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ISOLATION AND CHARACTERIZATION OF ALPHA-AMYLASE PRODUCING BACTERIAL ISOLATES FROM DETERIORATED ONION (*Allium cepa*) BULBS

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ABSTRACT
Postharvest losses due to microbial spoilage of vegetables continue to increase in developing countries. Onion (Alium cepa) bulbs and several other vegetables are highly prone to spoilage because they are composed of living tissues hence, the need for alternative uses for these vegetable wastes. This study was designed to isolate, characterize bacterial species from deteriorated onions and screen them for alpha-amylase production. Five onion bulbs were moistened with sterile distilled water and allowed to deteriorate for three weeks. Bacterial populations isolated from the deteriorated onion bulbs were cultured on Nutrient agar plates for 24hr and 48hr. The plates were sub-cultured to obtain pure cultures. Bacterial populations isolated from the deteriorated onion bulbs were screened for alpha-amylase production on nutrient agar supplemented with 1% (w/v) starch. Alpha-amylase production was determined at 37^{o}C for 48hr. All isolates which were positive for alpha amylase production were identified by biochemical characteristics. Isolates showing the highest zones of hydrolysis from each onion bulb were further characterized by molecular techniques. A total of twenty-seven bacterial isolates were obtained. Biochemical characterization revealed members of the Genera Pseudomonas, Bacillus and Staphylococcus. Isolate codes A3, B3, C4, D3 and E4 from Onion A, B, C, D, E showed the highest zones of hydrolysis at 23, 4, 9, 18 and 23 mm respectively. Molecular characterization revealed that all the five isolates were Pseudomonas spp. This result established that deteriorated onion bulbs plays host to a number of alpha-amylase producing bacteria which may be exploited for industrial production of alpha-amylase.

Keywords: *Alpha-amylase, Onion, deterioration, Bacterial isolates, Starch*

INTRODUCTION

Allium cepa (L.), commonly known as onion, is a perennial crop grown for consumption as food and also for medicinal purposes (Kumar *et al.*, 2010). Onion is rated as the second most valuable vegetable crop with a coverage area of 3.44million hectares used for its cultivation and annual production of 61.6 million tonnes (Shaibu *et al.*, 2015). Like other vegetables, onions are highly prone to spoilage because they are composed of living tissues (Rawat, 2015). Spoilage can occur

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at any stage between farm to fork; which may be as a result of insect damage, physical injury, enzyme activity of plant or microbial activity (Rawat, 2015). Farmers have reported losses of approximately 40% of total yield during onion-growing season when weather conditions are most favorable for bacterial growth (Zaid *et al.,* 2012). Numerous bacterial diseases of onions cause huge challenges for farmers globally because they cause spoilage problems and loss of bulbs at harvest and post-harvest (Schwartz and Mohan, 2008; Zaid *et al.,* 2012). Besides the economic implication of losses due to microbial deterioration of onions, Orpin *et al.* (2017) reported that onion deteriorated by microbes could cause severe damages to health if consumed. There is therefore a need to create valuable products from this waste thus maximizing its use as a cheap source of valuable products. Amylases (EC 3.2.1.1) are extracellular enzymes which catalyzes the hydrolysis of starch (Obafemi *et al*, 2018). They constitute a major group of industrial enzymes being the second largest group in the market after proteases (Vadiya *et al.,* 2015). Although derived from other sources, microbial amylases have been reported to provide better advantages over other sources because they are economical and easy to manipulate (Anbu *et al.,* 2013). Bacteria also generate optimum amount of enzyme within a very short period of time than fungi. They are also preferred for their extracellular production attribute thus making recovery and purification process easier, cheaper and faster (Abbas, 2009; Samanta *et al.,* 2013). Bacteria such as *Bacillus subtilis, B. cereus, B. polmyxa, B. amyloliquefaciens, B. coagulans, Lactobacillus, Escherichia, proteus, B. licheniformis, B. steriothermophilus B. megaterium, Strepotmyces sp., Pseudomonas sp.* etc. have been reported to produce amylase (Padhiar and Kommu, 2016). Spoilage of raw onions may be as a result of increased enzymatic action or entry and proliferation of microorganisms, thus serving as a cheap substrate from which amylase-producing microorganisms may be isolated (Onuorah and Obika, 2015). Thus microorganisms that cause spoilage in onions can be isolated and their potentials for use in industrial production of amylase exploited hence this study.

RESEARCH METHODOLOGY

Sample Collection

Five fresh onion bulbs were purchased from an open market in Ota, Ogun (7°00'0.00" N 3°34'59.99" E) State, Nigeria. The onion bulbs were transported in sterile plastic bags to the microbiology laboratory of Covenant University, Ota, Ogun State.

Sample Preparation

Sterile distilled water was intermittently sprinkled daily on the onion samples to encourage microbial growth. The samples were kept at room temperature and allowed to deteriorate for three weeks. After deterioration, the samples were crushed. One (1) gram of each onion bulb was weighed and serial dilutions carried out using sterile distilled water.

Isolation of Bacterial Population

One (1) milliliter of the dilution factors (10^{-4} – 10^{-6}) was dispensed aseptically using a syringe and needle into a sterile petri dish and sterile warm nutrient agar was aseptically added to the

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isolate. This was repeated for each dilution factor of the five (5) different onion bulb samples. The plates were allowed to solidify then inverted and incubated at 37ºC for 24 h. The colonies obtained were sub-cultured to obtain pure cultures.

Screening for Alpha-Amylase Production
Bacterial populations isolated from the deteriorated onion bulbs were inoculated into nutrient agar supplemented with 1% (w/v) starch by streaking. The plates were incubated at 37ºC for 48 h. After 48 h, the plates were flooded with iodine solution and a characteristic blue-black colour was observed. Iodine solution was prepared by weighing 3 g of potassium iodide (KI) into a dark bottle and dissolved with 100 ml of distilled water. This was allowed to warm and 0.3 g of iodine was added and allowed to dissolve by mixing and warming. Isolates which produced zones of hydrolysis after the iodine solution was added were selected as alpha-amylase producing bacteria and the zones of hydrolysis measured.

Identification of Isolates

Morphological Characterization
All isolates which tested positive for alpha-amylase production were inoculated into freshly prepared nutrient agar plates by streaking and incubated at 37ºC for 24 h. The appearance of the isolates on nutrient agar plates was observed. The gram reaction of isolates was determined.

Biochemical Characterization
The Bacterial isolates which tested positive for alpha-amylase production were subjected to various biochemical test; motility test, hydrogen sulphide production, utilization of urease, catalase test, oxidase test, utilization of citrate and indole production. The results obtained were compared with the Bergey's manual of systematic bacteriology in order to identify the organisms.

Molecular Characterization
One isolate with the highest zone of hydrolysis from each onion bulb were selected for molecular characterization.

DNA Isolation
DNA Extraction was carried out on the samples using the Jena Bioscience Bacteria DNA Preparation Kit according to manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a Nanodrop (ND 1000) Spectrophotometer (Thermo Scientific, USA).

PCR Amplification of the Pseudomonas 16s gene
The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP) (Solis

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Biodyne), 25pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), proofreading enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) for an initial denaturation of 95°C for 5 minutes followed by 30 amplification cycles of 30 seconds at 95°C; 1 minute at 55°C and 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker.

Isolation of Plasmid DNA

Plasmid DNA was extracted using the TENS prep kit protocol. The plasmid was separated on a 0.8% agarose gel and electrophoresis was carried out at 100V for 1 h. After electrophoresis, DNA bands were visualized by ethidium bromide staining. Hind III Lamda (Jena Bioscience) was used as DNA molecular weight marker.

RESULTS

A total of twenty-seven different isolates were obtained from the deteriorated onion (Table 1). Ten isolates were positive for alpha amylase production when screened on nutrient agar supplemented with starch (Table 2). Gram staining revealed three gram positive bacteria and seven gram negative bacteria (Table 3). Morphological and Biochemical characterization suggested members of the genera *Bacillus*, *Pseudomonas* and *Staphylococcus* (Table 4; Table 5; Table 6). Five isolates producing the highest zones of hydrolysis were confirmed as *Pseudomonas sp* following molecular characterization (FIG 1). None of the five isolates were observed to carry any plasmid (Fig 2).

Table 1: Frequency of distribution of bacterial isolates from deteriorated onion-bulb samples

DETERIORATED ONION-BULB	NO OF ISOLATES	ISOLATE CODES
Onion-bulb 1 (A)	4	A1, A2, A3, A4.
Onion-bulb 2 (B)	3	B1, B2, B3.
Onion-bulb 3 (C)	4	C1, C2, C3, C4.
Onion-bulb 4 (D)	7	D1, D2, D3, D4, D5, D6, D7.
Onion-bulb 5 (E)	9	E1, E2, E3, E4, E5, E6, E7, E8, E9.

Table 2: Diameter of zones of hydrolysis of alpha-amylase producing bacteria

S/N	ISOLATE CODES	ZONES OF HYDROLYSIS (mm)
1	A1	-
2	A2	-
3	A3	23
4	A4	-
5	B1	-
6	B2	-
7	B3	4
8	C1	-

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9	C2	-
10	C3	5
11	C4	9
12	D1	-
13	D2	-
14	D3	18
15	D4	-
16	D5	18
17	D6	-
18	D7	-
19	E1	-
20	E2	-
21	E3	-
22	E4	23
23	E5	15
24	E6	20
25	E7	17
26	E8	13
27	E9	-

Table 3: Gram Reaction of Bacterial Isolates

S/N	ISOLATE CODE	GRAM REACTION
1	A3	Negative
2	B3	Negative
3	C3	Negative
4	C4	Positive
5	D3	Negative
6	E4	Negative
7	E5	Positive
8	E6	Positive
9	E7	Negative
10	E8	Negative

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Table 4: Morphology of Bacterial Isolates

S/N	ISOLATE CODE	MORPHOLOGICAL PROPERTIES
1	A3	Light green, convex, smooth and opaque colonies
2	B3	Large, light green , flat and translucent colonies
3	C3	Small, cream, convex, shiny smooth, distinct colonies with entire margins.
4	C4	Golden yellow, convex, smooth and opaque
5	D3	Bluish green, mucoid, translucent, shiny colonies with undulating margins rods
6	E4	Green, convex, smooth and opaque colonies
7	E5	Golden yellow, convex, smooth and opaque colonies
8	E6	Cream, convex, smooth and opaque colonies
9	E7	Large, cream, low convex, rough and opaque colonies
10	E8	Large, cream, low convex, rough and opaque colonies

Table 5: Biochemical Test Result for Bacterial Isolates

S/N	CODE	MOTILITY	CATALASE	OXIDASE	CITRATE	UREASE	H_2S	INDOLE
1	A3	+	+	-	+	-	-	-
2	B3	+	+	+	+	+	-	-
3	C3	+	+	-	+	+	-	-
4	C4	-	+	-	+	+	+	-
5	D3	+	+	+	+	+	-	-
6	E4	-	+	+	+	+	-	-
7	E5	-	+	-	+	+	-	-
8	E6	+	+	-	-	-	-	+
9	E7	+	+	-	-	-	-	-
10	E8	+	+	-	-	-	-	+

Table 6: Presumptive Identification of Bacterial Isolates

S/N	ISOLATE CODES	PRESUMPTIVE IDENTIFICATION
1	A3	*Pseudomonas spp.*
2	B3	*Pseudomonas spp.*
3	C3	*Pseudomonas spp.*
4	C4	*Staphylococcus spp.*
5	D3	*Pseudomonas spp.*
6	E4	*Pseudomonas spp*
7	E5	*Escherichia coli*
8	E6	*Bacillus spp*
9	E7	*Staphylococcus spp*
10	E8	*Escherichia coli*

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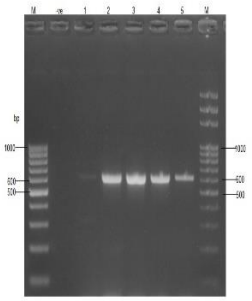

Fig. 1: DNA bands of bacterial isolates code 1 to 5 from deteriorated onion bulb on gel

M = 100bp Ladder
-ve = control
1- Isolate code A3
2- Isolate code B3
3- Isolate code C4
4 -Isolate code D3
5 -Isolate code E4

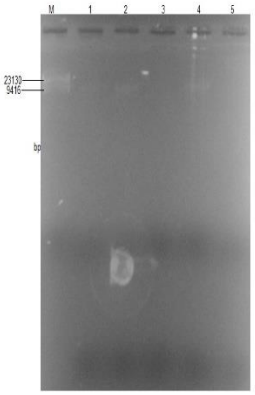

Fig. 2: Gel Image for Plasmid DNA Isolation
M- Hind III Lamda molecular weight marker
1- Isolate code A3
2- Isolate code B3
3- Isolate code C4
4 -Isolate code D3
5 -Isolate code E4

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DISCUSSION

This study established the deterioration of onion by species of the genera Bacillus, Pseudomonas, Staphylococcus. *Bacillus spp, Pseudomonas spp, Staphylococcus spp and Esherichia coli* have been previously isolated from deteriorated onion (Orpin *et al.*, 2017). A previous research by Tomlinson (1987) reported *Pseudomonas spp* as the primary microbe involved in onion deterioration. Following screening on nutrient agar supplemented with 1% (w/v) starch, only ten of the twenty-seven isolates were positive for alpha-amylase production. Certain species are capable of hydrolyzing starch better than others and some are not even capable of producing alpha-amylase for hydrolysis of starch. This result corroborated a previous research which noted that many microorganisms are able to produce amylases including *Bacillus spp., Lactobacillus spp, Escherichia, Proteus spp, Streptomyces spp, Pseudomonas spp* (Padhiar and Kommu, 2016; Vaidya and Rathore, 2015). From this research, all five (5) highest producers from each of the bulbs showed zones of hydrolysis at 23mm, 4mm, 9mm, 18mm, 28mm for onion A, B, C, D, E respectively. The highest zone of hydrolysis inhibition seen in all onions 28mm was by *Pseudomonas spp.* However, Olanbiwoninu and Fasiku (2015) reported highest frequency of alpha-amylase production by *Bacillus spp* (seven) followed by *Pseudomonas spp* (three) isolated from decaying sweet potato peels.

CONCLUSION

This investigation reveals that deteriorated onion bulbs harbors a number of alpha-amylase producing bacteria species which may be exploited for industrial production of alpha-amylase. Thus, onion waste may be utilized as substrate for alpha-amylase producing bacteria. The result also established the potentials of Pseudomonas for industrial production of alpha-amylase.

In all cases the absence of plasmids reveals that the alpha-amylase genes of the isolates were encoded within the bacteria chromosomes. Tao *et al.* (2008) had previously amplified chromosomal DNA harboring the amylase gene (amyA).

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