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PHYCOCYANIN PRODUCTION FROM *Galdieria sulphuraria* 009 IN PALM OIL MILL EFFLUENT: GROWTH, EXTRACTION, AND ANTIOXIDANT ACTIVITY

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Abstract

Palm oil mill effluent (POME), a major by-product of the palm oil industry in Indonesia, is generated in large volumes and poses environmental risks due to its high organic content. Microalgae offer a promising approach to reduce this waste while simultaneously producing value-added biomass products. This study aimed to determine the optimal POME concentration for microalgal growth of *G. sulphuraria* 009, to evaluate phycocyanin yield, and to assess its antioxidant activity. This study was initiated with a preliminary screening using 5–50% POME to identify optimal microalgal growth conditions; cultivation in bioreactors with selected concentrations (2.5%, 5.0%, and 7.5%) to evaluate growth performance and chemical yields; and analysis of antioxidant activity and pigment content in both fresh and residual biomass. The preliminary stage revealed 5% POME as the upper threshold for growth, with 2.5% supporting optimal biomass comparable to control (Allen pH 2). Higher POME levels inhibited growth due to light attenuation and ammoniacal nitrogen toxicity. 2.5% POME recorded the highest phycocyanin yield per liter, while 7.5% POME yielded the highest antioxidant activity, likely due to oxidative stress. Antioxidant assays confirmed significant antioxidant activity in all phycocyanin extracts, with the highest activity in 7.5% POME, likely due to oxidative stress. Carotenoid and chlorophyll contents were evaluated in both fresh and residual biomass. Carotenoids were more abundant in fresh biomass, while chlorophyll-A was higher in residual biomass post-extraction, emphasizing the importance of extraction techniques in bioactive compound recovery. This study highlights *G. sulphuraria* 009 as a viable source of phycocyanin in POME-based cultivation, offering insights into industrial wastewater valorization and sustainable bioproducts.

Keywords: biomass, carotenoid, microalgae, pigment, POME

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Produksi Fikosianin dari *Galdieria sulphuraria* 009 dalam Limbah Cair Pabrik Kelapa Sawit: Pertumbuhan, Ekstraksi, dan Aktivitas Antioksidan

Abstrak

Limbah cair industri kelapa sawit (*palm oil mill effluent* atau POME) di Indonesia menghasilkan volume yang tinggi dan berpotensi mencemari lingkungan. Mikroalga dapat dimanfaatkan untuk mengurangi limbah sekaligus menghasilkan produk biomassa yang bernilai tambah. Penelitian ini bertujuan menentukan konsentrasi optimum POME bagi pertumbuhan mikroalga *Galdieria sulphuraria* 009, mengevaluasi produksi fikosianin, serta menilai aktivitas antioksidannya. Penelitian ini diawali dengan penapisan awal menggunakan 5–50% POME untuk mengidentifikasi kondisi pertumbuhan mikroalga yang optimum; budi daya dalam bioreaktor dengan variasi konsentrasi (2,5; 5,0; dan 7,5%) untuk mengevaluasi kinerja pertumbuhan; dan analisis aktivitas antioksidan serta kandungan pigmen dalam biomassa. Penapisan awal menunjukkan bahwa POME 5% merupakan konsentrasi optimum, sedangkan konsentrasi lebih tinggi menghambat pertumbuhan akibat berkurangnya penetrasi cahaya. Pada budidaya lanjutan, mikroalga dalam POME 2,5% menunjukkan hasil sebanding dengan kontrol (Allen pH 2), sedangkan konsentrasi lebih tinggi menghambat pertumbuhan akibat penaungan dan toksisitas amonia. Produksi fikosianin per volume kultur tertinggi diamati pada mikroalga yang dibudidayakan dalam POME 2,5%, dengan hasil yang sebanding dengan media kontrol. Uji aktivitas antioksidan mengonfirmasi bahwa semua ekstrak fikosianin memiliki aktivitas antioksidan yang signifikan, dengan aktivitas tertinggi pada POME 7,5%. Kandungan karotenoid dan klorofil a dievaluasi dalam biomassa segar dan residu setelah ekstraksi. Karotenoid lebih melimpah dalam biomassa segar, sedangkan klorofil a lebih tinggi dalam residu biomassa. Penelitian ini menunjukkan bahwa *G. sulphuraria* 009 berpotensi sebagai sumber fikosianin yang layak dalam budi daya berbasis POME, serta memberikan wawasan mengenai pemanfaatan limbah industri dan pengembangan produk bioteknologi berkelanjutan.

Kata kunci: biomassa, karotenoid, mikroalga, pigmen, POME

INTRODUCTION

Indonesia is known as the world's largest palm oil-producing country, contributing approximately 60% of the world's palm oil needs (Nabila *et al*., 2023). Its high production is related to its wide application in the food and energy fields as a source of vegetable oil and biodiesel feedstock, respectively (Iskandar *et al*., 2018). Such tremendous industrial activity will bring significant environmental impact due to the release of waste, such as palm oil mill effluent (POME), which accounts for the largest portion of all waste generated from the extraction process of fresh fruit bunches. POME is an acidic colloidal suspension (pH 4-5) with a high content of organic materials, such as carotene, phenolics, lignin, and pectin. Thus, POME has high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) contents, which could cause water pollution if discharged into the environment in an untreated form (Iskandar *et al*., 2018; Sundalian *et al*., 2021). Therefore, the treatment of POME is obligatory prior to its discharge. However, 85% of palm oil mill facilities have treated POME by adapting biological techniques, including anaerobic, facultative, and aerobic treatments (Resdi *et al*., 2016).

The treatment process of POME using microalgae is a known concept involving the inclusion of POME as an additional component into the microalgal growth medium (Fernando *et al*., 2021; Nur *et al*., 2023; Sabilil & Agus, 2021). The utilization of POME wastewater employing microalgal cultivation could serve as a means for nutrient removal because microalgae have the capability to assimilate organic carbon, nitrogen, phosphorus, and other nutrients for biomass production (Cai *et al*., 2019). This process was aimed at several purposes, such as providing nutrient removal in POME, serving as a low-cost nutrient source for microalgal cultivation, and producing microalgal biomass for use as a feedstock for valuable metabolite production, such as carbohydrates, proteins, lipids, and pigments (Fernando *et al*., 2021; Nur *et al*., 2023; Resdi *et al*., 2016). Nur *et al*. (2023) reported that POME can be used in microalgal cultivation,

namely *Chaetoceros calcitrans* and *Arthrospira platensis*, for fucoxanthin and c-phycocyanin production, respectively, under outdoor cultivation.

Phycocyanin, as a natural compound, is in greater demand and is expected to have a global market of around $245.5 million in 2027. Microalgal species from the *Spirulina* genus are commonly used as feedstock for phycocyanin production, mainly *Spirulina platensis* (Yuliani *et al*., 2023) and *Spirulina maxima* (Fernandes *et al*., 2023). Besides *Spirulina, Rhodophyta* microalgae species from the *Galdieria* genus have also been reported as phycocyanin producers. Moreover, its phycocyanin yield was 20-287 times higher than that in the photoautotrophic cultures of *Spirulina platensis* (Graverholt & Eriksen, 2007). Furthermore, phycocyanin produced from *Galdieria* algae can suffer elevated temperatures up to 73°C, which is higher than that produced by *Spirulina* (46°C) (Moon *et al*., 2014).

POME has gained significant attention as a sustainable medium for microalgae cultivation due to its rich organic content, including 2,726 mg/L of COD, 1,270 mg/L of TOC, and 257.6 mg/L of phosphate (Basra *et al*., 2023). Recent studies highlight the use of POME for microalgae cultivation not only addresses the growing challenge of POME disposal but also contributes to the circular economy by converting waste into high-value products. Microalgae such as *Chlorella vulgaris* and *Spirulina platensis* have been successfully cultivated in POME-based media, demonstrating enhanced growth performance compared to conventional synthetic media (Saria *et al*., 2022; Fernando *et al*., 2021). However, the utilization of POME as an additional component in the culture media for microalgal phycocyanin production and other metabolites of *Galdieria sulphuraria* has yet to be investigated. *G. sulphuraria* is a highly versatile microalga known for its ability to grow under extreme environmental conditions, including high temperatures, acidic pH, and varied nutrient availability. This species has a flexible metabolism, capable of utilizing a wide range of carbon sources, and is recognized for its high phycocyanin production (Rahman *et al*., 2020). Therefore, the objective of this study was to determine the optimal POME concentration for the growth of *Galdieria sulphuraria* 009, evaluate phycocyanin production, and assess its antioxidant activity. The preliminary findings presented in this study offer valuable insights into the optimization of microalgal cultivation in POME, highlighting the key factors that influence growth and biochemical composition. We investigated the impact of varying POME concentrations on the growth of *G. sulphuraria* 009 and delved into the intricate relationship between POME dilution, light penetration, and microalgal viability. Furthermore, this study explored the potential of *G. sulphuraria* as an alternative source of phycocyanin, a sought-after pigment with numerous applications, including food coloration and antioxidant properties. Finally, we examined the carotenoids and chlorophyll a content of both fresh and residual biomass after phycocyanin extraction.

MATERIALS AND METHODS

Microorganism and Cultivation Media

Galdieria sulphuraria 009 was collected from the Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN). Seed cultures of microalgae were maintained in Allen medium, which contained 1 mM $MgSO_4.7H_2O$, 2 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 0.5 mM $CaCl_2.2H_2O$, 46 µM H_3BO_3, 71 µM $FeCl_3$, 9.1 µM $MnCl_2.4H_2O$, 760 nM $ZnSO_4.7H_2O$, 310 nM $CuSO_4$, 200 nM NH_4VO_3, 100 nM $Na_2MoO_4.2H_2O$ (Allen, 1959). The pH of the medium was amended to 2.0 using H_2SO_4 before sterilization. The bioreactor used for the experiments was a 500 mL laboratory glass bottle containing 400 mL of medium, which was continuously aerated and maintained at room temperature.

Preliminary Cultivation of *G. sulphuraria* 009

POME was obtained from PTPN Cikasungka Bogor, West Java. POME was filtered through a microfiber mesh to remove suspended solids and diluted to the required

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concentrations with Allen medium before adjusting the pH to 2.0. A preliminary growth experiment was performed using 5, 10, 20, 30, and 50% (v/v) concentrations of POME in Allen medium to identify the range of dilution corresponding to the optimum growth and phycocyanin production of *G. sulphuraria* 009. Preliminary cultivation was performed in 24 wells microplate with continuous light with total volume about 5 mL. The growth was monitored everyday using Varioscan (ThermoFisher Scientific) at 800 nm for 10 days.

Cultivation of Microalgae in POME

Based on the preliminary cultivation results, the concentrations of POME used for the cultivation were 2.5, 5.0, and 7.5% (v/v) in Allen medium pH 2, with Allen medium pH 2.0 used as the control medium. Microalgae were cultivated in a 500 mL glass-type vessel flask with a working volume of 400 mL. The culture was maintained at room temperature (25±2 °C) with continuous aeration. Light intensity was continuously provided at 3,000 lx using a tubular lamp.

Growth Curve and Biomass Production

The growth of microalgae was monitored at an optical density of 800 nm using a spectrophotometer (UV-Vis, Genesys, Thermo Fisher Scientific). The specific growth rate (μ) during the exponential growth phase was determined based on the method outlined in our previous paper (Rahman *et al.*, 2020), using the following equation:

$$\mu = \frac{\ln(N_i)-\ln(N_0)}{t_i-t_0}$$

Where N_0 is the absorbance value of the microalgae culture on the initial cultivation day (t_0) and N_i is the absorbance value of the microalgae culture on the i^{th} day of cultivation (t_i). Biomass was harvested at the end of the exponential phase by centrifugation at 8,000 rpm for 5 min and rinsed with distilled water to displace the residual culture medium. The wet microalgae biomass was lyophilized and weighed to obtain a dry biomass. The dried biomass was present in gram per liter (g/L).

Chemical Characterization of *G. sulphuraria* 009 Biomass

The carbohydrate content of the biomass was determined using a modified phenol-sulfuric acid method (Dubois *et al.*, 1956). Ten milligrams of dried biomass sample were added to 10 mL H_2SO_4 2% and heated at 90°C for 1 h. After cooling, the supernatant was reacted with 0.5 mL phenol 5.0% and 2.5 mL sulfuric acid (98 %). The absorbance of the mixture was measured at 490 nm and plotted using a glucose standard curve. The protein content was measured using the Bradford Protein Assay Kit. Dried biomass samples (10 mg) were resuspended in phosphate buffer and a bead beater for 300 s at 4°C. The supernatant was added to 1 mL of Bradford reagent, and the absorbance was measured at 595 nm. Meanwhile, the protein concentration was calculated using a BSA standard curve. Total lipid content was determined gravimetrically using a modified method described by Ryckebosch (Ryckebosch *et al.*, 2012).

Extraction and Determination of Phycocyanin

The extraction of phycocyanin was conducted by diluting five milligrams of dried biomass in 0.1 mM phosphate buffer pH 7. The extraction was carried out using a bead beater for 300 s with 15 s on and 15 s off. The solution was subsequently centrifuged at 4°C for 30 min at 10,000 rpm. The residual biomass from this step was then used continuously for chlorophyll *a* and carotenoids extraction.

The spectrum of phycocyanin was determined using a spectrophotometer in the 400–800 nm range. The phycocyanin concentration was estimated using a Spectrophotometer at 624 and 652 nm. Phycocyanin content was determined using the following equation (Silveira *et al.*, 2008):

$$\text{Phycocyanin } (\frac{mg}{mL}) = \frac{(A_{620}-(A_{652}\times 0.474))}{5.34}$$

$$\text{B (mg/g dried biomass)} = \frac{(PC \times \text{Vol. C})}{D}$$

$$\text{E (mg/L culture)} = B \times \text{Total F}$$

B = yield of phycocyanin (mg/g dried biomass)
PC = phycocyanin
Vol.C = vol. extract (mL)
D = sample (g)
E = yield of phycocyanin (mg/L culture)
Total F = total dried biomass per L culture

Antioxidant of Phycocyanin

The antioxidant activity of phycocyanin was presented as DPPH radical scavenging activity using the method described previously (de Marco Castro *et al.*, 2019). One hundred microlitres of the phycocyanin sample was placed in the wells of a 96-well microplate. Subsequently, the fresh DPPH solution (100 µL) was added to the sample, and the aqueous solution was used as a blank. The mixture was incubated for 30 min at 25°C in the dark. The absorbance of the sample was then measured spectrophotometrically at 517 nm. The obtained data were subsequently calculated using the DPPH scavenging equation below, which represents the inhibitory activity of the compound against DPPH radicals. A calibration curve was constructed using standard solutions of ascorbic acid at known concentrations. The % inhibition values obtained from the sample were plotted against the standard curve to determine the ascorbic acid equivalent antioxidant capacity (AEAC). The antioxidant activity of the sample was compared to a standard curve of ascorbic acid, and the results were expressed in units of µg ascorbic acid per mg of sample. This value reflects the antioxidant capacity of the sample in terms of its equivalence to ascorbic acid.

$$\text{DPPH scavenging} = \frac{G-H}{G} \times 100$$

G = blank absorbance value
H = sample absorbance value

Extraction and Determination of Chlorophyll *a* and Carotenoids

After phycocyanin extraction, chlorophyll *a* and carotenoids content were evaluated using fresh biomass and residual biomass. As phycocyanin is a protein extracted only with phosphate buffer, we expected the biomass to contain chlorophyll *a* and carotenoids. Biomass was resuspended in 100% methanol (Merck) and extracted using a bead beater for 300 s. The supernatant was measured spectrophotometrically at different wavelengths for chlorophyll-a and carotenoids determination. Chlorophyll *a* and carotenoid contents were calculated using the following equation (Náhlík *et al.*, 2023):

$$\text{Chl a (µg/mL)} = 12.25 \times A_{664} - 2.79 \times A_{647}$$

$$\text{Carotenoid (µg/mL)} = \frac{I-J}{198}$$

$I = (1000 \times A_{470})$
$J = (1.82 \times \text{Chl a})$

Data Analysis

All experimental data were statistically analyzed using a completely randomized design (CRD) with IBM SPSS Statistics (Version 20). The growth rate, biomass production, chemical composition, phycocyanin yield, and antioxidant activity were subjected to one-way analysis of variance (ANOVA) to determine significant differences among the treatments. Post-hoc comparisons were conducted using Duncan's multiple range test at a confidence level of $p<0.05$ to assess the statistical significance of variations between treatment groups. Descriptive statistical analysis was used to report mean values and standard deviations for all measured parameters. The normality of the data distribution was assessed using the Shapiro-Wilk test, while Levene's test was used to evaluate the homogeneity of variance. In cases where data did not meet parametric assumptions, non-parametric Kruskal-Wallis tests followed by Dunn's multiple comparisons test were applied.

RESULTS AND DISCUSSION

Preliminary Cultivation of *G. sulphuraria* 009

In the preliminary experiment, several concentrations of POME were employed to determine the optimum concentration for the growth of *G. sulphuraria* 009. The spike ratios of POME were 5, 10, 20, 30, and 50%. Figure 1 shows the growth profile of *G. sulphuraria*

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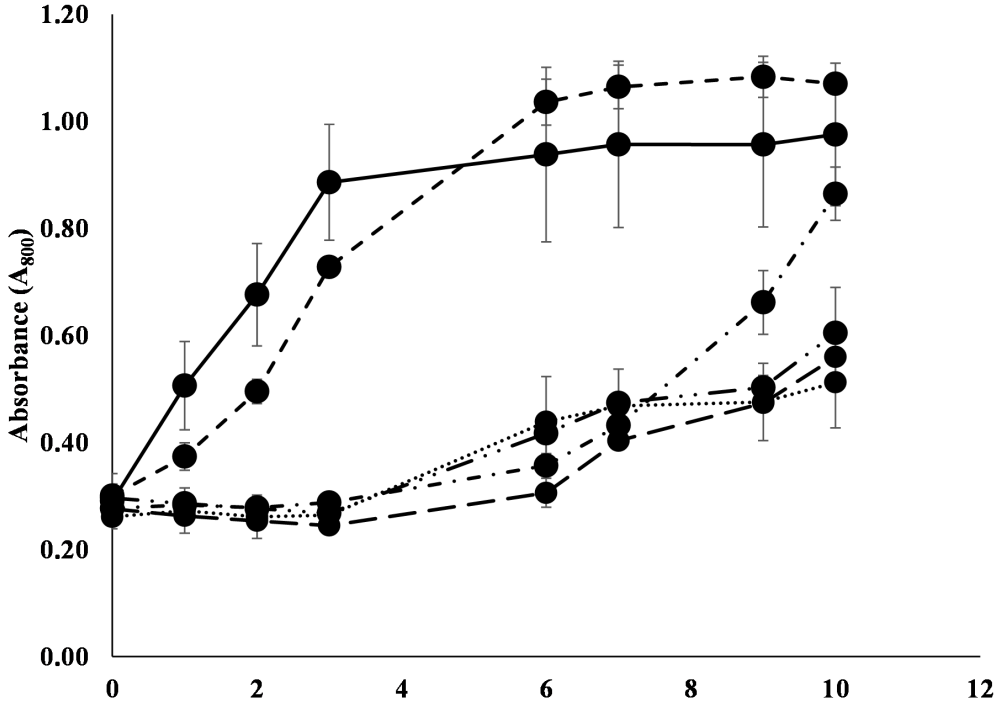

Figure 1 Growth curve of *G. sulphuraria* 009 in Allen medium (◇) and different concentrations of POME; (◆) 5% POME, (△) 10% POME, (▲) 20% POME, (□) 30% POME, (■) 50% POME

Gambar 1 Kurva pertumbuhan *G. sulphuraria* 009 dalam media Allen (◇) dengan berbagai konsentrasi POME; (◆) 5% POME, (△) 10% POME, (▲) 20% POME, (□) 30% POME, (■) 50% POME

009 in Allen medium as a control and POME at five different concentration levels. Under different concentrations of POME, 5% (v/v) showed maximum growth of *G. sulphuraria*. However, high concentrations of POME had a long lag phase and did not support microalgae growth. The growth of *G. sulphuraria* was inhibited at high concentrations of POME in the growth medium due to the typical dark brown liquor form of POME. The high concentration of POME had a shading effect on the culture as light penetration into the medium was blocked.

A previous study by Hadiyanto & Azimatun Nur (2014) showed that a high concentration of POME inhibited the growth rate and cause the lag phase into a longer time for growth adaptation due to light penetration limitation. Furthermore, the longer lag phase resulted in proloning the exponential and stationary phases, which extended the total cultivation time.

Growth of *G. sulphuraria* 009 in Addition of POME

Preliminary study data (Figure 1) showed that *G. sulphuraria* 009 grew well in 5% POME. The dilution of POME up to 5% (v/v) may have positively affected microalgae growth because of the lowered concentration of ammoniacal nitrogen in POME, which could be toxic to microalgae at high concentrations (Collos & Harrison, 2014; Wang *et al.*, 2019). Moreover, the dilution of POME would also enhance light penetration into the culture due to lessening POME's typical dark color liquor (Nur & Buma, 2019; Soleimaninanadegani & Manshad, 2014). Thus, the concentrations of POME for microalgae cultivation in the subsequent experiment were 2.5%, 5.0%, and 7.5%, with Allen medium pH 2 used as the control medium. The growth curves of *G. sulphuraria* 009 at three concentrations of POME and in the control medium (pH 2) are illustrated in Figure 2.

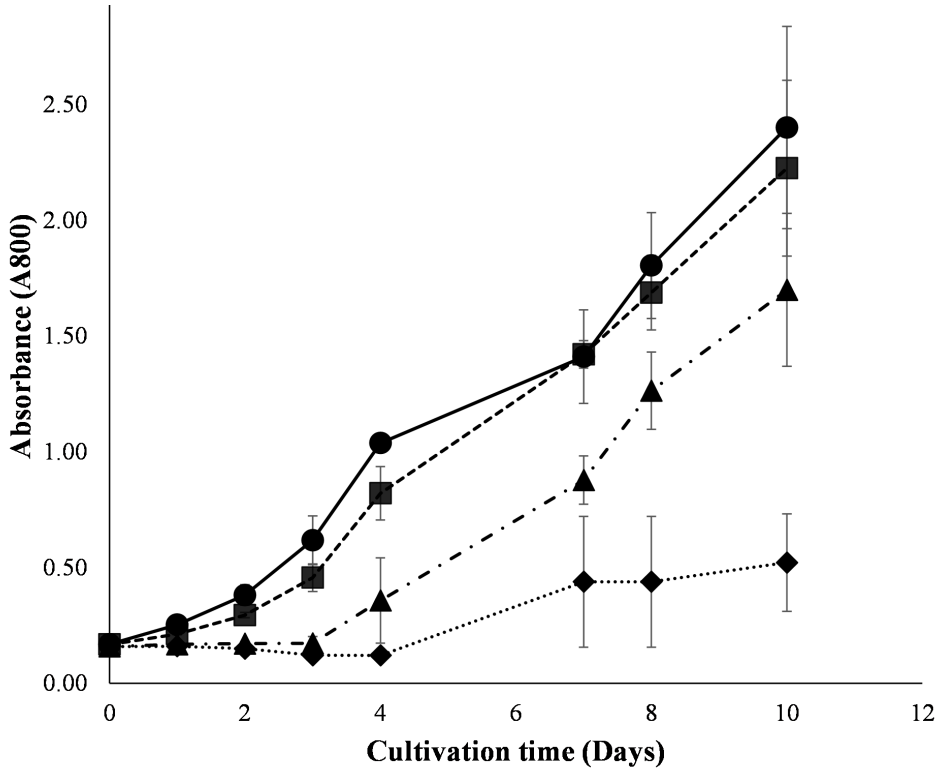

Figure 2 Growth curve of microalgae cultivated in Allen medium (●) and addition of 2.5% (■), 5.0% (▲), and 7.5% (◆) POME.

Gambar 2 Kurva pertumbuhan mikroalga yang dibudidayakan dalam media Allen (●) dengan penambahan 2,5% (■), 5,0% (▲), dan 7,5% (◆)POME.

As shown in Figure 2**,** *G. sulphuraria* grown in Allen medium and 2.5% POME had a slightly similar growth profile, which started increasing on day two. The 5.0% and 7.5% POME groups started the exponential phase on days three and four, respectively. The specific growth rate and biomass production in the Allen medium and the three different concentrations of POME are shown in Figure 3.

Microalgae grown in the control medium and 2.5% POME had similar specific growth rates and total biomass production. The total biomass of *G. sulphuraria* was 1.2066±0.1778 and 1.2182±0.1720 in the control medium (Allen pH 2) and 2.5% POME medium, respectively. This result confirmed that a high concentration of POME might inhibit microalgae growth because of its darker color with increasing POME concentration. *G. sulphuraria* is a highly acdaptable microalga with a flexible metabolism, apable of utilizing more than 50 different carbon sources and able to grow under autotrophic, mixotrophic, and heterotrophic conditions (Gross & Schnarrenberger, 1995). Since palm oil mill effluent (POME) contains various organic compounds, it can serve as an alternative carbon source for microalgal cultivation. In this study, *G. sulphuraria* successfully grew in POME-based media, confirming its potential as a cultivation medium. Among the tested concentrations, the lowest POME concentration (2.5%) supported the most favorable growth profile and highest total biomass production. In contrast, higher POME concentrations inhibited growth, likely due to reduced light penetration and ammoniacal nitrogen toxicity. This may occur as the strain limitation to utilize the POME and the exceeded ammoniacal nitrogen in higher concentrations of POME become toxic to the microalgae cells (Collos & Harrison, 2014; Wang *et al.*, 2019).

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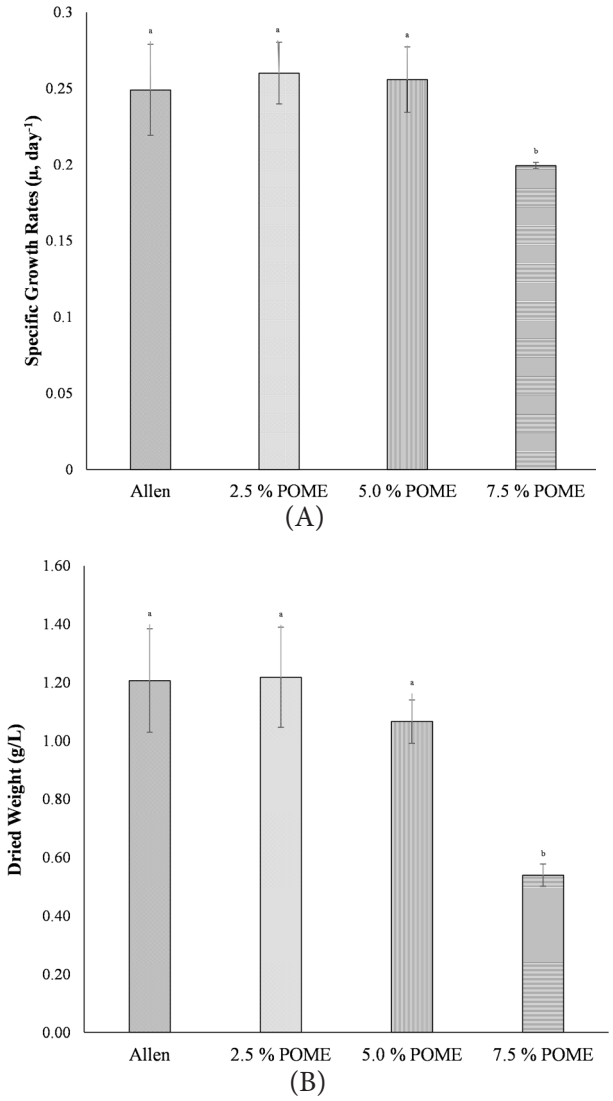

Figure 3 Specific growth rate (A) and biomass production (B) of *G. sulphuraria* 009 in Allen (▧) medium and addition of 2.5% (▨), 5.0% (▥), and 7.5% (▤) POME.

Gambar 3 Laju pertumbuhan spesifik (A) dan produksi biomassa (B) dari *G. sulphuraria* 009 dalam media Allen (▧) dengan penambahan 2,5% (▨), 5,0% (▥), dan 7,5% (▤) POME.

Table 1 Chemical composition of biomass from *G. sulphuraria* 009 in dry weight
Tabel 1 Komposisi kimia biomassa kering dari *G. sulphuraria* 009

Cultivation media	Carbohydrate (mg/g)	Protein (mg/g)	Lipid (mg/g)
Allen	93.91±26.98[a]	58.72±9.10[a]	97.74±7.52[a]
2.5% POME	123.22±28.18[a]	67.99±1.49[a]	95.28±1.27[a]
5.0% POME	132.14±18.14[a]	62.11±5.01[a]	90.90±9.09[a]
7.5% POME	106.81±7.33[a]	33.97±6.83[b]	111.09±3.66[a]

Values in the same column with different lower-case numbers are significantly different (p<0.05).
Values shown are the mean ± standard deviation.

Chemical Composition of *G. sulphuraria* 009 Biomass

The chemical compositions of *G. sulphuraria* 009 in three different POME concentrations and control media are shown in Table 1.

There are different chemical compositions between the three concentrations of POME and the Allen medium. Carbohydrate content increased with the concentration of POME up to 5.0%, where it reached 132.14±18.14 mg/g. However, at the highest concentration of 7.5% POME, carbohydrate content decreased to 106.81±7.33 mg/g, a reduction of 25.33 mg/g from 5.0% POME. This suggests that the optimal nutrient conditions for carbohydrate production are found at 5.0% POME, with higher concentrations potentially creating inhibitory effects due to factors such as nutrient imbalance or stress responses (Fernando *et al*., 2021). The lipid content displayed more fluctuation across the POME concentrations. At 2.5% POME, lipid content was 95.28±1.27 mg/g, slightly lower than that in Allen medium (97.74±7.52 mg/g). This trend continued at 5.0% POME, where lipid content further decreased to 90.90±9.09 mg/g. However, at 7.5% POME, lipid production increased significantly to 111.09±3.66 mg/g, a 20.19 mg/g rise from the 5.0% POME concentration. This suggests that *G. sulphuraria* may respond to higher POME concentrations by enhancing lipid biosynthesis, possibly as a stress-induced response. The fluctuation in lipid content indicates a complex interaction between nutrient availability, environmental stress, and metabolic regulation, with lipid accumulation potentially serving as a protective mechanism under higher POME concentrations (Rahman *et al*., 2020). In this study, lipid content was higher compared to data of this species reported previously by other authors about 46±3.2 up to 77±1.1 mg/g and (Graziani *et al*., 2013; Massa *et al*., 2019) but still lower than that of other studied microalgae, especially green microalgae. Our results showed that carbohydrates are the main chemical components of *G. sulphuraria* 009 biomass. This result is related to the findings of other authors, revealing that the main composition of *Galdieria* species is carbohydrates (Graziani *et al*., 2013). However, the protein content in this work was lower than that previously reported for *Galdieria* species. Information on the chemical composition is essential for identifying the application of the end products of microalgae biomass. Our results also indicate that the growth medium content regulates the chemical composition of microalgal biomass (Japar *et al*., 2021).

Phycocyanin Production and Antioxidant Activity

G. sulphuraria is currently known as an alternative source of phycocyanin in addition to *Spirulina platensis* (Moon *et al*., 2014; Rahman *et al*., 2020; Sloth *et al*., 2017). Phycocyanin production by *G. sulphuraria* growing in Allen and three different concentrations of POME are presented in Table 2.

The microalgae cultivated in 2.5% POME had the highest phycocyanin production based on the culture volume compare to other additional POME concentration, although it was not significantly different from the phycocyanin production of microalgae in the Allen medium. The yield of phycocyanin in Allen and 2.5% POME in this study was close to the results of Abiusi (Abiusi *et al*., 2021), who grew *G. sulphuraria* ACUF 064 under mixotrophic conditions. In this study, phycocyanin in Allen and 2.5% POME were 82.22±7.12 mg/g and 84.63±7.19 mg/g, respectively. A previous study by Abiusi *et al*. (2021) reported that phycocyanin from *G. sulphuraria* ACUF 064 in autotrophic and mixotrophic conditions was 97±14 mg/g and 87±3 mg/g, respectively. This finding is also in the range of what Hirooka & Miyagishima (2016) reported, about 40 to 100 μg/mg, depending on the nitrogen source type.

The characteristics of phycocyanin were confirmed by its typical optimum absorption at 620 nm (Patel *et al*., 2005), as illustrated in Figure 4. Phycocyanin from *G. sulphuraria* in Allen medium and 2.5% POME showed a sharp peak at 620 nm. In contrast, 5.0% and 7.5% were lower than both phycocyanin extracts mentioned previously. The spectrum of phycocyanin from this experiment is almost identical to those found for phycocyanin

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Table 2 Purity index and phycocyanin yield of *G. sulphuraria* 009 growing in Allen and different concentration of POME

Tabel 2 Indeks kemurnian dan produksi fikosianin dari *G. sulphuraria* 009 yang tumbuh dalam media Allen dengan berbagai konsentrasi POME

Cultivation media	Dried biomass (g/L)	Purity index	Yield of phycocyanin (mg/L culture)	Yield of phycocyanin on biomass (mg/g DW)
Allen	1.201±0.178[a]	0.79±0.02[a]	99.21±8.59[a]	82.22±7.12[a]
2.5% POME	1.218±0.172[a]	0.73±0.03[b]	103.09±8.76[a]	84.63±7.19[a]
5.0% POME	1.066±0.074[a]	0.54±0.05[c]	73.21±9.77[b]	68.66±9.17[a]
7.5% POME	0.539±0.039[b]	0.19±0.02[d]	13.13±1.37[c]	24.36±2.55[b]

Values in the same column with different lower-case numbers are significantly different (p 0.05).

Values shown are the mean ± standard deviation.

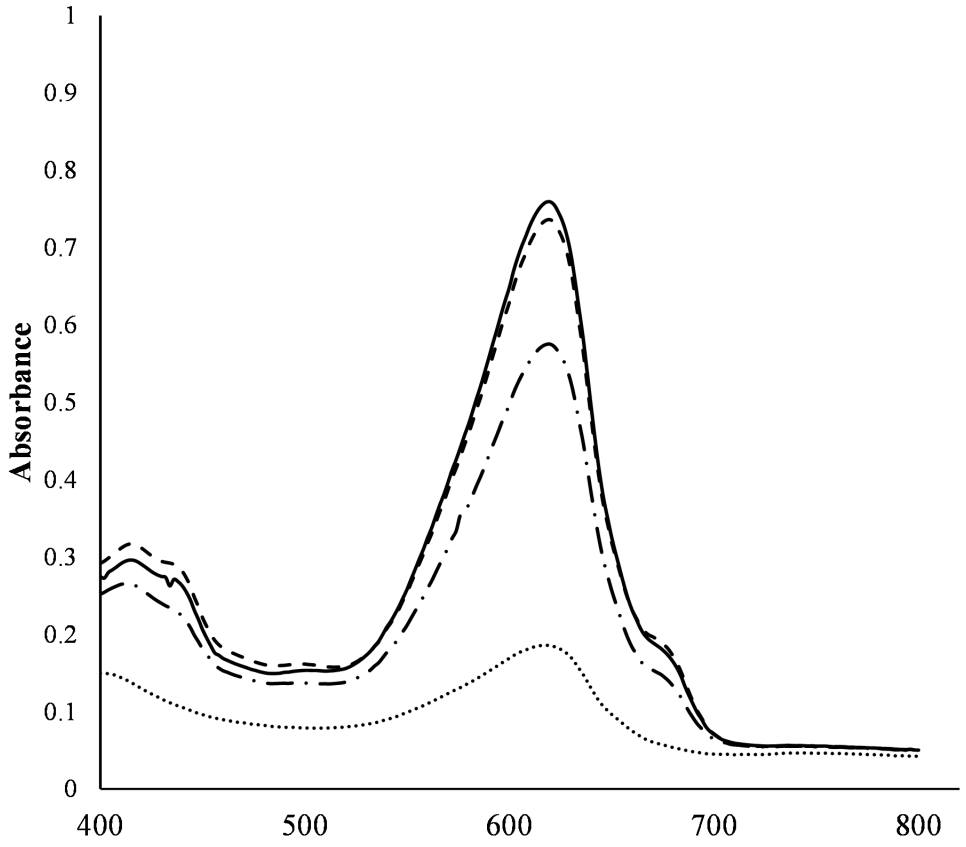

Figure 4 Visible spectrum of phycocyanin extracted from *G. sulphuraria* 009 in different medium; Allen (—), 2.5% (- -), 5% (-·-), dan 7.5% (····) POME.

Gambar 4 Spektrum tampak fikosianin yang diekstrak dari *G. sulphuraria* 009 dalam berbagai media; Allen (—), 2.5% (- -), 5% (-·-), dan 7.5% (····) POME

extracted from other microalgae strains, *G. sulphuraria* UTEX 2919, in autotrophic conditions (Moon *et al*., 2014), *Spirulina platensis* (Patel *et al*., 2005), and *Anabaena* sp. (Ramos *et al*., 2010).

The purity index of phycocyanin decreased with increasing concentrations of POME in the cultivation medium. Allen medium and 2.5% POME yielded the highest purity indices, 0.79 and 0.73, respectively, both within an acceptable range. However, at 5% and 7.5% POME, the purity index decreased significantly to 0.54 and 0.19, indicating a higher level of protein contamination or pigment degradation. This decline is likely due to environmental stress or excessive organic load from POME, which may adversely affect cell integrity and pigment stability during cultivation.

In this study, the extraction of the same amount of biomass from each medium composition resulted in different yields of phycocyanin. This may have occurred because of the shading effect of the high concentration of POME. In 7.5% POME, the medium became darker than the control medium and the other two lower concentrations of POME. The dark color of the medium blocked light penetration into the culture. A previous report by Perez-Garcia stated that photosynthetic pigments in *G. sulphuraria* under autotrophic conditions were higher than those cultivated in heterotrophic or light-limited cultivation. This lower phycocyanin content is caused by a decrease in phycocyanin synthesis in cells, as the energy in the limiting or without light penetration comes from the oxidation of the organic substrate and not from photosynthesis (Perez-Garcia *et al*., 2011; Salbitani & Carfagna, 2020).

The antioxidant activity of phycocyanin extracted from *G. sulphuraria* 009 cultivated in three different concentrations of POME (2.5%, 5%, and 7.5%) and Allen medium was evaluated using the DPPH assay (Figure 5). The results indicate that the antioxidant activity of phycocyanin ranged from approximately 100 µg AEAC/mg sample in Allen medium and 2.5% POME to higher values in 5% and 7.5% POME. Specifically, the phycocyanin extract from *G. sulphuraria* cultivated in 7.5% POME exhibited a strikingly high antioxidant activity of around 450 µg AEAC/mg sample, significantly higher than that of phycocyanin from *Spirulina platensis* (20.12±0.2 mg AEAC/g) reported by de Marco Castro *et al.* (2019). The antioxidant activity observed in this study is categorized as strong, indicating

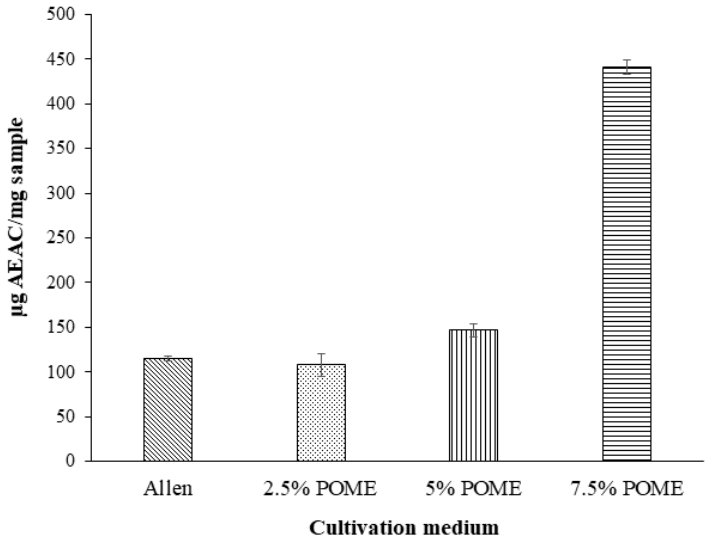

Figure 5 Antioxidant activity of phycocyanin extracted from *G. sulphuraria* 009 in Allen (▧) medium and addition of 2.5% (▩), 5.0% (▥), and 7.5% (▤) POME.

Gambar 5 Aktivitas antioksidan fikosianin yang diekstrak dari *G. sulphuraria* 009 yang dibudidayakan dalam media Allen (▧) dengan berbagai penambahan POME 2.5% (▩) , 5.0% (▥), and 7.5% (▤).

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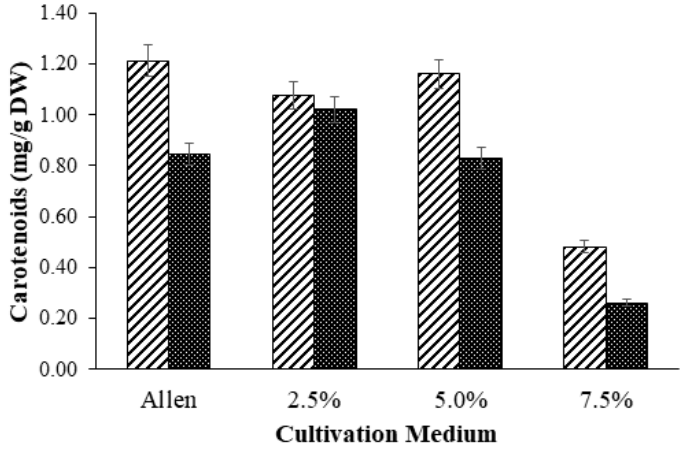
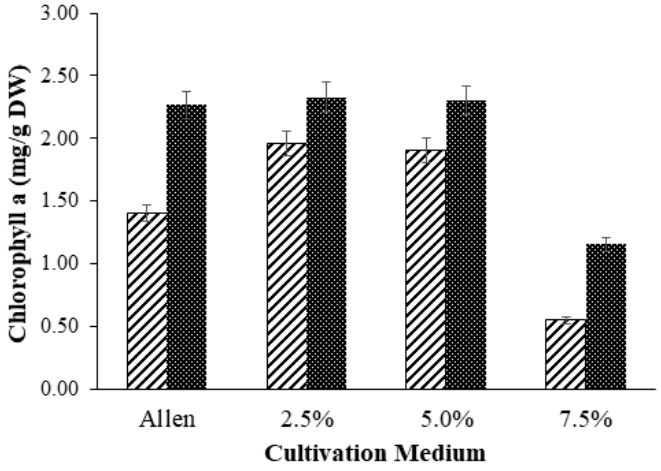


(A)



(B)

Figure 6 Carotenoids and chlorophyl a from *G. sulphuraria* 009 using fresh (▨) and continuously continuously extracted biomass (■).

Gambar 6 Karotenoid dan klorofil a dari *G. sulphuraria* 009 menggunakan biomassa segar (▨) dan yang diekstraksi secara kontinu(■).

that *G. sulphuraria* 009 may be a promising source of bioactive compounds with significant antioxidant potential.

The antioxidant activity of phycocyanin extracted from *G. sulphuraria* in 7.5 % POME was much higher than that of other phycocyanins extracted from *G. sulphuraria* at other POME concentrations and in Allen medium. Such high antioxidant activity might be caused by stress conditions related to the high concentration of POME (7.5%) in the medium. Thus, antioxidants serve as the protector for microalgal cells from oxidative stress (Ismaiel & Said, 2018).

Chlorophyll *a* and Carotenoids Content of Fresh and Residual Biomass

Galdieria, as red microalgae, contain phycocyanin and phycoerythrin (a light-harvesting complex protein specific for Rhodophyta and Cyanophyta), chlorophyll, and a high content of carotenes (Albertano *et al.*, 2000; Čížková *et al.*, 2021; Graziani *et al.*, 2013; Gross *et al.*, 2001; Marquardt, 1998). Carotenoids and chlorophyll are valuable bioactive compounds that can be extracted from microalgae biomass and utilized as natural food colorants and antioxidant agents

(Graziani *et al*., 2013; Halim *et al*., 2010). This study evaluated chlorophyll *a* and carotenoids contents using fresh biomass and residual biomass after phycocyanin extraction from *G. sulphuraria* cultivated in three concentrations of POME and Allen medium (Figure 6).

High carotenoids content was generally obtained from fresh biomass. In contrast, high chlorophyll *a* content was obtained from residual biomass. The yield of *G. sulphuraria* 009 carotenoids cultivated in 2.5% and 5.0% POME, and Allen medium were range at 1.2-0.8 mg/g dry biomass. These concentrations of POME seem to provide an optimal environment for carotenoid production, likely due to a balanced nutrient composition and favorable metabolic conditions that support both growth and secondary metabolite synthesis. The microalga may experience sufficient nutrient availability at these concentrations without overwhelming stress, leading to higher carotenoid accumulation. However, at the highest POME concentration of 7.5%, the carotenoid yield decreased significantly to 0.4 mg/g dry biomass. This reduction may be linked to several factors. Firstly, higher POME concentrations could introduce inhibitory effects, such as an excess of certain nutrients, particularly ammonium nitrogen, which could be toxic at elevated levels. Additionally, the darker color of POME could reduce light penetration in the culture medium, limiting the photosynthetic efficiency and consequently reducing the energy available for carotenoid biosynthesis. Inversely proportional to carotenoids content, the highest chlorophyll *a* content was found in the residual biomass. The results showed that the extraction protocol can affect the yield of bioactive compounds from microalgae biomass. Using fresh biomass and directly extracted with methanol 100%, the carotenoids content was higher, and chlorophyll *a* was lower. Extraction using residual biomass from phycocyanin resulted in a highest content of chlorophyll *a*.

CONCLUSION

The lowest POME concentration (2.5%) demonstrated the best growth profile and total biomass production. The highest phycocyanin production per culture volume was observed in microalgae cultivated in 2.5% POME. Phyocyanin extracts exhibited significant antioxidant activity, particularly in cultures with 7.5% POME, which could be attributed to the stress conditions induced by the high POME percentage, suggesting a role of antioxidants in protecting microalgal cells from oxidative stress.

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