

Screening for Protease Producing Bacteria from Agricultural Soils

Dienye, Blessing Nteimam^{1*}; Agwa, Obioma Kenekwku² and Worlu, Favour¹

¹Department of Microbiology, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Nigeria.

²Department of Microbiology, University of Port Harcourt, Rivers State, Nigeria.

*Corresponding Author: blessing.dienye@ust.edu.ng

ABSTRACT

Microorganisms are known to produce a variety of enzymes that have dominating applications in industrial sectors. Proteases are an important group of enzymes produced by many organisms and are well-known for their extensive distribution in industrial and medical uses. The study aimed to isolate and screen bacteria from soil samples with protease producing potential. Protease hydrolyzing bacteria were isolated from soil samples using standard microbiological procedures. The isolates were screened for protease producing potential using Skimmed Milk Agar (SMA) medium. Isolates with high Proteolytic activity index were selected and further screened quantitatively in liquid medium to determine protease enzyme activity. The efficient isolates were then identified through morphological and biochemical characteristics. Twenty-seven (27) bacterial isolates were isolated and screened, and sixteen (16) exhibited the ability to secrete protease with different potential. Based on the proteolytic index, three strains of bacteria; *Bacillus licheniformis*, *Bacillus cereus*, and *Bacillus subtilis*, showed high proteolytic index values of 2.8mm, 1.87mm, and 2.67mm respectively, with efficiency of protease enzyme activity of 8.46U/mL, 5.4U/mL, and 11.7U/mL, respectively. Therefore, it could be concluded that the *Bacillus* species isolated from soil source has protease production capacity. However, molecular characterization and optimization of protease production on conventional and agro-industrial waste as substrate for maximum utilization of the strains should be explored.

Keywords: Protease, Agricultural Soil, Bacteria, Enzyme activity, Skimmed milk agar

Introduction

Microorganisms contribute significantly to the production of various industrial enzymes (Usman *et al.*, 2023). Enzymes are specialized proteins with specific biocatalytic properties (Sukmawati *et al.*, 2020). Enzyme specificity and efficiency make them invaluable in various sectors, and their demand is increasing rapidly (Gurung *et al.*, 2013; Banerjee and Ray, 2017). Currently, different enzymes like amylases, cellulases, lipases, proteases, and xylanases are employed in industries; however, proteases are extensively utilized industrial enzymes with both degradative and synthetic activity (Mótyán *et al.*, 2013; Solanki *et al.*, 2021; Pawar *et al.*, 2023). Proteases are essential for metabolic and regulatory processes across cells, organs, and systems (Ekedegba *et al.*, 2022).

This hydrolase group of enzymes assists in cleaving protein into smaller peptides or amino acids through proteolysis (Rasheed 2021; Ullah *et al.*, 2022). Protease enzymes are categorized as either exopeptidases or endopeptidases depending on their site of action (Shaikh *et al.*, 2023).

They are further classified into aspartic, cysteine, metalloprotease, serine, and threonine proteases according to their catalytic mechanisms (Ratnaningrum *et al.*, 2022). Additionally, based on their acid-base behavior, they are classified as acid, neutral, or alkaline proteases (Thanoon *et al.*, 2021).

Proteases can be synthesized by microorganisms, animals, and plants, and the enzymes of microbial origin meet industrial demand (Razzak *et al.*, 2019; Fasim *et al.*, 2021; Adetunji *et al.*, 2023; Solanki *et al.*, 2021). Among the various proteases, bacterial extracellular proteases are more beneficial than other sources (Raju and Divakar, 2013).

Microbial proteases are gaining popularity in diverse applications like food, pharmaceuticals, detergents, leather processing, chemical, agricultural, and environmental (Shad *et al.*, 2024; Chimbekujwo *et al.*, 2020). However, protease use has expanded to include protein hydrolysate, brewing, waste treatment, and grain mashing (Maitig *et al.*, 2018). The use of proteases has widened in different industries and has placed greater stress on increasing indigenous protease production.

Hence, the potential exists to search for new proteases with new physicochemical characteristics from sources (Rupali, 2015; Idakwoji, 2022). Several microorganisms have been discovered for decades that can convert protease into amino acids, but the need for newly promising strains of protease producers is a continuous process (Lemenh *et al.*, 2021). This study was carried out to isolate and screen bacterial isolates with protease potential from agricultural soil, and to determine the protease activity.

Materials and Methods

Sample collection

Soil samples were obtained from farms where cassava, coconut, and vegetables were cultivated, including soil from piggery and slaughterhouse at Rivers State University from 10–15cm depth with the help of a soil auger into a sterile plastic bag. The samples were transported to the laboratory for analysis.

Isolation and Purification of bacteria isolates

Serial dilution method was adopted for the isolation of bacteria from the soil sample. One (1) gram of each soil sample was aseptically suspended in 9 mL sterile normal saline and serially diluted in the range of 10^{-1} to 10^{-5} . An aliquot of 10^{-4} was taken from each test tube and aseptically inoculated using spread plate method onto sterilized and solidified Nutrient agar plates. The plates were incubated at 30°C for 24 hours.

After incubation, morphologically contrasting colonies observed on the plates were selected and subcultured 2-3 times by streaking on nutrient agar plates to obtain a pure culture of the organism (Muzaifa *et al.*, 2023).

Maintenance of isolated microorganisms

The pure culture obtained was subsequently transferred into already sterilized nutrient agar slants. The slants were kept in the refrigerator at 4°C as stock culture.

Screening of the isolates for protease potential

Primary screening

The isolated colonies were screened for protease-producing potential using plate assay method described by Hamza and Woldesebet (2017).

Each pure bacterial isolate was spot inoculated on already prepared Skimmed Milk Agar medium containing (% (w/v)) peptone 0.1, NaCl 0.5, Skimmed milk 10, and Agar 2. The plates were incubated at 35°C for 24 hours. After incubation, the plates were observed for zones of hydrolysis. The production of protease was viewed by the appearance of a clear zone surrounding isolates, and the diameter of the clear zone and colony sizes were measured. The higher the protease index, the more protease enzyme is produced by the bacterial isolate. To identify isolates with higher protease potential, the Enzymatic Index (EI) was calculated for each bacterial culture using the formula described by Yang *et al.* (2021).

$$\text{Proteolytic index} = \frac{\text{Clear zone size} - \text{Colony Size}}{\text{Colony size}}$$

Morphological characterization of bacterial isolates

The isolated bacteria were identified by using standard morphological characteristics. Following a 24 hours incubation, morphological features such as shape, size, color, form, elevation, and margin were examined, and gram staining was performed. Different biochemical tests such as catalase, coagulase, indole, Voges Proskauer, citrate utilization test, starch hydrolysis, carbohydrate fermentation of glucose, sucrose, and Lactose were used as described by Fachrial *et al.* (2021).

Secondary screening

The potential isolates selected from primary screening were then evaluated for protease activity in a liquid medium.

Identification of protease producing bacteria

Potential isolates were tentatively identified by means of morphological and biochemical characterization according to Bergey's manual of determinative bacteriology (Holt 1994).

Inoculum preparation for protease production

Inoculum of isolates showing high proteolytic index was developed in broth containing (g/100ml) of 0.5 peptone and 0.5 NaCl, maintained at pH 7. 10 ml of broth was prepared, and potential bacterial colonies were inoculated under aseptic conditions, and flasks were incubated at 37 °C for 24 hours. After incubation, growth turbidity appeared in the inoculated broth preparation and was directly used as a source of inoculum (Tohoum and Hamza 2022).

Submerge fermentation

Protease production was carried out by submerged fermentation, adopting the method of Lakshmi *et al.* (2017). The bacterial isolates were inoculated into separate 250 mL Erlenmeyer flasks containing 50 mL fermentation media with the following composition (g/L): glucose 5.0, peptone 7.5, KH₂PO₄ 5.0, FeSO₄ 7H₂O 0.1, MgSO₄ 7H₂O 5.0. The flasks were incubated at 37°C at 160 revolutions per minute (rpm) for 48 hours.

Extraction of enzyme

After incubation, the Culture filtrate was centrifuged at 10000 rpm for 10 minutes, and the clear supernatant obtained was the crude enzyme solution, which was utilized for the determination of protease enzyme activity (Rupali, 2015)

Protease enzyme assay

Protease activity in the culture supernatant of each bacterial isolate was determined as described by Legesse (2017). The substrate solution was made by dissolving 1% (w/v) of casein in 50 mM sodium phosphate buffer (pH 7). To each test tube, 1 mL of 1% (w/v) casein in 0.1M phosphate buffer pH 7.0 was added, followed by 1 mL crude enzyme extract. The test tubes were incubated at 37 °C for 20 minutes. After incubation, 2 ml of 10% (w/v) trichloroacetic acid was added to each sample to stop the reaction and then kept at room temperature for 20 minutes. The reaction mixture was centrifuged at 10000 rpm for 15 minutes. 0.5 ml of supernatant was taken and mixed with 2.5 ml of 0.5M sodium carbonate. Then, 0.5ml folin phenol reagent was added, and the resulting solution was incubated at room temperature for 20 minutes.

The absorbance of all the test tubes was read at 660nm against a reagent blank using a tyrosine standard. The tyrosine standard graph was prepared following the protocol of Shad *et al.* (2024).

Calculation of protease activity

The concentration of tyrosine produced for each solution was obtained from the tyrosine standard curve, and then, protease activity was calculated using the formula of Mardina *et al.* (2018).

$$\text{Protease activity (U/ml)} = \frac{\mu \text{ mole of tyrosine released} \times \text{Reaction vol}}{\text{Enzyme vol (ml)} \times \text{Reaction time (minutes)} \times \text{Vol assay}}$$

Protease activity was defined as the amount of enzyme required to hydrolyze casein to give 1 µg of tyrosine per minute under the above assay conditions of supernatant.

Results

A total of twenty-seven (27) morphologically distinct bacterial isolates were isolated from the soil samples collected. Eleven (11) were isolated from the vegetable farm soil samples, five (5) from coconut farm soil, five (5) from slaughter site soil, three (3) from cassava farm soil and three (3) isolates from pig farm soil (Table 1). Of these, 16 (61.5%) isolates expressed protease producing potential haven been observed to give zone of clearance around their colonies, after they were spot inoculated on skimmed milk agar plate as shown in Plate 1. While 10 (38.5%) of the isolates showed no protease potential (Table 1).



Plate 1: Zones of clearance formed around bacterial colonies in skimmed milk agar plates

Result of the Primary screening protease producing bacterial isolates showed that, all sixteen (16) positive isolates selected for further investigation produced protease by showing different variation in the size of the clear zone of hydrolysis they produced on skimmed milk agar plates, which ranged from the least of 13mm to the largest 57mm (Table 2). Highest clear zone of hydrolysis, 57mm, was obtained from VF4, followed by SS2 with 55mm, while the lowest clear zone of hydrolysis, 13mm, was obtained from SS4.

The Largest colony size of 25mm was obtained from VF8, while the smallest 9mm was obtained from VF1. The selection of potent bacteria was done by comparing the isolates with each other in terms of both their diameter of clear zone of hydrolysis and colony size. Result shows that from the 16 isolates, the highest proteolytic index value was found in VF4 isolate, which was 2.8, followed by VF7 with proteolytic index value of 2.67, SS2 had proteolytic index value of 1.87, respectively, while the lowest proteolytic index value of 0.3 was obtained by CF4 (Figure 1).

Table 1: Clear zone formation of distinct bacteria colonies and screening result of the selected isolates for Protease producing potential

Isolate source	Isolate code	Clear zone formation for distinct colonies	Primary Screening for Selected bacteria for Protease potential	
			Clear zone size (mm)	Colony Size (mm)
Cassava farm	CAF1	-		
	CAF2	+	25	18
	CAF 3	+	27	20
Coconut farm	CF1	-		
	CF2	-		
	CF3	-		
	CF4	+	26	20
	CF5	+	30	22
Pig farm	PF1	-		
	PF2	-		
	PF3	+	30	15
Slaughterhouse	SS1	-		
	SS2	+	55	15
	SS3	+	20	13
	SS4	+	13	11
	SS5	-		
Vegetable farm	VF1	+	15	9
	VF2	+	17	10
	VF3	+	17	10
	VF4	+	57	15
	VF5	+	22	18
	VF6	+	26	17
	VF7	+	43	15
	VF8	+	50	25
	VF9	-		
	VF10	-		
	VF11	-		

Key: (+) = Colonies which form clear zone; (-) = Colonies with no clear zone formation

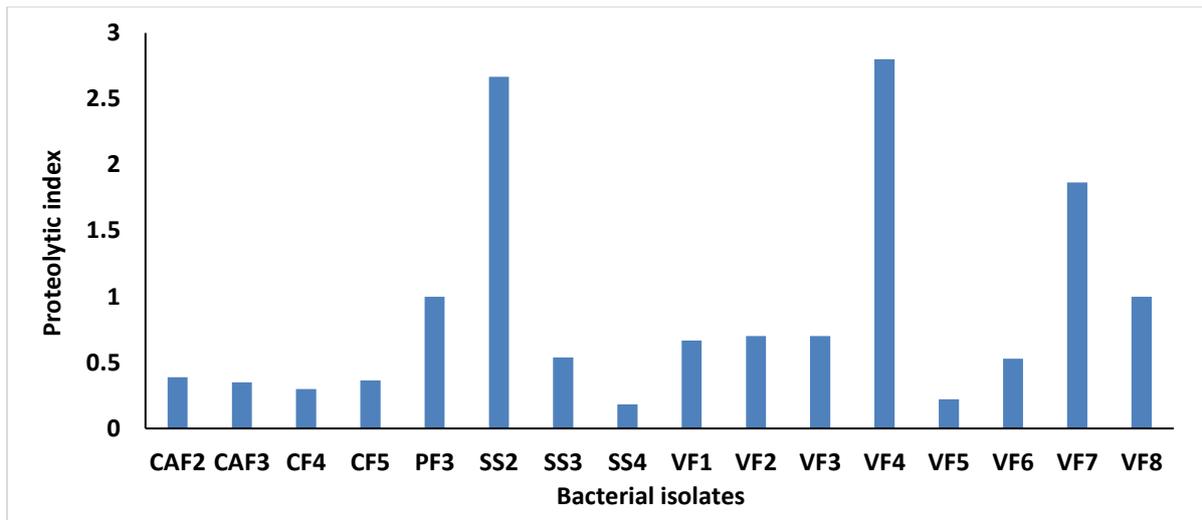


Figure 1: Clear zone size to colony size ratio (mm) of bacteria isolates

The cultural and morphological characteristics of the three potent protease producing isolates on nutrient agar medium are summarized in Table 2. The result reveals that all the isolates had cream, smooth, and round colonies. Two isolates had large colony sizes, while one had a medium colony size.

Microscopic observation indicated that the three strains of protease producing bacteria were rod-shaped bacteria. In gram staining technique, the isolates were all Gram-positive. The results of the biochemical test for the bacterial isolates are summarized in Table 2. According to presumptive identification, the isolates were tentatively identified to belong to the genus *Bacillus* and *Staphylococcus* with the following species; *Bacillus coagulans*,

Bacillus licheniformis, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus* and *Staphylococcus aureus*.

The result of the secondary screening or quantification of protease activity (U/mL) of the three potent protease-producing isolates is presented in Figure 2. The result revealed that, Protease enzyme activity was observed after 48 hours of incubation at 30 °C, and significant enzymatic activity of 11.7U/mL was exhibited by isolate SS2, followed by isolate VF4 with protease activity of 8.46U/mL, while the lowest protease enzyme activity of 5.4 U/mL was observed by isolate VF7, as shown in Figure 2.

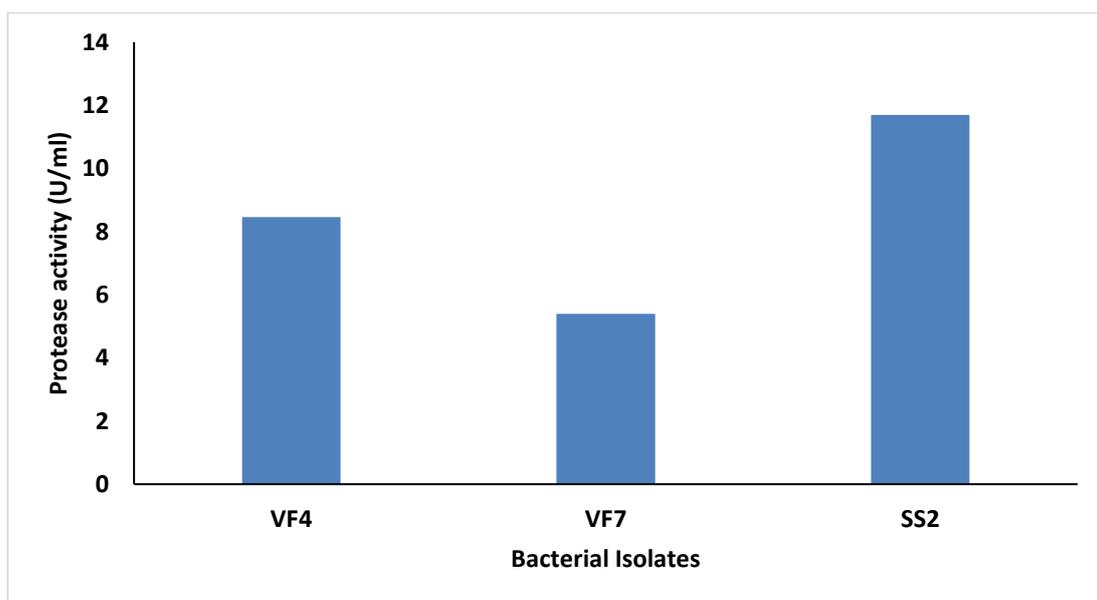


Figure 2: Protease enzyme activity of selected protease producing bacteria

Table 2: Cultural, morphological, biochemical characteristics and probable identity of protease producing bacteria isolated from agricultural soil

Isolate code	Cultural Morphology					Microscopy		Biochemical								Sugar fermentation				Probable organism	
	Colour	Elevation	Opacity	Size	Texture	Gram rxn	Shape	Catalase	Indole	Coagulase	Motility	Oxidase	MR	V.P	Starch	Citrate	Maltose	Glucose	Lactose		Sucrose
CAF2	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	-	+	+	+	-	<i>Bacillus coagulans</i>
CAF 3	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	-	+	+	+	-	<i>Bacillus coagulans</i>
CF4	Yellow	Convex	Opq	Small	Smooth	GPC	Cocci	+	-	+	-	-	+	+	+	+	+	+	+	+	<i>Staphylococcus aureus</i>
CF5	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	-	+	+	+	-	<i>Bacillus coagulans</i>
PF3	Cream	Flat	Opq	Medium	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	+	+	+	-	+	<i>Bacillus cereus</i>
SS2	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	+	-	+	+	+	+	+	-	+	<i>Bacillus subtilis</i>
SS3	White	Flat	Opq	Large	Smooth	GPC	Cocci	+	-	-	+	-	-	+	-	-	+	+	+	-	<i>Bacillus coagulans</i>
SS4	Cream	Flat	Opq	Small	Smooth	GPR	Rod	+	-	+	+	-	-	+	+	-	+	+	+	-	<i>Staphylococcus aureus</i>
VF1	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	-	+	+	+	-	<i>Bacillus coagulans</i>
VF2	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	-	+	+	+	-	<i>Bacillus coagulans</i>
VF3	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	+	+	+	-	+	<i>Bacillus cereus</i>
VF4	Cream	Flat	Opq	Large	Smooth	GPR	Rod	+	-	-	+	+	+	-	+	+	+	-	+	+	<i>Bacillus licheniformis</i>
VF5	Cream	Flat	Opq	Medium	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	-	+	+	+	-	<i>Bacillus coagulans</i>
VF6	Cream	Flat	Opq	Medium	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	-	+	+	+	-	<i>Bacillus coagulans</i>
VF7	Cream	Flat	Opq	Medium	Smooth	GPR	Rod	+	-	-	+	-	-	+	+	+	+	+	-	+	<i>Bacillus cereus</i>
VF8	Cream	Flat	Opq	Medium	Smooth	GPR	Rod	+	-	-	+	+	-	+	+	+	+	+	-	+	<i>Bacillus subtilis</i>

Key: Opq=Opaque; GPR = Gram positive rod; GPC = Gram positive cocci. + = positive; - = negative

Discussion

The present study aimed at isolating and screening potent protease producers from soil of Rivers State University. Bacteria producing protease were encountered and isolated from the soil, with vegetable farm having the highest number of isolates and the soil of cassava farm and piggery having the lowest. The occurrence of protease organisms in soil agrees with Vakilwala and Patel (2017), who isolated protease producing bacteria from soil samples collected from various regions. Parthasarathy *et al.* (2020) isolated protease bacteria from soil and sediment. Patel *et al.* (2020) reported the isolation of novel protease producing bacteria from rhizosphere soil. Balakrishnan and Sindhu (2019) isolated and identified 6 protease degrading bacteria from soil, and its protease production was studied. In addition, this could be isolated from places such as cow dung and abattoir effluent (Ogunnusi and Olorunfemi, 2018), coal mine (Afridi *et al.*, 2019), and fish sauce (Hang *et al.*, 2019).

Skimmed milk agar plate obtained many isolates with the potential to produce pectinase enzyme, marked by the formation of a clear zone around proteolytic bacteria. The creation of a hydrolytic zone on skimmed milk plates serves as the primary indicator in the screening procedure (Usman *et al.*, 2023). Skimmed milk contains casein (milk protein) consisting of phosphoproteins, which bind to calcium to form caseinate, giving it a white color (Ramadhan *et al.*, 2021). The disappearance of the white color and the formation of a clear zone were due to the breakdown of protein substrate contained in the skimmed milk into amino acid and peptide (Kabense *et al.* 2019; Mankge *et al.* 2024).

The use of skimmed milk agar for qualitative proteolytic activity of bacteria aligns with previous findings by Asha and Palnaiswamy (2018) and Kotb *et al.* (2023), and the property of clear zone around their colonies agrees with the findings of this study. The result is also in agreement with earlier findings of protease producing bacteria from different sources (Akshatha *et al.* 2020; Masi *et al.* 2021).

Recently, seven bacterial isolates were screened by Chakraborty *et al.* (2020) for their capabilities to produce protease using formation of clearing zone on skimmed milk agar, and bacterial colony with a measurable diameter of clear zone was selected.

During the screening of protease producing bacteria isolates in this study, it was noticed that the protease capacities of isolates vary quite considerably by the zone of clearing observed around the colonies of the isolates. This variation might be due to differences in the metabolic capabilities of the different isolates. This finding aligns with observations of Fashola *et al.* (2021), who reported 11 out of 18 isolates showing a clear zone diameter of more than 10mm. Patil and Jadhav (2017) reported that among 28 proteolytic bacteria isolated from dairy industry soils, three were considered good protease producers, exhibiting clearance zone higher than 18mm on skimmed milk agar plates incubated for 24-72h at 37 °C. Similarly, Artha *et al.* (2019) reported that the largest proteolytic zone obtained was 42mm from *Bacillus lentus*. A previous study by Hamza and Azmach (2018) documented clearance zones of 16mm and 26.5 mm, respectively, for two bacterial isolates. Additionally, studies by Ghumro *et al.* (2019) noted that one bacterial isolate exhibited maximum hydrolysis of 5.4mm out of nine bacteria screened for protease potential.

Enzymatic activity index (EAI) was used as a semi-quantitative parameter to select isolates as highly protease producers in solid media (Thongekkaew and Kongsanthia 2016). A higher EAI reveals the strength of each strain to secrete protease enzyme (Riady *et al.*, 2024). In this study, the bacterial isolates selected demonstrated variations in protease index value. These findings are consistent with the research of Santosa and Setiyaningrum (2024), who screened bacteria capable of producing protease with a PI of 1.12 to 2.89. Similarly, Mulyasari *et al.* (2018) successfully isolated bacteria from soil and identified potential protease producers with PI value of 0.3-3.3. In a similar work, Hamdani *et al.* (2019) reported that 2 out of 10 isolates exhibited clear zone with corresponding PI values of 0.561 and 0.625. However, the proteolytic index from our bacteria isolates was higher than those reported for nineteen lactic acid bacteria, which exhibited PI values ranging from 0.2 to 0.6 as documented by Ramadhan *et al.* (2021).

The three isolates with high EAI were provisionally identified as *Bacillus substilis*, *Bacillus licheniformis*, and *Bacillus cereus*. This study also corroborates the findings of Patil and Jadhav (2017) who identified 3 isolates (*Bacillus cereus*, *Bacillus licheniformis* and *Bacillus megaterium*) as significant protease producers.

Previous studies of Rupali (2015); Hadush *et al.* (2017); Su *et al.*, 2020) have also showed that most proteolytic bacteria belong to the genus *Bacillus*. Afrin *et al.* (2024) reported that from 7 bacterial strains identified as protease producers, four were *Bacillus* sp and two exhibited greater amount of protease activity than the other studied strains.

Similarly, *Bacillus* species have been reported to be dominant protease hydrolyzing bacteria in samples collected from soil, industrial waste, and processing sites (Hamza, 2017). Members of the genus *Bacillus* produce a large variety of extracellular enzymes (proteases), particularly significant for industrial applications (Hamza 2017)

The quantitative screening under submerged fermentation in this study revealed that *Bacillus substilis* accumulated maximum amount of protease, followed by *Bacillus licheniformis* and *Bacillus cereus*. The enzymatic activity of protease produced differs depending on the type of bacteria (Raigy *et al.*2022). This is in agreement with the study of Umeh *et al* (2015) who noted that *Bacillus substilis* SE 2 and SU 8 accumulated highest protease level.

This study is in contrast with the study of Gill *et al.* (2016) who reported that maximum protease activity was obtained by *Bacillus megaterium* (121.3U/mL) followed by *Bacillus substilis* (117.5U/mL). Protease production higher than the present findings was also reported earlier. Masi *et al.* (2021) successfully measured protease activity in *Bacillus cereus* isolated from leather effluent as 19U/mL.

Conclusion

In conclusion, strains isolated from soil samples of Rivers State University have the capability to produce protease. From the 26 isolates cultured on skimmed milk agar, 16 exhibited a halo of hydrolyzed substrate in the solid medium, and three stood out for having high enzymatic activity index.

Three bacteria species, *Bacillus substilis*, *Bacillus licheniformis*, and *Bacillus cereus*, exhibited maximum protease activity when assessed through liquid medium fermentation and measured using protease assay. These microorganisms with proteolytic activity could be further explored to optimize culture conditions to enhance protease production.

References

- Adetunji, A. I., Olaitan, M.O., Erasmus, M. & Olaniran, A. O. (2023). Microbial proteases: A next generation green catalyst for industrial, environmental, and biomedical sustainability. *Food Materials Research*, 3, 12.
- Afridi, M. I., Ali, N.1, Memon, A. R., Qasim, M., Jamal, Q., Khattak, B., Adnan, M., Khan, S. N., Ullah, A., Younas, F. & Ullah, F. (2019). Protease producing *Pseudomonas aeruginosa* strain (ibc-2) from coal mines of Orakzai agency, Pakistan, *applied ecology and environmental research*. 17(3), 6081-6093.
- Afrin, S., Tamann, T., Shahajadi, U. F., Bhowmik, B., Jui, A.H., Satter, M. A, Nazrul, M. & Bhuiyan, I. (2024). Characterization of protease-producing bacteria from garden soil and antagonistic activity against pathogenic bacteria. *The Microbe*, 4, (100123).
- Akshatha, J., Sura, N. K., Shwetha, H., Hiremath, L. & Srivastava, A. J. (2020). Isolation and screening of alkaline protease producing bacteria from fermented foods. *International Journal of Current Advanced Research*, 9(05), 22218-22222
- Artha, O.A., Sudarno, Pramono, H. & Sari, L. A. (2019). Identification of extracellular enzyme-producing bacteria (proteolytic, cellulolytic, and amylolytic) in the sediment of extensive ponds in Tanggulangrejo, Gresik, IOP Conference Series: *Earth Environment and Science*. 236 (012003).
- Asha, B. & Palnaiswamy, M. (2018). Optimization of alkaline protease production by *Bacillus* FT1 isolated from soil. *Journal of Applied Pharmaceutical Sciences*, 8(02), 119-127.
- Balakrishnan, D. & Sindhu, A. P. (2019). Optimization and Phylogenetic Analysis of Alkaline Protease Production by *Bacillus sonorensis* SNP3 Isolated from Soil. *International Journal of Advanced Biotechnology Research*, 9(1), 1-12
- Banerjee, G. & Ray, A. K. (2017). Impact of microbial proteases on biotechnological industries. *Biotechnology and Genetic Engineering Reviews*, 33(2), 119–143.

- Chakraborty, S., Roy, S., and Dutta, S. (2020). Isolation, optimization, and characterization of protease producing bacteria from soil collected from East Kolkata Wetland. *International Journal of Multidisciplinary Educational Research*, 9 (5), 212-226.
- Chimbekujwo, K. I., Ja'afaru, M. I. & Adeyemo, O. M. (2020). Purification, characterization, and optimization conditions of protease produced by *Aspergillus brasiliensis* strain BCW2. *Scientific African* 8, e00398
- Ekedegba, F. E., Ogbonna, A. I., Okoye, C. T., Ogbonna, U. S. A., Onyimba, I. A. & Madu, J. M. (2022). Optimization Studies on Extracellular Protease Production by *Aspergillus niger* and *Aspergillus terreus* Using Skim Milk Casein as Substrate. *Journal of Advances in Biology & Biotechnology*, 25 (7), 11-19.
- Fachrial, E., Krisdianilo, V., Harmileni, Lister, I. N. E., Nugroho, T. T. & Saryono. (2021). Isolation, characterization, activity test, and molecular identification of thermophilic bacteria producing proteases from Dolok Tinggi Raja Natural Hot Springs, North Sumatra, Indonesia. *Biodiversitas*, 22, 1725-1732.
- Fashola, F.A., Fadipe, O.T., Nwagala, P.N., Olatope, S.O., Augustine, C.P., Ibidapo, O.I., James, I.C., Aderinwale, F.B., Orji, F.A. & Lawal, A.K. (2021). Characterization of Novel Alkaline Protease Producing *Bacillus subtilis* C3a-FIIRO with Potential for Industrial Application. *Nigeria Journal of Biotechnology*, 38 (2), 56-66
- Fasim, A., More, V. S. & More, S. S. (2021). Large-scale production of enzymes for biotechnology uses. *Current Opinion in Biotechnology*, 69, 68-76.
- Gerlicz, W., Sypka, M., Jodlowska, I. & Bialkowska, A. M. (2024). Isolation, Selection and Identification of Keratinolytic Bacteria for Green Management of Keratin waste. *Molecules*, 29 (14), 3380.
- Gill, S. S., Shrivastav, A. & Jana, A. M. (2016). Isolation and identification of Protease producing bacteria through biodegradation of protein content of kitchen wastes in Gwalior, India. *International Journal of Current Microbiology and Applied Science*, 5 (10), 204-211.
- Gurung, N., Ray, S., Bose, S. & Rai, V. (2013). A Broader View: Microbial Enzymes and their Relevance in industries, medicine, and beyond. *Biomed Research International*, 2013, 329121
- Hadush, M., Andualem, B., Kebede, A., Gopalakrishnan, V. K., & Chaithanya, K. K. (2017). Isolation of Protease Producing Bacteria (*Bacillus* spp.) From Soil and Water Samples of Gondar Town, Ethiopia. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 8(5), 211-222
- Hamdani, S., Asstiyani, N, Astriany, D., Singgih, M. & Ibrahim, S. (2019). Isolation and identification of proteolytic bacteria from pig sludge and protease activity determination. IOP Conference Series: *Earth Environment and Science*, 230, 012095.
- Hamza, T. A. (2018). Isolation and Characterization of Protease Producing Bacteria from Soil, in Arba Minch University, Abaya Campus. *American Journal of Biological and Environmental Statistics*, 4(1), 10-14.
- Hamza, T. A. (2017). Isolation and screening of protease producing bacteria from local environment for detergent additive. *American Journal of Life Sciences*, 5(5), 116-124.
- Hamza, T.A. & Azmach, N. N. (2018). Studies on protease producing bacterial Biodiversity from soil collected from Arba Minch University, Abaya Campus, Southern Ethiopia. *Agricultural and Biological Science, Journal*, 4(1), 1-6.
- Hamza, T.A. & Woldesenbet, F. (2017). Optimization of culture growth parameters for production of protease from bacteria isolated from soil. *Bioscience and Bioengineering*, 3(1), 1-10.
- Hang, N. T., Thu, N.M., Ha, N.N. and Ha, L.T (2019). Isolation, Screening and Identification of Protease Producing Bacteria from Fish Sauce. *Journal of Science and Technology*, 134 (2019), 064-068.
- Holt, J., Krieg, N., Sneath, P., Staley, J. & Williams, S. (1994). *Bergey's Manual of Determinative Bacteriology*, Williams & Wilkins, Baltimore, MD, USA, 9th edition
- Idakwoji, J. A. (2022). Exploration of Moulds for Extracellular Protease Production. *Dutse Journal of Pure and Applied Sciences*, 8(4a), 1-8.

- Kabense R, Ginting E L, Wullur S, Kawung N J, Losung F & Tombokan J L (2019). Screening of the proteolytic bacteria symbiont with algae *Gracillaria* sp. *Jurnal Ilmiah Platax*. 7, 413–418.
- Kotb, E., Alabdallal, A. H., Alsayed, M. A., Alghamdi, A. I., Alkhalidi, E., AbdulAzeez, S., & Francis Borgio, J. (2023) Isolation, Screening, and Identification of Alkaline Protease-Producing Bacteria and Application of the Most Potent Enzyme from *Bacillus* sp. Mar64. *Fermentation*, 9(7), 637.
- Lakshmi, B.K.M, Ratna Sri, P.V., Ambika Devi, K., & Hemalatha, K. P. J. (2014). Media optimization of protease production by *Bacillus licheniformis* and partial characterization of Alkaline protease. *International Journal of Current Microbiology and Applied Science*, 3(5), 650-659
- Legesse, D.Y. (2017). Optimization and Partial Characterization of *Bacillus* Protease Isolated from soil and agro-industrial waste. *International Journal of Nitration and Food Sciences*, 6(1), 31-38.
- Lemenh, Y. A., Biru, T. G., Chernet, A. Z. & Lema, F. B. (2021). Isolation and identification of protease-producing bacteria from sludge and sediment soil around Adama, Ethiopia. *Indonesian Journal of Biotechnology*, 26(4), 159-165.
- Maitig, A. M. A., Alhoot, M.A.M.& Tiwari, K. (2018). Isolation and Screening of Extracellular Protease Enzyme from Fungal Isolates of Soil. *Journal of Pure and Applied Microbiology*, 12(4), 2059-2067.
- Mankge, M.E, Maela, M. P., Abraham, A. M. & Serepa-Dlamini, M. H. (2024). Screening of *Bacillus* spp. Bacterial endophytes for protease production and application in feather degradation and bio-detergent additive. *Heliyon*, 10(2024) e30736
- Mardina, V., Harmawan, T., Fitriani, Hildayani, G.M., & Yusof, F. (2018). Screening of Protease and Lipase Sources from Viscera of *Euthynnus affinis* IOP Conference Series: *Materials Science and Engineering* 420 (012083), 1-6
- Masi, C., Gemechu, G. & Tafesse, M. (2021). Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent. *Annals of Microbiology* 71, 24 (2021)
- Mótyán, J. A., Tóth, F. & Tőzsér, J. (2013). Research Applications of Proteolytic Enzymes in Molecular Biology, *Biomolecules*, 3(4), 923-942.
- Mulyasari, M., Sunarno, M.T.D. & Suryaningrum, L.H. (2018). Isolation, characterization, and identification of proteolytic bacteria to improve protein digestibility of fish feed ingredients. *Indonesian Aquaculture Journal*, 13 (2), 79-89.
- Muzaifa, M., Murlida, E., Nilda, C., Rozali, Z.F. & Rahmi, F. (2023). Isolation and identification of Protease producing bacteria from *belacan depik*, a traditional fermented fish of the Gayo tribe. IOP Conference Series: *Earth and Environmental Science*, 1177(012038), 1-5.
- Ogunnusi, T. A. & Olorunfemi, O. (2018). Isolation and Identification of Proteolytic and Lipolytic Bacteria in Cow Dung and Abattoir Effluent from Ekiti General Abattoir, Ekiti State, Nigeria. *Journal of Advances in Microbiology*, 11 (4), 1-10.
- Parthasarathy, M. & Gnanadoss, J. J. (2020). Purification and characterization of extracellular alkaline protease from *Streptomyces* sp. LCJ12A isolated from Pichavaram mangroves. *Journal of Applied Biology and Biotechnology*, 8(1), 15.
- Patel, A. R., Mokashe, N. U., Chaudhari, D. S., Jadhav, A. G. & Patil, U. K. (2019). Production optimisation and characterisation of extracellular protease secreted by newly isolated *Bacillus subtilis* AU-2 strain obtained from *Tribolium castaneum* gut. *Biocatalysis and Agricultural Biotechnology*, 19, 101122.
- Patil, R.C. & Jadhav, B.L. (2017). Isolation and characterization of protease producing *Bacillus* species from soil of dairy industry. *International Journal of Current Microbiology and Applied Science*, 6(6), 853-860.
- Pawar, K. S., Singh, P. N., & Singh, S. K. (2023). Fungal alkaline proteases and their potential applications in different industries. *Frontier Microbiology* 14, 1138401.
- Raju, E. V. N. & Divakar, G. (2013). Screening and Isolation of Keratinase Producing Bacteria from Poultry Waste. *International Journal of Pharmaceutical Research & Allied Sciences*, 2(1), 70-74.

- Ramadhan, A. R., Bachruddin, Z., Widodo, Erwanto, Y. & Hanim, C. (2021). Isolation and selection of proteolytic lactic acid bacteria from colostrum of dairy cattle. The 3rd International Conference of Animal Science and Technology IOP Conference Series: *Earth and Environmental Science* 788 (2021) 012077IOP
- Rasheed, H. T. (2021). Analyzing the Impact of a Formula Including a Partial Purified *Aspergillus niger* Protease. *Bionatura*, 13, 1-6
- Ratnaningrum, D., Kosasih, W., Endah, E. S., Lathifa, A. K. N., Diwan, A. M., Nida, V., Saraswati, V. & Risdian, C. (2022). Protease production by soil bacteria for green technology: Screening and optimization. IOP Conference Series: *Earth and Environmental Science*, 1201 (2023) 012094
- Razzaq, A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A. & Ashraf, M. (2019). Microbial proteases applications. *Frontier Bioengineering and Biotechnology*, 7, 110.
- Riady, R. M., Fitriadi, R., Kasprijo., Ryandini, D., Palupi, M., Sukardi. P., Nurhafid, M., Musa, A. & Asaf, R. (2024). Isolation and Screening of Proteolytic Bacteria from Rice-Fish Farming System Sediments. *International Journal of Design & Nature and Ecodynamics*, 19(3), 795-803.
- Rupali, D. (2015). Screening and Isolation of Protease Producing Bacteria from Soil Collected from Different Areas of Burhanpur Region (MP), India. *International Journal of Current Microbiology and Applied Science*, 4 (8), 597-606.
- Santosa, S. & Setyaningrum, E.N. (2024). Isolation and characterization of potential proteolytic and amylolytic bacteria from Bayanan hot spring as bioremediation agents. *Biogenesis: Jurnal Ilmiah Biologi*. 12(1), 66–73.
- Shad A. A., Ahmad, T., Iqbal, M.F., Asad, M. J., Nazir, S., Mahmood, R.T. & Wadood, W. A. (2024). Production, Partial Purification, and Characterization of Protease through Response Surface Methodology by *Bacillus subtilis* K-5. *Brazilian Archives of Biology and Technology*, 67, e24210355
- Shaikh. I.A., Turakani, B., Malpani, J., Goudar, S. V., Mahnashi, M. H., Al-Serwi, R. H., Ghoneim, M. M., El-Sherbiny, Mohamed., Mannasaheb, B. A.,
- Alsaikhan, F., Sindagimath, V., Khan, A.A., Muddapur, U.M., Azzouz, S., Mohammed, T. & Shakeel Iqbal, S. M. (2023). Extracellular Protease Production, Optimization, and Partial Purification from *Bacillus nakamurai* PL4 and Its Applications. *Journal of King Saud University-Science*, 35 (1),102429
- Solanki, P., Putatunda, C., Kumar, A., Bhatia, R. & Walia, A. (2021) Microbial proteases: ubiquitous enzymes with innumerable uses. *3 Biotech*, 11(10), 428
- Su, H., Xiao, Z., Yu, K., Huang, Q., Wang, G., Wang, Y., Liang, J., Huang, W., Huang, X., Wei, F. & Chen, B. (2020). Diversity of cultivable protease-producing bacteria and their extracellular proteases associated to Scleractinian corals. *Peer Journal*, 3, 1-17.
- Sukmawati, Sunari, A., Akbari, T.S., Ardhiani, A. Z., Afifah, Z. N., Dellanerra, D., Nurjayadi, N. & Enshasy, H. E. (2020). Isolation and selection of fungi Amylolytic and Cellulolytic originated from *Rhododendron* sp Flower. *AIP Conference proceedings*, 2242, 050023
- Thanoon, R.D., Mahmood, H. M., Makky, E. A. & Homady, M. H. (2021). Quantitative methods for Alkaline Protease Determination and its applications: A comprehensive review. *International Journal of Clinical Case Reports and Reviews*, 6(1)
- Thongekkaew, J. & Kongsanthia (2016). Screening and identification of cellulase producing yeast from Rongkho forest, Ubon Ratchathani University. *Bioengineering and Bioscience*, 4(3), 29-33.
- Tohoum, U. M. & Hamza, W.T. (2022). *Bacillus subtilis* and *Pseudomonas aeruginosa* as potent protease enzyme producers isolated from the aquatic environment. *Egyptian Journal of Aquatic Biology & Fisheries*, 26(4), 197-213.
- Ullah, N., Rehman, M. U., Sarwar, A., Nadeem, M., Nelofer, R., Shakir, H. A., Irfan, M., Idrees, M., Naz, S., Nabi, G., Shah, S., Aziz, T., Alharbi, M., Alshammari, A., & Alqahtani, F. (2022). Purification, Characterization, and Application of Alkaline Protease Enzyme from a Locally Isolated *Bacillus cereus* Strain. *Fermentation* 8(11), 628.

Umeh, S. O., Onyeneto, T.C., Ubajekwe, C.C., Iheukwumere, I. & Okpalla, J. (2015). Isolation and screening for protease-producing *Bacillus* species from soils in Awka, Anambra State, South Eastern Nigeria. *International Journal of Agriculture and Bioscience*, 4(2), 75-77.

Usman, U., Sabo, A., Ibrahim, H., Ado, B., Suraka, B., Muhammad, H., Tijjani, A., Ismail, U.S & Ado, M. B. (2023). Protease producing bacteria from soil in Gumel metropolis, Jigawa State of Nigeria. *Eastern Journal of Agricultural and Biological Sciences*, 3(2), 19-23.

Vakilwala, M. and Patel, D. (2017). Isolation and Screening of Protease Producing Organisms from Soil Sample. *International Journal of Research and Scientific Innovation*, IV (IV), 75-78.

Yang, Xi., Wang, Z., Zhang, C., Wang, L., Pang, L., Zhang, D., Man C. & Jiang, Y. (2021). Assessment of the production of *Bacillus cereus* protease and its effect on the quality of ultra-high temperature sterilized whole milk. *Journal of Dairy Science*, 104 (6), 6577-6587.