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Optimization of *Rhizopus* sp. Growth Media for Biofoam Manufacture: Effect of Temperature and Substrate Composition

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Abstract

Biofoam is a biodegradable material that can substitute the use of styrofoam. Biofoam can be produced from *Rhizopus* sp. mycelia. In the development of biofoam from fungal mycelia, mycelia act as a reinforcement or binder for the substrate (media) because mycelia grow and spread throughout the substrate. Therefore, optimization of the growth medium of *Rhizopus* sp. by varying the substrate composition and incubation temperature had been done in this research. The substrate consisted of sugarcane trash (particle size 20, 40, and 60 mesh), starch (soybean, sago, and rice), $CaCO_3$, and distilled water. *Rhizopus* sp. inoculum that had been mixed with the substrate was incubated at certain temperatures (29 and 35°C) for 7 days. The FESEM-EDS analysis of starches showed that soybean has more nutrients than rice and sago starch. The optimum substrate for the mycelia growth of the *Rhizopus* sp. is TK 1, which consists of 20 mesh sugarcane trash, soybean flour, $CaCO_3$, and distilled water with an incubation temperature of 29°C. That mycelia grew well and evenly distributed throughout the TK 1 substrate with an average length of ± 1.3 cm. The mycelia distribution throughout the media can also be seen by morphology analysis using Kayence digital microscope.The suitable substrate composition and incubation temperature can optimize the growth of *Rhizopus* sp. mycelia.

INTRODUCTION

From 2015-2016, there was 32% of styrofoam from the total waste found in Jakarta Bay (Cordova & Nurhati, 2019). That phenomenon shows the high public consumption of the use of styrofoam, especially in food packaging. Whereas styrofoam is one of the non-biodegradable materials causing environmental pollution. One of the alternative solutions to reduce the use of styrofoam is to substitute it with biofoam. Biofoam is a biodegradable material expected to replace the role of styrofoam and its function. In recent years, there have been biofoam developments from fungal mycelia such as *Pycnoporus sanguineus, Pleurotus albidus, Lentinus velutinus* (Bruscato et al., 2019) and *Rhizopus* sp. (Indarti et al., 2021).

Rhizopus sp. is classified into the phylum Zygomycota and the order Mucorales. The *Rhizopus* sp. has a grey color and it has mycelium

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(stolons) and hyphae that are not septate or insulated (coenocytic hyphae). These hyphae will form rhizoids that stick to the growth medium (substrate) and turn into dark color when the fungus was going old (Dewi et al., 2014). The fungus *Rhizopus* sp. reproduces asexually (vegetatively) therefore it was the potential to be a reinforcement for the substrate due to its mycelium spreading over the substrate surface. *Rhizopus* sp is also classified as the non-pathogenic fungi therefore the biofoam produced is safer to be applied as food packaging material.

The substrate used as a growth medium for mycelia generally consists of lignocellulosic materials, mineral salts, and water. Sawdust, wheat bran (Bruscato et al., 2019), sugarcane bagasse, cocodust (Indarti et al., 2021), rice husk, and rice bran (Nashiruddin et al., 2022) are the agricultural waste containing lignocellulose and serves as carbon sources. The function of mineral salts, such as $MgSO_4$, K_2HPO_4, $CaCO_3$, $ZnSO_4$, dan $CuSO_4$, is as the micronutrients for the growth of fungal mycelia (Laksana & Waluyo, 2021) while water as a humidity regulator.

The substrate composition affected the growth of fungal mycelia and the physicochemical properties of the biofoam produced. Hence, the optimization of the *Rhizopus* sp. growth medium has been studied. Although using the same fungus, the substrate composition and the analysis used in this study were different from the research conducted by Indarti et al. (2021). In this study, the substrate used as a growth medium consisted of sugarcane fiber, flour, $CaCO_3$, and distilled water. There are several variations of treatment carried out in this study to obtain optimum growth media, namely variations in substrate composition, consisting of fiber size (20, 40, and 60 mesh) and types of flour (soybean flour, rice, and sago), as well as variations incubation temperature (29°C and 35°C). The chemical content of sugarcane trash and each type of flour was also carried out in this study to determine their effect on the growth of fungal mycelia. Observation of fungal mycelia growth was carried out directly using a Keyence digital microscope.

RESEARCH METHODOLOGY

Preparation of the starting material

Sugarcane trash was dried in the oven (105°C, 24 h), was milled using a grinder, then soaked in the water to separate it from dirt and dust. After that, the sugarcane trash was sieved using a sieve (20, 40, and 60 mesh) to obtain the required mesh size.

Growth Media Preparation

The cleaned sugarcane was then autoclaved at 121 °C for 15 minutes. The media was made by mixing the autoclaved sugarcane trash with starch, $CaCO_3$, inoculum *Rhizopus* sp. and distilled water with a mass ratio in percent is 5:2:5:12 (% w/w) of the sugarcane fiber used. The mixture was stirred evenly then put into a sterile polypropylene mold, inserted into heat-resistant plastic, and incubated for ± 7 days. The variations in media composition and incubation temperatures can be seen in Table 1.

The growth observation and mycelia distribution of the fungus *Rhizopus* sp. in media is carried out directly on the 7th day after incubation. Media that can be overgrown with mycelia is evenly distributed throughout the surface, continued analysis of the media will be carried out. The advanced analysis is the length measurement of the *Rhizopus* sp. mycelia and sample morphology.

The measurement of the *Rhizopus* sp. mycelia is carried out on days 3, 5, and 7, under microscope and using the ruler following the method made by Suryani & Carolina (2017). This measurement was carried out by 3 replications starting from the surface of the media until a certain depth of the longest mycelium growth. After 7 days, media samples are dried in the oven (60°C, 2 days). After drying, the media sample was characterized by morphology using a digital Keyence microscope.

Chemical Content Analysis

The chemical content analysis of sugarcane trash including extractive (TAPPI, 1996), total lignin consists of acid-soluble lignin (ASL) and acid-insoluble lignin (AIL) (Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, 2011), holocellulose (Wise et al., 1946) and α- cellulose (Rowell et al., 2012). The measurement was done by 3 replications.

Morphology and the Composition of Starch

Morphology of *Rhizopus* sp. inoculum, soybean, sago, and rice starch was analyzed using Field Emission Scanning Electron Microscope (FE-SEM Thermo Scientific - Quatro S). The element content in starch was determined using Energy Dispersion Spectroscopy (EDS) which include in

Table 1. Variations of media composition for the growth of *Rhizopus* sp. fungi

No.	Sample	Particle size of SCT (mesh)			The type of starch			Incubation temperature (°C)		$CaCO_3$	Inoculum *Rhizopus* sp.	Distilled water
		20	40	60	Soybean	Sago	Rice	29	35			
1.	TK1	√			√			√		√	√	√
2.	TS1	√				√		√		√	√	√
3.	TB1	√					√	√		√	√	√
4.	TK2	√			√				√	√	√	√
5.	TS2	√				√			√	√	√	√
6.	TB2	√					√		√	√	√	√
7.	TK3		√		√			√		√	√	√
8.	TS3		√			√		√		√	√	√
9.	TB3		√				√	√		√	√	√
10.	TK4		√		√				√	√	√	√
11.	TS4		√			√			√	√	√	√
12.	TB4		√				√		√	√	√	√
13.	TK5			√	√			√		√	√	√
14.	TS5			√		√		√		√	√	√
15.	TB5			√			√	√		√	√	√
16	TK6			√	√				√	√	√	√
17	TS6			√		√			√	√	√	√
18.	TB6			√			√		√	√	√	√

the FESEM devices. The sample preparation was done by taking the sample on the carbon tape, then analyze the sample using FESEM-EDS with condition high vacuum (1 kV), spot size 3.0, and magnification of 1000x for FESEM, and mapping for EDS.

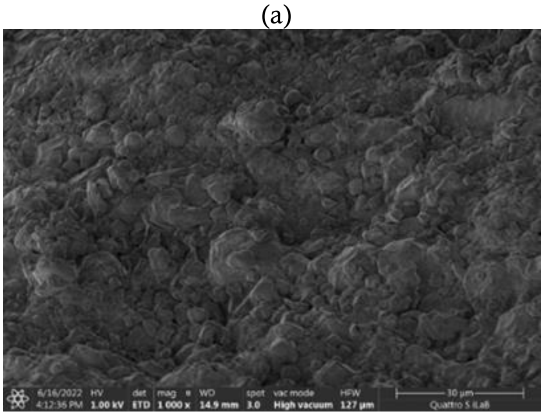
Morphology of samples was determined using digital microscope Keyence, in which samples are cut by 1x1 cm then its morphology will be analyzed on magnification 250X-500X. Morphological analysis of the media was carried out to determine the distribution of the *Rhizopus* sp. mycelium which grows on the surface and inside the sample.

RESULTS AND DISCUSSION

Preparation of Growth Media for The Mycelia of The Fungus *Rhizopus* sp.

Rhizopus sp. is a mold used in the tempeh fermentation process. In this study, the inoculum of *Rhizopus* sp. fungi was obtained from tempeh yeast "Raprima". Figure 1 is the morphology of tempeh yeast as seen using FESEM. Figure 1 shows that tempeh yeast is composed of thousands of immobilized inoculums of the fungus *Rhizopus* sp. The inoculum can grow well in media that contains elements needed in the growth process,

(a)



(b)

Figure 1. (a) The fungus *Rhizopus* sp. on the tempeh inoculum and (b) the mycelia morphology of the fungus *Rhizopus* sp. were analyzed by FESEM.

such as carbon, nitrogen, calcium, potassium, and other mineral elements (Aurelia et al., 2022).

The growth media of *Rhizopus* sp. that was used in this study consisted of sugarcane trash,starch, and $CaCO_3$ which functions as a source of nutrients and the function of distilled water is to regulate the moisture. In addition, $CaCO_3$ is also a mineral whose availability is abundant and economical. The media is used as a place to inoculate tempeh yeast.

The Effect of Sugarcane Trash Particle Size on The Growth of The *Rhizopus* sp. Mycelia

Sugarcane trash is one of the biomass wastes containing high lignocellulose. The content of lignin, hemicellulose, and α-cellulose in sugarcane trash were 19.03%, 34.64%, and 32.76%, respectively (Table 2). The lignin content in sugarcane trash from PTPN X is slightly higher and the cellulose and hemicellulose content is slightly lower than the sugarcane trash from Rajawali PG, which is lignin 17.44%, cellulose 35%, and hemicellulose 35.55% (Thontowi et al., 2020).

Table 2. The chemical component of sugarcane trash.

Chemical content	Percentage (%)
Extractive	13.44 ± 1.70
Total lignin	19.03 ± 2.01
Holocellulose	67.40 ± 1.05
Alpha cellulose	32.76 ± 2.13
Hemicellulose	34.64 ± 3.18

The particle sizes variation of sugarcane trash used in the media manufacture is 20, 40, and 60 mesh. The size of the sugarcane trash affects the media compaction and porosity, where is the smaller of particle size cause the compaction of the media was greater and the porosity was lower. Table 3 shows that the *Rhizopus* sp mycelia grew more evenly on the surface of the media containing sugarcane trash with a particle size of 20 mesh compared to 40 and 60 mesh. This caused the *Rhizopus* sp. mycelia easier to bind the substrates with a larger size (20 mesh).

The particle size of the substrate is very influential on the ability of fungal mycelia in utilizing nutrients and attachment to the substrate. Zhang et al. (2013), stated that the availability of surface area in solid substrate fermentation has an important role in microbial attachment and mass transfer. Smaller substrate particles cause substrate compaction and can interfere with aeration due to the lack of space between particles (Esa et al., 2014). Larger particles provide better aeration efficiency therefore the mycelia of *Rhizopus* sp. are more easily attached to the 20 mesh substrate. Esa et al. (2014) observed that filamentous fungi were able to grow and produce the pigments optimally on 18 mesh substrates, compared to 35 mesh substrates.

Effect of Starch Type on The Growth of The Rhizopus sp. Mycelia

The growth of the *Rhizopus* sp. mycelia was also influenced by the use of the starch type. Table 3 shows that the *Rhizopus* sp. mycelia grew well and evenly on media using soybean starch compared to sago and rice starch. On the media containing rice starch, mycelia grew unevenly and in less quantity. Mycelia even grow very little on the media containing sago starch.

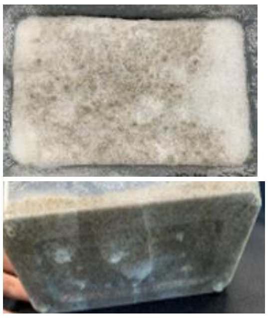
The starch type is closely related to the element content in it, and the element content in one starch can be different from other starch. Figure 2 shows that the three types of starch used in this study have different morphologies, in which soybean starch has an irregular shape (a combination of round and rectangular shapes), sago starch is round, and rice starch is square.

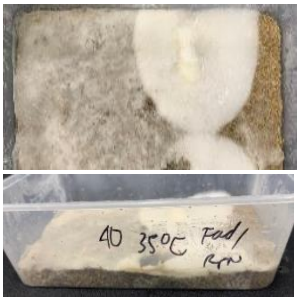
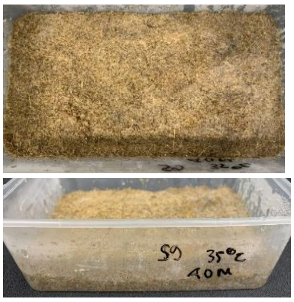
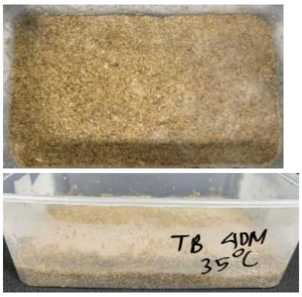
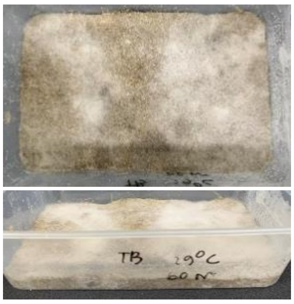
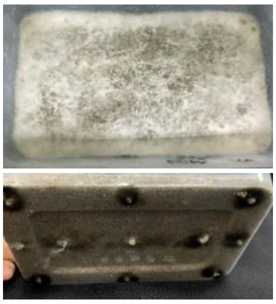
Not only the morphology, but the element content of the three types of starch is also different. Table 4 shows that sago starch contains only carbon and oxygen elements while soybean and rice starch have additional elements, such as potassium, phosphorus, sulfur, etc. The elements of carbon and oxygen in sago starch will form a glucose compound which is bonded to each other through glycosidic bonds to form amylose and amylopectin (Okazaki, 2018). The highest carbon content is found in soybean starch, while the highest oxygen content is found in sago starch.

The results study of the Ikechi–Nwogu (2012) showed that the fungi *Aspergillus niger, Aspergillus flavus, Aspergillus glaucus, Aspergillus terreus, Penicillium chrysogenum, Fusarium oxysporium,* and *Rhizopus* stolonifer grew well on Soybean Dextrose Broth (SDB) media because soybeans were known to contain carbohydrates, protein, fiber, fat, and other nutrients. The growth of fungi in this medium was better than in the Potato Dextrose Broth (PDB), Sawdust Sucrose Broth (SSB), and Ofor Sucrose Broth (OSB).

Hence it can be seen that the growth of *Rhizopus* sp. is strongly influenced by the nutrients in the growth media such as elements of carbon,

Table 3. The growth of the *Rhizopus* sp. mycelia

Simple Description	Sample Code		
SSF media which made from 20 mesh sugarcane trash with variations of soybean, sago, and rice starch at an incubation temperature of 29 °C.	TK 1  *Rhizopus* sp mycelia has covered the entire fiber surface	TS 1  *Rhizopus* sp mycelia grows very little on the growth media	TB 1  *Rhizopus* sp mycelia has not covered the entire fiber surface
SSF media which made from 20 mesh sugarcane trash, with variations of soybean, sago, and rice starch at an incubation temperature of 35 °C.	TK 2  Mycelia of Rhizopus sp has not covered the entire fiber surface	TS 2  Mycelia of *Rhizopus* sp grows very little on the sugarcane trash fiber	TB 2  Mycelia of *Rhizopus* sp grows very little on the sugarcane trash fiber
SSF media which made from 40 mesh sugarcane trash, with variations of soybean, sago, and rice starch at an incubation temperature of 29 °C.	TK 3  Mycelia of *Rhizopus* sp has not covered the entire fiber surface	TS 3  Mycelia of *Rhizopus* sp grows very little on sugarcane trash fiber	TB 3  Mycelia of *Rhizopus* sp has not covered the entire fiber surface

Simple Description	Sample Code		
SSF media which made from 40 mesh sugarcane trash, with variations of soybean, sago, and rice starch at an incubation temperature of 35 °C.	TK 4  Mycelia of *Rhizopus* sp has not covered the entire fiber surface	TS 4  Mycelia of *Rhizopus* sp grows very little on sugarcane trash fiber	TB 4  Mycelia of *Rhizopus* sp grows very little on sugarcane trash fiber
SSF media which made of 60 mesh sugarcane trash, with variations of soybean, sago, and rice starch at an incubation temperature of 29 °C.	TK 5  Mycelia of *Rhizopus* sp has not covered the entire fiber surface	TS 5  Mycelia of *Rhizopus* sp grows very little on sugarcane trash fiber	TB 5  Mycelia of *Rhizopus* sp has not covered the entire fiber surface
SSF media made of 60 mesh sugarcane trash, with variations in soybean, sago, and rice starch at 35 °C incubation temperature	TK 6  Mycelia of *Rhizopus* sp has not covered the entire fiber surface	TS 6  Mycelia of *Rhizopus* SP grows very little on sugarcane trash fiber	TB 6  Mycelia of *Rhizopus* sp grows very little on sugarcane trash fiber

oxygen, sulfur, phosphorus, potassium, bromium, magnesium, and aluminum.

Effect of Incubation Temperature on The Growth of The *Rhizopus* sp. Mycelia

Temperature is one factor that is very influential on the growth of the mycelia of the fungus *Rhizopus* sp due to if the incubation temperature is not suitable, then the microorganism (in this study is *Rhizopus* sp) cannot grow properly or even die. Incubation temperature also affects the activity of enzymes produced by these microorganisms. As we know that during incubation, microorganism produce enzymes (such as laccase, cellulose, amylase, etc) to degrade macromolecules (such as lignin, cellulose, hemicellulose, etc) into their monomer (such as glucose, fructose, etc). Then, these monomers will

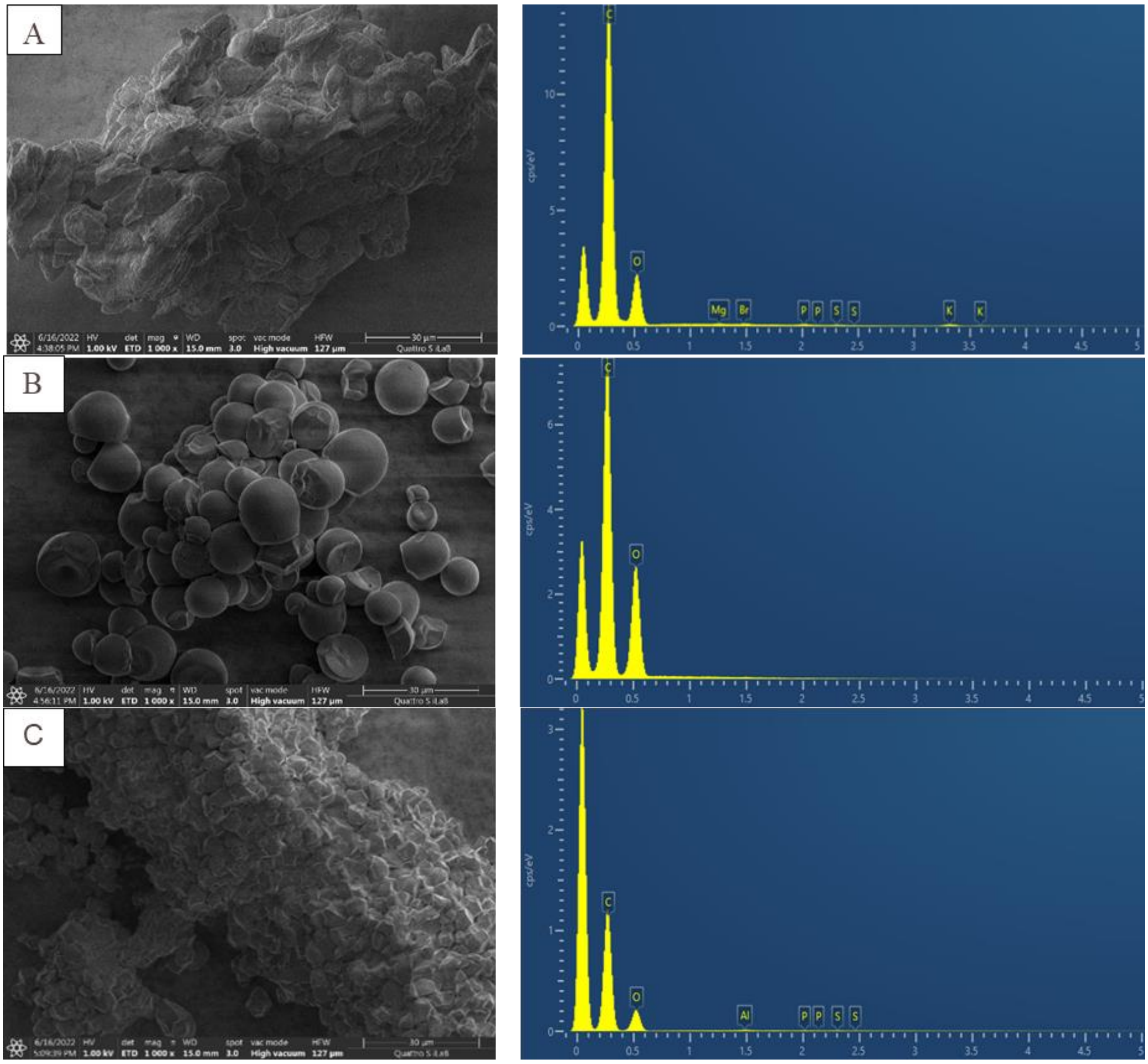


Figure 2. Morphology and elements content in A) soybean starch B) sago starch dan C) rice starch.

Table 4. The elements content in soybean, sago, and rice starch.

Element (%)	Soybean starch	Sago starch	Rice starch
Carbon (C)	74.0 ± 0.2	68.6 ± 0.2	72.0 ± 0.2
Oxygen (O)	24.1 ± 0.2	31.4 ± 0.2	27.7 ± 0.2
Sulphur (S)	0.1 ± 0.0	-	0.1 ± 0.0
Fosfor (P)	0.3 ± 0.0	-	0.1 ± 0.0
Calium (K)	1.0 ± 0.0	-	-
Bromium (Br)	0.3 ± 0.1	-	-
Magnesium (Mg)	0.1 ± 0.0	-	-
Aluminium (Al)	-	-	0.2 ± 0.0

be absorbed by microorganism or the fungus *Rhizopus* sp and used for the growth of mycelia (Manan et al., 2021).

According to Table 1, known that the mycelia of the fungus *Rhizopus* sp. grow better and more evenly at an incubation temperature of 29 °C compared to 35 °C. Other studies also gave the same results, which is the optimum temperature for incubation of *Rhizopus* sp. is 29 °C (Indarti et al., 2021) and 30 °C (Pervez, 2011). That condition is related with the research conducted by Nugraha et al. (2022) which *Rhizopus oligosprorus* able to produce enzymes optimally in a temperature 25-30 °C, a pH of 3-7, and an optimum incubation period of 72 h. In that temperature, the enzymes protease (Rauf et al., 2010) and lipase (Waseem et al., 2018) are produced by the fungus *Rhizopus* sp.

The Length Measurement of The *Rhizopus* Sp. Mycelia and The Morphology of Optimum Media

Table 1 shows that only one sample from the 18 media samples will be continued for the length measurement of mycelia and surface morphology. Sample TK 1 is the selected media because the entire surface of the media has been overgrown with *Rhizopus* sp. mycelia. TK 1 was composed of sugarcane trash with a particle size of 20 mesh, soybean starch, $CaCO_3$, and distilled water which was incubated at 29°C.

Figure 3 shows that the mycelia experienced an increase in length of 0.1 cm, from 1.2 cm on the third day to 1.3 cm on the fifth day, then did not experience an increase in length from the fifth to the seventh day. The growth of mycelia on the fifth to the seventh day was spread over the entire surface of the media. Manan et al. (2021) revealed that in the first phase of growth (the 3rd day), the mycelia produced a large number of hyphae to form a network on the substrate surface, which continued to grow at the tip through the elongation of the tip and formed a tubular structure.

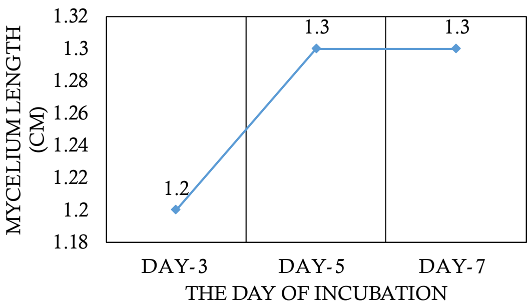


Figure 3. *Rhizopus* sp. mycelium length growth rate graph.

The growth and spread of *Rhizopus* sp mycelia on the biomass substrate could be observed by a Kayence digital microscope. Figure 4 shows that the mycelia of the fungus *Rhizopus* sp. (white color) are scattered on the surface of TK 1 media (brown yellow color). According to research by Bruscato et al. (2019), regarding composites made from a biomass substrate based on fungal mycelia, the resulting mycelia can produce an elongated and well-distributed hyphae morphology into the substrate particles and their gaps. Composites made from mycelia-based biomass substrates can form a matrix on the fibers and compact the material (Manan et al., 2021).

(a)

(b)

Figure 4. The morphology of the *rhizopus* sp. mycelia distribution at (a) 50x and (b) 500x magnification.

CONCLUSION

Sugarcane trash contains lignin 19.03%, hemicellulose 34.64%, cellulose 32.76%, and extractives 13.44 %. The element content of soybean starch is more complete and its carbon content is also higher than rice and sago starch. Factors affecting the growth of the fungus *Rhizopus* sp. are fiber particle size, nutrients (contained in starch), and incubation temperature. The best particle size of sugarcane trash, the type of starch, and the incubation temperature for the growth medium of *Rhizopus* sp. is 20 mesh, soybean starch, and 29 °C respectively. The optimum conditions were obtained in the TK 1 sample. This result study was very important because able to use to manufacture a biofoam-based mycelia of *Rhizopus* sp in the next research.

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