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Aquafeed Biofloatation through Mycelial Hydrophobic Coating

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Abstract

Aquafeed biofloatation through tempeh mold fermentation on sinking aquafeeds produces water-floating property, an alternative to the expensive extrusion technique. However, the role of the fungal mycelium in this biofloatation remains unclear. This study aimed to investigate the role of surface mycelium of the fermented feed in buoyancy. Commercial sinking feed was fermented using a tempeh starter at ambient temperature (28–33 °C) for 48 h. Freshly fermented feeds were produced, some of which were peeled to remove the surface mycelium, while the others were left intact. After 24-h oven-drying at 50 °C, physical tests were done on the peeled and unpeeled fermented feeds, plus unfermented feed as a negative control. Results showed that the unpeeled fermented feed had the highest floatability (48% at the 60th minute), and continued floating until the 120th minute with 36% floatability. In contrast, the unfermented feed did not float at all, while the peeled fermented feed sank within the first 2 minutes. Only the unpeeled fermented feed showed hydrophobic characteristics (> 90° contact angle and 20.16 s water absorption time). Thus, the hydrophobic surface mycelium might prevent rapid water infiltration into the fermented feed matrix, enabling the intact fermented feed to float longer.

INTRODUCTION

Edible molds of the genus *Rhizopus*, the fermenting microorganisms in the preparation of the Indonesian food "soybean tempeh", have found applications beyond human nutrition, and have been studied for their potential application in fish feeds (Langeland *et al.,* 2016; Vidakovic *et al.,* 2016; Abro *et al.,* 2014; Chen *et al.,* 2020). The fungal biomass is rich in protein as well as essential amino acids comparable to those of aquafeed-grade protein sources, except phenylalanine and methionine (Nitayavardhana *et al.,* 2013). Moreover, *Rhizopus oligosporus* biomass contains an exceptionally high lysine and has high *in vitro* protein digestibility (~80%) similar to that of commercial fish feed (Batsaikhan, 2017).

A recent promising application of *Rhizopus* is providing buoyancy to sinking aquafeed (Zaman *et al.,* 2018). The floating characteristic of feeds is important in aquaculture to ensure the feeds being consumed by fish and not wasted (Robb and

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Crampton, 2013; Robb *et al.,* 2017) which could lead to feed decomposition, dissolved oxygen depletion, and fish suffocation (Dahal, 2018). Feeds float on water due to their density being lower than that of water and are usually made using an extruder machine, triggering the thermal expansion of the starch component of the feed (Robb and Crampton, 2013). This technique is costly (Robb *et al.,* 2017) and requires specialized technology (Ahmed, 2015). Thus, biofloatation of fish feeds through bioconversion using tempeh mold could provide an alternative solution.

Some studies have demonstrated that the non-extruded, tempeh-mold fermented feeds were able to float with the highest floatability of 100% for 5 hours (Zaman *et al.,* 2018; Suliswati *et al.,* 2018). However, the exact mechanism of how the mycelium of the tempeh fungi produced the floating ability has not been investigated. Therefore, this study aimed to demonstrate the possible role of the surface mycelium in the fungal-induced biofloatation. We hypothesized that the surface mycelium might provide hydrophobic covering to the fermented feed surface, thus preventing the rapid infiltration of water molecules into the feed matrix, maintaining the feed density to below that of water, and keeping the feed afloat.

METHODOLOGY

Place and Time

The studies were conducted at the Agromicrobiology Laboratory, National Research and Innovation Agency (BRIN), South Tangerang, Banten, Indonesia. The research was carried out from February to June 2018.

Research Materials

The materials used in this research were Commercial sinking aquafeed pellets (100 g) (MS Feng Li, Finisher FL B, Matahari Sakti Ltd., Indonesia) and tempeh mold starter (local tempeh manufacturer in Serpong district, South Tangerang, Banten Province, Indonesia).

The tools used were household blender (Philips Cucina, HR 1741, China), glass Petri dishes (9 × 1.5 cm, and 9 × 1.9 cm) containing 4 rectangular rigid cardboard frames (3.27 × 2.72 × 1.37 cm).

Research Design

At a laboratory scale, solid substrate fermentation of pulverized sinking fish feed pellets was undertaken in a non-sterile condition (without sterile materials and equipment). This non-sterile procedure was to make it more applicable and financially less demanding for later implementation in the farms. Following the fungal bioconversion, the oven-dried fermented feeds were then subjected to physical and chemical tests.

Work Procedure

Fermentation using Tempeh Mold Starter

Commercial sinking aquafeed pellets (100 g) with the stated nutritional content (Table 1, Figure 1) were pulverized using a household blender at the lowest speed for 2 minutes to destroy their compactness and integrity. Afterward, 2 g of tempeh mold starter was added and mixed thoroughly. Next, the mixture was added with water up to 75% moisture content, mixed homogeneously, and transferred into glass Petri dishes (9 × 1.5 cm, and 9 × 1.9 cm) containing 4 rectangular rigid cardboard frames (3.27 × 2.72 × 1.37 cm) (Figure 2) to produce fermented feed blocks with rectangular or cuboid shape. The 48-h fermentation was carried out at an ambient temperature (28–33 °C), with some petri dishes left unfermented (or 0-h fermentation) as negative controls.

Fermented feed blocks with a sliceable cheese-like texture were finally produced. Some of the freshly fermented feeds had all their surfaces thinly sliced or peeled using a sharp cutter to remove the mycelial outermost covering (surface mycelium). Thus, the 3 different treatments resulted in 3 feed samples: unfermented feed (negative control), unpeeled fermented feed (fermented feed with intact

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surface mycelium), and peeled fermented feed (fermented feed with surface mycelium removed). All these samples were subsequently oven-dried at 50 °C for 24 hours.

Table 1. Nutritional and physical properties of aquafeed pellets MS Feng Li, Finisher FL B according to the manufacturer (Matahari Sakti Ltd., Surabaya, East Java, Indonesia).

Protein	Fat	Fiber	Ash	Moisture	Stability in Water
min. 38%	min. 5%	max. 2%	max. 13%	max. 11%	min. 90%

Composition : wheat flour, fish meal, soybean meal, fish oil, squid oil, squid meal, cholesterol, lecithin, vitamins, and minerals.

Figure 1. Commercial sinking pellets in intact form before pulverization and fermentation.

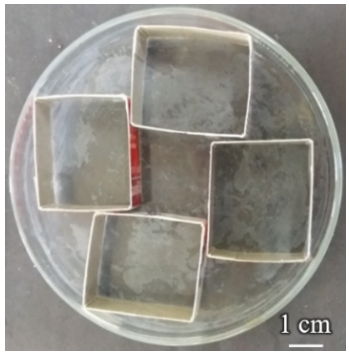

Figure 2. Petri dish with rectangular frames inside.

Physical Tests

All the feed samples were subjected to physical tests, namely density measurement, floating ability in water, stability in water, water absorption rate, and surface hydrophobicity. For clarity, from here onwards the term “feed block” is used to refer to the individual cuboid cake or slab obtained from the oven-drying of the unfermented feed, unpeeled fermented feed, and peeled fermented feed as mentioned previously.

Density is related to the volume and weight of a sample. The formula for calculating cuboid volume was used for the unpeeled fermented and peeled fermented feeds. As for the control unfermented feed, the volume could not be determined since the shape integrity could not be maintained after oven-drying (the sample disintegrated into grainy particles as the rectangular frames were lifted). Therefore, for the control, the density of the cylindrical sinking pellets before being pulverized was determined instead. A 0.01-cm accuracy vernier caliper was used to measure the dimensions (length, width, thickness, and diameter). The density (ρ in g cm^{-3}) calculation was undertaken based on the sample weight (w in g) and the sample volume (v in cm^{-3}) using the following formula and repeated 10 times for each sample.

$$\rho = \frac{w}{v}$$

The buoyancy, floating ability, or simply floatability was determined based on Leiskayanti *et al.* (2017) with a modification on the number of samples being

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tested per batch. Five feed blocks of each sample were transferred into a 500 mL glass beaker containing 400 mL autoclave-sterilized tap water. Counting was then carried out on those feed blocks which were still floating at the 0th, 1st, 2nd, 3rd, 4th, 5th minute, and, subsequently, every 5 minutes until the 120th minute. The procedure was repeated 5 times for all samples. Based on the initial number of the feed blocks (n) being tested, and the number of the feed blocks still afloat (b), floatability (f in %) at a given time was calculated using the formula below.

$$f = \frac{b}{n} \times 100\%$$

Water stability or compactness in water was tested using the method of Aas *et al.* (2017) with modifications. Five feed blocks of each sample were weighed out, put into 400 mL autoclave-sterilized tap water in a 500 mL beaker glass, and soaked for 120 minutes. The undisintegrated feed blocks were then filtered out using a ± 200 mesh tea strainer, drained, oven-dried for 24 hours at 50 °C, and finally weighed. The procedure was repeated five times. The dry weight of the feed blocks before (w_i in g) and after soaking in the water (w_t in g) were used to calculate the water stability (s in %) according to the equation:

$$s = \frac{w_t}{w_i} \times 100\%$$

Water absorption rate was measured as the time required by the surface of the feed blocks to absorb water. A previous method (Da Silva *et al.*, 2018) was used with modification. Onto the surface of a feed block sample, 20 μL tap water was pipetted out using a 20-200 μL microtip from the vertical distance of 5.79 ± 0.53 mm between the surface and the tip of the micropipette. The procedure was recorded using the video camera of a Samsung J5 handphone and repeated 10 times. The time elapsed between the water droplet touching the surface of the feed block and its complete absorption by the surface was determined as the water absorption time and expressed in seconds (s).

Hydrophobicity or water-repellency of the surface of a feed block was determined according to Goldsmith *et al.* (2017) by measuring the contact angle (θ) of a water droplet on the surface of a feed block (Figure 3). The procedure was similar to that of water absorption time. As a water droplet just reached the surface, a spherical cap formed, and the contact angles were measured through image analysis using MS PowerPoint's rotating tool on the snapshot image of the water spherical cap. For each snapshot image, the contact angle (θ) of both the left and right sides of a spherical cap was measured for calculating the average value.

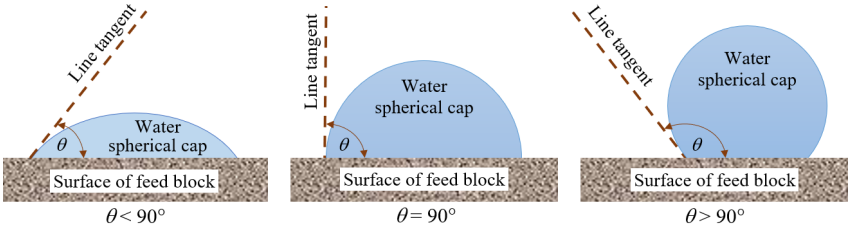

Figure 3. Surface hydrophobicity determination by measuring the contact angle (θ) of a water droplet forming a spherical cap on the feed block surface (Goldsmith *et al.*, 2017). A surface is considered hydrophobic when the contact angle ≥ 90° (Moriizumi *et al.*, 2020).

Soluble Protein and Carbohydrate Tests

After the water stability was tested and the residual feeds were filtered out, the remaining soaking water was filtered using filter paper for soluble protein and soluble carbohydrate measurement. This procedure was to determine the extent of nutrient leaching.

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To quantify soluble protein content, a previous method (Janairo *et al.,* 2015) was used with a slight adjustment on the protein standard solution series. The following protein standard concentrations were used: 0, 0.5, 1.0, 1.5, 2.0, 2.5, dan 5.0 mg mL^{-1}.

Soluble carbohydrate concentration was estimated from the reducing sugar content quantified using the DNS reagent method (Sumner and Graham, 1921). A standard solution series of 0, 150, 200, and 250 ppm were prepared from a 2 g L^{-1} d-glucose stock solution. Sample and standard solutions were mixed with DNS reagent at the volumetric ratio of 1:3, homogenized using a vortex, and heated for 5 minutes at 100 °C. After having cooled to room temperature, the solutions were measured spectrophotometrically at λ 515 nm.

Data Analysis

Data analysis was carried out using the statistical analysis method on the interval of 95% confidence (alpha=0.05). The design used is Complete Random Design (CRD) with one factor using IBM Statistical Software SPSS statistics version 16.0.

RESULTS AND DISCUSSION

The tempeh mold grew well on the moistened, pulverized sinking aquafeed. It was evident from the white-grey mycelium overgrowing the entire surface of the feed (Figure 4).

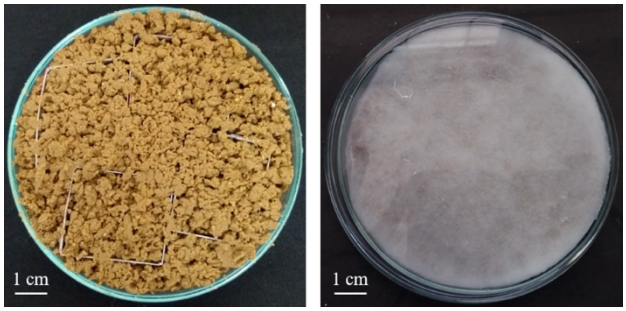

Figure 4. The moistened, pulverized sinking feed before (left) and after 48-h fermentation (right) on Petri dishes using tempeh starter as the fermenting microorganism.

Compared to those of individual cylindrical pellets of the commercial aquafeeds before being pulverized, the fermented feed blocks had much larger dimensions and surface areas (Figure 5). This relatively large surface area eased the peeling of the external mycelium layer coating all 6 faces of the cuboidal fermented feed blocks to produce the peeled fermented feed samples. In addition, the greater surface area was also deliberately designed to magnify the effect of interaction between the surface mycelium with the soaking water.

The ability of tempeh mold to grow well on commercially manufactured sinking fish feeds (Sriherwanto *et al.,* 2021a; Sriherwanto *et al.,* 2021b) and self-formulated fish feeds (Hariyono *et al.,* 2021; Maulana *et al.,* 2020) was reported previously. This was expected since the feed contained high nutritional components, especially protein and fat (Table 1).

Fermented Feed

The 3 feed samples showed different physical appearances after oven-drying (Figure 5). The unfermented feed crumbled immediately and could not maintain its integrity when the rectangular frames were lifted and detached from the moistened feed grits. The pulverization had mechanically destroyed the initial integrity of the sinking aquafeed, converting the cylinder-shaped pellets into grainy particles. These grits could not re-aggregate to form a single compact block through the sequential process of moisturization, placement inside the rectangular

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frames, and oven-drying. In contrast, the fermented feeds took the shape of rectangular frames, forming fermented feed blocks. These blocks remained firm and did not disintegrate when the rectangular frames were removed.

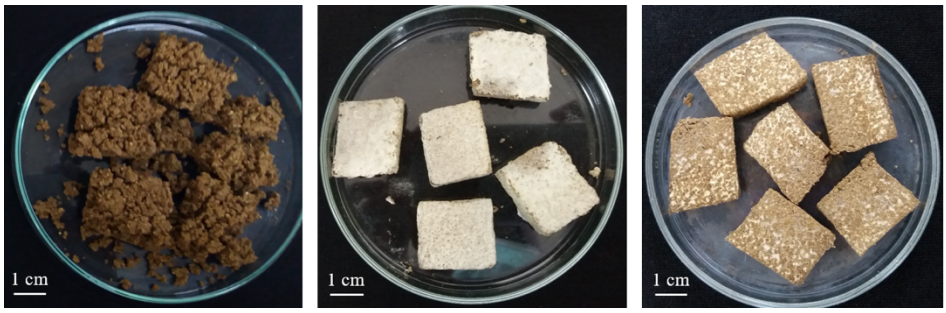

Figure 5. Three different feed samples produced after oven-drying: unfermented feed (left), unpeeled fermented feed (middle), and peeled fermented feed (right).

Peeling off the fermented feeds did result in the disappearance of the white color on the surface of the fermented feed blocks, indicating the absence of the mycelial outer layer (surface mycelium). This slicing off the mycelial coating, however, did not cause the fermented feed to fall apart as was the case with the unfermented feed. This was due to the presence of penetrative hyphae of the fungus which filled in the intra-particle space of the feed grits seen as white patches (Figure 5 (right)). These penetrative hyphae were also binding the feed grits together, forming a solid block that was not easily disintegrating..

The binding agent, which is an important component in commercial fish feed production, lost its effectivity after pulverization. Without adding an external binding agent, the sequential procedure of moisturization, molding in the rectangular frame, and oven-drying of the feed grits did not produce the feed particle re-aggregation. Thus, this means that the formation of a compact solid block in the case of the fermented feed could no longer be attributed to the pre-existing binding agent or the cohesiveness of the feed particles. Rather, it was indeed the work of the fungal filamentous hyphae that knitted the feed particles into a single solid aggregate as described in earlier studies (Cantabrana *et al.,* 2015). The fungal hyphae, seen as cottony white filamentous mass, grew by penetrating deeply into the substrate particles and by filling up interparticle spaces, forming a massive interlocking mycelial mass, that bound together and united the feed particles into a solid block with a compact, cake-like texture, just like soybean tempeh (Kustyawati *et al.,* 2020).

This particle-binding mode by fungal hyphae works differently from the conventional binding agent such as celluloses, alginates, collagen, starches, wood processing side-products, pectin, carrageenan, gelatins, and synthetic polymers used in the manufacturing of aquafeed pellets (Tacon *et al.,* 2009). It was also different from the binding effect produced by proper extrusion of feed formula containing sufficient quantity and quality of the starch component (Robb and Crampton, 2013).

The fact that peeling off the mycelial surface did not lead to the disintegration of the fermented feed blocks implies that surface mycelium, hence aerial and surface hyphae, did not play a major role in the integrity of the fermented feed block. Instead, it was the penetrative hyphae that were responsible for binding the feed particles together, thus maintaining the feed block integrity. It is well-known that fungal colonization of a substrate occurs by mycelium formation in which penetrative hyphae grow in and around individual substrate particles, forming a dense mycelial mass embedded with the substrate par-

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ticles. The mycelium formed by the penetrative hyphae acts like an adhesive agent that glues the discrete substrate particles, forming a solid composite (Manan *et al.,* 2021).

Physical Properties

Both types of fermented feeds, with or without surface mycelium peeled off, possessed density values below that of water, whereas the unfermented feed density was higher than water density (Table 2). This explains why the unfermented feed sank immediately since the start of the floatability test. The peeled fermented feed floated briefly at the beginning but sank within the first two minutes (0% floatability). In contrast, the unpeeled fermented feed underwent slow gradual sinking, with 48% floatability in the 60th minute, and continued afloat until the 120th minute with 36% floatability (Figure 6, 7, and 8). The fungal fermentation modified the way the fish feeds behaved in water. Unfermented feed grits, which instantly sank in water, were transformed by the colonizing fungal hyphae into a compact cake having density lower than that of water, thus capable of floating on water.

When the surface mycelial sheath was sliced off, the floatability of the peeled fermented feed blocks decreased considerably, unlike those with intact surfaces. Thus, the presence of surface mycelium strongly suggested its role in preventing the rapid sinking of the unpeeled fermented feed. The mycelium layer might work by impeding a sudden intrusion of water deeply into the porous spaces of the unpeeled fermented feeds. In contrast, the absence of the mycelium coating seemed to enable water to be in direct contact with the porous surface of the peeled fermented feed, rapidly filling the empty spaces inside the feed matrix. As a result, the density rose quickly to above that of water, causing the peeled fermented feed to sink.

The 3 feed samples behaved differently when their surfaces were subjected to the water droplet treatment (Table 2). A contact angle greater than 90° was observed on the surface of the unpeeled fermented feed, whereas both unfermented feed and peeled fermented feed showed contact angles smaller than 90°. The surface of the unpeeled fermented feed took the longest time (20 s) to absorb a 20-μL water droplet. This was in contrast to both unfermented feed and peeled fermented feed, whose surfaces absorbed water droplets within 2 s. This experiment demonstrated the important role that the surface mycelium played in providing a water-resistant barrier between the aquatic environment and the fermented feed matrix underneath. To be able to inhibit water intrusion, the surface mycelium must be water-repellent or hydrophobic. Indeed, the hydrophobic property of this surface mycelium was confirmed by the data obtained from the tests on hydrophobicity (contact angle) and water absorption time (Table 2). Amongst the 3 feed samples, the unpeeled fermented feed was the only sample having a hydrophobic surface (contact angle > 90°) and the longest water absorption time (a 20-μL droplet in 20 s). In contrast, the other two feed samples, with no surface mycelium covering, had hydrophilic surfaces (contact angles < 90°) and rapidly absorbed a water droplet in less than 2 s.

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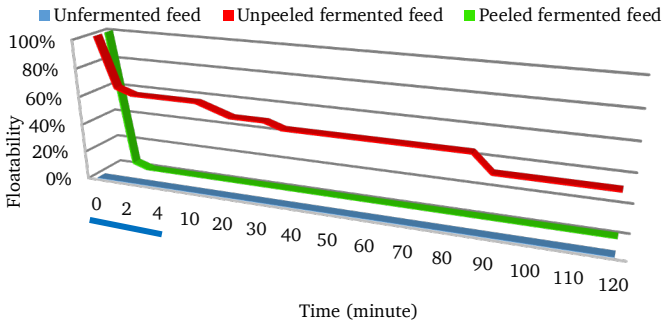

Figure 6. Floatability profile. (The underlined numbers on the *x*-axis is the time increment in the first 5 minutes which is stretched 5 times its original length for clarity purpose).

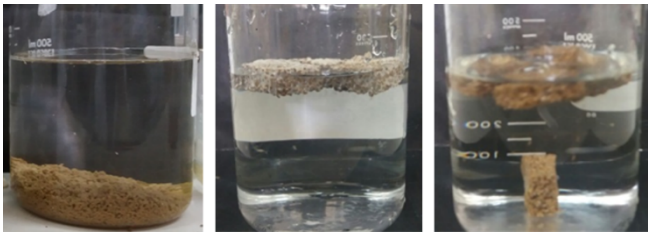

Figure 7. The 1st minute of floatability tests on the unfermented feed (left), unpeeled fermented feed (middle), and peeled fermented feed (right).

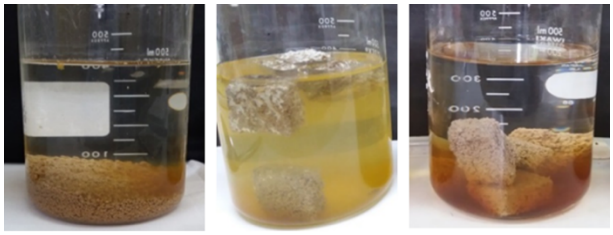

Figure 8. The 120th-minute floatability tests on the unfermented feed (left), unpeeled fermented feed (middle), and peeled fermented feed (right).

Table 2. Physical properties of feed samples (average ± standard deviation).

Physical Properties	Unfermented Feed			Unpeeled Fermented Feed			Peeled Fermented Feed		
Density (g cm^{-3})	1.27	±	0.18	0.47	±	0.06	0.58	±	0.05
Floatability[1,2] (%)	00.00	±	0.00^{b}	48.00	±	1.67^{a}	0.00	±	0.00^{b}
Water stability[1,3] (%)	58.07	±	10.36^{a}	86.66	±	11.24^{a}	86.31	±	4.59^{b}
Water absorption time[1] (s)	0.63	±	0.63^{a}	20.16	±	15.71^{b}	1.62	±	0.51^{c}
Hydrophobicity[1] (°)	56.75	±	9.06^{a}	94.30	±	4.22^{b}	66.05	±	2.97^{c}

[1] Data compared within the same row only, the same alphabetical superscript indicating no significant difference.
[2] Data recorded at the 60th minute of the floatability test.
[3] Data obtained from the 120-minute water stability test.

The hydrophobic nature of the filamentous fungal surface was demonstrated by previous studies on the water-repellency of soil contributed by fungi (Chau *et al.,* 2012; Zheng *et al.,* 2014), including *Rhizopus* (Lin *et al.,* 2006). In another research, a positive relationship between fungal presence and hydrophobicity was found in managed soils (Young *et al.,* 2012). The fungal contribution in soil water repellency has even been applied successfully in producing a water-repellent soil layer with a very high hydrophobicity (Salifu and El Mountassir, 2020). As proposed in the case of soil (Rillig, 2005), the hydrophobicity of the surface mycelium in this study might be attributed to the protein called hydrophobins which have a

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large proportion of hydrophobic amino acids, and are surface-active proteins synthesized by filamentous fungi (Linder *et al.,* 2005). Other hydrophobic components of fungal cell walls such as lipids, glucans, chitins, and pigment molecules may have contributed to this water-repellent property as well (Feofilova, 2010; Kang *et al.,* 2018).

Results of the 120-minute test on the stability in water showed that both the unpeeled fermented feed and peeled fermented feed had no significant difference in their water stability (circa 86-87%). Both maintained their compactness well in water (Table 2). Although having an impact on floatability, the presence or removal of the surface mycelium, however, did not contribute to structural advantage or disadvantage to the integrity of the fermented feed as indicated by the results of the water stability tests (Table 2). Rather, it was the deeply penetrative, vast intertwined network of hyphae joining the feed grits into a single solid block that was responsible for this structural integrity of the feeds in the aquatic environment. Without this filamentous fungal binder, the feed grainy constituents would readily disperse in water as happened with the unfermented feed (Figures 7 and 8).

Soluble Protein and Carbohydrate

At the end of the floatability test, the soaking water of the unfermented feed remained relatively clear, whereas those of the unpeeled fermented feed and peeled fermented feed turned murky yellowish-brown. Upon analysis, the soaking water of the unfermented feed contained the least concentration of both soluble carbohydrates and protein. These nutrients were found at concentrations that were higher in the soaking water of both peeled and unpeeled fermented feeds (Table 3).

The coloration observed in the soaking water of the fermented feeds was very likely caused by the water-soluble compounds of various molecular sizes, notably short-chained carbohydrates, polypeptides, and proteins produced by the enzymatic activity of the growing mold. Tempeh mold is known to produce various enzymes including amylase and protease (Londoño-Hernández *et al.,* 2017). Thus, these low molecular compounds would facilitate better digestion for fish. However, as far as the environmental aspect is concerned, this side effect of nutrient leaching is undesirable (Debnath *et al.,* 2020). Therefore, quantification of the feed's main components leaching to the water, namely soluble reducing sugars (as an estimate of soluble carbohydrate) and soluble protein was conducted and analyzed. Both nutrients were released at the lowest concentration by the unfermented feed.

The highest concentration of soluble protein was released by the unpeeled fermented feed, followed by the peeled fermented feed. Both of the fermented feeds caused reduced sugar leaching about 40 times higher than that of unfermented feed. The outermost surface of the fermented feed, which contained the mycelium layer, might be the zone where proteolytic activity was the highest. In other words, it was the layer which contained the highest concentration of soluble polypeptides, which leached readily and dissolved in the soaking water. This proposition, however, needs further investigation.

Table 3. Soluble reducing sugars and protein content of the residual water of the 120-minute floatability tests (values expressed as average ± standard deviation).

Nutrient Leaching	Unfermented Feed	Unpeeled Fermented Feed	Peeled Fermented Feed
Soluble protein (g 100 g^{-1})	1.44 ± 0.23^{a}	4.17 ± 0.21^{b}	3.25 ± 0.14^{c}
Soluble reducing sugar (mg g^{-1})	0.10 ± 0.13^{a}	4.00 ± 0.79^{b}	4.29 ± 0.27^{b}

Data compared within the same row only, the same alphabetical superscript indicating no significant difference.

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CONCLUSION

Physical tests conducted in this study showed strong water-repellent properties (94.3° contact angle and 20.16 s water absorption time) on the surface mycelium of the unpeeled fermented feed. In contrast, the unfermented and peeled fermented feeds had surfaced with more hydrophilic characters (less than 90° contact angle and less than 2 s water absorption time). It has been demonstrated that the surface mycelium played its role as a hydrophobic barrier in the aquatic setting, slowing down the infiltration of water into the substrate matrix of the unpeeled fermented feed. This allowed the mycelium-coated feed to maintain its density below that of water, hence staying afloat in water for a longer time, outperforming the unfermented feed and peeled fermented feed. The latter two feed samples were devoid of surface mycelium layer and sank immediately, unable to float at all after 2 minutes in water.

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