Recent potential biotechnological applications of the tempeh mould *Rhizopus*. A short review

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Abstract. The last 10 years have seen innovative researches worldwide on the potential use of the edible tempeh mould *Rhizopus* for various applications other than for human consumption. This is owing to the fungal ability to utilize various organic substrates alone or in combination with supplemented inorganic compounds to produce valuable biomolecules, as well as to generate other desired nutritional, chemical, biological, and physical properties. In addition, although the conventional biotechnological method of solid fermentation is still widely used, other ways of culturing the fungi have also been studied. Thus, fungi from the genus *Rhizopus* have now found their way for potential state-of-the-art applications in much wider contexts, for example in animal nutrition, environment and aquaculture, as well as in biomedical fields.

1. Introduction
The edible fungi from the genus *Rhizopus* are widely known for their important role in the fermentation of cooked soybeans (*Glycine max* L.) to traditionally prepare the indigenous Indonesian food called tempeh [1,2]. Soybean tempeh is known for its rich protein content [3], and has been hailed as a meat alternative [4]. This food has also been subjected to numerous decade-long studies on its potential health benefits such as healing effect on skin lesion [5], antihypertension [6], reversal of scopolamine-triggered memory damage, enhancement of brain cholinergic activities, neuroinflammation reduction [7,8], hepatoprotective improvement and antioxidant properties [9], oxidative stress reduction [10], anti-biofilm activity against oral bacteria [11], antioxidant, antidiabetic, and anticancer activities [12].

As affirmation to the nutritional and health benefits of tempeh outside Indonesia, *Rhizopus*-based tempeh fermentation has been extended to develop similar tempeh using non-soybean materials, especially agricultural products locally available in abundant in those countries. For example, studies have been conducted world-wide to produce tempeh using red bean (*Phaseolus vulgaris*) in Taiwan [13], carioca common bean (*Phaseolus vulgaris* L.) in Brazil [14], chickpea (*Cicer arietinum*), green lentil (*Lens culinaris*), red lentil (*Lens culinaris*), white bean (*Phaseolus vulgaris*), black bean (*Phaseolus vulgaris*), and broad bean (*Vicia faba* L.) in Turkey [13], barley (*Hordeum vulgare*) in China [15], stale bread and brewers spent grain in Sweden [16].

In the last decade, studies have been expanded further to explore the benefits of the moulds from genus *Rhizopus* beyond its use in human diets. Thus, this brief review aims to bring out some recent studies on the potential biotechnological application of *Rhizopus* in the fields of animal nutrition, hydrophobic coating for aquafeed, and wound healing.
2. Alternative quality protein source for aquaculture

The world aquaculture production reached 114.5 million tons in live weight in 2018, and in the year 2030 it is estimated to increase by 32% to 109 million tons [17]. This growing trend is however not supported by the increase in the supplies of fish meal and fish oil [18], which are the important ingredients of aquafeed as both are deemed as the most digestible and the most nutritious feed components. Thus, alternative ingredient that can substitute fish meal and fish oil are needed for the continuation of aquaculture industries. Amongst those alternative protein sources are fishery and aquaculture byproducts, insect meals, microbial biomass, macroalgae, and food wastes [19].

Edible fungi *Rhizopus*, used in the making of soybean tempeh for human consumption [20], have been intensively studied for their feasibility to replace fish meal as protein alternative for aquafeed (Table 1). The fungi were cultured in submerged fermentation system, which is different from solid substrate fermentation used in the traditional soybean tempeh fermentation. Submerged or liquid fermentation would ease separation of the fungal biomass from the liquid substrate.

**Table 1.** Production of protein-rich *Rhizopus* biomass using various organic by-products as substrate through submerged fermentation.

| Fermentation Substrate and Bioreactor | *Rhizopus* Strain Used in the Fermentation | Protein Content of *Rhizopus* Biomass | Reference |
|--------------------------------------|-------------------------------------------|-------------------------------------|-----------|
| Volatile fatty acids from food waste, bubble-column bioreactor, fed batch | *R. microsporus* var. *oligosporus* CBS 112586 | 39.28 ± 1.54% | [21] |
| Fish industry side-stream, bubble-column bioreactor | *R. oryzae* CCUG 28958 | 33% and 62% | [22] |
| Vinasse, 250 mL Erlenmeyer shake-flasks | *R. oryzae* CCUG 61147 | 50.9% | [23] |
| Wheat-starch plant waste water, 250 mL baffled Erlenmeyer flasks | *R. oryzae* CCUG 28958 | 50.76 ± 0.71% | [24] |
| Lignocellulosic wastes from pulp mills, 4-L airlift bioreactor, batch | *R. oryzae* CCUG 28958 | 44.90 ± 0.71% | [25] |
| Pea processing by-product, 250-mL Erlenmeyer flask | *R. oryzae* CCUG 61147 | 50.03 ± 0.09% | [26] |
| Potato protein liquor, 4-L airlift bioreactor | *R. oryzae* CCUG 28958 | 49.86% | [27] |
| Citrus waste free sugars, airlift bioreactor with 3.5 L working volume | *R. oryzae* CCUG 28958 | 51 ± 7% | [28] |
Mostly wheat-derived thin stillage, 250-mL Erlenmeyer flask

| Substance                  | Organism      | Protein (%) | Reference |
|----------------------------|---------------|-------------|-----------|
| Mostly wheat-derived stillage | Rhizopus sp.  | 49-55%      | [29]      |
| Spent sulphite liquor     | Rhizopus sp.  | 30-50%      | [30]      |

Figure 1. Pellet fungal biomass (A) and microscopic morphology (B) of *R. oryzae* after 72-h submerged fermentation in an airlift bioreactor containing free sugars squeezed from citrus waste as the sole substrate [28].

Amongst the advantage of using *Rhizopus* is its ability in utilizing wide range of organic byproducts, high protein content (30-55%) of the fungal biomass (Table 1), and easy separation of the biomass from the fermentation substrate as the fungal biomass can be conditioned to form mycelial pellets (Figure 1). In addition, amino acid content of *Rhizopus* was found to be fairly well represented when benchmarked against those of fish meal [25]. The fungal protein was also reported to have high digestibility (circa 80%) similar to that of protein sources available in the market [31].

The feeding trial on fish using *Rhizopus* biomass as one of the feed ingredients was already undertaken to assess its physiological response to the consuming fish. A feed formula containing protein mostly from *Rhizopus oryzae* biomass was fed to Arctic char (*Salvelinus alpinus*) for 4 weeks. $^1$H NMR spectroscopy technique was then used to analyze the liver tissue patterns of the fish. The results showed that the fish showed similar physiological response to that fed with a diet whose protein source came from fish meal only [32].

3. Alternative binding and coating agents for aquafeeds

In aquaculture, good stability or integrity in water is one of important physical properties that must present in quality aquafeed pellets [33]. This is to provide sufficient time for the consuming fishes or shrimps to eat the pellets [34]. Besides, pellets with poor water stability easily break down in water into smaller particles that sink into the bottom of water, and less likely to be consumed by the cultured fishes. Moreover, unconsumed aquafeed causes nutrient release to the aquatic environment, which could lead to reduced water quality [35], such as dissolved oxygen depletion [36] and eutrophication [37].

Commercial mass production of aquafeed has little problem with water stability as extruder machines can produce feed pellets with high stability in water [34]. However, for small and middle-scale aquafarmers, who have no access to the costly extruder machine, water stability of their self-produced feed pellets remains a challenge. With the intention of reducing the use of expensive commercial pellets,
these farmers manufacture their own feed using cheap, locally available, feed ingredients, and aided with simple pelleting machine. As a result, their self-made fish pellets crumble immediately in water. Thus, there is a need to develop a simple binding technique which is universally applicable for any feed ingredients, especially cheap, varying quality, and locally available feed raw materials.

In soybean tempeh solid fermentation, the role of the *Rhizopus* hyphae in joining the soybean cotyledons into a single, undivided compact and solid mass is already known [38]. Thus, this filamentous fungal behavior must be able to be used as bio-binder or bio-adhesive to increase water stability in non-extrusion preparation of fish feed pellets [39].

The use of tempeh mould hyphae for bio-binding agent in aquafeed preparation to increase stability and floatability in water has been reported in previous studies (Table 2 and Figure 2). These studies used various agro-industrial by-products [40–43] as well as commercial sinking aquafeeds [39,44–46] whose water stability was enhanced by the tempeh mould fermentation. The types of feeds or raw materials, as well as their composition in the mixture, did not seem to be the factors that determined whether the fermented products would have enhanced water stability or not. Rather, it was the filamentous mould that played the key role. As long as the tempeh mould grew well on the organic materials, which was indicated by the formation of dense cottony mycelial mass, the resulted fermented products would maintain their integrity in water very well and stayed afloat on water. This water stability might also be contributed by the water-repellent property of the outer surface of the fungal cell wall of filamentous fungi which contains the hydrophobic molecules such as chitins [47] and hydrophobins [48]. Thus, a thin sheath of surface mycelium might have provided a water-proof coating, resisting water penetration into the inside parts of the fermented pellets. In this way, further disintegrative effect of water molecules could be prevented. This mechanism has already known in other fungi, and has been applied to produce, amongst other, water-proof coating for papers [47] and marble-stones [49].

Table 2. Studies on the use of tempeh mould in the fermentation of sinking fish feed and/or organic by-products intended for fish feed through solid state fermentation.

| Feed Materials as Fermentation Substrate | Fermentation Treatment and Subsequent Drying | Water Stability and Floatability | Reference |
|----------------------------------------|---------------------------------------------|---------------------------------|-----------|
| Black soldier fly maggot, agricultural waste, tapioca flour, additive | *Rhizopus* sp. inoculum, 24-h incubation at 30 °C, 24-h oven drying at 40 °C | 80-95% water stability at 60th minute, highest floatability of 100% at 15th minute | [43] |
| Coconut testa, cassava bagasse | R. oryzae inoculum, 96-120-h incubation at 28-30 °C, 24-h oven drying at 50 °C | Increased stability in water, but not determined quantitatively | [41] |
| Rice bran, coconut bagasse | R. oryzae inoculum, 96-120-h incubation at 28-30 °C, 24-h oven drying at 50 °C | Increased stability in water, but not determined quantitatively | [40] |
Commercial sinking aquafeed

*Rhizopus* sp. inoculum, 24-h incubation at 29 ± 1 °C, 24-h oven drying at 50 °C

80.3-87.1% water stability and ε 95% floatability, both at 60th minute

Sinking fish feed, cassava bagasse, duck weed (*Lemna minor*)

*R. oryzae* inoculum, 40-h incubation at ambient temperature, 24-h oven drying at 50 °C

81% water stability at 30th minute, 100% floatability for 3 h

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The ability of tempeh moulds *Rhizopus* to act as mycological adhesive agent, that can knit all the organic particles they colonize through their penetrative hyphal network, is an interesting property shared by other filamentous fungi. This property has been studied for its application in the production of biomaterial called mycelium composites which have been commercialized in EU and US to be used in construction works. These low cost and environmentally friendly materials, which are derived from ample agricultural by-products and wastes, can substitute plastics, foams, and woods [50].

4. **Alternative chitin and chitosan source for biomedical use**

Chitin is a biopolymer found in the largest amount in this planet after cellulose [51]. Chitin, present as a major component of fungal cell walls [52], crustacean shell, insect, arthropod, and mollusk exoskeletons, can be processed further chemically by deacetylation reaction to produce a cationic

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**Figure 2.** Cassava bagasse (A), duck weed (B), sinking fish feed (C), and *R. oryzae* inoculum were mixed, moisturised with a mineral solution, and placed in Petri dishes (D). After 40-h incubation at 28-30 °C, the fermented feeds (E) were diced into small cubes, and oven-dried at 50 °C for 24 h (F). The resulted pellets were highly stable and afloat in water [42].
biopolymer called chitosan [53], which is 70-90% deacetylated [54]. Chitin is a poorly-soluble linear bio-polymer, consisting of the monomer β-1,4-N-acetyl-D-glucosamine. Chitin can be transformed to a soluble bio-heteropolymer chitosan that is made up of the monomer β-1,4-N-acetyl-D-glucosamine and D-glucosamine through deacetylation. Deacetylation of chitin can also occur in nature by the action of chitin deacetylases [55]. Chitosan antibacterial activity, non-toxicity [52], biodegradability and biocompatibility lends its prospect for pharmaceutical, cosmetic, and biomedical applications [53].

Current production of chitosan from crustacean shell waste result in massive amount of dangerous effluents. Therefore, non-animal, fungi-derived chitosan offers promising substitutes [56]. Amongst fungal species recently investigated for its chitin and chitosan bioproducts are from the genus Rhizopus. Rhizopus biomass, from which chitin and chitosan were extracted, could be cultivated on agro-industrial by-products such as corn steep liquor [57,58], potato chip processing waste [59], whey by-product, molasses [60], wheat hydrolysate [61], lignocellulosic pre-hydrolysate [62], as well as using specifically defined media [63–70].

Chitin and chitosan extracted from fungi have been demonstrated to have biological activity against microorganisms [71]. Amongst those fungi, whose chitin and chitosan were demonstrated to have such activities, were from the genus Rhizopus (Table 3). A study was conducted by culturing R. oryzae NCIM 877 in a broth medium followed by the extraction of chitin and chitosan from the dried fungal biomass using two-stage extraction system, namely dealkylation (1 N sodium hydroxide), and then deacetylation (1% hot sulphuric acid) [67]. The chitosan obtained was shown to have stronger antibacterial action against the tested gram-positive bacteria (Staphylococcus aureus and Bacillus subtilis) relative to the gram-negative counterparts (Escherichia coli and Salmonella typhimurium). In another study, R. arrhizus biomass was produced through cultivation on agro-industrial by-products (corn steep liquor and molasses). The harvested fungal biomass was subjected to drying and alkali-acid treatment to obtain the chitin and chitosan. The chitin and chitosan obtained had minimum inhibitory and bactericidal concentrations against all the assayed bacteria, namely gram-positive (Listeria monocytogenes and S. aureus) and gram-negative bacteria (E. coli, Salmonella enterica, Pseudomonas aeruginosa, and Yersinia enterocolítica) [58]. Antifungal activity of chitosan extracted from R. oryzae was also reported against the pathogenic yeast Candida albicans [68].

Table 3. Studies on the chitin, chitosan, and mycelial biomass derived from the fungal genus Rhizopus, the methods used, and the potential application in biomedical field.

| Rhizopus strain | Cultivation and Extraction Method | Results of Potential Application | Reference |
|----------------|----------------------------------|---------------------------------|-----------|
| R. oryzae NCIM 877 | culture: 100 mL Sabouraud’s dextrose broth and incubation at 25°C at 150 rpm; 2-phase extraction: NaOH dealkylation and H₂SO₄ deacetylation | antimicrobial activities against tested gram-positive bacteria stronger than gram-negative bacteria | [67] |
| R. arrhizus UCP/WFCC 0402 | culture: 200 mL medium with molasses and corn steep liquor, incubated at 28 °C for 96 h; extraction: 1 M NaOH deproteinization, 2% acetic acid treatment, alkaline precipitation | minimum inhibitory concentrations and minimum bactericidal concentrations against gram-positive and gram-negative bacteria tested | [58] |
An interesting study has been conducted in which wound healing property of chitin and chitosan was applied directly using the mycelial biomass of *Rhizopus stolonifer* without extracting the chitin and chitosan [70]. In the first part of that study, UV light-assisted mutation was carried out on the fungus to obtain a mutated strain that grew and maximized mycelial biomass formation while undergoing late sporangiospore development. In addition, cultivation condition was optimized which enabled maximum production of mycelial sheet and the least formation of sporangia. This *R. stolonifer* membrane, named Rhizochitin, had a sponge-like property and was evaluated its wound-healing capacity on an animal model (Figure 3). The results was promising as the wound zone in the rat model treated with Rhizochitin sheet-coating was 40% smaller than that of the control (wound not covered by Rhizochitin) [70].
Figure 3. Delayed spore-forming mutant of *R. stolonifer* was cultivated statically in a tray or erlenmeyer flask produced a thin mycelial membrane called Rhizochitin (A). The dried membranes of the mutant (B) and wild type (C) were presented here with deproteinization (left column) and without deproteinization (right column). Rhizochitin, together with two other similar products Sacchachitin, Beschitin-W, and the cotton gauze control, were tested on animal model (D and E). Rhizochitin healed the wound faster than the control, comparable to that of Sacchachitin, and better than that of Beschitin-W [70].

5. Conclusion
*Rhizopus*, which has been known for long as the fermentative microorganism in the preparation of soybean tempeh, has found novel applications in the fields other than human nutrition. Recent development regarding the fungal potential as alternative replacement of fish meal in fish feed production, bio-binding agent to enhance aquafeed stability in water, and wound healing enhancement are just three of them. Nevertheless, the few examples reviewed in this paper would hopefully drive other yet-unthinkable product innovations based on fungi from the genus *Rhizopus*.

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