Altering Physical Characteristics of Sinking Fish-Feed through Sub-Optimal Fermentation Using Tempeh Mould without Mechanical Extrusion

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Abstract. Sinking pellets disintegrates easily in water, hence reducing water quality and fish productivity due to oxygen-consuming biodegradation of the unconsumed feed. Modifying sinking pellets into more stable floating pellets using an edible tempeh mould through solid fermentation has previously been studied as an alternative to the more expensive extrusion method. However, the fermentation resulted in the uncontrolled growth of the fungal mycelium, causing the individual pellets to aggregate into a single compact mass. The fermentation also contributed to the dry weight loss of the initial pellets. Thus, using a laboratory-scale bioreactor, this study aimed at generating water-stable and floating properties on sinking-pellets through sub-optimal fermentation using tempeh mould. The conditions varied were the amount of sinking pellets substrate, fermentation duration, and aeration-humidification. Results showed that less pellet aggregation was observed with shorter fermentation time. Based on 40-minute laboratory tests in water, the best 80% floating ability was achieved for 10 g sinking pellets fermented for 72 h with humidified bubble aeration. The fermented pellets showed 42% water stability and 4.86% dry weight loss.

Keyword: tempeh mould, floating, fish, sinking pellets, solid fermentation, bioreactor

1. Introduction
A significant increase in aquaculture production [1] has been achieved with an unwanted detrimental effect in the aquatic environment [2]. The problem is partly caused by a significant amount of nutrient leaching and unconsumed feed released into aquaculture environment, causing algal blooms and/or eutrophication. Massive algal growth causes oxygen depletion which can endanger and even kill aquatic life, including fish [3].

The use of sinking pellets in aquaculture has drawbacks as they easily disintegrate, thus releasing nutrients into the water and preventing utilization by fish [4]. Sinking pellets are cheaper
compared to the highly stable floating feed [5]. Floating feeds are produced with higher production cost and advanced extrusion technology which is less accessible to farm-made pellet manufacturers [6].

Studies have been carried out to provide much simpler and cheaper alternatives for producing floating pellets without an extruder machine for small and middle scale aquaculture farmers, such as using feed mincer machine with rice bran and wheat offal as binders as well as yeast as a stabilizer [7]. Other studies used a combination of local starch and Saccharomyces cerevisiae [8], Baobab leaf meal (Adansonia digitata) [9], as well as a combination of tapioca starch and Rhizopus spp. [10] to provide stability and floatability to pellets.

The use of the filamentous fungi from the genus Rhizopus deserves particular attention as the edible fungi have been well known in the making of the fermented soybean tempeh, which is an Indonesian traditional food [11]. A recent study showed that Rhizopus oryzae biomass with up to 62% protein content [12] and amino acid profile similar to those of fishmeal [13] could be produced with potential application in fish feed. Solid fermentation using Rhizopus or tempeh mould has also been shown to be capable of producing desired physical qualities such as stability and buoyancy in water to fish feed made from local agro-industrial by-products [14, 15]. Similar studies using commercial sinking feeds demonstrated that better stability and floatability in water was achieved through tempeh mould fermentation without using an extruder machine, and that these physical properties were comparable to those of industrially-manufactured floating feeds [16].

Fungal fermentation using Rhizopus or tempeh fungi to provide floating property and water stability to fish feed is however not without disadvantages. The uncontrolled growth of the fungal aerial hyphae during fermentation has caused the initially-separated individual pellets to be aggregated into a single compact mass [17, 18]. This relatively big size compared to the fish mouth opening is undesirable as small-sized pellets facilitate better feeding [19]. Besides, the fermentation resulted in significant dry weight loss [18], producing a lower output product. Thus, this study aimed to generate the property of floating and stability in water on commercial sinking-pellets through fungal solid-fermentation. The fermentation was to be carried out at sub-optimal conditions to suppress the fungal growth, hence preventing the individual pellets from being tightly aggregated into a larger compact cake, as well as reducing the dry weight loss.

2. Materials and Methods

The studies were carried out at a laboratory scale, consisting of designing bioreactor, preparing tempeh mould inoculum, solid fermentation, and physical tests of the fermented feed. All materials and equipment used were not sterilized, and no sterile conditions were required during the preparation and experiments. This was to make the procedure less complicated and less costly for future implementation in fish farms.

2.1. Design of experiment

A randomized factorial design was used in this study using 3 factors. The first factor was the quantity of sinking pellet substrate (S): 5 g (S5), 10 g (S10), dan 15 g (S15). The second factor was fermentation duration (D), which was carried out for 24 h (D24), 48 h (D48), and 72 h (D72). The last factor was related to bubble aeration and humidification through water placement at the bioreactor base (A): without both aeration and water (A1), without aeration but with water (A2), and with both aeration and water (A3) (Figure 1 and Figure 2). Twenty-seven combinations were studied from those 3-factors, and each combination was repeated four times. A unique designation for each combination was given with the following example: a sample designated as S5-D24-A1 was for 5 g sinking pellets fermented for 24 h in a bioreactor without both aeration and water. The unfermented sinking pellets were used as control.

2.2. Bioreactor design

A modified bioreactor based on the previous design [20] was prepared using cylindrical jars made of transparent polyethylene terephthalate (PET) plastic with 9 cm diameter and 21 cm height. In the upper part inside each of the jars, a smaller cylindrical basket (8 cm diameter × 8 cm height) made of
aluminium gauge was placed. The basket contained 3 polyvinyl chloride (PVC) pipes (3 cm diameter × 7 cm height) into which the sinking pellets were placed and fermented (Figure 1).

2.3. Inoculum preparation
Inoculum to be used for the fermentation was prepared using a previously described method [17] with modification of adding teak (*Tectona grandis* L.) leaf. Teak leaves are normally used in preparing the traditional *usar* tempeh mould starter [21]. Tempeh mould inoculum was purchased a small-size tempeh factory in Serpong district, Tangerang Selatan city, Banten. A mixture containing 4 g of the purchased inoculum and 20 g of mashed teak-leaf was added with 250 mL of tap water, mixed homogenously, and then the whole mixture was poured into a plastic tray (23 × 19 × 3 cm). The tray was covered with a transparent plastic sheet having needle-sized pores separated at 1–1.5 cm distance between each other. Fermentation was carried out at ambient temperature for 48 h, followed by 24-h drying in a 40 °C oven.

2.4. Sinking pellets
Fish feed in the form of pellets was not self-produced using certain formula and nutrition considerations since this study emphasized the physical aspects of the pellets that changed after fungal fermentation. Sinking pellets (Muse Farm, Karawang, West Java) were purchased locally with known nutritional value (Table 1). The pellets sank immediately when placed on water, and could not float on water.

2.5. Solid fermentation
Sinking pellets (5, 10, and 15 g) was soaked in tap water equivalent to the weight of the pellets, left for 20 seconds to allow the water to be absorbed by the pellets. The remaining unabsorbed water was drained off, and the moistened pellets were mixed with the tempeh mould inoculum (2% of the pellet weight). The inoculated pellets were transferred into the PVC pipes inside the bioreactor (Figure 1). Three different treatment was given at the base of bioreactors: with neither water nor active aeration, with water but no active aeration, and with both water and active aeration (2 mL air bubble per second) (Figure 2). The placement of 400 mL of tap water at the base of the reactor was meant to provide more humidity, whereas aeration to provide air replacement. Fermentation duration was carried out for 24, 48, dan 72 h, after which the fermented pellets were observed in terms of their integrity and ability to be separated from each other before oven-drying at 50°C for 24 h for further physical analyses.

Table 1. Nutritional composition of sinking pellets according to the manufacturer (Muse Farm, Karawang, West Java).

| Component | Content (%) |
|-----------|-------------|
| Protein   | 28–30       |
| Fat       | 6–8         |
| Fiber     | 4–6         |
| Ash       | 10–13       |
| Water     | 11–13       |

Figure 2. Three different treatments of bioreactor: without water and without aeration (left), with water and without aeration (middle), and with water and with aeration (right). Water was placed in the bottom part of the jar to provide humidification.
2.6. Physical tests
All the pellet samples, fermented and unfermented (the sinking pellet control), were subjected to physical tests including dry weight loss, floatability, and water stability. In all formulae, the unit of weight was expressed in gram (g).

2.6.1. Dry weight loss. Dry weight loss ($\Delta DW$) was determined to know how much the fermentation could reduce some solid materials in the sinking pellets and transformation them into volatile compounds. After oven drying procedure at 50°C for 24 h, dry weight loss was calculated based on the dry weight of the pellets before ($DW_o$) and after ($DW_t$) fermentation using the following formula:

$$\Delta DW = \left( \frac{DW_o - DW_t}{DW_o} \right) \times 100\%$$  \hspace{1cm} (1)

2.6.2. Water stability. The stability or integrity of the pellets in water was determined according to the previously described method [22] with modification. Pellet sample (1 g) was transferred into a ±200 mesh plastic tea strainer [23], immersed in 400 mL of tap water contained in a 500 mL beaker glass. The water was aerated 16 mL s$^{-1}$ for 45 minutes. The filtered-out sample was drained off, dried 19 hours in 50ºC oven. Water stability ($WS$) was calculated based on the dry weight of the sample in the tea strainer before ($DW_{i0}$) and after ($DW_{it}$) immersion using the following formula:

$$WS = \left( \frac{DW_{it}}{DW_{i0}} \right) \times 100\%$$  \hspace{1cm} (2)

2.6.3. Floatability. The ability of the pellets to float on water was tested in two conditions: without and with water agitation through bubble aeration at the rate of 16 mL s$^{-1}$. Twenty pellets were tested on 400 mL water contained in a 500 mL glass beaker. The number of the pellets that were still afloat were counted on minute 0, 1, 3, 5, 10, 20, 30 and 40. Floatability ($F$) was determined based on the number of pellets still afloat ($F_t$) and the total initial number of pellets ($F_0$) using the formula below:

$$F = \left( \frac{F_t}{F_0} \right) \times 100\%$$  \hspace{1cm} (3)

2.7. Crude protein and fiber
Crude protein and fiber were determined before and after fermentation to know whether the fermentation would cause a significant change in those two components. The analysis was provided by the nationally-accredited (ISO 17025) analytical laboratory of Biotechnology Laboratory, Agency for the Assessment and Application of Technology (BPPT), located in National Science and Technology Park (Puspiptek), South Tangerang, Banten using gravimetry and Kjeldahl procedures based on Indonesian National Standard SNI 01-2891-1992.

2.8. Data analysis
ANOVA (Analysis of Variance) Univariate of SPSS IBM 25 program was used to analyze the data obtained from dry weight loss, floatability, and water stability determination. Significantly different results were verified using Duncan’s test.

3. Results and Discussion
Qualitative and quantitative assessment on the fermented pellets showed that the tempeh mould caused sinking pellets to have acquired physical characteristics different from its initial forms. These characteristics varied depending on the factor combinations.

3.1. Macroscopic observation
Macroscopic observation on the pellets fermented for 24, 48, and 72 hours, regardless of the other two fermentation factors (weight of pellets and aeration-humidification), showed the progressive mycelial formation of the tempeh mould (Figures 3 and 4). The 24-hour fermentation virtually had not
produced any physical change on the original sinking pellets with fungal growth hardly observed, except when aeration and humidification were given. After 48 h, for all treatments, the white cottony mycelium of the mould was seen and the pellet aggregation was also observed. The mycelium covering the pellets was not yet dense, therefore the individual pellet could still be separated from the aggregate using a tweezer. Fermenting for 72 h produced much denser fungal growth with grey colour, knitting the pellets into a solid compact mass which was almost impossible to separate. Effort in picking up individual pellets disintegrated the pellet initial shape (Figure 3 and Figure 4).

Similar previous studies produced fermented pellet aggregates with much denser mycelium and more compacted texture [16–18, 24]. In those studies, the fermented fish pellets resembled those of soybean tempeh, in which the mould grew as white and dense mycelium occupying interparticle spaces, connecting the beans tightly and forming a cheese-like texture. The fermented feeds necessitated slicing or crushing to reduce their size. The fermentation carried out in this study also produced similar texture, but with sparser mycelium, binding the pellets less tightly. It was still possible to separate the individual pellets post-fermentation, although the original pellet shapes were hardly preserved. This was an indication that the fermentation occurred in sub-optimal conditions.

Figure 3. The physical appearance of the freshly fermented pellets after having been fermented for 72 h (D72) with aeration-humidification (A3), using 3 different weights of pellets: 5 g (left, S5-D72-A3), 10 g (middle, S10-D72-A3), and 15 g (right, S15-D72-A3).

Figure 4. The physical appearance of the oven-dried, separated pellets resulted from the fermentation of 10-g pellets (S10) in the bioreactor with aeration and water humidification (A3). The incubation was carried out for 24 h (left, S10-D24-A3), 48 h (middle, S10-D48-A3), and 72 h (right, S10-D72-A3). The individual pellets were separated using a tweezer before oven-drying.

3.2. Dry weight loss
Dry weight loss is undesirable as it amounts to a reduction in feed production efficiency. Fermentation using the bioreactor used in this study caused dry weight loss as much as 0.60-11.70% (Table 2). Interaction between the three factors, namely weight of the sinking pellets, fermentation duration, and aeration-humidification, gave significant influence ($p < 0.05$) on the dry weight loss of the pellets (Table 2). Thus, this study achieved lower dry weight loss compared to the previous ones, which were 16-24% [24] and 10-14.1% [17]. Solid fermentation using tempeh mould or fungi from the genus *Rhizopus* were known to produce various volatile compounds from different substrates [25–26], which could be the main reason for the dry weight loss. The high standard deviation values shown in Table 2 could be due to the uneven growth of the fungal mycelium on the surface of each pellet.

### Table 2. Dry weight (DW) loss of the fermented pellets.

| Sample* | DW Loss* (%) | Sample* | DW Loss* (%) | Sample* | DW Loss* (%) |
|---------|--------------|---------|--------------|---------|--------------|
| S5-D24-A1 | 2.25 ± 1.05<sup>d</sup> | S5-D48-A1 | 0.90 ± 1.81<sup>d</sup> | S5-W72-A1 | 2.30 ± 1.14<sup>d</sup> |
| S10-D24-A1 | 4.30 ± 2.20<sup>abcd</sup> | S10-D48-A1 | 2.72 ± 0.38<sup>abcd</sup> | S10-W72-A1 | 4.52 ± 2.16<sup>abcd</sup> |
| S15-D24-A1 | 1.80 ± 0.92<sup>d</sup> | S15-D48-A1 | 2.66 ± 0.61<sup>abcd</sup> | S15-W72-A1 | 6.25 ± 4.61<sup>abcd</sup> |
| S5-D24-A2 | 0.70 ± 2.72<sup>d</sup> | S5-D48-A2 | 3.65 ± 1.86<sup>abcd</sup> | S5-W72-A2 | 4.80 ± 2.42<sup>abcd</sup> |
| S10-D24-A2 | 2.50 ± 1.83<sup>d</sup> | S10-D48-A2 | 3.82 ± 2.78<sup>abcd</sup> | S10-W72-A2 | 6.60 ± 4.27<sup>abcd</sup> |
| S15-D24-A2 | 3.00 ± 2.04<sup>abcd</sup> | S15-W48-A2 | 2.18 ± 0.49<sup>d</sup> | S15-W72-A2 | 2.66 ± 2.77<sup>abcd</sup> |
| S5-D24-A3 | 2.35 ± 0.96<sup>d</sup> | S5-W48-A3 | 11.70 ± 4.94<sup>e</sup> | S5-W72-A3 | 0.60 ± 3.09<sup>d</sup> |
| S10-D24-A3 | 3.97 ± 1.76<sup>abcd</sup> | S10-W48-A3 | 5.50 ± 4.37<sup>abcd</sup> | S10-W72-A3 | 5.80 ± 4.85<sup>abcd</sup> |
| S15-D24-A3 | 2.90 ± 2.47<sup>abcd</sup> | S15-W48-A3 | 3.93 ± 1.18<sup>abcd</sup> | S15-W72-A3 | 8.83 ± 2.75<sup>abcd</sup> |

* Sample designation: S5, S10, and S15 denote pellet quantity (5, 10, and 15 g, respectively); D24, D48, and D72 represent fermentation duration (24, 48, and 72 h, respectively); A1, A2, and A3 designate aeration-water treatment (without both aeration and water humidification, without aeration but with water humidification, and with both aeration and water humidification, respectively).

* Values of dry weight (DW) loss with the same alphabetical superscript indicate insignificant difference ($p > 0.05$) according to Duncan’s multiple range test.

### 3.3. Water stability

It is important that fish feeds must be stable in water, and not easily disintegrate and leach into the aquatic environment, thus consumed by the fish and lowering the quality of the aquatic environment. Overall, the fermentation increased the stability of the sinking pellets from 0% (not stable at all, readily disintegrating in water) to 8.5-54.25% (Figure 5). Both the weight of the sinking pellets and the use of aeration-humidification in the fermentation did not contribute significantly ($p > 0.05$) in producing better stability in water. On the other hand, fermentation duration did play a significant role ($p < 0.05$) in increasing the stability, with the best stability achieved for 72-h fermentation duration. By contrast, all the 24-h fermented samples and 2 of the 48-h fermented samples had poor stability, disintegrating readily in water just like the unfermented sinking pellets (Figure 5). Nevertheless, the water stability value in this study was still much lower than those of previous studies which achieved 80-95% for 60-minute stability tests in water [10]. Sub-optimal growth of the fungal mycelium resulted in the sparse mycelium formation, hence less mycelium to bind the pellet particle together.
3.4. Floatability

The sinking pellets used in this study possessed 0% floatability, meaning they sank immediately in water. The sub-optimal fermentation provided limited growth of tempeh mould; thus, the first 24-h of mould growth had not generated floating property to the pellets (Figure 6). The floating property began to be noticeable after 48-h fermentation. Most of the floating pellets were produced when the fermentation lasted for 72 h, with up to 80% floatability for 40 minutes in unagitated water. The later was achieved when 10 g sinking pellets were used in the aerated-humidified bioreactor, producing 42% water stability and 4.86% dry weight loss to the fermented pellets (Figure 6).

When the water was agitated using air bubbles, the floatability of almost all of the fermented pellets decreased. Agitation seemed to facilitate better absorption of water by the pellets, causing their density to increase rapidly to above that of water which eventually led to the sinking of the pellets. The weight of pellets and the aeration-humidification did not contribute significantly ($p > 0.05$) to the floatability of the pellets after sub-optimal fermentation. In contrast, the fermentation duration had significant ($p < 0.05$) influence on the floatability. This result was still lower than 100% floatability for 5 h achieved by previous authors [16].

![Figure 5. Water stability of the fermented pellets. Higher stability was shown mostly by the pellets fermented for 72 h, whereas 24-h fermented pellets showed no stability.](image-url)
Sample designation: S5, S10, and S15 denote pellet quantity (5, 10, and 15 g, respectively); D24, D48, and D72 represent fermentation duration (24, 48, and 72 h, respectively); A1, A2, and A3 designate aeration-water treatment (without both aeration and water humidification, without aeration but with water humidification, and with both aeration and water humidification, respectively).

**Figure 6.** Floatability of the fermented pellets. Higher floatability was demonstrated mostly by the pellets fermented for 72 h, whereas 24-h fermented pellets showed no buoyancy at all. Agitated water lowered the floatability. Sample designation: S5, S10, and S15 denote pellet quantity (5, 10, and 15 g, respectively); D24, D48, and D72 represent fermentation duration (24, 48, and 72 h, respectively); A1, A2, and A3 designate aeration-water treatment (without both aeration and water humidification, without aeration but with water humidification, and with both aeration and water humidification, respectively).
humidification, without aeration but with water humidification, and with both aeration and water humidification, respectively).

3.5. Crude protein and fiber
Analyzing the content of crude protein and fiber of the pellets before and after fermentation was intended to find out the extent of the fungal metabolization of the pellet substrate. Before fermentation, the sinking pellet was subjected to proximate analysis and found to contain 23.86% protein and 5.72% fiber, whereas after fermentation these values were 23.79% and 5.75%, respectively. The two values show no significant difference based on a one-way ANOVA test. It means, the mould did not metabolize a significant amount of the pellet substrate, hence it grew sub-optimally due to the conditions set up during fermentation such as humidity and aeration, which were known to affect the metabolic activities of *Rhizopus* [27].

4. Conclusion
This study successfully created sub-optimal growth of the tempeh mould on the commercial sinking pellets. The 72-h fermentation in a humidified and aerated bioreactor using 10 g sinking pellets was found to be optimum, producing fermented pellets with 80% floating ability, 42% water stability, and 4.86% dry weight loss.

Statement of authorship
The principal contributor for preparing and submitting this manuscript was Catur Sriherwanto, who was also the principal person designing and supervising the whole works from beginning until the end. Hardianingrum Rahmanisa contributed significantly by conducting the whole experiments in the laboratory, designing the bioreactor, data acquisition, data analysis, and preparing the first draft of the manuscript. Elyn Yunita was the co-supervisor for the project from the beginning until the end, and contributed significantly in examining the manuscript. Imam Suja’i and Amira Nadaviana were important technical assistants when the study was being conducted in the laboratory.

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