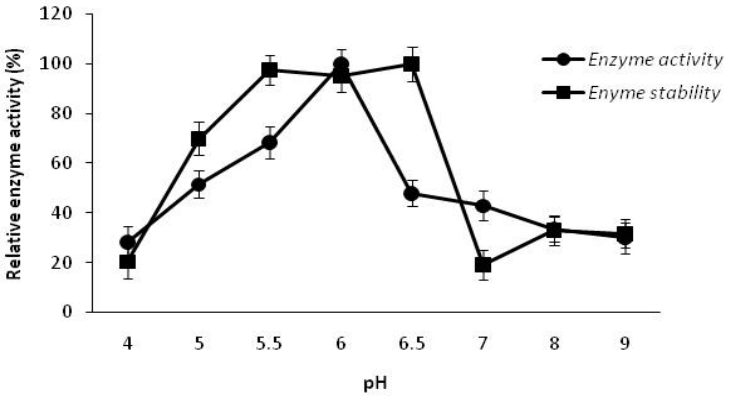

Fig. 6. Effect of pH on crude enzyme activity and stability. Relative activity is expressed as a percentage of the optimum

3.6 Effect of Various Temperatures on Enzyme Activity

The endoglucanase activity of the crude enzyme was measured at temperatures ranging from 30 to 80^{o}C and the result obtained is depicted in Fig. 7. The optimum temperature for 1, 4-β-endoglucanase activity was 40^{o}C. The result obtained indicates that about 81% of activity was retained at temperature of 50^{o}C whereas further increase in temperature resulted to loss of enzyme activity of upto 90.81% at temperature of 80^{o}C after 30 min of incubation. One way ANOVA showed that there is significant difference (P<.05) in the effect of various temperature values on 1,4-β-endoglucanase activity after 30 min and 1 h, respectively. Our result agreed with the work of Trinh et al. [60] who reported optimum 42^{o}C as the optimum temperature for the activity of endoglucanase for *Peniophora* sp. NDVN01. Carolina et al. [39] reported the presence of multiple cellulases from *Streptomyces thermocerradoensis* 13 with 35^{o}C, 50^{o}C and 70^{o}C as activity peaks. This current result indicates the potential of the enzyme for successful application in industries. Nizamudeen and Bajaj [31] reported that endoglucanase of a highly alkali tolerant and moderately thermotolerant *Bacillus* sp. NZ was highly active over a broad range of temperature (50–100^{o}C), with maximum activity at 50^{o}C and 90^{o}C and significantly high activity was observed even at 100^{o}C.

3.7 Effect of Various Temperatures on Enzyme Stability

Thermostability is an essential property for potential utilization of various enzymes in biotechnology. The result of the effect of various temperatures (30, 35, 40, 45,50,60,70 and 80^{o}C) on enzyme stability of *Pseudomonas aeruginosa* is shown in Fig. 7. The result revealed that the enzyme was moderately stable at wide range of temperature (40–80^{o}C) for 2 h of incubation. The optimum enzyme stability was obtained at 40^{o}C after 2 h incubation. The result indicated that over 75% of enzyme activity was still retained at 80^{o}C after incubation for 2 h. Analyses of variance (one way ANOVA) indicated that there was significant difference (P >.05) in the effect of various temperature values on 1,4-β-endoglucanase activity and stability. The 1,4-β-endoglucanase of *Pseudomonas aeruginosa* have thermostability in the temperature range of 30–80°C. This study demonstrated that endoglucanase from *Pseudomonas aeruginosa* is highly thermostable unlike most of the cellulases reported in previous studies. This result may confer an economical advantage for this bacterial strain. According to [31] industrially important enzymes must be capable of withstanding such harsh and hostile conditions for prolonged time periods or at least during the process time. Highly thermotolerant enzymes are required for industrial applications, for which either the natural microflora may be screened or the enzyme may be tailored by protein engineering so that it can withstand and work at elevated temperatures during process conditions [70]. Interestingly, our study has shown that *Pseudomonas aeruginosa* can be tailored to utilize cheap lignocellulosic substrates such as corncob and pawpaw fibre to mass produce 1,4-β-endoglucanase under harsh industrial environments.

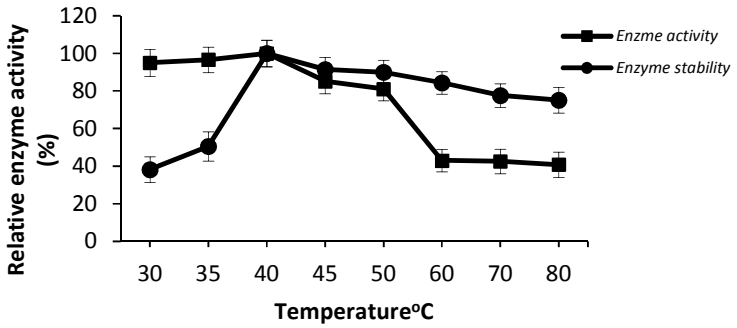

Fig. 7. Effect of temperature on enzyme activity and stability. Relative activity is expressed as a percentage of the optimum

4. CONCLUSION

The result obtained in this study indicated that *Pseudomonas aeruginosa* have good innate potentials of producing 1,4-β-endoglucanase using inexpensive lignocellulosic substrates such as corncobs and pawpaw fibres under unoptimized and optimized cultural conditions. Characterization studies on crude β-1,4-endoglucanase shows that is endowed adapt and function at and above ambient pH, temperature and in the presence of various cations of industrial importance. The crude endoglucanase investigated in this study is thermostable and active in a wide acidic pH range. This is a rare property which is lacking among most aerobic bacteria for industrial importance. The data obtained in this study can be used to model the preliminary process parameters and make economical evaluations of the *Pseudomonas aeruginosa* for 1,4-β-endoglucanase production process using cheap lignocellulosic substrates such as corncobs and pawpaw fibres. Therefore, this bacterium and its intrinsic metabolic capacity for endoglucanase production have great potential to be utilized in industrial processes for the conversion of biomass into biofuels and associated chemicals. A complete sequence and molecular analysis of the bacterium as well as purification and properties of 1,4-β-endoglucanase is ongoing in our laboratory.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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