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Determination of Proteolytic Activities of *Bacillus* species Isolated From Traditional Fermented Oil Bean Seed (UGBA: *Pentaclethra macrophylla,* Benth)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VO, CNO and JNO designed the study, author VO performed the statistical analysis, authors VO and CNO wrote the protocol and the first draft of the manuscript and managed literature searches. Author CNO managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Eighty samples of traditional fermented "ugba" (*Pentaclethra macrophylla*, Benth) wraps collected from eight markets in Umuahia were analyzed for proteolytic activities (by zone of inhibition in mm) of *Bacillus* species using two protein-based substrates: Skim Milk Agar and Nutrient Agar supplemented with 1% gelatin. 51 isolates were identified as *B. subtilis*, 18 as *B. licheniformis* and 11 as *B. pumilus* with Ariam Market having the highest mean count (mm) of 1.93±0.16. *B. subtilis* had the highest proteolytic activity (28.00±0.55) on Skim Milk Agar (P=.05) but together with *B. licheniformis* had the same level of proteolytic activity (26.00±0.00 and 26.00±0.55 respectively at P=.05) which is higher than *B. pumilis* (13.00±0.55) on Nutrient agar supplemented with 1% gelatin.

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However, *B. licheniformis* and *B. pumulis* had better proteolytic activities (26.00±0.55 and 13.00±0.55: P=.05) respectively on Nutrient agar supplemented with 1% gelatin than on Skim Milk Agar. The two potential protease producers that showed higher proteolytic activity were incubated at high temperature and result showed that *B. subtilis* proteolytic activity was optimum at (29.00±0.71) at 50°C while that of *B. licheniformis* was optimum (28.00±0.00) at 40°C (P=.05). Antimicrobial susceptibility of the *Bacillus* species showed that the three *Bacillus* species were most sensitive to Chloramphenicol (21.00±0.51) without any statistical difference followed by Gentamycin (20.00±0.31) but completely resistant to Rifampicin (0.00±0.0; P=.05). *B. subtilis* showed better proteolytic activity at a higher temperature and will be better suited for production processes in industries where thermophilic temperatures are needed.

Keywords: Antibiotics; Bacillus spp; fermentation; proteolytic activity; "Ugba".

1. INTRODUCTION

"*Ugba*", a fermented product of African oil bean seed (*Pentaclethra macrophylla,* Benth) is one of the common, fermented legumes, predominantly consumed by the Ibos and other smaller ethnic groups of the South Eastern Nigeria [1]. Oil bean seed belongs to the leguminous family mimosa cease found mostly in tropical Africa, it is cultivated in forest areas, with about eight flat glossy brown edible seeds per pod. The pods explode at maturity and disperse the seeds [2].

Fermentation detoxifies the African oil bean seed with subsequent increase in nutrient availability and digestibility [3]. Ugba is widely consumed as a snack, side dish or food condiment [4]. Ugba production is locally done through a mixed wild bacteria fermentation of the sliced, boiled and soaked African oil bean seeds are bitter and possess anti-nutritional factors amongst which pancine, cyanide, oxalates, saponin, phytic acid, phytate and tannins [5,6]. Microbial population of Ugba is introduced through the air, water, utensil, banana leaves or the handler; no starter culture is used for the traditional method [3]. Microorganisms excrete a wide variety of proteolytic enzymes, which are also found in systems. They are molecules of small size, compact, spherical structures that catalyzes the peptide bond cleavage in protein [7]. Commercially they are very important and isolated from various living sources such as plants, animals, bacteria and fungi [8].

Proteases from microbial sources are preferred over the enzymes from plant or animal sources since they possess all most, all the characteristics desired for their biotechnological application [9]. Among bacteria, *Bacillus* species are specific producers of extra-cellular proteases. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries [10].

The main fermenting microorganisms in the fermentation of African oil bean seeds have been identified to be proteolytic *Bacillus* species, which include *B. subtilis* (most predominantly), *B. licheniformis, B. megaterium, B. macerans, B. circulans* and *B. pumilus* [11,2]. During fermentation, these microorganisms use the nutritional components of the seeds, converting them into products that contribute to the chemical composition and taste of the condiment/ugba [12]. Although, yeasts and other bacteria are also seen, only part of them can be considered to play a substantial role in fermentation processes. For instance, non-fermenting species may just be ubiquitous contaminants, although they may affect the flavour of the final product when occurring in high numbers. The *Bacillus* species is of particular interest due to its prevalence in these fermented foods [13]. In addition, use of *B. subtilis* in the food industry is recommended by the US FDA as one of the GRAS (Generally Recognized as Safe) organisms [14]. This research work initiated in 2014 was aimed at:

i. Isolation and identification of proteolytic *Bacillus* species from traditional fermented oil bean seed (ugba);
ii. Comparing the proteolytic activities of *Bacillus* species on two different media and
iii. Determining the effect of temperature on the proteolytic activities of the isolates and their antibiotic sensitivity profile.

1.1 Sample Collection

The fermented oil bean condiments (*ugba*), were purchased from eight markets in Abia State namely: Ahiaeke, Ahiankwo, Ahia ohuru, Aforule, Ariam, Ndioru, Ubani and Umungasi markets

respectively. The 80 samples collected (10 from each market) were labeled A_1-A_{10}; B_1-B_{10}; C_1-C_{10}; D_1-D_{10}; E_1-E_{10}; F_1-F_{10}, G_1-G_{10} and H_1-H_{10}, respectively and immediately taken to the microbiological laboratory for analyses.

1.2 Sample Preparation and Inoculation

The samples were aseptically transferred to a sterile porcelain mortar, mashed to paste and 1g was serially diluted using sterile peptone water. 1ml aliquot of appropriate dilution was then inoculated on nutrient agar in triplicates using pour plate method. The plates were incubated at 30°C for 24 hours [1]. Thereafter, the mean colony forming unit per gram (cfu/g) was calculated. Representative colonies were isolated, purified by repeated streaking. The cultures were identified using biochemical methods according to Bergey's Manual of Determinative Bacteriology. Bacterial colonies were isolated and characterized by their morphological and physiological properties [15].

1.3 Growth in 5% and 7% NaCl

Five percent NaCl was prepared by adding 2.8 g of nutrient agar and additional 5g of NaCl to 100 ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes. 7% NaCl was also prepared by adding additional 7 g of NaCl and 2.8 g of nutrient agar to 100 ml of distilled water, sterilized by autoclaving at 121°C for 15 minutes, and allowed to cool. The two media were poured on different Petri dish and labeled. The bacterial isolates were spread onto surface of the two different media by repeated streaking and incubated at 37° for 24 hours [16,17]. Growth on the plates was determined by the presence of colonies on the plates.

1.4 Growth at 50°C

The bacterial isolates were sub-cultured onto Nutrient agar and incubated at 50°C for 24 hours [1].

2. DETERMINATION OF PROTEOLYTIC ACTIVITIES

2.1 Growth on Skim Milk Agar (SMA)

This was used for the demonstration of coagulation and proteolysis of casein. Proteolytic bacteria hydrolyze casein to form soluble nitrogenous compounds indicated as clear zone surrounding the colonies. The medium was prepared as instructed by the manufacturer (Himedia) and sterilized by autoclaving at 121°C for 15 minutes. Medium composition include: 2.8% skim milk powder, 0.5% casein enzymic hydrolysate, 0.28% yeast extract, 0.1% dextrose, 1.5% agar. A loopful of cultures of each of the identified species: *B. subtilis, B. pumilus* and *B. licheniformis*, was placed on the centre of the protein based substrates (SMA). The zone of clearance observed on each plate was recorded and the mean width of clear zone diameter was also determined.

2.2 Growth on Nutrient Agar Supplemented with 1% Gelatin

Gelatin was used to investigate the presence of proteolytic microorganisms as evidenced by the liquefaction of gelatin. The nutrient agar was supplemented with 1% (1 g) gelatin and sterilized in autoclave at 121°C for 15 minutes. The Nutrient gelatin composition include: 12% gelatin, 0.3% beef extract and 0.5% gelatin peptone [17]. A loopful of the culture growth was placed on the centre of Nutrient agar supplemented with 1% gelatin (Na+G). The plates were then incubated at 37°C for 24 hours. The bacterial growth and enzymatic activity were monitored on the media (agar plates) from the first 12, 16 and 24 hours of incubation. Proteolytic activities of the bacterial isolates were detected by observing the presence of a clear zone around the growing colony [13] in replicates of five: R_1, R_2, R_3, R_4, R_5, on each medium.

2.3 Effect of Temperature on Proteolytic Activity

This was determined by growing the isolates that showed the highest enzyme activity at different temperatures (20, 30, 40, 50 and 60°C) at a constant pH 8.0 in skim milk agar. The protease activity was recorded according to the diameter of the clear zone on the media (agar plates) after overnight incubation [18].

2.4 Preparation of Turbidity Standard Equivalent to McFarland 0.5

One percent (1%) v/v solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of water. 1% of barium chloride was also prepared by dissolving 0.5 g of dehydrated Barium chloride in 50ml of distilled water. 0.6ml of Barium chloride solution was added to 99.4 ml of the sulphuric acid solution

and mixed properly. The solution was preserved in the fridge.

2.5 Determination of Antimicrobial Activity

Antimicrobial activity was carried out on the isolates on Mueller Hinton Agar. The overnight bacterial cultures were spread onto surfaces of the set Petri dishes using sterile swab sticks. Then, antimicrobial impregnated discs were placed on the surface of the agar using a sterile forcep and incubated at 37°C for 24 hours [19]. The presence of zones of inhibition of growth were observed and recorded by measuring the diameter (mm) and the susceptibility was interpreted using Interpretative Range Table.

3. RESULTS AND DISCUSSION

In this study, eighty samples of traditional fermented oil bean seed (ugba: *Pentaclethra macrophylla*) collected from eight different market places in Abia State were analysed for the presence of *Bacillus* species with proteolytic activities on Skim Milk Agar (SMA) and Nutrient Agar supplemented with 1% gelatin. Eighty *Bacillus* species were isolated from the samples out of which 51(63.8%) were identified as *B. subtilis,* 11(13.8%) were identified as *B. pumilus* while 18(22.5%) isolates were identified as *B. licheniformis* (Table 1). The three *B*. species were isolated from the 80 samples analysed and they represent a group of phenotypically related species known as the *B. subtilis* spectrum. When subjected to a battery of tests, strains representing these species share many common properties and show relatively few characteristics by which they can be separated [20]. Based on cell morphology, all the eighty isolates were rods and endospore formers. [12] reported that the endospore forming bacteria were present throughout the 72 hours of fermentation period of "ugba", whereas the non-spore forming bacilli were found only at the beginning of the fermentation.

The colony forming units per gram result showed that the highest values was obtained from the Ariam Market (coded "E"; $1.93x10^5$) while the least value ($1.70x10^5$) was from Ahiaeke Market (coded "A"; Table 2). Variation in the mean count values recorded against the eight markets could be due to differences in the varieties of the "ugba" seeds used in the fermentation. In earlier works, [21] found that other bacteria progressively decreased in total count and the disappearance of these bacteria could be due to the production of the antibiotic (Bacitracin) by *B. subtilis*, which may have inhibited the growth of these bacteria. It was noted that the disappearance of *Micrococcus* species especially at 96 hours of fermentation may be due to the presence of this antibiotic. [21] noted that although, there were other bacteria identified in the fermenting slices of "ugba" such as coagulase negative *Staphylococcus* species, *Micrococcus* species, *Leuconostoc mesenteriodes, Lactobacillus plantarum, Streptococcus lactis, Proteus* species, *Enterobacter* species and *E. coli*, their counts decreased after 72 hours of fermentation while the main fermenting microorganisms have been identified to be proteolytic *Bacillus* species. [22] demonstrated that proteolytic microorganisms have been detected in appreciable numbers from 10^4 to 10^6 cfu/g and the highest mean count result from this work fell within this range of values. However, previous reports suggested that the predominance of the *Bacillus* species may be due to the fact that the other bacteria grow on the little carbohydrate present in the seeds, majority of which have been lost due to leaching during preparatory stages. Additionally, pre-fermentation treatment (boiling) must have assisted in initiating the hydrolysis of the complex proteins found in the seeds.

Two different protein-based media were used to evaluate the proteolytic activity (PA) of the three species and *B. subtilis* had the best PA on SMA (*P=.05*) among the three. Although *B. subtilis* and *B. licheniformis* had the same level of PA (*P=.05*) on Nutrient agar supplemented with 1% gelatin (NA+G), this value is higher (*P=.05*) than that of *B. pumilis* (12mm) on NA+G. The proteinase enzyme is considered the most important enzyme in ugba fermentation. This result suggested that the ability of the three species of *Bacillus* to produce enzyme (protease) at varying ranges (according to the zone of clearance on the media) depends on the genetic constituents of each species. By this result, *B. subtilis* has better proteases that could hydrolyse the complex proteins found in the seeds of oil bean plant. Serine and metallo-proteases are the principal classes of proteases found in several species of *Bacillus*. We noticed (Fig. 1) that SMA had the same level of effect on the proteolytic activities of *B. subtilis* (*P=.05*), but has less effect on the proteolytic activities of *B. licheniformis* and *B. pumilis* (*P=.05*). It is possible that gelatin, as a high molecular weight protein, induces an

increase in the protease production to degrade the substrate to an available form for the microorganism. [23] noted that the use of gelatin in the culture media provided a qualitative assay, simple, inexpensive, straight forward method to assess the presence of proteolytic activity of a given colony. [17] evaluated the proteolytic activities of *Bacillus* using five different protein-based media to evaluate whether this might affect the proteolytic activity of *Bacillus* species. These media include: skim milk agar; skim milk agar supplemented with 0.02% sodium azide; soya protein agar; nutrient agar supplemented with soya protein; nutrient agar supplemented with 1% gelatin. They suggested that skim milk agar and nutrient agar supplemented with soya protein or gelatin were good for the detection of protease activity, but also suggested that sodium azide can prevent microbial growth, thus reducing protease production.

During the *Bacillus* growth, cell morphology was examined; all bacterial cells appeared in vegetative forms during the first 12 hours, but spore formation was observed between 16 and 24 hours of the incubation period. According to [13] this suggested that protease production reached the highest level during the exponential phase and continued constant level when spores were formed (stationary phase).

Evaluation of the effect of temperature on the proteolytic activities of the isolates was carried out on the two isolates that performed best *ab initio* on SMA and NA + G respectively (*B. subtilis* and *B. licheniformis*) and result showed that the proteolytic activity of *B. subtilis* peaked at 50°C (28 mm) while that of B. *licheniformis* was maximum (28.00 mm) at 40°C (Fig. 2). Both species had the same proteolytic activities (*P=.05*) at the two different optimum temperatures. This result implies that *B. subtilis* will perform better in industries that operate at temperatures above 40°C. Similar result was obtained by [18] with the maximum activity at 40°C for *B. licheniformis* and 50°C for *B. subtilis* with a reduction in activity for both species at 60°C, respectively. [18] commented that temperature is one of the critical parameter which has to be controlled and maintained in an optimum condition for maximal enzyme production. [24,25] showed that 50°C and 35±2°C was optimum for *B. subtilis* and *B. licheniformis* respectively. It was clear that the *Bacillus* species, usually being mesophilic can survive high temperatures by forming spores. This result suggested that one feature of an extreme survival strategy employed by these species is the formation of endospores. This allowed the bacterium to produce a dormant and highly resistant cell to preserve the cell's genetic material in times of extreme stress. Therefore, alkaliphilic *Bacillus* species have important industrial application due to their ability to produce alkaline enzymes such as protease. These species produce extracellular enzymes that are resistant to high temperature conditions. Many obligately or facultatively alkaliphilic *Bacillus* strains have been isolated for use in industries and biotechnology.

The antibiotic susceptibility test (Table 3) showed that *B. pumilis* had the same level of sensitivity (the highest) to Gentamycin and Chloramphenicol (*P=.05*) respectively followed by Amoxil (19 mm) and is statistically different (*P=.05*). However, *B. pumilis* was completely resistant to Norfloxacin and Rifampicin (*P=.05*). *B. licheniformis* had the same level of sensitivity (the highest) to Gentamycin, Chloramphenicol and Ampiclox (20.00 mm, 20.00 mm and 20.00mm respectively; *P=.05*) but was completely resistant to Rifampicin. *B. subtilis was* also completely resistant to Rifampicin but most sensitive to Chloramphenicol (*P=.05*). It's noteworthy that the three *Bacillus* species were completely resistant to Rifampicin but most sensitive to Chloramphenicol followed by Gentamycin (*P=.05*). The high level of resistance exhibited to Rifampicin by the three species of *Bacillus* could be genetically mediated as there was no inhibition from the antibiotic-an indication that the three species are closely related in genetic make-up. The high performance of Chloramphenicol and Gentamycin to the three isolates is highly appreciated especially at a time like this when many bacteria are developing resistance to many antibiotics. Although the three Bacillus species are "GRAS", (generally recognized as safe) in food industry and agriculture; they can cause disease in the debilitated or immuno-compromised individuals. Some recent studies revealed that these species of *Bacillus* spp can cause infectious disease to man and animals ranging from skin infection to life threatening bacteremia in the immuno-compromised individual [26].

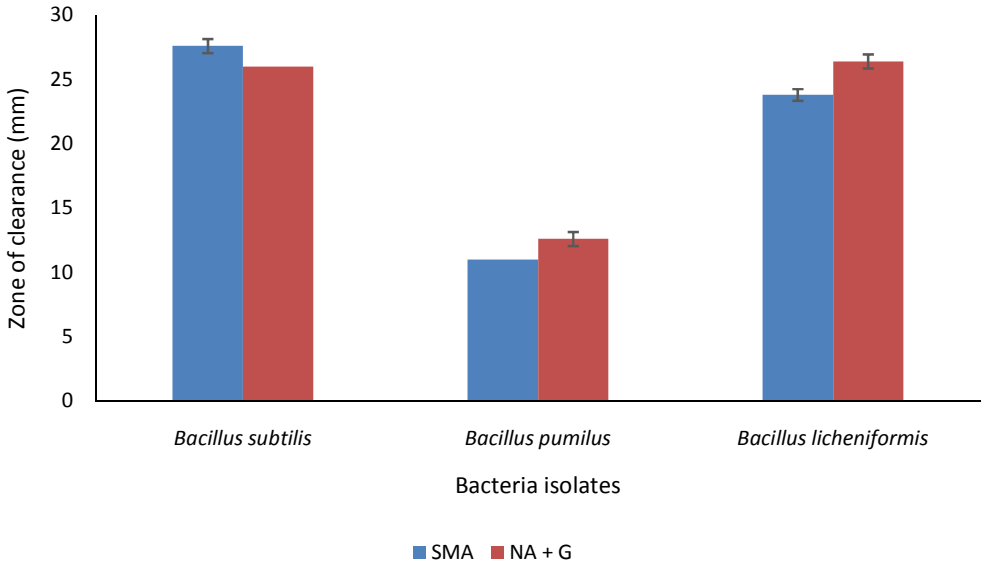

Fig. 1. Proteolytic activities of three *Bacillus* species on two media (mm)

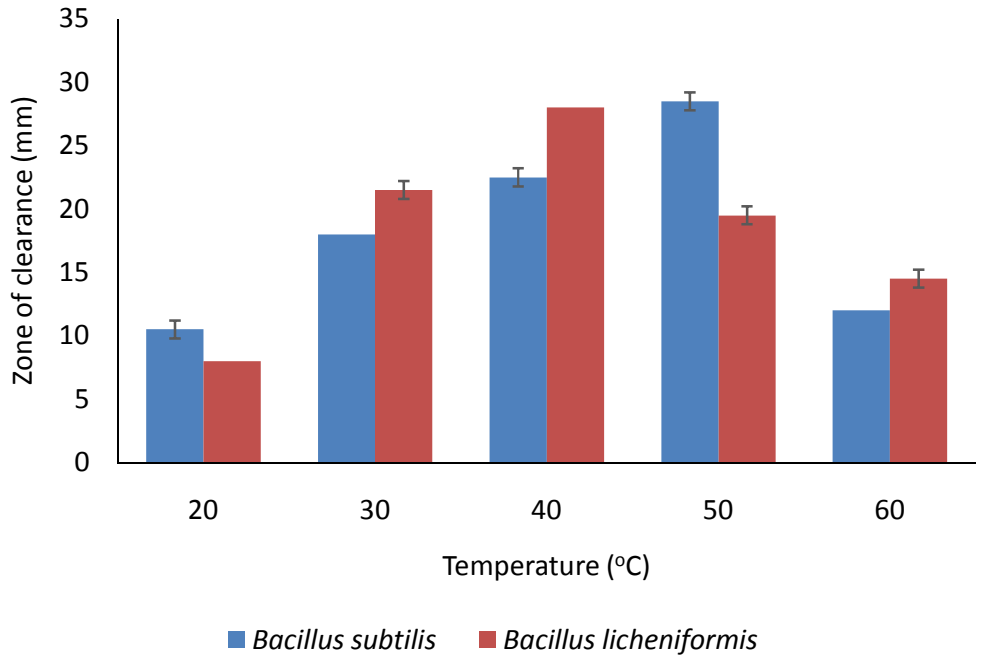

Fig. 2. Effects of temperature on the proteolytic activities of two *Bacillus* species (mm)

Table 1. Colonial and biochemical identification of the *Bacillus* species

Colonial morphology	Gram reaction	Catalase	Coagulase	Motility	Spore stain	Sucrose	Fructose	Maltose	Methyl red	Voges Proskauer	Starch hydrolysis	Nitrate reduction	Growth at 50°C	Growth in 5% NaCl	Growth in 7% NaCl	Isolate
Whitish, flat entire	+ rods in clusters	+	+	+	+	A	A	-	-	+	+	+	+	+	+	*B. licheniformis*
Creamy, convex	+ long rods	+	+	+	+	A	A	A	-	+	-	-	+	+	+	*B. pumilus*
Creamy, pin head, rough on agar surface when streaked.	+ single rods	+	+	+	+	A	A	-	-	+	+	+	+	+	-	*B. subtilis*

Key: + = Positive; - = Negative. A = acid production

Table 2. Microbial counts of "ugba" samples from eight markets (cfu/g x10^5)

Sample no	Markets							
	A	B	C	D	E	F	G	H
1	1.80	1.90	1.50	1.51	2.18	1.86	1.85	2.00
2	1.72	1.75	2.02	1.65	1.80	2.06	1.51	1.52
3	1.60	2.00	2.19	1.89	2.08	1.90	1.99	1.72
4	1.50	1.88	1.62	2.17	1.88	1.96	2.00	1.80
5	2.20	2.21	1.57	1.76	2.11	1.67	1.92	1.86
6	1.69	1.80	1.81	1.85	1.90	1.80	1.68	1.88
7	1.79	1.73	1.51	1.89	1.82	1.52	1.86	2.19
8	1.60	1.89	1.88	1.78	2.01	1.70	2.13	1.85
9	1.95	2.20	2.01	2.00	1.86	2.04	1.76	1.57
10	1.85	1.85	1.79	2.00	1.67	1.89	2.00	1.80
Mean Count	1.70±0.30	1.92±0.17	1.79±0.24	1.85±0.19	1.93±0.16	1.84±0.17	1.87±0.18	1.82±0.19

A = Ahiaeke; B = Ahiankwo; C = Ahia Ohuru; D = Afor Ule; E = Ariam; F = Ndioro; G = Ubani; H = Umungasi.

Table 3. Antimicrobial susceptibility of the three *Bacillus* species

Antimicrobial agent	Zone of inhibition (mm)		
	B. pumilus	***B. licheniformis***	***B. subtilis***
Ciproflox	3.00^{f}±0.30	3.00^{d}±0.22	2.00^{e}±0.06
Norfloxacin	0.00^{g}±0.00	2.00^{e}±0.18	0.00^{f}±0.00
Gentamicin	20.00^{a}±0.31	20.00^{a}±0.22	20.00^{b}±0.18
Amoxil	19.00^{b}±0.17	19.00^{b}±0.11	20.00^{b}±0.09
Streptomycin	5.00^{e}±0.16	5.00^{c}±0.28	3.00^{d}±0.15
Rifampicin	0.00^{g}±0.0	0.00^{f}±0.00	0.00^{f}±0.00
Erythromycin	6.00^{d}±0.11	5.00^{c}±0.17	6.00^{c}±0.10
Chloramphenicol	20.00^{a}±0.16	20.00^{a}±0.26	21.00^{a}±0.51
Ampiclox	18.00^{c}±0.09	20.00^{a}±0.12	20.00^{b}±0.18
Lavofloxacin	3.00^{f}±0.11	2.00^{e}±0.06	0.00^{f}±0.00

4. CONCLUSION

The present study has shown that *Bacillus* species are the predominant organisms in the traditional fermented ugba as they persisted throughout the fermentation stages. The isolates performed well in protease production while the two media tested showed good protease producing activity and *B. licheniformis* having best activity at 50°C. *B. licheniformis* and *B. subtilis* exhibited good abilities to survive high thermal temperatures and this has made them useful in the industries where thermophilic condition is needed especially as these isolates are sourced locally from traditional fermented foods which are readily available. The isolates may be useful in the industries such as foods, medicals, pharmaceutical and cosmetics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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