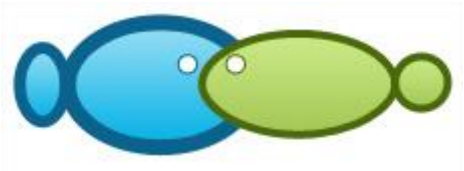

Optimization of *Euglena* sp. biomass harvesting using *Skeletonema* sp. bioflocculant under varying salinity conditions

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Abstract. *Euglena* spp. is a promising organism for industrial applications due to its ability to produce a variety of bioactive compounds. However, challenges arise in harvesting microalgae biomass because of its microscopic size and light biomass density. One effective harvesting strategy involves using bioflocculants such as *Skeletonema* sp., which contains valuable nutrients, including carbohydrates, proteins, fats, omega-3 fatty acids, and highly unsaturated fatty acids (HUFA). This research investigates the effect of water salinity and the mixing ratio of *Euglena* sp. and *Skeletonema* sp on biomass production. *Euglena* sp. was cultivated in CM (Cramer-Myers) medium with salinity stress conditions of 5, 10, and 20 g L^{-1}, while *Skeletonema* sp. was grown in modified F/2 medium without silicate in 500 mL culture bottles. The results indicated that cultivating *Euglena* sp. under 10 g L^{-1} salinity stress treatment enhanced growth, and a mixing ratio of 1:0.25 with *Skeletonema* sp. significantly increased metabolite production, meeting industrial requirements.

Key Words: bioactive compounds, bioflocculants, industrial applications, salinity stress, sustainable biotechnology.

Introduction. *Euglena* spp. comprise photoautotroph, heterotroph, and mixotroph microalgae species. These cosmopolite species living primarily in freshwater, are highly tolerant to external stress, such as acidic and heavy metal-contaminated environments. Some *Euglena* spp. accumulate paramylon polysaccharide reserves. β-1,3-glucan establishes 80% of its dry weight stored as granules inside the cytoplasm. The biomass of *Euglena* spp. is formed by essential amino acids, vitamins, lipids, carbohydrates, and proteins (Gissibl et al 2019). Paramylon is a type of carbohydrate contained in *Euglena* sp. Paramylon (β-1,3-glucan) is a glucose polymer with a β-1,3-glucan structure similar to the structure of carbohydrates. It belongs to the active polysaccharide group, and lentinan is a glucan produced by fungi, schizophyllan, or paclyman. Lentinan and schizophyllan suppress tumors, whereas fungal glucans can act as immunostimulators. It is reported that paramylon and its derivatives can be modified for medical applications. Paramylon grains are believed to be broadly dispersed inside the cytoplasm, surrounded by a membrane, or found inside a membrane-less cavity (Barsanti et al 2001).

Challenges arise in harvesting microalgae biomass due to its microscopic size and light biomass density. Various harvesting strategies, such as mechanical, electrical, chemical, and biological methods, are commonly used to harvest microalgae biomass. In the mechanical method, microalgae are harvested with centrifugation or filtration. However, this method is disadvantageous because of its high cost (Li et al 2022). The electrical method is rarely used because it can contaminate the microalgae biomass with metal ions, reducing the quality of the algae biomass (Branyikova et al 2018). Thus, this necessitates the recycling of the algae culture media. The chemical method is considered

to be dangerous to apply since it would cause secondary environmental pollution (Li et al 2018). Compared with mechanical, electrical, and chemical methods, the biological method, which uses bioflocculants, is considered more effective because it involves natural biological polymers, such as microorganisms, allowing the efficient retrieval of microalgae yields from biomass without contamination (Rakesh et al 2020). Various bioflocculants, such as proteins, polysaccharides, and positively charged extracellular polymers can harvest algae biomass (Li et al 2018). This bioflocculant is driven by extracellular polymers produced by microorganisms such as bacteria, fungi, and algae. It occurs because bacteria, fungi, and algae are products of cellular proliferation. The products are sugars, glycoproteins, amino acids, and nucleic acids, which can build long molecule chains that allow particles to coagulate (Mohammed & Dagang 2020). *Skeletonema* sp. is a type of diatom microalga belonging to the filamentous unicellular algae group. It has a distinct box-like cell structure comprising the larger and smaller epitheca. *Skeletonema* is classified as a pennate diatom, which means it has a bilateral symmetry and elongated shape. This species reproduces through isogamy, a form of sexual reproduction where the gametes are of similar size and shape. The dominant pigments in *Skeletonema* sp. are carotenoids and diatoms (Rudiyanti 2011; Armanda 2013). *Skeletonema* sp. is commonly used in shrimp cultivation due to its high nutritional content, approximately 22.3% protein and 2.55% fat (Rudiyanti 2011; Supriyantini 2013). Diatoms generally contain polysaccharides, which comprise most of their carbohydrate content. These polysaccharides play crucial roles as storage molecules and cell wall components. In diatoms, these essential polysaccharides coat the silica structures that form their cell walls.

Salinity is a critical factor influencing microalgae growth, productivity, and pigment content, including chlorophyll and carotenoids. Salinity stress adaptation occurs in microalgae, depending on the species' physiological characteristics and origin. According to Loeblich (1982), salinity stress occurs on *Bunaliella* sp., which shows that salinity affects the growth and pigments of *Dunaliella* sp. Furthermore, research about salinity stress on *Chlorella* sp. shows that this species experiences optimum growth while given a low salinity concentration. This study aims to optimize the biomass harvesting of *Euglena* sp. using *Skeletonema* sp. as a bioflocculant under varying salinity conditions. By investigating the effects of salinity stress and mixing ratios on metabolite production, the research seeks to enhance the efficiency and scalability of *Euglena* sp. cultivation for industrial applications.

Material and Method

Experiment design and treatment. A two-way factorial research with three repetitions was employed. The first factor was a treatment with four salinity levels, in concentrations of 5, 10, and 20 g NaCl L^{-1} and control (0 g NaCl L^{-1}). The other factor was the harvesting method using bioflocculation.

Culture preparation. In this research, fifteen 500 mL culture bottles were used. Cramers-Myers medium was used as the *Euglena* sp. culture media, followed by salinity stress treatment in concentrations of 5, 10, 20 g L^{-1}, and a control. Culture media was prepared using 1 L of distilled water as a solvent, based on a recommended prescription. *Skeletonema* sp. culture media was prepared by mixing F/2 medium with seawater as a solvent. A 400 mL medium was poured into culture bottles, followed by 100 mL of microalgae stock.

Maintenance and sampling. A plastic tube was installed to a culture bottle, connecting the bottle with an incubated Resun LP 100 aerator under a TL 1800 lux lamp as a lighting source. Then, the culture bottle was capped with cotton to reduce contamination risks. The bottle was stored in a culture chamber, controlled at a room temperature of 25 to 30°C to maintain optimum microalgae growth. Cell amount and bioflocculation potency were measured as *Euglena* sp. and *Skeletonema* sp. growth parameters by measuring their carbohydrate, chlorophyll, and carotenoid contents as a metabolite test. Cell amount and bioflocculation potency were measured in the stationary phase.

Cell amount. Microalgae cells were counted using a hemacytometer by putting 1 mL of sample into a microtube. Furthermore, 70% alcohol was added to the Neubauer Improved hemacytometer by 1 mm with the help of a microscope, until the chamber was full. Cell amount in the four chambers was counted using the following formula (Jakob 2013):

$$Cell\ Count\ \left(\frac{cell}{mL}\right) = \frac{Counted\ Cells\ Quantity}{4} x\ 10^4$$

Specific growth rate. The specific growth rates (SGR) of *Euglena* sp. and *Skeletonema* sp. were calculated using the following formula (Jakob 2013):

Growth Rate=(lnN1-lnN0)/(T1-T0)

Where: N1 - density at time t; N0 - initial density; T1 - observation time; T0 - initial time.

Bioflocculation. Bioflocculation potency was measured using a spectrophotometer with a 750 nm wavelength. Ratio constants of 1:0.25, 1:0.5, and 1:1 between *Euglena* sp. and *Skeletonema* sp. were used, with three repetitions each. Measurement was performed by inserting 2 mL of sample into a polystyrene cuvette. This inserted sample was taken from a sample stock in which the individual cell and the cell flock had been separated. Measurement was performed on 0 hours and the next 24 hours. Precipitation percentage was counted using the following formula (Salim et al 2012):

$$\%Optical\ Density = \frac{OD750(t0) - OD750(t)}{(OD750(t0))}$$

Where: OD750(t0) - cell density measured at hour 0; OD750(t) - cell density measured at hour t.

Carbohydrate contents. Carbohydrate contents were measured using a phenol-sulfate acid assay. This method uses colorimetry principles to calculate the total carbohydrates in a sample. The advantage of this method is its ability to detect all classes of carbohydrates. Carbohydrate molecules in the sample reacted with the strong acid to form a derived furan. This derived furan reacted with 5% phenol and showed a golden-yellow to brownish color. This strong sulfate acid broke the carbohydrate complex into a simple monosaccharide. The form of pentose or 5-carbon compounds leads a dehydrating process to form furfural, while hexose or 6- 6-carbon compounds lead a dehydrating process to form hydroxymethylfurfural (Nielsen et al 2010). The method starts by inserting a 15 mL sample into a conical tube. The sample was then centrifuged at 3300 rpm for 15 min. Afterward, the formed supernatant was separated and discarded. A 0.5 mL of phenol 5% and 1 mL H_2SO_4 were added. The sample was incubated for 30 min and homogenized. The 2 mL sample was moved to a glass cuvette. Subsequently, the absorbance was measured with a 490 nm wavelength. Standard curve was applied with the concentrations of 0.025, 0.05, 0.1, 0.25, and 0.5 g L^{-1} (Suyono et al 2016).

Chlorophyll and carotenoid contents. Chlorophyll and total carotenoid contents were measured using spectrophotometry by adding an organic solvent such as acetone. A 15 mL microalgae sample was inserted into a conical tube and centrifuged at 3300 rpm for 15 min. The formed supernatant was discarded, and acetone was inserted. Then, 2 mL of the sample was inserted into a glass cuvette to measure its absorbance using spectrophotometry with 470, 645, and 662 nm wavelengths. Chlorophyll and carotenoid contents were determined using the following formula (Dere et al 1998):

Chorophyll a (Ca)

Ca=(11.75 x A662 - 2.35 x A645)/1000

Chorophyll b (Cb)

Cb=(18.61 x A645 - 3.96 x A662)/1000

Total chlorophyll (Ca+b) = Ca + Cb

Total carotenoids (Cx + c)

Cx+c = (1000 x A470 - 2.27 x Ca - 81.4 x Cb/227)/1000

Where: Ca - chlorophyll a concentration expressed in g L^{-1}; Cb - chlorophyll b concentration expressed in g L^{-1}; Cx + c - total carotenoids concentration in g L^{-1}; A662 - chlorophyll a-specific absorbance of the sample at 662 nm; A645 - chlorophyll b-specific absorbance of the sample at 645 nm; A470 - carotenoids-specific absorbance of the sample at 470 nm; a,b,x - empirical coefficients obtained from the absorbance at particular wavelengths that are used to compute concentrations.

Statistical analysis. Data were analyzed using the Two Way ANOVA and LSD statistic test with a 5% significance level within SPSS software. The analysis was performed in three repetitions.

Results. Figure 1A shows that the control gave the highest cell density among other treatments. Cell density trends stably increased in all treatments, with the average cell density being lower than the cell density of the control. Figure 1B shows that the growth curve was stable because *Skeletonema* sp. had sequentially passed the lag, log, stationary, and mortality phases. *Euglena* sp.'s highest SGR was obtained in the control, with a value of 8.282±0.80 ind day^{-1}. This was followed by the values of 7.973±0.60 ind day^{-1} in the 5 g L^{-1} salinity, 7.629±0.64 ind day^{-1} in the 10 g L^{-1} salinity treatment, and 7.254±0.33 ind day^{-1} in the 20 g L^{-1} salinity treatment. Moreover, the specific growth rate of *Skeletonema* sp. was 9.699±0.980 ind day^{-1}. Salinity stress influenced the cell density, with a p-value of 0.009.

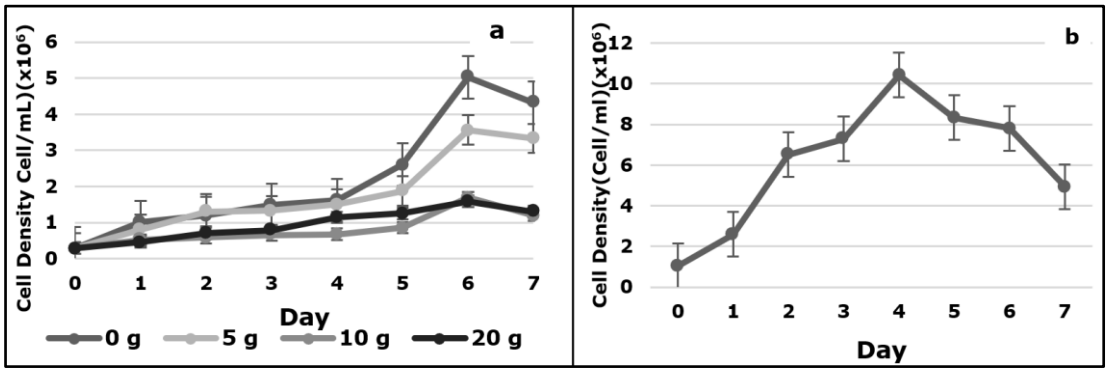

Figure 1. The change of cell density growth affected by salinity stress of (A) *Euglena* sp. and (B) *Skeletonema* sp. (Setiawardani 2022).

Figure 2 shows that the highest flocculation potency was achieved at a 1:1 ratio between *Euglena* sp. and *Skeletonema* sp. Salinity stress of 20 g L^{-1} had produced the highest bioflocculation potency among other treatments. The bioflocculation method on *Euglena* sp. with salinity treatment and *Skeletonema* sp. had significantly influenced bioflocculant potency with a p-value of 0. Figure 3 shows that the highest flocculation potency was achieved at a 1:0.25 ratio between *Euglena* sp. and *Skeletonema* sp. A salinity stress of 10 g L^{-1} produced the highest carbohydrate contents among other concentrations. The bioflocculation method used in *Euglena* sp. with salinity treatments and *Skeletonema* sp. affected the carbohydrate contents and served a real influence with a p-value of 0.0001.

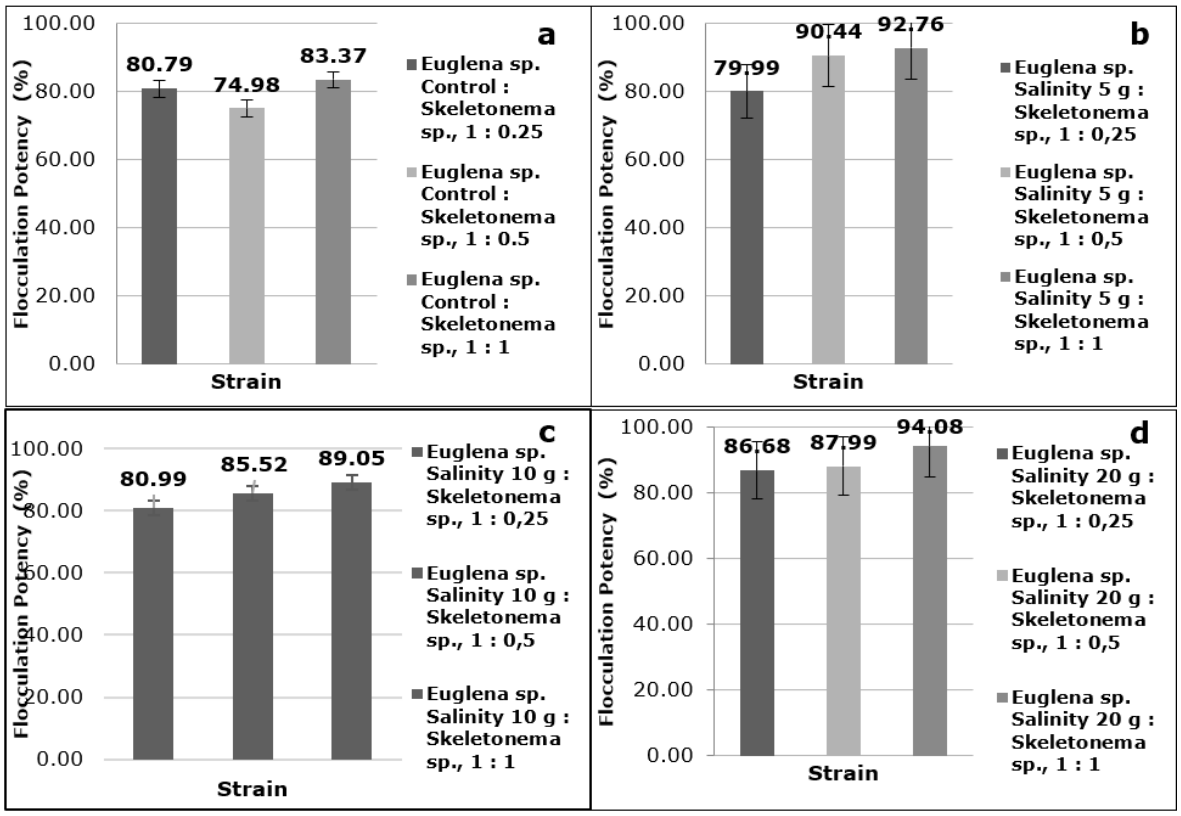

Figure 2. Flocculation potency between *Euglena* sp. and *Skeletonema* sp. in: (a) control; (b) 5 g L^{-1}; (c) 10 g g L^{-1}; (d) 20 g g L^{-1} salinity treatments (Setiawardani 2022).

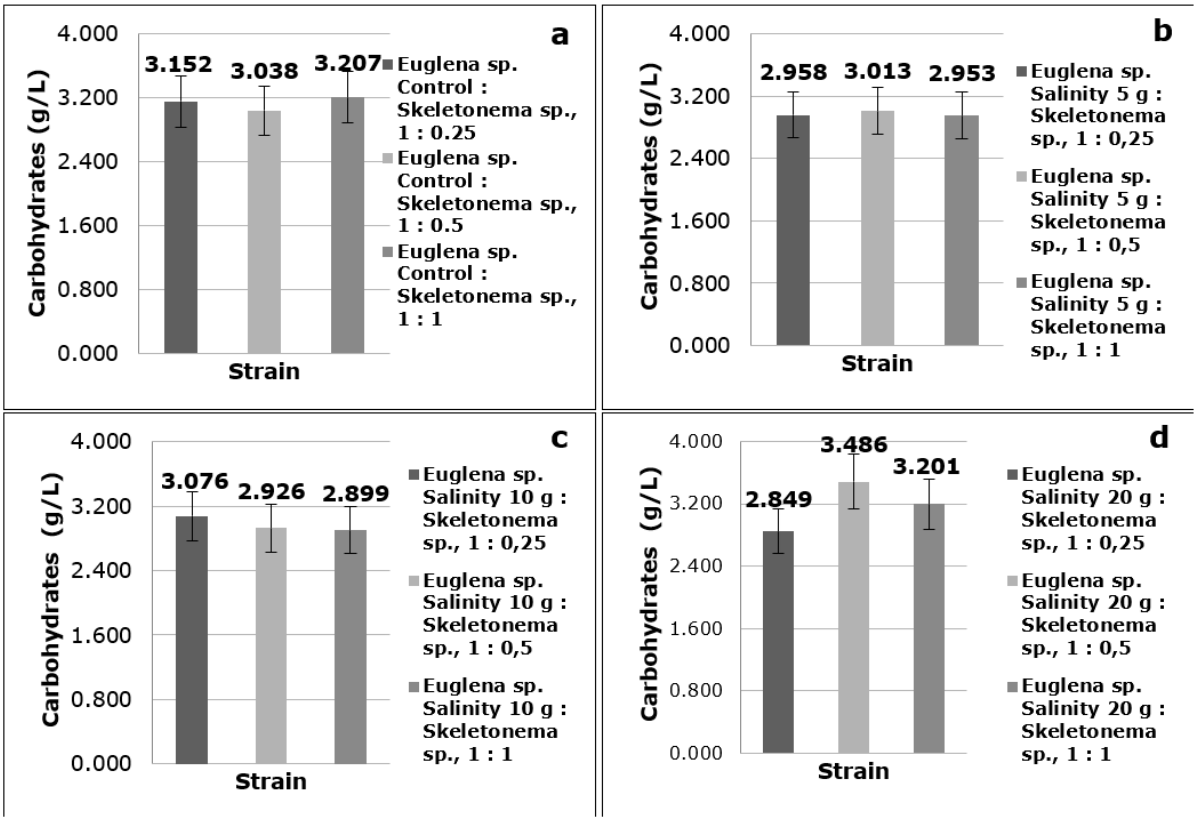

Figure 3. Mixture of carbohydrate contents between *Euglena* sp. and *Skeletonema* sp. in: (a) control; (b) 5 g L^{-1}; (c) 10 g L^{-1}; (d) 20 g L^{-1} salinity treatments (Setiawardani 2022).

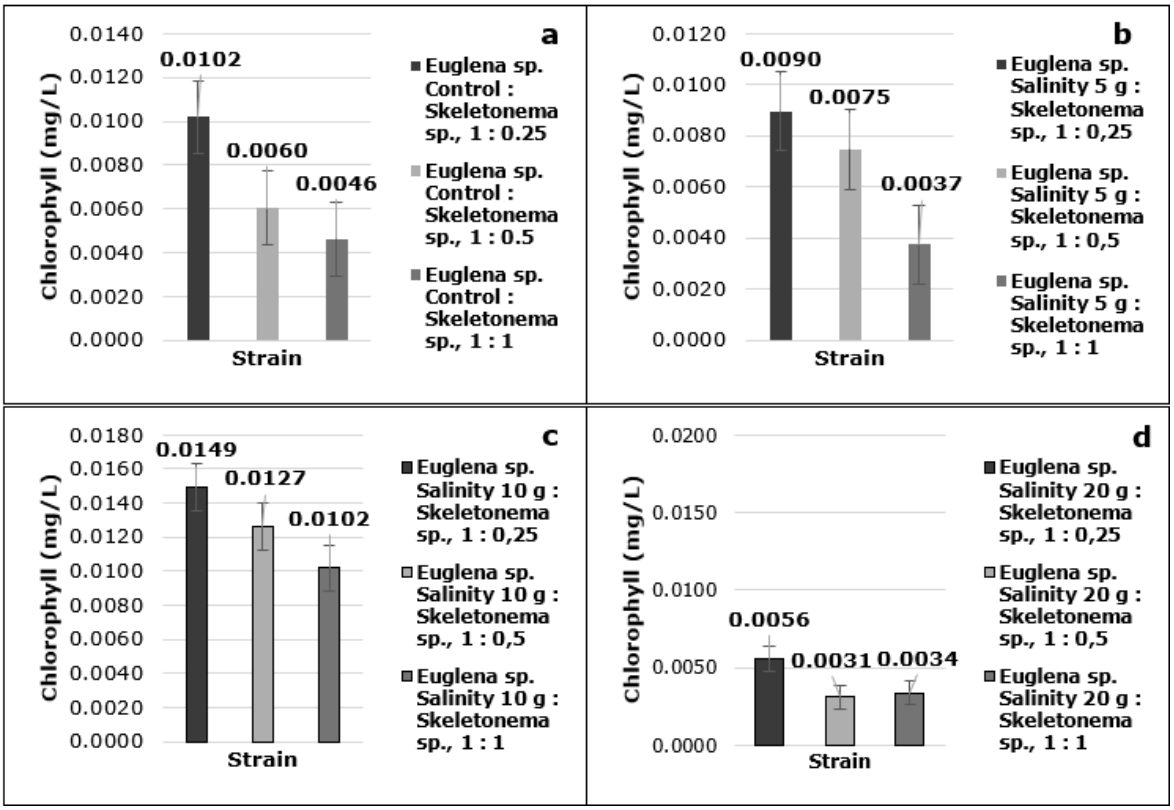

Figure 4. Mixture of chlorophyll contents between *Euglena* sp. and *Skeletonema* sp. in: (a) control; (b) 5 g L^{-1}; (c) 10 g L^{-1}; (d) 20 g L^{-1} salinity treatments (Setiawardani 2022).

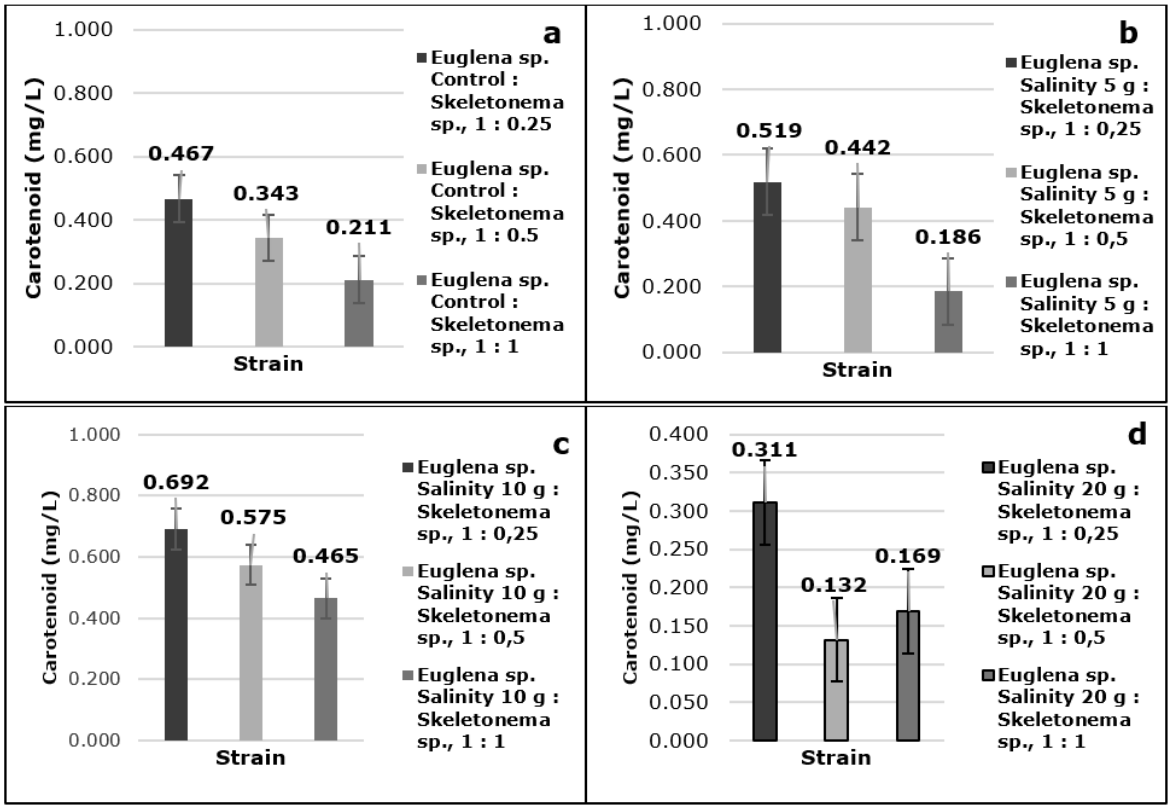

Figure 5. Mixture of carotenoid contents between *Euglena* sp. and *Skeletonema* sp. in: (a) control; (b) 5 g L^{-1}; (c) 10 g L^{-1}; (d) 20 g L^{-1}salinity treatments (Setiawardani 2022).

Moreover, Figure 4 shows that the highest chlorophyll contents was achieved at a 1:0.25 ratio between *Euglena* sp. and *Skeletonema* sp. A salinity stress of 10 g L^{-1} produced the highest chlorophyll content among other concentrations. The bioflocculation method used in *Euglena* sp. with salinity treatments and *Skeletonema* sp. affected the carbohydrate contents and had a real influence with a p-value of 0. Lastly, Figure 5 shows that the highest ratio obtained was 1:0.25. A salinity stress of 10 g L^{-1} had the highest carotenoid content among other concentrations. The bioflocculation method used in Euglena sp. with salinity treatments and *Skeletonema* sp. affected the carbohydrate contents and had a real influence with a p-value of 0.

Discussion. The density of *Euglena* sp. cell culture showed a higher cell density in the control than in the salinity treatments. This condition happened because the given salinity stress provoked microalgae to emit more energy to maintain the Turgor pressure. It involves a decrease in productivity or growth. There are two possible responses of microalgae when they are under salinity stress: (1) a rapid change in Turgor stress occurs, triggering cell volume change due to the entering or exiting water fluctuation, and (2) cell concentration stabilization to attain stable condition (Fakhri et al 2020). Higher salinity stress decreased cell growth in *Euglena* sp. due to stunting. Although the salinity of the growing media is controlled to be similar to its natural conditions, the minimum nutrient availability and environmental factors trigger an intraspecies competition for spaces and nutrients (Rudiyanti 2011). In his research on *Dunaliella* under salinity stress, Loeblich (1982) found that salinity affects *Dunaliella* sp.'s growth and pigment production. Research on *Chlorella* sp. shows that low salinity concentration serves optimum growth in this species (Adenan et al 2013).

The growth curve in the *Skeletonema* sp. cell culture density was considered stable. In the lag phase, the cells did not show significant cell amounts due to cell adaptation to utilize existing nutrients in the media. In the log phase, logarithmic phase, or exponential phase, the cell had been adapted to use the available nutrients. This phenomenon resulted in a significant increase in the cell count trend observed in this research. In the stationary phase, the amount of reproducing and mortal cells was considered even. However, the trend was declining due to the beginning of cell death. Cell death occurred more intensely in the death phase than in the stationary phase. Factors that promote cell death include nutrient deficiencies and the accumulation of secondary metabolite products, which naturally inhibit cell growth (Armanda 2013).

Euglena sp. and *Skeletonema* sp. were harvested using the bioflocculation method by adding self-bioflocculating microorganisms, or microorganisms that contain exopolysaccharides (EPS), which is usually used to harvest microalgae that cannot perform auto-flocculation. Cultures of *Euglena* sp. and *Skeletonema* sp. were cultivated separately. This method relies on the function of extracellular biopolymer reactivity or EPS that will flocculate on non-flocculant microalgae, which in this research was delegated by *Euglena* sp. (Matter et al 2019). It is also known that this bioflocculation harvesting method could increase the growth rate of microalgae (Branyikova et al 2018). *Skeletonema* sp. does not have locomotion organs. As a result, *Skeletonema* sp. tends to adhere to the substrate, and its silicate cell walls allow it to form chains of arranged cells. *Skeletonema* sp. exhibited a higher bioflocculation potential compared to *Euglena* sp. However, *Euglena* sp. can actively move due to its whip-like flagella, fringed with laterally arranged cilia in a feather-like pattern. Cell walls do not protect *Euglena* sp., but its cell is coated by a strip protein, forming a spiral encircling the cell. This protein strip is associated with microtubules forming a membrane coating known as a pellicle. This pellicle allows *Euglena* sp. to have high flexibility and contractibility in its movement (Gissibl et al 2019; Inwongwan et al 2019).

The flocculation potency of *Euglena* sp. in the 20 g L^{-1} salinity stress was higher than in other salinity treatments. It was known from this research that flocculant microalgae and non-flocculant microalgae need a ratio of 1:1 to have the highest flocculation potency. Results show that adding flocculant microalgae into non-flocculant microalgae inducted faster non-flocculant microalgae sedimentation, triggering increased harvesting efficiency.

Euglena sp. contains paramylon carbohydrate (β-1,3-glucan), a polymer glucose compound similar to starch. It belongs to the active polysaccharide group and lentinan, a glucan produced by fungi, schizophyllan, or paclyman. Paramylon content in *Euglena* sp. disperses inside its cytoplasm, surrounded by a membrane (Barsanti et al 2001). *Skeletonema* sp. is a diatom (Bacillariophyceae) that generally contains polysaccharides that may develop into broad carbohydrate compounds and a major cellular group in the form of storage polysaccharides and cell walls. Diatom has a β-1,3-glucan storage polysaccharide, a (1→3)-linked β-D-glucopyranose (DP 20-80) with C2 and C6 branchings. In dark conditions, microalgae consume β-1,3-glucan as a source for respiratory needs. Polysaccharides are vital compounds that form organic sheaths to coat silica components. This organic sheath contains glucomannan sulfate and heteropolysaccharides (Granum et al 2002).

Results show that 10 g L^{-1} salinity treatment to *Euglena* sp. served higher carbohydrate content than other salinity levels. *Euglena* sp. in salinity stress responded to osmotic pressure by accumulating intracellular low molecular weighted carbohydrates such as trehalose, sucrose, etc., which act as osmotic agents. Microalgae prevent salinity stress by increasing their carbohydrate contents, although salinity stress tolerance relies on utilized species (Markou et al 2012; Suyono et al 2016; Yuarrina et al 2018). Thus, the bioflocculation method was needed to maximize carbohydrate contents from the association of *Euglena* sp. and *Skeletonema* sp. The carbohydrate value was represented in a 1:0.25 ratio, which was higher than other observed ratios.

Chlorophyll is a typical green pigment in vascular plants and algae. Chlorophyll comprises porphyrin rings containing a magnesium center and long hydrocarbon phytol, which bond in an ester bond. There are two significant absorption ribbons: the blue or green ribbon for 450–475 nm wavelength and the red ribbon for 630–675 nm wavelength that produces the green color. There are two significant chlorophylls: chlorophyll a and chlorophyll b. Chlorophyll is the primary harvesting pigment that turns light energy into chemical energy. Chlorophyll b acts indirectly in photosynthesis by transferring light to be absorbed by chlorophyll a (Dharma et al 2017).

Results in chlorophyll testing in 10 g L^{-1} salinity stress treatment showed higher chlorophyll content than in other levels of salinity stress. The given salinity stress decreased chlorophyll production when salinity was increased. Theoretically, salinity stress affects pigment production in microalgae. The discharge of phycobilisomes in the thylakoid membrane triggers sodium ions to enter the cell rapidly, decreasing the photosynthetic process and affecting pigment production. The osmotic condition occurs when microalgae are cultivated in a high-salinity environment. A high concentration of NaCl causes cell shrinking, cell damage, and cell components due to hypertonic solvents (Begum et al 2016; Elloumi et al 2020). A chlorophyll value ratio of 1: 0.25 was considered higher than other ratios.

Carotenoids are secondary pigments essential in supporting chlorophyll as a primary pigment that transfers energy during photosynthesis. Carotenoids prevent damage due to photooxidation of the chlorophyll and the thylakoid membrane. Furthermore, this absorbed energy is transferred to chlorophyll for photosynthesis. It is known that a high carotenoid content may protect lipids from peroxidative damage (Deb 2015; Merdekawati et al 2017).

Carotenoid content showed that *Euglena* sp. in 10 g L^{-1} salinity stress served higher carotenoid contents than in other levels of salinity treatments. The osmotic condition occurs when microalgae are cultivated in a high-salinity environment. A high concentration of NaCl leads to a hypertonic environment that causes the shrinking and damage of cells and organelles. Salinity stress also causes ionic and oxidative stress in culture. Ionic stress occurred as the cause of the imbalance of Na^+ and K^+ ionic homeostasis. Osmotic conditions drive the decline of living cells because high salt concentrations exchange water with salt in the cytosol. The shrinking of the intracellular cavity leads to reversible photosynthetic electron transport inactivity. Reactive oxygen species (ROS) drive an oxidative stress condition due to salt addition. These three major types of stress occur because salinity stress forces microalgae to adjust themselves with various types of antioxidant enzymes. This drives compatible metabolites such as carotenoids and glycerol to act as antioxidants

by capturing free radicals and protecting cells from other damages caused by salt (Begum et al 2016; Elloumi et al 2020).

Conclusions. This research demonstrates that applying salinity stress significantly enhances cell density and positively influences the physiological responses of *Euglena* sp. cultures. The findings indicate that a salinity level of 10 g L^{-1} notably increases the production of critical metabolites such as carbohydrates, chlorophyll, and carotenoids, essential for various industrial applications. By optimizing the salinity conditions, we can maximize the growth and productivity of *Euglena* sp., making it a more viable and efficient source of bioactive compounds. Additionally, employing a mixing ratio of 1:0.25 (*Euglena* sp. to *Skeletonema* sp.) improves growth efficiency and optimizes metabolite production. *Skeletonema* sp. is an effective bioflocculant, aiding *Euglena* sp.'s aggregation and easier harvesting. This combined approach streamlines biomass harvesting and ensures a higher yield of valuable metabolites, fulfilling industrial requirements. Overall, integrating controlled salinity stress and the strategic use of *Skeletonema* sp. as a bioflocculant presents a promising method for enhancing the cultivation and harvesting of *Euglena* sp. This method can significantly contribute to the scalability and economic feasibility of using *Euglena* sp. in various biotechnological and industrial applications. The results of this study provide a strong foundation for further research and development in optimizing microalgae cultivation systems for improved productivity and cost-effectiveness.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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