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Optimization of Alkaline Protease Production in Submerged Fermentation Using *Bacillus cereus* Isolated from an Abattoir Wastewater in Ile-Ife, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author YJA carried out the bench work and wrote the first draft of the manuscript. Authors YJA and RKO carried out the statistical analysis. Author FOO managed the literature searches and references and author KOA designed the study, supervised the work and proofread the whole edited manuscript. All authors read and approved the final manuscript.

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ABSTRACT

This study isolated and screened protease-producing bacteria from abattoir wastewater, it determined the effect of physical and nutritional components on the production of the crude protease produced by a selected bacterium in order to obtain a bacterium capable of producing protease with properties for industrial applications. Abattoir wastewater samples were collected from different locations in Ile-Ife, Nigeria and using pour plate method, one milliliter each of the aliquot was inoculated into skimmed milk agar medium (SKMA) to obtain colonies of protease-producing bacteria after proper dilution. All the protease-producing bacterial isolates were further screened for protease activity by submerged fermentation technique. The growth curve and protease activity of the best isolate was determined. The effect of different components of the growth medium on protease production was also determined. Forty-three isolates were obtained, five of the isolates

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gave appreciable diameter of clear zones of hydrolysis by rapid plate assay technique, out of which *Bacillus cereus* gave the highest enzyme activity of 75.79 Units/ml/min by submerged fermentation process. The optimal production of the enzyme was obtained after 44 hours of incubation with an inoculum volume of 2.0 ml. The optimal pH and temperature of production were 10.0 and 50°C respectively, while the best carbon and nitrogen source was sucrose and NH_4Cl respectively. The study concluded that abattoir wastewater served as a good media for the growth of proteolytic bacteria while the strain of *Bacillus cereus* isolated could be applied in the industries for protease production.

Keywords: Protease; abattoir wastewater; Bacillus cereus; submerged fermentation; optimization.

1. INTRODUCTION

Insufficiency in the production level of protease to meet various industrial demands is one of the main reasons that has led to the search for alternative source from bacterial isolates for the production of protease. Proteases are industrially crucial enzymes constituting about 25% of the total world production of enzyme [1]. Although proteases are naturally well-known, microorganisms function as an ideal source of these enzymes, chiefly due to the little space needed for their cultivation, fast growth, their ease of genetic manipulation to generate novel enzymes having improved properties that are suitable for their diverse applications [2]. Moreover, the isolation of proteases from plant and animal tissues usually involves a series of extraction and purification steps that consumes a lot of time [3] and resources.

Proteases synthesized from microbes are one of the most important classes of protease enzymes that occupy about 60% of total protease enzyme and they can be synthesized using submerged and solid-state fermentation [4]. In fact, the available commercial proteases are mostly of bacterial origin [5]. Important role is played by cultural conditions (physical, chemical and nutritional factors) in the biological production of protease by microorganisms. The conditions of the environment of the fermentation medium takes a vital role both in the growth and metabolic production of a population of microorganisms. The microbial production of proteases from bacteria particularly depends on the following factors; the strains involved, medium composition, cultivation method, temperature, cell growth, nutrient requirements, co-factors, pH, incubation period and thermal stability [3].

Currently, a large proportion of commercially available proteases are obtained from strains of *Bacillus*. Species of *Bacillus* involved in protease production include *B. megaterium*, *B. cereus*, *B. stearothermophilus*, *B. mojavensis* and *B. subtilis* [6]. Microbes from diverse habitats have been examined by many researchers to obtain industrially suitable proteases; Kusuma and Pavan [7], have reported the optimization of protease produced from *Bacillus cereus* isolated from soil samples in protein rich from different regions of the world.

This study was carried out to isolate, screen, identify protease producing bacterium from abattoir wastewater samples and to determine the effect of physical and nutritional components on the growth and production of alkaline protease by *Bacillus cereus* isolated from the abattoir wastewater.

2. EXPERIMENTAL

2.1 Collection of Samples

Wastewater samples were aseptically collected into sterile sampling bottles from randomly selected abattoirs which are: Ola-Inukan (7.522775N, 4.517228E), God's Mercy (7.476147N, 4.540173E), Ola-Oluwa (7.475819N, 4.539414E), Anu-Oluwa (7.476196N, 5.43331E), all located around Modakeke area along Ondo road axis of Ile-Ife, and University campus gate abattoir (7.495749N, 4.518664E), Ile-Ife, Nigeria. The samples were transported in ice pack to the Department of Microbiology laboratory for microbiological assessment and bacterial isolation.

2.2 Isolation of Protease-producing Bacteria

One milliliter each of the samples collected was added to 9 ml of sterile distilled water and a serial dilution of 10-fold was aseptically carried out to reduce the microbial population and obtain distinct colonies. Using pour plate method, one

milliliter each of the dilution factors was aseptically inoculated into different sterile Petri dishes using sterile pipettes. Fifteen milliliters each of prepared sterile molten skimmed milk agar (SKMA) containing in g/250 ml: Casein (1.25%), Yeast extract (0.625%), Glucose (0.25%), NaCl (0.125%), Agar (3.75%) and skimmed milk powder (7%) was poured into the inoculated dishes under aseptic condition in duplicates. The inoculated plates were incubated at 37°C for a period of 18 – 24 h, after which the plates were observed for growth. The colonies were sub-cultured onto fresh nutrient agar plates and incubated at 37°C for 18 – 24 h. The pure colonies were selected and stored in nutrient agar slant [8] for further microbiological analysis.

2.3 Rapid Plate Assay

Pure colonies from the stock cultures were each point-inoculated on freshly prepared skimmed milk agar plates and incubated at 37°C for 24 – 48 h. The colonies showing halo zones of clearance around them confirmed primary protease production. The diameters of each of the zones were measured and recorded [9]. The bacterial colonies with significant high zone of diameter were selected for further studies by sub-culturing on fresh nutrient agar plates and thereafter, the cultures were stored on nutrient agar slant and kept in the refrigerator at -4°C where sub-culturing was done once a month.

2.4 Quantitative Assay for Protease

Further screening of the selected isolates with significant clear zone of hydrolysis on Skimmed milk agar plates was carried out by submerged fermentation. Freshly grown cultures (18 – 24 h) of the selected bacterial isolates were standardized to 0.5 McFarland standard and inoculated into 50 ml of skimmed milk medium (SKM) containing (g/50 ml): Casein (0.25%), Yeast extract (0.125%), Glucose (0.05%), NaCl (0.025%), Agar Agar (0.75%) and Skimmed milk (1.4%): at pH 9.0. Submerged fermentation was carried out at 37°C using incubator shaker at 180 rpm for 48 hours. Ten milliliters of the solution were withdrawn from the fermentation broth and centrifuged at 3,500 rpm for 15 mins to obtain the crude protease. The supernatant obtained was considered as crude enzyme and was used to determine the protease activity and protein concentration [10]. The isolate with the highest enzyme activity was then selected for further studies and identification.

2.4.1 Enzyme assay

Protease activity was determined using the reaction mixture consisting of 1 ml of the crude enzyme solution with 1% casein substrate solution in glycine-NaOH buffer of pH 10.5. Both the experimental and the control were incubated at 60°C for 10 minutes in a water bath. The reaction was terminated by adding 4 ml of 5% trichloroacetic acid. The reaction mixture was centrifuged at 3000 rpm for 10 minutes and to 1 ml of the supernatant, 5 ml of 0.4M Na_2CO_3 was added, followed by the addition of 0.5 ml Folins-Ciocalteu reagent. The absorbance was read at 660 nm. One unit (1 U) of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine ml/min under the specified assay conditions [11].

$$\text{Protease activity } (\mu/\text{ml}) = \frac{\Delta \text{ Abs } 660 \text{ nm/min}}{\text{m} \times \text{v} \times \text{t}}$$

Δ Abs 660 nm/min = change in absorbance at 660 nm
m= slope
v= enzyme volume
t= incubation time

$$\text{Specific Activity } (\mu/\text{mg}) = \frac{\text{enzyme activity}(\mu/\text{ml})}{\text{Protein concentration}(\text{mg/ml})}$$

2.4.2 Protein content determination

The protein concentration was determined by the method of Bradford [12] using Bovine Serum Albumin (BSA) as the standard protein. To 0.2 ml of crude enzyme, 0.2 ml of Bradford reagent and 0.8 ml of distilled water was added and the absorbance was read at 595 nm after 5 minutes. The amount of protein in the sample was determined by extrapolation from the protein standard curve. The protein content of the blank was also determined by adding 0.2 ml of the Bradford reagent to 1 ml of distilled water and the absorbance was read at 595 nm after 5 minutes. The absorbance of the blank was then subtracted from that of the samples and the protein concentration of the sample was determined.

2.5 Identification of Bacterial Isolate

The bacteria isolate with the highest enzyme activity was identified using its morphological, biochemical and molecular characteristics. The Bergey's Manual of Determinative Bacteriology was used for the primary identification of the

isolate [13]. The identity of the isolate was further confirmed by 16S rRNA sequence analysis as described by Priyanka et al. [14].

2.6 Optimization of Production Conditions

The effect of various nutritional and physical factors on the enzyme production and protein content was studied.

2.6.1 Effect of incubation time on protease production

Forty-eight tubes each containing 10 ml of the basal medium for protease production was sterilized by autoclaving at 121°C for 15 mins, allowed to cool and inoculated with 1 ml of the standardized inoculum. It was later incubated at 37°C in an incubator shaker set at 180 rpm for 48 h. Each of the test tubes containing the growth medium was taken at interval of 2 h and centrifuged at 3,500 rpm for 15 minutes each, in order to obtain the cell free supernatant which was then assayed for enzyme activity and protein content.

2.6.2 Effect of inoculum volume on protease production

One hundred milliliters conical flasks each containing 50 ml of the growth medium for protease production was inoculated with the standardized inoculum of varying volumes ranging from 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml respectively. The inoculated flasks were incubated at 37°C for 48 h after which the flasks were each centrifuged at 3,500 rpm for 15 minutes, in order to obtain the cell free supernatant which was assayed for enzyme activity and protein content [15].

2.6.3 Effect of temperature on protease production

One hundred milliliters conical flasks each containing 50 ml of the growth medium for protease production was inoculated with 1 ml of the standardized inoculum. The inoculated flasks were then incubated at varying temperatures ranging from 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C and 65°C and each of the inoculated flasks was incubated in an incubator shaker set at 180 rpm for 48 h. The contents of these flasks were centrifuged at 3,500 rpm for 15 minutes each. For each of the temperatures, the cell free supernatant was assayed for enzyme activity [15].

2.6.4 Effect of pH on protease production

The effect of varied pH ranging from 3 to 11 at a pH interval of 1.0 was determined in which the adjustment was done with 1M sodium hydroxide (NaOH) and 1M of hydrogen chloride (HCl) to adjust hundred milliliters conical flasks each containing 50 ml of the growth medium for protease production. The contents of these flasks were centrifuged at 3,500 rpm for 15 minutes each. For each of the pH values, the cell free supernatant was assayed for enzyme activity [15].

2.6.5 Effect of different carbon sources on protease production

Carbon sources such as sucrose, fructose, maltose, starch and carboxyl-methylcellulose were used as alternative replacements for glucose in the basal medium. These carbon sources were used at a concentration of 0.5% in substitute to glucose in the production media and other components were added. These separate production media were thereafter centrifuged at 3,500 rpm for 15 minutes each. For each of the carbon source, the cell free supernatant was assayed for enzyme activity and protein content [16].

2.6.6 Effect of different nitrogen sources on protease production

The nitrogen sources used include urea, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, Yeast extract and KNO_3. The nitrogen sources were added at 1% concentration to replace casein. One hundred milliliters conical flasks containing 50 millilitres of the modified medium of nitrogen source was inoculated with 1 ml of the standardized inoculum and were incubated at 37°C for 48 h. The culture filtrate was then assayed for enzyme activity [16].

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Bacterial Isolates for Protease Production

In this study, forty-three bacteria were isolated from abattoir waste water and twenty were confirmed as protease-producing bacteria, although the degree of protease production varied among these isolates. The isolates with substantial wide zone of clearance on the plates were selected for further screening by submerged fermentation process, where the clear zone of casein hydrolysis on skimmed milk

Table 1. Primary quantitative screening for protease producing bacteria (Rapid plate assay)

Isolate	Zone of hydrolysis (mm)
A1	No clear zone (0)
A2	9
A3	33
A4	13
A5	No clear zone (0)
A6	No clear zone (0)
A7	No clear zone (0)
A8	25
A9	32
A10	Overgrown
A11	Overgrown
A12	No clear zone (0)
A13	No clear zone (0)
A14	No clear zone (0)
A15	No clear zone (0)
A16	No clear zone (0)
B19	14
B20	No clear zone (0)
B21	No clear zone (0)
B22	20
B23	28
B24	23
B25	10
C26	24
C27	No clear zone (0)
C28	12
C29	24
C30	No clear zone (0)
C31	Overgrown
C32	No clear zone (0)
C33	25
D34	31
D35	27
D36	28
D37	32
D38	Overgrown
D39	Overgrown
E40	No clear zone (0)
E41	38
E42	No clear zone (0)
E43	30
G44	29
G45	29

Table 2. Secondary screening of the isolates by submerged fermentation

Isolate	Activity (Units/ml/min)
A3	2.174
A9	15.263
B22	5.965
C33	9.123
E41	75.789

Table 3. Morphological and biochemical characterization

Characteristics	Morphological characteristics						Grams staining and biochemical characterization										Sugar fermentation						Probable identity
Isolate E41	Shape	Size	Elevation	Surface	Opacity	Colour	Grams Reaction	Spore StainRxn Staini	Cell Shape	Catalase	Triple Sugar Reaction	Indole	Starch Hydrolysis	Citrate	MR Test	Voges-Proskauer	Glucose	Maltose	Mannitol	Galactose	Fructose	Lactose	
	Circular	Large	Flat	Glistering	Opaque	Cream	+	+	LR	+	+--	+	+	-	-	+	+	-	+	+	+	-	*Bacillus cereus*

Key: MR – Methyl red; LR – long rod; + = positive; - = negative

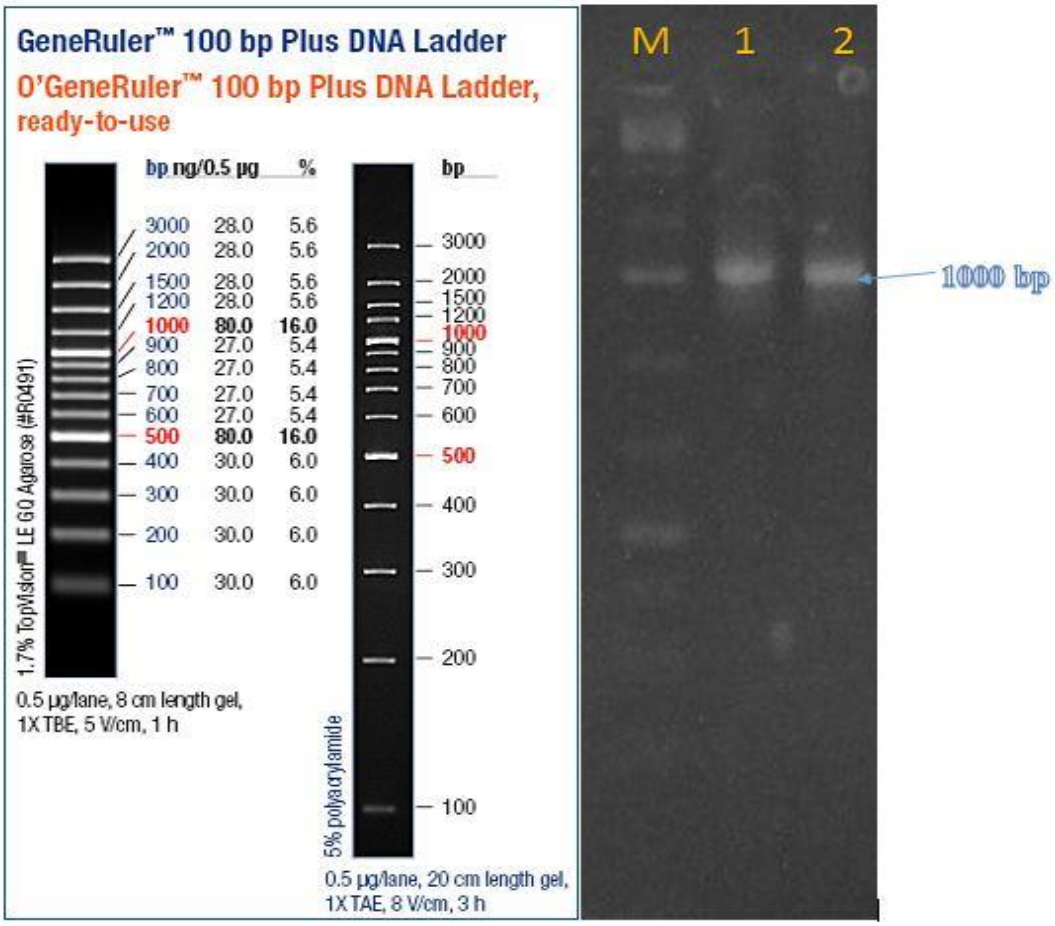

Plate 1. Agarose gel showing 16S rRNA amplicon band of the isolate

around the bacterial colony was an indication of good growth with the ability to secrete protease as reported by Olajuyigbe and Ajele [17], and Boominadhan et al. [18]. The isolate "E41" was the best isolate with the highest enzyme activity of 75.79 units/ml/min as shown in Tables 1 and 2, respectively. This organism which showed the highest activity by submerged fermentation was adopted as the organism of interest in this study.

3.2 Identification of the Bacterial Isolates

The results of the morphological characteristics of the isolates E41; such as shape, opacity, elevation, surface, size and color as well as the Gram staining and biochemical tests of the isolate "E41" was shown in Table 3.

3.3 Molecular Analysis of the Bacterial Isolate

Agarose gel showing 16S rRNA amplicon band of the isolate is shown in Plate 1. Lane M shows the standard DNA size marker ladder, while Lane 1 and 2 show the 1000bp of the 16S rRNA amplicon bands of the isolate. Analysis of the nucleotide sequence of the bacterial isolate revealed similarities between 99% and 100% with several corresponding sequences of *Bacillus cereus* available on the Genbank database. The bacterial isolate was therefore identified as *Bacillus cereus*. The BLAST hits and significant sequence alignments are presented in Plate 2.

3.4 Optimization Conditions for Protease Production from *Bacillus cereus*

3.4.1 Effect of incubation time on protease production

The growth curve and enzyme production by *Bacillus cereus* was studied, and according to the result obtained in this study, the enzyme production was evident after 4 h of growth, with

protease activity of 10.61 units/ml/min. The peak for the production of the protease was observed to be at 44 h, with an activity of 80.00 units/ml/min, after which the enzyme activity decreased drastically with time as shown in Fig. 1. A successful fermentation process depends largely on diverse parameters that influence the microbial growth and metabolite production [19]. The incubation period is directly related with the production of enzyme, up to a certain extent; after which the enzyme production starts to decrease, which can be attributed to the decreased supply of nutrients to the microorganisms, because the microorganisms would keep using up the available nutrients in the medium [20]. Submerged fermentation is widely used for the industrial production of microbial enzymes, and the availability of good quantity of utilizable substrate in the fermentation medium supports high production of the needed enzyme.

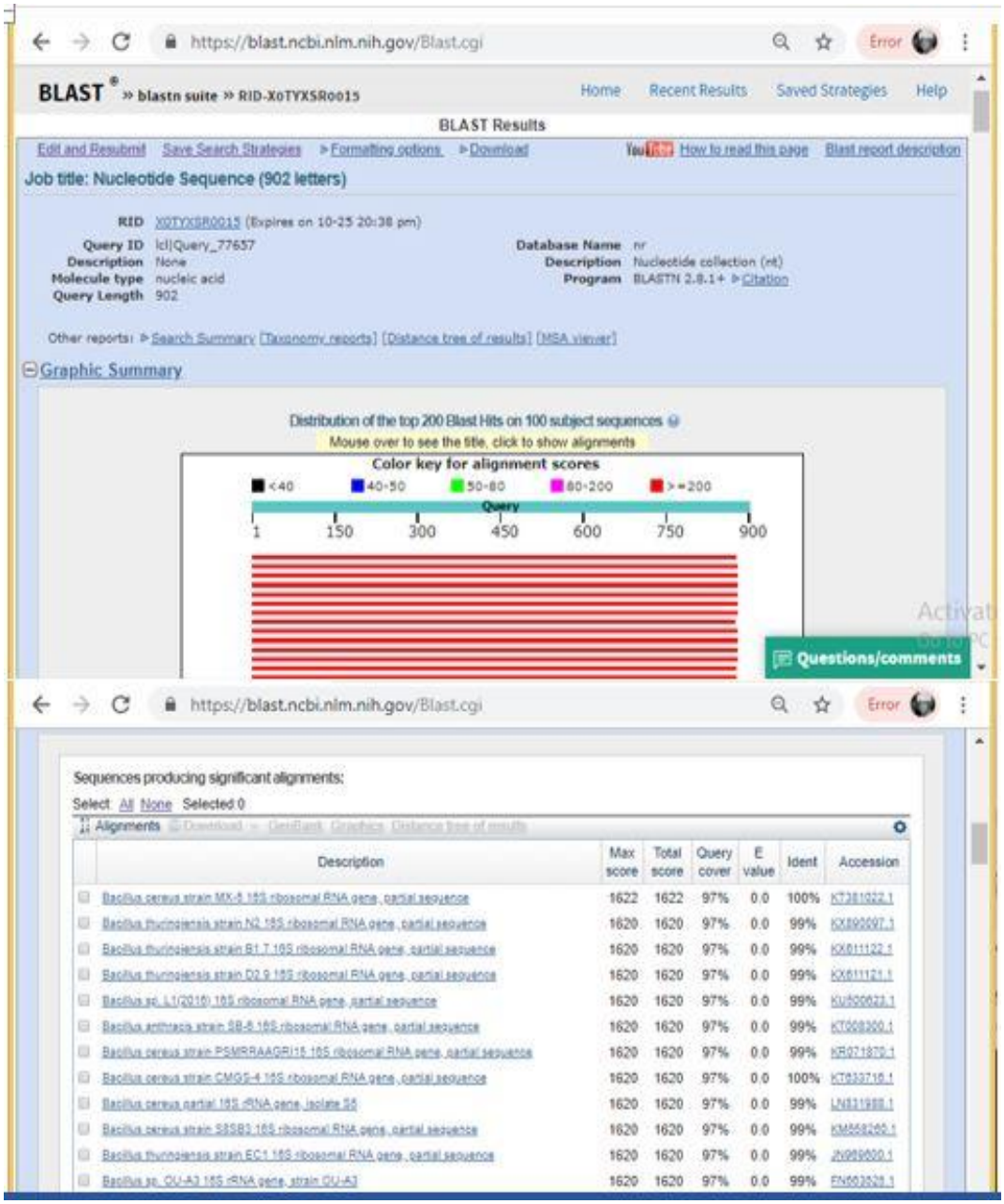

Plate 2. The BLAST hits and significant sequence alignments for the bacterial isolate

3.4.2 Effect of inoculum size on the production of protease from *Bacillus cereus*

The optimum production of protease was observed when 2.0 ml of the standardized inoculum was aseptically inoculated into the production medium, which gave an enzyme activity of 34.211 units/ml/min. The inoculum size that showed the least enzyme activity was 3.0 ml, which had an enzyme activity of 11.404 units/ml/min. An increase in the enzyme activity was observed between 0.5 ml and 2.0 ml inoculum volume but was followed by a drastic decrease after the optimum, till the last inoculum volume of 3.0 ml as shown in Fig. 2. The result obtained is slightly different from that of Kusuma and Pavan [7], who obtained 5% inoculum volume as the optimum volume when *Bacillus cereus* was used, however, the results obtained by Lakshmi et al. [21], revealed that maximum protease production was observed at 2% inoculum volume by B*acillus licheniformis* which is similar to the result obtained in this study. The result obtained here is also similar to that of *Bacillus brevis* which was reported by Odu and Akujobi [22]. The earlier reduction in protease production at lower inoculum volume between 0.5 ml and 1.5 ml could be due to insufficient number of the bacterial cells. The drastic decrease in enzyme activity at higher inoculum size beyond the optimum may have occurred due to the reduction in the available dissolved oxygen and increase in the competition for available nutrients by the bacterial cells [21].

3.4.3 Effect of incubation temperature on the production of protease from *Bacillus cereus*

The effect of different temperatures on protease production showed that the optimum temperature was observed at 50°C with an activity of 15.789 units/ml/min, while the least enzyme activity was observed at 60°C with an enzyme activity of 4.211 units/ml/min as illustrated in Fig. 3. The incubation temperature greatly affects the microbial growth rate, enzyme secretion, enzyme inhibition and protein denaturation [23]. Although, the optimum temperature obtained in this study slightly disagrees with the findings of Mohanapriya et al. [24] and the report of Nisha and Divakaran [25], where the maximum activity was recorded at 40°C for *Bacillus subtilis* in both studies, yet, the result obtained from this study agrees with the result obtained during the production of protease by *Bacillus licheniformis*, where maximum protease production was observed at 50°C [26]. Maximum protease activity was obtained between the temperature of 37°C and 50°C during the optimization of protease-producing bacteria from soil and water in Gondar town, North West Ethiopia. It was observed that the enzyme activity declined at temperature greater than the optimum temperature, which might be due to growth reduction, enzyme inactivation or the suppression of cell viability.

3.4.4 Effect of pH on the production of protease from *Bacillus cereus*

An irregularity was initially observed in the enzyme activity of the protease between pH 3.0 and pH 9.0, but the highest enzyme activity was observed at an optimum pH of 10.0 which had an enzyme activity of 26.404 units/ml/min. There was a marked decrease in enzyme activity at pH 11.0 which had an enzyme activity of 4.912 units/ml/min as revealed in Fig. 4. The pH of the fermentation medium plays a vital role in determining the level of metabolite synthesized. The stability of the microbial metabolite synthesized is likewise dependent on the hydrogen ion concentration of the medium [27]. This result is in accordance with the result of Johnvesly et al. [28] during their investigation of thermo-stable alkaline protease from thermo-alkalophilic *Bacillus* sp, where it was reported that *Bacillus* sp are highly stable at pH 9 – 10. The result is also in accordance with that of [24], where optimum protease production was obtained at pH 10.0. Bajaj and Jamwal [29] reported that *Bacillus pumilus* isolated from the soil sample of a dairy plant using submerged fermentation had high protease activity at pH 8 – 12. Ghasemi et al. [30] recorded optimum pH for proteolytic activity of protease-producing bacteria between pH 8 and 10, suggesting alkaline environment for the growth of protease-producing bacteria to be more suitable than acidic environment; this idea had also been supported earlier by Kuberan et al. [31].

3.4.5 Effect of different carbon sources on the production of protease from *Bacillus cereus*

The production of protease was observed to be highest with sucrose as the sole carbon source at 37°C and pH 9.0 for 48 h with an enzyme activity of 10.877 units/ml/min while the least was lactose with an enzyme activity of 1.930 units/ml/min with fructose and galactose having

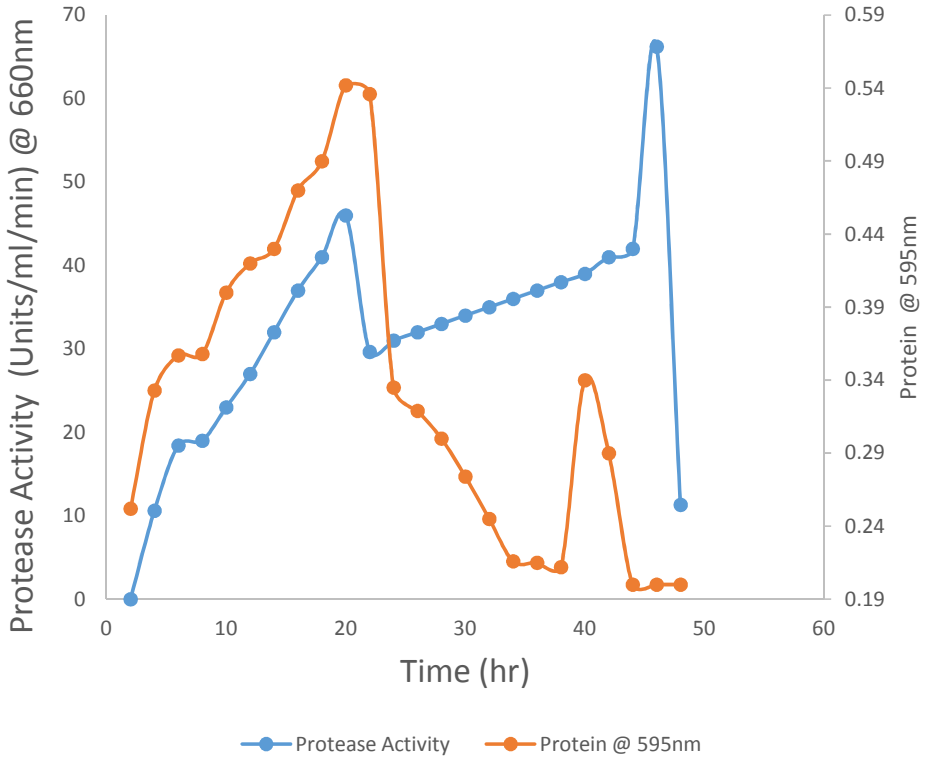

Fig. 1. Growth curve and production of protease from *Bacillus cereus*

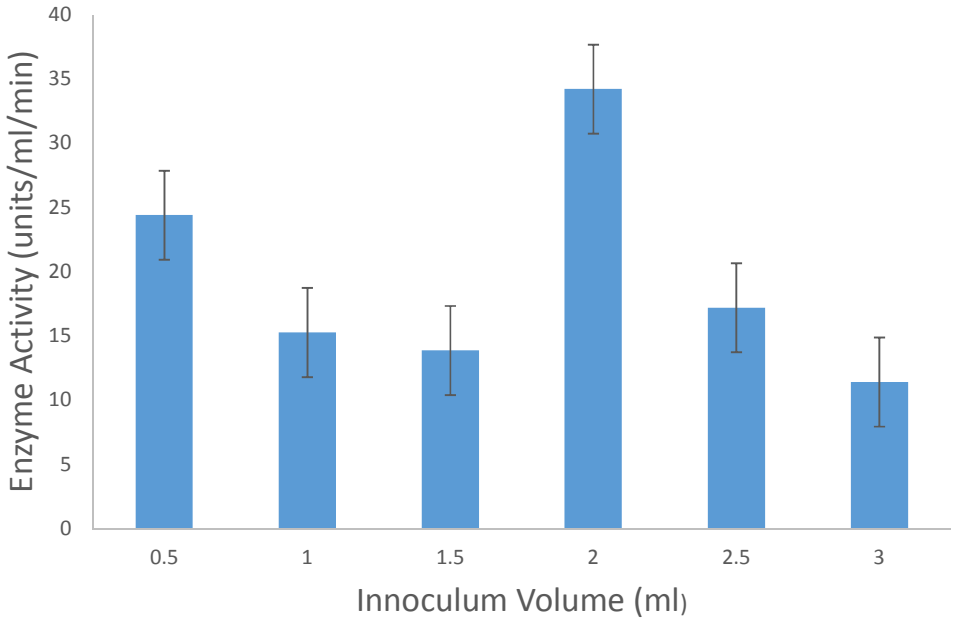

Fig. 2. Effect of inoculum size on the production of protease from *Bacillus cereus*

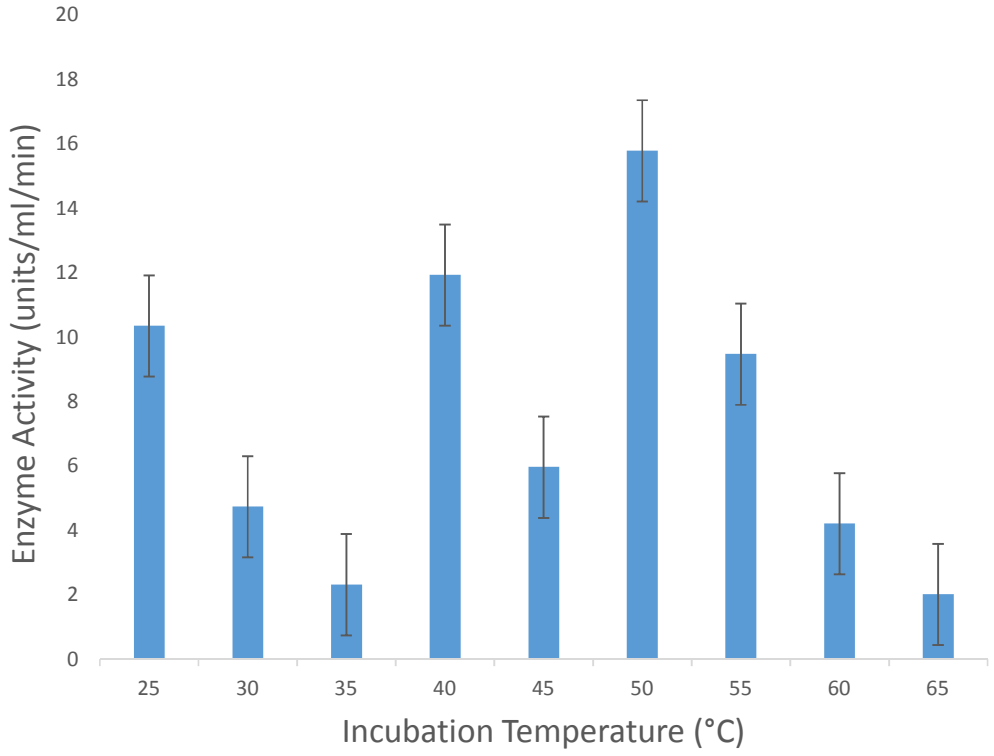

Fig. 3. Effect of incubation temperature on the production of protease from *Bacillus cereus*

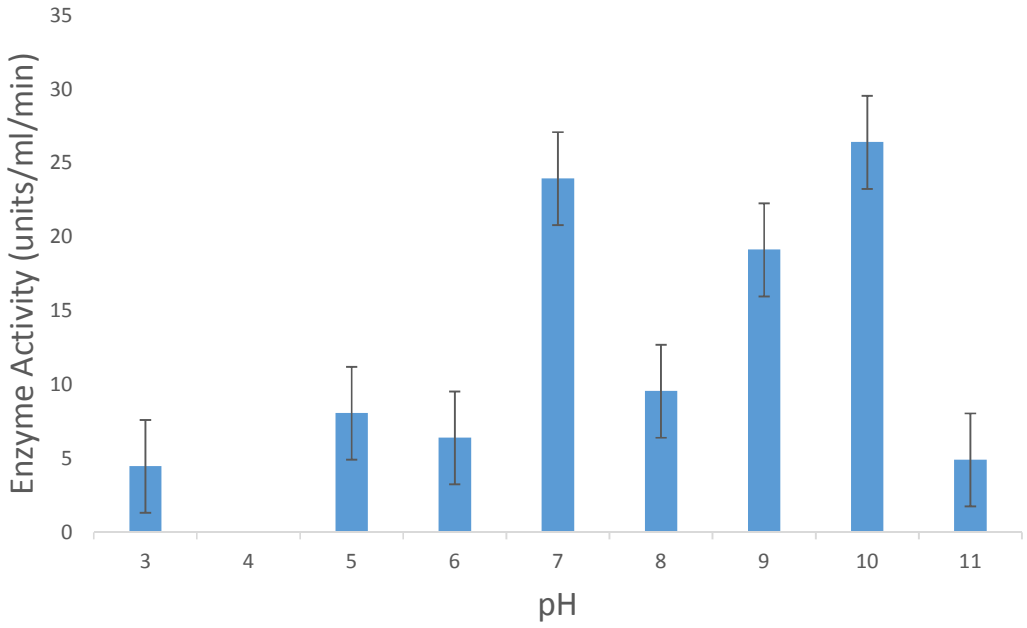

Fig. 4. Effect of pH on the production of protease from *Bacillus cereus*

no enzyme activity as shown in Fig. 5. The reason for obtaining highest yield of protease by the use of sucrose as a carbon source may be due to the secretion of the enzyme invertase by the microorganism, which causes the breakdown of sucrose to glucose and fructose which could be used effortlessly by the microorganism for its growth and enzyme production as well [32]. It had been proven by Sutar et al. [33] that sucrose, glucose and fructose are adequately good as carbon sources for the production of protease by *Condiobolus coronatus*. Another researcher reported that there was maximum yield of alkaline protease in the presence of lactose, maltose, sucrose and fructose obtained from different protease-producing *Bacillus* species [34], but, here, the least protease activity was observed with lactose. The findings of Nascimento and Martins [8], during their investigation on the effect of carbon on the production and properties of an extracellular protease from thermophilic *Bacillus* sp observed that glucose was a ready source of carbon, due to a moderate amount of protease production. According to Sharma et al. [35], protease production by *Bacillus* sp recorded maximum production when glucose was used as source of carbon with enzyme activity of 1.289 units/ml/min, followed by starch with an enzyme activity of 0.731 units/ml/min. Starch and glucose being the second-best carbon source for protease production here, suggested that glucose is a natural carbon source incorporated into most media for the growth of microorganisms, however, the presence of high glucose in the growth medium may also result in the reduction in protease production, which could be attributed to catabolite repression by high glucose available in the medium [24].

3.4.6 Effect of different nitrogen sources on protease production from *Bacillus cereus*

The effect of different sources of nitrogen on protease production showed that NH_4Cl (ammonium chloride) gave the highest enzyme activity of 38.772 units/ml/min, which was followed by $NH_4(SO_4)_2$ (ammonium sulphate) with an enzyme activity of 32.632 units/ml/min. Urea had the least enzyme activity of 20.702 units/ml/min, while KNO_3 (potassium nitrate) had no enzyme activity when used as a nitrogen source for protease production (Fig. 6). The incorporation of nitrogen supplements into the production medium facilitated better biomass production and more metabolites were subsequently secreted. Microbial cells use

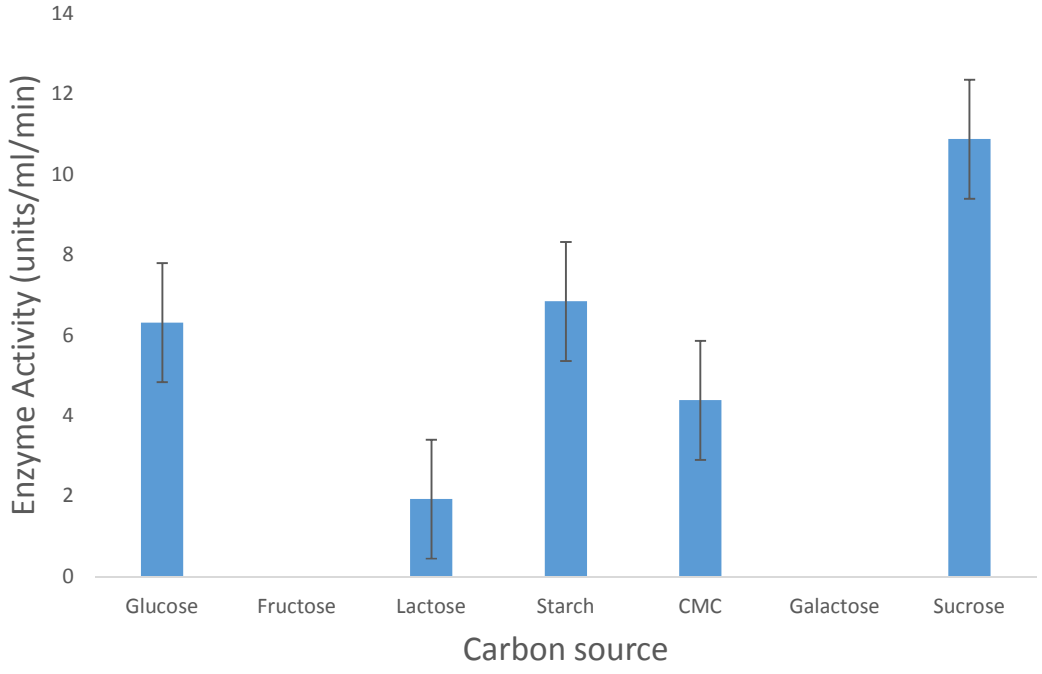

Fig. 5. Effect of different carbon sources on the production of protease from *Bacillus cereus*

Table 4. Summary of optimization condition for protease production

Optimization condition	Results
Effect of incubation time (h)	44
Effect of inoculum volume (ml)	2.0
Effect of temperature (°C)	50
Effect of pH	10.0
Effect of carbon source	Sucrose
Effect of nitrogen source	NH_4Cl

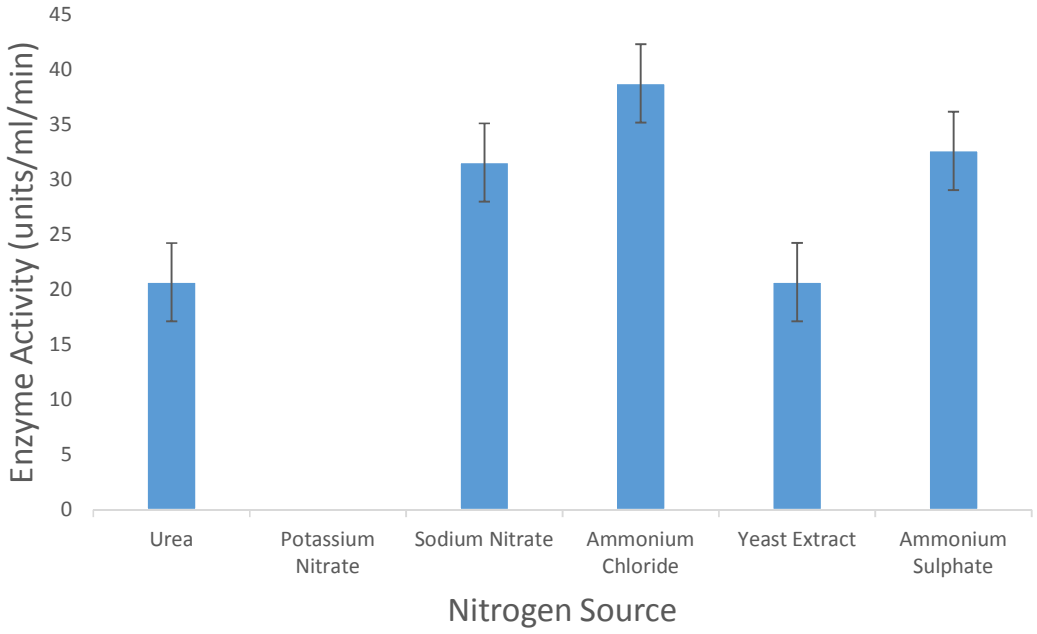

Fig. 6. Effect of different nitrogen sources on the production of protease from *Bacillus cereus*

nitrogenous compounds for the synthesis of nucleotides, amino acids, proteins, enzymes and other metabolites [36]. The effect of various organic and inorganic nitrogen supplements in the production of protease was examined. The result obtained in this study corresponds with that of Nascimento and Martins [8], who obtained a moderately good level of enzyme activity using NH_4Cl as a source of nitrogen for the production of an extracellular protease from thermophilic *Bacillus* sp. Similar result was also obtained by Banerjee et al. [37] using *Bacillus brevis*. Other inorganic sources of nitrogen like ammonium sulphate contributed to protease production, which is also similar to the findings of Nascimento and Martins [8], who reported that ammonium sulphate gave good yields of protease production. The summary of the optimization condition for protease production is shown in Table 4.

4. CONCLUSION

The results obtained in this study suggested that abattoir waste water from university gate abattoir Ile-Ife, Nigeria served as a good source for proteolytic bacteria. Since the currently available proteases are not sufficient in meeting various industrial demands, this study has provided a better knowledge of the other bacterium strain isolated from another source that has not been explored yet. The effect of some physical and nutritional components on the production of this protease has also been provided to give insight into its optimization for industrial applicability.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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