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Isolation and identification of amylase-producing microorganisms

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Abstract

The research was aimed at isolation and characterization of α-amylase producing microorganisms. Palm wine and soil samples and cassava processing wastewater were obtained from cassava processing sites in Port-Harcourt, River state, Nigeria. A total of 12 isolates comprising of 7 bacterial and 5 fungal strains respectively were obtained. After screening using starch hydrolysis, only 3 bacterial and 2 fungal strain displayed amylase activity. The 3 bacterial isolates, FS-10, PW-4, and PW-1 were identified as Gram-positive rods microscopically while the fungal strains SGA and SGB was identified as a mold through macroscopic examination. Quantification of amylase production showed that FS-10 had the highest amylase activity with 49.88 U/ml closely followed by PW-4 with 49.50 U/ml while the fungus SGB had the least enzyme activity with 10.57 U/ml. Amplification and sequencing of the 16S rRNA gene and the ITS region of the bacterial and fungal isolates respectively was used to identify the isolates. FS-10 was identified as *Bacillus flexus* (MW522619), PW-4 was identified as *Bacillus cereus* (OK384566) and SGA as *Aspergillus aculeatus* (MW577297). The isolates can be used for industrial production of α-amylase.

Keywords: Hydrolysis; Bacteria; Amylase; Starch; Molecular characterization; Fungi

1. Introduction

Amylases are crucial enzymes that have been massively used in industries. Amylases are divided majorly into alpha-amylase and gluco-amylase, they hydrolyze starch into smaller units such as glucose and dextrins [1]. The industrial applications of amylases include liquefaction of starch [2], baking and brewing of alcohol [3], production of high fructose syrups [4] paper production and textile industries [5]. Amylases are obtained from different sources including plant origin, animal origin and from microorganism. The production of amylase using microorganisms has been reported to be more efficient than the other sources of amylase production as the method is economical, fast, and consistent [6]. Amylase producers including *Bacillus sp.* obtained from soil have been reported to produce high yield using starch hydrolysis and biochemical technique [7]. Thermophilic bacterium identified as *Bacillus licheniformis* with amylase producing characteristics has been isolated from hot spring with optimum amylase production of over 200 U/ml [8]. Aside bacteria being one of the popular microbial sources of amylase production, fungi have been reported to possess this attribute as well. Cindy et al. [5] has reported the production of amylase from *Aspergillus niger* isolated from fermented cassava product and cultured in potato peel medium. Ire et al. [9] reported the isolation of *Aspergillus flavus* from soil and wastewater in cassava processing site which had the ability of producing amylase up to 2.3 U/ml/min after 96 h of incubation. However, amylase-producing bacteria are more preferred over fungi due to their ability of quick cell growth, multiplication, and ease of production process scalability [10]. This study was aimed at isolating, screening, and identifying amylase producing bacteria and fungi.

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2. Material and methods

2.1. Sample Collection

Soil and waste fermentation broth was collected from cassava processing site and palm wine in Port-Harcourt, River state using clean polythene bag for the soil and sterile capped tubes for the wastewater. The samples were immediately transported to Osaks Integrated Laboratory Services for storage and analysis.

2.2. Isolation of Bacteria and Fungi

One gram of the soil sample and 1 ml of the waste fermented broth and palm wine were transferred from the stock obtained into three 9 ml separate tubes labelled accordingly. This was dilution 10^{-1} and the contents were further serially diluted to dilution 10^{-5} for each tube. Bacteria isolation was done on starch medium which was prepared according to the method of Kanimozhi *et al.* [11]. Five hundred milliliters of the fermentation broth were prepared by weighing and mixing starch 10 g, peptone 5 g, yeast extract 2 g, $MgSO_4$ 0.25 g, $CaCl_2$ 0.1 g and agar 13.5 g in distilled water and sterilized at 121 °C, 15 psi for 15 min**.** One milliliter each of dilutions 10^{-2}, 10^{-3} and 10^{-4} were plated using spread plate method and incubated at 37 °C for 24 h. Fungi isolation was carried out according to the modified method of Ogbonna *et al.* [12]. About 19.5 g of potato dextrose agar (PDA) containing 10 % starch was prepared in 500 ml distilled water and sterilized by autoclaving at 121 °C, 15 psi for 15 min**.** One milliliter each of dilutions 10^{-2}, 10^{-3} and 10^{-4} were pipetted for both samples respectively and spread on Potato dextrose agar (PDA) prepared in Petri dishes with the aid of a sterile hockey stick. The PDA plates were incubated at 28 °C for 5 days.

2.3. Obtaining Pure Culture

The bacteria isolate on the starch medium after incubation were further purified by streaking on new starch medium plates. The plates were incubated at 37 °C for 24 h. The fungi isolates were further purified to obtain pure cultures on starch medium via streaking and incubated at 28 °C for 48 h.

2.4. Screening of isolates for amylase activity

Bacteria isolates were screened for amylase activity by flooding the starch plates with Lugol's iodine, this shows a clear zone around amylase producing microorganisms. The zones of clearances were measured as an indication of the degree of amylolytic activity [13]. Fungal isolates were screened according to the method described by Ire et al. [9]. The plates were flooded with Lugol's iodine and zones of clearances were measured to ascertain the hydrolytic activity.

2.5. Microscopic Examination of Isolates

Gram staining technique was carried out to group the bacteria isolates according to their cell morphology. Smears of pure isolates were made on labelled sterile slides and heat fixed, Crystal violet dye was added and allowed to stand for 60 s. and rinsed. Gram's iodine was added for 60 s, rinsed and decolourized with 95 % alcohol. Following rinsing with water, safranin was added and allowed for 30 sec. The slides were rinsed, blotted dry and viewed under oil immersion objective.

The fungal isolates were stained were stained with lactophenol cotton blue dye and viewed using 40 X magnification.

2.6. Crude Amylase Extraction

The modified method of Kanimozhi *et al.* [11] was used for extraction of crude amylase from bacterial isolates. Ninety milliliter of starch broth was prepared, the pH was adjusted to 7 and the flasks were autoclaved at 121 °C, 15 psi for 15 min. A 24 h old culture of each isolate was standardized according to Mc Farland's standard. Approximately 10 ml of each was added to the sterile 90ml starch broth. The flasks were placed on a rotary shaker and incubated at 37 °C for 24 h.

A modification of the method as described by Ire *et al.* [9] was used for the extraction of crude amylase from fungal isolates. Five hundred milliliter of the basal medium containing 10 g soluble starch, 7 g KH_2PO_4, 5 g NH_4NO_3, 0.25 g KCl, 0.05 g $MgSO_4.7H_2O$, and 0.01 g $FeSO_4.7H_2O$. The medium pH was adjusted to 7 and the medium was divided into 5 flasks containing 100 ml each and sterilized at 121 °C, 15 psi for 15 min. A 48 h old culture of each isolate was standardized according to Mc Farland's standard, 10 ml of each was used to inoculate each sterile basal medium. The flasks were incubated at 37 °C for 3 days on a rotary shaker.

2.7. Enzyme Assay

2.7.1. Bacterial Isolates

After 24 h, the flasks were centrifuged at 4000 rpm for 20 min at 4 °C. The supernatant (0.5 ml) was used as the crude amylase for each flask. This was pipetted into test tubes and 0.5 ml of starch phosphate buffer was added to all the tubes respectively. The combination was mixed and incubated at 25 °C for 3 min. Approximately 1 ml of DNSA solution was added and all tubes were incubated in a boiling water bath for 5 min and allowed to cool at room temperature. Dilution of the sample was done when necessary and absorbance read at 540 nm. The amount of reducing sugar released by the enzyme and enzyme activity was generated from maltose standard curve.

2.7.2. Fungal Isolates

After fermentation, the flasks were centrifuged at 7000 rpm for 20 min at 4 °C. The assaying method of Ire *et al.* [9] was used with slight modification. the supernatant (0.5 ml) was used as the crude amylase. The crude enzyme was added to the mixture 1 ml of potassium phosphate buffer and 1 ml of 1 % starch solution. The mixture was incubated at 37 °C for 15 min. DNSA solution (1 ml) was added and all tubes were incubated in a boiling water bath for 10 min and allowed to cool at room temperature. Spectrophotometric readings (absorbance) of each test tube were taken at 540 nm. The standard for reducing sugar released by the enzyme from the assay was generated from maltose standard. The enzyme activity was calculated with the formula: IU/ml = Activity of enzyme x 1000 / Molecular wt. of maltose [7].

2.8. Molecular Identification for Bacterial and Fungal Isolates

Two bacteria isolates and one fungal isolate respectively with the highest amylase activity were sent to the National Biotechnology Development Agency (NABDA) laboratory in Bayelsa state for molecular and phylogenetic analysis. The bacterial isolates were identified by PCR amplification of 16S rDNA using the universal primers 27 F (5′ AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′TACGGTTACCTTGTTA CGACTT3′), while the fungal isolates were identified by amplification of the ITS region using ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3, primers, followed by sequencing. The resulting nucleotide sequence (Accession No. MK106155.1, OK384566 and MW577297 for FS10, PW4 and SGA) has been submitted to the NCBI gene bank data base. The isolated strain's identity was confirmed by phylogenetic analysis of the 16S rRNA gene using the software Mega-6.

3. Results and Discussion

3.1. Isolation and Screening of Isolates

A total of 12 isolates comprising of 7 bacteria and fungi were screened for production of amylase, only 3 of the isolates including 2 bacteria and 1 fungal isolate had the highest hydrolytic activity as indicated by the hydrolysis ratio (Table 1).

The various zones of clearances indicate the ability of the bacteria to utilize starch on the plate having secreted amylase [14]. Isolates FS-10 and PW-4 had the widest zone of clearances with 34 mm and 25 mm respectively while FB-4, FB-5, FB-6 and FS-1 lacked the ability to secrete amylase thereby showing no clear zones. The result obtained in this study is consistent with the work of Singh *et al.,* [15] and Garba *et al.,* [16] who have reported the use of starch medium with iodine to identify amylase-producers based on cleared zones around their colonies.

The result shown in Table 2 shows the zones of clearance obtained from the fungal isolates. Two isolates SGA and SGB displayed amylase activity however SGA had higher hydrolysis ratio. The result obtained is consistent with the work of Ire *et al.* [9] who isolated fungi strains with cleared zone as high as 52 mm. Three fungal strains with cleared zones as high as 2 cm after flooding with iodine has been isolated [17]. Isolate SGC, SGD and SGE lacked the ability to produce amylase and hence did not utilize the starch.

Table 1 Bacteria isolates with their zones of clearances

Isolate code	Colony diameter (mm)	Hydrolysis zone (mm)	Hydrolysis ratio (mm)
PW-1	11	18	1.63
PW-4	13	25	1.92
FB-4	-	-	
FB-5	-	-	
FB-6	-	-	
FS-1	-	-	
FS-10	15	34	2.26

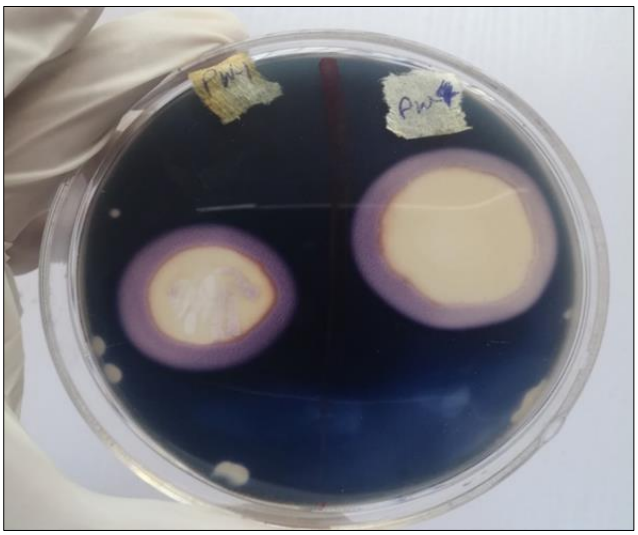

Plate 1 Starch hydrolysis plate assay using iodine

Table 2 Fungal isolates with their zones of clearances

Isolate code	Colony diameter (mm)	Hydrolysis zone (mm)	Hydrolysis ratio (mm)
SGA	11	20	1.81
SGB	13	17	1.30
SGC	-	-	
SGD	-	-	
SGE	-	-	

3.2. Preliminary identification of isolates

The bacterial isolates were identified according to their gram morphology. The result in Table 3 revealed that all three isolates with confirmed amylase production during screening where gram positive rods with no obvious distinct microscopic morphology. Comparison of the microscopic result with reference standards identified the three isolates as belong to the genus *Bacillus*.

Morphological characteristics of SGA with amylase production on PDA showed that SGA appeared as brownish, dark-centered, wooly and spore producing. SGA was identified as a mold from its morphology. Microscopic examination after

staining with lactophenol cotton blue revealed septate hypha with fluffy conidia with green centre and blue rough edges. SGA was identified as *Aspergillus* sp. from the characteristics.

Table 3 Gram morphology of bacterial isolates

Isolate code	Gram morphology	Microscopic characteristics
PW-1	Gram +	Rods
PW-4	Gram +	Rods
FS-10	Gram +	Rods

3.3. Enzyme Assay

The amylase activity and the concentration of maltose released by the bacterial and fungal isolates were calculated after 24 h and 72 h of fermentation respectively. The amylase activity result for the four isolates is shown in Table 4. FS-10 had the highest amylase activity with 49.88 U/ml closely followed by PW-4 with 49.50 U/ml while the fungus SGB had the least enzyme activity with 10.57 U/ml. Although the result obtained in this study are higher, the amylase activity of the bacterial isolates obtained in this study correlates with that work reported by Pranay, *et al.* [18] who isolated 6 strains of *Bacillus* sp. from soil with peak enzyme activity of 18.20 U/ml. Das et al. [17] in his study isolated and screened three fungal strains with amylolytic activity as high as 87 U/ml.

Table 4: Amylase activity of screened isolates

Isolates	Maltose released (µmole/mL)	Amylase activity (U/mL)
PW-4	74.25	49.50
FS-10	74.82	49.88
SGA	29.84	18.56
SGB	17.01	10.57

3.4. Molecular Identification of Isolates

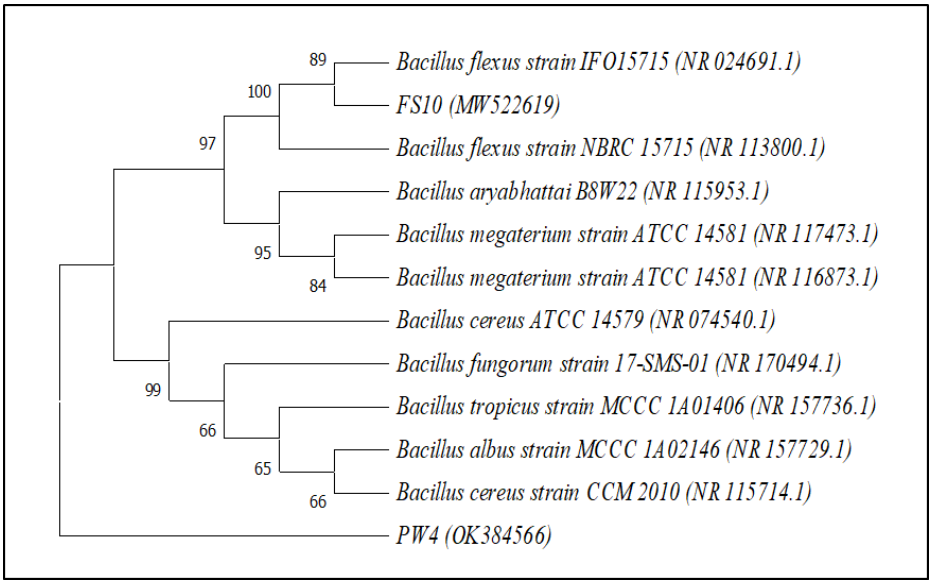

Figure 1 Phylogenetic tree of the bacteria isolates

All the isolates with the highest amylolytic activities FS-10, PW-4 and SGA were subjected to molecular analysis to identify their genetic sequences after which bioinformatics was conducted to create the phylogenetic tree and finally identify the isolates. FS-10 was identified *Bacillus flexus* while PW-4 as *Bacillus cereus* and SGA was identified as

Aspergillus aculeatus. The phylogenetic tree of the bacterial and fungal isolates is presented in Figure 1 and 2 respectively.

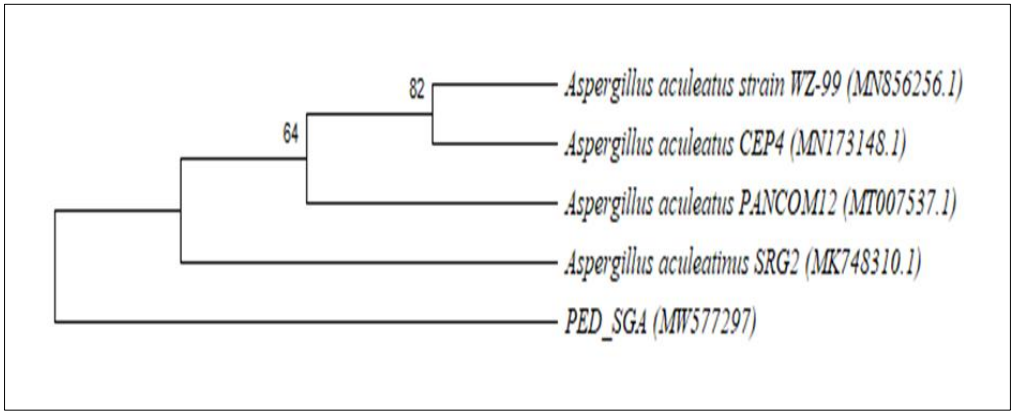

Figure 2 Phylogenetic tree of isolate SGA (*Aspergillus aculeatus*)

4. Conclusion

In this present study, 12 isolates comprising of 7 bacteria and 5 fungi were isolated and screened for amylase production. Three isolates FS-10, PW-4 and SGA had highest α-amylase activity. The two bacterial strains FS-10 and PW-4 were identified as *Bacillus flexus* and *B. cereus* respectively. The results obtained in this study demonstrated the amylolytic potential of *Bacillus cereus, Bacillus flexus* and *Aspergillus aculeatus* isolated from cassava processing site and palm wine. The α-amylase production potential of these isolates can be enhanced by optimization process. These isolates can be used industrially for amylase production.

Compliance with ethical standards

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Disclosure of conflict of interest

No known conflict of interest exists among the researchers.

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