Physico-chemical characteristics of *Rhizopus* sp.-fermented fish feed pellets containing black soldier fly larvae (*Hermetia illucens*) meal

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**Abstract.** In aquaculture, feed cost contributes the highest share in the total production cost. The increase in fishmeal price and the high cost of commercial fish feeds have prompted the search for alternatives. Thus, considerable research has been carried out to develop a farm-made feed that requires simpler methods and cheaper production costs. This study aimed at developing farm-made fish feed using simple equipment, locally available materials, and inexpensive fungal-based bio-floating technique. A combination of using black soldier fly (*Hermetia illucens*) larvae meal as a fishmeal replacement and *Rhizopus* sp. fermentation to provide floatability was used. An experiment using three different *Rhizopus* sp. inoculum doses (1, 2, and 3% w/w) and 3 different fermentation duration (30, 40, and 50 h) were designed, with unfermented feed as control. Results showed that fermented floating feeds gained better nutritional value and physical characteristics (p<0.05). The optimized treatment resulted in a fermented feed with the protein content of 29.72%; fibre 12.13%; fat 26.57%; and ash 7.83%. Relative to the unfermented control, all of the fermentation treatments produced feeds with high average values of floating ability (83.33%) and water stability (92.38).

**Keywords:** black soldier fly; feed; fermented; Rhizopus

1. **Introduction**

Fish feed is very important for the aquaculture industry, it generally constitutes 60-70% of the total production cost [1]. Fishmeal is widely used as the main protein source in fish feed production and is an increasingly expensive animal protein due to its limited resources and rising demand. Thus, the aquaculture industry is forced to search for alternatives to replace fishmeal to reduce fish feed production costs.

A promising alternative of protein sources to replace fishmeal is from insects. The use of insects as feed raw material has been widely studied because protein derived from insects can reduce the costs of
animal feed [2], environmental friendly [3], as well as easy to mass produce [4]. Another advantage is that the insect-derived protein does not compete with human food so that it is suitable for use as raw material for fish feed [5]. An insect protein source used in this study was Black Soldier Fly Larvae (Hermetia illucens). Black Soldier Fly Larvae (BSFL) meal contains a high level of crude protein (44.26%), lipid (29.65%), and crude fibre 7%. Besides, BSFL has balanced essential amino acids, and fatty acids profiles [6].

The production of floating fish feed requires high investment and operational costs. Solid fermentation using the tempeh mold from the genus Rhizopus sp. could provide an alternative solution to this problem. Fermentation using Rhizopus sp. strain can improve nutritional qualities such as higher contents of protein, fat, vitamins. Besides, Rhizopus sp. can be used as an agent for producing buoyancy property in floating fish feed production in a non-extruded way through a solid fermentation process [7]. This technology would be widely applicable when proven to be low in cost, easy, and profitable to low and middle-level fish farmers. This non-extrusion method has also been studied by previous authors using yeast [8, 9].

Despite the potential, manufacturing floating feed using Rhizopus sp. as a biological floating agent has several other issues to be solved. The fermented feed obtained caused the initial fish pellets to clump together, producing fermented feed with heterogeneous shapes and sizes which were often bigger than the initial pellet size. Moreover, the fermentation duration lasted for several days, which was time-consuming. Good physical quality of fish pellets is needed to minimize feed waste and maximize feed intake and utilization for fish. Hence, this research aimed to determine the physical and chemical characteristics of BSFL-containing fish feed fermented using Rhizopus sp. The fungal fermentation was meant to give the feed floating property and was carried out with variations in fermentation time and inoculum dose. The physical characteristics tested included density, water stability, and floatability in aerated and unaerated water. The chemical characteristics tested were protein, moisture, fat, carbohydrate, and ash content, as well as amino acid profile.

2. Material and methods
The production of fish feed was carried out at the Nano Centre Indonesia, Pamulang, South Tangerang, Banten, Indonesia. Feed fermentation was carried out at the Laboratory of Feed Biotechnology, Agency for the Assessment and Application of Technology (BPPT), South Tangerang, Banten.

2.1. Fish feed production
The fish feed components consisted of BSFL meal (60%), tofu dregs (21%), rice bran (9%), and tapioca starch (10%). BSFL was oven-dried at 70 °C for 60 minutes. The high inclusion level (60%) of BSFL was based on the previous study with modification of fermentation using Rhizopus sp. BSFL can substitute as much as 50% of trash fish without giving any negative impact [10]. Tofu dregs and rice bran were sun-dried for 2 days. All of the materials were grounded finely to facilitate easier mixing and molding, as well as to increase the digestibility of the fish feed produced. Tapioca starch was suspended in 1000 mL water and heated to produce a clear sticky solution as the binding agent. Tapioca starch is a natural adhesive, locally wide available, and cheap [11] so it was suitable for feed binder. All of the raw materials were then mixed homogenously and pelleted using a pelleting machine.

2.2. Fermented feed preparation
Feed pellets fermentation and feed tests were carried out at the Feed Biotechnology Laboratory-BPPT, South Tangerang, Banten. Fish feed pellets (200 g) in rectangular tray bioreactors were mixed with Rhizopus sp. inoculum at 3 different doses (1%, 2%, and 3%), added with 50 mL water, and mixed homogeneously before the trays were covered using a perforated plastic sheet. Next, incubation was done at room temperature (28-30 °C) with 3 different fermentation times (30, 40, and 50 hours). Regular stirring was done to prevent the hyphae from knitting the pellets and forming large-sized aggregates. At the end of the fermentation, the surface of the feed pellet substrates was physically transformed into a white cottony-appearance. The fermented feeds were dried at 50°C for 24 hours and then subjected to
2.3. Chemical analysis
Chemical analyses were carried out to analysed any nutritional changes brought about by the tempeh mold fermentation. Crude protein analysis was carried out at Testing Laboratory, Marine and Fisheries Faculty, Bogor Agricultural University, whereas amino acids profile was determined using UPLC (Ultra Performance Liquid Chromatography) method in the nationally accredited Saraswanti Indo Genentech (SIG) Laboratory. Moisture, crude lipid, crude fibre, and ash were determined in the nationally accredited analytical service of Biotechnology Laboratory, Agency for the Assessment and Application of Technology, BPPT.

2.4. Physical analysis
The floating ability test [13] was conducted using triplicate samples. Floating ability was carried out with different treatments, using aeration and no aeration to imitate agitated and unagitated waters, respectively. Ten fermented pellets (1-2 g) were put into a 500-mL beaker glass containing 400 mL of water. The pellets were dropped into the beaker glass and observed for 60 minutes at a 5-minute interval, at which the number of the pellets afloat were recorded accordingly. The floating ability was calculated using the following formula:

\[
\text{Floating ability (\%)} = \frac{\text{Final number of feeds afloat}}{\text{Initial number of feeds afloat}} \times 100
\]  

The water stability test [14] was conducted using triplicate samples. Ten samples (1-2 g) were placed into a tea strainer (±200 mesh), both of which were then immersed in 500 mL of water, with and without aeration. After 60 minutes, the remaining pellet residues were drained and oven-dried at 50 °C for 19 hours. Water stability was calculated using the following formula:

\[
\text{Water stability (\%)} = \frac{\text{Weight of retained whole pellets}}{\text{Initial total weight of pellets}} \times 100
\]  

2.5. Statistical analysis
The data obtained were statistically processed by Two-way analysis of variance (ANOVA) using MINITAB 16, in which differences of the means were determined at a 5% level using Tukey Post-Hoc Test. The most optimal combination of time and dosage was determined using the Zeleny method.

3. Results and discussion

3.1. Feed appearance
Unfermented fish feed was brown coloured, sized 0.36 mm diameters and 0.7 mm length. The limited hyphae growth of Rhizopus sp. on the substrate began to be observed after 24 hours. The fermented fish feed showed a white and the surface coated by fungal white dense. This colour change was caused by the presence of mycelia growing on the surface of the feed. Fish feed before and after fermentation using Rhizopus sp. can be shown in figure 1.
3.2. Chemical analysis

The parameters observed in this research include chemical compounds contained in the fish feed before and after being fermented through the proximate test (moisture, crude protein, crude fat, ash, and crude fibre content). The proximate analysis of feed samples after fermentation using *Rhizopus* sp. is shown in table 1.

**Table 1. Proximate analysis of feed samples after fermentation using *Rhizopus* sp.**

| Fermentation Treatment | Protein (%) | Fat (%) | Ash (%) | Crude fibre (%) | Moisture (%) |
|------------------------|-------------|---------|---------|-----------------|--------------|
| Unfermented (control)  | 26.77^b     | 28.95^bc| 6.75^e  | 9.12^c          | 5.60^d       |
| D1W1                   | 27.96^c     | 29.92^a | 7.30^d  | 10.43^bc        | 4.57^d       |
| D1W2                   | 28.12^c     | 26.87^d | 7.42^c  | 10.49^bc        | 6.13^bc      |
| D1W3                   | 29.88^b     | 23.48^e | 7.75^ab | 12.30^a         | 6.19^bc      |
| D2W1                   | 27.76^d     | 29.01^ab| 7.21^d  | 10.23^bc        | 6.57^b       |
| D2W2                   | 29.33^c     | 27.34^cd| 7.58^bc | 10.19^b         | 6.36^bc      |
| D2W3                   | 30.09^e     | 26.13^d | 7.57^bc | 11.52^ab        | 8.52^a       |
| D3W1                   | 27.37^g     | 28.34^cd| 7.23^d  | 10.43^bc        | 5.52^d       |
| D3W2                   | 29.11^d     | 27.43^cd| 7.29^d  | 11.34^ab        | 6.99^b       |
| D3W3                   | 29.72^b     | 26.57^bc| 7.83^a  | 12.13^a         | 6.06^bc      |

Table 1 showed that *Rhizopus* sp. fermentation increased the crude protein content of the feed for all of the treatments. The highest result occurred after the 50-hour fermentation. Protein content increased by approximately 2.21-12.40%. Variation in the protein content result might be due to different inoculum dosage and fermentation time. This result was similar to those of previous studies. *Rhizopus*-fermented sago flour also produced a higher protein content (3.4%) compared to the unfermented one (1.6%) [15]. A similar study on fermented rice bran increased the protein content by 58.5%. Glucose from polysaccharides becomes a source of energy in the metabolism of *Rhizopus* sp. cells which produce enzymes that can increase protein content [16]. Fungal dry biomass contains high protein content, which can be in the range between 31-50%. The more fungi growth, the more substrate protein content will be formed by the growing fungal body [17]. Besides, the composition of fish feed also gave high protein content. BSF larvae contain high protein levels (from 42.1% to 56.1%, dry matter), and other macro- and micronutrients that are important for animal feed [18]. BSF larvae can be used as an alternative protein source as fishmeal replacement since it showed a good profile of amino acids. In general,
fermented feeds resulted in better protein content, considering the standard values of fish feed which is 25-30% [18].

The fat content of fermented feed decreased with increasing inoculum dose and fermentation time. The lowest fat content result occurred on the 50 hours fermentation. Fat content decreased by approximately 0.9-19.38%. The fermentation treatment resulted in lower fat content than non-fermentation treatment. *Rhizopus* sp. produces lipase enzymes which can break down fat into fatty acids. During the fermentation process, the mold will synthesize the lipase enzyme which will hydrolyse the triacylglycerol into free fatty acids. This fat is used by fungi as a source of energy for growth so that the fat content decreases [18]. However, the fat content in this feed is still too high when compared to standard fish feed, which is a maximum of 14%. The high-fat content is due to the BSF larvae which have a high fat content. The fat content of BSF larvae ranges between 26-34.80% [19].

The fermentation also changed the crude fibre content of the fish feed. Table 1 showed that *Rhizopus* sp. fermentation increased the crude fibre content of the feed for all of the treatments. The crude fibre increased by 6.55-43.09%. The longer the fermentation time, the thicker mycelia formed. The more inoculum dosage used the crude fibre becomes higher. It resulted in the fibre content of fermented fish feed higher. The fermentation process of *Rhizopus* sp. will produce mycelia formed a drove of hyphae. This hypha has a cell wall containing cellulose and chitin components. The formulated feed, both fermented and non-fermented resulted in high crude fibre content. Crude fibre produced in this study ranges from 9.12 - 22.92%, which was too high since maximum fibre content for fish was 5-8% [18]. Crude fibre is needed in the fish diet to increase intestinal peristalsis, but too high crude fibre level may reduce the digestibility and the efficiency ratio of other nutrients. Fish are unable to digest cellulose because due to the absence of endogenous enzymes that catalyse the hydrolysis of cellulose [19].

Increased ash content occurred in this study. Ash content increased in the various range, between 6.02-16.29%. The increased ash content was due to the result of the growth of *Rhizopus* sp. mycelium. The more inoculum dosage used, the ash content became higher. *Rhizopus* sp. may increase ash content. The high carbohydrate content of cellulose, which can be converted into organic acids and hydrolysed into other compounds, including minerals, which will increase the ash content [20]. Fermentation using fungi can increase the inorganic mineral content [21]. Fungi also contain minerals, which can increase the ash content by about 4-10% [22]. The ash content in this study still too high compared to the fish feed standard, which maximum ash content of 13% [18].

Moisture content is one of the factors which was important to the fungal growth and production. If the moisture content of the feed is too high, it easier for the feed to spoil during the storage process. The increase in moisture content due to the presence of water vapor which was created during the metabolic activity of fungi. The longer the fermentation time, the more moisture content is formed. Similar results are shown by research conducted [23], which states that barley was using *Rhizopus* sp. experienced an increase in water content from 10.12% to 14.25% after 36 hours. Based on SNI for fish feed, the maximum moisture content is 12% [18]. In this study, the water content of the fermented feed was still below the fish feed standard by SNI.

3.3. Feed density
The density of the fermented feeds ranged from 0.73 to 0.87 g cm\(^{-3}\), all of which were below water density (1 g cm\(^{-3}\)), so it floated in the water. Density reduction occurred in feed after fermentation. This was likely due to the fungus *Rhizopus* sp. which grew well all over the feed surface, forming entrapped air cavities and thus, lowering the overall density of the fermented feeds. The decrease in density might also be caused by the loss of substrate dry weight into volatile compounds including water vapor which was released as a metabolic product. Besides, this metabolism causes biochemical processes and products to evaporate [24].

3.4. Floating ability
The pellet quality of the fermented feeds was also evaluated through their floating ability. The floating ability test was carried out in two different ways, without aeration (figure 2) and with aeration (figure 3).

The fermented feed D3W3 demonstrated the best floating ability in the 60-minute tests with the highest average values of 83.33% (with aeration) and 66.67% (without aeration) (figure 4). All fermented feeds were able to float in aerated and unaerated water, indicating the Rhizopus sp. fermentation did cause a change in the density of the feed. In contrast, the sinking time in the control treatment showed no floating ability by the first 5 minutes. In this study, the most optimal treatment is the fermentation time of 50 hours. The floating ability values of the buoyancy test using aeration were lower than those without aeration. Aeration might have caused the water surface to be agitated, causing repeated water impacts on the surface of the tested pellets. These impacts might have facilitated faster water infiltration into the feed matrix, rising the density above water, and then sank the feed. The effect might have been more severe on the pellets with uneven growth of fungal mycelia on their surface due to regular stirring during the fermentation.
3.5. Water stability

The fermented pellet quality was also evaluated through a water stability test without aeration (figure 4) and with aeration (figure 5).

![Figure 4. Water stability without aeration.](image)

![Figure 5. Water stability with aeration.](image)

Compared to the unfermented control, all fermented feed had higher water stability with the highest value of 92.38% (in unaerated water) and 89.55% (in aerated water) in the 60-minute test. Sample D3W3 demonstrated the highest water activity in unaerated water (92.38%). The fermented feed that shows the lowest stability in water of 75.95% was D1W1. Similar results of 80.3-87.1% water stability were already reported previously [25].

It clearly showed that higher water activity corresponds with higher inoculum doses, longer fermentation time, and more formation of *Rhizopus* sp. mycelium. Dense mycelium might provide surface coating, strengthening the pellet structure against disintegration in water. In addition, the use of tapioca flour as a binder in the feed manufacturing process might also affect the stability of the feed. The addition of 10% tapioca was reported to result in the highest feed stability [11]. This is because
tapioca flour contains amylose and amylopectin which, when heated, will gelatinize, acting like glue that sticks feed particles together.

The best combination of fermentation time and inoculum dose was determined based on “Multiple Attribute Analysis” or Zeleny’s method. Thus, a combination of 50-hour fermentation time and an inoculum dose of 3% produced the best result as shown in Table 2.

### Table 2. Determination the best treatment of fermented feed.

| Parameter                  | 50-hour Fermentation Time, 3% Inoculum Dose |
|----------------------------|---------------------------------------------|
| Crude protein              | 29.72 %                                     |
| Crude fiber                | 12.13 %                                     |
| Lipid                      | 26.57 %                                     |
| Ash content                | 7.83 %                                      |
| Floating ability (aeration)| 66.67 %                                     |
| Floating ability (without aeration) | 83.33 %                  |
| Water stability (aeration) | 92.38 %                                     |
| Water stability (without aeration) | 89.55 %                           |

#### 3.6. Amino Acids Profiles

The best sample fermented feed D₃W₃ showed an increase in amino acid content after fermentation (Figure 6). The contents of all amino acids increased except for proline, which decreased. The order of the largest to the smallest amino acids was Glu > Asp > Leu > Gly > Ala > Ser > Val > Arg > Thr > Tyr > Phe > Ile > His. Amino acids were metabolites produced during mold fermentation as a result of hydrolysis of protein and polypeptides, as well as the synthesis of new amino acids.

![Figure 6. Amino acids before and after fermentation for sample D₃W₃.](image)

#### 4. Conclusion

*Rhizopus* sp. fermentation was shown to improve the physical and chemical characteristics of fish feed containing BSLF meal as a protein source. The best fermentation results were obtained in the D₃W₃ treatment, namely 3% *Rhizopus* sp. inoculum dose and 50 hours fermentation time. The combination of...
these treatments resulted in the fermented feed having protein, fibre, fat, and ash content of 29.72, 12.13, 26.57, and 7.83%, respectively. The fermented feed also showed high floating ability (83.33%) and water stability (92.38%). Amino acids profile of the fermented feed was in general higher than that of the unfermented control.

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