

# Biochemical and Molecular Characterization of Eel Fish Trypsin (*Anguilla bicolor* McClelland) as Potential Candidates Protease Enzyme

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**Abstract.** Trypsin is one alkaline protease type widely used in various industry fields. One type of potential fish trypsin source is *Anguilla bicolor*. This study aims to characterize biochemical and molecular characterization of eel fish trypsin (*Anguilla bicolor* McClelland) as a possible candidate protease enzyme. The method used in this research is experimental research consisting of biochemical and molecular characterization. Fish Trypsin Extract was isolated from the digestive organs and then crushed using an electric homogenizer. During the pulverization process, 50 mM Tris-HCl buffer was added at a ratio of 1: 8 (w/v). The supernatant was then collected and can be stored at -80°C to measure enzyme activity. The treatment was given to juveniles and adults with stadia of *Anguilla bicolor*. While the molecular method was carried out using In Silico analysis in the analysis of the diversity of trypsin sequences in various fish species, preparation of specific primers, and analysis of Whole Genome Sequencing diversity of different species of *Anguilla* Spp. After that, extraction of *Anguilla bicolor* DNA, optimization of primer annealing temperature, DNA amplification, fish trypsin DNA fragments using the Sanger and Nanopore methods, and analysis of sequencing and phylogenetic results. The result of the protein content of the trypsin extract in the juvenile stage of *Anguilla bicolor* had an average of  $0.488 \pm 0.004$  g/dL, and the adult stage of *Anguilla bicolor* had an average of  $1.778 \pm 0.080$  g/dL. The highest trypsin activity was obtained in the juvenile stadia,  $0.529 \pm 0.016$  (U/mL), and in the adult stadia,  $0.399 \pm 0.009$  (U/mL). Trypsin activity increases with increasing temperature used and reaches a maximum of 40°C. The molecular character of the fish enzyme *Anguilla bicolor* shows that the sequence analyzed tend to be close to the Trypsinogen and Trypsin-like genes from *Anguilla japonica*, *Anguilla anguilla*, and *Megalops cyprinoides*.

**Keywords:** *Anguilla bicolor*; biochemical; Characterization; Enzyme; Molecular; Trypsin

## 1 Introduction

The demand for environmentally friendly products in the modern world requires that enzymatic methods replace industrial products produced by chemical processes. One of the most important groups of enzymes used for various industrial developments is alkaline proteases. Alkaline proteases are one of the most important groups of enzymes which industrially and scientifically account for about 65% of the annual enzymes market. Alkaline proteases have a history of application in the food and detergent industries, holding the largest share of the enzyme market worldwide [1], [2]. One type of alkaline protease that is generally used in various fields is trypsin.

Trypsin is a serine protease group digestive enzyme that hydrolyzes proteins on the carboxyl side of the amino acids lysine or arginine. Trypsin is responsible for protein hydrolysis in the digestive system into smaller peptides or even amino acids [3]. Alkaline proteases such as trypsin can be used as ingredients in detergents, the leather industry, medical diagnostics, vaccines, textiles, mining, the food and feed industry,

and many more. Due to its widespread application, many industries have started their production at a commercial level. Enzymes are needed to meet the increasing global demand in industrial markets [4], [5]. Meeting the needs of the enzyme can be explored from the existing biological resources in Indonesia.

Indonesia's biological resources are abundant, one of which is fish. According to data from the Ministry of Maritime Affairs and Fisheries in 2015, Indonesia has around 400,000 species of animals and fish. It is estimated that 8500 species of fish or strata, with a total of 45% of the number of species scattered in various parts of the world, live in Indonesian waters. The data shows that around 1,300 species from the data presented occupy areas in Indonesian freshwaters.

On the other hand, the high level of aquatic biodiversity, especially fish resources, also creates environmental problems such as increased water waste, one of which is fish offal waste [6], [7]. In contrast, fish offal as a by-product of the fishery industry has been recognized as a potential source of different enzymes, especially protease enzymes. One is trypsin, also known

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as fish trypsin [8], [9]. Only a little is known about fish trypsin, so its use in various fields is minimal.

Fish trypsin of various species and its potential applications in multiple industries have been developed. Fish trypsin applications include as an ingredient in detergents, extracting carotene proteins from shrimp waste, producing protein hydrolysates as food ingredients, and various applications in the food industry [10]. The number of potential fish species in Indonesia creates an opportunity to develop fish enzymes. According to [11], this predatory fish has excellent potential to produce high-quality alkaline proteases. One example of a superior predatory fish in Indonesia is the eel, or one of its scientific names, *Anguilla bicolor*.

The eel (*Anguilla bicolor*) is a type of fish with a catadromous nature; its life cycle occurs in two environmental conditions, namely being born in the sea and then growing in freshwater areas and when the maturation phase for reproduction returns to the sea [12]. Information on the enzyme activity of eel (*Anguilla bicolor* McClelland) using specific substrates is still unavailable. According to [13], when enzymes are used in industrial processes and analytical procedures, evaluation of enzyme activity becomes very important because it relates to the amount of enzyme needed to carry out the process correctly, the duration of the reaction, the amount of substrate to be converted, the number of conditions where the reaction occurs, and the overall cost of the process. So it is necessary to research the molecular characterization and enzyme activity of fish trypsin in eel (*Anguilla bicolor* McClelland) to develop its use in various applications.

## 2 Method

### 2.1 Experimental Design

The method used in this research is experimental research consisting of biochemical and molecular characterization. The biochemical method uses a completely randomized design (CRD) with six types of treatment in the form of temperature differences and four repetitions. Determination of the number of repetitions is based on the repetition calculation formula according to [14] as follows:

$$t(r-1) \geq 15 \quad (1)$$

Information:

t = treatment (number of treatments)

r = replication (number of repetitions)

15 = general degrees of freedom

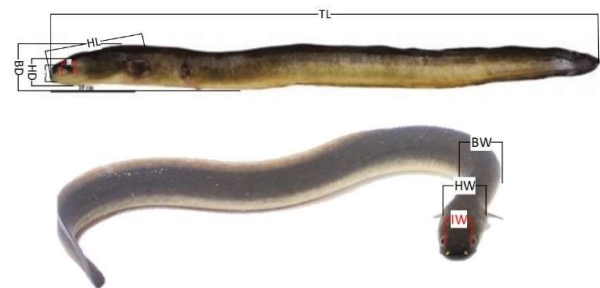
### 2.2 Object of Research

The sample of research is *Anguilla bicolor* obtained from PT. Laju Banyu Semesta (LABAS Sidat) Bogor City, West Java. The selected fish have different body sizes and weights based on juvenile and adult life stages. The number of each type of fish used in this study was 48 individuals representing juveniles (24 fish) and

adults (24 fish). Each fish is placed in a different container according to the type of stadia and quarantined for one week by giving a commercial feed.

### 2.3 Sample Preparation and Morphometric Measurements

Body morphometric measurements of *Anguilla bicolor* were carried out on each eel sample in two stadia. Measurements were made using a ruler and calipers with an accuracy of 0.01 mm, while the weight of the fish was weighed with a digital scale with an accuracy of 0.001 gram. The morphometric characters measured included 11 characters. Terms and abbreviations of the characters measured include standard length (SL), total length (TL), head length (HL), head height (HD), head width (HW), muzzle length (SNL), the distance between eyes (IW), eye diameter (ED), body height (BD), body weight (TW) and body width (BW). *Anguilla bicolor* morphometric scheme can be seen in Figure 1.



**Fig. 1.** Schematic of measuring the morphometric characters of *Anguilla bicolor*

Eel samples were isolated from the digestive organs by dissecting the ventral part of the eel's body. It begins with anesthetizing the fish by immersing it in ice water. Then the digestive organs are separated from the body, followed by the separation of the intestine and pyloric caeca and put into a particular container. The intestine that has been taken is then cleaned, and measurements are taken, including the intestine's width, the intestine's weight, and the intestine's length. Measurements were made using a ruler and calipers with an accuracy of 0.01 mm, while the weight of the intestine was weighed with a digital scale with an accuracy of 0.001 gram. Next, the intestine is cut into smaller parts of about 2 to 4 cm. After that, put it in a film bottle to be stored in the refrigerator at a temperature of -20°C to minimize protease autolysis.

### 2.4 Preparation of Fish Trypsin Extract

Samples of intestinal organs were isolated and then crushed using an electric homogenizer. During the pulverization process, 50 mM Tris-HCl buffer was added at a ratio of 1: 8 (w/v). The resulting homogenate was collected and transferred to a 1.5 mL tube, then centrifuged for 15 minutes at a speed of 12,000 rpm at a temperature of 4°C. The supernatant was then collected and transferred to another tube. The supernatant can be

stored at -80°C and measured to measure trypsin enzyme activity [15].

## 2.5 Determination of Protein Content

The working reagent (R1), albumin calibrators solution, and samples to be tested are stored at room temperature before use. Enter the albumin calibrators solution and working reagent solution into each well on the 96-well plate microplate to make a standard curve with the proportions according to Table 1.

**Table 1.** Preparation of standard albumin curve

Well No.	Working Reagent (uL)	Albumin Calibrators Solution (uL)	Final Concentration (g/L)
1	150	0	0
2	150	0,25	2,16
3	150	0,5	4,32
4	150	1	8,64
5	150	2	17,28

A total of 0.5 µL of the sample was put into the well, and 150 µL of the Working Reagent Solution was added to each sample. Then the solution was incubated at 15-25°C for 5 minutes. The absorbance of the solution was measured at a wavelength of 630 nm using the Multimode reader TECAN Infinite M200.

## 2.6 Fish Trypsin Activity Test

Trypsin enzyme activity test can be determined using a specific type of trypsin substrate, namely BAPNA. This activity test was applied to all supernatants from three types of stadia. Following the procedure from [16], making a BAPNA solution with the composition in 1 mL of DMSO dissolved 0.0435 g of BAPNA powder. Then it was dissolved in 100 mL with 0.05 M Tris-HCl containing 0.02 M CaCl<sub>2</sub>.2H<sub>2</sub>O. Then 0.05 mL of the sample was added with 2.5 mL of BAPNA solution. Then the sample tubes were incubated at 10°C, 20°C, 30°C, 40°C, 50°C and 60°C for 10 minutes. Then after completion of the incubation period, the reaction was stopped by adding 1 mL of 30% acetate solution. The incubation process was continued for 10 minutes at 37°C. The same procedure was carried out on the blank tube except that adding the enzyme extract was carried out after administration of 1 mL of 30% acetate solution. The reaction mixture was mixed until homogeneous using a vortex. The absorbance of the solution was checked using a spectrophotometer at a wavelength of 410 nm. Enzyme activity is calculated based on the equation:

$$\text{Enzym Activity} = \frac{(\text{Asampel} - \text{Ablanko}) \times \text{total volume after reaction} \times 1000}{8800 \times \text{time of inkubation} \times \text{volume of the enzyme reacted}}$$

## 2.7 In Silico Primary Design for Trypsin Gene Amplification in Eel (*Anguilla bicolor*)

### 2.7.1 Nucleotide Sequence Retrieval and Gene Library Construction

The gene encoding the trypsin in *Anguilla bicolor* is currently unavailable in the GenBank database. Therefore, the primer design was carried out by referring to the trypsinogen gene sequences in other species. The number of sequences used for the preliminary design came from 2 species with ten sequence variations (Table 2). For this study, [17] suggested a gene nucleotide search be performed on the NCBI database (<https://ncbi.nlm.nih.gov>). The whole trypsinogen gene sequences obtained, both partial and full-length sequences, were downloaded in FASTA format. All sequences obtained were selected from authenticated species known to possess the trypsinogen gene. A Multiple Sequence Alignment process was performed using Mega-X Software to determine the conservation area as the primary candidate selection area. Alignment is made using two algorithms, CLUSTALW and MUSCLE, to know which algorithm is optimal. The sustainable area is determined, and a primary degenerate is designed to amplify the area. The primers used to strengthen the sustainable regions are shown in Table 2. In addition to MSA, all sequences were constructed to form a phylogeny tree to analyze the trypsinogen gene's affinity level.

**Table 2.** Sequence identity for primary design

No.	Taxa Name	Locality	GenBank Accession No.
1	<i>Anguilla japonica</i>	China	KR827547.1
2	<i>Anguilla anguilla</i>	Republik Ceko	XM_035422887
3	<i>Anguilla anguilla</i>	Republik Ceko	XM_035422739
4	<i>Anguilla japonica</i>	Japan	AB519643.1
5	<i>Anguilla japonica</i>	Japan	AB070720.1
6	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429595.1
7	<i>Anguilla anguilla</i>	Republik Ceko	XM 035428317.1
8	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429594.1
9	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429592.1
10	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429593.1

### 2.7.2 In-Silico Evaluation of Primary Properties with Primer3Plus

Forward and reverse primary candidates that have been determined based on the results of Multiple Sequence Alignment were analyzed using Primer3Plus at the address <https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>. The results are the three best primer candidates from forward and reverse primers (Table 3) with different nucleotide base arrangements. This device displays primary properties such as % GC, hairpin value, base length, self-dimer, melting temperature (T<sub>m</sub>), and cross dimer. To confirm the results of the primary property evaluation, check again at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>.

**Table 3.** Primer design of fish trypsin *Anguilla bicolor*

Primer Name	Sequence 5'→3'	Length (bp)	T <sub>m</sub> (°C)	GC%	SC
AF	GGCTCTGGATGATGATAAGATTG	23	59,9	43,5	0,0
AR	AGCTCACCGTTACACACCAC	20	58,6	55,0	0,0
BF	ATGAGGTCTCTGGTTTTTATTCTGC	25	61,2	40,0	2,0
BR	CGTATCCCAGGACACAACA	20	61,8	55,0	0,0
CF	CTGTGGCTCTGGATGATGATAA	22	60,1	45,5	3,0
CR	CGTATCCCAGGACACAACA	20	61,8	55,0	0,0

### 2.7.3 Isolation of DNA, Primer Optimization using Gradient PCR, and Electrophoresis Process

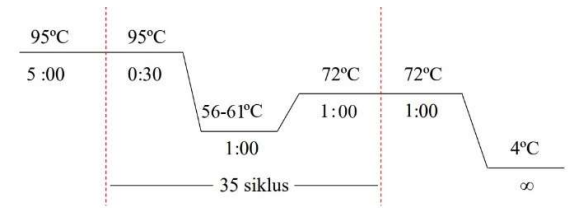
The Isolation of DNA procedure complies with TIANGEN's TiaNamp DNA Extraction KIT protocol. 1 mg of the sample was crushed using a mortar and put into a 1.5 mL Eppendorf tube. First, 500 ul of GMO1 buffer was added, then 20 µL of Proteinase K was added slowly and homogenized within 1 minute. Incubate the solution for 1 hour at 56°C and vortex every 15 minutes. 200 µL of GMO2 Buffer was added, then incubated at room temperature for 10 minutes. The sample was centrifuged for 5 minutes at 12,000 rpm and then transferred the supernatant to a new tube. Then add 0.7 volume of isopropanol and centrifuge again for 3 minutes at 12,000 rpm. Then discard the supernatant. For pellets, continue adding 700 µL of 70% alcohol, centrifugation for 1 minute at 12,000 rpm. Repeat twice in the previous step. After completion, the pellets were incubated at room temperature for 10 minutes. Dry the shells at room temperature, then add 50 µL of TE Buffer. Store DNA isolates at -20°C to -80°C.

The PCR amplification technique used a DNA template isolated from *Anguilla bicolor*. Optimization of the PCR profile at the primary annealing temperature is necessary to obtain the best conditions for amplifying the *Anguilla bicolor* trypsinogen gene. The composition of the PCR reaction (Table 4) is as follows:

**Table 4.** Composition of PCR reaction components using 2x PCR Master Mix Solution (i-MAXTM II)

No	Components	Concentration
1	Mastermix i-Max Intron (1X)	7,50 µl
2	Forward Primer (10 µM)	0,60 µl
3	Reverse Primer (10 µM)	0,60 µl
4	DNA Template	1,50 µl
5	Nuclease Free Water (NFW)	Up to 15 µl

The amplification process begins with an initial denaturation step for 5 minutes at 95°C, then 30 cycles of reaction are carried out consisting of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 56-61°C (temperature gradient for temperature optimization), and 1 minute. DNA elongation at 72°C. Then the final elongation was carried out for 60 seconds at 72°C (Figure 2).



**Fig. 2.** PCR profile using 2x PCR Master Mix Solution (i-MAXTM II)

Electrophoresis begins with the preparation of 2% agarose gel by dissolving 2 g of agarose in 100 ml of 1x TAE buffer. 100 µL of SYBR safe was added, homogenized, and poured into the Scie Plas HU10 Mini-Plus electrophoresis tray (10 x 11.5 cm). 5 µl of sample and 1 µl of loading dye were put into the electrophoresis chamber using a micropipette. The electrophoresis process was carried out using a power of 80 volts for 40 minutes. Then the electrophoresis results were visualized using a UV transilluminator.

### 2.7.4 Sanger Sequencing

The Sanger sequencing method used an Automatic DNA Sequencer with the dye terminator labeling method. The stages of DNA sequencing carried out in this study included preparing the DNA, followed by the amplification process via PCR using primers, DNA purification, electrophoresis, and electrophoretic reading of the sequenced results.

The data obtained shows the nucleoid sequence marked by a different color and is stored as an electropherogram in ABI file format. Green color indicates adenine base type, black color indicates guanine base type, red color indicates thymine base type and blue color indicates cytosine base type. The amplification of *Anguilla bicolor* trypsinogen DNA, which PCR had successfully confirmed, was then sequenced to determine the base sequence. Direct sequencing uses degenerate primers with two-way reading, namely forward and reverse. The entire base sequencing process was done using the Applied Biosystems 3730xl DNA Analyzer by Macrogen Inc. (Singapore).

## 2.8 Data Analysis

### 2.8.1 Analysis of Biochemical Data

The biochemical data obtained were analyzed using ANOVA at a 95% confidence level using SPSS version 21.0 to determine the effect of differences in incubation temperature on the activity of fish trypsin in the digestive tract of eels. A follow-up test followed the significantly different ANOVA results in the form of a DMRT (Duncan's Multiple Range Test).

### 2.8.2 Analysis of PCR Result Data

PCR amplification results were analyzed using GelAnalyzer 19.1 to determine the bp length of the bands obtained. Obtaining a number indicating the

length of bp will help analyze the differences between fish trypsin in eel (*Anguilla bicolor*) and other types of trypsin.

### 2.8.3 Data Analysis of Sanger Sequencing Results

Sequence alignment was performed using the Clustal W Program. The base sequencing results were analyzed using the BioEdit program to obtain the base sequence of the base-sorted fragments. Sanger sequencing results were then analyzed using the BLAST program available on the NCBI website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), which was used to look for the similarity of a nucleotide or protein sequence (query sequence) to the database sequence (subject sequence).

## 3 Results And Discussion

### 3.1 Body and Intestinal Morphometrics *Anguilla bicolor*

Fish morphometrics describes characters related to fish body parts or sizes, for example, standard length, total length, and other length measurements [17]. The eels used in the study were almost uniform in length and body weight. Morphometric measurements of each sample of eel from three stages were measured using calipers with an accuracy of 0.01 mm, and the weight of the fish was weighed with a digital scale with an accuracy of 0.001 gram which included 11 characters. The results of *Anguilla bicolor* morphometric measurements are as follows:

**Table 5.** Results of measurements of the body morphometric characters (mm) of *Anguilla bicolor*

Characters	Stadia	
	Juvenile	Adult
TL	310,3±16,792	464,08±27,673
SL	308,4±12,244	450,09±16,557
HL	21,2±5,241	40,9±2,511
HW	13,3±1,733	31,4±2,246
HD	8,3±1,542	26,4±3,275
SNL	4,1±0,016	6,8±0,031
ED	1,02 ± 0.094	1,81 ± 0.756
IW	2,01 ± 0.022	3,81 ± 0.633
BD	10,8±0,057	21,58±0,617
BW	16,8±0,254	27,58±0,179
TW (g)	66,08±12,497	186,79±35,413

The results of *Anguilla bicolor* body morphometric measurements (Table 5) show that the juvenile stage has a body weight (of 66.08 ± 12.497 g), and the adult stage has a body weight (of 186.79 ± 35.413 g). Currently, available information regarding the morphometrics of eels in Indonesia was provided by [11], who conducted research on local eels in the Segara Anakan area, Cilacap, by classifying the catches of collectors into eels with a size of 41.25 ± 0.898 g included in the elver stage, eels with a size of 319.8 ± 4.666 g were included in the yellow eel stage and eels with a size of 569.5 ± 9.150 g were included in the pre-silver stage.

The weight of the eel from PT. Laju Banyu Semesta (LABAS Sidat) is less valuable than local eels caught directly from the ocean. It is because the type of treatment for eels in captivity is different from eels that live in the ocean. In addition, the age difference between the samples taken from PT. Laju Banyu Semesta (LABAS Sidat) is different from the age of eel fish that live in the ocean. [18] states that the proportion of fish body parts has different values, influenced by several factors, including fish size, water conditions or habitat, fish species, fish physiological condition, and the handling process from catching to preparation. In contrast, differences in fish growth are influenced by several factors such as age, sex, food availability, and heredity.

**Table 6.** Results of measuring the morphometric characters (mm) of the intestines of *Anguilla bicolor*

Measurement Parameters	Stadium	
	Juvenile (n=24)	Adult (n=24)
Total Length (mm)	310,30±16,79	464,08±27,67
Body Weight (g)	66,080±12,500	186,790±35,413
Intestine Length (mm)	128,20±11,32	277,71±13,47
Intestine Weight (g)	0,014±0,373	0,038±0,914
The Ratio Intestinal Length/ Total Body Length (RGL)	0,413±0,674	0,598±0,0,486
The Ratio of Intestinal Length/Body Weight (ISI)	1,941±0,924	1,487±0,0813

The results of measuring the morphometric characters (mm) of the intestinal tract of *A. bicolor* (Table 6) show that the eel has a Gut Length Ratio (RGL) of 0.27 ± 0.99 at the larval stage, 0.58 ± 0.92 at the juvenile stage, and 0.62 ± 0.81 in the adult stage. The results of measuring the morphometric characters (mm) of the intestinal tract of *A. bicolor* showed that the eel had an Intestinal Somatic Index (ISI) of 2.106 ± 0.992 at the larval stage, 1.941 ± 0.924 at the juvenile stage, and 1.487 ± 0.0813 at the adult stage.

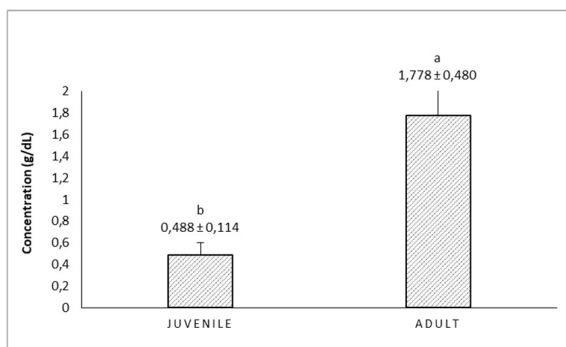
The older the stadium, the greater the Gut Length Ratio (RGL) value, but the older the stadium, the smaller the Intestinal Somatic Index (ISI). According to [19] Gut Length Ratio (RGL) is calculated as the ratio of intestinal length to total length. This ratio is used to determine the eating habits of fish, namely herbivores (RGL > 3), carnivores (RGL < 1), or omnivores (RGL = 1-3). Meanwhile, ISI is used to understand morphometric studies and research on fish physiology. This ratio can provide information about the adaptation and function of the fish's digestive system, mainly how the fish's intestine develops according to the body's metabolic needs as well as the health and nutritional conditions of the fish. Fish that have a high ISI have a more designed and functioning gut that has an excellent ability to digest and absorb nutrients. Conversely, a decrease in ISI can indicate a health problem or digestive disorder in the fish.

According to [20] based on how they eat, eel is a carnivore, so it has a shorter gut size when compared to other fish, which are herbivorous and omnivorous. [21] explained that one type of fish can be distinguished based on the morphology of the digestive system, which compares the ratio of digestive tract length to body

length for fish in different food categories. In addition, the quantification of these morphological measurements is assessed based on the trophic position of a species.

### 3.2 Protein Content of *Anguilla bicolor* Trypsin Extract

The protein content of the enzyme extract (Figure 3) in the larval, juvenile, and adult stages of *Anguilla bicolor* with three repetitions had an average of  $0.0893 \pm 0.106$  g/dL,  $0.488 \pm 0.114$  g/dL, and  $1.778 \pm 0.480$  g/dL. The results of the DMRT test showed that there was a significant difference in the average between the types of treatment (stadia) on protein content ( $P < 0.05$ ). *A. bicolor* belongs to a kind of fish with a high protein content. These results follow research from [22], which revealed that the protein content of *Anguilla* spp. as a food ingredient for fulfilling nutrition has a high protein content ranging from 15-20%.

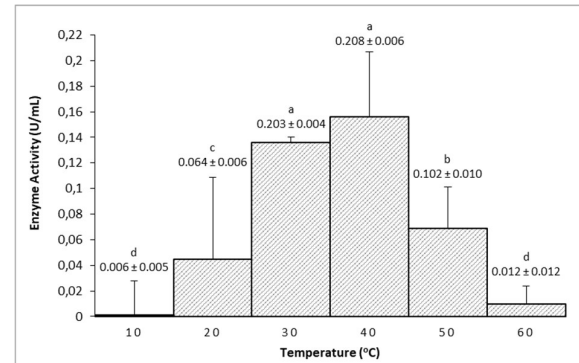


**Fig. 3.** Mean ( $\pm$ SD) intestinal protein levels of *Anguilla bicolor*

Compared with other types of eel, as stated by [22] the optimum protein content in the juvenile phase is 44% for the *Anguilla japonica* type. In contrast, for the *Anguilla marmorata* eel size 2.29 g, the optimum feed content is 50%, and a height of 21.97 g is 45%. According to [11], the protein requirement for carnivorous fish is more significant than for herbivorous and omnivorous fish due to the supporting factors described by [23], which stated that the protein content of fish is strongly influenced by age, stadia, type, size, feed protein quality, feed digestibility, and environmental conditions.

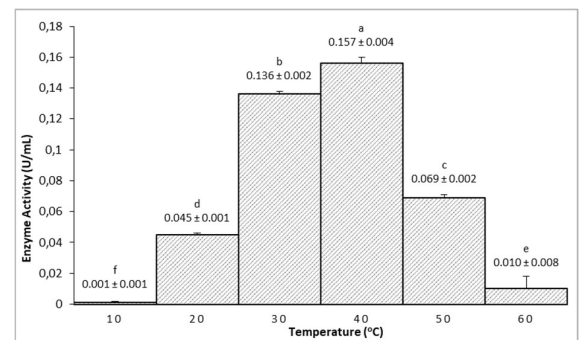
The body's need for protein is closely related to the work of enzymes because protein will be able to be absorbed by the body if enzymes have broken it down into simpler forms. Enzyme work control can take the form of regulation in producing active enzymes from inactive enzymes (inactive precursors). The precursors for proteases are called preprotein or proenzymes. Proenzymes can be activated into active enzymes by other already active enzymes [24].

### 3.3 Enzyme Activity of *Anguilla bicolor* Fish Trypsin



**Fig. 4.** Mean ( $\pm$ SD) trypsin activity (U/mL) in juvenile stadia at different incubation temperatures (°C)

The results of the measurement of enzyme activity (Figure 4) showed that the juvenile stadia *Anguilla bicolor* had an average enzyme activity at temperatures of 10°C ( $0.006 \pm 0.027$  U/mL), 20°C ( $0.064 \pm 0.064$  U/mL), 30°C ( $0.203 \pm 0.004$  U/mL), 40°C ( $0.208 \pm 0.051$  U/mL), 50°C ( $0.102 \pm 0.032$  U/mL), and 60°C ( $0.012 \pm 0.012$  U/mL). The results of the DMRT test showed that there was a significant difference ( $P < 0.05$ ) between the treatments (incubation temperature (°C)) in the juvenile stage.



**Fig. 5.** Average ( $\pm$ SD) trypsin activity (U/mL) in adult stadia at different incubation temperatures (°C)

The results of measurements of enzyme activity (Figure 5) showed that the adult stage of *Anguilla bicolor* had an average enzyme activity at temperatures of 10°C ( $0.001 \pm 0.001$  U/mL), 20°C ( $0.045 \pm 0.001$  U/mL), 30°C ( $0.136 \pm 0.002$  U/mL), 40°C ( $0.157 \pm 0.004$  U/mL), 50°C ( $0.069 \pm 0.002$  U/mL), and 60°C ( $0.010 \pm 0.008$  U/mL). The results of the DMRT test showed that there was a significant difference ( $P < 0.05$ ) between the treatments (incubation temperature (°C)) in the adult stage.

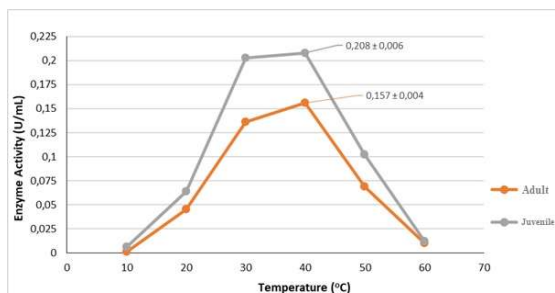
The enzyme activity measurements showed differences in trypsin digestion capacity, which was described as trypsin activity between eels with different stages. The younger the type of stadia or the smaller the size of the eel, the higher the value of enzyme activity and total activity. In line with research from [11], which informed that eels with a size of  $41.25 \pm 0.898$  g belong to the elver stage, which has higher trypsin-like activity

compared to the size of  $319.8 \pm 4.666$  g of the stadia yellow eel and  $569.5 \pm 9.150$  g pre-silver stage.

This high enzyme activity is related to the role of digestive organs and glands in secreting enzymes [26]. If the function of digestive organs and glands is optimal in secreting enzymes, then the digestive process will be optimal. The optimal digestive process will help to support growth [25]. Another factor that affects the activity of fish trypsin is feed, especially protein.

Protein is a source of energy for fish, especially if the availability of carbohydrates and fats in the feed cannot meet energy needs. Protein is needed continuously because the body needs amino acids to form new proteins during growth [26]. The development will run if energy and protein intake from the feed is sufficient or excessive. Optimum fulfillment of protein needs can affect fish growth [27]. In elver stadia eel, it is suspected that it requires protein to support its growth and encourages the digestive organs and glands to secrete large amounts of enzymes to compensate for the high protein requirements. As a result, protease activity, especially alkaline protease, was higher in the elver stage eel compared to the yellow eel and pre-silver stages [28].

### 3.4 Optimum Temperature of Fish Enzyme Activity *Anguilla bicolor*



**Fig. 6.** Optimum temperature (°C) of fish enzyme activity in larval (blue line), juvenile (grey line), and adult (orange line) stages of *Anguilla bicolor*

The optimum temperature for fish enzyme activity (Figure 6) in juvenile stadia *Anguilla bicolor* was 40°C with an enzyme activity value of  $0.208 \pm 0.006$  U/mL. The optimum temperature for fish enzyme activity in juvenile stadia *Anguilla bicolor* was 40°C with an enzyme activity value of  $0.157 \pm 0.004$  U/mL. According to [13], the temperature generally affects reaction rates, interfering with the solubility of reagents, enzyme stability, and kinetic constants. Two opposing mechanisms of activation (rate constant increases with increasing temperature) and denaturation (quaternary thermal opening and tertiary structure of the enzyme) coincide as the reaction temperature increases. Generally, at 50-60°C, the reaction rate (activation) increase exceeds the thermal denaturation limit. Except for thermophilic enzymes, denaturation predominates at temperatures above 60°C, and the reaction rate slows and stops around 80-90°C. The optimum temperature of an enzyme is the temperature where the amount of substrate is the most in a unit change of time.

The effect of temperature on enzyme activity is determined at temperatures ranging from 10 to 70°C. The optimum temperature for all protease sources was 40°C, with the highest activity of 90.61 U ml<sup>-1</sup> obtained from the viscera of yellow tuna. The protease activity decreased to 54 U ml<sup>-1</sup> at 50°C and sharply reduced at 60-70°C (13 U ml<sup>-1</sup>). However, proteases are more stable at lower temperatures (37-40°C) [28] which is supported by [29] who stated that proteases, especially trypsin, are active at 35-45°C, whereas protease activity is unstable at lower temperatures and extreme pH.

Enzyme activity can change depending on several factors, including pH, enzyme concentration, substrate concentration, and temperature. Enzyme and substrate concentrations affect the rate of enzymatic reactions. Changes in pH will involve changes in charge on substrates or enzymes, which can cause changes in the structure of enzymes and substrates [28], [30].

Enzymes with protein structures will be significantly affected by temperature. If there is an increase of 10°C above the minimum temperature, the enzyme activity will undoubtedly increase twofold until it reaches optimum conditions. An increase in temperature will generally increase the speed of enzymatic chemical reactions, but an increase in temperature that exceeds the optimum limit can cause enzyme denaturation [31]. Raising the temperature to the optimum point will cause an increase in the speed of the enzyme reaction due to the addition of kinetic energy of the molecules in the substrate and the enzyme so that contact between the enzyme and the substrate can occur. Too high a temperature causes the enzyme to lose its three-dimensional structure and catalytic ability [30]. [32] and [16] reported that fish belonging to the predatory species, for example, *Anoplarchus purpureus*, had a faster increase in trypsin activity when compared to other types. When enzymes are used in industrial processes and analytical procedures, the evaluation of their activity becomes very important. According to [13], focusing on the industrial scale, the decision to use or not an enzyme in a process must consider several things, such as the amount of enzyme needed to run the process correctly, the duration of the reaction, the amount of substrate to be converted, the number of conditions where the reaction occurs, and the overall cost of the process. The success of the enzymatic process depends on the optimization of three factors, namely the amount of enzyme needed, operating conditions (pH, temperature, and agitation), and the reaction results.

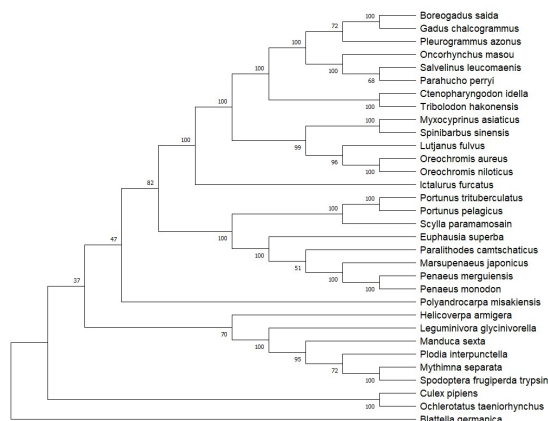
### 3.5 Molecular Characteristics of Enzyme Fish Trypsin *Anguilla bicolor*

#### 3.5.1 In-Silico Analysis of Trypsin of Various Species

Searching for the diversity of trypsin gene sequences for various species was carried out through the NCBI gene bank page (<https://www.ncbi.nlm.nih.gov/>) by writing the names of the targeted genes with the keywords trypsin and trypsinogen. Data were collected, including

accessions, nucleotide lengths, and nucleotide sequences in FASTA format. All DNA sequences collected from NCBI were aligned using CLUSTALW in the MEGA X software. It aims to determine the level of homology and identify sequences that have the potential to be used as barcodes. Sequences that have the potential to be barcodes are different and distinctive compared to the others.

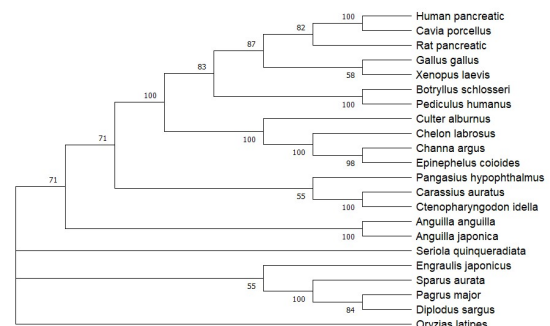
Search results for the trypsin gene of various species were carried out through the NCBI gene bank page, obtained 4-four full-length DNA sequences from four species, 16 partial-length DNA sequences from two species, 51 protein sequences from 40 species, 40 partial-length mRNA sequences from 37 species, and 32 full-length trypsin mRNA sequences from 32 different species.



**Fig. 7.** Phylogenetic tree showing the relationship between trypsin mRNA sequences of various species obtained from NCBI constructed using the Maximum Likelihood method with the GTR+G+I model.

After analyzing the alignment process, these sequences contain many gap areas and only have a few conservation areas. The phylogenetic tree showing the relationship between the trypsin mRNA sequences of various species (Figure 7) illustrates that the trypsin genes of multiple species have a very high sequence diversity. Only a few references provide information about the character sequences of the trypsin gene, so the information is still minimal.

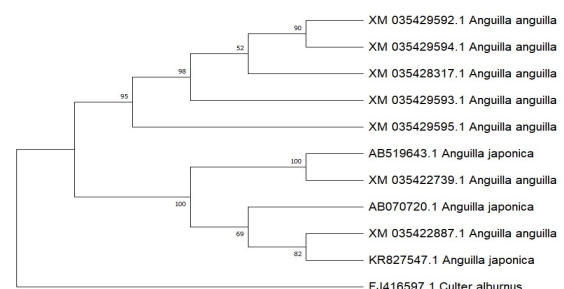
According to [33] and [34], the gene encoding the serine protease enzyme group is the PRSS1 gene. This gene encodes trypsinogen, a member of the serine protease trypsin family. This enzyme is secreted by the pancreas and converted to its active form in the small intestine. Several other trypsinogen genes are localized at the T-cell receptor beta locus on chromosome 7. These genes can provide instructions for making an enzyme called cationic trypsinogen. The PRSS1 gene is TRP1, TRY1, TRY4, and TRY1.



**Fig. 8.** Trypsinogen gene phylogeny tree for various species constructed using the Maximum Likelihood method with the GTR+G+I model.

Searching for the trypsin gene through the NCBI gene bank page has not shown any species close to the *Anguilla bicolor* type. So that the search was expanded by looking for trypsinogen genes of various species to obtain trypsinogen sequences that are closely related to *Anguilla bicolor*. It can be done to get specific degenerate primers by looking for the closest reference sequence. According to [35], designing a primer for a new gene or organism can be done by aligning the most relative species in one genus and determining the primary candidate in a consensus conservation area. Fragmented reads showing low query cover ( $\leq 30\%$ ) of the entire length of each sequence can be retrieved to minimize poor alignment quality. Species-specific genes are determined based on the query cover level (%) and then used to design primary sets through various existing software.

The results of searching the trypsinogen genes of various species were carried out through the NCBI gene bank page, obtained 4-four full-length DNA sequences from four species and 22 trypsin full-length mRNA sequences from 22 different species. The phylogenetic tree showing the relationship between the trypsinogen mRNA sequences of various species (Figure 8) illustrates that the trypsinogen genes of multiple species have a very high sequence diversity. After analyzing the alignment process, these sequences contain a lot of gap areas and only have a few conservation areas



**Fig. 9.** Trypsinogen gene phylogeny tree of *Anguilla* spp. was constructed using the Maximum Likelihood method with the GTR+G+I model.

Searching for the trypsinogen gene through the NCBI gene bank page shows that some species are close to the *Anguilla bicolor* type, namely *Anguilla japonica* and *Anguilla anguilla*, which are in the same genus. Therefore, the mRNA sequence of the trypsinogen gene

of the *Anguilla* genus was chosen (Figure 9) to be aligned in forming the *Anguilla bicolor* trypsinogen primary candidate. There are ten full-length trypsinogen mRNA sequences from two *Anguilla* species which show few gap areas and many conservation areas with nucleotide lengths between 800-900 bp. According to [36], one type of enzyme that plays an essential role in the growth process is the trypsin, where the appearance of trypsin and trypsinogen has been identified in the early stages of larval development of fish species.

### 3.5.2 Fish Trypsin Primer and Phylogenetic Analysis

The conventional PCR process requires a pair of specific primers to amplify certain parts of the genome. Primers are oligomeric components designed to limit the template and complete the target PCR amplicon's final sequence and the initial site for DNA chain synthesis [37], [38]. According to [39], primers generally have 50-60% guanine-cytosine content and a base length of 15-25 nucleotides. The primers used in PCR are oligonucleotides identical to one of the template DNA chains (5'-phosphate) and oligonucleotides identical to the other template (3'-OH). Each of the PCR primers can complement a single strand that is different from the double-stranded target.

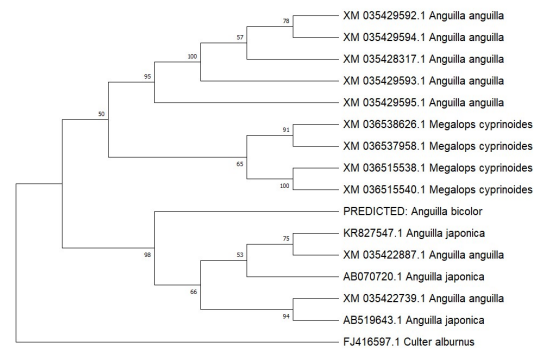
Data processing results with trimming, contig, and alignment stages using the BioEdit program[32]. Data processing based on these steps resulted in the DNA sequence of the *Anguilla bicolor* trypsinogen gene. Alignment was performed on the DNA of the *Anguilla bicolor* trypsinogen gene with the sequences in the GenBank database using the BLAST program. The assembly results gave a sequence length of 1030, 1422, and 1459 bp for the same sample using primer options A, B, and C.

The resulting contig sequences were identified taxonomically using BLAST with the "nucleotide collection" database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sample sequences A, B, and C with the highest percentage of similarity and total score are referred to as the taxon for the input sequences. Next, the top 14 taxa with the highest similarity values were selected to be used in phylogenetic construction. The sequence identities used for phylogenetic construction are as shown in Table 7.

**Table 7.** Sequence identity for phylogenetic construction

No.	Taxa Name	Locality	GenBank Accession No.
1	<i>Anguilla japonica</i>	China	KR827547.1
2	<i>Anguilla anguilla</i>	Republik Ceko	XM 035422887
3	<i>Anguilla anguilla</i>	Republik Ceko	XM 035422739
4	<i>Anguilla japonica</i>	Japan	AB519643.1
5	<i>Anguilla japonica</i>	Japan	AB070720.1
6	<i>Megalops cyprinoides</i>	Singapura	XM 036515540.1
7	<i>Megalops cyprinoides</i>	Singapura	XM 036538626.1
8	<i>Megalops cyprinoides</i>	Singapura	XM 036537958.1
9	<i>Megalops cyprinoides</i>	Singapura	XM 036537957.1
10	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429595.1
11	<i>Anguilla anguilla</i>	Republik Ceko	XM 035428317.1
12	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429594.1
13	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429592.1
14	<i>Anguilla anguilla</i>	Republik Ceko	XM 035429593.1

Analysis of the maximum likelihood method of the GTR+G+I model with Bootstrap 1000 has been carried out and confirmed using Molecular Evolutionary Genetics Analysis (MEGA 11) software to obtain cross-species reconstruction based on branch line length. Different line lengths indicate each species' evolution level [40]. Based on the phylogram, it looks like a tree showing the distance in evolutionary time. The longer the line, the farther the evolutionary distance, while the shorter the line, the closer the evolution of the species.



**Fig. 10.** The tree topology was constructed using the Maximum Likelihood method with the GTR+G+I model.

The *Anguilla bicolor* trypsinogen gene sequences analyzed were in the same group. They tended to be close to the trypsinogen and trypsin-like genes from *Anguilla japonica* and *Anguilla anguilla* with a bootstrap value at the node of 92 (Figure 10). This shows that the *Anguilla bicolor* kinship based on the trypsin gene sequence has the closest kinship with other *Anguilla* species in the same genus. The phylogenetic tree shows that the in-group groups are grouped within each clade based on the similarity of the trypsin gene sequence.

## Conclusion

1. The protein content of the enzyme extract in the juvenile stage of *Anguilla bicolor* had an average of  $0.488 \pm 0.004$  g/dL, and the adult stage of *Anguilla bicolor* had an average of  $1.778 \pm 0.080$  g/dL. The highest enzyme activity was obtained in the juvenile stage,  $0.529 \pm 0.016$  (U/mL), and in the adult stage,  $0.399 \pm 0.009$  (U/mL). Enzyme activity increases with increasing temperature used and reaches a maximum of 40°C.
2. Trypsin sequences of *Anguilla bicolor* analyzed tend to be close to the Trypsinogen and Trypsin-like genes from *Anguilla japonica*, *Anguilla anguilla*, and *Megalops cyprinoides*.

## References

1. R. S. Prakasham, C. Subba Rao, R. Sreenivas Rao, and P. N. Sarma, "Alkaline protease production by an isolated *Bacillus circulans* under solid-state fermentation using agroindustrial waste: Process parameters optimization," *Biotechnol. Prog.*, vol.

- 21, no. 5, pp. 1380–1388, 2005, doi: 10.1021/bp050095e.
2. D. S. Ningthoujam, P. Kshetri, S. Sanasam, and S. Nimaichand, “Screening , Identification of Best Producers and Optimization of Extracellular Proteases from Moderately Halophilic Alkalithermotolerant Indigenous Actinomycetes,” *World Appl. Sci. J.*, vol. 7, no. 7, pp. 907–916, 2009.
  3. B. S. Kaphalia, “Biomarkers of acute and chronic pancreatitis,” *Biomarkers Toxicol.*, pp. 279–289, 2014, doi: 10.1016/B978-0-12-404630-6.00016-6.
  4. H. Sundus, H. Mukhtar, and A. Nawaz, “Industrial Applications and Production Sources of Serine Alkaline Proteases: A Review,” *J. Bacteriol. Mycol. Open Access*, vol. 3, no. 1, pp. 191–194, 2016, doi: 10.15406/jbmoa.2016.03.00051.
  5. Y. Zhang, Q. Liang, C. Zhang, J. Zhang, G. Du, and Z. Kang. “Improving production of *Streptomyces griseus* trypsin for enzymatic processing of insulin precursor,” *Microb. Cell Fact.*, vol. 19, no. 1, pp. 1–11, 2020, doi: 10.1186/s12934-020-01338-9.
  6. T.- Nurhayati, E.- Salamah, - - Cholifah, and R.- Nugraha, “Optimasi Proses Pembuatan Hidrolisat Jeroan Ikan Kakap Putih,” *J. Pengolah. Has. Perikan. Indones.*, vol. 17, no. 1, pp. 42–52, 2014, doi: 10.17844/jphpi.v17i1.8136.
  7. R. G. La Apu, “PEMANFAATAN LIMBAH JEROAN IKAN CAKALANG (Katsuwonus pelamis) SEBAGAI BAHAN SUBSTITUSI TEPUNG IKAN PADA PERTUMBUHAN IKAN NILA (*Oreochromis niloticus*),” *J. Sains dan Teknol. Perikan.*, vol. 1, no. 2, pp. 13–24, 2021.
  8. V. Venugopal, “Enzymes from Seafood Processing Waste and Their Applications in Seafood Processing,” *Adv. Food Nutr. Res.*, vol. 78, pp. 47–69, 2016, doi: 10.1016/bs.afnr.2016.06.004.
  9. S. P. Kumari and R. Reshma, “Effect of alkaline protease produced from fish waste as substrate by bacilluclusi on destaining of blood stained fabric,” *J. Trop. Life Sci.*, vol. 11, no. 1, pp. 59–66, 2021, doi: 10.11594/jtls.11.01.08.
  10. K. Jesús-de la Cruz, C. A. Álvarez-González, E. Peña, J. A. Morales-Contreras, and Á. Ávila-Fernández, “Fish trypsins: potential applications in biomedicine and prospects for production,” *3 Biotech*, vol. 8, no. 4, 2018, doi: 10.1007/s13205-018-1208-0.
  11. F. A. Larassagita, Hana, and U. Susilo, “Aktivitas Tripsin-Like dan Kimotripsin-Like pada Ikan Sidat Tropik *Anguilla bicolor* McClelland,” vol. 5, no. 1, pp. 55–60, 2018, doi: <https://doi.org/10.20884/1.SB.2018.5.1.789>.
  12. H. Y. Sugeha and M. U. Genisa, “External and internal morphological characteristics of glass eels *Anguilla bicolor bicolor* from the Cibaliung River Estuary , Banten , Indonesia,” *Oseanologi dan Limnol. di Indones.*, vol. 41, no. 1, pp. 37–48, 2015.
  13. K. A. Gaidhani, M. Harwalkar, and P. S. Nirgude, “World Journal of Pharmaceutical ReseaRch SEED EXTRACTS,” *World J. Pharm. Res.*, vol. 3, no. 3, pp. 5041–5048, 2014, doi: 10.20959/wjpr20202-16660.
  14. N. Salkind, “American Statistical Association,” *Encycl. Res. Des.*, vol. 51, no. 276, pp. 667–669, 2012, doi: 10.4135/9781412961288.n9.
  15. K. Rungruangsak-Torrissen, R. Moss, L. H. Andresen, A. Berg, and R. Waagbø, “Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.),” *Fish Physiol. Biochem.*, vol. 32, no. 1, pp. 7–23, 2006, doi: 10.1007/s10695-005-0630-5.
  16. T. Nurhayati, R. Nugraha, and D. Lihuana, “Characterization of Ammonium Sulphate Fraction Tripsin Isolated from Intestine of Little Tuna,” *Jphpi*, vol. 23, pp. 372–382, 2020.
  17. C. Turan, “A note on the examination of morphometric differentiation among fish populations: The Truss System,” *Turkish J. Zool.*, vol. 23, no. 3, pp. 259–263, 1999.
  18. A. B. Kusuma, “Komposisi Nutrisi Ikan Sidat *Anguilla bicolor bicolor* dan *Anguilla marmorata*,” *Jphpi*, vol. 21, no. 3, pp. 504–512, 2018.
  19. F. S. Kertikasari, N. Cokrowati, and A. W. Puspitasari, “Gut content analysis of tilapia (,” vol. 040007, 2019.
  20. Y. Sugianti, M. R. A. Putri, and S. . Purnamaningtyas, “Eel fish species (*Anguilla* spp.) and its migratory habitat characteristics in Cikaso River, Sukabumi, West Java.,” *Limnotek Perair. darat Trop. di Indones.*, vol. 27, no. 1, pp. 39–54, 2020.
  21. D. L. Kramer and M. J. Bryant, “Intestine length in the fishes of a tropical stream: 2. Relationships to diet - the long and short of a convoluted issue,” *Environ. Biol. Fishes*, vol. 42, no. 2, pp. 129–141, 1995, doi: 10.1007/BF00001991.
  22. B. C. Sungchul Bai, K. Katya, and D.-J. Kim, “Japanese eel aquaculture in Korea,” vol. 2011, no. November 2012, pp. 1–6, 2012, [Online]. Available: <https://www.aquaculturealliance.org/advocate/japanese-eel-aquaculture-in-korea/?headlessPrint=AAAAPIA9c8r7gs82oWZBA>
  23. D. Sanjayasari and Kasprijo. “Estimasi Nisbah Protein-Energi Pakan Ikan Senggaringan (*Mystus Nigriceps*) Dasar Nutrisi Untuk Keberhasilan Domestikasi,” *J. Perikan. dan Kelaut.*, vol. 15, pp. 89–97, 2010.
  24. L. D. Nelson and M. M. Cox, “Principle of Biochemistry,” *Cell Biol. Physarum Didymium*, pp. 393–435, 1982, doi: 10.1016/b978-0-12-049601-3.50017-1.
  25. D. Y. . Airin and C. Lumenta, “Pakan diameter berbeda bagi pertumbuhan benih sidat (*Anguilla* sp),” *e-Journal Budid. Perair.*, vol. 3, no. 3, pp. 30–41, 2015, doi: 10.35800/bdp.3.3.2015.10409.

26. C. D. Webster and W. Oxon, "Nutrient requirements and feeding of finfish for aquaculture C.D.," vol. 214, pp. 419–420, 2002.
27. Z. Yandes, R. Affandi, and I. Mokiginta, "Pengaruh pemberian selulosa dalam pakan terhadap kondisi biologis benih ikan gurami (*Osphronemus gourami* Lac)," *J. Iktiologi Indones.*, vol. 3, no. 1, pp. 27–33, 2003.
28. E. Liviawaty and E. Afrianto, "Pakan Ikan," *Yogyakarta: Kanisius*, no. September, pp. 69–82, 2012.
29. A. Bougatef, "Trypsins from fish processing waste: Characteristics and biotechnological applications - Comprehensive review," *J. Clean. Prod.*, vol. 57, pp. 257–265, 2013, doi: 10.1016/j.jclepro.2013.06.005.
30. K. Arief, F. Nisa, dan U. Murdiyatmo, J. Teknologi Hasil Pertanian, F. Teknologi Pertanian, and U. Brawijaya, "Partial Characterization of Crude Protease Extracted from *Bacillus amyloliquefaciens* NRRL B-14396," *J. Teknol. Pertan.*, vol. 7, no. 2, pp. 96–105, 2006.
31. L. M. Shuler and F. Kargi, "Edition, Bioprocess Engineering Basic Concepts Second," *Prentice Hall books Up. Saddle River, NJ 07458*, vol. 22, no. 3, p. 293, 2022, doi: 10.1016/0168-3659(92)90106-2.
32. M. A. K. Bahrin, M. F. Othman, N. H. N. Azli, and M. F. Talib, "Industry 4.0: A review on industrial automation and robotic," *J. Teknol.*, vol. 78, no. 6–13, pp. 137–143, 2016, doi: 10.11113/jt.v78.9285.
33. C. Férec, O. Raguénès, R. Salomon, C. Roche, J. P. Bernard, M. Guillot, I. Quéré, C. Faure, B. Mercier, M. P. Audrézet, P. J. Guillausseau, C. Dupont, A. Munnich, J. D. Bignon, L. Le. Bodic "Mutations in the cationic trypsinogen gene and evidence for genetic heterogeneity in hereditary pancreatitis," *J. Med. Genet.*, vol. 36, no. 3, pp. 228–232, 1999.
34. Hu C, Wen L, Deng L, Zhang C, Lugea A, Su HY, Waldron RT, Pandol SJ, Xia Q, "The Differential Role of Human Cationic Trypsinogen (PRSS1) p.R122H Mutation in Hereditary and Nonhereditary Chronic Pancreatitis: A Systematic Review and Meta-Analysis," *Gastroenterol. Res. Pract.*, vol. 2017, 2017, doi: 10.1155/2017/9505460.
35. I. You and E. B. Kim, "Genome-based species-specific primers for rapid identification of six species of *Lactobacillus acidophilus* group using multiplex PCR," *PLoS One*, vol. 15, no. 3, pp. 1–9, 2020, doi: 10.1371/journal.pone.0230550.
36. A. Kvåle, A. Mangor-Jensen, M. Moren, M. Espe, and K. Hamre, "Development and characterisation of some intestinal enzymes in Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae," *Aquaculture*, vol. 264, no. 1–4, pp. 457–468, 2007, doi: 10.1016/j.aquaculture.2006.12.024.
37. D. A. Hewajuli and N. Dharmayanti, "The Advance of Technology of Reverse Transcriptase-Polymerase Chain Reaction in Identifying the Genome of Avian Influenza and Newcastle Diseases," *Indones. Bull. Anim. Vet. Sci.*, vol. 24, no. 1, pp. 16–29, 2014, doi: 10.14334/wartazoa.v24i1.1022.
38. M. Ehtisham, F. Wani, I. Wani, P. Kaur, and S. Nissar, "Polymerase Chain Reaction (PCR): Back to Basics," *Indian J. Contemp. Dent.*, vol. 4, no. 2, p. 30, 2016, doi: 10.5958/2320-5962.2016.00030.9.
39. R. R. Garafutdinov, A. A. Galimova, and A. R. Sakhabutdinova, "The influence of quality of primers on the formation of primer dimers in PCR," *Nucleosides, Nucleotides and Nucleic Acids*, vol. 39, no. 9, pp. 1251–1269, 2020, doi: 10.1080/15257770.2020.1803354.
40. C. Lambré J. M. Barat, C. Bolognesi, and P. S. Cocconcelli, "Safety evaluation of food enzyme trypsin from porcine pancreas," *EFSA J.*, vol. 19, no. 6, 2021, doi: 10.2903/j.efsa.2021.6637.