

Purification and Preliminary Characterization of Biopigments From *Nitzschia closterium*

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ABSTRACT

Biopigments are important bio-based compounds used in food, pharmaceutical, and cosmetic industries. *Nitzschia closterium*, a marine diatom microalga, is known for its potential to produce various pigments. This study aimed to purify and preliminarily characterize biopigments extracted from *Nitzschia closterium*. The research involved microalgal biomass cultivation, harvesting, pigment extraction, preliminary identification using thin-layer chromatography (TLC), and purification through column chromatography. After six days of cultivation, the average biomass density reached 1.67 g/L. TLC analysis of the crude pigment extract showed nine distinct pigment bands. Chromatographic purification and comparison with the TLC profile indicated four major pigment groups. Fractions 16 and 17 were predicted to be β -carotene (band 9); fractions 18 and 19 likely represented pheophytins (band 8); fractions 20 to 30 contained chlorophylls and their derivatives (bands 5–7); and fractions 31 to 45 showed orange-yellow hues associated with xanthophyll-type carotenoids (bands 2–4). These results demonstrate that *Nitzschia closterium* is a promising natural source of multiple biopigments, and that TLC and column chromatography effectively supported their purification and initial characterization.

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Introduction

Biopigments, or natural colorants, are important compounds widely used in the food, cosmetic, and healthcare industries. In the food industry, biopigments enhance product appeal and are considered indicators of product quality, as they are safe to consume and possess antioxidant properties that can inhibit lipid oxidation [4]. In cosmetics, biopigments not only serve as natural colorants but also have multiple functional roles due to their photoprotective, antioxidant, and anti-aging properties [8]. In the health sector or pharmaceutical industry, various biopigments are utilized as sources of vitamins and have been shown to exhibit antioxidant activity, delaying oxidation processes induced by free radicals [12], as well as demonstrating natural antibacterial effects against pathogenic bacteria [7]. Owing to their lower risk to human health

and the environment, microalgae-derived biopigments represent a promising alternative to synthetic pigments, which have been associated with health risks and environmental pollution. With increasing awareness of the importance of safety and sustainability for human health and the environment, there is a global shift from synthetic pigments to natural biopigments [13].

Indonesia, as a maritime country, possesses abundant biodiversity. One of the biological resources that has attracted increasing attention in recent years is marine microalgae, which continue to be actively explored [10]. Microalgae are autotrophic eukaryotic microorganisms, with millions of species that hold great potential for diverse applications. In addition to their roles as animal feed supplements and human nutrition, microalgae are being developed as alternative

energy sources and for cancer therapy [6]. Microalgae are considered valuable biological resources and have recently gained significant global attention. Their economic importance spans multiple sectors, including the food and pharmaceutical industries, immunostimulant development, biofuels, cosmetics, and agriculture. Microalgae are capable of synthesizing proteins, polysaccharides, lipids, fatty acids, vitamins, pigments, enzymes, phycobiliproteins, and more [5]. Compared to plants and other organic sources, microalgae are regarded as superior sources of biopigments due to their rapid growth rates and year-round production capability. Furthermore, microalgae cultivation does not require agricultural land, making it more sustainable than terrestrial plant-based sources [13].

Nitzschia closterium, a marine diatom microalga belonging to the class Bacillariophyceae, represents one of the functional groups of marine phytoplankton characterized by high species diversity. This microalga plays an essential role in marine ecosystems, particularly in primary productivity, marine food webs, and global carbon sequestration [3]. Diatoms are predominantly unicellular organisms, although some species may form chains or colonies. They are also commonly referred to as silica algae due to the presence of silica (SiO_2) in their cell walls [1]. In addition, diatoms possess well-developed chloroplasts containing various biopigments that function in light absorption during photochemical reactions [2]. These characteristics highlight their potential as natural sources of valuable biopigments.

Despite this potential, studies focusing on biopigments from *N. closterium* remain limited, and this species has been more extensively utilized as a natural feed source in aquaculture rather than as a biopigment resource. Previous research has reported the preliminary isolation and characterization of biopigments from *N. closterium* using thin-layer chromatography (TLC), which provided initial information on pigment composition based on color and R_f values [14]. However, such preliminary studies did not include purification of individual pigment groups, and therefore the separation and characterization of purified biopigments

from this species remain insufficiently explored. Purification is an important step, as individual biopigments possess distinct physicochemical properties and functional roles, which influence their potential applications in food, cosmetic, and pharmaceutical industries.

In this context, the novelty of the present study lies in extending beyond preliminary pigment isolation by performing purification using column chromatography. This approach enables the separation of biopigments into distinct groups based on their differential interactions with the stationary phase and their elution behavior in the selected solvent system. Furthermore, the grouping of biopigments according to their elution profiles provides improved resolution compared to preliminary TLC analysis alone, allowing a clearer differentiation of pigment classes. Therefore, this study contributes to advancing the understanding of biopigments from *N. closterium* by providing a purification-based approach and systematic grouping of pigment fractions, which represents an important step toward more detailed characterization and potential utilization of these natural pigments.

Material and Methods

Material

The microalgae used in this study was *Nitzschia closterium*, a pure culture obtained from the culture collection of the Research Center for Oceanography, Indonesian Institute of Sciences, Ancol, North Jakarta. Other materials used in this research included Walne medium, seawater, distilled water, 70% ethanol, cotton, filter paper, aluminum foil, acetone p.a (Merck, Germany), hexane p.a (Merck, Germany), technical acetone, and silica gel 7731 (Merck, Germany).

Cultivation of Microalgae and Biomass Harvesting

The cultivation of *Nitzschia closterium* was carried out in batch culture using 1 L glass bottles at room temperature with Walne medium. The initial cell density was maintained at a minimum of 1×10^5 cells/mL, and aeration was controlled throughout the cultivation period. Fluorescent lamps supplied light at an intensity of $67.5 \mu\text{mol}/\text{m}^2\cdot\text{s}$ under a 24-hour

photoperiod (24 hours light : 0 hours dark). Biomass harvesting was conducted via centrifugation at 6000 rpm for 35 minutes, followed by freeze-drying of the wet biomass.

Biopigments Extraction and Preliminary Identification

The dried biomass was extracted using analytical-grade acetone and homogenized with a mechanical homogenizer. During extraction, the biomass-acetone mixture was placed in an ice bath to prevent pigment degradation. The homogenate was filtered, and the resulting filtrate was stored in dark bottles to minimize light exposure. Preliminary identification of biopigments was conducted using thin-layer chromatography (TLC) with silica gel plates and a mobile phase consisting of hexane p.a. and acetone p.a. (7:3 v/v).

Purification of Biopigments from *N. closterium*

The crude extract was purified using column chromatography with 19 grams of silica powder 7731 as the stationary phase and a mobile phase of hexane p.a. and acetone p.a. (7:3 v/v). The eluate was collected in fractions, with each tube containing approximately 120 drops per fraction.

Results and Discussions

Biomass of *Nitzschia closterium*

Nitzschia closterium biomass was obtained through cultivation in Walne medium, a premixed formulation providing the complete nutrient profile necessary for microalgal growth. Based on a previously established growth curve [11], *N. closterium* requires 6 days for optimal growth, comprising 2 days of adaptation phase and 4 days of exponential growth in Walne medium. The growth of *N. closterium* was observed through the increasing intensity of culture color and rising cell density. On day 0 (Figure 1(a)), the culture appeared clear, while on day 6 (Figure 1(b)), it turned yellowish-brown. A darker culture color indicates higher microalgal cell density. In this study, the initial inoculum density was 1.0×10^5 cells/mL, which increased to 1.65×10^6 cells/mL on day 6. Based on the change in culture color (as shown in Figure 1) and the increase in cell

number, it can be concluded that the cultivation of *N. closterium* was successfully achieved.

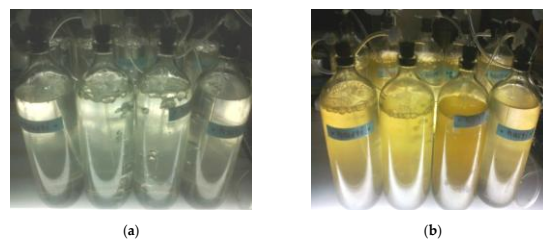


Figure 1. Cultivation of marine microalga *Nitzschia closterium*: (a) Day 0, (b) Day 6

Nitzschia closterium is classified as a microalga that is relatively susceptible to contamination and environmental changes. Prior to cultivation, microscopic examination was conducted using a light microscope to ensure that the *N. closterium* isolate to be cultured was axenic, consisting solely of a single isolate. The results showed that the initial culture contained only *N. closterium* microalgae, with no other microorganisms such as viruses or bacteria observed, as illustrated in Figure 2. The cells of *N. closterium* appeared flattened, with two central nuclei, which is a characteristic feature of diatom microalgae.



Figure 2. Microscopic image of *N. closterium* cells (Scale 1:1500)

Wet biomass of *Nitzschia closterium* was obtained by harvesting the microalgae through centrifugation. This method enabled the sedimentation of cells at high rotational speeds without compromising cellular integrity. The cumulative wet biomass from eight harvesting cycles amounted to 65.696 grams, with a mean yield of 8.212 ± 3.407 grams per harvest. Prior to biopigment extraction, the harvested wet biomass was dried using a freeze dryer. The duration of freeze-drying was adjusted according to the amount of wet biomass. The

total dry biomass obtained from eight harvests was 4.630 grams, with an average of 0.579 ± 0.254 grams per harvest.

Crude Extract and Preliminary Identification of Biopigments

The crude extract of biopigments from *Nitzschia closterium* microalgae was subjected to preliminary identification using thin-layer chromatography (TLC) with an n-hexane:acetone (7:3 v/v) mobile phase. The chromatogram revealed nine distinct pigment bands with varying colors and Rf values (Figure 3), indicating the presence of multiple biopigments with different polarity characteristics. In TLC separation, biopigments with lower polarity generally exhibit higher Rf values due to stronger interaction with the mobile phase, while more polar biopigments tend to migrate more slowly due to stronger retention on the stationary phase.

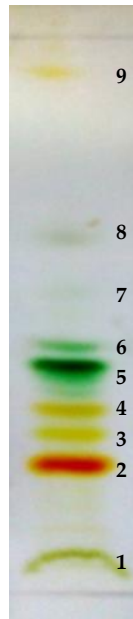


Figure 3. Chromatogram of biopigments from *N. Closterium*

Based on the observed color and Rf values (Table 1), the detected biopigments were tentatively classified into several groups, including chlorophyll c (Rf 0.04), xanthophylls (Rf 0.24–0.35), chlorophyll a (Rf 0.42), chlorophyll derivatives (Rf 0.38 and 0.46), pheophytin a (Rf 0.64), and β -carotene (Rf 0.94). This classification initially refers to the study by Kondoririk (2017), who reported similar

pigment separation behavior using TLC with solvent systems dominated by hexane and acetone. Although Kondoririk investigated pigments from marine macroalgae, the similarity in solvent polarity characteristics allows for comparable separation of major biopigment classes, particularly chlorophylls and carotenoids, which exhibit characteristic Rf ranges depending on their polarity.

Table 1. Biopigments separation results of *N. closterium* crude extract

Band	Color	Rf	Predicted Pigment
1	Light green	0.04	Chlorophyll c
2	Orange	0.24	Xanthophyll
3	Light yellow	0.29	Xanthophyll
4	Yellow	0.35	Xanthophyll
5	Pale green	0.38	Chlorophyll derivative
6	Green	0.42	Chlorophyll a
7	Pale green	0.46	Chlorophyll derivative
8	Gray	0.64	Pheophytin a
9	Yellow	0.94	β -Carotene

In addition, the biopigment profile observed in *N. closterium* is consistent with those reported in other marine diatoms. Telussa et al. (2022) reported the presence of chlorophyll c, xanthophylls, chlorophyll a, pheophytin, and β -carotene in the marine diatom *Navicula* sp., with Rf values distributed across low to high polarity ranges. The similarity in pigment groups and their relative migration behavior suggests that *N. closterium* possesses a characteristic diatom biopigment composition, consisting of primary photosynthetic pigments (chlorophyll a and c) and accessory pigments such as carotenoids and their derivatives. These accessory pigments play important roles in light harvesting and photoprotection, enabling diatoms to adapt to fluctuating light conditions in marine environments.

However, it should be emphasized that pigment identification based on TLC is preliminary and tentative, as it relies on migration behavior, color characteristics, and

comparison with previously reported R_f values. Therefore, further purification and characterization using more advanced analytical techniques are necessary to obtain more accurate identification and to better understand the properties of individual biopigments. Consequently, column chromatography was performed in the present study to further separate and purify the biopigments based on their polarity and chromatographic behavior.

Purified Biopigments from *Nitzschia closterium*

Purification of biopigments was achieved by subjecting the crude extract to column chromatography employing silica gel 7731. The purification process (Figure 4(a)) was carried out in a dark and cool environment to preserve the stability of the biopigments. The selection of the stationary and mobile phases was based on the previously conducted TLC method, using hexane:acetone (7:3 v/v) as the eluent and silica as the stationary phase. The length of the stationary phase used for purification ranged from 37 to 40 cm. A longer stationary phase increases purification time but allows for better separation. In contrast, a shorter stationary phase results in faster processing but poorer separation. With a stationary phase length of 37 cm, the average flow rate during purification was maintained at 0.35 mL/min. These conditions provided satisfactory separation. The purification process yielded 30 colored fractions (Figure 4(b)).

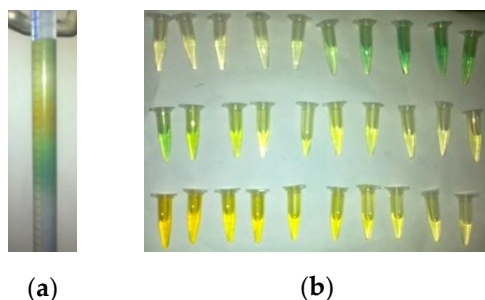


Figure 4. Purification of biopigments using column chromatography (a), and the resulting colored fractions after purification (b).

The purification process yielded 30 colored fractions, which were compared to the bands observed on the TLC plate. Fractions 1–15 were

colorless and presumed to be eluents; therefore, they were not collected. The first colored fraction appeared at fraction 16, displaying an orange-yellow hue, while the last-colored fraction appeared at fraction 45 with a pale orange shade. These colors varied depending on the type of biopigment; however, similar visible colors do not necessarily indicate identical biopigments. For instance, fractions 16 and 45 appeared visually similar but are likely different compounds due to differences in polarity and elution behavior.

Column chromatography using silica gel and a hexane:acetone (7:3 v/v) eluent operates under the same principle as TLC, in which less polar biopigments elute earlier than more polar ones. Based on elution order, TLC comparison, and observed color, fractions 16 and 17 are tentatively assigned to band 9 on the TLC plate and are presumed to be β -carotene. Fractions 18 and 19, which exhibited a greyish tone, are tentatively associated with pheophytins, corresponding to band 8. Fractions 20 to 30, displaying green coloration, are tentatively attributed to chlorophylls and their derivatives, corresponding to bands 5, 6, and 7. Meanwhile, fractions 31 to 45, which showed a gradient from orange to yellow, are tentatively predicted to be xanthophyll-type carotenoids, as indicated by bands 2, 3, and 4, consistent with their relatively higher polarity due to the presence of oxygen-containing functional groups.

However, the light green pigment observed as band 1 on the TLC plate was not detected in the purified fractions. This absence may be attributed to several factors, including possible pigment degradation during the purification process, strong retention on the stationary phase, or incompatibility between pigment polarity and the selected eluent system.

It is important to note that the identification of biopigments in this study is based primarily on visual color observation, TLC migration behavior, and elution order during column chromatography. While these approaches are appropriate for preliminary separation and exploratory characterization, they do not provide definitive structural identification. Therefore, the pigment assignments presented in this study should be

considered tentative. Further confirmation using advanced analytical techniques, such as UV-Vis spectroscopy, high-performance liquid chromatography (HPLC), or liquid chromatography-mass spectrometry (LC-MS), is necessary to accurately determine the molecular identity and purity of the isolated biopigments.

In addition to demonstrating effective chromatographic separation, the pigment groups tentatively identified in this study are known to have important functional and industrial relevance. β -carotene and xanthophylls are widely recognized for their antioxidant properties and are commonly used as natural colorants and bioactive compounds in food and cosmetic applications. Chlorophylls and their derivatives are also utilized as natural green pigments and have potential health-related benefits. Meanwhile, pheophytins, as chlorophyll degradation products, have been reported to exhibit antioxidant activity. Therefore, the successful separation of these pigment groups highlights the potential of *Nitzschia closterium* as a promising natural source of biopigments for applications in the food, cosmetic, and pharmaceutical industries. Nevertheless, the successful separation of distinct pigment fractions demonstrates the effectiveness of column chromatography in purifying and grouping biopigments from *Nitzschia closterium*.

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Conclusions

This study demonstrates the successful purification and fractionation of crude biopigment extract from the marine microalga *Nitzschia closterium* using column chromatography. The separation resulted in distinct pigment fractions that correspond to four major groups of biopigments previously observed in TLC analysis. Based on comparison of color characteristics, TLC migration behavior, and elution order, fractions 16 and 17 are tentatively assigned as β -carotene, fractions 18

and 19 as pheophytins, fractions 20 to 30 as chlorophylls and their derivatives, and fractions 31 to 45 as xanthophyll-type carotenoids.

However, it should be emphasized that these identifications remain tentative, as they are based on preliminary analytical approaches, including visual observation, TLC comparison, and chromatographic behavior. These methods are useful for initial separation and classification but do not provide definitive structural confirmation. Therefore, further analysis using advanced analytical techniques such as UV-Vis spectroscopy, HPLC, or LC-MS is required to confirm the molecular identity and purity of the isolated biopigments.

Despite this limitation, the present study successfully demonstrates the capability of column chromatography to purify and systematically group biopigments from *Nitzschia closterium* based on their elution behavior. This work represents an important step toward more comprehensive characterization and future utilization of purified biopigments from marine diatoms.

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