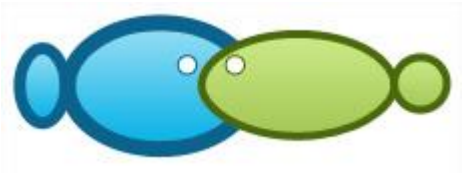

Evaluation of peanut shell and okara extracts as alternative culture media for *Nannochloropsis oculata*

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Abstract. The high cost of synthetic culture media poses a significant challenge for large-scale microalgae production. This study evaluated extracts from agricultural by-products, specifically peanut shells (PN) and okara (OKR) extracts, as alternative nutrient sources for cultivating microalgae *Nannochloropsis oculata*, a species valued in aquaculture for its nutritional profile. *N. oculata* was cultured under various conditions: individual extracts, extracts supplemented with 50% or 100% f/2 medium, and combined extracts with cane molasses (CM). Results demonstrated that *N. oculata* could grow in all extract-based media, with positive specific growth rates (SGR) observed across treatments. The combining OKR and PN extracts with CM (OKR + PN + CM) exhibited the highest SGR (0.12 day^{-1}) among treatments ($p < 0.05$) and reached peak cell density earlier (12.5×10^6 cells mL^{-1} on day 8). This improved performance of *N. oculata* is likely attributable to micronutrients from the combined extracts and the additional organic carbon from cane molasses. These findings confirm that PN and OKR extracts can serve effectively for *N. oculata* cultivation, offering a sustainable alternative to synthetic media. Further research into nutritional composition and mixotrophic potential of these by-products is recommended to optimize their application in large-scale microalgae production systems.
Keywords: agricultural by-products, algae, microalgae production, *Nannochloropsis oculata*.

Introduction. Microalgae, as unicellular organisms, utilize sunlight to convert CO_2 into valuable biomolecules, including lipids, proteins, carbohydrates, and sources of bioenergy (Lee et al., 2020; Ardo et al., 2022). It is a rich source of polyunsaturated fatty acids, including linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3), as well as pigments and proteins (NRC, 2011). These components provide significant nutritional and biomedical benefits, thereby making it highly suitable for aquaculture and other biotechnological applications (Converti et al., 2009; Zhu et al., 2013; Ma et al., 2014; Rasdi & Qin, 2015). Among marine microalgae, *Nannochloropsis oculata* contains high levels of eicosapentaenoic acid (EPA, 20:5 n-3, 215 g kg^{-1} total fat) and some docosahexaenoic acid (DHA, 22:6 n-3; 32 g kg^{-1} total fat) (Hulatt et al., 2017), along with high protein content (34%) and a fast growth rate (Fakhri & Arifin, 2016). Collectively, it is considered an ideal microalgal species for hatcheries and large-scale aquaculture production.

A number of studies have shown that carbon, nitrogen, and phosphorus are the most influential nutrients for biomolecule production in microalgae (Ilavarasi et al., 2011; Badar et al., 2017; Ghosh et al., 2017). Thus, the cultivation medium is one of the key factors controlling microalgal growth rate, and modifications to the culture medium can enhance the production of desired biomolecules, such as pigments, lipids, proteins, and biomass (Chakraborty et al., 2023). Currently, synthetic culture media are widely used for the cultivation of microalgae. These media are formulated from chemical ingredients and typically contain organic nutrients (e.g., amino acids, proteins, sugars, biotin, thiamine, riboflavin), inorganic nutrients (e.g., salts, trace metals), and non-nutrient factors (e.g., growth-stimulating factors, hormones). Synthetic media have a fixed composition, are commercially available, and are widely used in microalgae production and research; examples include BG11 (Ilavarasi et al., 2011; Choudhary et al., 2016),

BBM (Ilavarasi et al., 2011; Mtaki et al., 2020), acidified BBM (Ilavarasi et al., 2011), Zarrouk's medium (Madkour et al., 2012), half strength Chu 10 (Ilavarasi et al., 2011), Fog's medium (Pathak et al., 2016), modified Chu 13 (Yamaguchi et al., 1987; Massa et al., 2017), and f/2 (Naito et al., 2001; Kang et al., 2011; Li et al., 2013; Jiang et al., 2022). However, due to the high cost of synthetic media, microalgae cultivation is often economically non-viable at the industrial scale.

Moreover, global agricultural systems generate vast quantities of residues each year, representing an abundant yet underutilized biomass resource. Recent estimates indicate that approximately 1.44 billion tons of crop residues remain sustainably available worldwide (Nosenzo & Kelman, 2025). When efficiently utilized, these residues can serve as substrates for microalgal biomass production, thereby contributing to the mitigation of environmental pollution associated with agro-industrial waste. Accordingly, low-cost agricultural by-products rich in essential nutrients have attracted increasing attention as alternative culture media for microalgae cultivation (Chakraborty et al., 2023). Taken together, this study aims to investigate the potential of using extracts from agricultural by-products, including peanut shells and okara, as an alternative nutrient source for the growth of microalgae *N. oculata*.

Material and Method

Microalgae strain and stock culture. The microalgae *N. oculata* was obtained from the Aquaculture Research Centre, Hue University. The cell culture was maintained as a stock culture in 250 mL Erlenmeyer flasks containing f/2 medium (Table 1; Guillard, 1975) at a salinity of 20‰. Cultures were kept at 27°C, under a 12:12 h light/dark cycle with a light intensity of 60 µmol photons m^{-2} s^{-1}. The stock culture was routinely subcultured every 7-10 days to maintain cell viability and exponential growth.

Table 1

Composition of f/2 medium used for *N. oculata* cultivation

Components	*Concentration*
Macronutrients	
$NaNO_3$	8.83×10^{-4} M
$NaH_2PO_4.H_2O$	3.63×10^{-5} M
Trace metals	
$FeCl_3 \cdot 6H_2O$	1×10^{-5} M
$Na_2EDTA \cdot 2H_2O$	1×10^{-5} M
$CuSO_4 \cdot 5H_2O$	4×10^{-8} M
$Na_2MoO_4 \cdot 2H_2O$	3×10^{-8} M
$ZnSO_4 \cdot 7H_2O$	8×10^{-8} M
$CoCl_2 \cdot 6H_2O$	5×10^{-8} M
$MnCl_2 \cdot 4H_2O$	9×10^{-7} M
Vitamins	
Vitamin B12	1×10^{-10} M
Biotin	2×10^{-9} M
Thiamine HCl	3×10^{-7} M

Extracts from agricultural by-products. Peanut shells (PN) are lignocellulosic agricultural residues generated from peanut processing, whereas okara (OKR) is a protein-rich agro-industrial by-product derived from tofu manufacturing processes. The extracts from PN and OKR were prepared using a modified method adapted from Malakar et al., (2020). Briefly, the PN and OKR were dried at 80°C until a constant weight was achieved. The dried materials were then ground into a fine powder using a blender. Subsequently, 20 g of powder from either PN or OKR was added to 100 mL of distilled water. The mixture was autoclaved at 121°C and 15 psi in the presence of 1% (v/v) H_2SO_4. After treatment, the flasks were allowed to cool to room temperature, and the mixture was filtered to obtain the final extract. The obtained extracts were analyzed for the contents of organic carbon, total nitrogen, and total phosphorus. Organic carbon and total phosphorus were determined using the Walkley–Black and colorimetric

spectrophotometric methods, respectively, following Vietnamese Ministry of Science and Technology, (2011a, b), while total nitrogen was measured by the Kjeldahl method according to Vietnamese Ministry of Science and Technology, (1999) (Table 2).

Table 2

Contents of organic carbon, total nitrogen, and total phosphorus in peanut shells and Okara extracts

Agricultural by-product extracts	*Organic carbon (%)*	*Total nitrogen (%)*	*Total phosphorus (%)*
Okara (OKR)	4.97	0.59	0.20
Peanut shells (PN)	3.74	0.45	0.10

Effects of agricultural by-product extracts on the growth of Nannochloropsis oculata. All cultivations of *N. oculata* were performed in 2 L transparent plastic tanks at an initial microalgal density of 1×10^5 cells mL^{-1}. Cultures were maintained at 27°C with a salinity of 20‰, under a 12:12 h light/dark cycle at a light intensity of 60 µmol photons m^{-2} s^{-1}, with continuous gentle aeration.

Effects of individual extracts on the growth of Nannochloropsis oculata. Two separate experiments were conducted to evaluate the effects of OKR and PN extracts on the growth of *N. oculata*. Both experiments were arranged in a similar manner, each including four treatments: f/2 medium as the control (CT); the respective extract alone (OKR or PN); the extract supplemented with f/2 medium at 50% of its standard nutrient concentration (either OKR or PN + 50% f/2); and the extract supplemented with f/2 medium at full (100%) nutrient concentration (either OKR or PN + 100% f/2). In all treatments, the extract was added at an amount of 100 mL L^{-1}, and the culture medium pH was adjusted to 8 prior to microalgal inoculation. Each treatment was conducted in triplicate.

Effects of combined extracts on the growth of Nannochloropsis oculata. The experiment was conducted to evaluate the effects of combined agricultural by-product extracts on the growth of *N. oculata*. Four treatments were established with all extracts added at 50 mL L^{-1}: f/2 medium as the control (CT); PN extract supplemented with 1 mL cane molasses (CM) and 50% f/2 medium (PN + f/2 + CM); OKR extract supplemented with 1 mL CM and 50% f/2 medium (OKR + f/2 + CM); and a combination of OKR extract with PN extract plus 1 mL CM (OKR + PN + CM). Each treatment was conducted in triplicate, and the pH was adjusted to 8 before microalgal inoculation. Industrial grade CM supplied by SFARM was used as a supplemental carbon source, with a high sugar content and a stable carbon fraction exceeding 45%.

Density and growth rate determinations. Cell density of microalgae (cells mL^{-1}) was determined daily by taking 1 mL samples from each experimental treatment and fixing them with Lugol's solution. The samples were appropriately diluted prior to counting. Microalgal cells were counted using a Sedgewick–Rafter chamber under a microscope (Olympus™) at 10 × magnification (Moheimani et al., 2013). The cell density of *N. oculata* was calculated using the following equation: $((1000 \times A)/n) \times D$. In this equation, A is the total number of cells counted, n is the number of fields counted, D is the dilution factor, and 1000 represents the total number of fields in the Sedgewick–Rafter chamber (corresponding to a total chamber volume of 1 mL). The specific growth rate (SGR) of microalgae (K, day^{-1}) was calculated according to Levasseur et al., (1993) as the following equation: $K = (\ln N_t - \ln N_0)/T$. In this equation, N_0 represents the initial cell density (cells mL^{-1}), N_t represents the cell density at time T (cells mL^{-1}), and T denotes the culture period in days.

Data analysis. Differences in cell density and SGR of *N. oculata* among treatments were analyzed using one-way analysis of variance (ANOVA). When significant differences were detected, post hoc multiple comparisons were performed using Tukey's honestly

significant difference (HSD) test at a 95% confidence level. Prior to conducting ANOVA, data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. All statistical analyses were performed using IBM SPSS Statistics. Data are presented as mean±standard error (SE), and figures were generated using GraphPad Prism (GraphPad Software, CA, USA).

Results

Growth performance under okara extract treatments. The growth patterns and SGR of *N. oculata* cultured in different media, including f/2 medium as the control (CT), okara alone (OKR), OKR supplemented with f/2 medium at 50% of its standard nutrient concentration (OKR + 50% f/2), and okara supplemented with f/2 medium at full (100%) nutrient concentration (OKR + 100% f/2), are illustrated in Figures 1 and 2, respectively.

All treatments exhibited similar growth dynamics, with cell density increasing gradually over the 15-day culture period and reaching a maximum on Day 10 at approximately 12-13 × 10^6 cells mL^{-1} (Figure 1). No significant differences in cell density were observed among treatments on most sampling days, except on Days 6 and 8 ($p < 0.05$). On these days, the OKR treatment showed lower cell densities (9.7 and 11.2 × 10^6 cells mL^{-1}) compared with the control treatment (10.22 and 11.9 × 10^6 cells mL^{-1}), respectively.

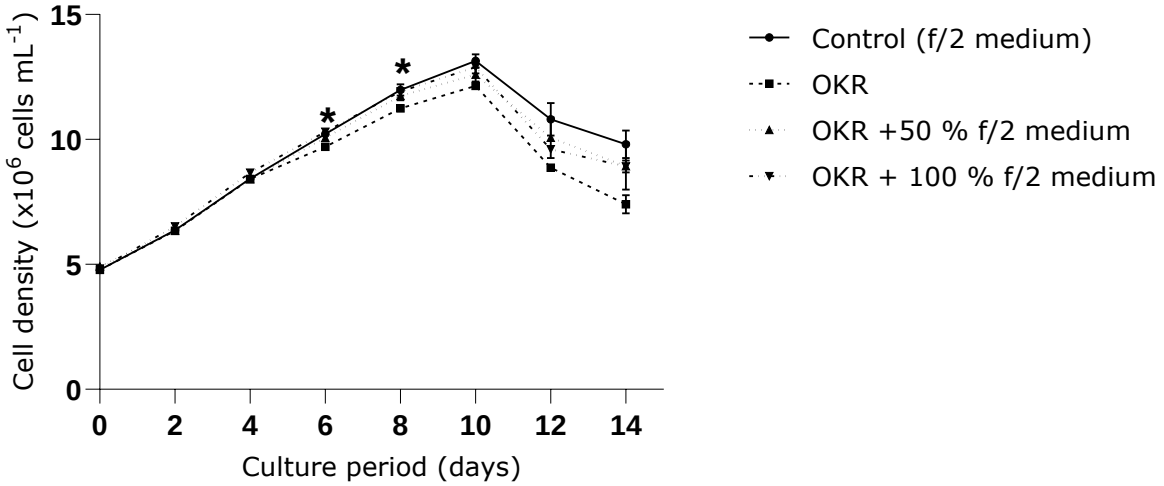

Figure 1. Cell density of *Nannochloropsis oculata* cultured in f/2 medium, okara extract (OKR), OKR + 50% f/2, and OKR + 100% f/2 over time. Values are mean±SE; asterisks indicate significant differences compared with the f/2 control ($p < 0.05$).

Regarding the SGR of *N. oculata*, all treatments exhibited positive growth throughout the experimental period, with an overall SGR of approximately 0.10 day^{-1} across all culture media. A significant difference in SGR was observed among treatments ($p < 0.05$). The OKR treatment showed the lowest SGR (0.10±0.00 day^{-1}), which was significantly lower than that of the control (0.11±0.00 day^{-1}) ($p < 0.05$). However, no significant differences were observed among the control, OKR + 50% f/2, and OKR + 100% f/2 treatments. In addition, significant differences were not detected among the OKR-containing treatments (OKR, OKR + 50% f/2, and OKR + 100% f/2) (Figure 2).

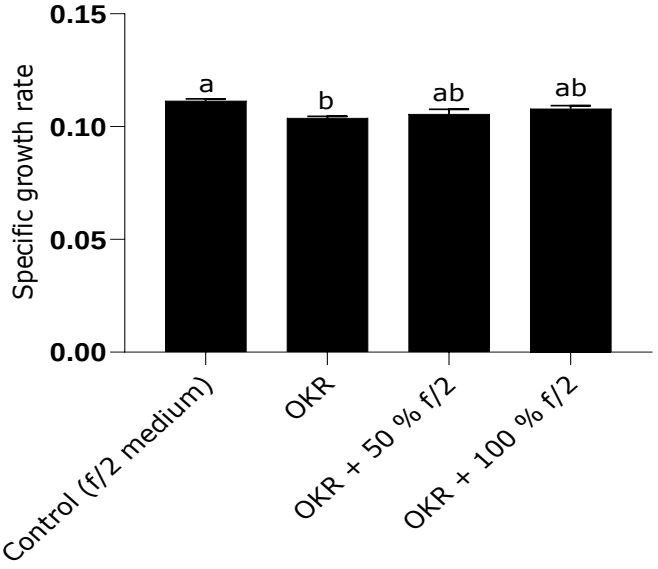

Figure 2. Specific growth rate of *Nannochloropsis oculata* cultured in f/2 medium, okara extract (OKR), OKR + 50% f/2, and OKR + 100% f/2. Values are mean±SE; different letters (a, b) indicate significant differences among treatments ($p < 0.05$).

Growth performance under peanut shell extract treatments. Similar to the okara extract treatments, a continuous growth pattern of *N. oculata* was observed when the microalgae were cultured in media containing PN shell extract. Cell concentration in all treatments peaked on Day 10 at approximately 6-7 × 10^6 cells mL^{-1} (Figure 3).

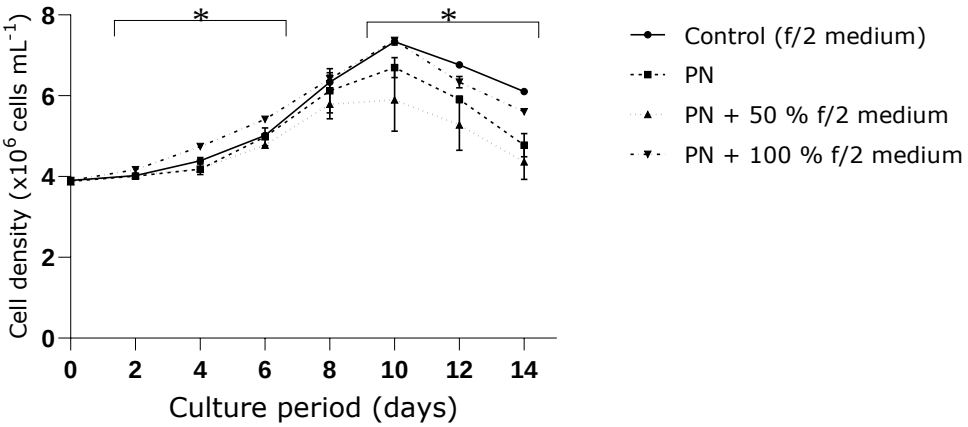

Figure 3. Cell density of *Nannochloropsis oculata* cultured in f/2 medium, peanut shell extract (PN), PN + 50% f/2, and PN + 100% f/2 over time. Values are mean±SE; asterisks indicate significant differences compared with the f/2 control at each sampling day ($p < 0.05$).

Except for Day 8, significant differences in cell density were observed among treatments on all other sampling days ($p < 0.05$). In particular, the highest cell concentrations were observed in the control and PN + 100% f/2 treatments, reaching approximately 7.3 × 10^6 cells mL^{-1}, which were significantly higher than in the PN + 50% f/2 treatment (5.8 × 10^6 cells mL^{-1}), the lowest among all treatments ($p < 0.05$). At the peak of cell density, no significant differences in cell density were observed when comparing the PN treatment with the control, PN + 50% f/2, or PN + 100% f/2 ($p > 0.05$).

For SGR, the highest values were–recorded in *N. oculata* cultured in f/2 medium (0.08) and in the PN + 100% f/2 treatment (0.07), followed by the PN-only treatment (0.069) and PN + 50% f/2 (0.06). A significant difference in SGR was detected only between the control and PN + 50% f/2 ($p < 0.05$) (Figure 4).

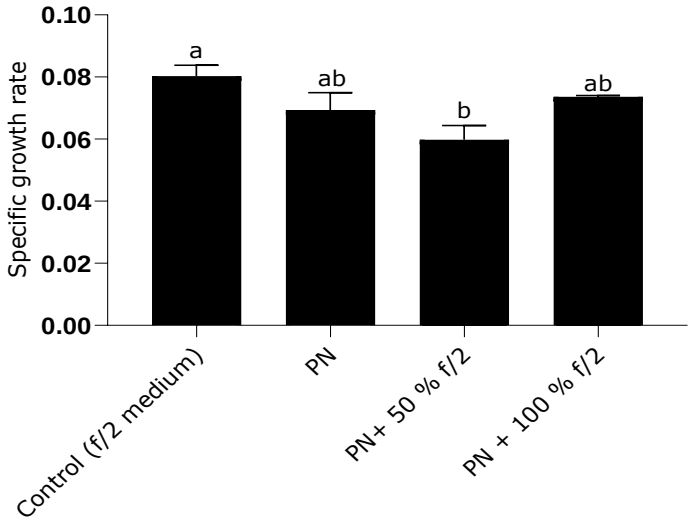

Figure 4. Specific growth rate of *Nannochloropsis oculata* cultured in f/2 medium, peanut shell extract (PN), PN + 50% f/2, and PN + 100% f/2. Values are mean±SE; different letters (a, b) indicate significant differences among treatments ($p < 0.05$).

Effects of combined extracts on the growth of Nannochloropsis oculata. The effects of the combined plant extracts on the growth of *N. oculata* are presented in Figures 5 and 6. A slight variation in the growth pattern of *N. oculata* was detected in the OKR + PN + CM treatment compared with the other treatments. In this treatment, cell density peaked earlier, reaching its maximum on Day 8, whereas the remaining treatments reached their peaks on Day 10 (Figure 5). Statistical analysis revealed significant differences in cell density among treatments from Day 4 onward ($p < 0.05$). In particular, on Day 8, the highest cell density was observed in the OKR + PN + CM treatment, reaching approximately 12.5×10^6 cells mL^{-1}, which was significantly higher than those in the other treatments ($p < 0.05$). However, by Day 10, the cell density in this treatment slightly decreased to 12.1×10^6 cells mL^{-1} and was significantly lower than those in the OKR + f/2 + CM and PN + f/2 + CM treatments (approximately 12.6×10^6 cells mL^{-1} in both treatments), but did not differ significantly from the control treatment (12.4×10^6 cells mL^{-1}). Furthermore, no significant differences were detected among the control, OKR + f/2 + CM, and PN + f/2 + CM treatments on Day 10 ($p > 0.05$).

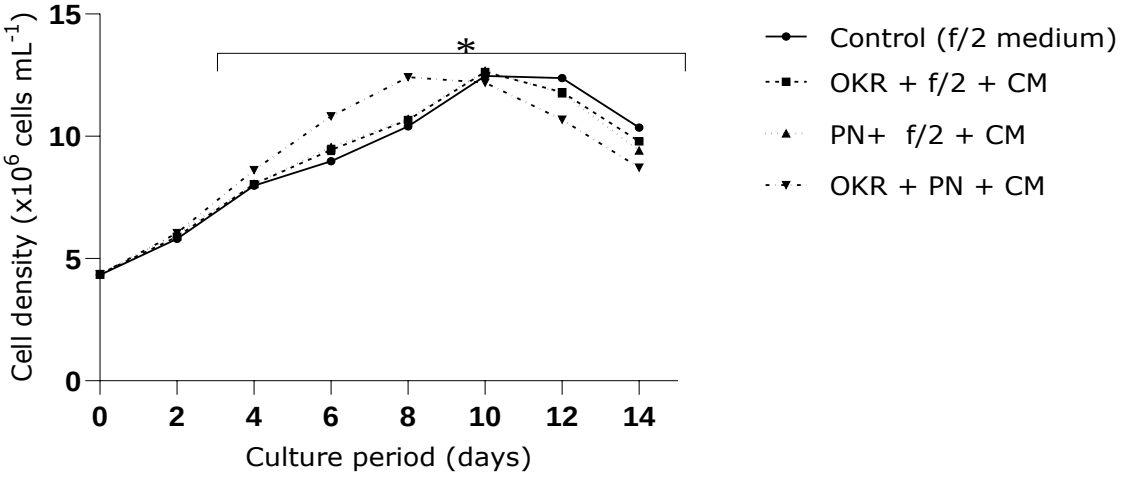

Figure 5. Cell density of *Nannochloropsis oculata* cultured in f/2 medium, okara extract + 50% f/2 + cane molasses (OKR + f/2 + CM), peanut shell extract + 50% f/2 + cane molasses (PN + f/2 + CM), and okara extract + peanut shell extract + cane molasses (OKR + PN + CM) over time. Values are mean±SE; asterisks indicate significant differences compared with the f/2 control at each sampling day ($p < 0.05$).

The SGR ranged from approximately 0.09 to 0.12 day^{-1} across treatments. The highest SGR was obtained in the OKR + PN + CM treatment (0.12 day^{-1}), followed by the control and the treatments supplemented with single extracts. Statistical analysis indicated significant differences among treatments ($p < 0.05$), with the OKR + PN + CM treatment showing significantly higher SGR than the remaining treatments (Figure 6).

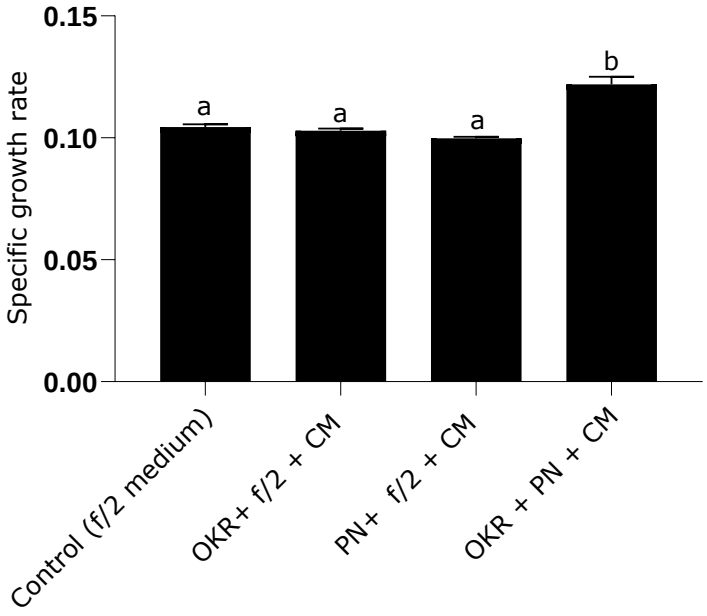

Figure 6. Specific growth rate of *Nannochloropsis oculata* cultured in f/2 medium, okara extract + 50% f/2 + cane molasses (OKR + f/2 + CM), peanut shell extract + 50% f/2 + cane molasses (PN + f/2 + CM), and okara extract + peanut shell extract + cane molasses (OKR + PN + CM). Values are mean±SE; different letters (a, b) indicate significant differences among treatments ($p < 0.05$).

Discussion. The high cost of synthetic culture media remains one of the major challenges limiting the economic feasibility of large-scale microalgae production (Ilavarasi et al., 2011; Choudhary et al., 2016). Consequently, increasing attention has been directed toward the use of nutrient-rich agricultural by-products as low-cost alternative substrates for microalgae cultivation. These residues often contain considerable amounts of nitrogen, phosphorus, organic carbon, and other essential nutrients that can support microalgal growth and biomolecule synthesis (Chakraborty et al., 2023). In this context, the present study evaluated the potential of extracts derived from PN and OKR as alternative nutrient sources for the cultivation of *N. oculata*, one of the most important microalgae used in aquaculture, particularly in hatcheries (Kargin, 2023).

The results indicated that *N. oculata* was able to grow in media containing PN and OKR extracts under different formulations, including individual extracts, their combination, or supplementation with f/2 medium. This was evidenced by the positive SGRs and the occurrence of an exponential growth phase in all treatments from day 4 onwards. These findings suggest that PN and OKR extracts can support growth of *N. oculata* and may serve as alternative nutrient sources for microalgae cultivation. Nitrogen and phosphorus are well known as essential macronutrients for microalgal growth. Particularly, nitrogen supports the synthesis of proteins, lipids, and carbohydrates (Yodsuwan et al., 2017; Zarrinmehr et al., 2020), while phosphorus contributes to algal growth, lipid accumulation, and key metabolic processes such as energy transfer and photosynthesis (Ota et al., 2016; Yang et al., 2018). In the present study, the total nitrogen and phosphorus contents in OKR extracts were 0.59% and 0.2%, respectively, while those in PN extracts were 0.45% and 0.1%, respectively, indicating relatively high nutrient levels (Vietnamese Ministry of Science and Technology, 1999; 2011a, b). Therefore, in the present study, the good growth of *N. oculata* observed in media containing these extracts under different formulations is reasonable. This observation is supported by previous studies reporting that agricultural by-products derived from

soybean processing including okara can serve as alternative nutrient sources for microalgae cultivation. For example, soybean processing wastewater has been successfully used to cultivate *Chlorella* sp. (Hu et al., 2020). In addition, okara has been explored as a potential substrate for microalgae cultivation, with *Phaeodactylum tricornutum* grown in fermented okara medium achieving a biomass concentration of 0.52 g L^{-1}, approximately twice that obtained in the conventional f/2 medium (0.25 g L^{-1}) (Kim et al., 2023).

The nutrient composition of the f/2 medium, as detailed in Table 1, has been optimized to support the growth of *N. oculata* by providing appropriate levels of macronutrients, trace metals, minerals, and vitamins. It is therefore not surprising that the highest cell density and SGR were observed in this medium across experiments. However, it is noteworthy that, except for the treatments with OKR or PN alone and PN supplemented with 50% f/2, the remaining media formulations showed cell density and SGRs comparable to those of the f/2 control. This confirms the successful proliferation of *N. oculata* in media containing OKR or PN extracts supplemented with f/2 medium.

Regarding the low cell density and SGR observed in the OKR or PN alone treatments and in PN supplemented with 50% f/2, this may be attributed to the absence or insufficient concentrations of essential micronutrients, particularly trace metals and vitamins. These micronutrients are known to be crucial for the proper functioning of the photosynthetic apparatus and other cellular processes (Camacho-Rodríguez et al., 2013). Indeed, several studies have demonstrated that the micronutrient composition of culture media, including trace metals and vitamins, directly influences microalgal growth performance. For example, copper (Cu), cobalt (Co), iron (Fe), manganese (Mn), nickel (Ni), and zinc (Zn) function as essential cofactors in numerous metabolic processes in microalgae, including lipid biosynthesis and other cellular activities (Liu et al., 2024; Manic et al., 2024; Tan et al., 2024). Similarly, Faé Neto et al., (2018) evaluated five different low-cost culture media based on fertilizers and aquaculture effluents for the cultivation of *N. oculata* and reported significantly lower cell abundance compared with the conventional f/2 medium. The authors suggested that the reduced growth was likely associated with the absence of essential trace metals and vitamins in those media formulations. Our hypothesis was supported by the results obtained in the present study, where the combined extracts of OKR and PN in the presence of cane molasses resulted in improved growth performance of *N. oculata* compared with the other treatments. This improvement was reflected by a significantly higher SGR and a shorter time required to reach peak cell density (8 days in the OKR + PN + CM treatment compared with 10 days in the other treatments). Therefore, it is likely that the micronutrient components present in OKR and PN extracts may complement each other, thereby enhancing the growth of *N. oculata*. To further clarify this assumption, future studies should investigate in greater detail the nutritional composition of OKR and PN extracts and their specific roles in supporting microalgal growth.

Microalgae require carbon for growth, which can be supplied either as inorganic carbon (e.g., CO_2) or as organic carbon (e.g., sugars or other carbon-containing compounds). This fixed carbon is utilized for cellular respiration, energy production, and biomass synthesis, supporting overall cell growth and metabolism (Wang et al., 2014; Mohan et al., 2015). It is worth noting that the supplementation of cane molasses (CM) and the relatively high organic carbon content of OKR and PN extracts (4.97% and 3.74%, respectively) may have contributed to the enhanced growth of *N. oculata*. This is supported by the observation that no significant differences in cell density or SGR were detected between the control and the treatments supplemented with 50% PN shell extract + 1 mL cane molasses + 50% f/2 (PN + f/2 + CM) or 50% okara extract + 1 mL cane molasses + 50% f/2 (OKR + f/2 + CM). Notably, Lin et al., (2017) reported that *N. oculata* grown under mixotrophic conditions with acetate as a carbon source exhibited enhanced biomass and lipid production compared with autotrophic controls, indicating that the alga can utilize organic carbon to support growth and metabolism. Taken together, these findings suggest that the potential role of organic carbon in promoting microalgal growth cannot be ruled out in the present study.

Conclusions. The present study demonstrates that PN and OKR extracts can effectively support the growth of *Nannochloropsis oculata* under various formulations, either alone or in combination with f/2 medium. The combined extracts with cane molasses notably enhanced growth performance, likely due to complementary micronutrients and additional organic carbon. These results highlight the potential of agricultural by-products as low-cost alternative nutrient sources for microalgae cultivation. Further investigations into the detailed nutritional composition and mixotrophic potential of these extracts are warranted to optimize their use in large-scale production.

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Authors Contributions. ATN: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft; HTTN: Investigation, Validation, Data curation; TTHH: Conceptualization, Supervision, Project administration, Methodology, Writing – review & editing.

Conflict of Interest. The author(s) declare that there are no competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Data Availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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