Detection of Amylolytic Bacteria from Starch-Based Agro Wastes and Optimisation of Culture Conditions for Enhanced Production of Extracellular Amylase

Sunday Babatunde Akinde, Deborah Adepeju Ojewande and Janet Olubukola Olaitan
Department of Microbiology, Faculty of Basic and Applied Sciences, Osun State University, P.M.B. 4494, Osogbo, Nigeria

Abstract: The search for novel microorganisms with enhanced amylase production is a continuous process, and the starch-based agrowastes (SBAW) hold such potential. This study investigated the ability of bacteria isolated from SBAW obtained locally to produce extracellular amylase with varying cultural conditions. A total of 41 bacteria isolated by spread-plate from the SBAW samples were identified using their morphological, biochemical and 16S rRNA gene characteristics. Preliminary screening by starch hydrolysis revealed three most-promising amylolytic isolates with 5.0 – 6.0 mm clear zone diameter. They were *Myroides odoratimimus* BY-F1, *Enterobacter asburiae* YP-F1 and *Bacillus cereus* CP-A4 with the amylase production of 308.75 ± 1.41 U/ml, 274.44 ± 0.68 U/ml and 205.83 ± 0.18 U/ml respectively. Their optimal culture conditions were BY-F1 [pH 7.0 at 40°C for 72 h], YP-F1 [pH 9.0 at 40°C for 24 h] and CP-A4 [pH 9.0 at 35°C for 72 h]. The FTIR characterisation of the amylase purified by *Calotropis procera* latex and activated charcoal revealed the presence of -OH, -COOH and -NH_2 group, which are essential for amylase activity. The findings suggest the exploitation of *Myroides odoratimimus*BY-F1, *Enterobacter asburiae* YP-F1 and *Bacillus cereus*CP-A4 for industrial-scale production of extracellular amylase.
Keywords: amylase, amylolytic activity, starch-degrading bacteria, agricultural wastes, enzyme

INTRODUCTION

Among different types of enzymes obtained from microbial sources, amylases are the most commercially produced and widely used in industries (Souza, 2010). Amylase production from bacteria is economical as the enzyme production rate is higher compared to other sources. Additionally, bacteria are highly manipulable genetically for cheap bulk production and have a broad spectrum of industrial applications as they are more stable than those derived from plant and animal sources (Pandey *et al*., 2000; Singh *et al*., 2012; Sundarram and Murthy, 2014).

Isolating amylolytic bacteria from their natural environment is very useful for addressing many biotechnological problems, especially in the developing nations where there is a shortage of local production in commercial quantity. More importantly, extensive potentials of amylases to be used in a broad range of industries, including pharmaceutical, food, brewing, detergents, textile, paper, etc., have placed a significant stress on researchers to search for a stable and more efficient ones (Pandey *et al.,* 2000; Dash *et al*., 2015; Souza, 2010).

In the developed world, characteristics of bacteria with amylolytic potential obtained from diverse sources were well documented and genetically modified strains exist for improved production (Pandey *et al.,* 2000; Souza, 2010).Such progress in amylase production is still a major challenge faced by the contemporary industries in many developing countries.Presently, manufacturers in developing nations spend huge sums importing this vital enzyme to meet local needs. This practice constitutes a substantial economic loss to the developing nations, especially Nigeria, an agrarian country, with unlimited natural sources of microorganisms with amylase production potential. One of such potential sources of amylolytic bacteria is the tons of wastes generated annually from agricultural production processes.

In this study, we seek to explore the amylase-producing capabilities of bacteria isolated from starch-based agricultural wastes (SBAW), a cheap and sustainable source, as the first and important step in local large-scale amylase production.

[1*]Correspondence:
akindesb@uniosun.edu.ng;
+234 8033798688

MATERIALS AND METHODS

Sample Collection

SBAW including cassava wastewater, cassava peel, rot yam tuber, yam peel, rot solid corn porridge ("*eko*" in Yoruba) and soil chronically contaminated with cassava wastewater samples served as the sources of amylase producing bacteria used in this study. The samples were sourced locally inOsogbo, Nigeria. Sterile zip-lock and screw-capped bottles were used to collect the respective solid and liquid samples. All samples were appropriately-labelled and transported at 4 ± 2°C to the laboratory.

Isolation and Characterisation of Bacteria from SBAW

Heterotrophic bacteria from the SBAW samples were isolated using the spread-plate method (Akinde and Obire, 2008). Following ten-fold serial dilution of the samples, a 100 µl of appropriate dilution factor (10^1 – 10^6) wasspread ontonutrient agar plates.All plates were kept incubated at 37°C for 24 h. Discrete colonies were selected based on their different morphological characteristics. The colonies were further purified by repeated streaking on nutrient agar plates. The pure isolates were stored on sterile nutrient agar slant at 4± 2°C for further analysis.

Preliminary Screening of Bacterial Isolates for Amylolytic Activity

Primary screening for the amylolytic activity of all the isolateswas carried out by starch hydrolysis test on starch agar plates (Himedia, India) (Roslan*et al*., 2018). Axenic colonies were aseptically picked from each plate and streaked in straight lines on a separatestarch agar plate. After incubation at 37°C for 48h,the surface of each plate was flooded with Gram's iodine to producea starch-iodine complex of deep blue colour indicating amylase production. The clear zone around colonies of amylase-producing bacteria (after addition of iodine) is indicative of amylase activity.

Production of Amylase by Amylolytic Isolates

Amylase production by the amylolytic isolates was done using submerged fermentation (Vidyalakshmi *et al*. 2009).

Bacterial Inocula and Fermentation Medium

The bacterial inocula were prepared by the addition of sterile phosphate buffer to the freshly grown nutrient agar cultures, from this 0.5 ml of each bacterial cell suspension was inoculated into 100 ml of sterilised fermentation/production medium [bacteriological peptone, 6.0 g/L; $MgSO_4.7H_2O$, 0.5 g/L; KCL, 0.5 g/L; starch, 10 g/L; pH 7.0] and incubated in SI600 shaker incubator (Stuart) at 35°C for 24 h.

Amylase Extraction from Fermentation Medium

After incubation, the fermentation medium was harvested by centrifugation in Beckman Coulter Allegra X-22R refrigerated centrifuge at 5000 rpm and 4°C for 15 min. The cell-free supernatant (enzyme extract) was collected to estimate the amylase produced.

Amylase Assay and Estimation using UV Spectrophotometer

Amylase was assayed by incubating a 1:1 mixture of each enzyme extract(2 ml) and 0.5% of soluble starch solution (Prepared in 0.1 M phosphate buffer) (2 ml) in a water bath at 35°C for 15 min in triplicates. The reaction was terminated by adding 2.0 ml of 3,5-dinitrosalicylic acid (DNS) (Sigma) reagent and kept in a boiling water bath for 5 min. The final volume was made up to 12 ml with distilled water, and optical density was taken at 540 nm (OD_{540}) (Sankaralingam *et al*., 2012) using Spectrumlab752S UV-Vis Spectrophotometer (China).

Expressed as Units/ml (1 U/ml = amount of enzyme which releases 1 µM of glucose under the assay condition), the enzyme activity was calculated using the formula:

$$\text{Amylase activity} = \frac{E_0 - E_t \times A \times 1000}{E_0 \times T \times V}$$

Where,$E_0 = OD_2 - OD_1$ (assay system starch);$E_t = OD_3 - OD_1$ (residual starch of the assay);T= Incubation period (15 min);A= 12.35 (constant) micromole of amylase in assay culture;V= Volume of 0.5% soluble starch used (2 ml); 1,000 = To convert to litre; OD1 = Optical density of fermentation medium only; OD2 = Optical density of fermentation medium + soluble starch; OD3 = Optical density of fermentation medium + soluble starch + enzyme extract.

Identification of Bacteria and 16S rRNA Characterisation of Amylolytic Isolates

All isolates were presumptively identified based on their morphological and biochemical characteristics according to the Bergey's Manual of Systematic Bacteriology (Logan and DeVos, 2009).

Genomic DNA of the amylolytic isolates was extracted using ZR Fungal/Bacterial DNA MiniPrep™ kit from 24-hour old cultures incubated at 37°C in nutrient broth.The pure DNA was amplified using the primers 16SF (5'-GTGCCAGCAGCCGCGCTAA-3') and 16SR (5'-AGACCCGGGAACGTATTCAC-3'). PCR amplification was performed in a total volume of 10 µL containing 1.0 µL of 10x PCR buffer, 0.5 µL of 5 pMol forward primer, 0.5 µL of 5 pMol reverse primer, 1.0 µL of 25 mM $MgCl_2$, 1.0 µL of DMSO, 0.8 µL of 2.5 Mm dNTPs, 0.1 µL of Taq 5u/ul, 2.0 µL of 10 ng/µl DNA and 3.1 µL of DNase-free H_2O. Thermal cycling reaction conditions in GeneAmp® PCR System 9700 consisted of initial denaturation (94°C for 5 min), followed by 36 cycles of denaturation (94°C for 30 s), annealing (56°C for 30 s) and extension (72°C for 45 s), and finishing with a single extension (72°C for 7 min).A 2 µl of PCR product was electrophoresed on 1.5% agarose gel containing 0.5µg/ml EtBr with Hyper Ladder™ 1kb (Bioline). The product was visualised by UV trans-illuminator and photographed. Purification of the PCR product was byethanol/ EDTA precipitation.

The purified product was sequenced with the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Britain).The sequencing reaction mixture contained 4µL of ready reaction 2.5x premix, 2 µL of Big Dye 5x sequencing buffer from the kit,3.2pmol of the sequencing primer and 2 µL of purified PCR product in a total volume of 20 µL.The amplification primers were used for sequencing in GeneAmp® PCR System 9700by 25 cycles of denaturation (96°C for 10 s), annealing (50°C for 5 s) and extension (60°C for 4 min) at athermal ramping rate of 1°C/s. The sequencing products werepurified and then prepared for running on an ABI 3500 Genetic Analyzer (Applied Biosystems) by the manufacturer's instructions.

BioEdit 7.2 sequence alignment software was used to analyse the sequences obtained, and BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out to confirm the sequenced data with the standard strains and to determine the percentage homology.

Optimisation of Culture Conditions for Improved Amylase Production

The culture conditions such as pH, temperature and incubation period affecting amylase production were optimised by varying the parameters one at a time. The experiments were conducted in 100 ml Erlenmeyer flask containing sterilised fermentation medium. The flasks were cooled and inoculated with bacterial culture and maintained under separate operational conditions of pH (5, 6, 7, 8, 9, 10), temperature (35°C, 40°C, 45°C along with arbitrary control at 37°C) and incubation period (24 h, 48 h and 72 h). After 72 h (except for incubation period optimisation), the culture filtrate was assayed in triplicate for amylase production.

Purification and FTIR Characterisation of Amylase fromAmylolytic Isolates

Purification of amylase from the cell-free supernatant obtained from the fermentation medium was done using two methods. (1) Purification with *Calotropis Procera*latex: the latexwasobtained locally as exudates from lucked leaves of *C. procera* plant, stored at 4°C until use.*C. procera* latex was diluted serially with sterile distilled water, and 1ml of each dilution was added to 9 ml of crude amylase and then stored at 4°C to precipitate, the purified amylase was collected in sterile test tubes and stored at 4°C until use (Kareem *et al.,* 2002). (2) Purification with activated charcoal: 3% activated charcoal was added to crude amylase extract and incubated at 30°C for 30 min with constant stirring (Kareem *et al.,* 2011). The mixture was centrifuged for 10 min at 2,500 rpm, and the supernatant was stored at 4°C to precipitate, then the purified amylase was collected and stored at 4°C (Kareem *et al.,* 2011).

The purified amylase extract was characterised using Fourier Transform Infrared (FTIR) spectrophotometer to determine the functional groups of the biopolymers. The sample was blended with KBr and passed into a disc for FTIR analysis. The spectrum of the sample was recorded on the spectrophotometer over a wavelength range of 4000 - 400 cm^{-1} under ambient conditions (Kumar *et al.,* 2004).

RESULTS

Characteristics of Bacteria from SBAW Samples

A total of 41 heterotrophic bacteria belonging to 18 sub-species were isolated from the SBAW samples (Figure 1). The genus *Bacillus* spp. had the highest occurrence (46.34%) in the SBAW samples followed by *Staphylococcus* spp. (21.95%). Other isolated bacterial genera included *Aeromonas* sp. (2.44%), *Corynebacterium* sp. (2.44%), *Enterobacter* spp. (4.88%), *Micrococcus* sp. (2.44%), *Myroides* sp. (2.44%), *Neisseria* spp. (9.76%), *Proteus* spp. (4.88%) and *Streptococcus* sp. (2.44%).

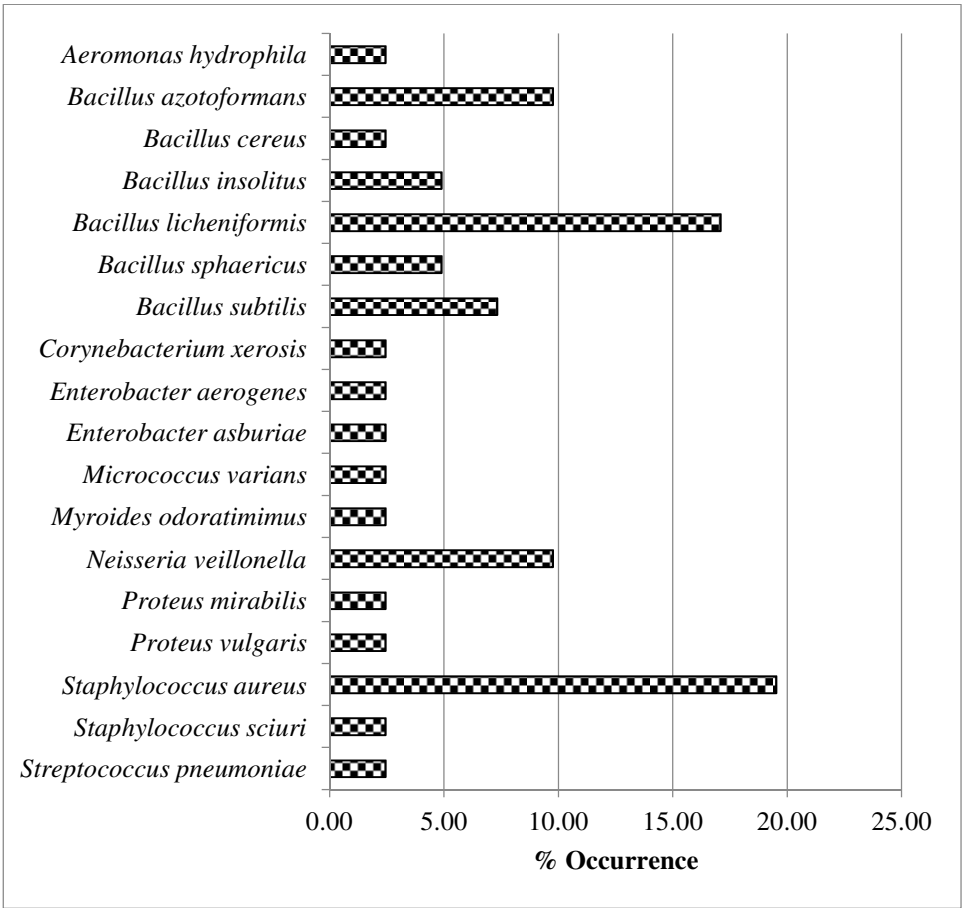

Figure 1: Presumptive Identity and % Occurrence of Bacteria in SBAW Samples

Amylolytic Activity and Estimation of Amylase Production by Isolates

Amylolytic Activity of Isolates on Starch Agar Plates

Out of the 41 bacteria isolated from the SBAWsamples, 24 strains were positive with amylolytic activity on starch agar plates (Table 1). The clearance zone diameter on starch agar plates, which is an indication of the amylolytic activity of the isolates ranged between 1.0 mm and 6.0 mm. The distribution of the positive strains based on agro-waste type included: cassava wastewater (2 of 3 isolates), cassava peel (6 of 10 isolates), rot yam tuber (2 of 5 isolates), yam peel (3 of 7 isolates), rotten solid corn porridge (0 of 3 isolates) and soil chronically contaminated with cassava wastewater (11 of 13 isolates).

The bacterial strains with the highest clearance zone diameter (≥ 5 mm) on starch agar plates included BY-F1, YP-F1 and CP-A4 from rot yam tuber, yam peel and cassava peel samples respectively. The three bacteria were selected as amylolytic isolates for further studies.

Estimation of Crude Amylase using UV-Vis Spectrophotometer

Figure 2 shows the result of the crude amylase produced by the amylolytic isolates based on the OD_{540} measurement. The amylase concentration in crude extract of BY-F1, YP-F1 and CP-A4 were 308.75 ± 1.41 U/ml, 274.44 ± 0.68 U/ml and 205.83 ± 0.18 U/ml respectively.

Table 1: Amylolytic Activity of Isolates Measured by Clearance Zone Diameter on Starch Agar Plates (mm)

Cassava Wastewater		Cassava Peel		Rot Yam Tubers		Yam Peel		Rot Solid Corn Porridge		Soil Chronically Contaminated with Cassava Wastewater	
Isolate Code	Clearance Zone	Isolate Code	Clearance Zone	Isolate Code	Clearance Zone	Isolate Code	Clearance Zone	Isolate Code	Clearance Zone	Isolate Code	Clearance Zone
CWW-A1	0.0	CP-F3	0.0	BY-A3	0.0	YP-A3	0.0	BSP-F1	0.0	SCW1-A1	1.3
CWW-A2	2.5	**CP-A4**	**5.0***	BY-A2	0.0	YP-A6	0.0	BSP-F2	0.0	SCW1-A2	4.0
CWW-F1	4.8	CP-A2	0.0	**BY-F1**	**6.0**	YP-A2	0.0	BSP-A1	0.0	SCWI-A3	2.2
		CP-A1	3.2	BY-F2	4.9	YP-A1	4.5			SCW2-A1	1.5
		CP-A5	4.0	BY-A1	0.0	**YP-F1**	**5.6**			SCW2-A2	2.5
		CP-A3	3.7			YP-A5	0.0			SCW2-A3	3.2
		CP-A6	0.0			YP-A4	4.1			SCW1-F1	3.0
		CP-F1	3.0							SCW2-F3	4.3
		CP-F2	1.0							SCW2-A4	3.9
		CP-F4	0.0							SCW2-F2	3.6
										SCW2-F4	0.0
										SCWI-F2	0.0
										SCW2-F1	3.0

*Isolates in bold with hydrolysis ≥ 5 mm selected for further investigations.

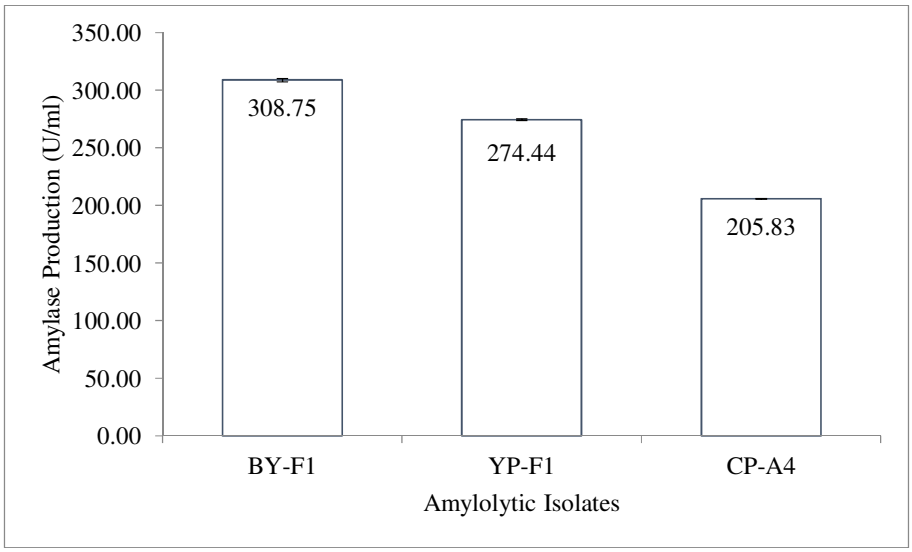

Figure 2: Estimation of Amylase Produced in Crude Extracts of Amylolytic Isolates. BY-F1 from Rot Yam Tuber; YP-F1 from Yam Peel and CP-A4 from Cassava Peel. Error bar represents differences between three independent measurements.

Identity of Amylolytic Isolates Based on their 16S rRNA Genes

The 16S rRNA gene sequence revealed that BY-F1 belongs to the genus *Myroides* and is closely related to *Myroides odoratimimus* (95%), YP-F1 belongs to the genus *Enterobacter* and is closely related to *Enterobacter asburiae* (98%) while CP-A4 belongs to the genus *Bacillus* and is closely related to *Bacillus cereus* (100%). Their DNA band pattern on theagarose gel is shown in Figure 3while theirsequence similarities to the nearest relatives ispresented in Table 2.

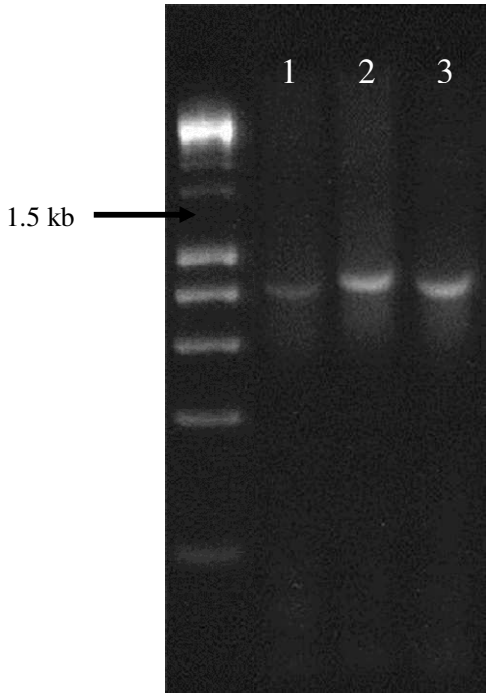

Figure 3: Agarose Gel Electrophoresis of the PCR Products Obtained after Amplification of 16S rRNA gene (~1.5kb) from Amylolytic Isolates. Lane 1: *Myroides odoratimimus* BY-F1; Lane 2: *Enterobacter asburiae* YP-F1; Lane 3: *Bacillus cereus* CP-A4.

Table 2: Sequence Similarity of Amylolytic Isolates to Nearest Relatives

Isolate Code	Blast Search Request ID	Sequence length blasted (bp)	Highest coverage (%)	E Value	% identity (accession no.)	Nearest Relative
BY-F1	2Z8M4261014	540	100	0.0	95 (KF742685.1)	*Myroides odoratimimus*
YP-F1	2Z7HSZJC015	597	99	0.0	98 (KY363410.1)	*Enterobacter asburiae*
CP-A4	2Z93MXV6015	596	100	0.0	100 (MK192044.1)	*Bacillus cereus*

Optimum Culture Conditions for Improved Amylase Production

The amylase production by YP-F1 and CP-A4 were optimal at pH 9 (403.59 and 329.33 U/ml respectively) and minimal at pH 6 (16.47 and 43.33 U/ml respectively) whereas production by BY-F1 was optimal at pH 7 (308.75 U/ml) and minimal at pH 9 (164.67 U/ml) (Figure 4).

Optimal temperatures of 37°C and 40°C gave the highest amylase yield (247 U/ml) with BY-F1 whereas 40°C gave the highest yield (411.67 U/ml) with YP-F1 while 35°C produced the highest amylase (411.67 U/ml) with CP-A4. Amylase production was minimal at 35°C with BY-F1 (41.17 U/ml) as well as 45°C withYP-F1 and CP-A4 (137.22 and 102.92 U/ml respectively).The result obtained at different incubation period shows that the production of amylase enzyme by BY-F1 and CP-A4 increases as the incubation period increases whereasfurther increase in incubation period showed a considerable decrease in productionwith YP-F1.

Characteristics of Purified Amylase with FTIR Spectrophotometer

FTIR spectrum analysis revealed the presence of different functional groups in the amylase extracts from the amylolytic isolates (Figure 5).The spectrum showed broad stretching peaks at 3445.05 cm^{-1}, 3446.91 cm^{-1} and 3441.12 cm^{-1} for BY-F1, YP-F1 and CP-A4 respectively, suggestive of the presence of the hydroxyl and amino groups.

The absorption peaks at 2949.26cm^{-1} and 2945.40 cm^{-1} show C-H stretch for BY-F1 and CP-A4 respectively, but none for YP-F1. The absorption peaks of 2017.61 cm^{-1} for BY-F1 and 2360.95 cm^{-1} for YP-F1 as well as 2004.11 $cm^{-1}$and 2370.59 cm^{-1} for CP-A4 are indicative of C-H aliphatic bond. The peaks, 1637.62cm^{-1} for BY-F1, 1639.55cm^{-1} for YP-F1 and 1635.69 cm^{1} for CP-A4 displayed an asymmetrical stretching band which is suggestive of the presence of carboxyl groups with C=O in an amide group.

Theasymmetrical stretching band near 1340.57 cm^{-1} to 1560.40 cm^{-1} in all the isolates were consistent with the presence of carboxylate ions. The small absorption peaks exhibited at 1026.16 cm^{-1} to 1199.76 cm^{-1} are attributed to the asymmetrical stretching vibration of a C-O-C ester linkage indicating the presence of uronic acids. The small/weak peaks identified at 414.71 cm^{-1} to 925.86 cm^{-1} for all the isolatesare indicative of sugar derivatives. The spectrum revealed the presence of carboxyl, hydroxyl, and amino groups in the amylase extracts.

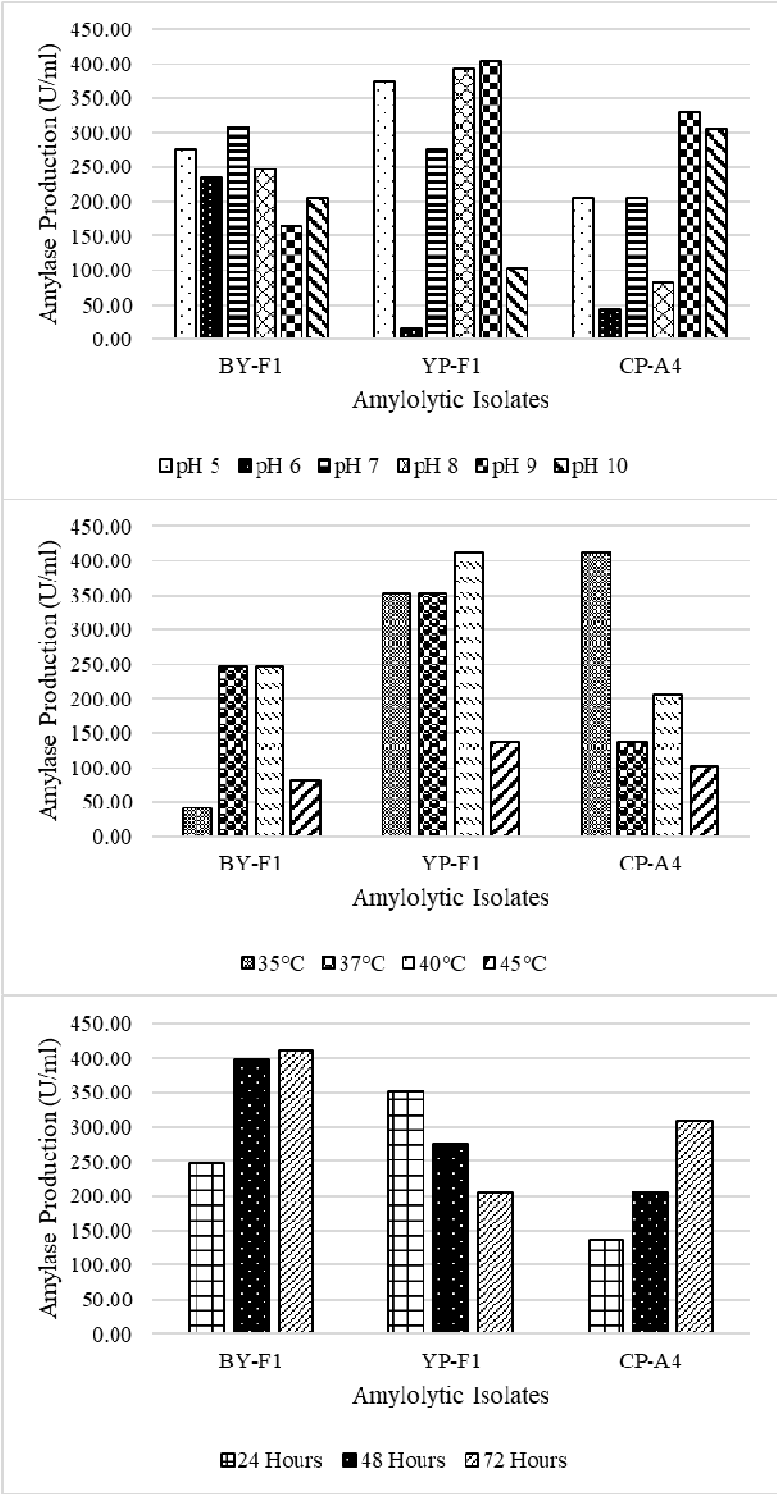

Figure 4: Effect of pH, Temperature and Incubation Period on Amylase Production by*Myroides odoratimimus*BY-F1, *Enterobacter asburiae*YP-F1 and*Bacillus cereus*CP-A4.

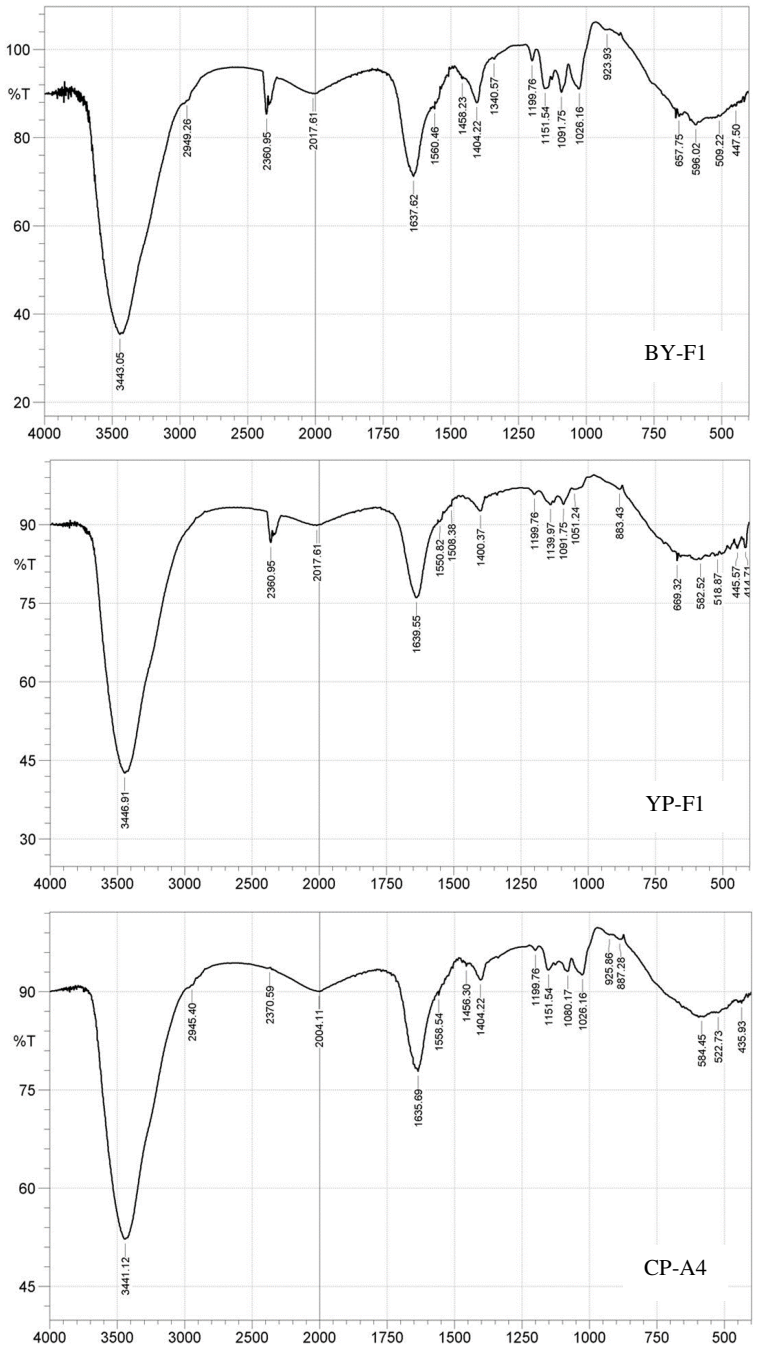

Figure 5: FTIR Characteristics of Amylase Extracted from *Myroides odoratimimus* BY-F1, *Enterobacter asburiae* YP-F1 and *Bacillus cereus* CP-A4.

DISCUSSION

Forty-one bacterial isolates with distinct characteristic features were isolated from cassava wastewater, cassava peel, rot yam tuber, yam peel, rotten solid corn porridge ("*eko*" in Yoruba) and soil chronically contaminated with cassava wastewater samples. Due to their ubiquitous nature and high versatility in their adaptability to the environment, amylase producing bacteria could be present in different starchy and non-starchy places (Mishra and Bereha, 2008; Khan and Priya, 2011).

The genus *Bacillus* constituted 46.34%, making it the most encountered of the total bacteria isolated from the SBAW samples (Figure 1). Various *Bacillus* spp. are reported to produce approximately 60% of commercially available enzymes including the α-amylase (Burhan *et al*., 2003; Dash *et al*., 2015; Rai *et al*., 2017, Sewalt *et al*., 2018). This capability could be due to their (1) high growth rates leading to short fermentation cycle times (2) capacity to secrete proteins into the extracellular medium and (3) GRAS (generally regarded as safe) status with the Food and Drug Administration for species, such as *B. subtilis* and *B. licheniformis* (Alcaraz *et al*., 2010; Sundarram and Murthy, 2014; Dave *et al*., 2015).Various authors have reported the potential other isolated bacterial genera to produce amylase and other industrially essential enzymes(Gupta *et al*., 2003; Souza, 2010; Sundarram and Murthy, 2014; Gopinath *et al*., 2017).

Altogether, 58.5% of the 41 bacterial isolates exhibited amylolytic activity on starch agar plates(Table 1). None of the threebacteria isolated from rot solid corn porridge showed amylase production potential.Soil chronically contaminated with cassava wastewater supported more bacteria with amylolytic potential.Previous studies had reported clear zone diameters similar to 1.0 – 6.0 mm range observed in this study (Akpomie*et al*., 2012; Ogbonnaya and Odiase, 2012).

The selected amylolytic isolates identified with their 16S rRNA genes as *Myroides odoratimimus* BY-F1, *Enterobacter asburiae* YP-F1 and *Bacillus cereus* CP-A4 (Table 2) are known for their polysaccharide degradation (Vijayaraghavan *et al*., 2015; Blanco *et al*., 2016; Thanh *et al*., 2016; Zhang *et al*., 2018).

Changes in pH, temperature and incubation period of the production medium influenced the amylase production by the amylolytic isolates (Figure 4). The observed optimum pH in the alkaline region (pH 7.0 – 9.0) agrees with the reports from earlier studies (Behal *et al*., 2006; Meenakshi *et al*., 2009; Ashwini *et al*., 2011; Sivakumar *et al*., 2012; Singh *et al*., 2015). The mesophilic temperatures of 37°C and 40°C favoured improved amylolytic activityby *Myroides odoratimimus* BY-F1 and *Enterobacter asburiae*YP-F1.

Meenakshi *et al.* (2009) and Jogezai *et al.* (2011) reported 37°C as the optimum temperature for α-amylase production by their respective *Bacillus* spp. while Ashwini *et al.* (2011) reported 40°C on the other hand. For *Bacillus cereus* CP-A4, the maximum amylase produced (411.67 U/ml) was obtained at 35°C, which agrees with the report by Sonia *et al.* (2013).Amylase production by *Myroides odoratimimus* BY-F1 and *Bacillus cereus* CP-A4 increased with the increasing incubation period up to 72 h, whereas amylase yield by *Enterobacter asburiae*YP-F1 decreased with the increasing incubation period. In previous studies, maximum amylase production was shown to occur between 36 and 48 h (Santos and Martins, 2003; Swain *et al*., 2006; Oyeleke *et al*., 2010; Jogezai *et al*., 2011; Deb *et al*., 2013). Decreased amylase production by *Enterobacter asburiae*YP-F1 might be due to the depletion of nutrients, death phase of the bacteria or accumulation of by-products such as proteases, toxins, inhibitors and proteolytic activities in the medium(Teodoro and Martins, 2000; Aiyer, 2005; Sivakumar *et al*., 2012).

FTIR spectra (Figure 5) revealed the presence of carboxyl, hydroxyl, and amino groups as the main functional groups in the amylase extracts, which confirms the presence of α- amylase. The presence of additional peaks implies interference due to other functional groups (Mohanasrinivasan *et al.,* 2014).The carboxyl groups may act as binding sites for the metal ions present in surface particles, hence forming chemical bonds, which allow improved amylase activity (Kumar *et al.,* 2004). For example, the OH, COOH, and COO- groups may link with the H^+ and OH^- present on the surface of the particles forming hydrogen bonds when the amylase chains approach the particles' surface. The result of this study also revealed that the amylase contains functional groups similar to those reported in other studies (Wang *et al.,* 2007).

CONCLUSION

The study showed that the amylolytic isolates, *Myroides odoratimimus* BY-F1, *Enterobacter asburiae* YP-F1 and *Bacillus cereus* CP-A4, obtained from SBAW samples, could produce a considerable yield of amylase at optimal culture conditions. Their ability to produce this important enzymeover a range of pH profile, temperature and incubation period will make them suitable for industrial scale production.

Acknowledgements

Tertiary Education Trust Fund (TETFUND), Nigeria funded this work (Institutional Grant). The authors are thankful to Osun State University Management for providing research facilities to carry out thestudy. The authors also acknowledge the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, for the sequencing of 16S rRNA genes of the amylolytic bacterial strains.

REFERENCES

Aiyer, P.V., (2005). Amylases and their applications. *African Journal of Biotechnology,* 4(13): 1525–1529.

Akinde, S.B. and Obire, O. (2008). Aerobic heterotrophic bacteria and petroleum-utilising bacteria from cow dung and poultry manure. *World Journal of Microbiology and Biotechnology*, *24*(9): 1999–2002.

Akpomie, O.O., Akponah, E. and Okorawhe, P. (2012). Amylase production potentials of bacterial isolates obtained from cassava root peels. *Agricultural Science Research Journal*, 2(2): 95–99.

Alcaraz, L.D., Moreno-Hagelsieb G., Eguiarte L.E., Souza V., Herrera-Estrella L., and Olmedo,G. (2010). Understanding the evolutionary relationships and major traits of *Bacillus* through comparative genomics. *BMC Genomics*, 11: 332.

Ashwini, K., Kumar, G., Karthik, L. and Rao, B.K. (2011). Optimisation, production and partial purification of extracellular α- amylase from *Bacillus* sp. *marini. Archives of Applied Science Research*, 3 (1): 33–42.

Behal, A., Singh, J., Sharma, M.K., Puri, P. and Batra, N. (2006). Studied thermostable amylase producing *Bacillus* spp. *International Journal of Agriculture and Rural Development*, 8: 80–83.

Burhan, A., Nisa, U., Gökhan, C., Ömer, C., Ashabil, A. and Osman, G. (2003). Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. *Process Biochemistry*, *38*(10): 1397–1403.

Dash, B.K., Rahman, M.M. and Sarker, P.K. (2015). Molecular identification of a newly isolated *Bacillus subtilis* BI19 and optimisation of production conditions for enhanced production of extracellular amylase. *BioMed Research International*, *2015*: 1–9.

Dave, B.R., Parmar, P., Sudhir, A., Panchal, K. and Subramanian, R.B. (2015). Optimisation of process parameters for cellulase production by *Bacillus licheniformis* MTCC 429 using RSM and molecular characterisation of cellulase gene. *Journal of Bioprocessing and Biotechniques*, *5*(3): 1–9.

Deb, P., Talukdar, S.A., Mohsina, K. Sarker, P.K. and Sayem, S.M. (2013). Production and partial characterisation of extracellular amylase enzyme from *Bacillus amyloliquefaciens*P-001. *Springer Plus Open Journal*, 2:154.

Gopinath, S.C., Anbu, P., Arshad, M.K., Lakshmipriya, T., Voon, C.H., Hashim, U. and Chinni, S.V. (2017). Biotechnological processes in microbial amylase production. *BioMed Research International*, 2017.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K. and Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process Biochemistry*, 38(11): 1599–1616.

Jogezai, N., Raza, A., Abbas, F., Bajwa, M., Mohammad, D., Kakar, W., Saeed, M. and Awan, A. (2011). Optimisation of cultural conditions for microbial alpha amylase production. *Journal of Microbiology and Antimicrobials*, 3(9): 221–227.

Kareem, S.O., Akpan, I. and Osho, M.B. (2002). *Calotropis procera* (Sodom apple)− a potential material for enzyme purification. *Bioscience Technology,* 87: 133–135.

Kareem, S.O., Akpan, I., Popoola, T.O. and Sanni, L.O. (2011). Activated Charcoal– A potential material in Glucoamylase Recovery. *Enzyme Research,* 483943: 4.

Khan, J.A. and Priya, R. (2011). "A study on partial purification and characterisation of extracellular amylases from *Bacillus subtilis". Advances in Applied Science Research*, 2 (3): 509–519.

Kumar, C.G., Joo, H.S., Kavali, R., Choi, J.W. and Chang, C.S. (2004). Characterisation of an extracellular biopolymer flocculant from a haloalkalophilic *Bacillus* isolate. *Journal of Microbiology and Biotechnology,* 20: 837–843.

Logan, N.A. and DeVos, P. (2009). Genus *Bacillus*. In De- Vos, P., Garrity, G. M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B. *Bergey's manual of Systematic Bacteriology*, Second Edition, Volume III (The Firmicutes). Springer Science + Business Media publishing, New York, USA. pp. 21–127.

Meenakshi, C., Narender, K.,Vikrant, A., Karupothula, S., Shobhana, B. and Sushma, S. (2009). Detection of alpha amylase producing bacteria (*Bacillus* sp. 5250) from soil: Isolation and optimization of various conditions of growth. *Research Journal of Biotechnology,* 4(1): 50–56.

Mishra, S. and Behera, N. (2008). "Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes". *African Journal of Biotechnology*, 7 (18): 3326–3331.

Mohanasrinivasan, V., Diksha, D., Suganthi, V, Selvarajan, E. and Devi, C.S. (2014). Process optimization for enhanced production of α-amylase by submerged (SmF) and solid-state fermentation (SSF) using *Bacillus pumilus* VITMDS2. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 5(2):1785

Ogbonnaya, N. and Odiase, A. (2012). "Influence of media composition on the production of alkaline α-amylase from *Bacillus subtilis* CB-18". *Acta Scientiarum Polonorum Technologia Alimentaria*, 11(3): 231–238.

Oyeleke, S.B., Auta, S.H. and Egwim, E.C. (2010). Production and characterisation of amylase produced by *Bacillus megaterium* isolated from a local yam peel dumpsite in Minna, Niger State. *Journal of Microbiology and Antimicrobials,* 2(7): 88–92.

Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D and Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry*, 31: 135–152.

Rai, A.K., Sanjukta, S., Chourasia, R., Bhat, I., Bhardwaj, P.K. and Sahoo, D. (2017). Production of bioactive hydrolysate using protease, β-glucosidase and α-amylase of *Bacillus* spp. isolated from kinema. *Bioresource Technology*, *235*: 358–365.

Roslan, R., Rehan, M.M., Kamarudin, K.R., Noor, H.M., Huda-Faujan, N. and Radzi, S.M. (2018). Isolation and identification of amylolytic bacteria from Ragi. *Malaysia Applied Biology*, 47(2): 83–88.

Sankaralingam, S., Shankar, T., Ramasubburayan, R., Prakash, S. and Kumar, C. (2012). Optimisation of culture conditions for the production of amylase from *Bacillus licheniformis* on submerged fermentation. *American–Eurasian Journal of Agricultural and Environmental Science*, *12*: 1507–1513.

Santos, E.O. and Martins, M.L. (2003). Effect of medium composition on formation of amylase by *Bacillus* sp. *Brazilian Archives of Biology and Technology*, 46(1): 129–139.

Sewalt, V.J., Reyes, T.F. and Bui, Q. (2018). Safety evaluation of two α-amylase enzyme preparations derived from *Bacillus licheniformis* expressing an α-amylase gene from *Cytophaga* species. *Regulatory Toxicology and Pharmacology*, *98*: 140–150.

Singh, P., Gupta, P., Singh, R. and Sharma, R. (2012). "Factors affecting α- amylase production on submerged fermentation by *Bacillus* sp". *International Journal of Pharmacy and Life Sciences*, 3(12): 2243–2246.

Singh, V., Sharma, R. and Sharma, P. (2015).Isolation, screening and optimisation of amylase producing *Bacillus* sp. from soil.*Asian Pacific Journal of Health Sciences,* 2(3): 86–93.

Sivakumar, T., Shankar, T., Vijayabaskar, P., Muthukumar, J. and Nagendrakannan, E. (2012). Amylase production using *Bacillus cereus*isolated from a vermicompost site. *International Journal of Microbiological Research,*3(2): 117–123.

Swain, M.R., Kar, S., Padamaja, G. and Ray, R.G. (2006). Partial characterisation and optimisation of production of extracellular α-amylase from *Bacillus subtilis* isolated from culturable cow dung microflora. *Polish Journal of Microbiology*, 55(4): 289–296.

Sonia, S., Shyam, S. A., Sakhsam, G. and Gupta, B.L. (2013). Amylase activity of a starch degrading bacteria isolated from soil. *Archives of Applied Science Research*, 5(1): 15–24.

Souza, P.M. (2010). Application of microbial α-amylase in industry – Areview. *Brazilian Journal of Microbiology*, *41*(4): 850–861.

Sundarram, A. and Murthy, T.P.K. (2014). α-Amylase production and applications: A review. *Journal of Applied and Environmental Microbiology*, *2*(4): 166–175.

Teodoro, C.E. and Martins, M.L. (2000). Culture conditions for the production of thermostable amylase by *Bacillus* sp. *Brazilian Journal of Microbiology*, 32: 298–302.

Vidyalakshmi, R., Paranthaman, R. and Indhumathi, J. (2009). Amylase production on submerged fermentation by *Bacillus* spp. *World Journal of Chemistry*, *4*(1): 89–91.

Wang, S., Gong, W., Liu, X., Tian, L., Yue, Q. and Gao, B. (2007). Production of a novel bioflocculant by culture of *Klebsiella mobilis* using dairy wastewater. *Biochemistry England Journal,* 36: 81–86.