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RANDOM MUTAGENESIS OF *Lipomyces maratuensis* InaCC Y720 USING COMMERCIAL UV LAMP TO INCREASE LIPID PRODUCTION

MUTAGENESIS ACAK *Lipomyces maratuensis* InaCC Y720 MENGGUNAKAN LAMPU UV KOMERSIAL UNTUK MENINGKATKAN PRODUKSI LIPID

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Abstract

Oleaginous yeasts are capable of accumulating high lipid concentration up to 20% of dry cell weight. High lipid content, a shorter life cycle, and similar a fatty acid composition to vegetable oils makes oleaginous yeast a potential lipid producer. *Lipomyces maratuensis* InaCC Y720 is a novel species isolated from Maratua Island, East Kalimantan, which has been reported as a potential yeast lipid producer. However, lipid productivity of the yeast is needed to be increased to make it suitable for an industrial scale. The aim of this study is to obtain potential mutant strains for the biodiesel industry. Random mutagenesis was applied by using commercial UV-C lamp on the strain which resulting in an 80% death rate after three hours irradiation. Subsequent treatment was carried out using cerulenin as a selection agent for mutans, yielding six mutant strains. Among these strains, mutant 1 produced the highest lipid production, with a lipid concentration of 0.072 g/L and a lipid percentage of 8.603%. Nevertheless, when compared to the wild type, the lipid productivity of mutant 1 is low. Based on these results, the mutagenesis approach using commercial lamp UV-C has not obtained the expected mutants, so it is recommended to use different methods for future study.

Keywords: Cerulenin; *Lipomyces maratuensis*; Mutation; Oleaginous yeast; Productivity

Abstrak

Khamir oleaginous memiliki kemampuan dapat mengakumulasi lipid hingga 20% dari berat kering selnya. Tingginya kadar lipid yang diproduksi, siklus hidup yang pendek serta komposisi lipid yang mirip dengan minyak tumbuhan dapat menjadikan khamir sebagai alternatif penghasil lipid. Lipomyces maratuensis InaCC Y720 merupakan spesies baru yang diisolasi dari Pulau Maratua, Kalimantan Timur yang dilaporkan sebagai khamir penghasil lipid potensial. Namun, produktivitas lipid khamir tersebut perlu ditingkatkan agar sesuai untuk skala industri. Tujuan dalam studi ini adalah mendapatkan strain mutan yang potensial untuk industri biodiesel. Metode mutagenesis secara acak dilakukan dengan menggunakan lampu UV-C komersial pada strain yang menghasilkan tingkat kematian 80% selama tiga jam penyinaran. Setelah itu, dilakukan perlakuan lebih lanjut dengan penggunaan serulenin sebagai agen seleksi mutan. Proses seleksi menghasilkan enam strain mutan. Di antara keenam strain mutan, mutan 1 menghasilkan jumlah lipid tertinggi dengan berat lipid 0,072 g/L dengan persentase lipid yaitu 8,603%. Namun, dibandingkan dengan wild type, produktivitas lipid mutan 1 lebih rendah. Berdasarkan hasil ini, mutagenesis menggunakan lampu UV-C komersial belum mendapatkan mutan yang diharapkan sehingga disarankan penggunaan metode yang berbeda untuk penelitian selanjutnya.

***Kata kunci**: Khamir oleaginous; Lipomyces maratuensis; Mutasi; Produktivitas; Serulenin*

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INTRODUCTION

Biodiesel is a type of renewable alternative fuel typically derived from plant oils. However, this has led to rapid increase in the price of vegetable oils due to the growing global consumption of biodiesel (Guo et al., 2019).Furthermore, utilizing vegetable oils for biodiesel production can create competition for essential raw material. Consequently, there is a pressing need for alternative materials that are more efficient for biodiesel production, but also do not interfere upon food crops resources. One such alternative is the utilization of microorganisms.

Microorganisms that can accumulate lipids up to 20% of their cell'dry weight of their cells are known as oleaginous microorganisms (Huang et al., 2010; Meng et al., 2009). Yeast protrudes as one of the promising oleaginous microorganisms. The lipids produced by yeast contain fatty acids such as palmitic, oleic and linoleic, rendering them suitable as raw material for biodiesel production. Noteworthy, yeast genera that have been extensively studied and are recognized for high lipids accumulation include *Lipomyces, Rhodosporidium*, *Cryptococcus*, and *Yarrowia* (Sitepu et al., 2014). Among these, *Lipomyces maratuensis* InaCC Y720 is a yeast species originally isolated from soil on Maratua Island, East Kalimantan. Notably, *Lipomyces maratuensis* InaCC Y720 exhibited a lipid production of 3.696 g/L. Consequently, *Lipomyces maratuensis* is considered as having substantial potential as a lipid-producing yeast (Yamazaki et al., 2017).

There are various approaches that can be employed to enhance lipid production, including the optimization of growth conditions and media, as well as the refinement of yeast strains. The enhancement of yeast strains can be achieved through either random or targeted genetic manipulation. Tachioka et al. (2016) have suggested that randomized mutagenesis proves effective, especially for strains possessing limited genetic information. UV light is recognized for generating mutations across a broad spectrum (transition and transversion mutations) in yeast cells. The resulting mutants can then be selected based on the desired character using certain selective agents such as cerulenin (Winston, 2008). Cerulenin is an antibiotic that inhibits the process of fatty acid synthesis. The use of cerulenin as a selective agent is hypnotized to enable the selection of mutants showcasing higher fatty acid production capabilities than the wild-type strains.

Therefore, in this study, optimization of lipid production was carried out by improving the quality of *Lipomyces maratuensis* InaCC Y720 by random mutation of UV light with a selective agent of cerulenin. The results of our study aim to provide information regarding the lipid production by the mutant isolate in comparison with wild-type *Lipomyces maratuensis* InaCC Y720, as well as the differences in the fatty acid profiles that they produced.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Lipomyces maratuensis InaCC Y720 is a part of the Indonesian Culture Collection (InaCC) and was originally isolated from Maratua Island, East Kalimantan, Indonesia (Yamazaki et al., 2017). The strain was cultivated on Potato Dextrose Agar (PDA) and stored in refrigerator. A seed culture was prepared by subculturing the yeast in Yeast Peptone Dextrose Broth (YPD Broth) at 28 °C *with 130 rpm agitation for 48 hours.*

Mutagenesis and ***Selection***

Lipomyces maratuensis InaCC Y720 was cultured on liquid YPD media for 24 hours in 50 mL Erlenmeyer. Subsequently, the cultures were exposed to UV-C-irradiation using UV-C mini box (Phillips) 4.3 W and a wavelength of 275 nm until approximately 80% of the population was deceased. Following irradiation, the cultures were incubated at 30 °C until OD_{600} reached 2. The cells were then inoculated into YPD liquid supplemented with 5 µmol/L cerulenin, which served as a mutant selective agent. Subsequent to the initial treatment, the cultures were subjected to another round of UV-C-irradiation and then inoculated into liquid YPD media with varying concentrations of cerulenin (5, 10, 20, and 40 µmol/L). The resulting mutant isolates were subsequently inoculated onto YPD agar medium containing 40 µmol/L cerulenin. From separate colonies, a random selection of 6 mutant isolates were obtained.

Growth Profile

The growth profile was assessed by cultivating yeast in 50 mL of YPD Broth medium at 28 °C with agitation at130 rpm. Cell growth was monitored by creation of a growth curve. This growth curves were constructed by measuring OD_{600} every 24 hours for duration of 168 hours. Biomass and lipid measurements were conducted at the 120-hour mark. All experiments were replicated three times.

Lipid Extraction and Analysis

Biomass and lipid levels were determined the gravimetric method (Bligh & Dyer, 1959). The culture grown on 35 mL of YPD media was subjected to centrifugation at 5,000 rpm for 10 minutes. Afterward, the pellet was rinsed twice with 10 mL distilled water. Subsequently, 10 mL of 4M HCl was added to the pellet, and the mixture was incubated for 2 hours with agitation. Following this, a 10 mL of methanol and a 10 mL of chloroform were added at room temperature and the solution was incubated for additional 2 hours. The sample was then centrifuged at 5,000 rpm for 10 minutes, then the organic phase was carefully collected and subjected to evaporation. The resultant dry biomass was weighed, allowing for the calculation of lipid content.

Fatty acid profile analysis involved lipid transesterification, followed by GC-MS analysis, following the method outlined by Ichihara and Fukubayashi (2010). An 8% (w/v) HCl solution was prepared by diluting in 41.3 mL of methanol to make a final volume of 50 mL. The lipid sample was transferred to a lidded test tube d and dissolved in 0.2 mL of toluene. This was followed by the addition of 1.5 mL of methanol and 0.3 mL of 8% HCl. The mixture was vortexed and incubated at 45 °C for at least 14 hours, or alternatively, it could be heated at 100 °C for 1 hour. After the sample was cooled to room temperature, 1 mL of hexane and 1 mL of distilled water were added for fatty acid extraction. The resulting mixture was vortexed, and the hexane layer were separated for injection into the GC-MS. Helium gas was utilized as the carrier gas, with a sample injection volume of 1 µL. The lipid profile was determined using an external database set as a reference (Ichihara & Fukubayashi, 2010).

RESULTS

Mutagenesis and Selection of Mutant Yeasts Based on Cerulenin

In this study, random mutagenesis was conducted utilizing a UV-C mini box with a power of 4.3 Watt and a wavelength of 275 nm. Selection was also performed using antibiotic cerulenin against *Lipomyces maratuensis* InaCC Y720 isolate, with the aim of obtaining a mutant isolate capable of producing higher lipid levels compared to the wild type. Upon optimizing the irradiation time with UV-C, it was determined that an irradiation duration of 3 hours resulted in a total colony mortality rate of 80%. Consequently, the colony count was reduced to 20% of the total colonies observed on the control plate. Figure 1 depicts the effects of UV radiation on the *Lipomyces maratuensis* InaCC Y720 colonies.

Following the UV irradiation, mutant selection was undertaken by gradually introducing cerulenin in increment up to 40 µmol/L. The application of cerulenin up to 40 µmol/L effectively suppressed the growth of cultures. As result of the selection process, six distinct mutant strains were successfully isolated, denoted as mutant 1, mutant 2, mutant 3, mutant 4, mutant 5, and mutant 6.

Cultivation of Lipid Producing by Mutants in Comparison to Wild Type

The six mutant isolates that had been obtained from the cerulenin-based selection, along with the wild type, were subsequently cultivated in liquid medium to assess their respective growth rate and lipid accumulation capability. Each experiment was replicated three times and conducted over a period of approximately 120 hours, maintaining a temperature of 28 °C and an agitation of 130 rpm. During the production process, noticeable distinction emerged between wild-type and mutant isolates. The variation in the growth rate and the resulting lipid production are outlined in Table 1 and Figure 2.

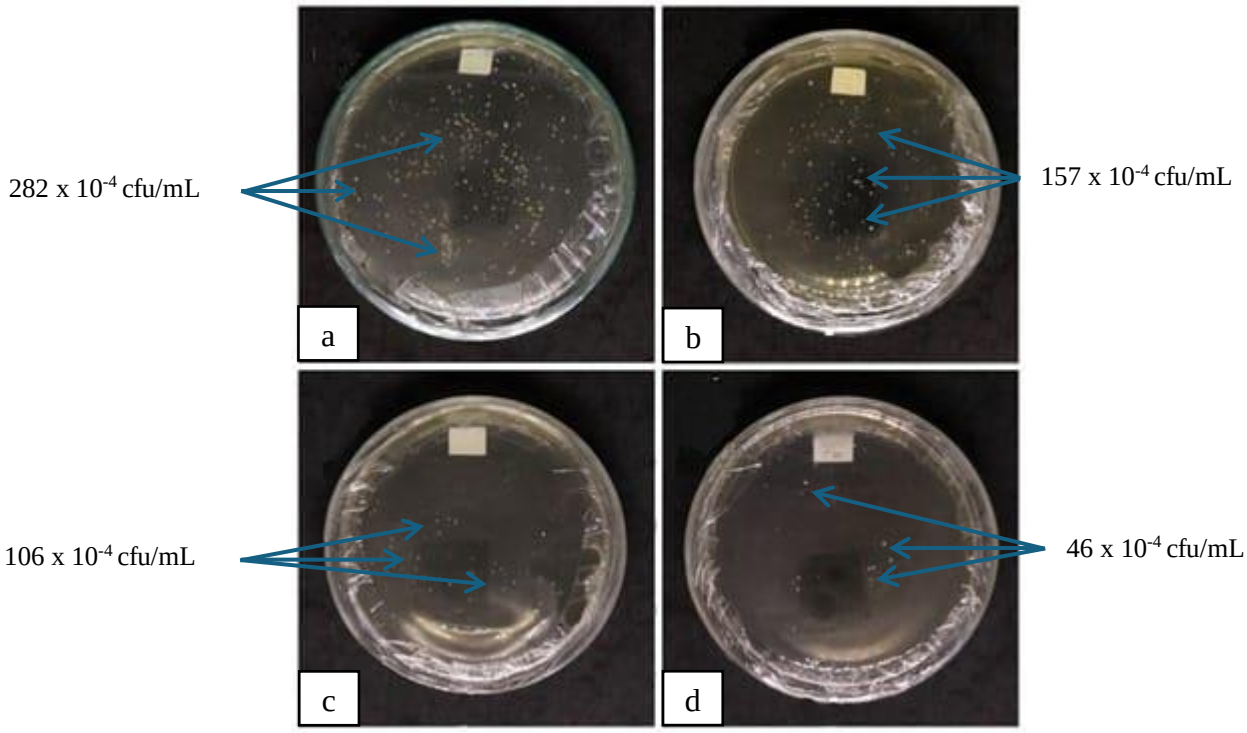

Figure 1. *Lipomyces maratuensis* InaCC Y720 in YPD after irradiating with UV, control (a); 1 hour (b); 2 hours (c); and 3 hours (d)

Table 1. Biomass and production of mutant and wild-type lipids

Strain	Biomass (g/L)	Lipid (g/L)	Lipid content (%)
Wild type	2.69	0.148	5.02
Mutant 1	0.863	0.072	8.603
Mutant 2	1.03	0.046	4.437
Mutant 3	0.933	0.042	4.463
Mutant 4	1.05	0.048	4.553
Mutant 5	0.89	0.041	4.616
Mutant 6	0.946	0.044	4.722

From the data, it is evident that mutant 1 yields a higher lipid content or percentage of lipids in comparison to the wild type. This is reflected in its lipid percentage of 8.603%, calculated as the ratio of the lipid dry weight to biomass weight. However, it's important to note that in this experiment, no mutant isolates were identified capable of surpassing the wild type in terms of both dry biomass weight of biomass and lipid production.

Lipid Composition in Wild Type and Mutants

Fatty acid composition analysis was conducted using GC-MS on wild type and mutant 1. The identification of fatty acids relied on retention time and mass spectrum characteristics with the method proposed by Gomaa et al. (2021). From the analysis, various fatty acids were successfully detected, and a comparison between the wild-type and the mutant can be made based on the total detected fatty acids. This comparison is detailed in Table 2.

Based on the fatty acid composition present in the wild-type isolate and mutant 1 isolate, difference exist in the fatty acids produced between the two types. The most prominent disparity lies in the lipid isolate mutant 1, where no linoleic acid was detected. In contrast, in the wild-type isolate exhibited a linoleic acid content of 11.64%. Furthermore, the wild-type contained 35.03% oleic fatty acid, a proportion significantly higher than the more 2.71% found in mutant isolate 1. Additionally, the wild-type displayed 10.19% adipic fatty acids, whereas in mutant 1, this figure was notably elevated, constituting 38.58% of the total detected fatty acids.

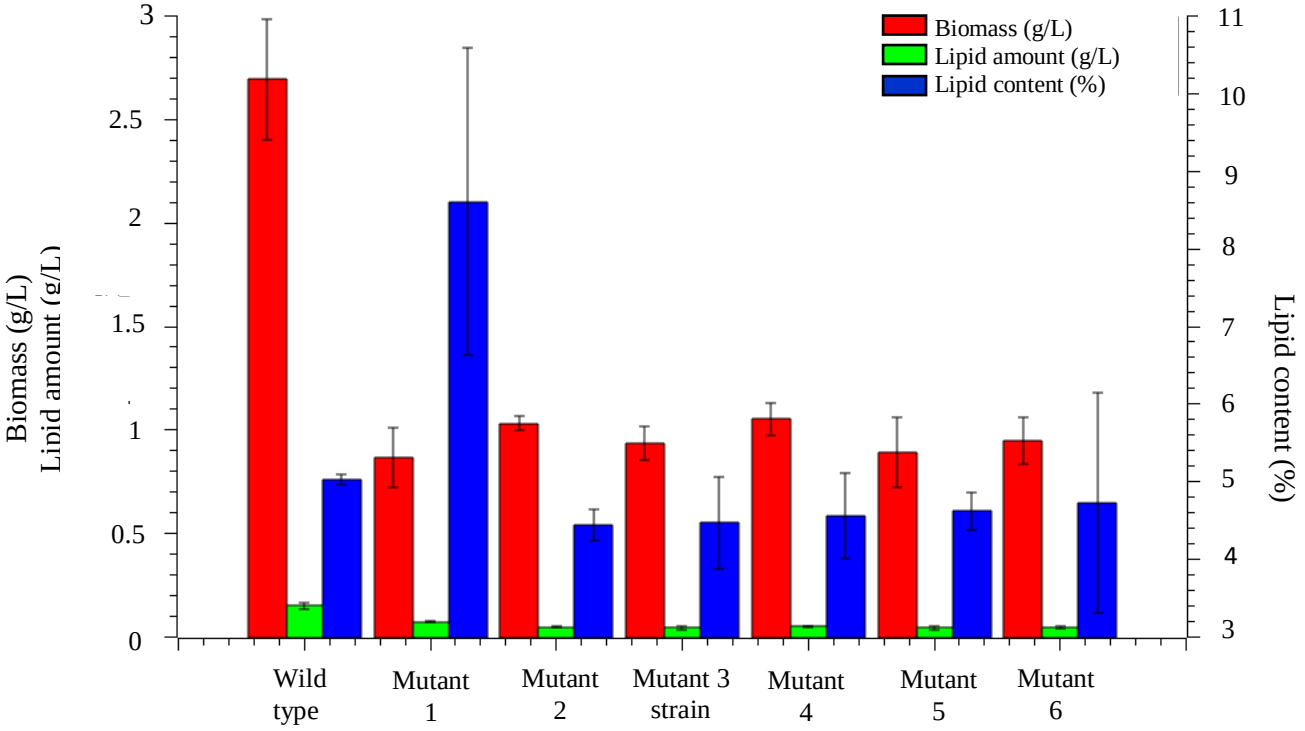

Figure 2. Comparison of biomass, lipid amount, and lipid content from wild type and mutant strains

Table 2. Fatty acids identified from lipid produced by wild type and mutant 1 strains

Fatty acids	Chemical formula	Content (%)	
		Wild type	Mutant 1
Palmitic acid	$C_{17}H_{34}O_2$	13.48	13.42
Linoleic acid	$C_{19}H_{34}O_2$	11.64	-
Oleic acid	$C_{19}H_{36}O_2$	35.03	2.71
Stearic acid	$C_{19}H_{38}O_2$	7.37	8.15
Adipic acid	$C_{22}H_{42}O_4$	10.19	38.58

DISCUSSION

The availability of nitrogen can influence the process of lipid accumulation in yeast. This in line with Ratledge (2004), which discuss the substantial lipid accumulation exhibited by oleaginous yeasts under nitrogen-limited conditions. However, in this particular experiment, the mutant exhibited inadequate growth and were unable to produce lipids where subjected to limited nitrogen conditions (C/N= 80:1; 28:1; and 10:1). This outcome suggests that lipid accumulation during the yeast growth is influenced by factors beyond just nitrogen limitation (Papanikolaou & Aggelis, 2011). Subsequently, in subsequent experiment, cultivation was conducted using nitrogen-rich medium (C/N= 5:1) to stimulated lipid production. Despite these efforts, no potential lipid production results were obtained. This lack of lipid production potential is evident in Table 1, where the minimum biomass generated by the mutants appeared to impact the levels of lipids levels produced even in experiments using a nitrogen-rich medium (C/N= 5:1).

Aside from nitrogen availability, several factors could potentially contribute to mutant's failure to produce higher lipid levels. UV-C irradiation, as employed by Lee et al. (2016) and Reed (2010), is known to be utilized for the inactivation various pathogenic microorganisms through DNA damage. In the context of this study, the randomized UV-C treatment lasting three hours could induce mutations in genes unrelated to unintended modifications, leading to unintended and uncontrollable genes modifications. Consequently, this extended UV-C irradiation period is hypostatized to induce mutations in genes crucial to lipid metabolism in mutant derived from *Lipomyces maratuensis* InaCC Y720. Such mutations may initiate alterations in lipid metabolism pathways, consequently influencing the lipid accumulation process. It's worth nothing that gene

mutations affecting lipid metabolism pathways could also be attributed to cellular damage resulting from excessive UV-C irradiation. The biological effects of UV radiation encompass reduced growth and viability, protein impairment, and generation of free radicals that damage cell components, including membranes (De Gruijl & Van Der Leun, 1994; Sinha & Häder, 2002).

The finding of the research conducted by Leung et al. (2013) indicated the impact of UV-C radiation exposure on *Caenorhabditis elegans*, specifically affecting mitochondria and ATP production. This observation holds potential relevance to the lipid metabolism within yeast mitochondria, particularly in context of isocitrate accumulation process occurring during yeast growth under nitrogen-limited conditions. Isocitrate accumulation in mitochondria tends to occur when yeast experiences limited nitrogen conditions. The accumulated isocitrate is subsequently balanced through its conversion to citric acid, leading to an increase in citric acid concentration. Once the citric acid concentration of the citric acid within surpasses a certain threshold, citrate is transported to the cytosol while malate is imported. Subsequently, citric acid ise converted to acetyl-CoA and oxaloacetate by ATP:citrate lyase. Acetyl-CoA then initiates a condensation reaction, eventually leading to fatty acid synthesis (Papanikolaou & Aggelis, 2011; Ratledge & Wynn, 2002; Ratledge, 2004). These fatty acid synthesis could occur if there is no cellular damages which cause alterations in lipid metabolism pathways.

The fatty acid composition plays significant role in determining the quality of biodiesel. As reported by Aslam et al. (2018), the optimal raw materials for biodiesel production encompass C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), and C18:2 (linoleic acid). While all four fatty acids were detected in the wild type isolate, linoleic acid notably was absent in mutant 1. The presence of these specific fatty acids presents a potential opportunity for their utilization as raw materials for biodiesel. However, despite anticipation that mutant 1 would accumulate higher levels of lipids compared to the wild type, it did not yield more favourable outcomes. It worth noting that the fatty acid composition greatly influences biodiesel quality due to its impacts on various characteristics such as oxidative stability. Biodiesel with higher composition of unsaturated fatty acids tends to exhibit improved oxidative stability compared to biodiesel rich in polyunsaturated fatty acids (PUFA).

CONCLUSION

In this study, efforts to enhance the quality of the yeast strain *Lipomyces maratuensis* InaCC Y720 through random UV-C radiation did not produce a promising mutant strain. The lipids production exhibited by mutant isolate 1 indicated an increase in percentage, but this enhancement did not render an increase in biomass and dry weight of lipids. The fatty acid composition produced by mutant 1 isolate differed from that of the wild type, spesifically due to the absence of linoleic acid. These observed variations can be potentially attributed by the difference in the type of UV-C radiation used and the extended duration of irradiation. These factors likely induced gene mutations and cellular damage, thereby leading to alterations in lipid metabolism pathways. It's essentially to underscore the importance of several considerations moving forward. Firstly, the selection of the appropriate UV-C type and the irradiation duration should align with pertinent references to ensure controlled and targeted outcomes. Additionally, the determination of the appropriate medium requires careful consideration from the outset. The choice of medium is crucial because the carbon-to-nitrogen (C/N) ratio can influence the yeast ability to accumulate lipids.

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