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Optimization of *Rhizopus* spp. Production as Mycoprotein using Soymilk Media

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Mycoprotein is food with high protein content, fiber, and low in cholesterol made from fungal mycelium. In this research, mycoprotein was produced by *Rhizopus* spp. isolated from tempeh with soymilk as growth media. This research aims to determine the best strain of *Rhizopus* spp. and optimum carbon to nitrogen ratio for mycoprotein production. Two parameters were applied which were inoculum selection and carbon to nitrogen ratio treatment in media. The best inoculum was selected from four strains of *Rhizopus* spp., ATH 1, ATH 24, ATH 40, and ATH 53. On the other hand, carbon to nitrogen ratio treatment used were as follows 20:1, 20:2, and 40:2. Mycelium dry weight and protein content were measured, as well as reduction sugar, dissolved protein and total volatile base nitrogen concentration in media. The best strain for producing biomass was ATH 24 with 0.60 g of mycelium dry weight per 50 mL of media and the protein content was 0.236 g. The best carbon to nitrogen ratio treatment was 20:1 with 0.57 gram of mycelium dry weight per 50 mL of media and the protein content was 0.20 g. Thus, our data indicate that strain ATH 24 with 20:1 of carbon to nitrogen ratio in media were highly potential for producing mycoprotein.

Key words: fermentation, mycelium, mycoprotein, *Rhizopus*, soymilk

Mikoprotein merupakan pangan yang tinggi protein, serat, dan rendah kolesterol yang terbuat dari miselium fungi. Produksi mikoprotein pada penelitian ini menggunakan *Rhizopus* spp. yang diisolasi dari tempe dengan media pertumbuhan berupa susu kedelai. Tujuan penelitian ini adalah menentukan galur *Rhizopus* spp. terbaik dan perbandingan karbon dan nitrogen optimum dalam memproduksi mikoprotein. Penelitian ini terdiri dari dua tahap, yakni seleksi inokulum dan perlakuan perbandingan karbon terhadap nitrogen pada media. Inokulum diseleksi dari empat galur *Rhizopus* spp. yang ATH 1, ATH 24, ATH 40, dan ATH 53, sedangkan variasi perlakuan perbandingan karbon terhadap nitrogen terdiri dari 20:1, 20:2, dan 40:2. Bobot kering dan protein biomassa ditentukan, serta kadar gula reduksi, protein terlarut, dan total nitrogen basa yang mudah menguap dalam media. Galur terbaik dalam memproduksi mikoprotein adalah ATH 24 dengan biomassa sebesar 0,60 gram bobot kering per 50 mL media dan kandungan protein sebesar 0,236 g. Perlakuan perbandingan karbon dan nitrogen terbaik adalah 20:1 dengan produksi biomassa sebesar 0,57 gram bobot kering per 50 mL media dan kandungan protein sebesar 0,20 g. Oleh karena itu, pilihan terbaik untuk memproduksi mikoprotein adalah menggunakan ATH 24 dengan perbandingan karbon terhadap nitrogen pada media sebesar 20:1.

Kata kunci: fermentasi, mikoprotein, miselium, *Rhizopus*, susu kedelai

Indonesia has been known for its traditional food called tempeh, which is soybeans fermented by *Rhizopus* spp.. *Rhizopus* spp. isolated from tempeh is considered safe and potentially used for mycoprotein production. Eight Rhizopus spp species were identified: *R. caepitosus*, *R. delemar*, *R. homothallicus*, *R. microsporus*, *R. oryzae*, *R. reflexus*, *R. schipperae,* and *R. stolonifer* (Abe *et al*. 2010). Several studies had proven that *Rhizopus* spp. can be grown on synthetic or natural media to produce biomass. *R. microsporus* var. *oligosporus* could be grown in media consist of soybean meal and glucose, which produced yield ($Y_{x/s}$) of 0.5 g/g substrate (Iftikhar *et al*. 2010). *R. microsporus* var. *azygosporus* could produce chitosan by using media consist of soybean meal, peptone, glucose, and corn starch (Chen *et al*. 2002). Various types of media such as Yeast Nitrogen Base (YNB) and Sabouraud Broth (SAB) can be used to grow *R. microsporus* var. *rhizopodiformis* (Meletiadis *et al*. 2001). *R. delemar* could produce 8 g/L biomass in glucose media (2%) and 8 g/L biomass in soybean peptone media (0.6%) (Zhou *et al*. 2011).

Based on previous research by Chen *et al*. (2002), Iftikhar *et al*. (2010), and Zhou *et al*. (2011), *Rhizopus* spp. likely to grow in glucose and soybean containing media. Therefore, in this research, soymilk will be used as the mycoprotein production media. Soymilk, which is a natural media, has a lower cost rather than rich synthetic media, such as Potato Dextrose Broth (PDB).

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Soymilk also consists of 17.7% carbohydrate and 2.7% protein (Hajirostamloo 2009). Carbohydrate is the source of carbon, used as an energy source to produce cell wall polysaccharides. On the other hand, protein is the primary source of nitrogen used as an energy source to produce mycelium building blocks and shorten the adaptation phase of the cell (Maulida *et al*. 2014). Therefore, the amount of carbon and nitrogen in growth media is very important for fungal growth. It is worth noting that a high amount of carbon and nitrogen may interfere growth since optimum carbon to nitrogen ratio for fungal growth are vary (Yalemtesfa *et al*. 2010). Different strains of *Rhizopus* spp. may also have different capabilities in producing mycoprotein. Hartanti *et al*. (2015) had isolated several strains of *R. microsporus* and *R. delemar* from fresh tempeh in Indonesia, which will be used in this research to observe their potential as a mycoprotein source. Thus, in this research, knowing the nutritional requirements for each *Rhizopus* strain can lead to optimum biomass production, therefore, this study aims to determine the ability of *Rhizopus* spp. at different carbon to nitrogen ratio in producing mycoprotein.

MATERIALS AND METHODS

Materials. *Rhizopus* spp. strains used in this research consist of ATH 1 (IPBCC 13.1102), ATH 24 (IPBCC 13.1110), ATH 40 (IPBCC 13.1120) and ATH 53 (IPBCC 13.1126) obtained from Atma Jaya Catholic University of Indonesia culture collection. ATH 1 is *R. microsporus* like var *oligosporus*, ATH 24 is *R. microsporus* like var *azygosporus*, ATH 40 is *R. microsporus* like var *rhizopodiformis* and ATH 53 is *R. delemar* (Hartanti *et al*. 2015). All of them were maintained at 4°C on Potato Dextrose Agar (PDA) (Oxoid, Hampshire, GB). The fermentation media used in this research was soymilk (Yeo's, Singapore, SG). For the carbon to nitrogen ratio treatment, glucose (MERCK, Darmstadt, DE) and ammonium sulfate ($(NH_4)_2SO_4$) (MERCK, Darmstadt, DE) were used.

Inoculum Preparation. All *Rhizopus* spp. strains were grown on PDA slant at 30°C for 48 hours. Then, 5 mL of natrium chloride (NaCl) solution with a concentration of 0.85% (w/v) was added into the slant to make a culture suspension.

Media Preparation. The fermentation was divided into two stages, which were inoculum selection and carbon to nitrogen ratio treatment. For both stages, the growth media used was 50 mL. In inoculum selection, empty flasks were sterilized, and soymilk was poured in sterile conditions. It was similar to inoculum selection in carbon to nitrogen ratio treatment, but there were variations of carbon to nitrogen ratio of 20:1, 20:2, and 40:2. Addition of glucose and $(NH_4)_2SO_4$ were done using microfilter (Axiva, Faridabad, IN) in sterile condition. The amount of carbon and nitrogen in soymilk was determined based on a calculation using data from literature and nutrition facts in the product (Appendix 1).

Fermentation Process. The amount of 2% culture suspension was inoculated into the media and incubated at 30°C and 125 rpm for 72 hours. Mycelium dry weight, reduction sugar, and dissolved protein were measured triplicate every 24 hours for inoculum selection. Simultaneously, they were done only once for carbon to nitrogen ratio treatment at the end of fermentation with the addition of total volatile base nitrogen (TVBN) measurement. Strain with the highest biomass production and protein content from inoculum selection was chosen to be used in carbon to nitrogen ratio treatment.

Mycelium Dry Weight Measurement. Grown mycelium from the culture was filtered using a stainless steel filter, then washing was done using sterile distilled water. Cleaned mycelium was put into a petri dish with filter paper (Sartorius Stedim, Göttingen, DE) pads and dried using the oven at 50°C for 24 until 48 hours. Dried mycelium was measured periodically until constant weight to obtain the mycelium dry weight.

Reducing Sugar Measurement. Reducing sugar was measured by using DNS method spectrophotometer (Thermo Fisher Scientific, Waltham, US) at 520 nm as reported by Miller (1959). Reducing sugar concentration was obtained by calculating absorbance value into the glucose standard curve equation.

Dissolve Protein Measurement. Dissolve protein was measured using spectrophotometer at 595 nm as reported by Bradford (1976). Dissolve protein concentration was obtained by calculating absorbance value into the bovine serum albumin standard curve equation.

TNVB Measurement. TNVB was measured using the Conway micro diffusion method. 1 mL of boric acid (4%) was pipetted into the inner ring. For the outer ring, 1 mL of potassium carbonate (20%) and 1 mL of fermentation culture was added opposite each other. The outer ring solution was slowly homogenized by using 8 movements, and then the dish was incubated at 37°C for 2 hours. The boric acid from the inner ring was

titrated with 0.02 N HCl and 2 drops of BCG-MR as an indicator. The titration was done when the solution's color change from green to red, and the TNVB concentration was calculated using this formula (Ng and Low 1992):

$$\%TVBN\ (w/v) = \frac{(V\ sample\ titration - V\ blank\ titration) \times N\ HCl \times 100 \times 14.008}{V\ sample \times 1000}$$

Total Nitrogen Measurement. Total nitrogen measurement was done by sending the dried mycelium to Livestock Research Institute in Bogor, West Java, Indonesia. The measurement was done using the Kjeldahl method, where the amount of sample was destructed using sulfuric acid. Then, the sample was diluted in distilled water and distilled. The distillate was accommodated in a flask contained boric acid (4%) and was titrated using chloric acid with BCG-MR as an indicator. The titration was done when the solution's color change from green to red, and the nitrogen concentration was calculated using this formula (AOAC 2005).

$$\%N = \frac{(V\ sample\ titration - V\ blank\ titration) \times N\ HCl \times 14.008}{mg\ sample} \times 100\%$$

$$\text{Total protein (wet base)} = \%N \times conversion\ factor\ (6.25)$$

$$\%Protein\ (dry\ base) = \frac{\%\ Protein\ WB}{1 - water\ content} \times 100\%$$

Statistical Analysis. Statistical analysis was performed in Statistical Package for the Social Sciences (SPSS) using analysis of variance (ANOVA) with Tukey's wholly significant difference (WSD) as post hoc test. All these tests were performed with $p<0.05$ as a significant level.

RESULTS

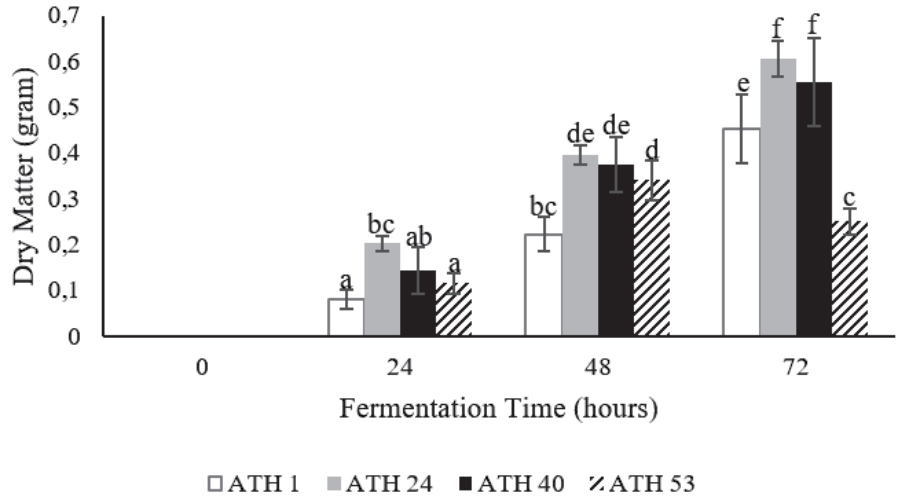
Inoculum Selection. Based on our data, longer fermentation time resulted in higher biomass. Inoculum ATH 1, ATH 24, and ATH 40 highest mycelium dry weight were produced after 72 hours fermentation, while ATH 53 was at 48 hours. ATH 24 had the best capability in producing biomass. ATH 24 produced 0.20 g, 0.40 g and 0.60 g of mycelium dry weight at 24, 48 and 72 hours, respectively, meanwhile ATH 53 produced 0.34 g of mycelium dry weight at 48 hours. ATH 24 biomass increased significantly from 24 hours to 48 hours and then 72 hours. It was also significantly higher than ATH 1 and ATH 53 (Fig 1).

It is indicated in our study that mycelium dry weight production was not correlated to sugar consumption. After 72 hours of fermentation, ATH 24 biomass was the highest while it consumed the least amount of sugar though it was not significant to ATH 1 and ATH 40. The initial concentration of reducing sugar in the media was 2259 µg mL-1. Reduction sugar ATH 24 was kept decreasing throughout the fermentation, and the final concentration was 323 µg mL-1. While ATH 1 and ATH 40 reduction sugar was decreasing at 24 hours, increased at 48 hours, and decreased again at 72 hours. The final concentrations were 232 µg mL-1 and 233 µg mL-1, respectively. ATH 53 had a different trend from the others because the reduction in sugar was decreasing at first 24 hours then, it increased until fermentation ended with final concentration was 1002 µg mL-1. (Fig 2).

Unlike the reducing sugar concentration, dissolved protein concentration decreased since the fermentation started until it ended. The initial dissolved protein concentration in the media was 9725 µg mL-1. At the end of fermentation, the concentration in ATH 53 was the lowest, which was 401 µg mL-1, followed by ATH 1, ATH 40, and ATH 24 with 1138 µg mL-1, 2404 µg mL-1, and 3988 µg mL-1, respectively. ATH 24 consumed the least amount of dissolved protein that was significant to the others and it could produce a high amount of biomass, while ATH 53 consumed the most dissolved protein but produced the lowest biomass (Fig 3).

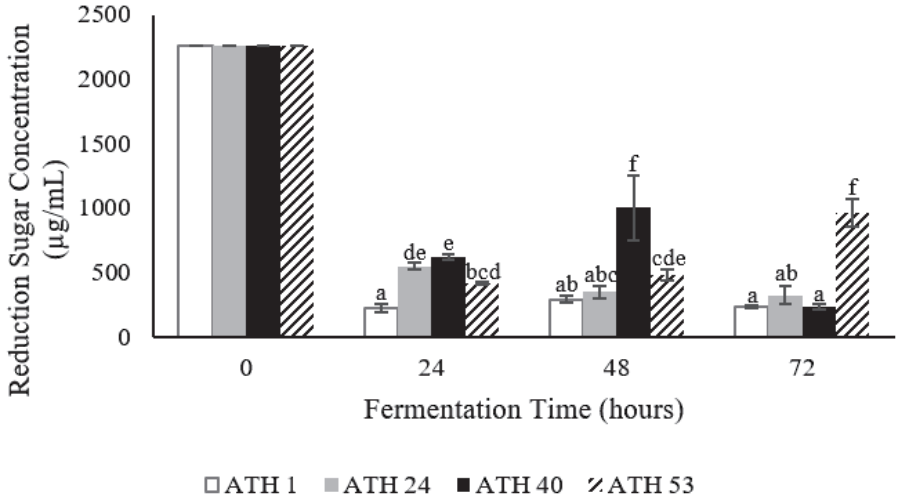
Based on total protein measurement, ATH 53 had the highest protein content, followed by ATH 24, ATH 1, and ATH 40 with 40.65, 38.98, 37.59, and 36.02% (w/w), respectively. However, after multiplication to the mycelium dry weight, ATH 24 had the highest protein content at 24, 48, and 72 hours of fermentation. At 24 hours, ATH 24 had 0.079 g of protein, which was not significant to ATH 40 with 0.052 g of protein, but significant to ATH 53 and ATH 1 with 0.047 and 0.03 g of protein, respectively. At 48 hours, ATH 24 produced 0.154 g of protein, which was not significant to ATH 40 and ATH 53 with 0.135 and 0.138 g of protein, respectively, but significant to ATH 1 with 0.084 g of protein. At 72 hours, ATH 24 produced 0.236 g of protein, which was significant to ATH 40, ATH 1, and ATH 53 with 0.2, 0.17, and 0.102 g of protein, respectively. Therefore, ATH 24 with the highest biomass and protein content was used for the carbon to nitrogen ratio treatment (Fig 4).

Carbon to Nitrogen Ratio Treatment. The initial carbon to nitrogen ratio in soymilk was 20:1. The addition of $(NH_4)_2SO_4$ in 20:2 treatment increased the biomass production, protein content in mycelium dry weight, dissolved protein, and TVBN consumption compared to 20:1 treatment. While the addition of



[a,b,c,d,e,f]Means with different superscript differ significantly ($p < 0.05$)
Experiment were done in six replicates

Fig 1 Biomass production of Rhizopus spp. in soybean milk medium incubated at 30°C and 125 rpm for 72 hours.

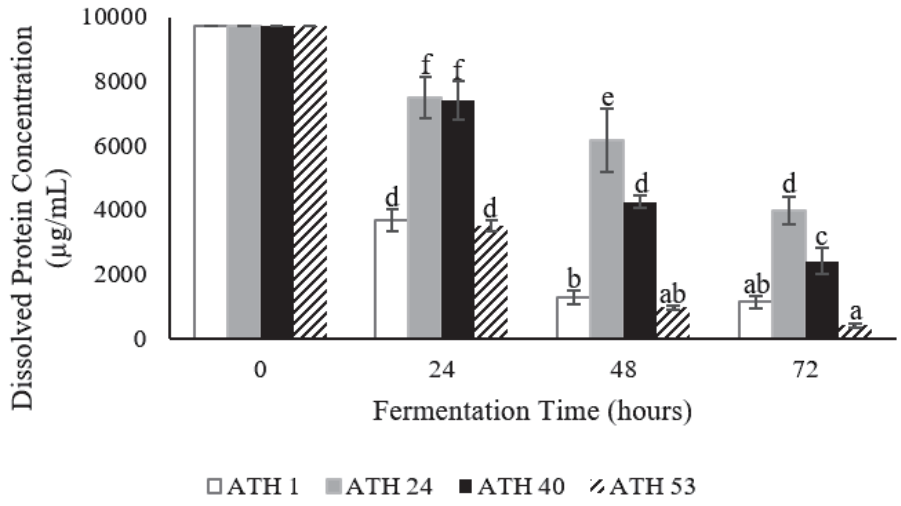


[a,b,c,d,e,f]Means with different superscript differ significantly ($p < 0.05$)
Experiment were done in six replicates
Fig 2 Reduction sugar concentration in soybean milk media during 72 hours fermentation by Rhizopus spp.

glucose and $(NH_4)_2SO_4$ in 40:2 treatment increased sugar and dissolved protein consumption, but decreased biomass production, protein content in mycelium dry weight and TVBN consumption compared to 20:1 treatment. The difference in biomass production and protein content in 20:2 and 40:2 treatments was not significant to 20:1 (Table 1).

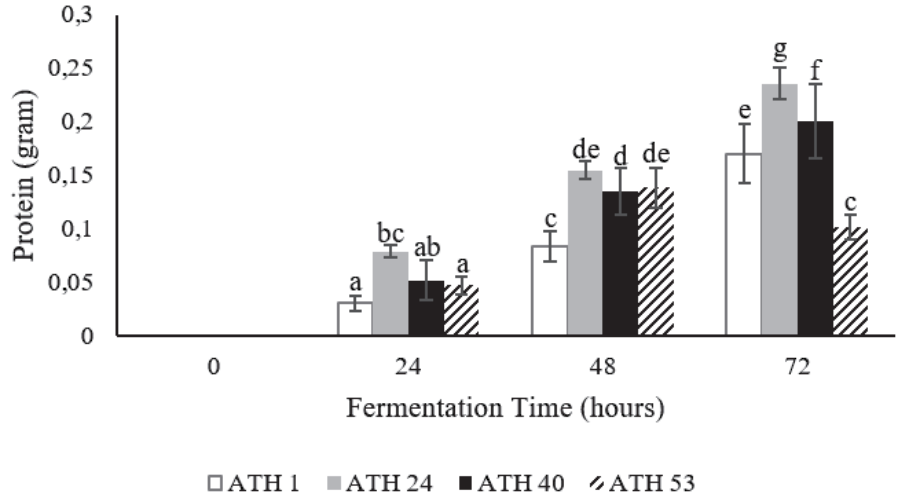
DISCUSSION

Our study indicates that ATH 24 has the highest biomass production. It could be due to the formation of azygospores (Hartanti *et al*. 2015). Azygospores is zygospores which can be formed without the mating process. Zygospores are round balls that are formed when mycelia in fungi interact and fuse. After the formation, zygospores will make sporangium that contains spores for reproduction. Zheng *et al*. (2007) stated that the formation of azygospores could be induced by lecithin in the growth media and there is lecithin in soymilk around 0.5% (Van Ee 2009). Zheng *et al*. (2007) also stated that *R. microsporus* var *rhizopodiformis* was heterothallic fungi that could form zygospores. On the other hand, *R. microsporus*



[a,b,c,d,e,f]Means with different superscript differ significantly ($p < 0.05$)
Experiment were done in six replicates

Fig 3 Dissolved protein concentration in soybean milk media during 72 hours fermentation by Rhizopus spp.



[a,b,c,d,e,f,g]Means with different superscript differ significantly ($p < 0.05$)
Experiment were done in six replicates

Fig 4 Protein content in Rhizopus spp. mycelium dry weight produced in soybean milk medium incubated at 30°C and 125 rpm for 72 hours.

var *oligosporus* and *R. delemar* could neither form zygospores nor azygospores. Homothallic fungi have male and female reproductive structures on the same mycelium, which is self-compatible. On the contrary, heterothallic fungi need mycelium from other fungi to reproduce because they only have one type of reproductive structure in its mycelium (Ulloa and Hanlin 2000). Therefore, ATH 1, ATH 40 and ATH 53 could only reproduce using an asexual system, which are spores. Based on this evidence, the only strain which has an advantage in reproduce was ATH 24. The difference in biomass production of ATH 1, ATH 40 and ATH 53 may be caused by the physiology characteristic and the capability to utilize nutrition in the growth media. On the other hand, protein content in mycelium dry weight was depended on the mold ability to produce amino acids (Ahmed *et al*. 2017). Therefore, ATH 24 was the best strain in utilizing nutrients to produce amino acids because it has the highest protein content.

Rhizopus spp. can utilize nutrition in media because they can produce specific amounts of enzymes like amylase, protease, and lipase (Wong 1995). There are many types of soluble carbohydrates in soybean,

Table 1 Effect of carbon to nitrogen ratio treatment to the fermentation parametres of ATH 24

Treatment (C:N)	Biomass (gram)	Rate of Reduction Sugar Consumption ($\mu g\ mL^{-1}\ h^{-1}$)	Rate of Dissolved Protein Consumption ($\mu g\ mL^{-1}\ h^{-1}$)	Δ [TVBN] ($\mu g\ mL^{-1}$)	Protein (gram)
20:1	$0.57\pm0.08^{(ab)}$	$31.49\pm0.35^{(a)}$	$83.94\pm6.13^{(a)}$	$0.0009\pm0.0011^{(ab)}$	$0.20\pm0.03^{(a)}$
20:2	$0.61\pm0.08^{(a)}$	$31.41\pm0.72^{(a)}$	$86.46\pm8.98^{(a)}$	$0.0022\pm0.0038^{(a)}$	$0.21\pm0.03^{(a)}$
40:2	$0.50\pm0.05^{(b)}$	$76.69\pm1.19^{(b)}$	$98.66\pm14.09^{(a)}$	$-0.0021\pm0.0023^{(b)}$	$0.17\pm0.02^{(a)}$

[a,b] Means in same column with different superscript differ significantly ($p<0.05$)
Experiment were done in six replicates

such as sucrose, raffinose, stachyose, sugar alcohols, etc (Obendorf and Kosina 2011). At the beginning of fermentation, the molds would begin to consume simple carbohydrates in media, but complex carbohydrates would also be digested when they run out of simple carbohydrates. The increment of reduction sugar concentration in ATH 1, ATH 40 and ATH 53 after 24 hours might be caused by their speed in hydrolyzing carbohydrates was greater than utilizing them. On the other hand, ATH 24 was faster in utilizing carbohydrates rather than hydrolyzing them. The decrement of reducing sugar concentration in ATH 1 and ATH 40 after 48 hours was because the media was run out of complex carbohydrates. However, reducing sugar in ATH 53 was increasing after 48 hours though the mold was already in the death phase. It might be caused by carbohydrase was still active in digesting the complex carbohydrates. This occasion could be because mold's enzyme was produced extracellular, which still works though the cell was dead (Sinsabaugh 1994). Dissolved protein concentration decreased throughout the fermentation process, indicating that the protein was consumed.

TVBN measurement using Conway micro diffusion method was done because Bradford method can only measure protein content, where $(NH_4)_2SO_4$ is not protein (Bradford 1976). From the result, it could also be seen that the Bradford method can only measure the dissolved protein content, while the Conway micro diffusion method cannot measure protein content, but it can measure simple molecule with nitrogen atom-like $(NH_4)_2SO_4$. The 20:2 treatment triggered ATH 24 to consume more dissolved protein and $(NH_4)_2SO_4$. However the same occasion did not happen in the 40:2 treatment because it triggered ATH 24 to consume more sugar and protein. The negative result of TVBN measurement in 40:2 might be caused by the degraded protein was also measured after 72 hours of fermentation, while the original $(NH_4)_2SO_4$ added into the media was not consumed yet. Therefore, the final TVBN concentration becomes higher than the initial concentration.

The 20:2 treatment produced the highest biomass and protein content in mycelium dry weight, but the difference was not significant to 20:1 ($p < 0.05$), while the 40:2 treatment had the lowest biomass and protein content. Yalemtesfa *et al*. (2010) stated that the addition of $(NH_4)_2SO_4$ as a nitrogen source produced a higher protein level in *Chaetomium* spp. and *Aspergillus niger*. It also applied to ATH 24 in 20:2 treatment. However, it did not happen in 40:2 treatment, which might be caused by the addition of sugar disturbed the growth and ability to produced amino acids of ATH 24. Ahmed *et al*. (2017) stated that a reasonable amount of carbon to nitrogen ratio is the key to high-quality harvesting biomass. It meant that a higher amount of carbon and nitrogen did not guarantee higher biomass and protein content production.

From all those results, ATH 24 with a C/N ratio of 20:2 could produce 0.61 g of mycelium dry weight with 0.21 g of protein, but the difference was not significant ($p > 0.05$) compared to ATH 24 with a C/N ratio of 20:1 which produced 0.57 g of mycelium dry weight with 0.20 g of protein. Therefore, using a 20:1 ratio will be more economical because it is not necessary to add any other nutrient. The mycoprotein had similar protein content with beef, chicken, chicken egg, soybean and tempeh. The protein content of 0.57 g of beef, chicken, chicken egg, soybean and tempeh were 0.15, 0.15, 0.07, 0.20, and 0.11 g, respectively (USDA 2019). On the other hand, if it was compared to the growth media, the mycelium dry weight protein content was much higher because 0.57 mL of soymilk only had 0.01 g of protein. Therefore, it was beneficial to produce mycoprotein using soymilk to increase the protein content by 20 folds.

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