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Optimization of Agitation Speed and Aeration Rate for Fungal Protein Production from Tofu Whey Using *Aspergillus oryzae* in a Stirred Tank Bioreactor

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ABSTRACT

Fungal protein, derived from microbial biomass, offers a sustainable protein source and can be produced through fermentation. However, the utilization of tofu whey, an abundant agro-industrial by-product in Indonesia, as a substrate for fungal protein remains underexplored. This study optimizing both agitation speed and aeration rate for *Aspergillus oryzae* fermentation in a stirred-tank bioreactor. Fermentation was conducted in a 5 L stirred-tank bioreactor with a 3 L working volume for 48 hours at an initial pH of 5 and a temperature of 35°C. Agitation speeds of 150, 200, 250, and 300 rpm were tested at a constant aeration rate of 1.0 vvm to determine the optimum mixing condition. The agitation speed that yielded the highest dry cell weight was then used as the basis for further aeration experiments (0, 0.5, 1.0, and 1.5 vvm). The optimum conditions were obtained at 150 rpm and 1.0 vvm, resulting in a dry cell weight of 7.1 g/L and a protein content of 6.83% (w/w). These findings demonstrate the potential of valorizing tofu whey into fungal protein while highlighting the need for further multi-parameter optimization to enhance protein levels toward single-cell protein standards.

Keywords: *Aspergillus oryzae*, biomass, fungal protein, stirred tank bioreactor, tofu whey.

1. INTRODUCTION

Fungal protein is a protein contained in fungal biomass and is produced through a fermentation process. It has attracted increasing attention as an alternative protein source due to its sustainability, nutritional quality, and potential to valorize agro-industrial wastes. The advantages of this protein are that it does not require a large area for production, is high in protein, rich in fiber, and low in fat [1]. The ideal substrate for fungal protein production must contain sufficient carbon, nitrogen, and micronutrients so that mycelium growth can proceed rapidly [2]. Previous studies have reported the use of potato starch wastewater and cheese industry wastewater for fungal protein production, as these substrates still contain valuable nutrients for microbial growth [3,4].

Tofu whey is another promising substrate that can be used for fungal protein production. In Indonesia, tofu whey is abundantly available yet remains underutilized for value-added applications. Indonesia has around 84,000 tofu industry units with a production capacity exceeding 2.6 million tons per year [5]. The wastewater generated is estimated at 800–2,400 L/day for every 100–300 kg of soybeans processed [6]. This volume is significant when multiplied by the national production capacity. Tofu whey contains 99.12% water; 0.39% protein; 0.14% carbohydrates; and 0.11% fat [7]. The presence of carbon and nitrogen elements makes tofu whey a potential substrate for microbial fermentation.

Aspergillus oryzae is a filamentous fungus widely used in biotechnology processes and recognized as safe by WHO and FDA. It has strong enzymatic capabilities, producing hydrolytic enzymes such as amylase and protease [8]. As a strictly aerobic

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microorganism, its growth is strongly influenced by oxygen availability, making bioreactor design and operating conditions crucial.

The stirred-tank bioreactor is one of the most commonly used systems for aerobic fermentation, as it ensures effective mixing and oxygen transfer. Two of the most critical operating parameters are agitation speed and aeration rate. Agitation speed influences nutrient mixing, shear stress, and fungal morphology [9], while aeration rate directly affects oxygen transfer efficiency and thus the metabolic activity of the culture [10]. Previous research has shown that both parameters must be optimized to achieve high productivity; excessive agitation may damage cells, while insufficient aeration can limit biomass accumulation [2].

However, few studies have investigated the combined influence of agitation speed and aeration rate on fungal protein production using tofu whey as a substrate, particularly in the context of Indonesian tofu industry waste. Moreover, the use of tofu whey for fungal protein production has not yet been reported at larger bioreactor scales [11,12]. Therefore, this study aims to determine the optimal agitation speed and aeration rate to maximize biomass and protein yield of *A. oryzae* in a 5 L stirred-tank bioreactor using tofu whey as a cost-effective substrate.

2. RESEARCH METHODS

This research was conducted at Bioprocess Laboratory and Industrial Waste Management Laboratory, Department of Chemical Engineering, Politeknik Negeri Bandung.

2.1. Preparation of Fermentation Media

Raw tofu whey (AMH Putri Cihanjuang, Indonesia) was filtered to remove particulates and pasteurized at 60°C for 30 minutes to reduce microbial load. A total of 2.7 L of pre-treated tofu whey was adjusted to pH 5 using NaOH and sterilized by autoclaving at 121°C for 15 minutes. Soybean oil was used as an antifoam agent and sterilized separately. Distilled water used in this study was Amidis (Indonesia), with a conductivity of 2,840 µS/cm, total dissolved solids (TDS) value of 1,830 ppm and pH 6.5-7.5. The same distilled water source was used for all subsequent media preparations.

2.2. Preparation of Stock Culture

Potato Dextrose Agar (PDA, Merck, Germany) was prepared using distilled water and autoclaved at 121°C for 15 minutes. *A. oryzae* spores were aseptically inoculated onto PDA slants and incubated at 35°C for 7 days to establish stock cultures.

2.3. Preparation of Inoculum

Potato Dextrose Broth (PDB, Himedia, India) was prepared by dissolving 7.2 g in 300 mL distilled water, then sterilized at 121°C for 15 minutes. Spores from PDA slants were transferred aseptically into the sterile PDB medium. The inoculum was incubated at 35°C and 150 rpm for 24 hours in a rotary shaker incubator.

2.4. Fermentation Process

Fermentation was carried out in a 5 L stirred-tank bioreactor (BioFlo®/Celligen® 115, Eppendorf, USA), equipped with a Double Rushton Turbine (DRT) impeller. The vessel was filled with 2.7 L of sterilized tofu whey, 0.3 L of *A. oryzae* inoculum, and 7 mL of sterile soybean oil, resulting in a 3 L working volume.

Fermentation was conducted for 48 hours at a controlled temperature of 35°C and an initial pH of 5. The optimization was performed in two stages:

Stage 1 – Agitation speed variation: Agitation speeds of 150, 200, 250, and 300 rpm were tested at a constant aeration rate of 1.0 vvm. The optimum agitation speed was determined based on the highest dry cell weight (DCW).

Stage 2 – Aeration rate variation: Using the optimum agitation speed obtained from Stage 1, aeration rates of 0, 0.5, 1.0, and 1.5 vvm were tested. The optimum aeration rate was determined based on DCW.

Only the final optimum condition (best combination of agitation speed and aeration rate) was further analyzed for protein content and biomass yield.

2.5. Analytical Methods

Samples were collected three times daily during fermentation. Biomass was separated by vacuum filtration and dried to constant weight to determine dry cell weight (DCW). Protein content was analyzed using the Kjeldahl method (SNI 01-2891-1992). Reducing sugar content was determined using the Luff-Schoorl method (SNI 01-2892-1992). Biomass yield was calculated as the ratio of biomass formed to substrate consumed (equation 1). All analyses were performed in duplicate, and the results are presented as mean values with standard deviation.

$$Y_{(X/S)}=\frac{\text{Biomass concentration}}{\text{Substrate consumed}}\times 100\% \qquad (1)$$

The overall experimental procedure is summarized in the flowchart (Figure 1).

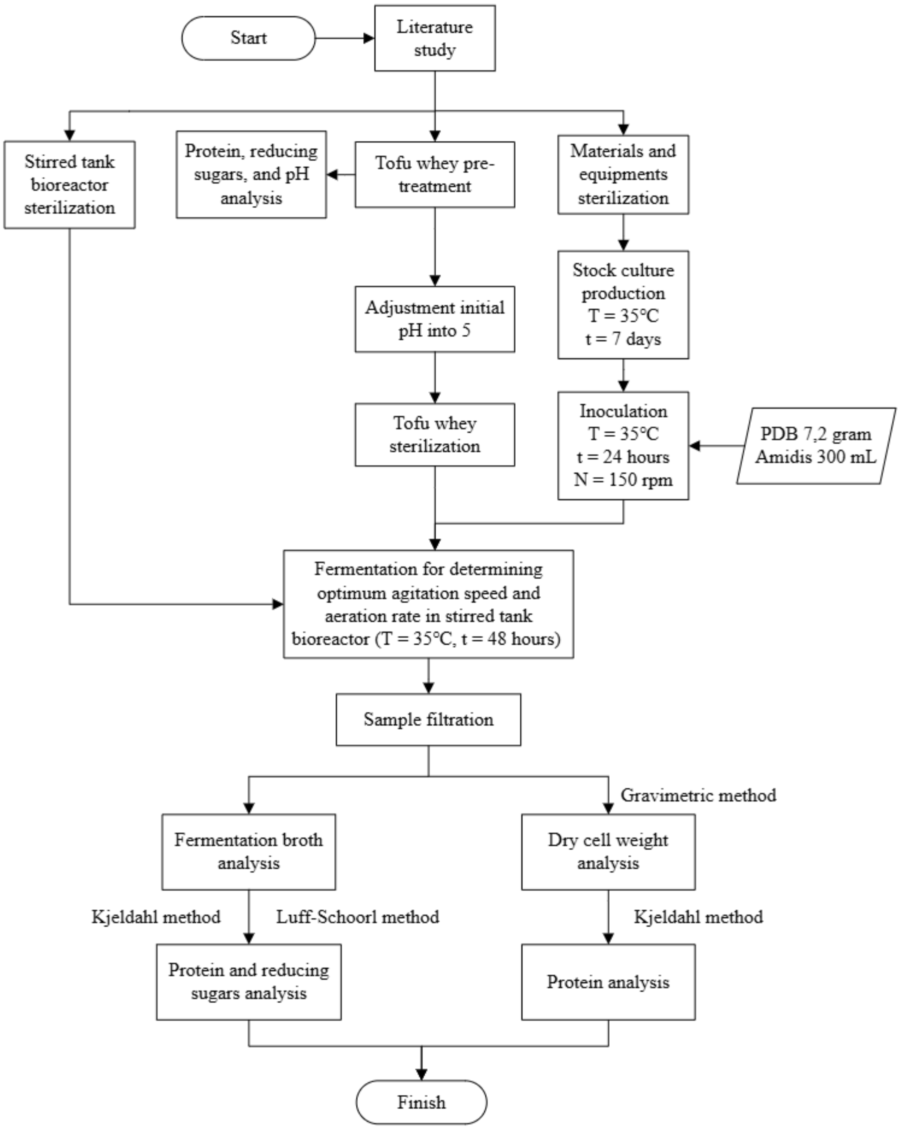

Figure 1. Flowchart of the research methodology.

3. RESULTS AND DISCUSSION

3.1. Characteristics of Tofu Whey

The pH value of the tofu whey used ranged from 3.7 to 4.0, making it too acidic and not optimal for fermentation of *A. oryzae*. This acidic pH value occurs due to the addition of acidic liquids such as vinegar during the soy protein coagulation process [9]. Therefore, it is necessary to adjust the initial pH to 5 using NaOH to support the fermentation conditions of *A. oryzae*. According to research by Natasa and Rahayu [10] and Pratama et al. [11], the initial pH of tofu waste that produces the highest biomass yield is pH 5. Characteristics of tofu whey are shown in Table 1.

Table 1. Characteristics of tofu whey.

Parameter	Content (%, w/v)	Concentration (g/L)
Reducing sugar	0.55	5.5
Protein	2.62	26.2

3.2. Effect of Agitation Speed on Biomass Production

Agitation is essential for promoting fungal growth as it ensures uniform mixing and nutrient distribution throughout the medium. Appropriate agitation speed also prevents excessive pellet formation by gently fragmenting hyphae, thus reducing medium viscosity and supporting consistent biomass productivity [12]. The results of dry cell weight obtained from variations in agitation speed are shown in Figure 2.

Based on Figure 2, an inverse relationship was observed between agitation speed and fungal biomass production. The highest dry cell weight of 7.1 g/L was achieved at an optimal agitation speed of 150 rpm. Conversely, agitation speed of 200 rpm and above demonstrably had a detrimental effect on the growth of *A. oryzae*, resulting in a significant decrease in dry cell weight. This indicates that excessive shear forces generated at higher agitation speeds negatively impacted fungal viability and growth within the fermentation medium.

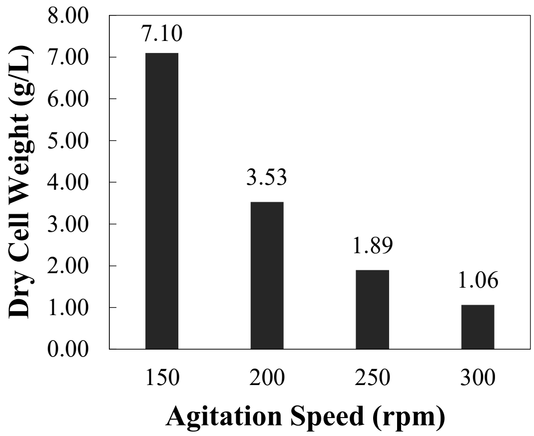

Figure 2. Effect of agitation speed on dry cell weight of *A. oryzae* after 48 h of fermentation.

According to Zhou et al. [13], agitation speeds that are too high can cause damage to microbial cells due to large shear forces and can affect the morphology and growth rate of microbes. Damage to fungal cells will reduce performance and biomass yield in the bioreactor. In addition, local shear stress and high shear rates around the DRT (Double Rushton Turbine) impeller blades cause a decrease in dissolved oxygen concentration, pellet diameter size, and enzyme activity [14].

Agitation not only promotes fungal growth by ensuring uniform nutrient distribution and controlling pellet formation, but its speed also critically affects energy consumption in stirred-tank bioreactors [14]. Optimizing agitation balances maximizing A. oryzae biomass yield while minimizing power requirements, which is particularly important for reducing operational costs during scale-up.

Previous research has been conducted by Miranti et al. [15] to observe the effect of agitation speed on the dry cell weight of *A. oryzae* using synthetic substrates. The highest dry cell weight was obtained at a agitation speed of 120 rpm, which was 5.59 g/L, then decreased when the agitation speed was increased to 170 rpm. Another research by Ghobadi et al. [14], reported that increasing the agitation speed from 300 rpm to 500 rpm also had a negative effect on the growth of *A. oryzae* in synthetic substrates.

The reactor used was a stirred tank bioreactor with a working volume of 1.5 L and the same type of impeller (DRT). An agitation speed of 300 rpm produced a dry cell weight of 5.01 g/L then decreased to 4.90 g/L when the agitation speed was increased to 500 rpm. The dry cell weight obtained from both research was relatively smaller when compared to the results obtained under the optimum conditions of this research.

3.3. The Effect of Fermentation Time and Agitation Speed on pH and Biomass Production

In this research, there was an increase in pH value as the fermentation time progressed for all variations of agitation speed. The results of the pH value observations are presented in Figure 3.

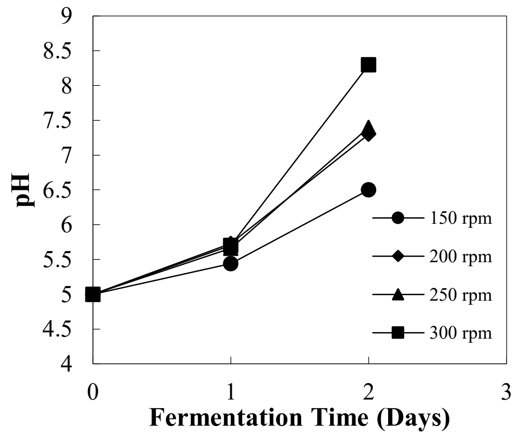

Figure 3. Observation of pH value at variations of agitation speed.

Based on Figure 3, it can be seen that the pH increase on the first day was not too significant with a pH value range of 5-6. Then a significant increase in pH occurred from the first day to the second day of fermentation with the highest final pH reaching 8.3. This increase in pH can be caused by deamination reactions that occur in protein metabolism. The deamination reaction produces ammonia which is alkaline as a source of nitrogen in the synthesis of proteins and nucleic acids [16]. An increase in media pH accompanied by an increase in ammonia concentration has been found in the production of *R. Oryzae* fungus using Potato Protein Liquor substrate [3]. The pH value increased significantly on the second day of fermentation, after which there was a slowdown in the growth of *A. oryzae* fungus. This indicates that fermentation media with alkaline pH can be one of the factors that limit biomass production [11].

The final pH increase under optimum conditions (150 rpm; 1 vvm) was not significant, reaching pH 6.5, which remained supportive for *A. oryzae* growth. This is consistent with the known optimal pH range of 5-6 for this fungus [17]. The stable pH suggests that metabolic processes at optimal conditions were balanced, preventing excessive ammonia accumulation, which could otherwise lead to a more substantial pH increase. The acidity or pH level of the fermentation medium is critical as it directly impacts the structure and function of enzymes, which act as biocatalysts in the fermentation process [18]. Variations in media pH can alter enzyme activity and modify the ionic form of their active sites, consequently affecting the rate of enzymatic reactions [16].

3.4. Growth Curve Analysis of *A. oryzae*

Based on the optimum agitation speed condition, a growth curve analysis of *A. oryzae* was performed, measured by dry cell weight. The results are presented in Figure 4.

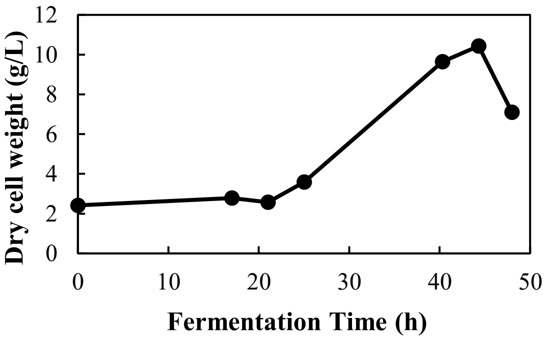

Figure 4. Growth Curve of *A. oryzae* in fermentation using tofu whey as substrate.

The growth curve of *A. oryzae*, as depicted in Figure 4, illustrates the characteristic phases of fungal proliferation based on dry cell weight over a 48-hour period at the previously determined optimum agitation speed. The initial lag phase (approximately 0-18 hours) shows minimal increase in biomass (around 2.5-2.8 g/L), representing the period where the fungal cells adapt to the new substrate and synthesize necessary enzymes for growth. This is followed by a robust exponential (log) phase (roughly 18-44 hours), where the dry cell weight rapidly and exponentially increases, indicating active growth and biomass accumulation, peaking at about 10.4 g/L. Subsequently, the curve enters a stationary phase around 44 hours, where the net growth becomes negligible as the rate of cell division approximates the rate of cell death, likely due to nutrient depletion or accumulation of metabolic waste products. Finally, a sharp decline into the death phase is observed after 44 hours, with the dry cell weight decreasing significantly to approximately 7.1 g/L by 48 hours, signifying a reduction in viable fungal biomass

3.5. The Analysis of Reducing Sugar Content and Yield Calculation

Analysis of reducing sugar levels was carried out to determine the intensity of the use of reducing sugars such as fructose and glucose in the fermentation process of *A. oryzae*. The concentration of reducing sugars in tofu whey and filtrate from the product filtration is shown in Table 2.

Table 2. Concentration of reducing sugars in tofu whey and fermentation broth sample (optimum condition).

Concentration of Reducing Sugars	
Tofu Whey (g/L)	**Fermentation Broth Sample (g/L)**
5.5	4.0

Based on Table 2, the tofu whey used in this research contains 0.55% (w/v) reducing sugar. According to Pratama et al. [11], soy whey or tofu whey contains about 1% carbohydrates (Stachyose, raffinose, sucrose, glucose, and fructose). Likewise, Devanthi et al. [19], stated that tofu whey contains about 1% carbohydrates.

Based on Table 2, there has been a decrease in reducing sugar in the medium from the initial 5.5 g/L to 4.0 g/L. This shows that reducing sugar has been consumed by A. oryzae as much as 1.5 g/L or only 27.3% of the total reducing sugar available. Concentration of biomass obtained under the optimum of this research was 7.1 g/L from 2.46 g/L at initial condition.

The biomass yield based on reducing sugar consumption obtained under the optimum condition appeared remarkably high (reported as 309%). This is not necessarily an indication of extraordinary metabolic efficiency but is more likely due to the limitations of using reducing sugar as the sole measurement basis. In addition to glucose and fructose, tofu whey contains sucrose, complex carbohydrates, and other organic components that are not detected by the reducing sugar method. *Aspergillus oryzae* is capable of hydrolyzing and utilizing these fractions, which increases biomass production without being reflected as a decrease in reducing sugar concentration. Furthermore, the accumulation of protein (organic nitrogen) and mineral components from the medium also contributes to the overall biomass mass. Therefore, a more representative calculation of yield would require measuring total carbohydrates (after hydrolysis) or performing a carbon balance analysis (TOC/HPLC) to confirm the actual ($Y_{x/s}$).

In the research of Pratama et al. [11] with the adjustment of the initial pH of tofu whey to 5, there was a decrease in sucrose levels on the first day but fructose levels increased significantly while glucose increased slightly from the initial conditions. The increase in fructose and glucose levels can come from the breakdown of more complex sugars including sucrose because of optimal enzyme production at a pH of around 5. Furthermore, on the second day, sugar consumption reached around 74% and tended to be constant towards the third day.

In this research, the remaining reducing sugar in the fermentation liquid was 4 g/L. When looking at the trend of sugars from previous studies, the remaining reducing sugars can also come from the breakdown of more complex sugars. This means that there are still many substrates that can be utilized and indicates that the growth of the fungus is not limited by substrate depletion, but there are other influential factors such as an increase in pH which will affect metabolism and enzyme performance. *A. oryzae* has a strong capacity to secrete hydrolytic enzymes such as amylase and protease [8]. In addition, *Aspergillus* sp. are prominent microbes in the production of invertase [20]. *A. oryzae* is capable of producing invertase enzymes but with lower activity than *A. foetidus* and *A. niger* [21]. These enzymes will show high activity when the pH of the media matches the working pH of the enzyme.

3.6. The Analysis of Protein Content and Comparison with Other Literature

Protein is a major component of fungal biomass, typically 50–55% depending on the microorganism and substrate [19]. Under optimum conditions (150 rpm, 1 vvm), A. oryzae biomass contained 6.83% protein (w/w), a 2.7-fold increase over tofu whey (2.62%, w/v) (Table 3). Protein in the medium decreased to 1.58% (w/v), indicating that approximately 40% of substrate protein was utilized for nitrogen metabolism. Despite this enrichment, the protein content remains relatively low compared to previous studies with alternative substrates. To contextualize these results, a comparison with selected literature is presented in Table 4.

Table 3. Proteins content in tofu whey, fermentation liquid sample (optimum condition), and dry cell of *A. oryzae* biomass.

Protein Percentage		
Tofu Whey (%, w/v)	**Fermentation Liquid Sample (%, w/v)**	**Biomass (%, w/w)**
2.62	1.58	6.83

Table 4. Comparison of fungal protein production from various industrial residues.

Substrate	Microorganism	Bioreactor Type/working volume	Operating Conditions	Main Results	Reference
Tofu whey (this study)	*Aspergillus oryzae*	Stirred-tank reactor (3 L)	Agitation: 150 rpm; Aeration: 1 vvm; Temp: 35 °C; pH: 5; Fermentation time: 48 h	Biomass yield: 309% (based on reducing sugar consumption); Protein content: 6.83%	**This study (2025)**
Cheese industry waste	*Aspergillus oryzae*, *Neurospora intermedia*	Bubble column reactor 3 L	Aeration: 1 vvm; Temp: 35 °C; pH: 5; Fermentation time: 48 h	Biomass yield: 7.05 g/L (*A. oryzae*), 6.39 g/L (*N. intermedia*); Protein content: 20.86–21.76%	[4]
Hydrolyzed aspen wood chips	*Pleurotus ostreatus*	Stirred-tank bioreactor (3L)	Glucose: 38 g/L; Xylose: 15 g/L; Inoculum: 5% (v/v); Agitation: 200–800 rpm; Temp: 28 °C; pH: 5; Fermentation time: 4 days	Biomass yield: 25 ± 3.4 g/L; Protein content: 54.5 ± 0.5%	[2]
Ethanol distillation stillage	*Neurospora intermedia*, *Aspergillus oryzae*	CSTR (1 L)	Aeration rate: 0.42 vvm; Agitation: 250 rpm; Temp: 35 °C; pH: 5; Fermentation time: 72 h	Biomass yield: 5.8 ± 0.8 g/L; Protein content: 42%	[22]

As shown in Table 4, the protein content obtained in this study is lower than that reported in previous research, despite the high apparent biomass yield (309%). This discrepancy is likely due to the compositional characteristics of tofu whey, which contains complex carbohydrates, sucrose, and other organic components not captured by reducing sugar measurements. *A. oryzae* can utilize these fractions, leading to substantial biomass accumulation without a proportional increase in measured protein content. In contrast, substrates such as lignocellulosic hydrolysates and nitrogen-rich industrial wastes provide a more balanced carbon–nitrogen ratio, resulting in higher protein percentages in fungal biomass [2]. Additionally, the use of antifoam and the presence of oils, such as soybean oil, may influence protein content, as these lipids can serve as secondary substrates. *A. oryzae* can degrade fatty acids like oleic, linoleic, and linolenic acids into glycerol and metabolites, which are either utilized for growth or stored in the cells [23,24]. Similarly, low protein content was reported by Al-Farsi et al. [25] when using industrial date waste as a substrate, achieving only 5.53% protein.

3.7. Characteristics of Fungal Protein Products

The product obtained is fungal protein biomass of *A. oryzae* in powder form. The color of the product is dark brown and slightly greenish due to color of *A. oryzae*. The visual appearance of the fungal protein product is shown in Figure 5.

Figure 5. Fungal protein of *A. oryzae* growth in tofu whey as substrate.

Fungal protein can be applied as protein supplement in animal feed. One of the brands on the market, PEKILO® produced by eniferBIO using *Paecilomyces variotii*. In addition, there is a fungal protein produced by Hebei Youngdo Import and Export Co., Ltd (China). Fungal protein from eniferBIO tend to be dark brown in color while the product from Hebei are greenish brown. This could be due to differences in terms of production and the type of fungus used. Visually, fungal protein from this research is more similar to Hebei product.

Fungal protein on the market have quite high protein levels. Both products mentioned have 65% (eniferBIO) and 66.25% (Hebei Youngdo) protein content while the product from this research has relatively low protein content of 6.83%. Therefore, fungal protein from this research have not met SNI 01-3136-1993 standard on Single Cell Protein (SCP) for feed ingredients that require minimum 40% of protein content. This means that the product needs to be improved and cannot be marketed commercially. Feed supplements require high protein levels to increase the weight of livestock [26].

4. CONCLUSION

This study successfully determined the optimum agitation speed for fungal protein production from *Aspergillus oryzae* in a stirred tank bioreactor to be 150 rpm at an aeration rate of 1 vvm. At this optimal condition, a dry cell weight of 7.10 g/L was achieved. Higher agitation speeds (200 rpm and above) were found to be detrimental, leading to a decrease in biomass yield. The resulting biomass produced under optimal conditions exhibited a protein content of 6.83% (w/w) demonstrating the potential of tofu whey as a substrate for fungal protein production.

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