

Isolation and Screening of Microbial Isolates from Decomposing Palm Kernel Shaft for Cellulase Production

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Abstract: This study was designed to screen microorganisms from decomposing palm kernel shaft for cellulase palm oil processing sites in Akoko area of Ondo State, Nigeria. Isolation of microorganisms was carried out by serial dilution and pour plate methods and identified using standard biochemical methods. The isolates were screened for cellulase production using standard assay methods. The microorganisms were grown in a mineral salt basal medium for maximum yield of cellulase production. The microorganisms isolated from the sample include *Bacillus subtilis*, *Micrococcus varians*, *M. luteus*, *Cellulomonas blazotes*, *C. flavigina*, *Sarcina ventriculi*, *B. cereus*, *C. fimi*, *Aspergillus niger*, *Rhizopus stolonifer*, *Saccharomyces cerevisiae* and *Trichoderma viridae*. The screened microorganisms exhibited varied cellulase activities. The diameters of zones of clearance of the isolates ranged from 1.45 to 1.83 for bacteria and 0.00 to 2.06 for fungi. The cellulase activity exhibited by bacteria ranged from 0.238 $\mu\text{mol/ml}$ to 0.590 $\mu\text{mol/ml}$ while fungal cellulase activity ranged from 0.452 $\mu\text{mol/ml}$ to 0.775 $\mu\text{mol/ml}$. The high cellulase activity exhibited by fungi isolated from decomposing palm kernel shaft suggested that their predominance as a potential source of cellulase could be more promising in various industrial processes for the degradation of cellulose containing organic substances.

Keywords: Cellulose, Wastes, Palm Kernel Shaft, Cellulose, Microorganisms

1. Introduction

The trend in human population and global quest for food for human sustenance in recent time through involvement in various agricultural practices has resulted in generation and accumulation of wastes in our environment [1]. Wastes generation from various agricultural practices, if not properly managed can contaminate or pollute the environment and water bodies which can result in disease outbreak. However, some of these wastes could serve as good substrate for microbial growth with potent of enzymes biosynthesis for various industrial uses [2].

Palm oil processing is one of the major economic activities that are common among large number of rural dwellers either for commercial or domestic purposes. Palm oil plays a key

role in Nigeria economy with the largest producers from the South-Western part of the country. Processing of palm oil can be achieved either manually or by using milling machine [3]. Various by-products are generated during palm oil processing. They include the kernel surrounded by the hard shell, the shaft and the processing waste water. Some of these by-products can as well serve as raw materials of domestic and industrial importance. For instance, the kernel can be used for the production of vegetable oil while the shell is important in the production of shoe soles.

Palm kernel shaft, a major by-products obtained during palm oil processing contains 20-30% cellulose, small amount of water and fibre. It has been used in the production of single cell protein, glucose, and chemicals such as liquids or gaseous fuel, citric acid and ethanol [4].

Cellulose is one of the most abundant components of plant

cell walls. It contains polysaccharides such as pectins, lignins and hemicelluloses. Plants and microorganisms can synthesize cellulose in the form of microfibrils that is made up of crystalline and non-crystalline region [5]. The derivatives of cellulose include methylcellulose, ethylcellulose, cellulose acetatephthalate and carboxymethylcellulose [6].

Enzymes play an important role in the production of different products and has been greatly used in in-line chain production process in industries. Environmental factors such as pH, temperature and cultural conditions affect the rate of enzyme biosynthesis in growth medium. The effectiveness of microorganisms in secretion of enzymes depends on their specificity to particular substrate needed for a desirable product formation [7].

Cellulase is one of the enzymes produced extracellularly by some group of microorganisms mostly bacteria and fungi which are capable of hydrolyzing cellulose to smaller sugar component such as glucose [8]. The catalytic action of most enzymes produced from microorganisms for low expensive substances could be promising in various industrial applications. Therefore, this study was designed to isolate and screen cellulase-producing microorganisms from decomposing palm kernel shaft.

2. Materials and Methods

2.1. Collection of Samples

The decomposing palm kernel shaft samples were collected from five different palm oil processing sites in Ikare-Akoko, Supare-Akoko, Oka-Akoko, Ayegunle-Akoko and Akungba-Akoko all in the northern part of Ondo State, Nigeria. The shaft samples were taken by employing a sterile hand trowel to dig out 0.5 m depth. The samples were immediately transferred into sterile sealable bags and then transported into Microbiology laboratory for further analyses.

2.2. Microbiological Analysis

Isolation of microorganisms from the decomposing palm kernel shaft sample was carried out by serial dilution and pour plate techniques [9]. A stock culture of each sample was prepared by measuring 5 g of the sample into a conical flask containing 45 ml of peptone water. The suspension was shaken vigorously and allowed to settle. One milliliter was taken from the suspension and dispensed into test tubes containing 9.0 ml of sterilized distilled water. The samples were further serially diluted up to the appropriate dilutions. From the diluents, 0.1ml was aseptically inoculated prepared Petri dishes containing Nutrient Agar and Potato Dextrose Agar. The bacterial plates were incubated at 37°C for 24 hours while fungal plates were incubated at 28±2°C for 72 hours. Representative colonies were sub-cultured on the appropriate plates while pure cultures were obtained by repeated streaking of fresh colonies on appropriate media.

The isolates were identified based on their morphological examination and biochemical characterizations [10-11]. Their respective pure cultures were maintained on agar slants containing nutrient agar and potato dextrose agar at refrigeration temperature 4°C for further use [12].

2.3. Screening of Microorganisms for Cellulase Production

Cellulase screening was done in a medium containing carboxymethylcellulose (CMC) only for bacteria and CMC containing streptomycin (100µg/ml) for fungi according to method of Saraswati *et al.* [13]. The microorganisms were inoculated into their media and incubated at 24 hours and 120 hours for bacteria and fungi respectively. The plates were then flooded with 0.1% Congo red solution and decanted after 30 minutes, and later flooded with 5M NaCl for 20-30 minutes and decanted again. The clear zone of hydrolysis around the colony indicates cellulase production. The bacteria and fungi with positive and larger zones were selected for further studies.

2.4. Cellulase Production

Cellulase production was carried out in submerged state fermentation in a medium containing (per 100 ml of distilled water): 1.0 g CMC, 1.0 g yeast extract, 1.0 g glucose, 1.7 g agar, 0.1 g (NH₄)₂SO₄, 0.01 g CaCl₂ · 2H₂O, 0.01 g MgSO₄ · 7H₂O, 1.3 g K₂HPO₄, 0.7 g KH₂PO₄ and 0.2 g NaCl (pH 6.8). The media were sterilized at 121°C for 15 minutes, allowed to cool and then incubated at 37°C for 2 days (bacteria) and 28±2°C for 7 days (fungi) using rotary shaker (Gallenkamp, Model RS-12, Singhla Scientific Industries, India). The media were centrifuged (Model KBM-70, Centurion Scientific Limited, Germany) at 6,000 rpm for 30 minutes at 4°C to obtain the supernatants (crude enzyme) [9]. The supernatants were used as crude extracellular cellulase sources.

2.5. Cellulase Assay

Cellulase assay was carried out using a modified method of [14]. The reaction mixture which contained 0.5ml culture supernatant (crude enzyme) and 0.5ml of 0.5% (w/v) CMC in 0.5M acetate buffer (pH 5.5) was pipetted into test tubes. The enzyme solutions were heated at 100°C in boiling water bath for 15 minutes to inactivate the enzyme and then cooled at room temperature. Both the experimental and control tubes were incubated in water bath (Lamfield Medical England Model DK600, Labnics Equipment, United Kingdom) at 37°C for 15 minutes for colour development and cooled in the ice. The reaction mixture was terminated by adding 3.0 ml 3,5-dinitrosalicylic acid (DNSA) reagent per tube. The activity of reaction mixture was measured against a reagent blank at 540 nm. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentration of glucose. One unit of cellulase activity was defined as amount of enzyme producing 1 micromole of glucose per minute under the experimental conditions.

2.6. Protein Determination

An aliquot of the culture filtrate with appropriate dilution was used for estimation of soluble protein content according to the method using bovine serum albumin (BSA) as standard. Suitable aliquots of the filtrate were mixed with 5 ml of alkaline solution. After 10 minutes, 0.5 ml of appropriate diluted Folin-Ciocalteu reagent was added. After 30 minutes, extinction was read at 550 nm using spectrophotometer (ELICO-SL164) [15].

3. Results

3.1. Microbial Counts

The total microbial counts from the decomposing palm kernel shaft are represented in table 1. The highest microbial load of 2.5×10^7 cfu/ml was obtained from Ayegunle-Akoko sample while Supare-Akoko sample had the lowest counts of 1.3×10^5 cfu/ml. The highest fungal count was also observed from Ayegunle sample (7.3×10^4 sfu/ml) while the lowest count was however obtained from Akungba Sample (7.3×10^4 sfu/ml). The microorganisms isolated from the samples include the bacteria *Bacillus subtilis*, *B. cereus*, *B. amyloliquefaciens*, *Cellulomonas blazotes*, *C. flavigina*, *C. blazotes*, *Micrococcus varians*, *M. luteus*, and *Sarcina ventriculi*, and the fungi *Aspergillus niger*, *Rhizopus stolonifer*, *Saccharomyces cerevisiae* and *Trichoderma viridae* (Tables 2 and 3). *Bacillus subtilis* and *A. niger* were the most frequently isolated organisms from the samples followed by *B. amyloliquefaciens* while *B. cereus*, *M. luteus* and *S. ventriculi* were least isolated (Table 4).

3.2. Screening of the Isolates for Cellulase Production

Varied cellulase activities were exhibited by the isolate microorganisms. The diameter zones of inhibition ranged from 33 mm to 55 mm, and 00 mm to 66 mm for bacteria and fungi respectively as *B. amyloliquefaciens* and *A. niger* recorded highest zones of hydrolysis (55 mm and 66 mm) while *C. fimi* and *T. viridae* had the least zone of hydrolysis (33 mm and 39 mm). *Saccharomyces cerevisiae* and *R. stolonifer* had no zone of clearance (Table 5).

3.3. Quantitative Screening of the Isolates for Cellulase Production

Table 6 showed the quantitative determination of cellulase activity of the isolated microorganisms. The cellulase activity ranged from 0.32 to 0.78 $\mu\text{mol}/\text{min}/\text{ml}$ as observed in *M. luteus* and *A. niger* respectively. *Rhizopus stolonifer* exhibited highest specific cellulase activity of 6.20 $\mu\text{mol}/\text{ml}$ while *T. viridae* had least specific cellulase activity 1.36 $\mu\text{mol}/\text{ml}$. The least protein content of 0.11 mg/ml was produced by *C. fimi* and *B. cereus* while the highest content of 0.33 mg/ml was obtained from *T. viridae*.

Table 1. Microbial loads of the palm kernel shafts from the communities.

Sample site	Bacterial counts loads (cfu/ml)	Fungal counts (sfu/ml)
Ikare	$1.8 \times 10^6 \pm 0.3^b$	$2.3 \times 10^4 \pm 0.3^{ab}$
Supare	$1.3 \times 10^5 \pm 0.4^c$	$5.4 \times 10^3 \pm 0.4^b$
Ayegunle	$2.5 \times 10^7 \pm 0.4^a$	$7.3 \times 10^4 \pm 0.4^a$
Oka	$1.9 \times 10^6 \pm 0.2^b$	$1.6 \times 10^4 \pm 0.2^{bc}$
Akungba	$1.6 \times 10^6 \pm 0.2^b$	$4.6 \times 10^3 \pm 0.2^c$
Eti Oro	$2.1 \times 10^6 \pm 0.2^{ab}$	$4.1 \times 10^4 \pm 0.2^{ab}$

Means with the same letters in each column are not significantly different ($p > 0.05$).

Table 2. Biochemical characteristics of the microorganisms isolated from palm kernel shafts.

Isolate	Shape	Gram's Reaction	Endospore Staining Test	Catalase Test	Coagulase Test	Manitol	Fructose	Glucose	Lactose	Sucrose
BB1	R	+	+	+	-	+	+	+	+	+
BB2	R	+	-	+	-	+	+	+	-	-
BB3	R	+	+	+	-	-	-	-	-	+
BB4	C	+	-	+	-	+	+	+	+	+
BB5	R	+	-	+	-	+	+	+	-	+
BB6	R	+	-	+	-	+	+	+	-	+
BB7	C	+	-	+	-	+	+	+	-	+
BB8	R	+	+	+	-	-	+	+	-	+
BB9	C	+	+	+	-	+	+	+	+	+

Table 2. Continued.

Isolate	Shape	Indole	Motility Test	Ornithine	Gelatin Test	Nitrate	Starch	CMC Test	Probable Microorganisms
BB1	R	-	+	+	+	+	+	+	<i>B. subtilis</i>
BB2	R	-	+	-	-	+	+	-	<i>C. blazotes</i>
BB3	R	-	-	-	-	+	+	-	<i>B. amyloliquefaciens</i>
BB4	C	-	-	-	+	-	-	-	<i>M. varians</i>
BB5	R	-	+	-	-	-	+	+	<i>C. fimi</i>
BB6	R	-	-	-	-	+	+	+	<i>C. flavigina</i>
BB7	C	-	-	-	+	-	+	-	<i>M. luteus</i>
BB8	R	-	-	-	+	+	+	+	<i>B. cereus</i>
BB9	C	-	-	-	+	-	+	-	<i>S. ventriculi</i>

Key: R – Rod, C – Cocci, S = Sample, + = Positive, - = Negative, CMC = Carboxymethyl cellulose.

Table 3. Morphological characteristics of fungi isolated from decomposing palm kernel shaft.

Isolate code	Cultural Characteristics	Colour	Suspected microorganisms
FF1	Black conidia, non septate mycelia	Black colonies	<i>Aspergillus niger</i>
FF2	Black sporangia, non septate mycelia	White cotton colonies	<i>Rhizopus stolonifer</i>
FF3	No mycelia	Creamy coloured colonies	<i>Saccharomyces cerevisiae</i>
FF4	Septate mycelia pigmented	Pigmented	<i>Trichoderma viridae</i>
FF5	Black sporangia non septate mycelia	White cotton colonies	<i>Rhizopus stolonifer</i>

Table 4. Occurrence of microorganisms from decomposing palm kernel shafts.

Isolates	Ikare	Supare	Ayegunle	Oka	Akungba	Etioro
<i>Bacillus subtilis</i>	+	+	-	+	+	+
<i>Bacillus amyloliquefaciens</i>	+	-	-	+	+	+
<i>Bacillus cereus</i>	-	-	-	-	+	+
<i>Cellulomonas blazotes</i>	+	-	+	+	+	-
<i>Cellulomonas flavigina</i>	-	-	+	+	+	-
<i>Cellulomonas fimi</i>	-	+	-	+	+	-
<i>Micrococcus varians</i>	+	+	+	-	-	-
<i>Micrococcus luteus</i>	-	-	+	+	-	-
<i>Sarcina ventriculi</i>	+	-	-	+	-	+
<i>Aspergillus niger</i>	+	+	+	-	+	+
<i>Rhizopus stolonifer</i>	+	-	-	-	-	-
<i>Saccharomyces cerevisiae</i>	+	-	+	-	+	-
<i>Trichoderma viridae</i>	+	-	-	-	+	+

Table 5. Cellulase screening of the microbial isolates.

Isolates	Colony (mm)	Clear zone (mm)	Ratio of clear zone/colony
<i>Aspergillus niger</i>	32	66	2.06
<i>Bacillus cereus</i>	26	42	1.62
<i>Bacillus subtilis</i>	30	50	1.67
<i>Cellulomonas amyloliquefaciens</i>	30	55	1.83
<i>Cellulomonas blazotes</i>	31	45	1.45
<i>Cellulomonas fimi</i>	23	38	1.65
<i>Cellulomonas flavigina</i>	24	33	1.38
<i>Micrococcus luteus</i>	30	46	1.53
<i>Micrococcus varians</i>	30	54	1.80
<i>Rhizopus stolonifer</i>	28	00	00
<i>Saccharomyces cerevisiae</i>	23	00	00
<i>Sarcina ventriculi</i>	22	38	1.73
<i>Trichoderma viridae</i>	34	39	1.15

Table 6. Cellulase activity of the microbial isolates.

Isolates	Cellulase activity ($\mu\text{mol}/\text{min}/\text{ml}$)	Protein content (mg/ml)	Specific enzyme activity ($\mu\text{mol}/\text{ml}$)
<i>Bacillus subtilis</i>	0.34	0.19	1.83
<i>Cellulomonas blazotes</i>	0.35	0.14	2.54
<i>Bacillus amyloliquefaciens</i>	0.39	0.20	1.94
<i>Micrococcus varians</i>	0.34	0.17	2.03
<i>Cellulomonas fimi</i>	0.44	0.11	3.86
<i>Cellulomonas flavigina</i>	0.48	0.17	2.82
<i>Micrococcus luteus</i>	0.32	0.17	1.94
<i>Bacillus cereus</i>	0.35	0.11	3.13
<i>Sarcina ventriculi</i>	0.39	0.12	3.37
<i>Aspergillus niger</i>	0.78	0.13	6.20
<i>Rhizopus stolonifer</i>	0.60	0.12	5.19
<i>Aspergillus niger</i>	0.61	0.14	4.36
<i>Aspergillus niger</i>	0.56	0.22	2.60
<i>Trichoderma viridae</i>	0.45	0.33	1.36

4. Discussion

Wastes generation from homes, industries and other sources could stand as main channel of contaminants into the environment. Wastes mismanagement and its indiscriminate

disposal could result in the release of obnoxious odours to the environment and distort in microbial ecological balance. In recent time, biotransformation of these wastes by the activities of microorganisms with recent biotechnological advancement in various products formation is on high demand [16]. Isolation and screening of microorganisms for

enzymes production from cheap, readily available, low cost and inexpensive waste materials in the submerged state fermentation at optimum conditions for maximum enzyme yield have been reported [17].

The high microbial loads obtained from Ayegunle-Oka Akoko sample could be attributed to the profuse discharge of the waste water from the palm oil processing site into the environment, ability of the microorganisms to utilize the substrate and suitable environmental conditions [18]. The variations observed in the microbial counts depend on the colonization and the activities of different kind of microorganisms in utilizing the available nutrients in the wastes, prevailing environmental conditions and physicochemical parameters [19]. Blibech *et al.* [20] had reported high microbial loads from large amounts of agricultural wastes from forestry and agricultural practices, paper pulp industries, timber industries and many agro-industries. The microorganisms isolated from this study agree with the findings of Saowapar *et al.* [21] who reported similar microorganisms from oil palm meal.

Different researchers have reported screening of enzymes from microorganisms from different agricultural wastes [22-24]. Abeer *et al.* [25] reported exopolygalacturonase production from Jojoba mill solid waste by *Aspergillus oryzae* FK-923. Similarly, amylase production by *Aspergillus niger*, *A. flavus*, *A. phoenicis* and *Penicillium granulatatum* from garden soil, rhizospheric soil, effluent of textile industry and apple fruit respectively had been reported [26]. Amir *et al.* [27] and Olaniyi *et al.* [28] reported cellulose production from corn cob using *Alternaria alternata* and mannanase production from orange peels. Besides, Damisa *et al.* [29] and Ekundayo and Arotupin [8] had reported cellulase production from cellulolytic wastes.

Production of extracellular enzymes by microorganisms into its culture medium is a critical aspect of enzyme biotechnology. The quantitative evaluation of cellulase produced by isolates under submerged state conditions could depend on the ability of the microorganisms to secrete the enzymes extracellularly [8]. The variation in the amount of cellulase activity displayed by the isolates might be attributed to the medium composition, genetic make-up and the fermentation parameters [30]. Abeer *et al.* [25] and Omemu *et al.* [31] reported 10.8 $\mu\text{mol/mg}$ and 150 $\mu\text{mol/mg}$ specific activities of exopolygalacturonase and glucoamylase from *Aspergillus niger* F-119 and *A. flavus*.

Different microorganisms had been reported to produce variety of enzymes (amylases, pullulanase, protease, mannanase, lipase, linamarase and xylanases) apart from the enzyme been examined for in this study. The protein from microbial cells might also interfere with cellulase enzyme causing variation in protein content, due to the secretion of other these enzymes (amylases, protease, mannose, linamarase and xylanases) in the fermentation medium, since the protein assay could only identify accumulated protein in solutions. This result obtained was in agreement with the findings of Muhammad *et al.* [32] who reported variation the cellulase activity and protein content from cellulase-

producing microorganisms from soil environment.

5. Conclusion

Fermentation medium containing adequate nutrients enhances enzymes biosynthesis from most microorganisms. The varied cellulase activities exhibited by the microorganisms suggested that the use of decomposing palm kernel shaft could serve as potential source of enzyme for various industrial processes which could also lead to novel bioprocesses and new products. Production of cellulases from microorganisms is still necessary to complement those that are commercially available which are used in food, feed paper and textile industries.

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