GROWTH OPTIMIZATION AND CHARACTERIZATION OF FUNGAL BIOMASS FROM *Rhizopus oligosporus* CULTIVATED IN TAPIOCA EFFLUENT

(Bachelor Thesis)

By

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ABSTRACT

GROWTH OPTIMIZATION AND CHARACTERIZATION OF FUNGAL BIOMASS FROM *Rhizopus oligosporus* CULTIVATED IN TAPIOCA EFFLUENT

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Protein alternative currently needed due to several reasons, namely the high prevalence of protein malnutrition, the high fat content in animal products, and livestock production is not environmentally friendly because it contributes to greenhouse gases. Biomass of filamentous presents potential as meat substitute due to its favorable nutritional profile, such as protein. Fungal biomass can be produced using agricultural residue, one of which is tapioca effluent. This study aims to assess the potential of tapioca effluent as substrate for fungal biomass production, determine the optimum growth conditions, and characterize of the fungal biomass produced. The research uses tapioca effluent as the fungal biomass substrate and Raprima starter as the *R. oligosporus* culture. The study commences with the characterization of tapioca effluent, optimization of growth conditions in terms of supplementation, pH, and agitation speed, followed by the chemical and nutritional characterization of the fungal biomass. The results of this study show that tapioca effluent holds potential as a fungal biomass production medium, containing 0.47% carbon, 0.04% nitrogen, and 1.36 mg/kg phosphate. *R. oligosporus* exhibits optimal growth and produces the highest biomass in supplemented media with yeast extract, at pH 5.5, and agitation speed of 125 rpm. Media supplementation does not significantly affect the protein content but affect the fat content of the fungal biomass, with yeast extract supplementation resulting in the lowest fat content. The nutritional composition of the fungal biomass cultivated on tapioca effluent contains 26.447% protein, 4.81% fat, 4.26% ash, 5.96% crude fiber, and a protein digestibility of 61.25%. The result of this study showed that tapioca effluent shows potential as a medium for high-quality mycoprotein production.

Keywords: Fungal biomass, *Rhizopus oligosporus*, tapioca effluent

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STATEMENT OF ORIGINALITY

I am Bella Amanda Iswahyudi, student identification number 2014051026.

I hereby declare that what is written in this work is my own original work based on the knowledge and information I have obtained. This work does not contain material that has been previously published or, in other words, is not the result of plagiarism from other people's work.

This statement is made and can be accounted for. Should there be any dishonesty in this work in the future, I am prepared to take full responsibility.

Bandar Lampung, August 6th, 2024

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AUTOBIOGRAPHY

The author was born in Serang, Banten on September 21, 2002. The author is the first child of Sri Wahyuni and Cahya Iswahyudi. Since childhood, the author has lived in Lampung. The author completed elementary education at SDN Berundung in 2014, junior high school at SMPN 3 Ketapang in 2017, and senior high school at SMAN 1 Kalianda in 2020.

In 2020, the author was admitted to the Agricultural Product Technology study program at the Faculty of Agriculture, University of Lampung. The author was involved in the University of Lampung Student Cooperative (KOPMA). The author participated in various KOPMA committees, such as being an event coordinator, as a mentor for MC and moderator Squad, and working as a business staff member managing KOPMA Unila's business. The author was also involved in the University of Lampung Research Student Association (UKM Penelitian). The author was joining committees and rganizing national events. In the second year with UKM Penelitian, the author served as the treasurer of the research department.

The author actively participated in various competitions, volunteer work, lecturer projects, as a teaching assistant, internships, training, scientific publications, entrepreneurship, and other activities. The most memorable achievements was participating in the National Scientific Week (PIMNAS) for three consecutive years by the Ministry of Education. The author has also won several international awards in scientific writing. This active involvement has led the author to become an outstanding student at the University of Lampung and win the prestigious LLDIKTI 2 (South Sumatra) Outstanding Student Award. As a result, the author has been invited to various events as a speaker. By mid-2024, the author has received a total of over 35 awards.

DEDICATION

Alhamdulillahi rabbil 'alamin. I express my gratitude and thanks to Allah SWT, who has granted blessings and grace, enabling me to complete this bachelor thesis entitled "Growth Optimization and Characterization of Fungal Biomass from *Rhizopus oligosporus* Cultivated in Tapioca Effluent" as a requirement for obtaining a Bachelor's degree in Agricultural Technology from the University of Lampung. I acknowledge that the completion of this thesis has received extensive guidance, support, and advice both directly and indirectly, and I would like to extend my thanks to:

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I hope that Allah SWT rewards the kindness shown and that this thesis may be beneficial to me, specifically, and to readers, generally.

Bandar Lampung, August 6th, 2024

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I. INTRODUCTION

1.1 Background

The global human population has increased to reach 8 billion people in 2022 and is predicted to increase (United Nation, 2022). The increasing human population is a challenge to meet the food and nutritional needs of each individual, including protein. The Daily Protein Adequacy Rate for adult men and women aged 19 and over, with an average body weight of 55 to 60 kg, are 60-65 grams (Indonesian Ministry of Health, 2019). However, the fact remains that there is still a significant prevalence of protein malnutrition with a prevalence of 14,767,275 cases of protein-energy malnutrition worldwide in 2019 (Zhang *et al*., 2022). Protein is one of the macronutrients that must be fulfilled for the structural and functional needs of body cells. Protein deficiency can lead to stunted growth, weak muscles, and weaken the immune system (Anggraeny *et al*., 2016).

Animal protein sources such as meat are the choice of most people as a source of protein and have a delicious taste. This is evidenced by the high consumption of meat, reaching 328 million tons in 2020 (FAO, 2021). However, alongside its popularity, meat contains high levels of saturated fat, which, if consumed excessively, may potentially lead to atherosclerosis or other degenerative diseases (Suarsana, 2016). Besides health concerns, further meat consumption is associated with climate change. Livestock farming produces significant methane gas emissions, contributing to greenhouse gases (FAO, 2021). Therefore, there is a need for healthier and environmentally friendly meat alternatives.

Filamentous fungi hold significant potential as a protein source, known as mycoprotein (Bakratsas *et al*., 2023). Filamentous fungi with potential as protein

sources include *Aspergillus oryzae, Fusarium venenatum, Monascus purpureus, Neurospora intermedia, and Rhizopus sp.* (Filho *et al*., 2018). Fungal biomass has the potential as a meat substitute because it contains various ingredients that are needed by the body and has characteristics similar to meat. One hundred grams of dry mycoprotein contains 13 g of fat, 45 g of protein, 10 g of carbohydrates, 25 g of fiber, several vitamins and minerals. In addition, mycoprotein can control cholesterol levels in the blood (Ruxton *et al*., 2010). Mycoprotein also contains aspartate and glutamate, contributing to meat-like aroma and umami taste (Bakratsas *et al*., 2023). In terms of texture, fungal biomass can be molded to resemble meat due to its fibrous texture, similar to meat fibers. There is a commercial mycoprotein product derived from fungal biomass of *Fusarium venetatum* and sold under the brand Quorn. The fungi are cultivated on glucose which is expensive and make the product become not economically attractive. Hence, a low cost media with high availability is of interest.

Lampung is one of the largest tapioca-producing regions in Indonesia. The cassava processing into tapioca results in a substantial amount of liquid waste, reaching 2-5 m^3/ton of cassava (Isdiyanto & Hasanudin, 2018). Tapioca effluent originates from the cassava starch extraction process and sedimentation waste water. The water from sedimentation is usually disposed of without being treated, then simply channeled to the disposal. Presently, many tapioca industries pay insufficient attention to tapioca effluent management, contributing to greenhouse gas emissions (Damiri & Santoso, 2022). In fact, tapioca effluent still contains organic compounds, including a total sugar content of 34.04 g/L, organic carbon of 1.42 g/L, and protein (total N) of 0.10 g/L (Wulan *et al*, 2017). These compounds in tapioca effluent can be utilized by microorganisms as an alternative substrate for filamentous fungi growth. This approach strongly supports the concept of green economy by harnessing the potential of waste to generate economical alternative of protein source.

The filamentous fungus with potential as a protein source grown on tapioca effluent substrate is *Rhizopus oligosporus*. *Rhizopus oligosporus* is an edible filamentous

fungus well-known in Indonesia for producing tempeh. This fungus possesses several advantages, including its ability to produce high digestibility protein, rapid growth, adaptability to various substrates, and safety for food applications (Canedo *et al*., 2016). Furthermore, from an economic perspective, this fungus is affordable and readily available in Indonesia.

The production of fungal biomass from *R. oligosporus* on tapioca effluent must consider several factors such as media nutrition, pH, and agitation speed to achieve high biomass yields. To date, no research has investigated the optimization of *R. oligosporus* growth on tapioca effluent. Therefore, this study aims to assess the potential of tapioca as a medium for fungal biomass production and determine the optimum conditions for achieving the highest biomass of *R. oligosporus*, considering media nutrition, pH, and aeration. Additionally, to assess the chemical characteristics and nutritional value of the produced fungal biomass, characterization is conducted, including protein, fat, carbohydrate, protein digestibility, crude fiber, and cyanide, and compare with other meat substitution.

1.2 Objectives

The objectives of this research are as follows:

1. To know the characteristics of tapioca effluent and its potential as a medium for fungal biomass production.
2. To determine the optimum conditions (media supplementation, media pH, and agitation speed) for achieving the highest *R. oligosporus* biomass production.
3. To characterize the chemical and nutritional properties of the fungal biomass.

1.3 Research Framework

Fungal biomass presents a promising alternative protein source in the food industry. This is due to its nutritional richness and ease of production on various media, both synthetic and natural (Baltork *et al*., 2020). Agricultural industrial residue can serve as a viable alternative medium due to its composition of several nutrient

components essential for fungal growth, thus enhancing the value of agro-industrial residue (Boˆas *et al*., 2002). Tapioca effluent holds considerable potential for use as a growth medium for *R. oligosporus*. This is attributed to its composition, including organic carbon content of 1.42 g/L and nitrogen content of 0.10 g/L (Wulan *et al*., 2017), which can serve as essential nutrients for fungi, particularly *R. oligosporus*. Jin *et al*. (1999) previously investigated starch processing wastewater for mycoprotein production using *R. oligosporus,* wherein starch processing wastewater without additional nutrients yielded biomass of 4.66 g/L and protein content of 45.56%. Therefore, tapioca effluent, being starch-rich and abundant in organic carbon content, holds significant potential as a medium for fungal biomass production.

The optimal growth of *R. oligosporus* fungi is significantly influenced by several factors, including media nutrition, pH, and oxygen requirements (Barzee *et al*., 2021). Based on Jin *et al*. (1999)'s study on *R. oligosporus* biomass production in starch processing wastewater, treatment without supplementation produced a mycoprotein biomass of 4.66g/L, while supplementation with dipotassium phosphate produced a higher fungal biomass of 5.21g/L compared to various nitrogen sources such as urea, ammonium sulfate, ammonium nitrate, sodium nitrate, and monopotassium phosphate. Similarly, Wikandari *et al*. (2023) revealed that supplementation of residual water of tempeh industry media with yeast extract nitrogen sources produced three times higher biomass compared to without supplementation and other supplementation types such as urea and minerals. These studies indicate that supplementation, particularly with nitrogen and phosphate, plays a crucial role in fungal growth as it is utilized as energy for fungal growth (Barzee *et al*., 2021). Garuba *et al*., (2012) analyzed the best type of nitrogen source supplementation, the result was that the inorganic nitrogen sources that produced the highest amount of *Rhizopus* biomass in basal medium were ammonium nitrate, sodium nitrate, and ammonium sulfate, respectively. Meanwhile, the sequence for organic nitrogen source supplementation produced total biomass of *Rhizopus sp.* the highest to lowest were urea, yeast extract, and malt extract, respectively. Therefore, this study aims to determine the optimal supplementation for *R.*

oligosporus growth in tapioca effluent using yeast extract, ammonium sulfate, sodium nitrate, ammonium nitrate, and dipotassium phosphate supplementation.

The degree of acidity or pH is crucial for fungal growth. Each fungus has its own optimal pH, if the fungus grows at an inappropriate pH, it will inhibit fungal growth (Sparringa *et al*., 2002). Based on research by Jin *et al*. (1999), pH influences the growth of the fungus *R. oligosporus* in starch processing wastewater, with the optimal pH ranging from pH 3-6. The initial pH conditions that produce the highest to lowest amounts of biomass respectively are 4; 3.5; 3; 4.5; 5; 5.5; 6; 2.5; 6.5; and 7. This is supported by Wikandari *et al*'s study (2023), stating that pH affects the growth of *R. oligosporus* in liquid tempeh waste media. The initial pH conditions that produced the highest to lowest amounts of biomass respectively 4.5; 4; and 5.5. Based on both studies, there is a difference in optimal pH to produce the highest biomass *R. oligosporus*. This is presumed to be influenced by the nutrient composition of the media and the absence of pH control during the fermentation process. Both studies conditioned the initial pH as a treatment without any pH control. During fermentation, pH increases due to the protease enzyme activity of *R. oligosporus*, which breaks down proteins into ammonia and nitrogen which are alkaline, thereby increasing pH (Wikandari *et al*., 2023). Therefore, in this study, to determine the most optimal pH for the growth of *R. oligosporus* in tapioca effluent media, pH variations within the optimal pH range from previous research 4; 4.5; 5; 5.5; 6; and 6.5, with controlled pH conditions.

Rhizopus oligosporus is an aerobic microorganism that requires oxygen for optimal growth (Ikasari & Mitchell, 1998). Agitation at a certain speed can supply the required oxygen for aerobic microorganisms (Barzee *et al*., 2021). Based on previous research, agitation speed significantly affects the biomass yield of the fungus (Ahangi *et al*., 2008; Manfaati, 2010; Wikandari *et al*., 2023). However, the optimal agitation speed in each study varies even when using the same method, as presented in Table 1.

Table 1. Previous research on optimal agitation speed

Fungi	Substrate	Variation of Agitation Speed	Optimal Agitation Speed	Source
Fusarium oxysporum	Glucose	150 rpm, 200 rpm, 250 rpm	150 rpm (lowest speed)	Ahangi *et al* (2008)
Rhizopus oryzae	Tofu effluent	100 rpm, 150 rpm, 200 rpm & 250 rpm	250 rpm (highest speed)	Manfaati (2010)
Rhizopus oligosporus	Tempeh residual water	110 rpm & 125 rpm	125 rpm (highest speed)	Wikandari *et al* (2023)

Based on Table 1, there are differences in the optimal agitation speed for biomass production, thus the optimal agitation speed for producing the highest biomass cannot be determined yet. The variations in agitation speed in each study are suspected to be related to the viscosity of the liquid media used, resulting in different levels of turbulence and affecting oxygen supply (Standbury *et al*., 1995). Therefore, this study employs agitation speeds within range 110 rpm, 125 rpm, and 140 rpm, to determine the optimal agitation speed for the production of *Rhizopus oligosporus* mycoprotein biomass in tapioca effluent.

1.4 Hypothesis

The hypothesis of this research is as follows:

1. Tapioca effluent contains essential nutrient for *R. oligosporus* growth, thus potentially serving as medium for fungal biomass production.
2. Optimization of conditions (media supplementation, media pH, and agitation speed) influences the biomass yield of *R. oligosporus*.
3. The produced fungal biomass exhibits favorable chemical and nutritional characteristics.

II. LITERATURE REVIEW

2.1. Tapioca Effluent

Tapioca effluent is a by-product generated from the processing of cassava (*Manihot esculenta*) into tapioca. This waste is produced from sedimentation process of starch. Fresh tapioca effluent typically exhibits characteristics of being whitish to yellowish in color with a distinctive cassava odor. The turbidity of tapioca effluent is caused by the presence of organic matter, such as soluble starch and other colloids that do not precipitate rapidly. Tapioca effluent also contains glucose, carbohydrates, vitamin C, and organic compounds such as proteins, fats, and carbohydrates which are easily perishable and contribute to unpleasant odors (Tjokroadikoesoemo, 1986).

Based on the research by Wulan *et al*. (2017), the components constituting tapioca effluent from the sedimentation process include water content, total solids (TS), Total Dissolved Solids (TDS), Total Suspended Solids (TSS), pH level, total sugar, total organic carbon, and protein content (total N), with amounts as presented in Table 2. Due to these components, tapioca effluent must be properly managed to prevent environmental pollution. Tapioca effluent also still contains cyanide at a concentration of 9.3 ppm (Nikmatusya'ban, 2016), thus efforts to utilize tapioca effluent must take into account the cyanide content. One of the efforts to utilize tapioca effluent is as a growth medium for microorganisms because it contains carbon and nitrogen that can be utilized as nutrients for microbes (Wulan *et al*., 2017). Ardhianto *et al*. (2013) also reported that fermentation can reduce cyanide levels in cassava.

Table 2. Characteristics of Tapioca Effluent

Parameter	Content
Water content	96.25% (%b/b)
pH	6.28
Total solid	35.51 g/L
TDS (*Total Disolved Solid)*	34 g/L
TTS (*Total Suspended Solid*)	1.51 g/L
Total sugar	3.404 %
Total C-organic	0.142 %
Protein (Total N)	0.01 %
C/N ratio	14.2

Source: Wulan *et al*., 2017

2.2 Mycoprotein

Mycoprotein is a protein produced by filamentous fungi. Mycoprotein has the potential as an alternative protein source for humans (Baltork *et al*., 2020). Mycoprotein contains various crucial nutrition for the body's needs, as presented in Table 3. Despite not being of animal product, mycoprotein possesses excellent protein quality with a biological value comparable to skim milk protein (Finnigan *et al*., 2017). Additionally, mycoprotein contains various amino acids, fatty acids, minerals, and vitamins in substantial amounts (Finnigan *et al*., 2017). Essential amino acids in mycoprotein, particularly aspartate and glutamate, serve as precursors in the maillard reaction to form meaty aroma and umami taste (Bakratsas *et al*., 2023). Based on research by Ruxton *et al*. (2010) mycoprotein can reduce total cholesterol and Low Density Lipoprotein (LDL) in the blood. This is the advantage of mycoprotein as a meat substitute.

The characteristics of mycoprotein, such as flavor, color, and texture, are determined by the cultivated fungus (Barzee *et al*., 2021). The texture of mycoprotein is dictated by the fungal mycelium, which forms branching filaments resembling protein fibrils in meat (Finnigan, 2011). Furthermore, mycoprotein

contains fiber, with one-third composed of chitin and two-thirds beta-glucan (Denny *et al*., 2008). The fiber content in mycoprotein induces a longer-lasting feeling of satiety compared to chicken meat (Williamson, 2006). Based on its characteristics and nutritional content, mycoprotein has the potential as meat substitute.

Table 3. Mycoprotein Nutritional Composition

Composition/100g	Content (Wet basis)	Amount (Dry basis)
Energy (Kcal)	85	340
Protein (g)	11.25	45
Total Carbohydrates (g)	3	10
Total Fat (g)	3.25	13
Fiber (g)	6.25	25
Dietary fiber (g)	6	25

Source: Finnigan *et al*., 2017

Mycoprotein is produced by various edible filamentous fungi, such as Fusarium venetatum, *Rhizopus oligosporus*, and *Aspergillus oryzae*. These fungi possess a range of enzymes capable of synthesizing proteins (Nursiwi *et al*., 2018). Mycoprotein production through aerobic fermentation requires maintaining conditions, including temperature, pH, nutrient availability, dissolved oxygen, and growth rate, specific to the type of fungus cultivated (Barzee *et al*., 2021). The fermentation media must be rich in carbon and nitrogen for optimal fungal growth (Wulan *et al*., 2017).

2.3 *Rhizopus oligosporus*

Rhizopus oligosporus is edible fungus that has long been utilized in the food industry. The following is the taxonomy of *R. oligosporus*:

Kingdom : Fungi
Phylum : Zygomycota
Subphylum: Mucoromycota
Ordo : Murocales

Family : Muroceae
Genus : *Rhizopus*
Species : *Rhizopus oligosporus*

R. oligosporus has the characteristics of white to gray-black thread-like hyphae that are non-separated, have rhizoids and sporangiospores. The growth of *R. oligosporus* is characterized by ash brown colonies with a height of 1 mm or more. The sporangiophores are solitary or in groups, with thin or slightly coarse walls, measuring over 1000 µm in length and 10-18 µm in diameter. The sporangia are globose, turning blackish-brown when mature, with a diameter of 100-180 µm. Chlamydospores are numerous, single or short chains, colorless, containing granules, formed in hyphae, sporangiophores and sporangia. The shape of chlamydospores is globose, elliptical or cylindrical with a size of 7-30 µm or 12-45 µm x 7-35 µm (Madigan and Martinko, 2006).

R. oligosporus grows optimally at temperatures ranging from 30-35°C, with a minimum temperature of 12°C and a maximum temperature of 42°C. The optimum growth time is 36 hours (Wahyudi, 2018). *R. oligosporus* can grow at slightly acidic to neutral pH conditions, specifically within the range of 4-7, although the optimal pH for *R. oligosporus* growth is around 5. The optimal pH for *R. oligosporus* growth is associated with the water activity (Aw) of the medium (Sparringa *et al*., 2002). *R. oligosporus* requires a nutrient-rich medium containing carbohydrates, proteins, and minerals, such as potato dextrose agar or medium from enriched agricultural waste (Surbakti *et al*., 2022).

R. oligosporus has long been utilized as a starter culture to produce tempeh. It possesses the ability to produce various enzymes that can hydrolyze macro components of the substrate into simpler components, which are more easily digestible by the body. *R. oligosporus* exhibits the capability to synthesize protease enzymes, thereby displaying proteolytic activity capable of breaking down proteins into amino acids and peptides from the substrate (Nursiwi *et al*., 2018). Additionally, *R. oligosporus* can produce lipase enzymes to hydrolyze the fat in the

medium and is capable of producing long-chain omega-3 fatty acids, particularly linoleate (Rahayu *et al*., 2003). These abilities render *R. oligosporus* has the potential to produce food with high-quality protein content.

2.4 Factors Influencing Fungal Biomass Production

The production of fungal biomass is influenced by several factors, including temperature, pH, nutrient availability, agitation, and dissolved oxygen (Barzee *et al*., 2021). The impact of these factors on the growth of *R. oligosporus* fungus is discussed in the points below.

2.4.1 Substrate Nutrition

The composition of the growth medium for fungi must be rich in nutrients required by the fungus because fungi are heterotrophic organisms (Barzee *et al*., 2021). The essential compounds needed for fungal growth are divided into macronutrients, required in large amounts, and micronutrients, required in small amounts. Macronutrients include carbon, nitrogen, oxygen, hydrogen, phosphorus, potassium, magnesium, and sulfur. Micronutrients include manganese, iron, zinc, copper, and molybdenum (Barzee *et al*., 2021). The main composition crucial for fungal growth is carbon and nitrogen (CN ratio) (Zhang & Elser, 2017). Ahmed *et al*. (2017) revealed that an appropriate carbon-to-nitrogen ratio enhances fungal growth and yields high-quality biomass.

Fungi metabolize carbon and utilize it to build cell mass and form products. Organic carbon sources that can be directly absorbed by fungi include glucose, fructose, and sucrose. Carbon sources can also be found in agricultural waste substrates, such as lignocellulose and starch. However, fungi must break down these substrates into simple sugars using extracellular enzymes (Barzee *et al*., 2021). Nitrogen is required by fungi as an energy source to accelerate the degradation process and the formation of organic compounds (Manfaati, 2010). Nitrogen sources that can be used for media supplementation include ammonium, urea, proteins, and peptides.

Complex nitrogen from natural media sources such as soybeans, peanuts, yeast extract, and fish products (Jin *et al*., 2005).

2.4.2 Acidity Level (pH)

The acidity level (pH) is a crucial factor in fungal growth. The concentration of hydrogen ions affects the absorption of nutrients into the cell, nutrient solubility, enzyme activity, and fungal morphology. These factors significantly influence fungal growth and productivity (Barzee *et al*., 2021). Each fungus has a different pH requirement for optimal growth, also known as the optimal pH. The optimal pH for a microorganism can vary depending on the fermentation purpose (Manfaati, 2010). The optimal pH for growth is not always the same as the optimal pH for product formation. The optimal pH for *Rhizopus* fungus growth is pH 3-6 (Jin *et al*., 1999). pH of the media that is too acidic or too alkaline will inhibit the growth of fungus. During fermentation, a gradual increase in pH is likely to occur due to the degradation activity of nutrients by fungi, resulting in the production of compounds that can change the acidity level of the medium (Purwoko, 2004).

2.4.3 Agitation Speed

The fermentation of a product occurs under aseptic conditions in a closed system to prevent contamination by other microbes. This closed condition causes limited oxygen supply to aerobic microorganisms. In this regard, agitation plays a crucial role in the fermentation of aerobic microorganisms to meet the dissolved oxygen requirements by creating agitation in the medium, thereby allowing the medium to come into direct contact with oxygen and dispersing oxygen into the medium alternately. Consequently, the oxygen requirements of aerobic microorganisms can be fulfilled, enabling optimal microbial growth (Standbury *et al*., 1995). For aerobic microorganisms, such as *R. oligosporus*, oxygen plays a significant role in various aspects of intracellular metabolism. The presence of dissolved oxygen affects respiratory rate, enzyme synthesis and activity, metabolite formation, thereby enhancing cell growth (Moehady & Djenar, 2018).

Agitation also plays a role in homogenizing fermentation conditions, including the mixing of nutrients, culture, and heat. Agitation can accelerate the transport of nutrients from the liquid medium into the cells (Mustafa *et al*., 2023). Agitation speed generates shear forces that influence growth, morphology, formation of metabolic products, and even cell structure damage (Kim *et al*., 2003). Therefore, the agitation speed for each fermentation condition varies depending on the purpose of the fermentation process and the microoganism used. The optimal agitation speed for obtaining high biomass of *Rhizopus* has been studied previously, but yielded different results even when using the same method. In the study by Manfaati (2010), the optimal agitation speed for producing R. oryzae fungus was 250 rpm, while in the study by Wikandari *et al*. (2023), the optimal agitation speed for producing *R. oligosporus* fungus was 125 rpm.

III. METHODOLOGY

3.1 Place and Time of Research

This research was conducted from October 2023 to January 2024 at the Biotechnology Laboratory, Training Laboratory, Food & Nutrition Laboratory, Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta.

3.2 Materials and Equipment

3.2.1 Materials

The materials used in this study included tapioca effluent from the sedimentation process obtained from PD. Semangat Jaya, Pesawaran, Lampung. The supplementation media used are yeast extract (Oxoid), ammonium sulfate (Merck), sodium nitrate (Merck), ammonium nitrate (Merck), and potassium dihydrogen phosphate (Merck). *Rhizopus oligosporus* fungus from Raprima brand tempeh starter developed by Lembaga Ilmu Pengetahuan Indonesia (LIPI), produced by PT. Aneka Fermentasi Industri, Bandung, Indonesia. Other materials used included H_2SO_4, NaOH, 70% alcohol, and distilled water.

Materials used for chemical analysis included $HgO.K_2SO_4$ catalyst, H_2SO_4, NaOH-$Na_2S_2O_3$, boric acid, BCG-MR indicator, HCl, distilled water, borax buffer, potassium sodium tartrate, Nessler's reagent, HNO_3, $HCLO_4$, AMV solution, PO_4 working solution, petroleum ether, pepsin, laphole buffer solution, trichloroacetic acid, picric acid, and Na_2CO_3.

3.2.2 Equipment

The research equipment used in this study included 250 mL Erlenmeyer flasks, shaker bath, filter cloth, a pH meter, analytical balance, 100 mL and 1000 mL volumetric flasks, micropipettes, macropipettes, 1 mL and 5 mL tips, laminar flow, autoclave, spatula, volumetric pipettes, cotton plugs, plastic wrap, zip plastic, heating plates, and filter paper.

The equipment used for chemical analysis included Kjeldahl flasks, distillation sets, burettes, clamps, statives, 250 mL Erlenmeyer flasks, 250 mL and 1000 mL beakers, 100 mL volumetric flasks, droppers, analytical balances, furnaces, ovens, desiccators, crucibles, UV spectrometer, heating plates, atomic absorption spectrophotometer, porcelain dish, Soxhlet extractor, fat flasks, Whatman filter paper No. 41, cotton, funnels, centrifuges, aluminum foil, water baths, and fiber sleeves.

3.3 Research Method

The study commenced with the characterization of tapioca effluent, including analysis for carbon, ammonium, phosphate, and cyanide levels to ascertain its potential as a medium for fungal biomass production. Subsequently, optimization research on fungal biomass growth was conducted in three stages of a Completely Randomized Design with one factor. The first stage is variations in supplementation tapioca effluent media with six variations, including yeast, ammonium sulfate, sodium nitrate, ammonium dihydrogen phosphate, dipotassium phosphate, and a control (without supplementation). The second stage is pH variations (4; 4.5; 5; 5.5; 6; 6.5). The third stage is variations in agitation speed (110 rpm, 125 rpm, 140 rpm). Data were analyzed using Analysis of Variance (ANOVA), and further tests were conducted using the Duncan Multiple Range Test (DMRT) at a significance level of 0.05. Treatments that produced the highest fungal biomass were subsequently characterized chemical analysis, including ammonium, phosphate, cyanide, and

proximate analysis comprising ash content, protein, fat, fiber, protein digestibility, and compared with other protein sources.

3.4 Research Procedures

3.4.1 Nitrogen Analysis

The nitrogen contents was analyzed by the Kjeldahl method. A sample weighing 0.1g was placed into a Kjeldahl flask, followed by the addition of 0.7g of $HgO.K_2SO_4$ catalyst and 3mL of H_2SO_4. Subsequently, the Kjeldahl flask was heated until the sample solution became clear. The cooled sample solution was then diluted with distilled water. The sample solution was distilled with 10-20mL of NaOH-Na2S2O3 solution. The distillate was collected in an Erlenmeyer flask containing 5 mL of 4% boric acid and 3 drops of BCG-MR indicator. The obtained distillate was then titrated with standardized HCl solution until a color change from blue to pink occurred to determine the required titration volume. The determination of the nitrogen content in the sample was calculated using the formula:

$$\text{Nitrogen (\%)} = \frac{(Y-Z)x\ (N \times 14.007)x\ 100\%}{W}$$

Note:

Y = ml HCl (titrant) sample

Z = ml HCl (titrant) blanko

W = Sample weight (g)

N = Normality of HCl (N)

3.4.2 Carbon Organic Analysis

The carbon organic contents was analyzed by the gravimetric method. The carbon analysis began with weighing an empty porcelain crucible. The homogenized sample was then weighed and placed into the porcelain crucible. The sample was heated in an oven at 105°C for 3 hours. After removal from the oven, the sample was placed into a desiccator and weighed. This process was repeated until a constant

weight was obtained. Subsequently, the porcelain crucible was covered and placed into a furnace, heated at 300°C for 1.5 hours until a constant weight was obtained. Upon removal from the furnace, the sample was placed into a desiccator, and the porcelain crucible was weighed without a cover. Next, the crucible was covered again and placed into the furnace, heated at 550°C for 2.5 hours until a constant weight was obtained. After from the furnace, the sample was placed into a desiccator, and the porcelain crucible was weighed without a cover. The organic carbon content was calculated using the following formula.

$$\text{Content at temperature } 300°C = \frac{d-a}{c-a} \times 100\%$$

$$\text{Content at temperature } 550°C = \frac{e-a}{c-a} \times 100\%$$

C-organic content = Content at temperature 300°C - 550°C

Note:

a = An empty porcelain crucible weight (g)

c = Sample weight after oven + porcelain crucible (g)

d = Sample weight after furnace 300^{o}C + porcelain crucible (g)

e = Sample weight after furnace 550^{o}C + porcelain crucible (g)

3.4.3 Ammonium Analysis

The ammonium contents was analyzed by the UV-Vis Spectrophotometry method. The analysis of ammonium content commenced by transferring 100 mL of the sample into a distillation flask, followed by the addition of 25 mL of borax buffer and 2 boiling stones. To the container erlenmeyer flask, 25 mL of borate acid was added. Subsequently, distillation was performed until the volume of distillate reached approximately 100 mL. Two milliliters of the distillate were mixed with 0.2 mL of potassium sodium tartrate solution, 0.4 mL of Nessler reagent, and distilled water to a total volume of 10 mL. The solution was then read using a UV spectrophotometer at a wavelength of 430 nm.

3.4.4 Phosphate Analysis

The phosphate content was analyzed by the UV-Vis Spectrophotometry method. The analysis commenced with the preparation by weighing approximately 1 gram of the sample. Subsequently, 10 mL of a mixture of HNO_3 and $HClO_4$ in a 1:1 ratio was added. The mixture was then heated on a hot plate until it became clear and white fumes appeared. Next, the solution was filtered, and distilled water was added up to 50 mL. Then, the prepared sample was combined with 3.2 mL of the PO_4 working solution and 1 mL of the AMV solution. The sample was then read using a UV spectrophotometer at a wavelength of 430 nm. The final determination of the PO_4 content was calculated using the following formula.

$$PO_4 \text{ Levels} = \frac{PO_4 \text{ from instrument x Distilate volume x Dilution factor}}{\text{Sample weight (g)}}$$

3.4.5 Optimization of Fungal Biomass Production

Growth optimization research was conducted in three stages: the first stage variations of supplementation, the second stage variations of pH, and the third stage variations of agitation speed. The production of fungal biomass in this research employed a fermentation method in Erlenmeyer flasks as conducted by Wikandari *et al*. (2023).

3.4.2.1 Stage 1 (Influence of Supplementation)

Fungal biomass produced from tapioca effluent using *R. oligosporus* from Raprima starter with a variety of additional treatments, 5 types of supplementations namely 5g/L of yeast extract, 2.5g/L of ammonium sulfate ($(NH_4)_2SO_4$), 3g/L of sodium nitrate ($NaNO_3$), 1.5g/L of ammonium nitrate (NH_4NO_3), 3.5g/L of dipotassium phosphate (K_2HPO_4) and control (without supplementation) (n=3). Production began with media preparation, 100 mL of tapioca effluent was added to each 250 mL Erlenmeyer flask. Then, supplementations were added in each Erlenmeyer flask and shaken to distribute the supplementations evenly. The media with added supplementations, then pH checking; if it is within the range of pH 4.5-5.5 no

adjustment is made. The Erlenmeyer flasks were covered with cotton plugs and plastic wrap for sterilization. Sterilization was performed using an autoclave at 121°C for 15 minutes. After sterilization, inoculation was carried out in the laminar. In the laminar, the cotton plugs of the Erlenmeyer flasks were opened, and Raprima starter containing $1x10^5$ spores/g were added as much as 3 grams. Then, the Erlenmeyer flasks were shaken to prevent clumping of Raprima in the media. Subsequently, the Erlenmeyer flasks were covered with cotton plugs and paper and placed on a shaker bath with agitation speed of 125 rpm at room temperature (28°C) for 48 hours. After 48 hours, harvesting was conducted by pouring the contents of the Erlenmeyer flasks onto a filter cloth, and the remaining biomass was rinsed with water and then squeezed. The flowchart of fungal biomass production stage 1 can be seen in Figure 1.

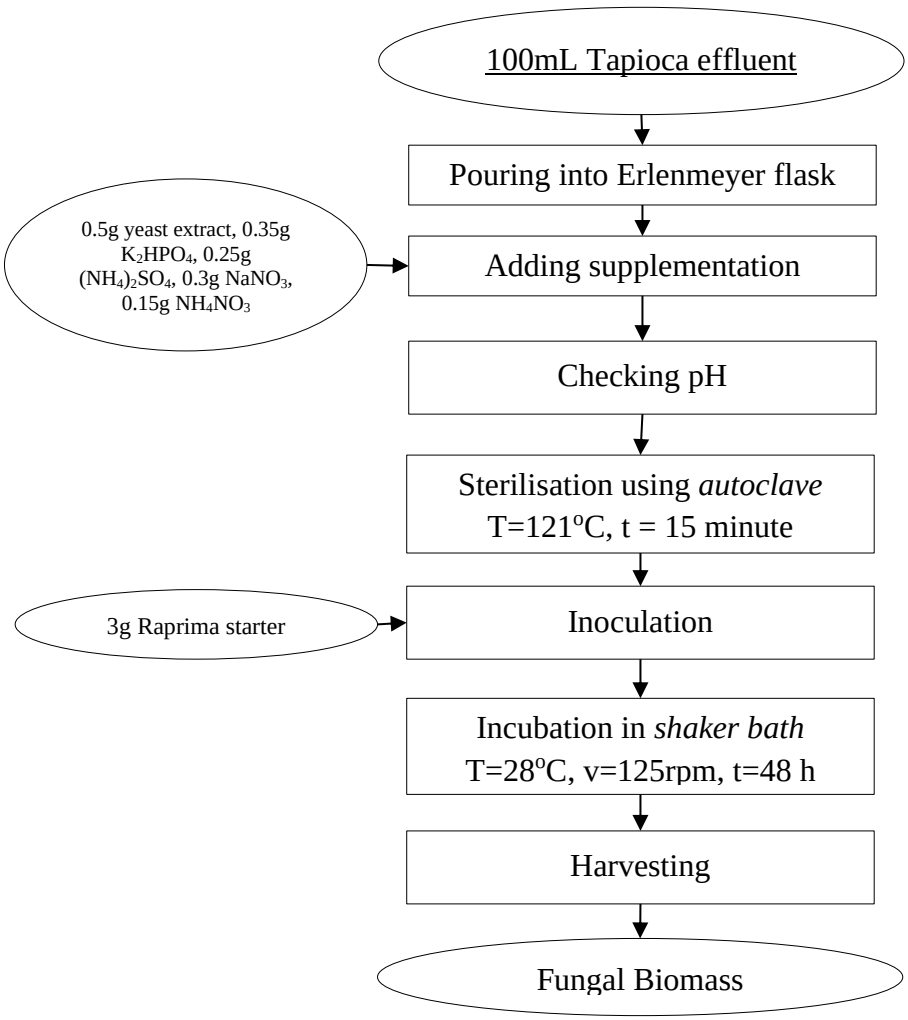

Figure 1. Stage 1 workflow diagram (Influence of Supplementation)

3.4.2.2 Stage 2 (Influence of pH)

Fungal biomass produced from tapioca effluent using *R. oligosporus* fungus from Raprima starter with pH variations of 4; 4.5; 5; 5.5; 6; 6.5 (n=3). The supplementation used were the best supplementation from stage 1. The fungal biomass production procedure is the same as in stage 1. Production began with media preparation, 100 mL of tapioca effluent was added to each 250 mL Erlenmeyer flask. Then, supplementations were added in Erlenmeyer flask and shaken to distribute the supplementations evenly. The media with added supplementation were then adjusted to the pH according to the treatment variation using 1N H_2SO_4 and 2N NaOH, measured using a pH meter. Subsequently, sterilization was conducted. Inoculation was carried out in the laminar, the cotton plugs of the Erlenmeyer flasks were opened, and Raprima starter containing $1x10^5$ spores/g were added as much as 3 grams. The Erlenmeyer flask was placed on a shaker at 125 rpm at room temperature (28°C) for 48 hours. Every 8 hours, pH checks were conducted, and if there were any changes in pH, adjustments were made to restore it to the initial treatment pH using 1N H_2SO_4 and 2N NaOH. These pH adjustments were carried out under aseptic conditions in the laminar. After 48 hours, harvesting and weighing were conducted. The flowchart of fungal biomass production stage 2 can be seen in Figure 2.

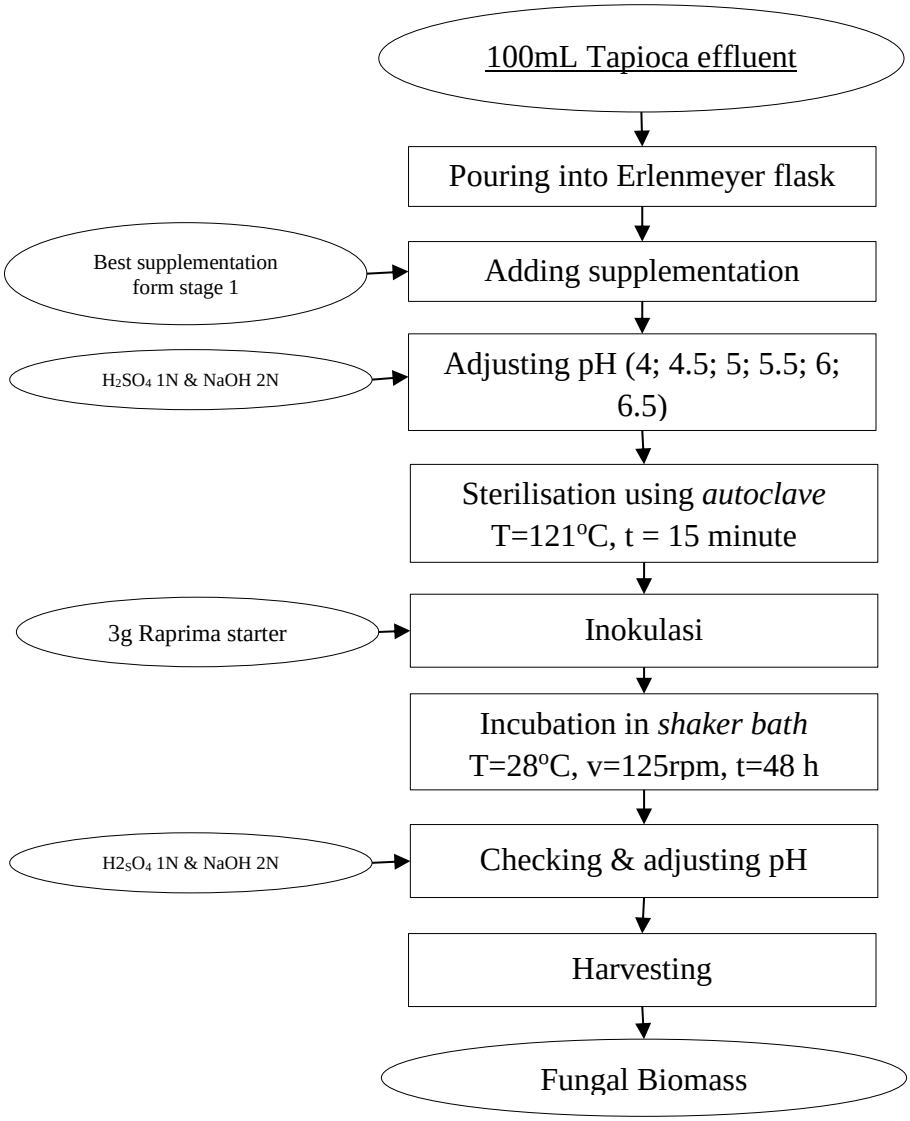

Figure 2. Stage 2 workflow diagram (Influence of pH)

3.4.2.3 Stage 3 (Influence of Agitation Speed)

Fungal biomass produced from tapioca effluent using *R. oligosporus* fungus from Raprima starter with agitation speed variations of 110 rpm, 125 rpm, and 140 rpm (n=6). The fungal biomass production procedure is the same as previous stage. Production began with media preparation, 100 mL of tapioca effluent was added to each 250 mL Erlenmeyer flask. The supplementation used were the best supplementation from stage 1. The supplementations were added in each Erlenmeyer flask and shaken to distribute the supplementations evenly. The media with added supplementations, then adjusted to pH according to the best treatment

results from stage 2 using 1N H_2SO_4 and 2N NaOH, using a pH meter. The Erlenmeyer flasks were covered with cotton plugs and plastic wrap for sterilization at 121°C for 15 minutes. After sterilization, inoculation was carried out in the laminar. Raprima starter containing $1x10^5$ spores/g were added as much as 3 grams. Then, the Erlenmeyer flasks were shaken to prevent clumping of Raprima in the media. The Erlenmeyer flask was placed on a shaker operated at speeds of 110 rpm, 125 rpm, and 140 rpm at room temperature (28°C) for 48 hours. After 48 hours, harvesting was conducted by pouring the contents of the Erlenmeyer flasks onto a filter cloth, and the remaining biomass was rinsed with water and then squeezed. The flowchart of fungal biomass production optimization stage 3 can be seen in Figure 3.

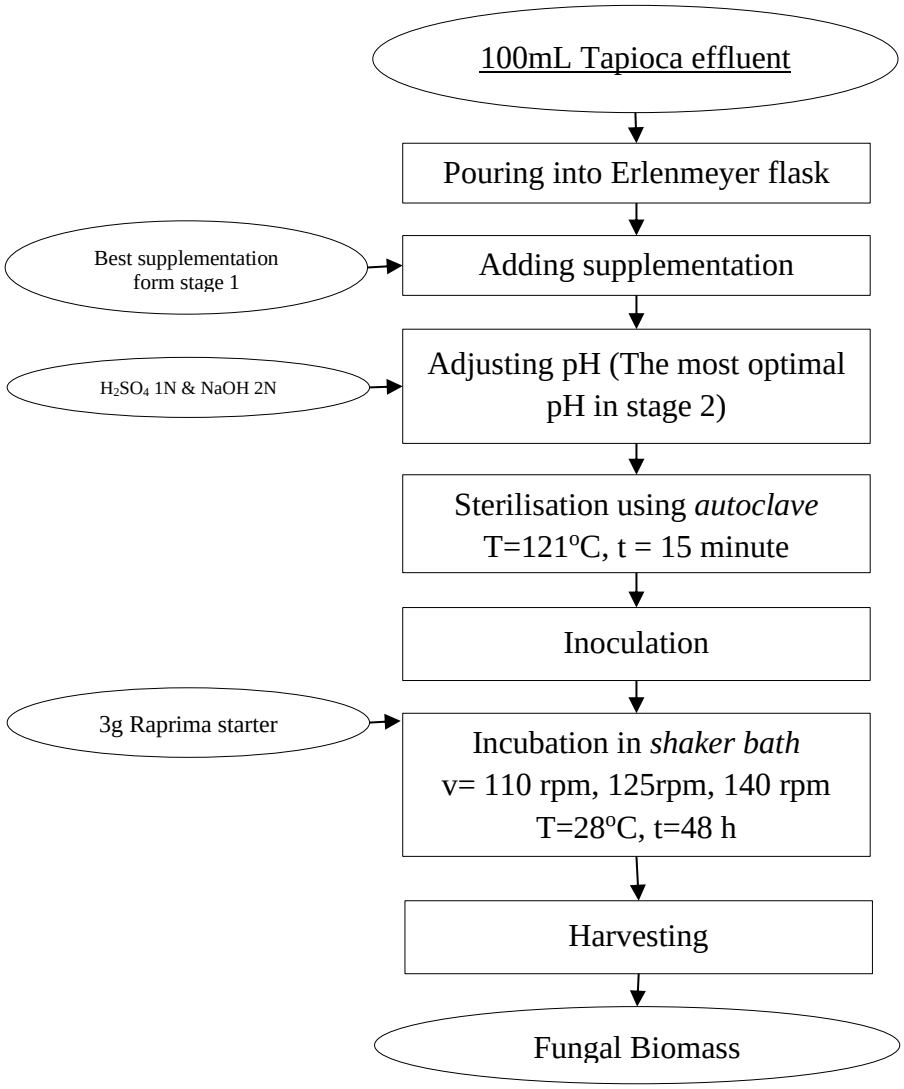

Figure 3. Stage 3 workflow diagram (Influence of agitation speed)

3.4.6 Determination of Biomass Quantity

The fungal biomass mycelium was harvested after 48 hours of cultivation, followed by washing with water three times. Subsequently, the biomass was squeezed using a filter cloth, then dried using an oven at 100°C for 24 hours and weighed to determine the weight of the fungal biomass.

3.4.7 Ash Content Analysis

The ash contents was analyzed by the gravimetric method AOAC (2016). The analysis commenced by oven-drying empty porcelain crucibles at 105°C for 30 minutes, followed by cooling and placement in a desiccator for 15 minutes. Subsequently, the crucibles were weighed. This process was repeated until a constant weight of the crucible was obtained. Next, dried sample (1g) was placed into a porcelain crucible and weighed. The crucible containing the sample was ignited over a flame of a stove until no smoke was produced. Then, ashing was carried out using a furnace at 550°C for 3 hours until white ash was formed. The sample was then placed in a desiccator for 15 minutes and subsequently weighed until a constant weight was obtained. The ash content was calculated using the following formula:

$$\text{Ash content (\%)} = \frac{(c-a)}{(b-a)} \times 100\%$$

Note:

a = An empty porcelain crucible weight (g)

b = Porcelain crucible weight + sample weight (g)

c = Porcelain crucible weight + ash weight (g)

3.4.8 Protein Analysis

The protein contents was analyzed by the Kjeldahl method AOAC (2016). A sample weighing 0.1g was placed into a Kjeldahl flask, followed by the addition of 0.7g of HgO.K2SO4 catalyst and 3mL of H2SO4. Subsequently, the Kjeldahl flask was destructed by heating in an acidic environment until the sample solution became

clear. The cooled sample solution was then diluted with distilled water. The sample solution was distilled with 10-20mL of NaOH-$Na_2S_2O_3$ solution. The distillate was collected in an Erlenmeyer flask containing 5 mL of 4% boric acid and 3 drops of BCG-MR indicator. The obtained distillate was then titrated with standardized HCl solution until a color change from blue to pink occurred, indicating the volume of titration required. The determination of the protein content of the sample was calculated using the following formula:

$$\text{Protein (\%)} = \frac{(Y-Z)x\ (N \times 0.014 \times 6.25)x\ 100\%}{W}$$

Note:

Y = ml HCl (titrant) sample

Z = ml HCl (titrant) blanko

W = Sample weight (g)

N = Normality of HCl (N)

6.25 = Conversion factor

3.4.9 Fat Content Analysis

The fat content was analyzed by the Soxhlet method AOAC (2016). The analysis commenced by sample preparation, the sample was dried at 100°C for 6 hours. Subsequently, the Soxhlet flask was oven-dried at 100°C for 1 hour. Then, the flask was placed in a desiccator and weighed. A dry sample of 1g was wrapped in filter paper with cotton placed on the top and bottom, forming a thimble. The Soxhlet setup included a heating mantle, fat flask, lead weight, Soxhlet extractor, and condenser. The sample was placed into the chamber of the Soxhlet apparatus, and petroleum ether solvent was added according to the size of the Soxhlet flask. The sample was then extracted for approximately 6 hours until the solvent returned through the siphon into the Soxhlet flask. After extraction, the Soxhlet flask was oven-dried at 105°C for 24 hours. Subsequently, the Soxhlet flask was placed in a desiccator and weighed until a constant weight was obtained. The determination of fat content was calculated using the following formula:

$$\text{Fat content (\%)} = \frac{(c-a)}{b} \text{ x } 100\%$$

Note :

a = An empty Soxhlet flask weight (g)

b = Sample weight (g)

c = Weight of extracted Soxhlet flask after oven-dried (g)

3.4.10 Protein Digestibility Analysis

The digestibility of fungal biomass was analyzed using in vitro method and continued with the Kjeldahl method previously described by Ketnawa *et al*., (2021) with modifications. The sample (0.2g) was put into a 250mL Erlenmeyer. The sample was dissolved in 9 mL of 0.2 N laphole buffer solution (pH 2) and 1 mL of 2% pepsin enzyme, then the Erlenmeyer was covered using alumonium foil. Subsequently, the sample was incubated in a water bath at 37°C for 1.5 hours. Afterward, the sample was centrifuged at a speed of 3000rpm for 20 minutes. Five milliliters of the supernatant resulting from centrifugation were transferred into a reaction tube and mixed with 5 mL of 20% trichloroacetic acid. The reaction tube was covered again with aluminum foil and left for 1.5 hours. Then, the supernatant was filtered using Whatman no. 41 filter paper. The filtered supernatant solution could then be analyzed for total filtrate nitrogen content using the Kjeldahl method. The determination of protein digestibility was calculated using the formula:

$$\text{Protein Digestibility (\%)} = \frac{\text{N total filtrate}}{\text{N total sample}} \text{ x dilution factor x } 100\%$$

3.4.11 Crude Fiber Analysis

The crude fiber was analyzed using the acid-base hydrolysis method referring to SNI 01-2891-1992 with modifications. The analysis began with the oven drying of empty fiber sleeves at 100°C for 30 minutes. Subsequently, the sleeves were placed in a desiccator and weighed. Then, samples (1-2g) placed into the sleeves. Place the sleeve containing the sample into the sleeve rack. Then, pour 500mL of 1.25% H_2SO_4 into a 1000mL beaker. Place the sleeve rack into the beaker. Position the beaker on a heating plate and place a cooling glass on top of the beaker. Heat the heating plate to 100°C for 30 minutes, rotating the sleeve rack to induce agitation

in the sleeves containing the samples. After 30 minutes, turn off the heating plate, discard the 1.25% H_2SO_4, and rinse the sleeve rack and beaker containing the sleeves with flowing water. Next, repeat the process using 1.25% NaOH. Then, remove the sleeves from the sleeve rack and oven dry them at 100°C for 1-3 hours until a constant weight. The determination of crude fiber content was calculated using the formula:

$$\text{Crude Fiber (\%)} = \frac{a-b}{W} \times 100\%$$

Note:

a = Residue weight in dried sleeve (g)

b = Weight of empty sleeve (g)

W = Sample weight (g)

3.4.12 Cyanide Content Analysis

The cyanide content was analyzed using method previously described by Egan *et al*., (1994) with modifications. The sample (3g) diluted with distilled water to a volume of 100 mL. The diluted sample was then incubated for 2 hours. Subsequently, the sample was filtered. One milliliter of the sample filtrate was mixed with 1 mL of 0.1N NaOH and added to 5 mL of alkaline picrate pH 11 solution (a mixture of 0.5g/100mL picric acid and 2.5g/100ml Na_2CO_3). The solution was then heated at 100°C for 30 minutes until the solution volume reached 10 mL. The solution was filtered and analyzed using a UV-Vis spectrophotometer at a wavelength of 250 ɲm.

V. CONCLUSION & SUGGESTION

5.1 Conclusion

The conclusions of this study are as follows:

1. Tapioca effluent has the potential as a fungal biomass growth medium because it contains essential nutrients required by filamentous fungi, namely nitrogen 0.04%, organic carbon 0.47%, phosphate 1.36 mg/kg, and ammonium 32.6 mg/L, although supplementation is still necessary.
2. Media supplementation, pH level, and agitation speed significantly affect the fungal biomass yield. The optimal conditions resulting in the highest fungal biomass are supplementation with yeast extract, pH 5.5, and agitation speed at 125 rpm.
3. Fungal biomass produced under optimal conditions exhibits chemical characteristics with higher nitrogen, ammonium, and phosphate levels compared to tapioca effluent, but lower cyanide content than tapioca effluent and does not exceed the cyanide consumption threshold. The nutritional content of fungal biomass from optimal conditions includes a protein content of 26.447% (db), fat content 4.81% (db), ash content 4.26% (db), crude fiber content of 5.96% (db), and a protein digestibility 61.25%. Fungal biomass from this study demonstrates the advantage of high protein digestibility.

5.2 Suggestion

Based on this study, the following recommendations should be considered for future research:

1. Further research is needed to effectively reduce cyanide levels in fungal biomass to ensure its safety even when consumed in large quantities.
2. Additional studies are warranted to explore the supplementation of other organic nitrogen sources such as peptone, glycine, and others for mycelium production.
3. Further investigation is necessary to understand the carbon, nitrogen, and phosphate consumption by *R. oligosporus* fungi.

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