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Increasing the water stability of sinking feed grits using edible fungal hyphae for reducing aquatic waste: A laboratory study

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Abstract. Binding agents and extruder machines are commonly used to produce aquafeeds with high water stability, preventing feed disintegration and wastage. This technique is complicated and costly. The alternative use of living microorganisms as the binding agent without a high-temperature extrusion has been studied. This research aimed at increasing the water stability of sinking-aquafeed grits using edible fungal hyphae as a binding agent through fungal fermentation, with and without subsequent oven-drying. Commercial sinking pellets were pulverized and subjected to 3 different treatments: fermentation and oven-drying, fermentation without oven-drying, and oven-drying without fermentation. Results showed that the oven-dried unfermented aquafeed disintegrated and sank in water. In contrast, the fermented feeds, with or without oven-drying, showed better stability and floatability in water. The combination of fermentation and oven-drying produced the highest water stability of $73.59 \pm 12.13\%$ as well as the highest floatability of $86.67 \pm 5.77\%$ at the 120th minute. These values were higher than the undried fermented feed (36.90 ± 0.83 water stability and $74.0 \pm 8.94\%$ floatability). Thus, the fungal hyphae possessed the ability to bind the aquafeed constituents, enhancing the water stability and floatability, which was further improved by oven-drying.

Keywords: aquafeed; fermentation; fungal; hyphae; stability

1. Introduction

In aquaculture, feeds make up most of the production costs, and thus it has a very significant influence on the profitability [1]. Increasing aquafeed prices, which means less profit to the aquaculture farmers, has driven the efforts of manufacturing cost-effective feeds with higher components of locally-available ingredients for small-scale fish farmers [2]. The aquafeeds produced however still have low quality in terms of their physical properties, notably water stability [3]. Lower stability in water means that the feeds disintegrate easily, releasing nutrients into the aquatic environment and being less available for fish consumption [4]. This highly nutritious waste has proven to lower the quality of the aquatic environment, especially in the area of intensive aquafarming [5, 6].

Water stability in aquafeed is achieved by incorporating binder or binding agents that can bind feed components during the feed manufacturing process. The binders create strong inter-particle interaction by the mechanism of bonding formation, coating, or filming, thus enhancing the feed integrity from the



disintegrating effect of water [7]. The most frequently used binding agents in aquafeed manufacture are derived from biomolecules such as carbohydrates (agar, alginate, pectin, chitosan, carrageenan, carboxymethylcellulose, guar gum) and proteins (gelatin) [7]. Besides, the binding effect can also be produced by gelatinization of the starch added as the binder and energy constituent of the aquafeed [8]. The gelatinization occurs through humidification and heating in pellet press or extruder machines [9].

The tendency to replace animal-derived components, notably fish, with other nutrient materials of plant-origins [10, 11] necessitates the optimization of the quantity and type of binders, as well as other parameters, in the manufacturing of the aquafeeds. For instance, a previous study showed that incorporating plant-derived materials up to 70%, which consisted of camelina meal, canola meal, and isolated soybean protein in a 5:5:65 ratio, produced aquafeed with water stability when wheat gluten protein (7%) and guar gum (%) were used together as the binding agents [11]. Other binders that have been studied for its suitability to enhance the structural integrity of aquafeeds containing plant-derived proteins were corn, cassava, and potato starches [12]. The search for and optimization of suitable binders for new aquafeed formulation incorporating more ingredients originated from plants seem to continue in the future. This is important as fish feed quality could impact the aquatic environment [13].

The ability of the filamentous fungi to colonize organic substrates while at the same time binding together the substrate particles [14, 15] offers their potential as bio-binder for aquafeed production. Indeed, several studies have been carried out in recent years and have demonstrated effectively the natural adhesive capacity of the moulds from genus *Rhizopus* in producing fermented aquafeeds with better water stability and floatability [16–20]. The water stability and floatability of this fungal fermented feed was indeed found to be comparable to those of commercial floating aquafeed [20]. These edible fungi, used in the preparation of tempeh (an Indonesian traditional fermented food) [21], showed their promising alternative as binders in aquafeed production without the use of pressing or extruder machines, merely involving fermentation, drying, and size-reducing steps. In some of these studies, the binding capacity of the filamentous fungi for aquafeeds in enhancing water stability and floatability was investigated using intact commercial sinking feeds, which might have already contained a binding agent [19,20]. Therefore, this study aimed to find out whether the integrity of the fungal fermented aquafeed, shown by its water stability and floatability values, is contributed mainly by the fungal colonization through their substrate-binding hyphae, or if there is any contribution from the drying step and the pre-existing binding agent in the commercial sinking feed.

2. Materials and methods

A study at a laboratory scale was conducted at several stages. At the first stage, fermentation using a tempeh mould starter was carried out on the sinking aquafeed grits. The aquafeed grits were obtained from commercial sinking aquafeed pellets which were then pulverized in the laboratory. The fermentation was conducted without sterilization of materials and equipment to simplify the procedure and reduce operational costs when applied in the field later. Following the solid fermentation step, some of the fermented aquafeeds were subjected to oven-drying treatments. Finally, fresh and oven-dried fermented aquafeed, as well as unfermented aquafeed grits (control) samples were subjected to analysis of their physical properties.

2.1. Solid fermentation

Aquafeed pellets of sinking type (Finisher FL B, MS Feng Li, Matahari Sakti Ltd., Indonesia) were obtained commercially (Figure 1). The pellets' ingredients consisted of wheat flour, fish meal, soybean meal, fish oil, squid oil, squid meal, cholesterol, lecithin, vitamins, as well as minerals, and had the stated nutritional content of protein (min. 38%), fibre (max. 2%), ash (max. 13%), fat (min. 5%), and moisture (max. 11%), as well as 90% stability in water. The aquafeed pellets (50 g) were transformed into grits form by pulverization using a household blender (Philips Cucina, HR 1741, China) to destroy their integrity and, hence, remove pre-existing stability in water. Afterward, 30 mL of tap water was added and mixed thoroughly. Then, the mixture was added with 1 g of tempeh mould inoculum (with an estimated 1.6×10^5 cfu g⁻¹) which was obtained commercially from a tempeh producer located in

Tangerang Selatan city, Banten Province, Indonesia. After being mixed homogenously, the humidified and inoculated aquafeed grits were transferred into glass Petri dishes (9 cm diameter \times 1.9 cm thickness) packed with plastic cut-straws in an upright position (1.2 cm diameter \times 1.9 cm length) (Figure 2). The cut-straws were placed inside the dishes to produce fermented feed with a cylindrical shape. The fermentation was carried out at room temperature (28-33 °C) for 36 hours. Some of the Petri dishes were left without fermentation (or 0-h fermentation), and proceeded with oven-drying at 50 °C for 24 h to obtain dried unfermented pellets as negative controls.

At the end of the fermentation, some of the fermented aquafeed pellets were oven-dried at 50 °C for 24 h. Thus, at the end of the fermentation and oven-drying processes, 3 different samples were obtained (table 1). The petri dish fermentation was replicated 5 times for each sample. These samples were then subjected to some physical and chemical tests.



Figure 1. Intact commercial aquafeed pellets of sinking type before being pulverized into grits form.

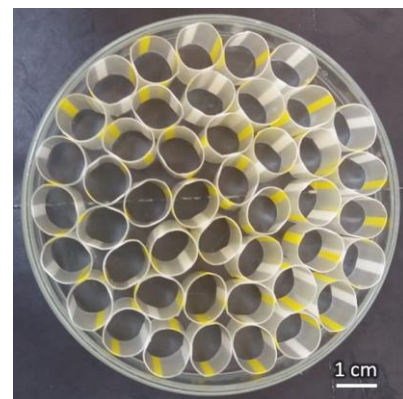


Figure 2. Petri dish containing cylindrical frames inside.

Table 1. Treatments of sinking aquafeed pellets.

Sample Designation	Treatment
Dried, unfermented sample	Pulverization, oven-drying
Undried, fermented sample	Pulverization, fermentation
Dried, fermented sample	Pulverization, fermentation, oven-drying

2.2. Physical tests

The physical tests on the aquafeed samples consisted of density, stability in water, and floating ability in water. In addition, chemical tests were also carried out on the soaking water used in the water stability test.

2.2.1. Density. Density is a value obtained by dividing the weight of a sample (g) by its volume (cm³). The sample weight was obtained using an analytical balance. To obtain the volume, the sample dimensions (length and diameter) were measured using a 0.01-cm accuracy vernier calliper. The mathematical formula for obtaining a cylindrical volume was used for both the undried and dried fermented samples, as well as the intact aquafeed pellets. As for the control, dried unfermented aquafeed sample, the density could not be determined since the individual samples disintegrated into smaller aggregates after oven-drying. That is, the sample broke down into smaller irregular particles when taken out from the cut-straws. Therefore, the density of the intact sinking aquafeed pellets was determined instead, and assumed to be the same as that of the dried unfermented aquafeed sample. For each sample, the measurement was repeated 10 times.

2.2.2. Stability in water. Stability or firmness in water was determined based on the previous method [22] with modifications. A quantity of 1.5-2.5 g sample was placed in a ± 200 mesh tea strainer and submerged for 120 minutes in 400 mL water contained in a 500 mL glass beaker. The residue was then drained, oven-dried at 50°C for 24 hours, and weighed on an analytical balance. The procedure was repeated five times. Water stability was expressed in percent and obtained by dividing the dry weight of the sample before soaking in water by the dry weight of the same sample after soaking in water. The procedure was repeated 5 times for each sample.

2.2.3. Floatability. The buoyancy or floating ability was measured using a method described in an earlier study [23] with a modification. Ten pieces of each sample were transferred into a 500 mL glass beaker already containing 400 mL water. The floating pellets were counted at the 0th, 1st, 5th, 10th, and then every 5-minute intervals until the 120th minute. The number of the still-floating pellets was divided by the initial number of the pellets to give the floatability value, which was expressed in percent. The procedure was repeated 3-5 times for each sample

2.3. Chemical tests

The soaking water resulted from the water stability test (2.2.2) was poured through a filter paper to remove the suspended particles and obtain a clear solution for the measurement of dissolved carbohydrate and protein. These procedures were undertaken as the soaking water underwent a colour change, indicative of nutrient leaching.

2.3.1. Dissolved carbohydrate. Dissolved carbohydrate was approximated by measuring the concentration of reducing sugar using DNS reagent *developed in* an earlier study [24], with some adjustments. A standard solution series with a concentration of 0, 150, 200, and 250 ppm were prepared from a 2 g L⁻¹ stock solution. Samples were added with DNS reagent at the ratio of 1:3 (v/v), vortexed homogeneously, and heated in a 100 °C water bath for 5 minutes. After having cooled to room temperature, subsequent spectrophotometric measurement at λ 515 nm was carried out to obtain the absorbance. The procedure was repeated 8-9 times for each sample.

2.3.2. Dissolved protein. Dissolved protein was determined spectrophotometrically using the Biuret method as described in a previous study [25]. An adjustment to the procedure was done on the protein standard solution series, namely 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0 mg mL⁻¹. The procedure was repeated 10 times for each sample.

2.4. Data analysis.

Statistical analysis was carried out on water stability and floatability data with a confidential level of 95% ($\alpha = 0,05$) using the Kruskal-Wallis test, with those showing significant differences examined further using the Mann–Whitney test. The analysis was performed using the IBM SPSS version 25 program. The density, dissolved protein, and dissolved carbohydrate measurement results were analyzed using the one-way ANOVA with the posthoc Tukey HSD Test.

3. Results and discussion

Solid fermentation on the sinking aquafeed grits as the substrate produced a physical appearance that was different from that prior fermentation. A typical white cottony mycelium was seen overgrowing the entire surface of the petri dish (figure 3). Although the filamentous mould was normally used for making tempeh from soybeans, the mould grew very well also on other organic substrates.

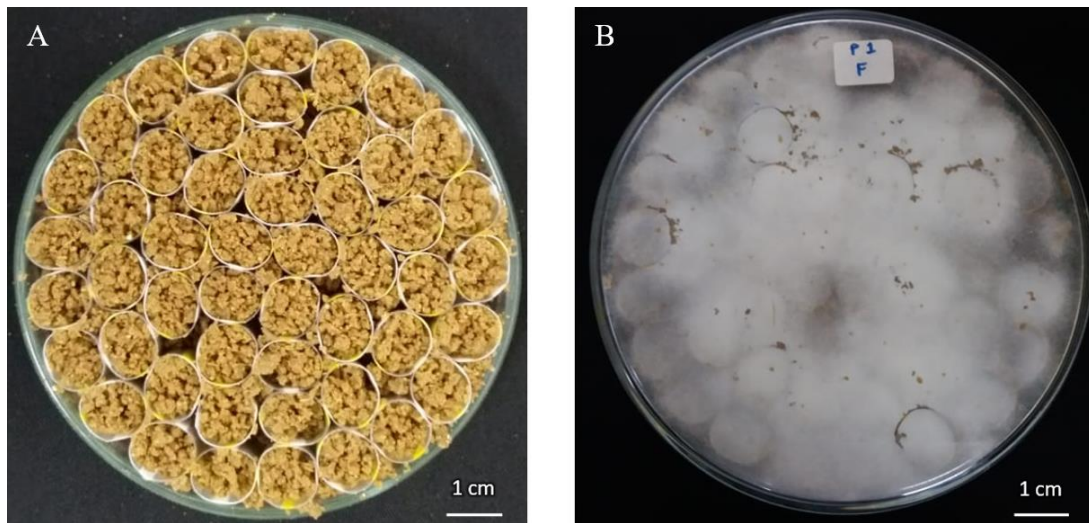


Figure 3. The moisturized aquafeed grits before (A) and after (B) 36-h fermentation at room temperature (28-33 °C) using a tempeh mould starter.

3.1. Fermented aquafeed

The 3 different treatments on the sinking aquafeed grits produced 3 different samples having different physical characteristics (Figure 4). The fermented aquafeed grits, with or without drying, took the cylindrical shapes of the cut-straws. The aquafeed particles were bound strongly together into a single cylindrical mass. This cylindrical shape was preserved and maintained its integrity even when the fermented pellets were taken out from the inside part of the cut straws. The undried fermented aquafeed was heavier than the oven-dried fermented feed as the former still contained a significant level of moisture. The oven-dried unfermented grits could not form compact cylindrical pellets, and easily disintegrated when touched. It indicated that the pulverization of the initial aquafeed pellets destroyed the effectivity of the pre-existing binding agent which provided an adhesive substance gluing the feed particles together.

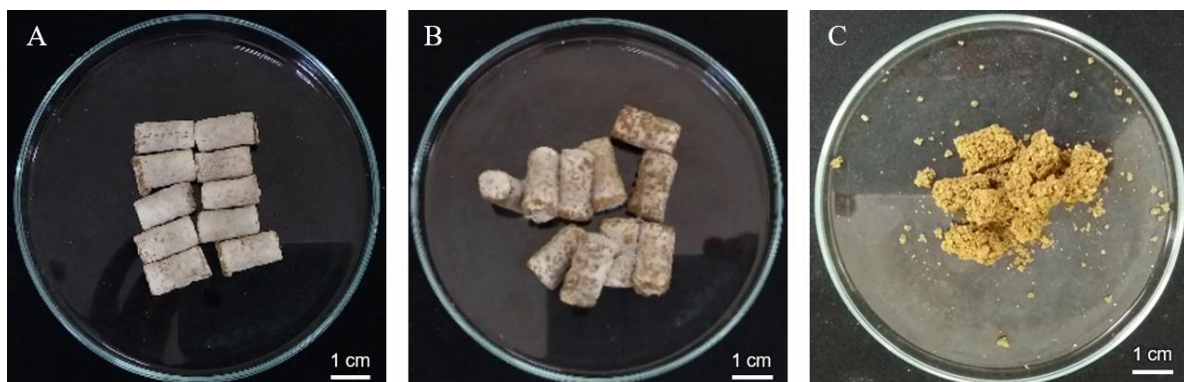


Figure 4. Three different samples obtained from the sinking aquafeed grits: oven-dried fermented aquafeed (A), undried fermented aquafeed (B), and oven-dried unfermented aquafeed (C).

It was the fungal colonization of the aquafeed grits during fermentation which was mainly responsible for the formation of the cylindrical compact pellets. The fungal filamentous hyphae formed a dense hyphal network that interconnected the aquafeed particles into a single solid mass. Indeed, this adhesive property of filamentous fungi has been known for its potential applications in the production of biomaterial for various purposes [26, 27].

3.2. Physical characteristics of the fermented aquafeed

Both types of fermented aquafeeds, with or without oven-drying, had density values below that of water. On the other hand, the oven-dried unfermented aquafeed (control) possessed a density value higher than water density (Table 2), thus sank immediately since the start of the floatability test. The decrease of density after the fermentation was likely caused by the formation of thick mycelium resembling cotton in the intra-particle spaces of the aquafeed grits. This mycelial mass entrapped air which had very low density. Hence, although the initial aquafeed grits had a higher density above that of water, the density of the aquafeed grits and the entrapped air combined would be below 1 g cm^{-3} .

Stability in water for all the 3 samples was significantly different from each other (table 2). Although the fresh, still-wet fermented aquafeed proved to have better stability compared to the unfermented one, oven-drying further increased the water stability. Both fermentation and oven-drying contributed significantly to maintaining the aquafeed integrity against the disintegrating effect of water molecules during submersion. Thus, filamentous fungal fermentation was shown here to act as a bio-binder that glued the aquafeed grit particles, forming stable pellets that did not easily break down in water, hence avoidance from becoming waste.

Table 2. Results of physical tests of density, water stability, and floatability of feed samples (values expressed as average \pm standard deviation).

Physical Properties	Oven-Dried Fermented Feed	Undried Fermented Feed	Oven-Dried Unfermented Feed
Density (g cm^{-3})	0.74 ± 0.06^a	0.91 ± 0.19^b	1.20 ± 0.13^c
Water stability ^{1,2} (%)	73.59 ± 12.13^a	36.90 ± 0.83^b	20.88 ± 4.59^c
Floatability ^{1,2} (%)	86.67 ± 5.77^a	74.00 ± 8.94^a	0.00 ± 0.00^b

¹ Data compared within the same row only, the same alphabetical superscript indicating no significant difference; ² Data recorded at the 120th minute.

Having its initial pellet structure and cohesiveness destroyed by pulverization, the unfermented aquafeed grits could not stick together to form a single compact structure. The aquafeed particles dispersed and sank immediately in water (figure 5), a poor quality that would cause aquaculture feeding inefficient and contribute to nutrient wastage and eutrophication [28].

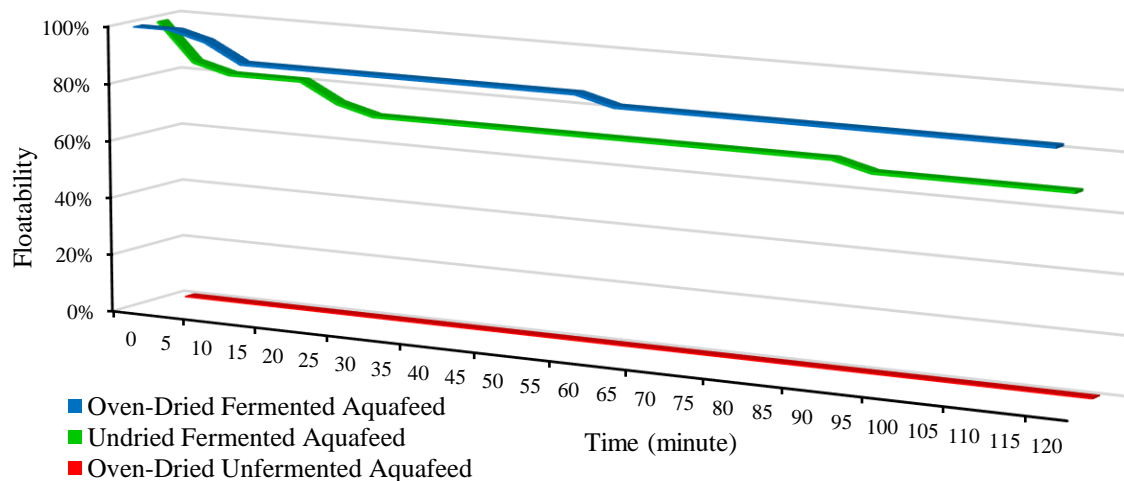


Figure 5. Results of 120-minute floatability test of 3 aquafeed samples: oven-dried fermented aquafeed, undried fermented aquafeed, and oven-dried unfermented aquafeed.

In addition to water stability, the fungal fermentation, with or without subsequent oven-drying, generated a buoyancy effect to the aquafeed with more than 60% floatability for 120 minutes (figure 5). The oven-drying seemed to have no significant contribution to the floating property of fermented aquafeed (table 2). Thus, it was the fungal fermentation which generated the floating ability to the previously sinking feed. The hyphal network of the tempeh mould knitted the aquafeed particles [29] as well as forming mycelial mass which filled in the empty intra-particle spaces. This produced fermented pellets consisting of a mixture of aquafeed grits, fungal biomass, and air cavities with the overall density below that of water. This mechanism is different from the extruded floating aquafeeds whose air cavities are generated from the thermal expansion caused by the evaporation of the trapped water as the aquafeed dough is forced through a die [30].

3.3. Nutrient leaching in the soaking water

The soaking water produced after the water stability test showed no differences between the 3 samples in the first minute of the test (figure 6). Practically no physical change was observed for the soaking water of the oven-dried unfermented aquafeed, thus the soaking water remained relatively clear from the beginning (figure 6C top) until the 120th minute of the test (figure 6C bottom). However, during 120-hour submersion of the oven-dried and undried fermented aquafeeds, murky brownish-yellow coloration (figure 6A and 6B bottom) developed in the soaking water which was initially clear (figure 6A and 6B top), indicating leaching of small size molecules from the fermented aquafeed into the soaking water. The leaching molecules were the products of the enzymatic activity of the growing mould on the complex carbohydrates and protein which were the main constituents of the feed. The fungal enzymes are known to hydrolyze carbohydrates and proteins of high molecular size into their smaller and soluble constituents such as simple sugars and peptides which are soluble in water.

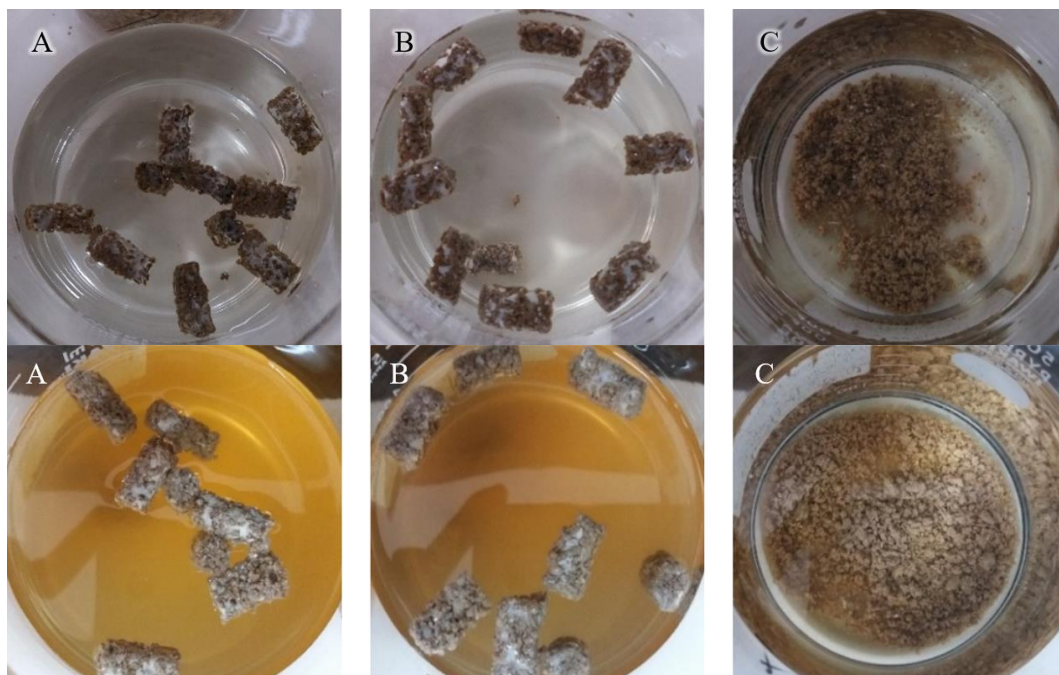


Figure 6. The physical appearance of 3 aquafeed samples before (top) and after (bottom) a 120-minute stability test in water: oven-dried fermented aquafeed (A), undried fermented aquafeed (B), and oven-dried unfermented aquafeed (C).

Measurement of the dissolved carbohydrate and proteins showed that the oven-dried unfermented aquafeed (control) underwent the least nutrient leaching (table 3). This was expected since no fungal enzymatic activities occurred to hydrolyze the protein and carbohydrate contents. The highest

carbohydrate and protein release into the soaking water was found in the undried fermented aquafeed. Thus, the oven-drying procedure following the fermentation helped reduced the nutrient loss in water of the fermented aquafeed. Further improvement to reduce this nutrient leaching could be suggested such as the use of water-resistant coating material [31] in order to minimize nutrient loss which could be detrimental to the aquatic environment.

Table 3. Dissolved carbohydrate and dissolved protein content of the soaking water of the 120-minute water stability test (values written as average \pm standard deviation).

Nutrient Leaching in The Soaking Water	Oven-Dried Fermented Feed	Undried Fermented Feed	Oven-Dried Unfermented Feed
Dissolved protein (g L^{-1})	0.74 ± 0.06^a	1.69 ± 0.22^b	0.52 ± 0.04^c
Dissolved carbohydrate (mg L^{-1})	30.28 ± 1.70^a	76.56 ± 5.18^b	6.56 ± 3.22^c

Data were compared within the same row, values having different alphabetical superscripts indicating significant different.

4. Conclusion

Using sinking aquafeed grits, it was shown that solid fermentation using a tempeh mould starter produced fungal hyphae that could act as bio-binder. As the edible fungal colonized the aquafeed substrate, a massive hyphal network joined the aquafeed particle together, forming a compact fermented pellet that had high stability in water, as well as generated floating characteristics. This water stability was enhanced further by oven-drying.

Statement of authorship

Catur Sriherwanto was the major contributor for preparing and submitting this finalized English manuscript. He was also the principal author planning, designing, and supervising the entire works from start until the end. Rizky Hastuti Purwaningsih had a very significant contribution by conducting the entire laboratory experiments, data acquisition, and analysis, as well as preparing the first manuscript draft. Etyun Yunita was the co-supervisor for this research and contributed to examining the first manuscript draft. Imam Suja'i was an important technical assistant and co-worker of Rizky Hastuti Purwaningsih in the laboratory activities.

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