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# Screening of protease producing bacteria from the gastrointestinal tract of Nile tilapia (*Oreochromis niloticus*)

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**Abstract**
This study was designed to identify and characterize protease-producing bacteria residing in the gastrointestinal tract (GIT) of Nile tilapia (*Oreochromis niloticus*) that optimizes feed utilization in the aquaculture system. For this purpose, a diverse collection of bacteria was isolated from different segments of GIT of the Nile tilapia. The isolated bacteria were screened for their ability to produce proteases, enzymes essential for the digestion of dietary proteins. In this study, approximately 60 samples of the Nile tilapia were used to analyze the bacterial species residing in the intestinal segments using culture techniques. The results showed that bacterial populations in different segments of the intestine, when cultured on skim milk agar, showed significant results ($P \leq 0.05$) for screening of protease-producing bacteria. From this study, it was concluded that the protease-producing bacteria present in the gut of the Nile tilapia have a significant impact on fish digestion and health. Our findings suggest that protease-producing bacteria present in GIT of the Nile tilapia, provide a valuable insight into the implications of fish aquaculture practices.

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## Introduction

The gastrointestinal system of fish contains high levels of nutrients which is a favorable habitat for

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microorganisms. These microbial communities influence significantly a wide variety of enzymatic capabilities in numerous fish species (Luan et al., 2023). Fish intestines release various substances including vitamins, riboflavin, and enzymes which break down proteins, carbohydrates, and lipids (Zhang et al., 2020). Bacteria that produce enzymes in fish have shown their ability to break down chitin, cellulose, protein, starch, phytate, and tannin (Armada and Simora, 2016). The microbiota present in the gastrointestinal tract of fish plays a pivotal role in both nutrition and immune function (Adeoye et al., 2016; Luan et al., 2023). Previous studies revealed that most scientists focused much on bacteria among all fish-related microorganisms (François-Étienne et al., 2023). As a primary microbial colonist in fish's gastrointestinal tract, bacteria play a vital role in influencing the complex digestion process of fish (Banerjee et al., 2014; Xiong et al., 2019).

Due to its great economic significance, Nile tilapia (*Oreochromis niloticus*) is a widely cultured species in the aquaculture sector worldwide. Various studies conducted on this species, focused on numerous aspects including breeding and culturing practices (Aryati et al., 2021). Tilapia is the most significant aquaculture species in terms of volume, and it holds a substantial economic value (Hossain et al., 2021). Due to its remarkably fast growth, easy cultivation and most importantly its adaptation to survive in a wide range of harsh environmental conditions, its capabilities make it favorable and most preferred species in the aquaculture sector. Significantly, Southeast Asia is the world's top producer, contributing up to 72% of the Nile tilapia production worldwide. It is important to remember that stress in the fish population, poor water quality, and excessive stocking densities are all major contributors to fish diseases (Mramba and Kahindi, 2023). These factors are significant for adopting proper management techniques in the aquaculture industry (Aryati et al., 2021).

Tilapia is an omnivorous fish, its ability to adapt to both freshwater and saltwater environments makes it a great candidate species for studying gut flora. The digestive tract of tilapia is rich in beneficial bacteria and is five to seven times longer than its body length (Thillaimaharani et al., 2012). The fish consumes a wide variety of feed that includes phytoplankton, zooplankton, macrophytes, insects, detritus and nematodes, among other things (Engdaw, 2023). Similar to the common carp, tilapia also lacks a stomach; hence, the microorganisms present in the fish's gut are crucial for the process of digestion, especially in the absence of the pepsin enzyme; thus, the function of pepsin is taken up by alkaline proteases, which is more common in alkaline environments (Nalinanon et al., 2010). Its growth appears to be greatly enhanced by the addition of extracellular protease-secreting bacteria to its diet (Hossain et al., 2021). It has been shown that fish gastrointestinal tracts are excellent sources of bacteria that produce extracellular hydrolytic digestive enzymes (Hossain et al., 2021).

Proteases activate the hydrolysis of peptide bonds between amino acid residues in proteins (Razzaq et al., 2019). Proteases exhibit variability in their catalytic efficacy, optimal pH, effective temperature, substrate selectivity resistance to the body's natural digestive enzymes and other characteristics (Razzaq et al., 2019). Microbial proteases, known for their environment-friendly characteristics, are of significant interest due to their commercial applications (Mienda et al., 2014). Because of their small culture area needs and quick genetic adaptation, microorganisms make an attractive reservoir for proteases (Arogundade et al., 2023). Because of their unique and specialized mode of action, proteases have several biotechnological uses. Various bacteria, including the species of *Penicillium*, *Vibrio*, *Streptomyces*, *Pseudomonas*, and *Bacillus*, *Rhizopus oryzae*, *Serratia marcescens*, and others have been shown to generate proteases (Armada et al., 2016).

While substantial information exists regarding the gut microbiota of warm-blooded animals and their role in the digestive process, there remains a lack of understanding concerning the bacterial population in the gut of cold-blooded animals. There haven't been a lot of studies done on fish, particularly in this field. However, a few researchers addressed the microbial origin of the digestive enzymes present in fish intestines (Bairagi et al., 2002; Luan et al., 2023). Studies on the utilization of probiotic bacteria have been shown to enhance the immune system and disease resistance in fish, as well as stimulate growth (Bandavong et al., 2016; Salam et al., 2021). Their potential value in various applications has been highlighted. However, no study has been conducted on the isolation of protease-producing bacteria from the gastrointestinal tract of the Nile tilapia, which would involve the identification of their purity and characteristics, as well as the distribution of digestive enzymes throughout the intestinal tract of *O. niloticus*. This project aimed to bridge this research gap by contributing valuable insights for the establishment of effective food safety protocols within the fish industry.

## Materials and Methods

### Study area

The current research project was carried out at the Department of Zoology, Wildlife and Fisheries,

University of Agriculture, Faisalabad.

## Stock management

Healthy fish samples of *O. niloticus* of various sizes were collected from freshwater earthen ponds at the University of Agriculture, Faisalabad. All samples were acclimatized for 12 days. The experimental design included tank size, aeration, fish type, water chemistry, and general design. Prior to adding the fish, the tanks were cleaned with warm water. Water chemistry was tested on regular basis using a test kit to ensure stability of environmental factors.

## Sample collection and bacterial isolation

Sixty (60) samples of *O. niloticus* were collected with care and placed in clean sterilized plastic bags. The samples were transferred to the laboratory with great care for further analysis. The fish intestine was dissected and divided into three segments; proximal (P), middle (M) and distal (D). Each excised segment was transferred to a sterile Eppendorf tube of 2 mL that was filled with 9% saline solution along with a small amount of fluid carrying sample segments. Total heterotrophic counts were carried out on tryptone soy agar and nutrient agar plates, and for detection of protease producing bacteria intestinal homogenates were diluted in a sterile 0.9% saline solution and placed on skim milk agar containing tryptone 1.0% (10 g/L); yeast extract, 0.5% (5 g/L); sodium chloride, 0.05% (0.5 g/L); skim milk, 1.6% and either 1.5% or 2% agar and incubated at 37 °C for 24 h. Distinct bacterial colonies were selected and streaked on the skim milk agar and kept at 37 °C under aerobic conditions for 5 to 7 days or until the growth was observed.

## Gram staining

Gram staining is used to characterize and identify bacterial populations found in the fish gut microbiota by distinguishing Gram-positive and Gram-negative bacteria. It is also used to detect and classify bacterial strains by examining their morphological characteristics under a microscope. It essentially differentiates cells by the fact that their cell walls absorb more dye than those with a thin cell wall made up of a few layers of peptidoglycan. Gram-positive bacteria maintain their primary color and appear purple or blue under a microscope, whereas Gram-negative bacteria appear red or pink.

## Slide preparation

After sterilizing the culture plates, colonies were selected using a flame-sterilized platinum loop. The samples were distributed, air-dried, and then heat-fixed by passing the slides over a flame. Gram staining was performed on the heat-fixed bacterial smear. Under the light microscope, a vast family of protease-producing Gram-positive and Gram-negative bacteria was observed.

## Gram staining protocol

Crystal violet was used for 60 seconds to thoroughly rinse the heat-fixed smear off the glass slide. The slides were thoroughly cleaned to remove any leftover discoloration. Iodine droplets remained on the slides for one minute. The slides were washed again to remove any remaining color from the markings. After a brief exposure to acid alcohol, the slides became discolored. The slides were cleaned as rapidly as possible because overly severe decolorization might produce inaccurate outcomes. Safranin counterstain was applied to the slide and washed after 60 s. A light microscope was used to examine the bacterial morphology and staining features on air-dried slides.

## Water quality analysis

### *Temperature*

Temperature has a significant influence on fish development and performance. Tropical fish thrive best at temperatures ranging from 24-27 °C. A digital thermometer was used to regularly measure the temperature of water.

### *pH*

The pH indicates the concentration of hydrogen ions. Every organism has an optimum pH and a pH tolerance range. The pH of the water was regularly measured using a digital pH meter.

### *Dissolved oxygen*

Dissolved oxygen (DO) levels were maintained at 5 mg/L to keep aquarium fish healthy. The aquarium's water was circulated continually using aerators. The splashing technique was chosen because it increases water flow and hence enhances oxygenation. The dissolved oxygen was measured using a DO meter (HI-9146) with a "ppm (mg/L)" range.

### Impact of physico-chemical parameters on protease production

Some physico-chemical parameters that influence protease production including growth at different pH (4, 5, 6, 7 and 8), incubation time (12, 24, 36, 48, 60 and 72 h) and temperature (20, 30, 40 and 50 °C) were examined on bacterial isolates.

### Statistical analyses

Using one-way analysis of variance (ANOVA) followed by Tukey's test, statistical analysis was performed to find out significant differences among the quantitative data of bacterial population present in the Nile tilapia's gastrointestinal tract.

## Results

### Total bacterial count from the gastrointestinal tract of *O. niloticus* cultured on 3 different media

For the bacterial count of gastrointestinal tract of *O. niloticus*, the intestine was divided into 3 segments, i.e., proximal, middle and distal. The heterotrophic bacterial count on the nutrient agar was observed from a minimum of 86 × 106 CFU to a maximum of 453 × 106 CFU and the heterotrophic bacterial count on tryptic soy agar was observed from a minimum of 92 × 106 CFU to a maximum 431 × 106 CFU. For screening of protease producing bacteria the proteolytic count was recorded on the skim milk agar, and it was observed from a minimum of 120 × 106 CFU to a maximum 399 × 106 CFU. **Table 1** shows the mean colony count and standard error of all the segments cultured on three different media. The mean colony count for each segment represents the average number of bacterial colonies that were detected. The TSA proximal segment had the highest mean count of approximately 297 million CFU (2.97E+08) followed by that of the distal segment with approximately 292 million CFU (2.92E+08). In case of NA, the distal segment had the highest mean count of approximately 312 million CFU (3.12E+08) followed by that of the middle segment with approximately 224 million CFU (2.24E+08) and for skim milk agar the highest mean count was observed in the proximal segment which was approximately 308 million CFU (3.08E+08) followed by that of the middle segment being approximately 176 million CFU (1.76E+08).

**Table 1. Comparison of means ± SE for the total bacterial count from the gastrointestinal tract of *O. niloticus***

| | Proximal | Middle | Distal |
|---|---|---|---|
| Nutrient agar | 2.97E+08 ± 4.37E+07 | 1.54E+08 ± 2.57E+07 | 2.92E+08 ± 5.75E+07 |
| Tryptic soy agar | 2.23E+08 ± 2.30E+07 | 2.24E+08 ± 4.27E+07 | 3.12E+08 ± 5.19E+07 |
| Skim milk agar | 3.08E+08 ± 4.24E+07 | 1.76E+08 ± 1.75E+07 | 1.74E+08 ± 1.94E+07 |

### Comparative analysis of protease-producing bacteria abundance across intestinal segments

For the investigation of protease producing bacteria abundance across three segments, the gastrointestinal tract of Nile tilapia we used for working out analysis of variance (ANOVA) and Tukey's test. **Table 2** shows that the results of ANOVA are significant which means there are significant differences among bacterial abundance among these segments. The one segment that differed significantly from the others is the proximal segment. Tukey's post-hoc test identified specific pairwise differences between the segments (**Table 3**; **Figure 1).**

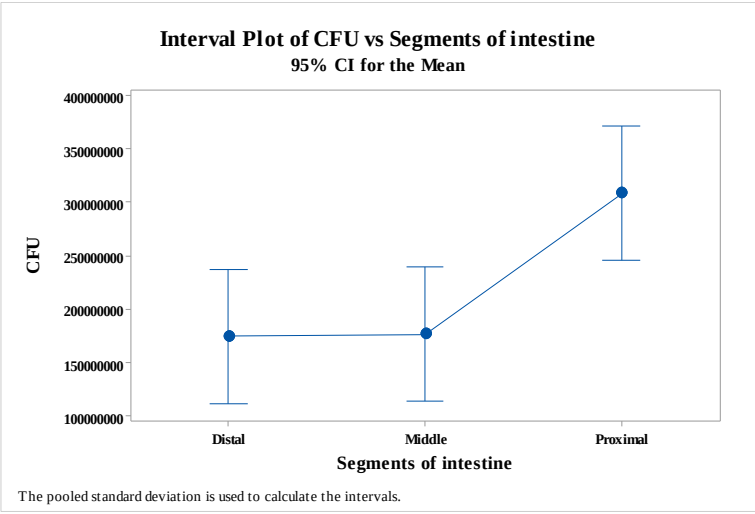


**Figure 1. Interval plot of CFU and intestinal segments**

**Table 2. Analysis of variance for protease producing bacteria abundance among segments of gut of *O. niloticus***

| Source | DF | Adj SS | Adj MS | F-Value | *P*-Value |
|---|---|---|---|---|---|
| Segments of intestine | 2 | 5.86192E+16 | 2.93096E+16 | 7.08 | 0.009 |
| Error | 12 | 4.97084E+16 | 4.14237E+15 | | |
| Total | 14 | 1.08328E+17 | | | |

**Table 3. Grouping information using the Tukey's method and 95% confidence**

| Segments of intestine | N | Mean | Grouping |
|---|---|---|---|
| Proximal | 5 | 308000000 | |
| Middle | 5 | 176400000 | B |
| Distal | 5 | 174400000 | B |

Means that do not share a same letter are significantly different.

## Morphological analysis

An overview of morphological characteristics of bacterial colonies grown on skim milk agar is given in **Table 4**. The characteristics involved shape, color, elevation, surface, edges and size and they played an important role in the identification of bacteria.

**Table 4. Morphological characteristics of bacterial colonies grown on skim milk agar**

| Isolate | Shape | Color | Elevation | Surface | Edge | Size |
|---|---|---|---|---|---|---|
| Isolate 1 | Circular | White | Flat | Rough | Entire | Small |
| Isolate 2 | Irregular | White | Flat | Rough | Entire | Small |
| Isolate 3 | Circular | Yellow | Raised | Smooth | Undulate | Small |
| Isolate 4 | Circular | Transparent | Flat | Rough | Entire | Small |
| Isolate 5 | Irregular | Blue | Raised | Smooth | Entire | Small |
| Isolate 6 | Circular | Cream | Raised | Smooth | Entire | Large |
| Isolate 7 | Circular | Grey | Flat | Smooth | Entire | Small |
| Isolate 8 | Irregular | Yellow | Flat | Smooth | Undulate | Small |
| Isolate 9 | Circular | Cream | Flat | Rough | Entire | Large |
| Isolate 10 | Circular | Pale yellow | Flat | Rough | Entire | Small |

The best growth of bacteria was observed at pH 7 and temperature 37 °C, while the incubation time for the growth of bacteria was observed to be 36-72 h. The following given **Table 5** provides the details of protease producing bacteria residing in the gastrointestinal tract of Nile tilapia that were identified based on their morphology.

**Table 5. Bacterial isolates that were identified after Gram staining**

| Isolate | Species name | Morphology | Gram stain | Motility | Oxygen requirement | Flagella |
|---|---|---|---|---|---|---|
| Isolate 1 | *Bacillus* spp. | Rod-shaped (bacilli) and occur singly, in pairs, or in chains | Gram positive | Motile | Aerobic | Peritrichous |
| Isolate 2 | *Clostridium* spp. | Rod-shaped (bacilli) | Gram positive | Motile | Anaerobic | Peritrichous |
| Isolate 3 | *Pseudomonas aeruginosa* | Rod-shaped bacterium (bacillus) | Gram negative | Motile | Aerobic | One or more polar flagella |
| Isolate 4 | *Lactobacillus* spp. | Rod-shaped bacilli, occur singly or in short chains | Gram positive | Non-motile | Anaerobic | No flagella |
| Isolate 5 | *Streptococcus* spp. | Spherical or oval-shaped cocci | Gram positive | Non-motile | Anaerobic | No flagella |
| Isolate 6 | *Escherichia coli* | Rod-shaped bacterium (bacillus) | Gram negative | Motile | Anaerobic | Peritrichous flagella |
| Isolate 7 | *Vibrio* spp. | Curved or comma-shaped bacteria (vibrios) | Gram negative | Motile | Anaerobic | Single polar flagellum |
| Isolate 8 | *Proteus* spp. | Rod-shaped bacteria (bacilli) | Gram negative | Motile | Facultative anaerobes | Peritrichous flagella |
| Isolate 9 | *Staphylococcus* spp. | Spherical or oval-shaped cocci occur in grape like clusters or irregular clusters | Gram positive | Non-motile | Anaerobic | No flagella |
| Isolate 10 | *Klebsiella* spp. | Rod-shaped bacteria (bacilli) | Gram negative | Non-motile | Aerobic | No flagella |

## Discussion

Considerable proteolytic bacteria were detected in the gastrointestinal tract of *O. niloticus*, particularly in the proximal region. Protease-producing bacteria in fish gut hydrolyze proteins from food and other digestive enzymes, including aspartic and serine proteases, trypsin, chymotrypsin, collagenase and elastase (Balti et al., 2009; Chen et al., 2024). We used skim milk agar for production of protease producing bacteria. Similarly, Olajuyigbe and Ajele (2005) and Prihanto et al. (2021) also suggested the use of skim milk for growing proteolytic bacteria. The bacterial isolates that were identified based on morphological analysis were *Bacillus*, *Clostridium, Pseudomonas aeruginosa, Lactobacillus, Streptococcus, Escherichia coli, Vibrio, Proteus, Staphylococcus* and *Klebsiella*. Previous studies reported that *Pseudomonas* bacteria are widely found in fish's gastrointestinal tract. Ariole and Kanu (2014) also reported *Pseudomonas* spp. in the tilapia digestive tracts with *Aeromonas, Staphylococcus, Bacillus, Vibrio, Salmonella, Flavobacterium, Enterobacter, Escherichia, Micrococcus* and *Lactobacillus.* **Table 5** shows bacterial isolates that were identified based on their colony morphology and cell morphology.

Microbial enzyme synthesis is heavily influenced by media components and physical conditions, including incubation duration, temperature, and pH. The incubation time varies from 24 h to 7 days according to the type of organism being cultured. Our study found that the optimal incubation time for protease synthesis was 36-72 h. pH and temperature are two key parameters that significantly influence enzyme synthesis. Temperature significantly impacts biological processes, affecting enzyme production (Uyar et al., 2011; Garofalo et al., 2024; Salas-Bruggink et al., 2024). The optimum temperature for protease production was observed to be 37 °C and the optimum pH for protease production was 7. According to Armada and Simore (2016) the best protease production was recorded at temperature 40 °C and pH 7-8. Similarly, Jobin and Grenier (2003) studied that *Streptococcus suis* produces four proteases with an optimal pH range of 6 to 8. At pH levels over 10, the bacterium's metabolic activity is reduced, leading to decreased protease synthesis.

Moreover, the rearing environment may also affect the microbiota present in the gastrointestinal tract of Nile tilapia. The ideal water temperature for growing Nile tilapia is 27 to 32 ℃, as it would be difficult for fish to survive the temperatures below 8 ℃. Previously, a substantial relationship was observed between the microbial populations in the rearing water and in the gastrointestinal tract (Giatsis et al., 2015). Seasonal fluctuations in water temperature were also reported to alter the makeup of the gut bacteria in Nile tilapia. In a previous study, Bereded et al. (2021) found that seasonal and regional variation affected both the composition and diversity of gut bacteria.

In summary, protease producing bacteria were found from the gastrointestinal tract of *O. niloticus*. By growing on specific agar mediums and using the Gram staining technique, we examined all the morphological properties of proteolytic isolates. Further research on the capacity of protease to digest proteins in aquaculture feed, explication of their biochemical characteristics for industrial applications, and the presence of other beneficial features in the proteolytic isolates is required. To further validate particular proteolytic strains, an extensive number of samples should be analyzed individually using both metagenomic and culture dependent approaches.

## Conclusion

In this project, *Bacillus* spp., *Clostridium* spp., *Pseudomonas aeruginosa*, *Lactobacillus* spp., *Streptococcus* spp., *Escherichia coli*, *Vibrio* spp., *Proteus* spp., *Staphylococcus* spp., and *Klebsiella* spp. were the protease-producing bacteria that were identified from the gastrointestinal tract of Nile tilapia. The need for protease enzymes is growing in the industrial, commercial, analytical, pharmaceutical and diagnostic sectors, because enzymes derived from plants and animals are not only more efficient but are also more expensive. Enzymes generated by microorganisms are chosen due to their quick development and less space requirements for culture. Protease can be employed in innovative protein engineering methodologies and procedures in the future, and the commercial protease industry will continue to expand.

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### Contribution of authors
Conceptualization and designing of the study: RN, SP, FR, ZAK. Conduction of experiments: RN, SP. Data collection, visualization, and interpretation: RN, SP, FR, ZAK, DR, MA. Formal statistical analysis: RN, SP, MA, AA, KM, MU, SM, GR. Writing of first draft: RN, SP, FR, GR, TH, MJK. Proof reading and approval of the final version: RN, SP, FR, ZAK, DR, MA, AA, KM, MU, SM, GR, TH, MJK.

### Ethical approval
This work was approved by Institutional Ethical Review Board/Committee (IERB/C) of University of Education, Lahore, under approval number ASRB/2023/Rafia-Naeem dated 12-12-2022.

### Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of the experiment, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

### Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

### Authors' consent
All authors have critically read this manuscript and agreed to publish in IJAaEB.

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It is declared that we the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

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