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Proteolytic Bacterial Activity from the Digestive Tract of Udikan Fish (*Tor* Sp.) Caught in Banjaran River

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Abstract

The type of feed commonly consumed by fish in nature can be predicted from the presence of microorganism communities in their digestive tracts. One type of bacteria that plays an important role in the digestive tract is proteolytic bacteria. The purpose of this study was to determine the activity index and proportion of proteolytic bacteria in the digestive tracts of udikan fish (*Tor sp*.) caught in the Banjaran River. The methods used in this study included sampling, bacterial isolation, calculation of bacterial colony counts, observation of bacterial morphology, 3% KOH Gram staining, and testing of proteolytic bacterial activity and proportion. The results of the study showed that the activity index of proteolytic bacteria from the digestive tract of udikan fish (*Tor sp*.) was relatively low, with an average value of 0.06 at 24 hours and 0.05 at 48 hours. The proportion of proteolytic bacteria obtained from the digestive tract of udikan fish was 33%.

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Introduction

Udikan fish (*Tor* sp.) is one of the endemic fish species in Banyumas Regency. Based on its morphology, udikan fish is included in the *Tor* sp. species, this genus is generally known as mahseer fish. Udikan fish has a high economic value, the price per kilogram reaches IDR 1,000,000. The high price of udikan fish causes excessive fishing so that the existence of udikan fish in the Banjaran River is increasingly rare (Rumondang *et al*., 2023). Dwirastina & Wibowo (2022) revealed that excessive fishing has a major impact on the decline in the population of udikan fish in nature.

The decline in the population of udikan fish in the Banjaran River has necessitated domestication efforts. However, the domestication process in several Tor sp. fish species is still constrained, one of which is the adjustment of feed (Yanto *et al*., 2014). Feed that does not match its eating habits in nature affects the growth rate. Additionally, Rahayu *et al*. (2019) stated that inappropriate feed also has an impact on the survival of fish. According to Icas *et al*. (2019), the

growth, reproduction, and survival of fish are greatly influenced by eating habits.

The type of feed commonly consumed by fish in nature can be predicted from the presence of microorganism communities in their digestive tract (Afrianto & Liviawaty, 2019). Bacterial communities in the digestive tract differ between herbivorous, omnivorous, and carnivorous fish (Ray *et al*., 2012). There is a relationship between feeding habits and bacterial activity in the digestive tract of fish. According to Hossain *et al*. (2021), the activity of proteolytic bacteria in carnivorous fish species is higher than in herbivorous fish. Meanwhile, Kar & Ghosh (2008) reported that the activity of amylolytic bacteria was higher in herbivorous fish species than in carnivorous fish species. Meanwhile, in omnivorous fish species, the activity of proteolytic and amylolytic bacteria is relatively balanced (Das *et al*., 2014).

Research related to the activity of proteolytic bacteria in the digestive tract of fish has been widely conducted, including in catfish (Kurniasih *et al*., 2014), tilapia, and carp (Nurhafid *et al*., 2021). Bacteria with proteolytic activity were also found in the digestive tract of Malaysian mahseer (*Tor tambroides*) (Asaduzzaman *et al*., 2018). However, information regarding the activity of proteolytic bacteria in the digestive tract of udikan fish in the native habitat of the Banjaran River is not yet known. Therefore, this study was conducted to estimate the tendency of the types of feed commonly consumed by udikan fish. During the domestication process, it is hoped that udikan fish will be able to grow and survive by feeding on species-appropriate feed.

Materials and methods

Time and Place of Research

The research implementation period starts from February to August 2022. Samples were collected from the Banjaran River, Baturraden District, Banyumas Regency (7°25'16“S 109°13'29”E). Bacterial isolation and proteolytic enzyme activity testing were conducted at the Research Laboratory of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University.

Sampling

This study uses observational methodology with purposive sampling. One Udikan fish (Tor sp.) was caught using a net in the Banjaran River. Then, the caught udikan fish (*Tor* sp.) were put into a cooler box and added with ice cubes to be taken to the research laboratory of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University.

Isolation of Bacteria

The udikan fish (*Tor* sp.) was dissected using surgical scissors aseptically. The digestive tract of the udikan fish was removed from the abdominal cavity and aseptically unraveled on a millimeter block to measure its length. Samples of the digestive tract were taken 1 cm each from the anterior, middle, and posterior parts. The samples were placed into a 1.5 mL microtube to be weighed, then 1 mL of sterile physiological solution was added and homogenized using a vortex.

The suspension of the digestive tract sample was taken at as much as 0.5 mL, then put into a test tube containing 4.5 mL of sterile physiological solution and homogenized to produce a 10^{-1} dilution. Furthermore, the suspension from the 10^{-1} dilution was taken as much as 0.5 mL and put into a 10^{-2} dilution tube containing 4.5 mL of sterile physiological solution and then homogenized. Furthermore, a multilevel dilution was carried out up to 10^{-5}. The results of the 10^{-3} to 10^{-5} dilutions were taken as much as 0.5 mL each using a micropipette aseptically and planted using the pour plate method. Furthermore, the Tryptic Soy Agar (TSA) media was poured into a petri dish containing the sample suspension, then homogenized by rotating the petri dish following a figure eight pattern. The sample incubation was carried out for 18-24 hours at a temperature of 28 ° C.

Calculation of the Bacterial Colonies Number

The calculation of the bacteria colonies number that grow is done using the Total Plate Count (TPC) method. The results of the calculation of the number of bacterial colonies are entered into the following formula. (Kadri *et al*., 2015).

$$\text{Bacteria count (CFU/g)} = \sum \text{Colony} \times \frac{1}{\text{Dilution x Culture Volume (mL) x sample weight (g)}}$$

Observation of Bacterial Colony Morphology

Observation of bacterial morphology was carried out macroscopically on Tryptic Soy Agar media. This morphological observation is based on the shape, elevation, edge, and color of the growing bacterial colonies (Doh *et al*., 2019). Bacterial colonies with different characteristics were taken for bacterial isolation using the streak plate technique on TSA media.

Gram Observation

Gram observation was carried out using 3% KOH. One drop of 3% KOH was dropped on the object glass. One loop of bacterial isolate was taken aseptically and then streaked on 3% KOH on the object glass. The absence of mucus formation indicated positive test results, whereas mucus

formation indicated negative test results (Anggraini *et al*., 2016).

Proteolytic Activity Test

Proteolytic activity testing is conducted by inoculating one loop of bacterial isolate at one point on the surface of the skim milk agar media. Furthermore, it is incubated for 48 hours at a temperature of 28 °C. Bacteria that have proteolytic activity will form a clear zone around the colony, while those that do not have proteolytic activity will not form a clear zone around the colony (Ardiansyah *et al*., 2021). A measure of the proteolytic activity index is made by measuring the clear zone diameter and the bacterial colony diameter. The proteolytic activity index is calculated using the following formula (Asril & Leksikowati, 2019).

$$\text{Proteolytic Index (IP)} = \frac{\textit{Clear zone diameter (mm)} - \textit{Colony diameter (mm)}}{\textit{Colony diameter (mm)}}$$

The proportion of proteolytic bacteria is calculated using the following formula (Sinatryani, 2014).

$$\text{Proportion of proteolytic bacteria (\%)} = \frac{\textit{number of proteolytic bacteria colonies obtained}}{\textit{total number of colonies observed}} \; x\; 100$$

Data Analysis

The research data obtained were in the form of abundance data, colony morphology, Gram properties, activity index, and proportion of proteolytic bacteria. The data were presented in the form of tables and graphs, then analyzed descriptively and compared with the literature.

Results and Discussion

Abundance of Digestive Tract Bacteria in Udikan Fish (*Tor* sp.)

Digestive tract samples of udikan fish (*Tor* sp.) were taken from the anterior, middle, and posterior parts. Bacterial abundance was calculated using the Total Plate Count (TPC) method. The bacterial abundance obtained was 3.8 x 10^6 CFU/g. This number was obtained from a dilution of 10^{-4} with 36 bacterial colonies. The abundance of bacterial colonies obtained in this study is in accordance with several previous studies. According to Hagi *et al.* (2004), The abundance of bacterial colonies from the digestive tract of several freshwater fish species is around 10^6 CFU/g. Al-Harbi & Uddin (2004) reported that abundance of bacterial colonies in the digestive tract of nila fish ranged from 5,5 x 10^6 to 9,8 x 10^9 CFU/g. The amount of different bacterial abundance is influenced by feed and the environment (Syukri *et al.,* 2021).

Characteristics of Digestive Tract Bacteria in Udikan Fish (*Tor* sp.)

The characteristics of colony morphology and Gram properties of digestive tract bacteria of udikan fish (*Tor* sp.) caught in the Banjaran River can be seen in Table 1.

Table 1. Colony Morphology Characteristics and Gram Properties

Isolate	Shape	Elevation	Edge	Colour	Gram
PU01	*Circular*	*Convex*	*Entire*	*White*	+
PU02	*Irregular*	*Umbonate*	*Entire*	*Cream white*	+
PU05	*Circular*	*Convex*	*Entire*	*Cream white*	-
PU09	*Irregular*	*Convex*	*Lobate*	*Cream white*	-
PU10	*Circular*	*Convex*	*Entire*	*Yellowish white*	-
PU11	*Irregular*	*Convex*	*Undulate*	*Yellowish white*	-

Based on the observation results, 6 isolates were obtained with different bacterial colony morphologies. In general, the morphology of the bacterial colonies obtained has a Circular and Irregular shape; Convex and Umbonate elevation; Entire, Lobate, and Undulate edges; and a white, creamy white, and yellowish white color. Based on the Gram characteristics, 2 isolates of Gram-positive bacteria and 4 isolates of Gram-negative bacteria were obtained.

The morphology of bacterial colonies obtained in this study is in accordance with the results of research conducted by Andriyanto & Yulianti (2020) on the digestive tract of the same fish. The morphology of bacterial colonies obtained in this study has a circular shape, convex elevation, and entire edges, and is cream-colored. Research conducted by Ginting *et al.* (2018) reported that the morphology of bacterial colonies in the digestive tract of milkfish is dominated by a circular shape, an entire edge, and a convex elevation. According to Kurniasih *et al.* (2013), the morphology of bacterial colonies in the digestive tract of catfish has a circular shape with clear, milky white, cream, and yellow colors.

The morphology of bacterial colonies can be used as a basis for identifying genus to species. Each bacterial species has a different colony shape and is characteristic of a particular species. The difference in colony shape can be influenced by several things such as bacterial age, environment, nutrition, and temperature (Agustina *et al.*, 2022). According to Fathoni *et al.* (2016), temperature plays an important role in bacterial growth because it is related to enzyme activity. Differences also occur in the color of bacterial colonies, the varying colors are influenced by the pigment content in them. Bacteria are known to be able to produce color pigments such as carotenoids, melanins, flavins, quinones, and prodigiosin

(Venil, 2009). These color pigments can be influenced by temperature and pH (Qayyum *et al.*, 2020).

Based on Gram characteristics, the bacterial isolates in this study were dominated by Gram-negative bacteria. The same results were also obtained in the study of Spanggaard *et al.* (2000) who isolated bacteria from the digestive tract of rainbow trout. According to Thune *et al.* (1993), Gram-negative bacteria are generally pathogenic when interacting with fish. However, recent studies have reported that Gram-positive bacteria are one of the triggers of infectious diseases in fish. *Lactococcus garvieae* and *Streptococcus iniae* are two of the Gram-positive bacteria confirmed to be agents of freshwater and marine fish diseases (Pękala-Safińska, 2018). The digestive tract of fish from rivers or lakes has the potential to contain Gram-negative *Aeromonas* and *Enterobacteriaceae* as the dominant microflora in it (Cahill, 1990).

Activity Index and Proportion of Proteolytic Bacteria

The proteolytic activity index of the digestive tract of udikan fish (*Tor* sp.) bacteria can be seen in Figure 1. Isolates that produced proteolytic activity at 24 and 48 hours of observation had codes PU02 and PU05. The highest proteolytic activity at 24 and 48 hours of observation was produced by isolate PU05 with values of 0.20 and 0.17. Isolates that did not produce proteolytic activity at 24 and 48 hours of observation had codes PU01, PU09, PU10, and PU11. The proteolytic index values for isolates PU01, PU09, PU10, and PU11 were 0, respectively. The average proteolytic bacterial activity at 24 and 48 hours of observation was 0.06 and 0.05, respectively. The standard deviation for each observation time was 0.09 and 0.08.

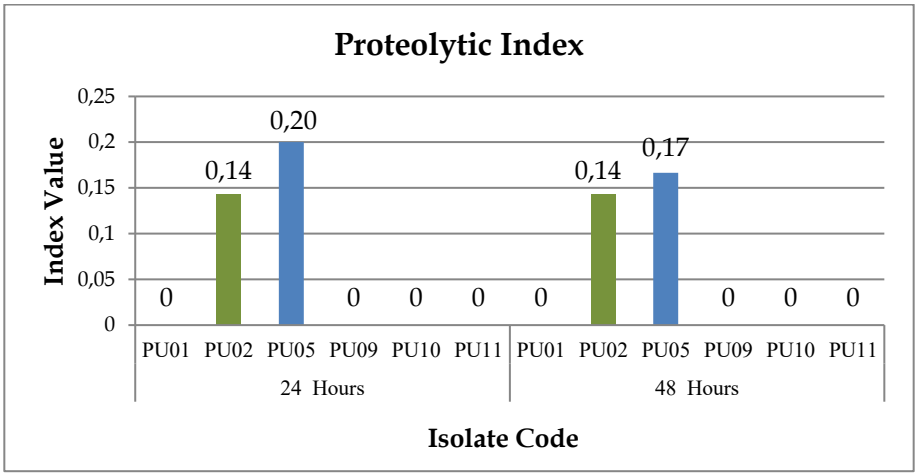

Figure 1. Proteolytic Activity Index

Table 2. Proportion of Proteolytic Bacteria

Types of Bacteria	Total Isolate	Proportion
Bacteria with proteolytic activity	2	33%
Bacteria without proteolytic activity	4	67%
Total	6	100%

The proteolytic activity index in the digestive tract of udikan fish (*Tor* sp.) caught in the Banjaran River is included in the low category. Proteolytic activity is stated to be high if it has an index ≥2.00, while low proteolytic activity has an index ≤2.00 (Wahjuningrum *et al.*, 2009; Hastuti *et al.,* 2017; Aguilera-Toro *et al.,* 2023). According to Setiawan *et al.* (2016), bacteria are stated to have proteolytic activity if the index value is ⩾ 1.00. Each species of bacteria has different protein hydrolysis capabilities. The presence of a clear zone around the bacterial colony indicates hydrolysis activity. The protease enzyme in the bacteria will hydrolyze casein in Skim Milk Agar media into amino acids (Hastuti *et al.*, 2017).

The increase and decrease in proteolytic activity occurs along with the incubation period of bacteria. This is due to the growth phase of the bacteria itself. According to Narvhus *et al.* (2021) the bacterial growth phase is related to the production of extracellular enzymes. High enzyme production occurs in the log or exponential phase (Sørhaug & Stepaniak, 1997). According to Risna *et al.* (2022) the rate of bacterial growth during the log phase is influenced by temperature, pH, nutrient content of the media, and genetic properties of the bacteria. The log phase occurs from the 4th to the 24th hour, after which the bacteria enter a constant growth phase. Bacteria enter the death phase at 28 to 56 hours (Cahyaningrum *et al.*, 2021).

The proportion of proteolytic bacteria in the digestive tract of udikan fish can be seen in Table 2. Bacteria that have proteolytic activity are 2 out of a total of 6 isolates with a percentage of 33%. While bacteria that do not have proteolytic activity are 4 out of a total of 6 isolates with a percentage of 67%.

The proportion of proteolytic bacteria in the digestive tract of udikan fish caught in the Banjaran River is a low percentage. This is thought to be because udikan fish are included in the omnivorous fish group. Omnivorous fish have an intestinal length ratio of 1-1.5 times longer than their bodies (Puspasari *et al.*, 2020). The result of this

study showed that the body length of udikan fish (*Tor* sp.) was 15.2 cm and the length of its digestive tract was 25.5 cm, which means that the digestive tract of udikan fish was 10.3 cm longer than its body. In nature, the natural food of the Tor genus is green algae, aquatic plants, worms, crustaceans, and small fish (Bami *et al.,* 2017; Sam *et al.,* 2021). According to Mohapatra *et al.* (2017) reported that fish of this genus have a large abundance of Chlorophyceae phytoplankton in their digestive tracts.

A low percentage of proteolytic bacteria was also obtained in the study of Hossain *et al.* (2021) from the digestive tract of tilapia, which was only 27% of the total 26 isolates tested. This is because tilapia have eating habits, namely herbivores tending to be omnivores. A study of carnivorous fish (*Bombay duck*) found 59% of the 17 bacteria to be proteolytic (Hossain *et al.*, 2020). Meanwhile, herbivorous fish have a higher density of amylolytic bacteria (Ray *et al.*, 2012). Mondal *et al.* (2008) reported that high populations of amylolytic bacteria were also obtained in herbivorous and omnivorous fish species.

Conclusion

The results of the study indicate that the proteolytic bacterial activity index of the digestive tract of udikan fish (Tor sp.) is low, with an average value of 0.06 at 24 hours and 0.05 at 48 hours. The proportion of proteolytic bacteria obtained from the digestive tract of udikan fish (Tor sp.) is 33%. Further research is needed to determine the types of bacterial species that have proteolytic activity.

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