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Low Temperature Preservation on Dense Biomass of *Nannochloropsis oculata*

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Abstract: The consistency supply of microalgae is crucial for hatchery as it plays important role as feed for larvae in the early stages. Thus, ensuring the availability of microalgae needs efficient management of feed, including the technique of biomass preservation. The purpose of this research was to observe the application of various low temperature to preserve *Nannochloropsis oculata* as feed. The source of *N.oculata* was filtered through a double set of 0.45 micron of cartridge which then preserved based on treatments A (5°C); B (-20° C); and C (15°C) for 30 days. After 30 days of preservation, each treatment as inoculum were cultured in the volume of 2 liter. Initial density of *N.oculata* was 23-24x106 cells.ml^{-1} placed into medium consisted of sterile sea water salinity 30 ppm, KW21™ fertilizer dose 1 ml.l^{-1}. Culture environment was set with illumination 3.000-4.000 lux, homogenized with constant aeration, with 15 days of culture period. The result showed that on the days 9, cell density on treatment A, B and C were significantly different each other (sig 0.001), where treatment A had the highest cell density at 57.42±6.95x10^6 cell.ml^{-1}, then followed with treatment C with 38.33±2.08x10^6 cell.ml^{-1} and B with 20.33±5.35x10^6 cell.ml^{-1}. The use of low temperature as preservation for *N.oculata* could help low-middle hatchery with remoted area to maintain the sustainability of *N.oculata* stocks.

Keywords: Low temperature; *Nannochloropsis oculata*; Preservation

Introduction

The sustainability of microalgae supply plays important role in the success of hatchery. Microalgae as live feed is essential for larvae in its early stages, as it easily captured and highly digestible (Conceição, Yúfera, Makridis, Morais, & Dinis, 2010).The long-term microalgae availability requires effective management of microalgae feed, including proper biomass preservation. Several techniques for preserving microalgae have been explored, including light exposure, pH treatment, freezing, cool packaging variations, thermal treatment (Castelló, Pariente, Andrés, & Ortolá, 2018), cryopreservation which involves chemical agent such as DMSO, sorbitol or proline, glycerol, or ethylene glycol (Nakanishi, Deuchi, & Kuwano, 2012). Preservation technique for storing microalgae is widely applied in aquaculture hatcheries, such as periodic transfer or sub culturing, freeze drying, lyophilization, cryopreservation (Arguelles, Gana, & Monsalud, 2020; Gwo, Chiu, Chou, & Cheng , 2005), flocculation, centrifugation, freezing and preserving in refrigerator (de la Peña and Franco, 2020) (Aléman-Nava, Muylaert, Bermudez, Depraetere, Rittmann, Parra-Saldivar, & Vandamme, 2017; Borges, Caldas, D'Oca, & Abreu , 2016).

In microalgal biomass preservation, factors such as storage duration, initial biomass concentration, light exposure, the use of preservatives, and atmospheric conditions are crucial as they impact cell viability, concentration, contamination levels, fatty acid content, and pigment stability (Camacho-Rodríguez, Cerón-García, Macías-Sánchez, Fernández-Sevilla, López-Rosales, & Molina-Grima, 2016). Low temperature preservation has been applied in microalgae cultivation. Freeze tolerance in microalgae not only vary among species but also in strains based on the environment it lives. Cellular structure of microalgae and the specific

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phase of their growth cycle play significant roles in determining their tolerance to freezing conditions (Foo, Mok, Ho, & Khong, 2023). In the long term, stable quality of microalgae concentrates is required even when stored for months (Camacho-Rodríguez *et al*., 2016), thus the issues of inconsistency and suboptimal performance in microalgae stored for extended periods need to be considered (Heasman, Diemar, O'Connor, Sushames, & Foulkes, 2000).

Nannochloropsis oculata is one of microalgae species that widely use as live feed in aquaculture, both for direct and indirect feeding. Many aquaculture species rely their early stages feed on *N.oculata* such as penaeid shrimp (de Moraes, Santos, Junior, Mota, Dantas, Bezerra, & Gálvez, 2022), nile tilapia for stimulating fish body weight (Salem, Adawy, Zaki, & Zahran, 2022) with direct feed or indirect feed through rotifer (Dinesh, Nandhakumar, & Padmavathy, 2024; Sales, Derner, & Tsuzuki, 2019). In aspect of nutrition, *N.oculata* displays high *eicosapentaenoic acid* (EPA) about 34% and arachidonic acid (ARA) about 7% and can be consumed directly to marine zooplankton (Brown & Blackburn, 2013). In addition, *N.oculata* also can be applied into green-water system in hatchery to improve water quality (Brown & Blackburn, 2013; (Ding *et al*., 2021). The conventional harvest method for *N.oculata* is on site-hatchery, with direct or indirect feeding to larvae. This technique is faced difficulty especially for remote hatcheries which one to another ponds is far away.

During off-seasons, the cultivation process can be halted so that microalgae can be concentrated and stored for future use in the next production cycle. This approach improves the efficiency of hatchery operations while also reducing costs (de la Peña and Franco, 2020). For small and remote hatcheries with a shorter cultivation process, the easiest and low-cost technique is more preferred. Camacho-Rodríguez *et al.* (2016) found that fresh *Nannochloropsis gaditana* samples with an initial biomass concentration of 5 $g.l^{-1}$, as well as paste samples with a concentration of 150 $g.l^{-1}$, could be stored for up to four months at 4°C under nitrogen or a controlled air atmosphere with minimal light exposure (35 µmol photons.$m^{-2}.s^{-1}$). However, small to mid-sized hatcheries with remote ponds prefer the easiest and low-cost technique to preserve microalgae, especially *N.oculata* as one of the most common live feed for larvae. It is important to apply affordable preservation method that capable of maintaining the density of *N.oculata*. Preservation at low temperatures is one of feasible approach to this condition, therefor this research focuses on assessing the response of *N.oculata* stored at varying temperatures.

Method

Biomass Production and Preservation

Biomass of *N.oculata* used as test material came from mass culture that had reached its peak density. The *N.oculata* was harvested by filtering it through a double set of 0.45 micron of cartridge that connected to polyvinyl chloride (PVC) pipes and submersible pump. The backwashing procedure carried out to retrieve the biomass from outlet pipes. The volume of 500 ml harvested biomass was placed into bottle and then stored for 30 days based on the temperature treatments: A (5°C); B (-20°C); and C (15°C).

Cell Culture

After 30 days of treatments preservation, biomass were prepared as an inoculum for a new *N.oculata* culture in the volume of 2 liter. Before being cultured, treatment A and B were adapted an hour at laboratory temperature (15-17°C). With density of 23-24x10^6 cells.ml^{-1}, inoculum from all treatment were being transferred into erlenmeyer of culture medium with three replication each. Culture medium consisted of sterile sea water with 30 ppm of salinity, KW21™ fertilizer (Daiichi Seimo Co., Ltd., Kumamoto Japan) with a dose of 1 $ml.l^{-1}$. Erlenmeyer were placed on the rack, illuminated with artificial light from an LED with intensity of 3.000-4.000 lux, and homogenized with constant aeration. The duration of the *N.oculata* culture was carried out for 15 days.

Cell Density Measurement

The parameter of the test was *N.oculata* cell density that observed and counted daily by using haemacytometer (LeGresley & Georgina McDermott, 2010). The optimum cell density among the treatment was compared and analyzed with one-way ANOVA followed by Tukey test (sig<0.005). The decision of the research was based on the highest cell density with short culture periods.

Result and Discussion

Biomass Production

Harvesting *N.oculata* biomass through double filtration using a 0.45-micron cartridge filter provided a denser result compared to conventional biomass harvesting methods. This was because the amount of accompanying water in the harvested product was less than in conventional methods. The method of concentrating microalgae may be related to a reduction in microbial and chemical contaminants after concentration process (Heasman *et al*., 2000). This method was suitable for low to middle class hatchery as it offered an easier compartment and lower cost. The

amount of cell density resulted from the biomass was in range of 200-250x10^6 cell.ml^{-1}. Filters with pore sizes ranging from 0.45 to 1 µm are efficient for filtering bacteria and algae on a small scale (Droppo, 2000). Creswell (2010) stated that cartridge filters are favored over methods like flocculation, centrifugation that is limited to process small volume, and coagulation that tend to leave residue on the biomass. However, filtration method was not a main topic of the research thus the effectivity of filtration compared to other method was not being observed thoroughly.

Cell Density

The entire treatments showed a similar growth pattern of *N.oculata* cells which was low at the beginning of inoculation, then progressively increasing and subsequently decreasing after reaching a peak density period. Cell density counting showed that treatment A had the densest biomass at 57.42±6.95x10^6 cell.ml^{-1} on days 9, followed by treatment C at 38.33±2.08x10^6 cell.ml^{-1}, and then B at 20.33±5.35x10^6 cell.ml^{-1} both on days 8. Both treatment B and C had its optimum growth at days 8 unlike treatment A which was on the days 9. Despite the fact that treatments B and C reached their peak earlier than treatment A, the cell density of *N.oculata* in treatment A was higher compared to treatments B and C. Nevertheless, during the 15 days of observation period, treatment A exhibited a higher trend of cell density increase compared to treatments B and C, especially on days 8 through 9 (Figure 1.).

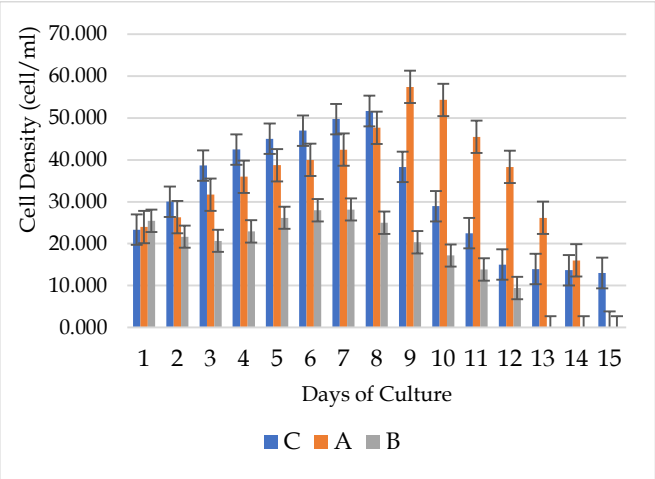

Figure 1. Cell density of *N.oculata* from treatment A, B and C during culture period

Furthermore, One-way ANOVA test was performed to all treatment in order to compare individual means. The result showed that on the days 9, cell density on treatment A, B and C were significantly different each other (sig 0.001), where treatment A had the highest cell density, then followed with treatment C and B. This result indicates inoculum that came from *N.oculata* that had preserved in 5°C of temperature yielded the highest cell density compare to another preservation method which were 15°C and -20°C. The difference of one additional day in culture time for treatment A can be disregarded, as treatment A not only produces higher biomass but also showed a better growth trend for *N.oculata* compared to treatments B and C. de la Peña and Franco (2020) noted that the viability of preserved microalgae cells can be determined by their capacity to divide.

Low temperature preservation reduces the rate of cell metabolism while maintaining cell viability, thus recovery and the activation of cells can be delayed with longer period of storage (de la Peña and Franco, 2020). The selection of the appropriate temperature for microalgae preservation varied. Several studies discussed the preservation of microalgae along with its harvesting techniques. The use of freezing to preserve microalgae stocks can improve hatchery efficiency due to its cost-effectiveness, but the challenge was the viability of the cells that would lose over time during preserved period (Ansari *et al*., 2021; Camacho-Rodríguez *et al*., 2016). This statement concurs with the result of the research that the cell density with inoculum derived from preservation at -20°C was low and suboptimal.

One of the disadvantages of microalgae culture was a high cultivation and processing cost (Ansari *et al*., 2021; Foo *et al*., 2023). Thus using low temperature to preserve *N.oculata* for a longer period was particularly necessary especially for small-scale hatcheries. Other preservation technique such as lyophilization and cryopreservation are not favorable due to expensive cost (Arguelles *et al*., 2020) and its toxicity (de la Peña and Franco, 2020). Nevertheless, many processes in microalgae preservation are unique, and the suitable technique for each type of aquaculture hatchery varies between species (de la Peña and Franco, 2020; Heasman *et al*., 2000; Brown & Blackburn, 2013), and to date, no standardized protocol has been established for preserving all microalgae classes (Foo *et al*., 2023; Arguelles *et al*., 2020).

Conclusion

Biomass of *N.oculata* that had been preserved in 5°C (treatment A) gave the best growth performance compared to preservation in 15°C (treatment B) and -20°C (treatment C). The highest cell density was 57.42±6.95x10^6 cell.ml^{-1} from treatment A with 9 days of culture, followed by treatment C with 38.33±2.08x10^6 cell.ml^{-1} and treatment B with 20.33±5.35x10^6 cell.ml^{-1}. The use of low temperature preservation was an

alternative to maintain the sustainability of *N.oculata* in the hatchery.

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Author Contributions
All authors contributed to the completion of this research. The first author also corresponding author initiated the idea and provided the necessary materials, while the second author structured the research result. Everyone was actively involved in data gathering, data analyzing, writing, and reviewing the manuscript.

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Conflicts of Interest
All authors declare that there is no conflict of interest during the research and preparation of the manuscript.

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