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Strengthening Microalgae Biodiesel Production Capacity Based on Strain Selection for *Chaetoceros amini*, *Nannochloropsis oculata* and *Nitzschia* spp.

Suripto[1]*, Lalu Japa[2]

[1]Department of Environmental Sciences, Faculty of Mathematics and Natural Sciences, Universitas Mataram, Mataram, Indonesia.
[2]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Mataram, Mataram, Indonesia.

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Corresponding Author:
Suripto
suriptobio@unram.ac.id

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Abstract: Increasing microalgae biodiesel production through improvement of biomass production is reversible and is often considered economically unprofitable. This research aims to determine the effect of various levels of media salinity stress in producing microalgae strains that have higher oil content than the original population. Three species of microalgae are known to be capable of producing biodiesel, namely *Chaeticheros amini*, *Nannochloropsis ocu*lata and *Nitzschia* spp. isolated from Sekotong coastal waters, West Lombok, then cultivated in bioreactor systems for seven days with salinity stress treatment to produce strains. The resulting strains were cultivated again, but without salinity stress. Microalgae cell density was observed every day and harvested on the seventh day. The resulting microalgae biomass was extracted in stages to produce biodiesel oil. The results showed that after cultivation under salinity stress, S_{20}, S_{25}, S_{30}, and S_{35} strains were produced from each of these species. Under salinity stress, all strains except S_{25} of *Nitzschia* spp. showed a higher maximum cell density compared to the original population and were reversible respectively. Each of the S_{25} strains of *C. amini* and *N. oculata*, S_{20} and S_{25} strains of *Nitzschi*a spp., had a higher oil content than the original population, each of which was permanent.

Keywords: Microalgae; Oil; Salinity stress; Strains

Introduction

Finding new energy sources to compensate for the decline in global oil reserves is a real challenge and the response to this challenge includes the discovery of first, second and third, even fourth generation biofuels, which are still being studied today. First generation biofuels were derived from edible materials and, therefore, created a new problem in the form of a "food" versus "fuel" dilemma. Second generation biofuels come from inedible materials such as agricultural and forest residues and plants grown for biofuel purposes (Demirbas & Demirbas, 2010; Nada et al., 2024).

However, the development of second generation biofuels still leaves the problem of food versus energy, especially in terms of land use competition. So, third generation biofuel using microalgae as a biodiesel source becomes more attractive and promising (Nada et al., 2024; Suparmaniam et al., 2019; Suripto et al., 2023).

The two main advantages of microalgae biodiesel are the high yields per hectare (up to 10 times higher compared to other biofuels), and the fact that microalgae does not compete for land or drinking water with agriculture or forestry (Faried et al., 2017; Hendro & Zahra, 2024; Sheehan et al., 2020). Research on the growth of microalgae using certain nutritional treatments or environmental conditions such as lighting, temperature and salinity has proven an increase in microalgae productivity (Chhandama et al., 2023). The final report states that genetic engineering may be necessary to overcome these and other natural limitations of algal strains, and that the ideal species may vary by place and season (Faried et al., 2017). However, there is a dilemma in increasing the productivity of

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microalgae, namely biomass productivity versus oil content. Among the most significant findings from the development of microalgae for biodiesel production is that rapid growth and high lipid production are "mutually exclusive." This is because fast growth requires high nutrition, while high lipid production requires low nutrition (Demirbas & Demirbas, 2010; Zhang et al., 2022).

Even though lipid production increases when algal biomass growth increases, much of the lipid produced is used to build membrane structures in cells and less becomes reserve lipid, which is generally stored in cell vacuoles. The measured oil content of microalgae from the extraction results usually comes from reserve lipids in the cell vacuoles. Moreover, structural membrane lipids are more difficult to separate so less can be extracted (Baqi et al., 2022). The description of the problem above provides inspiration, that increasing microalgae lipid production should not be through increasing plastic growth, but rather through permanently strengthening the capacity of the microalgae population based on natural selection. Natural selection in question is selection to obtain strains of microalgae that have a higher capacity to produce oil that is genetically permanent and is passed on to the next generation of descendants. Strengthening microalgae biodiesel production capacity through strain selection has never been studied and this is the novelty of this research, especially in terms of methods and choosing salinity stress as a treatment.

The high oil content in microalgae cells is associated with the size and number of oil-storing vacuoles (Suripto & Japa, 2021; Zhang et al., 2022; Suripto et al., 2023). In a population of microalgae there are various genetic variations in phenotype, including the size and number of oil-storing vacuoles (Russell et al., 2022). Thus, variations in the size and number of vacuoles between algal cells can be used as targets for adaptive selection to obtain generations that have larger vacuole sizes or a greater number of vacuoles. Treatment environmental conditions that are appropriate for selecting microalgae strains based on variations in these vacuoles include salinity stress in the growth medium. The salinity of the medium, in this case waters, is linearly related to its spesific mass (ρ).

Microalgae cells, which are often also known as phytoplankton (Greek, plankton = floating) have a specific mass approximately the same as the specific mass of the medium in which they live and grow. Adaptation (which is often confused with plasticity) of microalgae to changes in the salinity of their environment (changes in the specific mass of the media) occurs through a survival selection mechanism based on their body (cell) specific mass. Microalgae cells whose body specific mass is the same or around the same as the media specific mass will remain alive, grow and reproduce, while others (whose body specific mass is much smaller or larger) will die or cannot grow or cannot reproduce further (Pradana et al., 2024; Russel et al., 2022; Sachlan, 1982; Suripto et al., 2023). Thus, to produce a generation of microalgae that has a higher oil production capacity, this can be done by reducing the salinity of the medium, which selects to obtain a population (cells) of microalgae with a lower specific mass. The decrease in the specific mass of the body or microalgae cells is in line with the increase in the size or number of oil-storing vacuoles. Based on the description of the problem above, research was carried out with the aim of determining the effect of media salinity stress on producing strains of *C. amini*, *N. oculata* and *Nitzschia* spp. which have a higher biodiesel production capacity than the original population.

Method

Isolation of Microalgae and Analysis of Their Growth

Isolation is the first and important step to obtain the most suitable microalgae culture as a biodiesel source. Three species of microalgae, namely *Chaetoceros amini*, *Nannochloropsis oculata* and *Nitzschia* spp., have been known to be suitable for producing biodiesel (Suripto & Japa, 2018, 2021; Suripto et al., 2023). The three microalgae species were each isolated from Sekotong coastal waters, West Lombok using a modified technique from (Baqi et al., 2022; Chhandama et al., 2023; Japa et al., (2022). Identification of microalgae species was carried out using the phytoplankton atlas from (Japa et al., 2024).

Each microalgae species was purified by growing in Bold Basal Medium in a 20 L capacity bioreactor for seven days with varying medium salinity stress treatments, namely 20, 25, 30 and 35 ppt, resulting in strains S_{20}, S_{25}, S_{30}, and S_{35}. A salinity of 30 ppt is the salinity condition of the original waters where microalgae sampling was carried out. Purification and production of this microalgae strain was carried out using a modified bioreactor technique from (Chhandama et al., 2023; Japa et al., 2024).

Each of the resulting microalgae strains was cultivated again for seven days but without salinity stress. The growth of the microalgae population was observed every day for 7 days by calculating the cell density under a microscope using a modified technique from Japa et al. (2024). Microalgae cell density was calculated using the following formula:

$$D = N \times \frac{n_1}{n_2} \times \frac{V_1}{V_2} \times \frac{1}{V} \quad (1)$$

where

D = density = number of cells per litre

N = The number of cells counted in a number of visual fields is observed on a glass slide under microscopic observation
n_1 = Total number of visual fields under the cover glass (20)
n_2 = Number of visual fields observed
V = Volume of sample captured (in L)
V_1 = Volume of filtered sample (50 mL), that contained in a mesh bottle
V_2 = Volume of filtered sample used under a cover slip (1 drop = $\frac{1}{40}$ mL, depending on the pipette used)

The microalgae growth curve is divided into 4 phases, namely lag, log, stationary and death phases. Maximum growth is achieved at the end of the log phase. On the seventh day, the culture of each strain was harvested, then concentrated using a phytoplankton filter and dried to produce dry microalgae biomass. The resulting dry microalgae biomass is ready for extraction.

Extraction and Lipid Estimation

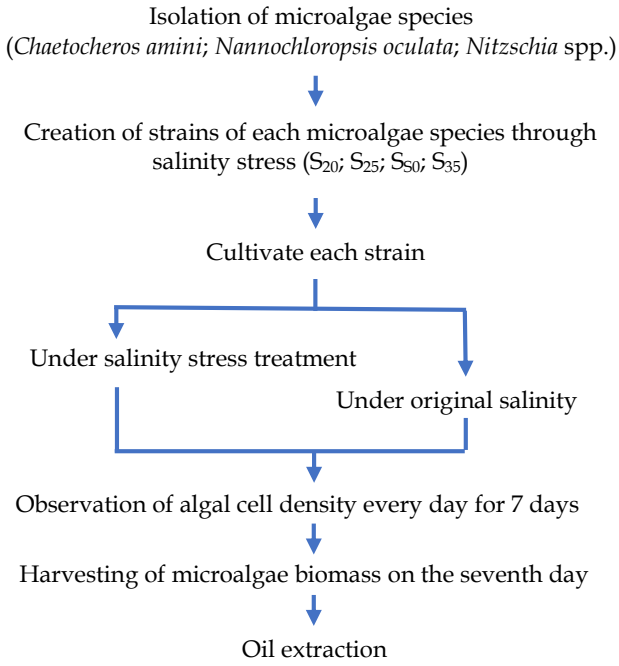

Figure 1. Work flow chart for strengthening microalgae production capacity based on strain selection

Dry microalgae biomass was extracted using the Soxhlet technique, using non-polar and polar organic solvents, in this case hexane and ethanol simultaneously to extract maximum lipid molecules from microalgae cells. According to Chhandama et al. (2023), lipid extraction with one solvent alone is not effective enough to break the strong interactions between neutral lipids and other biomolecules in cell membranes. So non-polar solvents are often used together with polar solvents.

Extraction of microalgae biomass and processing of the resulting crude oil into biodiesel, including the transacetylation process to reduce the viscosity of crude oil were carried out using techniques modified from Pôjo et al. (2021), Kumar & Choudhary (2023), Marnelisa et al. (2022), Mirzayanti et al. (2021), and Sekar et al. (2024). The oil content in % dry weight can be calculated using the following formula:

$$\text{Oil content (\%)} = \frac{\text{Weight of extracted lipids}}{\text{Dry microalgae biomass}} \times 100\% \quad (2)$$

In general, the work flow chart for the study of strengthening microalgae biodiesel production capacity can be seen in Figure 1.

Result and Discussion

Results

Acquisition of Strains and Analysis of Their Growth

Table 1. Maximum growth and oil content of various strains of *C. amini*, *N. oculata* and *Nitzschia* spp.

Microalgae species	Strains		Maximum growth 10^6 cells L^{-1}	Day	Oil content (%)
C. amini	S_{20}	a	3000.6	6	32.44
		b	3000	4	31.94
	S_{25}	a	3600.96	5	38.12
		b	3200	4	37.62
	S_{30}	a	3400.6	4	34
		b	3400.6	4	33.5
	S_{35}	a	3200.6	5	34.08
		b	2900.6	4	33.58
N. oculata	S_{20}	a	2500.005	6	31.56
		b	4100	5	31.06
	S_{25}	a	3012.8	5	72.55
		b	4200	5	72.05
	S_{30}	a	4375.36	5	68.00
		b	4375.36	5	67.5
	S_{35}	a	3240	5	31
		b	4000	5	30.5
Nitzschia spp.	S_{20}	a	2600	5	51.7
		b	2200	4	51.2
	S_{25}	a	3369.4	5	46.00
		b	2200	4	45.5
	S_{30}	a	2300.03	4	46
		b	2300	4	45.5
	S_{35}	a	2540	4	37.5
		b	2000	4	37

Information:
a. Cultivated under salinity stress
b. Cultivated under original salinity

Three species of microalgae isolated from Sekotong coastal waters, namely *Chaetoceros amini*, *Nannochloropsis oculata* and *Nitzschia* spp., after being purified and grown under salinity stress treatment, namely 20, 25, 30,

and 35 ppt produced strains S_{20}, S_{25}, S_{30}, and S_{35}, respectively. Peak growth or maximum cell density and oil content for each strain of each microalgae species grown for 7 days under salinity stress and under native salinity conditions can be seen in Table 1.

In general, variations in microalgae growth peak, in terms of maximum cell density and the time required to achieve it, were mostly caused by differences in media salinity. Meanwhile, variations in oil content were more determined by differences in strains within the same species.

Chaetocheros amini

All strains of *C. amini*, except strain S_{25} which was grown under the original salinity conditions, namely 30 ppt, showed growth peaks almost as high as those grown under salinity stress conditions. However, the peak growth of microalgae in native salinity conditions was reached more quickly (4 days) than in cultivation under salinity stress conditions (5 to 6 days) (Figure 2).

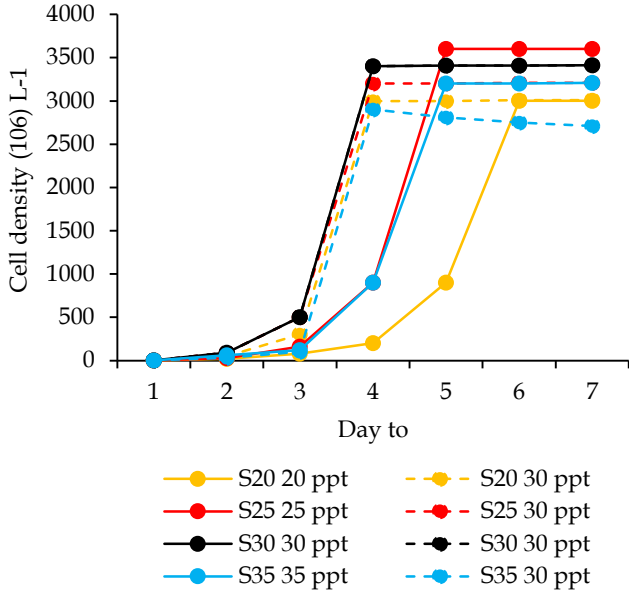

Figure 2. Growth of various *C. amini* strains under salinity stress treatment (full line) and under control treatment (original salinity) (dotted line).

Strain S_{25} grown under salinity stress showed a higher but slower growth peak than that grown under the original salinity conditions. Strains that are late in reaching peak growth are generally caused by a slow lag phase, before the log phase. An increase in the maximum growth capacity of *Chaetoceros* algae has also been reported by providing blue light treatment. However, the maximum growth of this culture was achieved more slowly than the growth of the control culture (Prasetyo et al., 2022; Sopian et al., 2019). The various *C. amini* strains, after being re-cultivated under the same initial salinity conditions of 30 ppt, showed almost the same productivity, namely peak growth ranging from 3000 x 10^6 to 3400 x 10^6 cells per liter (dotted line graph) were reached at the same time, namely on day 4. After reaching peak growth (maximum cell density) the next day growth enters the stationary phase and then the death phase.

The phenomenon of changes in the maximum growth capacity of algae caused by variations in environmental conditions has previously been observed, for example increasing microalgae productivity by providing blue light treatment (Prasetyo et al., 2022; Sopian et al., 2019), by providing medium composition treatment (Grubisic et al., 2024), by providing temperature and salinity (Antoni et al., 2020; Endrawati & Riniatsih, 2013) and by providing treatment of variations in the volume of cultivation containers (Ilhami et al., 2015). However, if the algae strain is grown again in natural light conditions, its maximum growth capacity decreases again, becoming the same as the original population. The facts above show that the adjustment of microalgae growth to variations in salinity stress is reversible or "plastic". In other words, it can be said that the mechanism of change in microalgae growth is not the result of permanent adaptation but is more precisely plasticity. In contrast to biomass productivity, the oil content of various strains of *C. amini* remained different, even though they were cultured under the same salinity conditions as the native salinity, namely 30 ppt. The S_{25} strain had the highest oil content (38.2%) than other strains, including the S_{30} strain as a native strain with an oil content of 34% (Figure 3).

Changes in oil content due to different strains are genetic, irreversible, or non-plastic because they are the result of natural selection. These facts were the information sought in this research. In the figure above, it can be seen that the S_{25} strain of *C. amini* has the highest oil content (38.12%) compared to other strains, including the native strain (S_{30} with an oil content of 34%). The results also showed that the oil content of these strains did not change even though they were cultured again at their native salinity conditions. Thus, variations in the oil content of these algae appear to be related to differences in strains and not to differences in the salinity of their media. As a comparison, another species from the genus *Chaetoceros*, namely *C. kalcitrans* which is cultivated in its natural conditions is known to have an oil content of 11.76% (Chhandama et al., 2023).

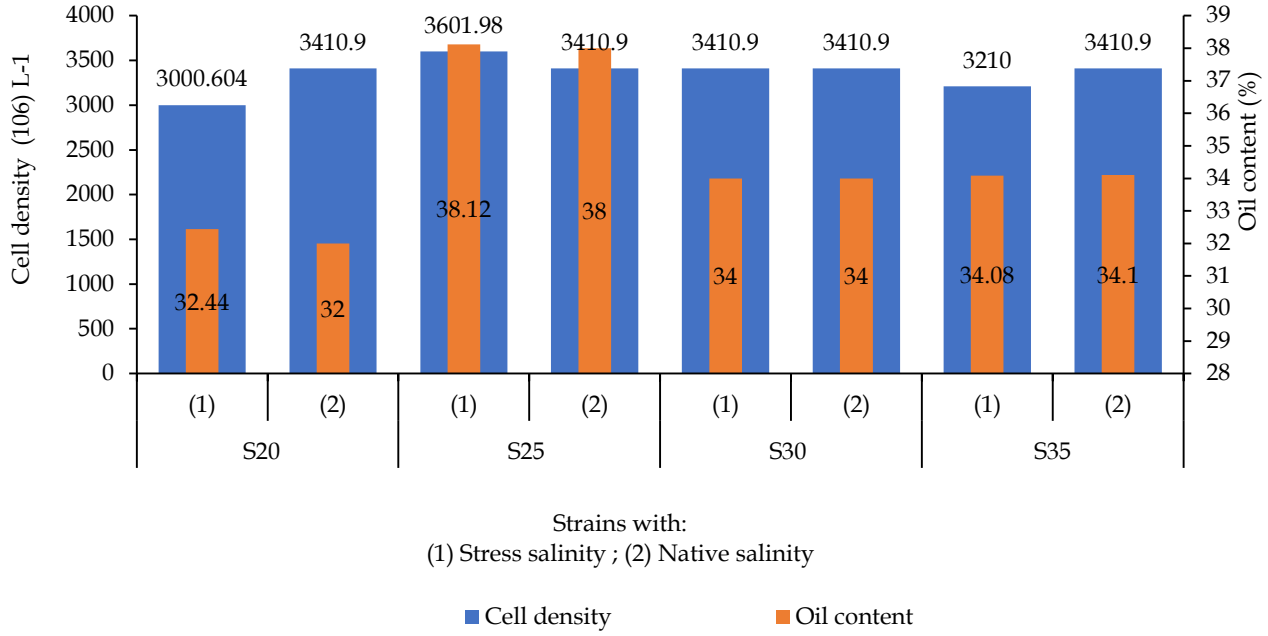

Figure 3. Maximum growth and oil content of various strains of *C. amini* cultured under salinity stress (1) and native salinity (2)

Nannochloropsis oculata

N. oculata culture with various media salinity stress treatments produced S_{20}, S_{25}, and S_{35} strains, whose productivity (maximum cell density of 3200 x 10^6 cells per liter or less) was lower than the native strain, S_{30} (maximum cell density of 4375 x 10^6 cells per liter). In addition, the S_{20} strain, apart from having the lowest maximum density (2600 x 10^6 cells per liter), was also the slowest in reaching peak growth (Maximum density was reached on day 7) (Figure 4). After reaching peak growth on day 5 (in the 25, 30 and 35 ppt salinity treatment) or day 6 (in the 20 ppt salinity treatment), the next day microalgae growth entered the stationary phase, and so on until the death phase. However, when these strains were cultured again at their original salinity conditions (30 ppt), they showed almost the same maximum cell density (ranging from 4000x10^6 to 4300x10^6 cells per liter) which was achieved in almost the same time.

These results show that different *N. oculata* strains produced by salinity stress treatment actually do not cause differences in growth capacity. In other words, it can be stated that the response of microalgae growth to changes in environmental salinity is reversible or plastic. Meanwhile, variations in the oil content of *N. oculata* were caused more by differences in strains and not by differences in environmental conditions, such as the salinity of the living medium (Figure 5).

In that figure, it can be seen that the S25 strain of *N. oculata* has the highest oil content (72.55%), followed by the S_{30} strain with an oil content of 68%, which was the ancestral strain isolated from coastal sea waters in Sekotong, West Lombok under native salinity condition of around 30 ppt. Another report states that *Nannochloropsis* sp. has an oil content of 40% (Septianto et al., (2020), 27.8% (Chhandama et al., 2023), and 35% (Mirzayanti et al., 2021).

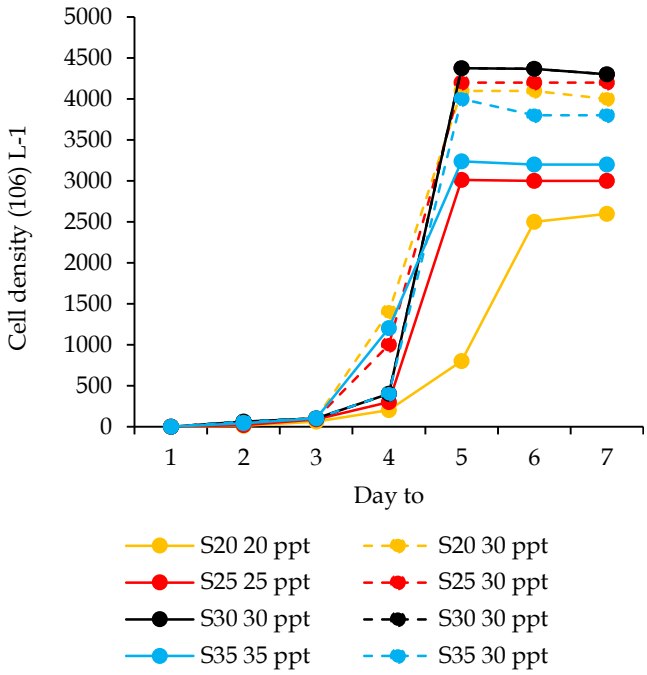

Figure 4. Growth of various *N. oculata* strains under salinity stress treatment (full line) and under control treatment (original salinity) (dotted line)

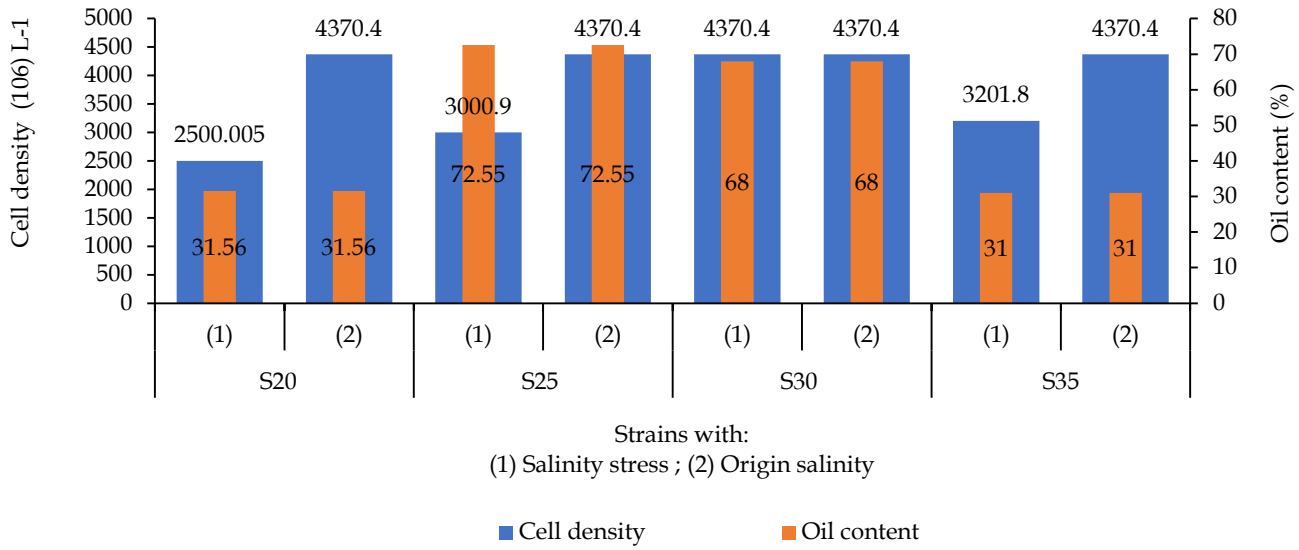

Figure 5. Maximum growth and oil content of various strains of *N. oculata* cultured under salinity stress (1) and native salinity (2)

Nitzschia spp.

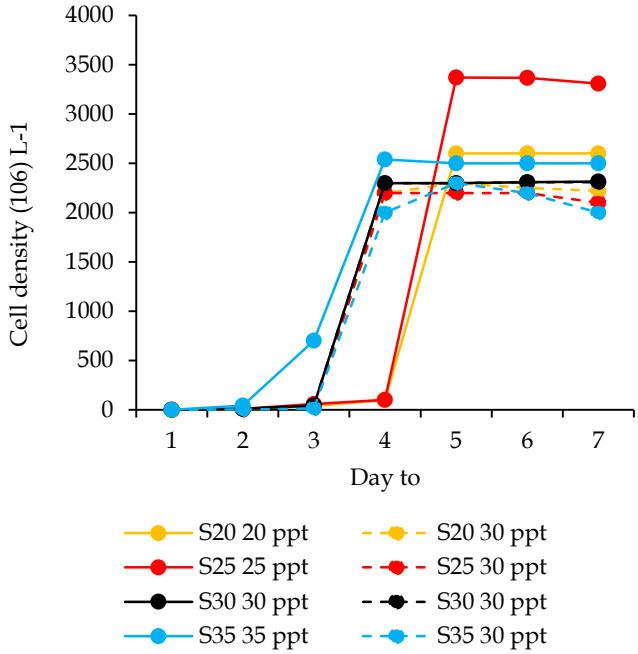

Figure 6. Growth of various *Nitzschia* spp. strains under salinity stress treatment (full line) and under control treatment (original salinity) (dotted line)

Salinity stress treatment on *Nitzschia* spp. also produced strains S_{20}, S_{25}, S_{30}, and S_{35}. Giving salinity stress treatment (reducing or increasing the salinity) to this algal species generally increased peak growth (maximum cell density), especially in cultures with a salinity stress of 25 pp with a maximum cell density of 3369 x 10^6 cells per liter, but this was achieved in a slower time (Maximum cell density was reached on day 5) when compared to cultures that were not subjected to salinity stress, namely culture with original salinity, 30 ppt (The maximum cell density was 2300 x 10^6 cells per liter, which was reached on day 4) (Figure 6).

The maximum growth capacity of a microalgae species from the genus *Nitzschia*, in this case the maximum density of cells per liter can also be increased through treatment of other environmental factors, such as variations in the volume of the cultivation container (Ilhami et al., 2015) and nutrient composition in the media (Grubisic et al., 2024). Similar to the salinity factor, the volume of the container and the composition of the media also influence the length of time needed to reach peak growth or maximum cell density for the microalgae. If strains of *Nitzschia* spp. mentioned above were cultivated again under the original salinity conditions, they showed a growth peak with almost the same height (in this case the maximum cell density was around 2300 x 10^6 cells per liter) and was achieved at the same time, namely on the fourth day after cultivation, whereas starting on 5th and so on, growth enters the stationary phase until the death phase.

Providing salinity stress, namely reducing salinity to 20 ppt, appeared to be able to select for the survival of the *Nitzschia* spp. thus producing the S_{20} strain which has a higher oil content (51.7%) than the other strains. The S_{25} and S_{30} strains have almost the same oil content, namely around 46%, while the S_{35} oil content was slightly lower, namely 37.5% (Figure 7). *Nitzschia* microalgae from elsewhere, cultured at their native salinity conditions, have been reported to have lower oil content, namely 1.57% (Ilhami et al., 2015).

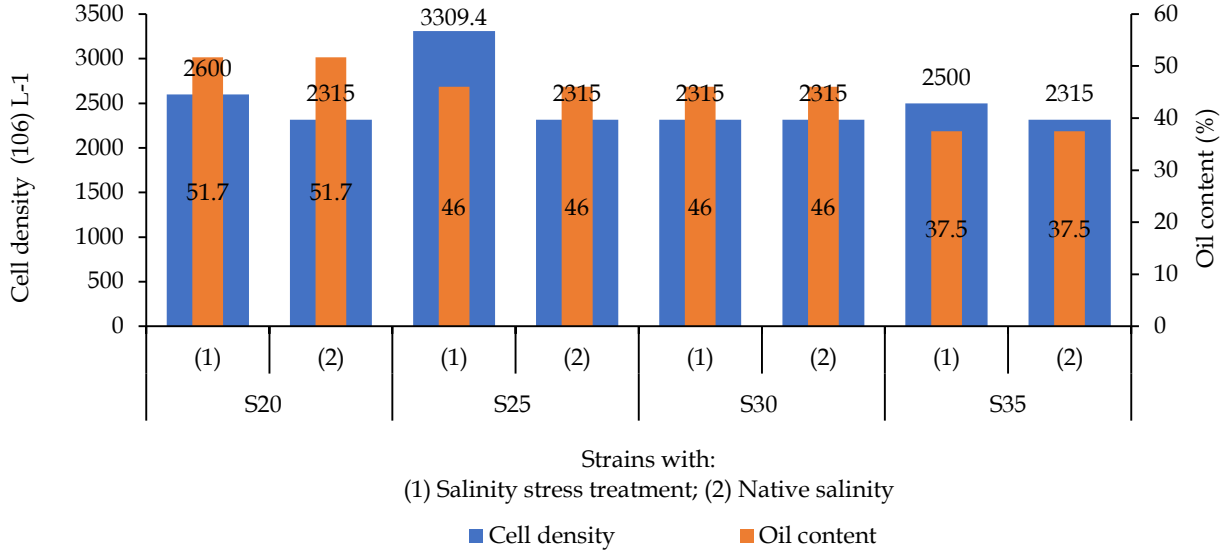

Figure 7. Maximum growth and oil content of various strains of *Nitzschia* spp. cultured under salinity stress (1) and native salinity (2)

In the figure above, it can be seen that the growth variations of *Nizschia* spp. influenced by differences in media salinity and not by differences in strains, however, the oil content of microalgae was more influenced by differences in strains. These results show the same fact as the two species mentioned previously, namely that differences in growth of *Nitzschia* spp. according to variations in media salinity were reversible, while changes in oil content in microalgae cells according to differences in strain were permanent. Thus, enhancing the oil production capacity of microalgae can be done through a strain selection mechanism, which the result is similar to the mutation results, as reported by Cheng et al. (2024), Ağbulut et al. (2023), and Santigosa & Milanese (2021). They reported that the *Nitzschia* strain resulting from the irradiation mutation had oil content that increased drastically by 47 to 51.2%.

Discussion

Reducing the salinity of the media from its native salinity can increase maximum growth for *C. amini* and *N. oculata*., but not so for *Nitzschia* spp. However, to reach peak growth under salinity stress conditions, it takes longer than under native salinity conditions. This phenomenon has previously been observed in another species, *N. palea* (Wang et al., 2024). Algal biomass production systems with higher yields but require longer time are not necessarily more economically profitable. This supports the opinion of Ahmed et al. (2023), that increasing the time for cultivating microalgae generally has the consequence of increasing operational costs which are relatively expensive. The conversion of the length of time from cultivation to harvest into economic value also depends on the intended use of the harvest, additional costs for treatment operations, equipment maintenance costs and wages (Febrinawati et al., 2020; Japa et al., 2022; Prasetyo et al., 2022; Wahyuni et al., 2020). Increasing microalgae production using environmental conditions treatments, such as salinity, temperature and lighting, has often been studied. However, the benefits from increasing microalgae biomass production are generally not enough to compensate for losses due to the increased time required for each harvest period, and increased maintenance operational costs (Faisal et al., 2024; Rakhmonov et al., 2024; Suripto et al., 2023).

Salinity stress treatment in microalgae cultivation, which can cause an increase in crop yields such as in *C. amini* and *N. oculata*, is not necessarily more economically profitable for the reasons outlined above, if the treatment aims to increase crop yields alone. In fact, the factor that most determines the conversion of microalgae cultivation time into economic value is the production systems, whether a closed system such as a bioreactor or an open system such as ponds and lagoons. In closed systems, the length of downtime generally determines the amount of production operational costs, whereas additional operating time in open systems does not result in a significant increase in operational costs (Chhandama et al., 2023; Endrawati & Riniatsih, 2013; Kwangdinata et al., 2013; Suripto & Japa, 2018; Rezania et al., 2019; Valdovinos-García et al., 2022).

Providing treatment with environmental conditions, such as temperature, salinity and lighting, which has been proven to increase microalgae biomass productivity, encounters obstacles, when this is applied to open systems. This is because these environmental factors are very difficult to control strictly in the field, and even if forced, it would certainly require very large operational costs. Thus, the use of any treatment should

not be designed to increase microalgae productivity directly, which is plastic in nature, but rather designed to select a "variant" of microalgae that has the most potential and set aside other "variants" that are not potential (Suripto et al., 2023; Zhang et al., 2022; Sardi et al., 2020; Valdovinos-García et al., 2022).

In this research, the salinity stress treatment was actually designed to produce the most viable microalgae strains in oil production through natural selection based on the similarity in density between the microalgae cells and their living medium. According to Manoharan et al. (2024), Suripto & Japa (2021), Uzwatania (2017), and Xue et al. (2020), in a population of plankton, including microalgae as phytoplankton, there are variations in the specific mass of cells that correspond to the range of tolerance to salinity of waters. If the salinity of the water changes, increases or decreases drastically, microalgae cells that have a specific mass that is the same or almost the same as the specific mass of the water will continue to live and reproduce, while cells that have a specific mass that is much lower or higher than that of the water will die or do not reproduce further.

The decrease in water salinity is in line with the decrease in specific mass. Variations in the specific mass of microalgae cells were in line with variations in the number and total volume of oil-containing vacuoles. The decrease in media salinity which is in line with the decrease in specific mass encourages selection of microalgae cells to survive or be eliminated. Thus, cultures treated with salinity stress, especially decreasing salinity, can produce groups or strains of microalgae that have a lower specific mass of cells (Antoni et al., 2020; Sachlan, 1982; Suripto et al., 2023; Gašparović et al., 2024).

Strain S_{25} of *C. amini*, strain S_{25} of *N. oculata* and S_{20} of *Nitzschia* spp., each of which survived under salinity stress conditions. Each of them may have a specific mass of cells equal or nearly the same as the specific mass of the medium in which they live, float around normally and reproduce. That is why microalgae are also known as phytoplankton (Greek: plankton = floating). These microalgae strains have a lower specific mass of cells than the native population (S_{30}). According to Russell et al. (2022), Suripto et al. (2023); Pradana at al. (2024), the decrease in the specific mass of plankton cells is in line with the increase in the number and volume of cell vacuoles containing oil. That is why strains S_{25} of *C. amini* and *N. oculata* and S_{20} of *Nitzschia* spp. in this study had a higher oil content compared to the oil content of the original population. Increasing the oil production capacity of microalgae through selection of strains that carry a permanent phenotype without mutation treatment can be considered a novelty discovered by this research.

Conclusion

Three species of microalgae isolated from Sekotong coastal waters, namely *Chaetocersos amini*, *Nannochloropsis oculata* and *Nitzschia* spp., after being cultivated under salinity stress conditions, respectively produced strains S_{20}, S_{25}, S_{30}, and S_{35}. Under salinity stress, all strains except S_{25} of *Nitzschia* spp. showed higher maximum cell density, but reversibly compared to the native population. The S_{25} strains of *C. amini* and *N. oculata*, the S_{20} and S_{25} strains of *Nitzschia* spp. had higher oil content and were permanent respectively, compared to the native population.

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Author Contributions

Conceptualization, formal analysis, resources, writing—original draft preparation, project administration, S.; methodology, investigation, funding acquisition, L.J. and S.; software, validation, data curation, writing—review and editing, visualization, supervision, L.J. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

All authors declare no conflict of interest.

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