Jurnal Riset Biologi dan Aplikasinya
https://journal.unesa.ac.id/index.php/risetbiologi

The Effect of Water Concentration on Growth Media on Lipid Production by Oleaginous Fungi Isolate BR 2.2

Herin Yoga Lesti[1], Miftahul Ilmi[2*]
Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Sel., Senolowo, Sinduadi, Kec. Mlati, Kabupaten Sleman, Daerah Istimewa Yogyakarta 55281
*Corresponding Author:
e-mail: m.ilmi@ugm.ac.id

Article History

Received : 1 July 2022
Revised : 4 August 2022
Approved : 12 September 2022
Published : 30 September 2022

Keywords
Oleaginous fungi, glucose, water content, lipid, biomass

ABSTRACT

Oleaginous fungi are one of the microorganisms that can accumulate a high number of biomasses quickly (within 96-130 hours) and are often used to produce lipids. The growth of fungi depends on the chemical composition of the environment in which it grows. The growth media of fungi must contain high carbohydrates as a source of nutrients and high nitrogen content. One of the carbon sources that fungi can use in the growth process is glucose. BR 2.2 isolate is an oleaginous fungus capable of accumulating 28.44% lipids from the total dry biomass with glucose as a carbon source in 50 mL of growth media. Therefore, this study was conducted to determine the effect of variations in the volume of media and incubation time on the production of biomass and lipid isolate BR 2.2. Biomass and lipid production were analyzed at media with additional water volumes of 10, 20, 30, 40, and 50 mL with 48, 96, and 144 hours of incubation times. The results showed that lipid accumulation and biomass production increased with the reduction of water content in the growth media and reached the highest number in the media volume of 20 mL with an incubation time of 144 hours, i.e., 0.87±0.04 g/L and 12.53±0.29 g/L. It can be concluded that biomass and fungal lipid increased along with incubation time and nutrient concentration.

How to cite: Lesti, H.Y & Ilmi, M. 2022. The Effect of Water Concentration on Growth Media on Lipid Production by *Oleaginous Fungi* Isolate BR 2.2. *Jurnal Riset Biologi dan Aplikasinya*, 4(2):51-56. DOI: 10.26740/jrba. v4n2.p.51-56.

INTRODUCTION

Fungi are a group of multicellular and filamentous eukaryotic microorganisms. Fungi cells do not contain chlorophyll, resulting in the inability of fungi to carry out the photosynthesis process. Therefore, fungi are chemo-organoheterotrophs that obtain energy through the oxidation of organic compounds. Fungi are an aerobic microorganism that requires oxygen to survive (Fifendy, 2017).

Microorganisms have a high productivity level with a low need for a growth media. Microorganisms that can accumulate lipid biomass above 20% are called oleaginous fungi. These microorganisms are often used in lipid production during the growth of secondary metabolites under conditions of excess carbon and limited nutrients. (Sergeeva et al., 2008). The high lipid accumulation can be up to 80% compared to bacteria or microalgae and is generally dominated by triglycerides (Dey et al., 2014).

Oleaginous fungi are widely used as a source of lipids in biodiesel production. The use of oleaginous fungi are based on several advantages in the industrial sector compared to other plants and microalgae, namely, oleaginous fungi are easy to grow in bioreactors and have a short life cycle. Its short life cycle indicates a fast growth rate of oleaginous fungi and is not affected by space, light, or climate change (Shafiq & Chechan, 2019). In addition to their short life cycle, these microorganisms have a high growth rate and biomass density and can be grown in conventional bioreactors to maximize yield and productivity. In addition, cells from filamentous fungi are generally easier to harvest than algae and yeasts, especially

when grown in the form of pellets or mycelia (Yang et al., 2019).

Growth media is divided into three types based on density: solid, semisolid, and liquid media (broth). Growth in a solid media generally shows the formation of colonies on the surface of the media, while growth in a liquid media is characterized by increasingly cloudy liquid. The turbidity is caused by the multiplication of microorganism cells (Murwani, 2015).

Each type of microorganism requires a different growth media adapted to each microorganism's nutritional needs. Microorganisms need about ten macro elements, namely Carbon (C), Oxygen (O), Hydrogen (H), Nitrogen (N), Sulfur (S), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), and Iron (Fe). The first six macro elements are used to synthesize carbohydrates, lipids, proteins, and nucleic acids, while the other four are present in cells as cations. In addition to macro elements, microorganisms also need several microelements such as Manganese (Mn), Zinc (Zn), Cobalt (Co), Molybdenum (Mo), Nickel (Ni), and Copper (Cu). Microelements are generally part of enzymes and cofactors (Basu et al., 2015).

In general, the growth of microorganisms can include two major processes: Solid-State Fermentation (SSF) and Submerged Fermentation (SmF). SSF is a process for the growth of microorganisms under uncontrolled conditions without the use of excess water during the process (Mienda & Idi, 2011). SmF is a process of growing microorganisms in a liquid media to the optimize appropriate nutrients. The process involves the growth of microorganisms in a closed reactor containing a fermentation media and a high oxygen concentration (Doriya et al., 2016). However, in lipid production, SmF method is considered too expensive due to the high cost of bioreactors and substrates (Meeuwse, 2011). The large quantity of substrate produced makes it difficult for the substrate disposal process and affects the cost used. SmF is known to be so sensitive that it is susceptible to infection, resulting in yield loss and total breakdown of individual batches (Manpreet et al., 2005).

The main object used in this study was the oleaginous fungi isolate BR 2.2, which was isolated from the Baturraden Botanical Gardens, Central Java, Indonesia. A previous study (Rizki & Ilmi, 2021) found that BR 2.2 isolate had the highest lipid concentration (28.44%) of the 19 isolates. In a previous study, lipid production by oleaginous fungi isolates BR.2.2 still used the SmF method. Therefore, with the high cost and the amount of air used by SmF, it is necessary to research reducing air in lipid production by BR.2.2 oil isolate to make it more economical. Departing from the production of isolates obtained, this research is expected to produce a high lipid content with water that is not excessive.

MATERIALS AND METHODS

The research started with the process of rejuvenation and manufacture of spore suspension of BR 2.2 isolate. Starting from the suspension, lipid production was carried out in the growth media using a shaker incubator

Subculture of BR 2.2 isolate

Subculture of BR 2.2 isolate was made by inoculation technique from culture stock available at the Microbiology Laboratory, Faculty of Biology, Gadjah Mada University. The isolates were inoculated into a Potato Dextrose Agar (PDA) growth media slant in a test tube and incubated for 14 days in an incubator at room temperature.

Suspension preparation and spore calculation of BR 2.2 isolate

The fungi spore suspension was prepared using the subculture isolates on slanted PDA grown for 14 days. The growth media was added with 10 mL of distilled water mixed with 0.01% Triton. Using a loop needle, the isolates were suspended by carefully threshing the spores immersed in 0.01% triton solution. The suspension was put into a sterile glass bottle, then tightly closed and stored until used.

The number of spores in the suspension was calculated by graded dilution. The dilution results were then calculated using a spectrophotometer at 550 nm. PDA was then inoculated using the spread plate method for 14 days based on the concentration obtained. Colonies that grow are counted to get total plate count (TPC). The number of microbes in Colony Forming Unit (CFU)/mL from each dilution was obtained. The data spectrophotometer and TPC were used to create a standard curve between Abs and CFU/mL values.

Making growth media with variations of water content

Fungi biomass production was carried out by the batch fermentation method. The production media is composed of KH_2PO_4 0.125 gr; $ZnSO_4.H_2O$

0.0005 gr; $CuSO_4.5H_2O$ 0.00005 gr; $MnSO_4$ 0.0005 gr; $MgSO_4.7H_2O$ 0.025 gr; $FeSO_4.7H_2O$ 0.001 gr; $CaCL_2$ 0.005 gr; yeast extract 0.05 gr; KNO3 0.05 gr; glucose 1.5 gr; and varied distilled water amounts (50, 40, 30, 20, and 10 mL). The pH in the media was adjusted to pH 5.5 using HCl and NaOH (Somashekar et al., 2002). The media was sterilized using an autoclave at a temperature of 121°C with a pressure of 1 atm for 15 minutes.

Lipid production from BR 2.2 with variations in water content

An amount of 3.85 mL spore suspension representing 10^2 CFU was inoculated into media with additional water of 50 mL, 40 mL, 30 mL, 20 mL, and 10 mL. Each media variation was carried out in three replications and grown for 48, 96, and 144 hours at 28 °C. The lipid production process was carried out using a shaker at 130 rpm. The formed biomass was separated from the media by filtration using Whatman filter paper no. 1. To ensure that no media was left, the biomass were washed with sterile distilled water twice.

Fungi biomass calculation and lipid extraction

The biomass calculation was carried out on the dry weight of the fungi mycelium. The fungi mycelium was filtered using filter paper (Whatman no. 1; diameter 15 cm/150 mm) which was dried in the oven for 24 hours and weighed. The dried biomass was weighed periodically until it reaches a constant weight. The difference between initial and final weight was taken as the dry weight of fungi biomass (Barboráková et al., 2012).

Lipid extraction was carried out by crushing the dry biomass produced by the BR.2.2 oleaginous fungi and then homogenized with acid sand in a ratio of 1:2. The homogenization results were then added with chloroform and methanol as much as 20 times the total weight of biomass and acid sand. The ratio of chloroform: methanol added to the mixture of biomass and acid sand was 2:1 (Axelsson & Gentili, 2014). The mixture was vortexed until each component mixed and then centrifuged for 10 minutes at a speed of 4000 rpm.

The supernatant containing lipid was transferred into a sterile 15 mL vial bottle previously weighed and then placed in the oven for the evaporation process. After all, the solvent was evaporated, and only the lipid remained, the bottle was weighed again. Lipid accumulation was expressed as grams of lipid per liter of growth media and the percentage of grams of lipid per dry biomass (Somashekar et al., 2002).

Data Analysis

The analysis was carried out using Two-Way ANOVA analysis followed by Duncan's Post-Hoc Test with the IBM SPSS Statistics 22 application to see a significant difference in the study results with $p< 0.05$.

RESULTS AND DISCUSSION

Judging from the average data testing results using Duncan's analysis test with a significance of $p<0.05$, it shows that the incubation times of 48 hours, 96 hours, and 144 hours are significantly different. This is indicated by a superscript symbol that differs between incubation times. The incubation time of 48 hours has a mean total biomass yield of 4.54 g/L marked with a superscript symbol a, and an incubation time of 96 hours has an average total biomass yield of 7.17 g/L with a superscript symbol b. An incubation time of 144 hours has a mean biomass yield of—total 9.97 g/L with superscript symbol c. In addition, that the increase in incubation time is directly proportional to the total biomass production produced by BR 2.2 isolates, which is indicated by a gradual increase in the average total biomass yield concerning incubation time. Viewed from the average total biomass yield based on variations in the volume of media, average biomass yields of 10, 20, 30, 40, and 50 mL are 6.20, 12.53, 9.85, 4.12, and 3.44 g/L, respectively.

Based on the Figure 1, it can be seen that the orange bar shows the results of the treatment for the production of BR 2.2 isolate at media with additional 20 mL volume of water. The orange diagram in the 144-hour incubation group had the highest yield compared to the total biomass production under other conditions, which is 12.53 g/L.

Based on the data shown in Figure 2, there is a gradual increase in lipid production in all variations of the water volume of the media. Like the results in the test to calculate total biomass, the results in this test, when viewed from the aspect of incubation time, it can be seen that the increase in incubation time is directly proportional to the lipid produced by BR 2.2 isolates in all variations of the media.

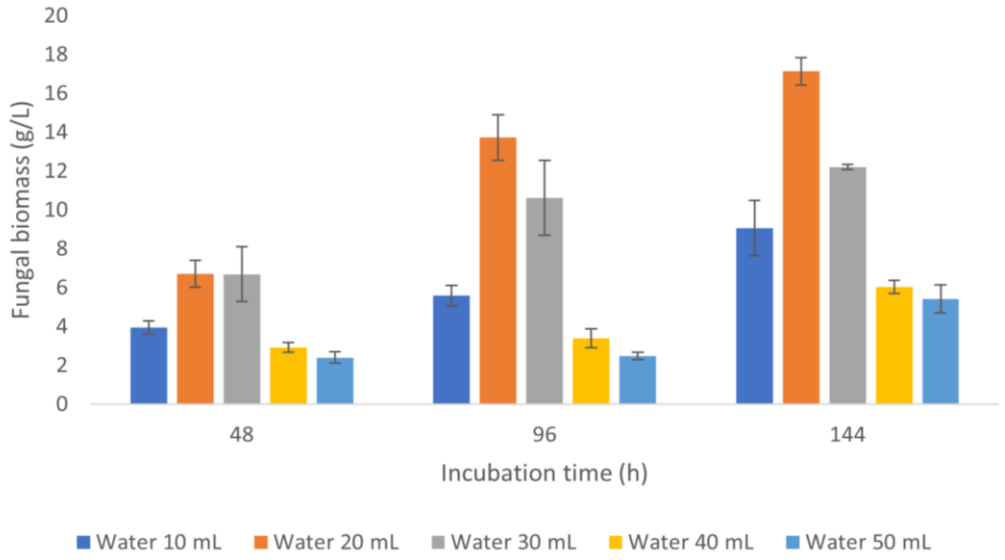

Figure 1. Comparison of water volume variations in medium and incubation time on biomass production of BR 2.2. isolates. a: group with confidence interval of 2.828-4.717; b: group with confidence interval of 5.6 - 6.8; c: group with confidence interval of 9.25 - 10.45; d: group with confidence interval of 11.93 - 13.22

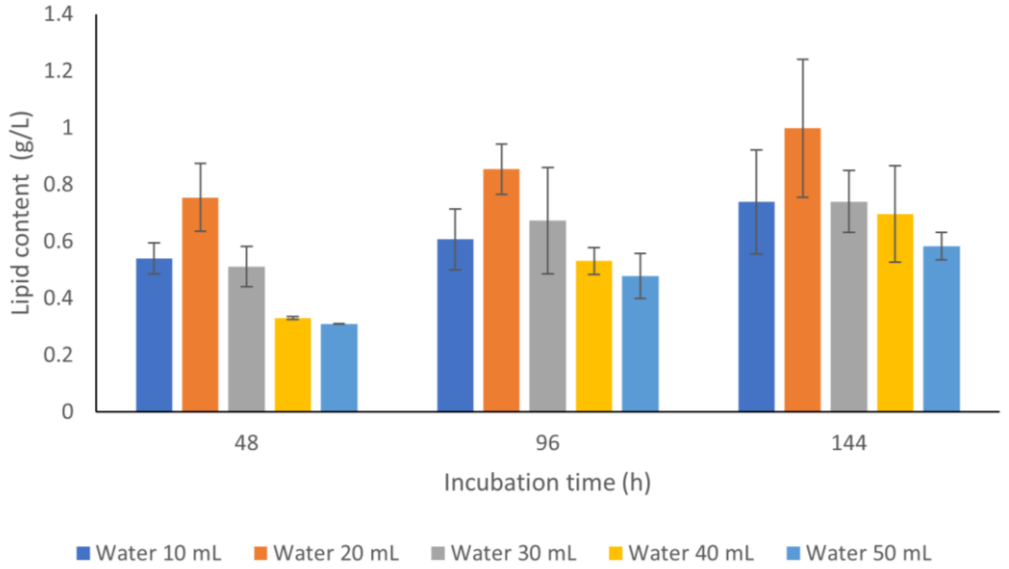

Figure 2. Comparison of water volume variations in medium and incubation time on lipid production of BR 2.2. isolates. a: group with confidence interval of 0.38 - 0.54; b: group with confidence interval of 0.55 - 0.72; c: group with confidence interval of 0.79 - 0.95

These results, it was also obtained through Duncan's test of significance p <0.05, showed that each incubation time was significantly different. The average lipid produced at an incubation time of 48, 96, and 144 hours was 0.49 g/L, 0.63g/L, and 0.75 g/L, respectively. Meanwhile, when viewed from the variations of water addition onto media, the 10, 20, 30, 40, and 50mL media produced an average lipid of 0.63 g/L, 0.87 g/L, 0.64 g/L, 0.52 g/L, and 0.46 g/L, respectively. Based on all the data presented in Figure 2, the treatment at the media volume of 20 mL water for 144 hours produced the most lipids compared to other treatment conditions, namely 0.87±0.04 g/L.

Filamentous fungi have an important role in the industrial production of biological products and the fermentation industry due to their ability to secrete proteins and enzymes, high growth rates, ease of handling in large-scale production, and low-cost requirements for production compared to other microorganisms. The fungi-growing process results in high-quality biomass (high protein and fat content) (Asadollahzadeh et al., 2018). Filamentous fungi grow by elongation and branching of hyphae

tips. The process involves the cytoplasm mass flow from the colony's center to the tip of the hyphae. In hyphae, there are porous septa that function to separate hyphae and can potentially control the movement of molecules within the colony (Daly et al., 2020).

Based on the data obtained, it can also be seen that the increase in incubation time is directly proportional to the total biomass production produced by the BR 2.2 isolate, which is indicated by a gradual increase in the average total biomass yield. Incubation time allows more fungi to grow, forming more biomass (Hosseinpour et al., 2017). The concentration of nutrients in the media has been shown to affect the activity of fungi, especially on sporulation and oxygen consumption. Increasing oxygen in culture can produce thicker cell walls than less oxygen, so the dry weight of the biomass formed will be greater. Generally, the biomass produced and fungal activity increased with incubation time and nutrient concentration (Fuentes et al., 2015).

The process of accumulation of lipids in oleaginous fungi is known to produce polyunsaturated fatty acids (PUFAs) by the SmF method and glucose as a source of C (Meeuwse, 2011). Based on the data shown in Figure 2, there is a gradual increase in lipid production in all variations of the water volume of the media.

This study resulted in data on the condition that the volume of 20 mL of media water for 144 hours had the most lipid accumulation compared to other treatment conditions, that is, 0.87±0.04 g/L. Lipid accumulation by oleaginous fungi mostly occurs when nutrients in the growth media are limited and excess carbon sources are converted to TAG storage. The limited supply of nitrogen (N), phosphorus (P), sulfur (S), iron (Fe), or zinc (Zn) is known to cause lipid accumulation in oleaginous fungi (Wu et al., 2010). During the growth phase, the carbon source is regulated for cell growth and, consequently, low lipid content. When nitrogen concentration becomes limited, cell growth stops, and microbial metabolism shifts to lipid accumulation (Vazquez-Duhalt & Greppin, 1987).

A rapid decrease in lipid accumulation is seen when cultures are grown in media containing higher inorganic and organic nitrogen sources. At the same time, there will be a higher accumulation of biomass but lower lipid content. The low lipid accumulation that occurs due to the availability of sufficient amounts of nitrogen for producing reproductive enzymes leads to an increase in biomass rather than lipid accumulation. In terms of stress level, lower nitrogen salt concentration creates higher metabolic stress conditions in microbes which triggers lipid accumulation in cells much faster than higher nitrogen concentration where the stress level is relatively low (Gohel et al., 2013).

CONCLUSION

Lipid accumulation and biomass production increased with the reduction of water content in the growth media. They reached the highest number in the media volume of 20 mL with an incubation time of 144 hours, i.e., 0.87±0.04 g/L and 12.53±0.29 g/L, respectively. The biomass produced and fungal activity increased with incubation time and nutrient concentration. Therefore, even with limited water but high nutrient content, it could still achieve high biomass and lipid production yields without excessive substrate waste.

ACKNOWLEDGMENT

This study was partially funded by Universitas Gadjah Mada through RTA project nr. 3143/UN1.P.III/DIT-LIT/PT/2021.

REFERENCES

Asadollahzadeh, M., Ghasemian, A., Saraeian, A., Resalati, H., & Taherzadeh, M. J. (2018). Production of fungal biomass protein by filamentous fungi cultivation on liquid waste streams from pulping process, *BioRes*, *13*(3), 5013-5031. https://bioresources.cnr.ncsu.edu/resources/production-of-fungal-biomass-protein-by-filamentous-fungi-cultivation-on-liquid-waste-streams-from-pulping-process/.

Axelsson, M., & Gentili, F. (2014). A single-step method for rapid extraction of total lipids from green microalgae. *PLoS ONE*, *9*(2), e89643. https://doi.org/10.1371/journal.pone.0089643.

Barboráková, Z., Labuda, R., Häubl, G., & Tančinová, D. (2012). Effect of glucose concentration and growth conditions on the fungal biomass, ph of media and production of fumagillin by a non-pathogenic strain *penicillium scabrosum*. *Journal of Microbiology*, *4*, 466–477.https://www.jmbfs.org/wpcontent/uploads/2012/01/Barborakova.pdf.

Basu, S., Bose, C., Ojha, N., Das, N., Das, J., Pal, M., & Khurana, S. (2015). Evolution of bacterial and fungal growth media. *Bioinformation*, *11*(4),182. https://doi.org/10.6026%2F97320630011182.

Daly, P., Peng, M., Mitchell, H. D., Kim, Y. M., Ansong, C., Brewer, H., de Gijsel, P., Lipton, M. S., Markillie, L.

M., Nicora, C. D., Orr, G., Wiebenga, A., Hildén, K. S., Kabel, M. A., Baker, S. E., Mäkelä, M. R., & de Vries, R. P. (2020). Colonies of the fungus Aspergillus niger are highly differentiated to adapt to local carbon source variation. *Environmental Microbiology*, *22*(3), 1154–1166. https://doi.org/10.1111/1462-2920.14907.

Dey, P., Mall, N., Chattopadhyay, A., Chakraborty, M., & Maiti, M. K. (2014). Enhancement of lipid productivity in *oleaginous colletotrichum* fungus through genetic transformation using the yeast ctDGAT2b gene under model-optimized growth condition. *PLoS ONE*, *9*(11), e0187171. https://doi.org/10.1371/journal.pone.0111253.

Doriya, K., Jose, N., Gowda, M., & Kumar, D. S. (2016). Solid-State Fermentation vs Submerged Fermentation for the Production of L-Asparaginase. *Advances in Food and Nutrition Research*, *78*, 115–135. https://doi.org/10.1016/bs.afnr.2016.05.003.

Fifendy, M. (2017). *Mikrobiologi*. Depok: Kencana.

Fuentes, M. E., Quiñones, R. A., Gutiérrez, M. H., & Pantoja, S. (2015). Effects of temperature and glucose concentration on the growth and respiration of fungal species isolated from a highly productive coastal upwelling ecosystem. *Fungal Ecology*, *13*, 135–149. https://doi.org/10.1016/j.funeco.2014.09.006.

Gohel, H., Ghosh, S., & Braganza, V. (2013). Yeast as a Viable and Prolonged Feedstock for Biodiesel Production. *Article in International Journal of Renewable Energy Research*, *3*(1), 126–131. https://doi.org/10.20508/ijrer.v3i1.488.g6115.

Hosseinpour, L., Zareei, M., Borjian Boroujeni, Z., Yaghoubi, R., Jamal Hashemi, S., & Author, C. (2017). Effect of Different Incubation Temperatures, Times, and Colored Lights on Fungal Biomass and Black Pigment (Melanin) Production in *Exophiala crusticola*. *Infect Epidemiol Microbiol*, *3*(3), 90–95. https://doi.org/10.18869/modares.iem.3.3.90.

Manpreet, S., Sawraj, S., Sachin, D., & Banerjee, S. (2005). Influence of Process Parameters on the Production of Metabolites in Solid-State Fermentation. *Malalaysian Jounal of Microbiology*, *1*(2), 1–9. https://agris.fao.org/agris-search/search.do?recordID=DJ2021067075.

Meeuwse, P. (2011). *Production of fungal lipids Kinetic modeling and process design*. The Netherlands.

Mienda, B., & Idi, A. (2011). Microbiological Features of Solid-State Fermentation and its Applications. An overview Model-Guided Metabolic Gene Knockouts Strategies for Enhanced Succinic Acid Production in Escherichia coli Strain K-12 View project. *Research in Biotechnology*, *2*(6), 21–26. https://www.researchgate.net/publication/236177189.

Murwani, S. (2015). *Dasar-dasar Mikrobiologi Veteriner*. Malang: UB Press. pp 151-151.

Rizki, M. A. A. H., & Ilmi, M. (2021). The Potential of Oleaginous Filamentous Fungi Isolated from Soil of Baturraden Botanical Garden, Central Java, Indonesia. *IOP Conference Series: Earth and Environmental Science*, *736*(1). https://doi.org/10.1088/1755-1315/736/1/012060.

Sergeeva, Y. E., Galanina, L. A., Andrianova, D. A., & Feofilova, E. P. (2008). Lipids of filamentous fungi as a material for producing biodiesel fuel. *Applied Biochemistry and Microbiology*, *44*(5), 523–527. https://doi.org/10.1134/S0003683808050128.

Shafiq, S. A., & Chechan, R. A. (2019). Influence of different natural media on production of myco-diesel. *IOP Conference Series: Earth and Environmental Science*, *388*(1). https://doi.org/10.1088/1755-1315/388/1/012054.

Somashekar, D., Venkateshwaran, G., Sambaiah, K., & Lokesh, B. R. (2002). Effect of culture conditions on lipid and gamma-linolenic acid production by mucoraceous fungi. *Process Biochemistry*, *38*(12), 1719–1724. https://doi.org/10.1016/S0032-9592(02)00258-3.

Vazquez-Duhalt, R., & Greppin, H. (1987). Growth and production of cell constituents in batch cultures of *botryococcus sudeticus*. *Pergamon Journals*, *26*(4), 885–889. https://doi.org/10.1016/S0031-9422(00)82311-0.

Wu, S., Hu, C., Jin, G., Zhao, X., & Zhao, Z. K. (2010). Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. *Bioresource Technology*, *101*(15), 6124–6129. https://doi.org/10.1016/j.biortech.2010.02.111.

Yang, Y., Heidari, F., & Hu, B. (2019). Fungi (mold)-based lipid production. *Methods in Molecular Biology*, *1995*, 51–89. https://doi.org/10.1007/978-1-4939-9484-7_3.