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Protease Production by Submerged Fermentation in Shake Flasks Using *Bacillus* sp. Isolated from the Soil

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Authors' contributions

This work was carried out in collaboration among all authors. Author JO designed the study, managed the literature searches and wrote the protocol. Author DAO managed the literature searches. Author IED wrote the first draft of the manuscript. Author TOM managed the analysis of the study. All authors read and approved the final manuscript.

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ABSTRACT

Proteases are one of the most industrially important enzymes, which account for about 60% of total enzyme market. Protease production by submerged fermentation in shake flasks using *Bacillus* sp. isolated from the soil was studied. Soil samples were collected from different locations within Chukwuemeka Odumegwu Ojukwu University, Uli, Anambra state. The soil samples were serially diluted and inoculated on sterilized skim milk agar plates. The plates were incubated at 30^{o}C for 72 h. A clear zone around the colonies gave an indication of protease-producing bacteria isolates. The selected protease producers were subsequently used for shake flask fermentation in 50 ml

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sterile medium. Optimization study was conducted to determine the effect of carbon sources, nitrogen sources, trace elements, agitation rates and pH. Twenty one bacteria isolates were found to be active protease producers and isolates RS-5 and OS-9 had the highest zone of clearance of 13.5 and 12.1 mm respectively. The result of submerged production of protease by the bacteria isolates revealed that the isolates RS-5 and OS-9 accumulated maximum protease yield of 3.23 and 2.71 U/ml respectively. The isolates were Gram positive and endospore formers, and were identified as *Bacillus* sp. RS-5 and OS-9.The addition of Starch and maltose stimulated optimum protease production of 3.47 and 2.77 U/ml by *Bacillus* sp. RS-5 and OS-9 respectively. Beef extract enhanced maximum enzyme yield of 3.35 and 2.90 U/ml for *Bacillus* sp. RS-5 and OS-9 respectively. Maximum protease yield of 3.28 U/ml for *Bacillus* sp. RS-5 and 2.85 U/ml for *Bacillus* sp. OS-9 was obtained by the supplementation of 0.4 g/l of $FeS0_4$ respectively. The maximum protease yield was observed at agitation rate of 200 rpm for *Bacillus* sp. RS-5 and 170 rpm for *Bacillus* sp. OS-9. At pH8, protease accumulation was highest for *Bacillus* sp. RS-5 and OS-9. The study revealed that the soil harbours some protease-producing bacteria strains and protease production can be greatly enhanced through optimization of process parameters.

Keywords: Soil; Bacillus species; protease; submerged; fermentation; trace elements.

1. INTRODUCTION

"Proteases (EC3.4.21-24) are degradative enzymes, which catalyze the total hydrolysis of protein [1] and are classified according to their structure or the properties of the active site" [2]. "Proteases are grossly subdivided into two major groups: exopeptidases and endopeptidases depending on their site of action. Exopeptidases cleave the peptide bonds proximal to the amino or carboxyl terminal of the substrate, whereas endopeptidases cleave peptide bonds distant from termini of the substrate. Based on the functional groups present at the active site, proteases are further classified into four prominent groups: serine proteases, aspartic proteases, cysteine proteases, and metalloproteases" [3-5] Proteases are (physiologically) necessary for living organisms; they are ubiquitous and found in a wide diversity of sources.

"Protease is one of the most important group of industrial enzymes accounting for about 60% of the total worldwide enzyme sales" [6-8]. "A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes is known to produce these enzymes" [9]. "Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. Among bacteria, Bacillus species are the main producers of extracellular proteases, and industrial sectors frequently use *Bacillus subtilis, B. licheniformis, B. horikoshii* and *B. sphaericus* for the production of various enzymes" [10]. "*Bacillus species* are found in soil and they are rod-shaped organism. They can form a tough, protective endospore and can withstand extreme environmental conditions. *Bacillus* species are obligate aerobes or facultative anaerobe and include both free-living and pathogenic species" [11,12]. "In view of their wide application in various industries, protease enzymes occupy an important position" [13].

"Microbial proteases are preferred to proteases from plant and animal sources for various reasons, which include the development of eco-friendly technology. Selection of efficient micro-organism plays an important role in higher yield of enzymes producing enzyme on industrial level, isolation and characterization of new promising strains with cheap carbon and nitrogen source is a continuous process" [9]. "The yield of extracellular enzymes is significantly influenced by physicochemical conditions" [14,15]. Hence physical parameters are optimized for the maximum production of protease.

"Proteases are one of the most important industrial enzymes and are used in a variety of industrial applications, such as laundry, detergents, pharmaceutical industry, leather industry in dehairing and bating of hides, manufacture of the protein hydrolysis, food industry like meat tenderizing, cheese flavor development, treatment of flour in the manufacture of baked goods, improvement of dough texture, flavor and colour in cookies [16-18], silver recovery from X–ray film and even in waste processing industry" [19,20].

As the need for proteases increases for industrial processes, there is need to search for bacteria protease producers in the presence of economical indigenous carbon and nitrogen sources. At present, the requirements of some Nigerian industries for protease is met through importation, which involves spending huge amount of foreign exchange. There is huge potential in the production of protease locally, by microbiological methods using available raw materials.

This study was undertaken to isolate protease-producing *Bacillus* sp. from the soil and to optimize the culture conditions for optimal protease production.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were randomly collected from ten different locations within Chukwuemeka Odumegwu Ojukwu University, Uli, Anambra state. The samples were transferred into sterile plastic containers and subsequently used for the screening of protease producing strains.

2.2 Isolation and Screening of Bacteria from Soil

One gram of each soil sample was transferred into a test tube containing 9 ml normal saline. A ten fold serial dilution was conducted and 0.l ml of 10^{-4} dilution was inoculated into skimmed milk agar plates. The plates were incubated at 30°C for 72 h. A clear zone around the colonies gave an indication of protease-producing bacterial isolates. The protease producers were subcultured severally into nutrient agar plates to obtain pure cultures. The pure isolates were inoculated on fresh skimmed milk agar plates and incubated 30°C for 72 h. Thereafter, the diameters of the clear zones around the colonies were measured.

2.3 Protease Production in Shake Flask Fermentation

2.3.1 Preparation of inoculum

A loopful (24 h) of each isolate was collected from agar slant and inoculated into 100ml Erlenmeyer flask containing 20ml of nutrient broth medium, which was already sterilized at 121°C for 15 min. The flasks were incubated for 24 h on a rotary shaker (150 rpm) at 30°C.

2.3.2 Fermentation

A 250ml Erlenmeyer flasks containing 50ml of fermentation medium g/l: glucose, 5; peptone, 7.5; casein, 1; $MgS04.7H_2O$, 5; KH_2PO_4, 5; $FeSO_4$ $7H_2O$, 0.1; pH 7.2 was sterilized at 121°C for 15 min. Each flask was inoculated with 2 ml (6.7×10^6 cfu/ml) of each selected isolate (24 h) . The flasks were incubated at 30 °C on a rotary shaker (160 rpm) for 72 h. After incubation, the fermentation medium was subjected to centrifugation at 5,000 rpm for 15 min to obtain the cell free supernatant which is the crude enzyme source. The cell free supernatant was used for protease determination.

2.4 Identification of the Bacterial Isolates

The bacterial isolates that showing the highest protease production were identified on the basis of cultural, morphological and biochemical characteristics, according to [21].

2.5 Determination of Protease Activity

Protease activity was measured using the casein digestion method of [22]. The reaction mixture containing 2 mL of 1 % casein solution prepared in 0.2 M Tris buffer (pH 8.5) and 1 mL of enzyme solution were incubated at 40 °C for 30 min and the reaction was then stopped with the addition of 3 mL of 10 % trichloroacetic acid. After 10 min the entire mixture was centrifuged at 9000 rpm for 10 min at 4 °C and absorbance of the liberated tyrosine was measured with respect to the blank at 280 nm. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 μg of tyrosine per min under assay conditions. One unit of protease activity was defined as the amount of enzyme that will release 10μg of tyrosine under the specified conditions (pH 8.5, 40 °C and 30min).

2.6 Optimization of Culture Conditions for Protease Production

2.6.1 Effect of carbon sources

The effect of carbon sources (0.5%w/v) which included maltose, glucose, starch, lactose and mannitol was studied by inoculating *Bacillus* sp. RS-5 and OS-9 in 50 ml fermentation media (in 250 ml Erlenmeyer flasks), containing various carbon sources. The flasks were incubated at 30°C on a rotary shaker (160 rpm) for 72 h. At the end of incubation, samples of the fermentation medium were aseptically dispensed into cuvettes using micropipettes. Thereafter, the cuvettes were placed in the spectrophotometer and the reading for bacteria growth was determined at 660nm.The cell-free supernatant was obtained as previously described and used for the determination of protease production. The experiments were conducted in triplicate.

2.6.2 Effect of nitrogen sources

The effect of nitrogen sources(1%w/v) which included gelatine, soybean, tryptone, peptone, beef extract, casein and yeast extract was studied by inoculating *Bacillus* sp. RS-5 and OS-9 in 50 ml fermentation media (in 250 ml Erlenmeyer flasks), containing various nitrogen sources. The flasks were incubated at 30^0C on a rotary shaker (160rpm) for 72h. At the end of incubation, samples of the fermentation medium were aseptically dispensed into cuvettes using micropipettes. Thereafter, the cuvettes were placed in the spectrophotometer and the reading for bacteria growth was determined at 660nm.The cell-free supernatant was obtained as previously described and used for the determination of protease production. The experiments were conducted in triplicate.

2.6.3 Effect of trace elements

Effect of concentrations of trace elements (3 – 10g/l) of $MgSO_4$ and KH_2PO_4 and (0.1 – 0.4 g/l) of $FeSO_4$ on protease production was determined by inoculating *Bacillus* sp. RS-5 and OS-9 in 50 ml fermentation media (in 250 ml Erlenmeyer flasks), containing various trace elements. The flasks were incubated at 30 ^{0}C on a rotary shaker (160rpm) for 72h. At the end of incubation, samples of the fermentation medium were aseptically dispensed into cuvettes using micropipettes. Thereafter, the cuvettes were placed in the spectrophotometer and the reading for bacteria growth was determined at 660nm.The cell-free supernatant was obtained as previously described and used for the determination of protease production. The experiments were conducted in triplicate.

2.6.4 Effect of agitation rates

Effect of agitation rates on growth and protease production was studied by inoculating *Bacillus* sp. RS-5 and OS-9 in 50 ml fermentation media (in 250 ml Erlenmeyer flasks), at various agitation rates. The flasks were incubated at 30^0C on a rotary shaker (80- 200 rpm) for 72h. At the end of incubation, samples of the fermentation medium were aseptically dispensed into cuvettes using micropipettes. Thereafter, the cuvettes were placed in the spectrophotometer and the reading for bacteria growth was determined at 660nm.The cell-free supernatant was obtained as previously described and used for the determination of protease production. The experiments were conducted in triplicate.

2.6.5 Effect of pH

Effect of pH values on growth and protease production by *Bacillus* species was studied by inoculating *Bacillus* sp. RS-5 and OS-9 in 50 ml fermentation media (in 250 ml Erlenmeyer flasks), at different pH values (6-9). The flasks were incubated at 30^0C on a rotary shaker (160 rpm) for 72h. At the end of incubation, samples of the fermentation medium were aseptically dispensed into cuvettes using micropipettes. Thereafter, the cuvettes were placed in the spectrophotometer and the reading for bacteria growth was determined at 660nm.The cell-free supernatant was obtained as previously described and used for the determination of protease production. The experiments were conducted in triplicate.

2.7 Statistical Analysis

The data obtained were analyzed by covariance matrix analysis using Microsoft excel 2013.

3. RESULTS

Among the protease-producing bacteria isolated, 9 of them were Gram positive rods, 3 were Gram negative rods, 4 were Gram positive cocci and 5 were Gram negative cocci. Nine (9) of the bacteria isolates were endospore formers. The result of screening for protease production revealed that, out of the 88 isolates that were screened, only 21 were found to be protease producers. The isolates RS-5 and OS-9 had the highest zone of clearance of 13.5 and 12.1 mm respectively (Table 1).The result of shake flask production of protease by bacteria as shown in Table 2, revealed that the isolates RS-5 and OS-9 accumulated maximum protease yield of 3.23 and 2.71 U/ml respectively.

The two bacterial isolates showing the highest protease production were identified on the basis of cultural, morphological and biochemical characteristics. They were identified as *Bacillus* sp. RS-5 and OS-9.

Table 3 shows the effect of carbon sources on growth and protease production by *Bacillus* sp. RS-5 and OS-9. Starch and maltose stimulated maximum protease production of 3.47 and 2.77 U/ml by *Bacillus* sp. RS-5 and OS-9 respectively, while mannitol encouraged the least accumulation of 2.62 U/ml and 2.25 U/ml in both species. The covariance matrix analysis shows that there was a significant high value of effect in starch and maltose for protease production in *Bacillus* sp. RS-5 and OS-9 respectively.

The effect of nitrogen sources on growth and protease production by *Bacillus* sp. RS-5 and OS-9 is shown in Table 4. Beef extract enhanced maximum growth and enzyme yield of 3.35 and 2.9 U/ml for *Bacillus* sp. RS-5 and OS-9 respectively, while soyabean meal stimulated the lowest production of 2.21 and 1.93U/ml in both species. The covariance matrix analysis shows that there was a significant high value of effect in beef extract for protease production in *Bacillus* sp. RS-5 and OS-9.

The effect of trace elements on growth and protease production (Table 5) showed that maximum protease yield for *Bacillus* sp. RS-5 and OS-9 was obtained by MgSO4 at a concentration of 10 and 7 g/l respectively. KH_2PO_4 at a concentration of 5 and 7 g/l stimulated optimum protease production in *Bacillus* sp. RS-5 and OS-9 respectively, while $FeSO_4$ at a concentration of 0.4 g/l stimulated protease production in both species. The covariance matrix analysis shows that there was a significant high value of effect in 0.4g/l of $FeSO_4$ for protease production in *Bacillus* sp. RS-5 and OS-9.

The result of the effect of agitation on growth and protease production by *Bacillus* sp. RS-5 and OS-9 is shown in Table 6. Maximum protease yield was obtained at 200 rpm (3.45 U/ml) for *Bacillus* sp. RS-5 and 170 rpm for *Bacillus* sp. OS-9 (2.86 U/ml).The covariance matrix analysis shows that there was a significant high value of effect at agitation rates of 200 and 170rpm for protease production in *Bacillus* sp. RS-5 and OS-9 respectively.

Table 7 shows the result of effect of pH on growth and protease production by *Bacillus* sp. RS-5 and OS-9 is shown in Table 4. The highest protease yield for *Bacillus* sp. RS-5 (3.40 U/ml) and *Bacillus* sp. OS-9 (2.90 U/ml) was achieved at a pH 8.0, there was decrease in protease yield at pH 8.5 and 9 respectively. The bacteria growth for both isolates decreased at pH 8.0. The covariance matrix analysis shows that there was a significant high value of effect in pH of 8 for protease production in *Bacillus* sp. RS-5 and OS-9.

Table 1. Screening for protease-producing bacteria using solid agar

Bacteria isolate code	Gram reaction	Spore test	Average zone of clearance (mm)
RS-2	- cocci	-	9.0
IS-10	- rods	+	7.2
US-9	+ cocci	-	3.0
US-6	+ rods	+	9.3
RS-1	- cocci	-	3.8
OS-13	+ cocci	-	3.5
RS-4	+rods	+	6.4
US-2	+ rods	+	11.0
RS-5	+ rods	+	13.5
RS-11	+ cocci	-	6.3
IS-3	- cocci	-	7.7
IS-6	+ rods	+	9.6
IS-2	- rods	-	5.3
OS-7	+ cocci	-	4.1
OS-9	+ rods	+	12.1
IS-5	- cocci	-	4.0
US-3	+ rods	+	9.0
RS-7	- rods	-	5.3
RS-8	- rods	-	8.2
OS-2	- cocci	-	4.0
US-4	+ rods	+	6.2

Key: + represents positive; - represents negative

Table 2. Shake flask production of protease by bacteria isolated from soil

Bacteria isolate code	Protease activity (U/ml)
RS-2	1.35
IS-10	0.85
US-9	0.27
US-6	1.74
RS-1	0.67
OS-13	0.44
RS-4	1.31
US-2	2.45
RS-5	2.45
RS-11	0.53
IS-3	0.77
IS-6	2.18
IS-2	0.41
OS-7	0.55
OS-9	2.71
IS-5	0.93
US-3	1.84
RS-7	1.10
RS-8	1.50
OS-2	0.37
US-4	1.11

Table 3. Effect of carbon sources on growth and protease production by *Bacillus* species RS-5 and OS-9

Carbon source(0.5%w/v)	*Bacillus* species RS-5		*Bacillus* species OS-9	
	Protease activity (U/ml)	Bacterial growth (OD660nm)	Protease activity (U/ml)	Bacterial growth (OD660nm)
Maltose	3.12	1.93	2.77	1.47
Glucose	3.04	1.88	2.56	1.40
Starch	3.47	1.97	2.60	1.32
Lactose	2.91	1.76	2.44	1.47
Mannitol	2.62	1.60	2.25	1.30

Table 4. Effect of nitrogen sources on growth and protease production by *Bacillus* species RS-5 and OS-9

Nitrogen Source (1%w/v)	*Bacillus* species RS-5		*Bacillus* species OS-9	
	Protease activity (U/ml)	Bacterial growth (OD660nm)	Protease activity (U/ml)	Bacterial growth (OD660nm)
Gelatine	2.76	1.75	2.44	1.24
Soyabean meal	2.21	1.58	1.93	1.05
Tryptone	3.03	1.84	2.58	1.36
Peptone	3.23	1.89	2.71	1.45
Beef extract	3.35	2.03	2.90	1.57
Casein	3.16	1.85	2.76	1.41
Yeast extract	3.28	1.80	2.64	1.35

Table 5. Effect of trace elements on growth and protease production by *Bacillus* species RS-5 and OS-9

Trace element	Concentration (g/l)	*Bacillus* species RS5		*Bacillus* species OS9	
		Protease activity (U/ml)	Bacteria growth (OD66onm	Protease activity (U/ml)	Bacteria growth(OD660nm)
MgS04	3.0	2.58	1.61	2.36	1.41
	5.0	2.86	1.67	2.49	1.45
	7.0	2.97	1.75	2.72	1.52
	10.0	3.25	1.86	1.85	1.57
KH_2PO4	3.0	2.70	1.57	2.03	1.38
	5.0	2.93	1.66	2.19	1.43
	7.0	2.26	1.70	2.28	1.46
	10.0	2.13	1.78	1.72	1.37
FeS04	0.1	2.43	1.67	2.51	1.33
	0.2	2.60	1.73	2.68	1.41
	0.3	2.86	1.84	2.79	1.46
	0.4	3.28	1.72	2.85	1.51

Table 6. Effect of agitation rates on growth and protease production by *Bacillus* species RS-5 and OS-9

Agitation Rate(rpm)	*Bacillus* species RS-5		*Bacillus* species OS-9	
	Protease activity (U/ml)	Bacteria growth (OD660nm)	Protease activity (U/ml)	Bacteria growth (OD660nm)
80	1.51	1.60	0.97	1.31
100	1.74	1.63	1.20	1.34
120	2.27	1.65	1.50	1.42
150	2.96	1.70	2.18	1.49
170	3.30	1.77	2.86	1.54
200	3.45	1.82	2.85	1.63

Table 7. Effect of pH on growth and protease production by *Bacillus* species RS-5 and OS-9

pH	*Bacillus* species RS-5		*Bacillus* species OS-9	
	Protease activity (U/ml)	Bacterial growth (OD660nm)	Protease activity (U/ml)	Bacterial growth (OD660nm)
6.0	1.97	1.750	1.88	1.24
6.5	2.81	1.78	2.44	1.31
7.0	3.03	1.84	2.58	1.36
7.5	3.23	1.89	2.71	1.45
8.0	3.40	2.03	2.90	1.46
8.5	3.16	1.85	2.76	1.41
9.0	2.90	1.80	2.14	1.27

4. DISCUSSION

A total of 21 bacterial organisms isolated from the soil were found to be protease producers. The occurrence of protease-producing organisms from the soil agrees with the report of [1], who isolated protease producers from soil. [23], isolated proteolytic bacteria from soil samples of Ikogosi warm spring.

In the study, the supplementation of starch stimulated maximum protease yield by *Bacillus* sp. RS-5 and OS-9.This is corroborated by the report of [24], who showed that starch was the best carbon source for growth and protease production by *Bacillus subtilis*. Similarly, [25] also reported that starch caused high level of expression in *Bacillus* species. [26], reported that among the ten carbon sources studied, starch, sucrose and lactose proved appreciably good for the protease production. “In contrast, [27], reported that wheat bran supported the maximum production of protease by *Bacillus* species”.

In the study, it was observed that the addition of beef extract stimulated enhanced protease production by *Bacillus* sp. RS-5 and OS-9.This is similar to the findings of [25], who observed that beef extract enhanced protease production by *Bacillus cereus* strain 146. Also, [27], reported that beef extract was the best nitrogen source for protease production by *Bacillus* species. In contrast, [28], found skim milk to have significant effect on the production of protease by *Bacillus cereus* strain CA15.Beef extract is believed to play an important role in enzyme production due to the presence of essential elements and growth factors.

Different concentration of trace elements ($MgSO_4$, KH_2PO_4, $FeSO_4$) used in the study encouraged growth and protease yield [1]. Reported that combination of Ca^{2+} and Mg^{2+} in medium stimulated the highest protease production by *Bacillus* sp. N-40 isolated from the soil, but noted that both ions were not effective alone. Ire et al. [29], reported that $FeSO_4$ favoured highest production of the protease by *Aspergillus carbonarius*, compared to other ones evaluated. Trace elements play a vital role in fermentation as they are required to activate enzymes [30], Fe^{2+} and Mn^{2+} seem to be the most important of the trace elements as they play a role in the excretion of primary metabolites.

In the study *Bacillus* sp. RS-5 and OS-9 were observed to produce maximum protease at agitation rate of 200 and 170 rpm respectively [2] and [31]. Reported maximum protease production at 200 rpm using *Bacillus licheniformis* NCIM-2042 and *B. subtilis* strain Rand respectively. For *Bacillus* species OS9, it was observed that at 200 rpm protease production reduced this could be as a result of excessive agitation which may lead to cell lysis and denaturation of enzymes. At the speed of 170 and 200 rpm, it is opined that aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media [32]. Nutrient uptake by bacteria also would be increased [7] resulting in increased protease production [25]. Pointed out that mixing is especially important because oxygen is a very low solubility nutrient. Agitation intensity provide homogeneity and influences the oxygen transfer rate in many bacterial fermentations thereby influencing growth and product formation.

The result of the study, revealed that optimum protease yield was achieved at pH 8.0 by *Bacillus* sp. RS-5 and OS-9. This corroborate the report of [33] who considered the pH of 8.0 as the best pH for protease production by *Bacillus subtilis.* Also [23], observed maximum protease production at pH 8.0. In contradiction, [27] observed maximum protease production at pH 9 for *Bacillus* species K-30 using rice bran. The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. [34], reports maximum enzyme production was observed in the culture medium of pH 9.0. “However, a relatively high enzyme yield was achieved between pH 7 and pH 10. A similar result (pH 9.0) and closely related one (pH 10) were obtained from proteases produced from a *Bacillus* species and *B. Halodurans”* [35,27].

Generally, the pH of a culture medium affects both the morphological and physiological characteristics of an organism [36] and [37] observed maximum lipase at a pH 8. “Microorganisms vary in their oxygen requirements. In particular, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities” [6].

5. CONCLUSION

In the study, two protease – producing *Bacillus* species were isolated from the soil. During the submerged fermentation of protease, it was revealed that *Bacillus* sp. RS-5 and OS-9 accumulated maximum protease yield of 3.23 and 2.71 U/ml respectively. The Optimization studies conducted on the bacteria isolates showed that the supplementation of starch and beef extract, some trace elements improved protease production. Agitation rates (200 and 170 rpm) and pH 8 encouraged enhanced protease production. The *Bacillus* species isolated in the study, have shown potential for protease production and can be used for large scale production of the enzyme, to meet present-day needs in the Nigeria’s industrial sector. The microbiological process of protease production, if well developed could lead to the availability of the product in Nigeria and this to some extent will reduce the importation of the product into the country. Further research is ongoing to study the effect of other parameters on protease production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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