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Digestive Enzyme Activities of *Osteochilus vittatus* with *Spirulina platensis* Feed Supplementation in Biofloc System

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Abstract

Osteochilus vittatus is a freshwater fish that needs to be developed because it tastes savory and can be used as a biocleaning agent. Research on the effect of supplementation of *Spirulina platensis* in nilem fish that is maintained in a biofloc system on digestive enzyme activity has never been done before. The objectives of this study were to determine the effect of *S. platensis* supplementation on *Osteochilus vittatus* digestive enzyme activity maintained in the biofloc system and to obtain the most optimum level of *S. platensis* supplementation in improving the digestive enzymes activity. The study was conducted experimentally with Completely Randomized Design with four treatments and five replications. *Osteochilus vittatus* were fed with *Spirulina platensis* level of 0, 2, 4, and 6 g kg^{-1} for 56 days. Enzyme activity was measured on days 0 and 56 with three kinds of buffer solutions. Amylase and protease activity was analyzed using ANOVA with a confidence level of 95%. The results showed that the highest amylase activity in the liver was 4.764 ± 1.705 U mg^{-1} protein and proximal intestine was 2.328 ± 0.838 U mg^{-1} protein. The highest protease activity was found in the liver and proximal intestine with activity of 6.536 ± 0.911 U mg^{-1} protein and 6.207 ± 2.195 U mg^{-1}protein. *Spirulina platensis* supplementation level of 6 g kg^{-1} feed is the most optimum level ($p<0.05$). Cultivation of fish with *S. platensis* supplementation in feed can increase enzyme activity and the fish maintenance in biofloc systems can improve water quality.

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INTRODUCTION

Osteochilus vittatus (O. vittatus) or known as, nilem fish is native to Indonesia. Nilem fish has been cultivated intensively (especially in Java) to meet the market demand. The increase in nilem fish production is closely related to feed given during the cultivation process. Giving different types of feed greatly affects to the growth and feed digestibility in the fish (Fujaya, 2004). German et al., (2004) stated that there is an activity of digestive enzymes in response to changes in the composition of food patterns. Relevant information regarding enzymes activity and digestive processes of each fish species is important for developing feed formulations. The pattern of digestive enzymes activity is also correlated with eating habits and digestive capacity (Candiotto et al., 2016). Substrates of protein and carbohydrates in feed are factors that greatly influence the activity of protease and amylase enzymes in fish, including nilem fish. Therefore, this study was conducted with feeding which was supplemented with *Spirulina platensis*.

S. platensis is a spiral green-blue algae that contains high nutritional value in form of, protein, β-carotene, phycocyanin, vitamins, essential minerals and amino acids (Lokapirnasari et al., 2011). Based on research conducted by Simanjuntak et al., (2018), supplementation of *S. platensis* with 6 g kg^{-1} feed level tested on gourami (*Osphronemus gouramy*) was able to increase growth, body composition, and blood biochemical parameters. It could support the increase of fish production.

Furthermore, Tietze (2004) said that blue-green algae contains protein (60-63%), carbohydrates (16%), fat (4%), β-carotene, fiber, essential and non-essential amino acids, vitamins, fatty acids and various kinds of micronutrients. In addition, *S. platensis* has a simple cell wall consisting of complex carbohydrates and proteins and it is not covered by cellulose that make it easily digested by the fish (Belay, 2002). This is what makes *S. platensis* often used as additional food for fish.

The metabolic waste and feed in the maintenance media greatly affects the quality of the water, so the water needs to be changed regularly. One cultivation system that converts ammonia to harmful metabolic waste results for fish into cell biomass (floc) as a source of natural fish food with the help of heterotrophic bacteria known as biofloc systems. This system utilizes cell growth in heterotrophic bacteria in the cultivation pond to utilize nitrogen waste to become high protein feed by providing a source of organic carbon to increase the C/N ratio. The biofloc technology/ system has advantages compared to other technologies because it combines the handling of waste to maintain water quality, while producing fish feed in situ (Ebeling et al., 2006; Salamah et al., 2015).

The biofloc system will maintain the quality of water while maintaining the conversion of ammonia to N2, which can be used by phytoplankton in the media (water) and not harmful to the fish, so that, the periodic change of water is not needed. In this study, *S. platensis* supplementation in feed of nilem fish was carried out using a biofloc system to improve the performance of digestive enzymes in the digestion channels (proteases and amylases) with an environmentally friendly maintenance system. The higher the enzyme activity, the better food digestion. The remaining feed and metabolic waste in maintenance media can be decomposed with the help of heterotrophic bacteria through the biofloc system, so that, the water quality is maintained as well. The aim of this study was to determine the effect of *S. platensis* supplementation on digestive enzyme activity of *Osteochilus vittatus* maintained in the biofloc system and to obtain the most effective dose of *S. platensis* for supplementation. Based on this study it was found that the improvement of maintenance pond water quality as well as increased enzyme activity in several digestive organs of *Osteochilus vittatus* fish.

METHODS

Preparation of biofloc systems and supplemented feed

Fish maintenance was carried out in a 20 pieces fiber tub measuring 60x40x60 cm^3, each with an aeration and a recirculation system. The biofloc system was carried out with the following procedure. In the fiber tub, was added 10 mL of probiotics EM4 and 40mL of molasses, then stirred for seven days using strong aeration. Fish was transferred on the eighth day to utilize the remaining feed and feces as a source of N. Environmental factors (pH, temperature and dissolved oxygen content) were routinely controlled every week to maintain optimal maintenance media conditions.

The feed supplemented with *S. platensis* for the treatment group was made with the following steps: 2 g of dried *S. platensis* were put into a beaker glass, then 100 mL of distilled water was added and stirred until homogeneous. Furthermore, 1 kg of commercial pellets LP1 were placed on a plastic tray, then the *S. platensis* solution was added into the pellet and flipped slowly. Feeds

that have been supplemented with *S. platensis* were dried in the sun. Dried foods were stored in tightly closed containers and labeled. The same procedures were carried out for *S. platensis* with 4 and 6 g kg^{-1} feed.

Treatment

The nilem fish used were 260 juvenile size weighing 9-15 gr. The fish were originated from the village of Cipaku, Mrebet Subdistrict, Purbalingga Regency. Nilem fish was put into a fiber tub with a density of 13 fish/tub. Before being treated, the fish was acclimated for 7 days. Feeding according to treatment with feeding rate (FR) 5% of the weight of the test fish biomass was given twice a day, for 56 days.

Data retrieval

Fish body weight measurements were carried out on days 14, 28, 42 and 56 for adjustments to feed. Sampling of protease and amylase enzymes was carried out at the beginning (day 0) and the end of the study (day 56) at three different buffer solution, i.e. pH 5, 7 and 10 for amylase activity, and pH 5, 8 and 10 for proteases activity in the liver, stomach and intestines (proximal intestine, mid intestine and distal intestine).

Preparation of the enzyme extracts

The digestive organs that have been taken and stored in the freezer -80^{0}C were crushed using an electric homogenizer in 50 mMTris-HCl buffer (pH 7.2-8.0) cold with a ratio of 1: 6 (w/v). Homogenate obtained was centrifuged using electric centrifuge at a speed of 12,000 rpm for 10 minutes, and the supernatant obtained was used to test the enzyme activity.

Reagents preparation for the measurement of supernatant proteins.

Reagent A: 2 g of NaOH plus 500 mL double distilled water, added 10 g of Na2CO3 and dissolved until homogeneous.

Reagent B: 50 mg CuSO4.5H2O plus 100 mg Na-K tartate.4H2O dissolved in 10 mL of double distilled water, dissolved until homogeneous.

Reagent C: 500 mL Reagent A plus 10 mL Reagent B.

Reagent D: 1 N FolinCiocalteu plus double distilled water with a ratio of 1: 1.

The supernatant protein was measured by inserting 2500 µL of reagent C on each test tube and added with 50 µL of the enzyme extract, shaken immediately then incubated for ± 10 minutes. After that, 250 µL of reagent D were added to each tube, then incubated at room temperature for ± 30 minutes. After 30 minutes, absorbance was measured at a wavelength of 660 nm.

The standard solution of maltose was made by weighing 72.0 mg of maltose and dissolving in double distilled water till the volume reached 10 mL, then a solution of 20 µmol mL^{-1} would be obtained. The standard solution was used to create a standard maltose curve with a regression equation between the concentration of maltose and absorbance obtained (wavelength of 540 nm).

Amylase activity was calculated by the Somogi-Nelson assay method (Hidalgo et al., 1999). Amylase activity was calculated using a standard maltose curve with a standard maltose concentration of 2.00, 4.00, 8.00, and 16.00 µmol / mL.

Protease activity was measured using the Furne et al., method. (2005) with modifications. The standard tyrosine curve was made with tyrosine concentrations between 50–400 µg/mL. Protease activity was calculated as the amount of enzyme needed to catalyze the formation of 1 µg tyrosine / mg protein / minute.

Data Analysis

Data on enzyme activity in the liver, stomach, proximal intestine, mid intestine and distal intestine were analyzed using analysis of variance (ANOVA) with a confidence level of 95%. If the results were significant, then they would be proceed with the Duncan test.

RESULTS AND DISCUSSION

Amylase enzyme activity in various digestive organs of *Osteochilus vittatus*

Measurement results of amylase activity in digestive organ of *Osteochilus vittatus* with different levels of *S. platensis* supplementation in feed at different buffer solutions (pH 5, 7 and 10) is presented in Figures 1, 2 and 3.

The results showed different enzyme activity. The pH value describes the optimal environmental conditions of the amylase enzyme in an organ. Amylase enzyme activity in buffer solution with pH 5 (Figure 1.) was only found in the liver, stomach and proximal intestine on day 56. Meanwhile, amylase enzyme activity on day 0 was not detected or had concentrations below 2.00 µmol/mL. Amylase enzyme activity was also only found in *S. platensis* supplementation level of 4 and 6 g kg^{-1} feed.

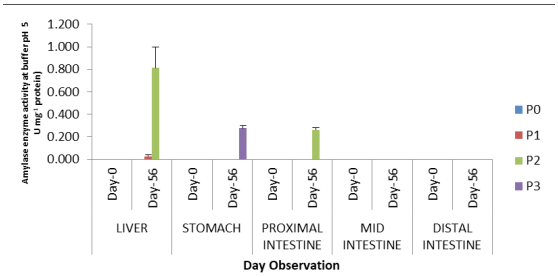

Figure 1. Amylase Enzyme Activity of *O. vittatus* at Buffer Solution with pH 5. Note: Amylase enzyme activity: the number of enzyme that hidrolyze starch (µg ml^{-1} minute^{-1} mg^{-1} protein). P0: *S. platensis* supplementation level of 0 g kg^{-1}feed in biofloc system, P1: *S. platensis* supplementation level of 2 g kg^{-1}feed in biofloc system, P2: *S. platensis* supplementation level of 4 g kg^{-1}feed in biofloc system, P3: *S. platensis* supplementation level of 6 g kg^{-1} feed in biofloc system. Values with the same letter shows no significant differences between treatments (Mean±SD, n=P>0.05), while the values with the different letter in the figure shows significant differences between treatments (Mean±SD, n=P<0.05).

S. platensis supplementation of 4 g kg^{-1} feed was able to increase the activity of amylase enzymes from previously undetectable (0.00 U mg-1 protein) to 0.812 ± 0.185 U mg^{-1} protein in the liver and 0.262 ± 0.016 U mg^{-1} protein in proximal intestine. Inside the stomach, amylase enzyme activity was also detected in *S. platensis* supplementation of 6 g kg^{-1} feed at 0.277 ± 0.019 U mg^{-1}protein.

Based on analysis of variance (ANOVA), the activity of the amylase enzyme was significantly different between treatments in acidic buffer solution. Providing *S. platensis* supplementation level of 4 g kg^{-1} feed becomes the best treatment for increasing amylase activity in the liver and proximal intestine. Meanwhile, in the gastric organs, amylase activity was significantly different between supplementation with level of 6 g kg^{-1} feed and other treatments because it was able to increase amylase activity. These results are consistent with the research of Gioda et al., (2017) in some herbivorous, omnivorous and carnivorous freshwater fish in various pH media, that at low pH (acids) amylase activity tends to decrease compared to neutral pH and the highest amylase activity was owned by herbivorous fish, *Ctenopharyngodon idella*.

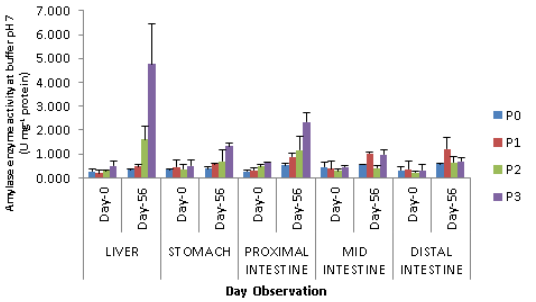

Figure 2. Amylase Enzyme Activity of *O. vittatus* at Buffer Solution with pH 7**.**

The results of measurement using neutral buffer solution (pH 7) showed amylase enzyme activities in liver, stomach, and intestine (Figure 2.). In general, the treatment of *S. platensis* supplementation at various levels in the biofloc system showed an increase in amylase enzyme activity measured on 56th day. Increased enzyme activity occurred in almost all digestive organs. The highest amylase enzyme activity (4,764 ± 1,705 U mg^{-1} protein) was found in the liver of fish fed with *S. platensis* supplementation with level of 6 g kg^{-1}.

The provision of 6 g kg^{-1} *S. platensis* has a considerable influence in increasing amylase activity in the liver, stomach, proximal intestine and distal intestine, except in the distal intestine. Amylase activity in the proximal intestine increased from 0.621 ± 0.037 U mg^{-1} protein to 2.328 ± 0.383 U mg^{-1} protein with *S. platensis* supplementation level of 6 g kg^{-1} feed. The similar condition happened to the stomach with an increase in amylase enzyme activity on day 56 from 0.500 ± 0.269 U mg^{-1} protein to 1.330 ± 0.133 U mg^{-1} protein with *S. platensis* supplementation level of 6 g kg^{-1} feed.

These results indicate that the amylase enzyme activity works optimally at neutral buffer solution (pH 7) and it occurs along the fish digestion channel. This is in accordance with the statement of Solovyev et al., (2015) that the pH ranging from 6.8-8 is optimum for the α-amylase enzyme in breaking down carbohydrates in the intestine. In addition, the pH value in the intestine increases compared to the stomach because of the neutralization of hydrochloric acid by bicarbonate ions in the intestinal mucus membrane.

The results of further tests showed that on day 56, amylase activity differed significantly. *S. platensis* supplementation of 6 g.kg^{-1}feed is more effective compared to the dose of 0, 2 and 4 g kg^{-1} feed in liver, stomach, proximal intestine and mid intestine. However, the most effective amyla-

se activity in the distal intestine was obtained by *S. platensis* supplementation of 2 g kg^{-1} feed level. The increase of enzyme activity was thought to be due to *S. platensis* supplementation which has a 16% carbohydrate content.

This is in accordance with the statement of Yuniati et al., (2018) that the higher carbohydrate content in the feed can increase the activity of the amylase enzyme because the more substrate will be broken down into simpler compounds to be utilized by the fish body. Hlope & Moyo, (2013) reported that amylase activity in the proximal intestine was higher than the back of the intestine in *Tilapia rendalli* and *Oreochromis mossambicus*. The increase in amylase enzyme activity (14-19%) was also occurred in previous studies by Hajarathaiah & Nagajyothi, (2018) by adding probiotics into maintenance media and feed for 30 days.

The results of the measurement of amylase activity at alkaline buffer solution (pH 10) is presented in Figure 3. *Spirulina platensis* supplementation level of 6 g kg^{-1} feed increased amylase activity in the mid intestine 0.073 ± 0.013 U mg^{-1}protein and distal intestine 0.206 ± 0.008 U mg^{-1} protein from no activity detected (0,000 U mg^{-1} protein). Different results were obtained by administering *S. platensis* dose of 4 g kg^{-1} feed that increased amylase activity in the liver to 0.242 ± 0.005 U mg^{-1}protein from the previously undetectable amylase activity measured on 56th day. It shows that the enzyme can work optimally in a specific environment especially with a certain range of pH. In addition, enzymes that work in each digestive organ also have a different optimum range of pH.

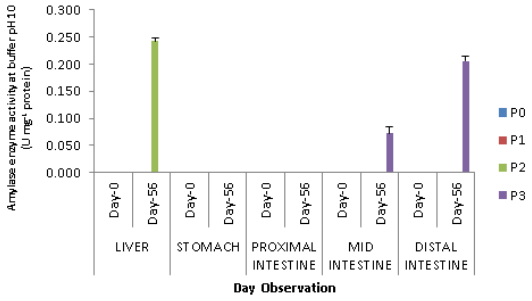

Figure 3. Amylase Enzyme Activity of *O. vittatus* at Buffer Solution with pH 10.

The results of statistical tests showed that there were significant differences between treatments in the liver, mid intestine and distal intestine. *Spirulina platensis* supplementation level of 6 g kg^{-1} feed is the best treatment in the intestine at alkaline pH. According to Ye et al., (2013), amylase activity is decreasing at pH 10 compared to neutral pH. Some digestive enzymes have a different optimum range of pH and in *Odontobutis obscurus* the pH suitable for digestion enzymes ranging from 7.5 to 8.0. Tian et al., (2019) also stated that the amylase activity detected in the hepatopancreas (liver) and proximal intestine had the maximum value after 2 hours of feeding on scaleless carp (*Gymnocypris przewalskii*), while the highest level of amylase enzyme activity in the rear intestine was at 6 hours after feeding.

Protease enzyme activity in various digestive organs of *Osteochilus vittatus*

Measurement results of amylase activity in *Osteochilus vittatus* digestive organ with different levels of *S. platensis* supplementation in feed and maintenance in the biofloc system at buffer solution with pH 5, 8 and 10 is presented in Figures 4, 5 and 6.

Measurements of protease enzyme activity in nilem fish with *S. platensis* supplementation in feed and biofloc system as maintenance media at acidic buffer solution (pH 5) were only found in the liver and stomach in all treatments on day 0 and day 56. The highest protease activity was found in *S. platensis* supplementation dose of 6 g kg^{-1} feed, (7.541 ± 0.596 U mg^{-1} protein) in the liver. The activity of the protease enzyme on day 0 was only detected by 0.186 ± 0.062 U mg^{-1} protein, but on day 56, protease activity increased.

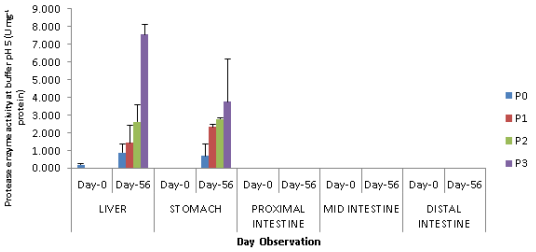

Figure 4. Protease Enzyme Activity of *O. vittatus* at Buffer Solution with pH 5. Note: Protease enzyme activity: the number of enzyme that hidrolyze casein (µg ml^{-1} $minute^{-1}$ mg^{-1} protein). P0: *S. platensis* supplementation dose of 0 g kg^{-1}feed in biofloc system, P1: *S. platensis* supplementation dose of 2 g kg^{-1} feed in biofloc system, P2: *S. platensis* supplementation dose of 4 g kg^{-1} feed in biofloc system, P3: *S. platensis* supplementation dose of 6 g kg^{-1} feed in biofloc system. Values with the similar letter in the figure shows no significant differences between treatments (Mean±SD, n=P>0.05), while the values with the different letter in the figure shows significant differences between treatments (Mean±SD, n=P<0.05).

The highest protease activity in gastric organ with a value of 3.758±2.399 U mg^{-1} protein was in the treatment with the supplementation of *S. platensis* 6 g kg^{-1} feed. Feed supplemented with *S. platensis* level of 2 and 4 g kg^{-1} feed in biofloc system also increased protease activity in liver organ to be respectively 1.422±1.019 and 2.608±0.997 U mg^{-1} protein. These results showed that there was an improvement in protease activity so it can support food digestibility and absorption of nutrients in the fish body.

Protease enzyme activity was significantly different between the treatment in liver and stomach (P<0.05). The effect supplementation of 6 g kg^{-1} feed *S. platensis* was significantly different among the other treatments. According to Sankar et al., (2014), proteolytic activity at low pH was found to be highest in the acidic region of the stomach. This founding was the result of research on *Etroplus suratensis* and *Oreochromis mossambicus*. However, in this research, protease activity was not only found in stomach but also in liver. Acidic protease works in acidic condition of stomach to digest protein at the initial phase then digested as a whole protein in the intestine (Sankar et al., 2014). Xiong et al., (2011) also stated that there was protease activity at acidic pH in stomach of carnivorous fish *Glyptosternum maculatum* and the initial breakdown of protein happened at stomach with the help of hydrochloric acid.

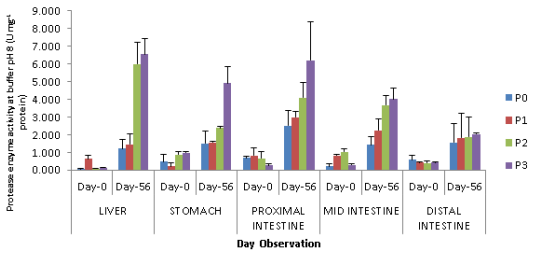

Figure 5. Protease Enzyme Activity of *O. vittatus* at Buffer Solution with pH 8.

Protease activity using buffer solution with pH 8 (Figure 5) was detected in all organs of digestion and all treatments on day 56. Protease activity in the liver increased almost sixfold from the previous 0.123±0.003 U mg^{-1} protein to 6.536±0.911 U mg^{-1} protein in treatment with *S. platensis* 6 g kg^{-1} feed supplementation. This result corresponds with Solovyev et al., (2016) that said the protease activity works optimally in pH ranging from 7.00 to 8.00 in intestine.

There was a significant difference between the *S. platensis* supplementation treatment on feed compared to control treatment (without *S. platensis* supplementation) in biofloc system after analized by statistical test (P<0.05) in liver, stomach, front intestine and middle intestine organ. The *S. platensis* supplementation of 6 g kg^{-1} feed was the level that is able to produce the highest protease activity in liver organ. The highest protease activity in the stomach and proximal intestine was also obtained by supplementation level of 6 g kg^{-1} feed compared to the dose of 0, 2 and 4 g kg^{-1} feed supplementation. The increases in enzyme activity was thought to due to feeding *S. platensis* supplementation which contain 60-63% protein (Tietze, 2004).

These results are in accordance with the previous studies by Simanjuntak et al., (2018) that S. *platensis* supplementation of 6 g kg^{-1} feed is the most optimum level given to gouramy (*O. gouramy*). Najdegerami et al., (2015) using carp (*Cyprinus carpio*) in the 75% biofloc system and maintained for 30 days, protease activity increased from 7.6 U mg^{-1} protein to 8.2 U mg^{-1} protein. Digestion of food in the digestive tract is strongly influenced by *endogenous* and *exogenous* enzyme (Liu et al., 2017). *S. platensis* supplementation contains high protein as substrates that can stimulate endogenous enzyme along with increase in protease activity. Other research by Zainal et al., (2016) found that the application of papain enzyme in the diet (dose 27.5 mg kg^{-1} feed) has significant effect on growth performance, feed utilization and survival rate of keureling fish (*Tor tambra*). It indicates that the better the work of enzymes, the more supportive of fish life.

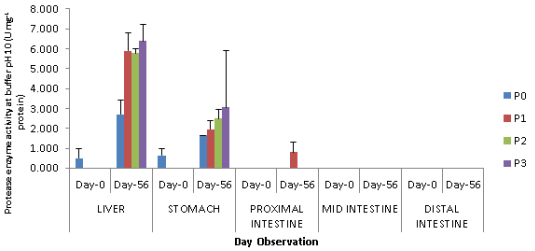

Figure 6. Protease Enzyme Activity *O. vittatus* at Buffer Solution with pH 10

The results of protease activity with alkaline buffer solution (Figure 6.) only show enzyme activity in the liver and stomach. Whereas in the proximal intestine, mid intestine and distal intestine no protease activity was detected. The undetectable activity was caused by the value of tyrosine concentration that were below 50 µg mL^{-1}. After the treatments for 56 days in the biofloc system with supplemented feed, protease activity increased to 6.372 U mg^{-1} protein in liver organ

when given *S. platensis* supplementation dose of 6 g kg $^{-1}$ feed. Meanwhile, protease activity was detected in stomach with supplementation dose of 2, 4 and 6 g kg^{-1} by 1.715±0.008 U mg^{-1} protein, 2.485±0.442 U mg^{-1} protein, and 3.042±2.846 U mg^{-1} protein respectively.

Results of ANOVA showed that the protease enzyme activity was significantly different between the treatment with only the provision of commercial feed and the treatment with supplemented feed in liver organ ($P<0.05$). Providing *S. platensis* supplementation of 6 g kg^{-1} feed becomes the best treatment for increasing protease activity in the stomach of nilem fish. The result of this study was supported by previous research by Simanjuntak et al., (2016) on gouramy (*O. gouramy*) that *S. platensis* supplementation on feed could improve body composition influenced by high nutrient content and it affects the protein and lipid content of the entire body of the fish. According to Solovyev et al., (2015) who examined the activity of gastrointestinal tract enzymes in five species of fish, such as *Carassius auratus gibelio*, *Leuciscus idus*, *Cyprinus carpio*, *Perca fluviatilis*, and *Sander lucioperca* found that the maximum alkaline protease activity was at pH 8-9; while the maximum acid protease activity is at pH 2-3.

Fish farming in biofloc systems can improve the quality of waters and grow natural food. Feed supplemented with *Spirulina platensis* can increase fish immunological. Therefore, aquaculture in biofloc systems along with the provision of feed supplemented with *Spirulina platensis* is very beneficial for fish farmers because it can reduce feed costs, environmental toxicity due to leftovers and improve the performance of fish digestion enzymes.

CONCLUSION

The enzymes activity of nilem fish (*Osteochilus vittatus*) digestive organs administered with various level of *Spirulina platensis* supplementation in feed and maintained in biofloc system increased on day 56 compared with fish fed with comercial pellet. *S. platensis* supplementation levels of 4 g kg^{-1} and 6 g kg^{-1} feed were the most optimum in improving the performance of amylase enzyme activity at buffer solution with pH 5 and 10 and protease activity at buffer solution with pH 10. The *S. platensis* supplementation level of 6 g kg^{-1} feed level was the most optimum for increasing protease activity at buffer pH 5 and 8 as well as amylase activity at buffer pH 7.

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