Contribution of Co\textsuperscript{2+} in increasing chlorophyll \textit{a} concentration of \textit{Nannochloropsis salina} in controlled Conwy medium

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\textbf{Abstract.} A research in determining the contribution of Co\textsuperscript{2+} on the increase of chlorophyll \textit{a} concentration of \textit{Nannochloropsis salina} has been carried out. The cultivation of \textit{N. salina} was conducted in the Conwy medium with a salinity of 5\% and 25\% and various Co\textsuperscript{2+} concentration (2, 4, and 8 ppm). In this research, Co\textsuperscript{2+} was exposed early in the cultivation of \textit{N. salina}. The growth of \textit{N. salina} was observed daily by counting the number of populations using a haemocytometer while the chlorophyll \textit{a} concentration was determined by a Uv-Vis spectrophotometer. The results showed that the growth of \textit{N. salina} in the control was higher than that in the medium containing Co\textsuperscript{2+}. The optimum growth time was achieved on 15\textsuperscript{th} days (5\%) and 8\textsuperscript{th} days (25\%). In the cultivation medium with a salinity of 5\%, Co\textsuperscript{2+} with a concentration of 2 ppm increased the chlorophyll \textit{a} level while Co\textsuperscript{2+} with concentrations of 4 and 8 ppm decreased it. In the medium of cultivation with a salinity of 25\%, the increase in chlorophyll \textit{a} level was observed at Co\textsuperscript{2+} concentrations of 2 and 4 ppm whereas the decrease in chlorophyll \textit{a} level was given at a concentration of 8 ppm. It can be concluded that at low concentrations, Co\textsuperscript{2+} increased the concentration of chlorophyll \textit{a} in \textit{N. salina}.

1. Introduction

Biosorption of bivalent metal ions of Zn\textsuperscript{2+} and Cd\textsuperscript{2+} by \textit{N. salina} has been implemented[1]. This process is very much affected by chemical components of the cell surface and the structure of cell walls. Various polysaccharide compounds such as cellulose, chitin, alginate, can be found in the cell wall of microalgae, as well as various proteins. Some of the functional groups, especially carboxyl –and other functional groups which contain –O, –N, –S, and –P have the potential to actively involved in bonding between heavy metals and biomass [2]. Chu and Hashim [3] reported that hydroxyl, carboxyl, amino, ester, carbonyl, phosphate, and sulphohydryl functional groups have the potential to bind metal ion by biomass.

Chlorophyll is a pigment produced by microalgae which plays an important role in photosynthesis. It utilizes energy from sun rays to turn carbon dioxide and water into glucose while releasing oxygen to the environment. One of the factors affecting the concentration of chlorophyll and microalgae growth is salinity [4]. \textit{Nannochloropsis salina} (Eustigmatophyceae) is one of the microalgae containing chlorophyll \textit{a} in its cell, other than violaxanthin, and ester vaucherioxanthin. The other minor pigments
found are β-karoten, zeaxanthin, and some other unknown chlorophyll a derivatives [5]. The formation of chlorophyll in plants requires an essential element.

Cobalt is heavy metal which is naturally found in various forms in the environment. It is also an essential element that affects plant or animals growth. However, in high concentration, Co\(^{2+}\) can be very toxic [6]. Cobalt can displace Fe, Mn, Zn, and Cu from physiologically important binding sites and thus might decrease uptake and translocation of essential micronutrient [7].

This research was conducted to investigate the role and contribution of Co\(^{3+}\) in increasing chlorophyll a concentration in N. salina cultivated in the medium with a salinity of 5‰ and 25‰.

2. Material and Methods

2.1. Materials

Materials used in this research were sterile seawater, sterile freshwater, the unialgal strain of N. Salina, and Conwy cultivated medium [1] was obtained from The Research Institute for Coastal Aquaculture, Maros, Indonesia; HNO\(_3\) (p.a) solution; double distilled water. Stock solutions of Co\(^{2+}\) (1000 ppm) were prepared by dissolving 0.4941 g of Co(NO\(_3\))\(_2\).6H\(_2\)O with a few of HNO\(_3\) (p.a) in a 100 mL volumetric flask diluted with double distilled water to 100 mL. The stock solutions were then diluted to the certain concentration.

2.2. Apparatus

Apparatus used in this research included haemocytometer Marienfeld LOT-No 4551, hand counter, aerator Amara, autoclave All American No. 1925X, phase contrast microscope Olympus IX71 magnification 40 times, stirrer Hettich Mikro 22R, and oven Memmert, cellulose nitrate membrane filter Millipore (0.45 µm), Uv-Vis spectrophotometer Shimadzu model Prestige-21.

2.3. Procedures

2.3.1. Optimum time of N. salina growth. Sterile freshwater with a salinity of 5‰ or sterile seawater with a salinity of 25‰ was put into a 1 L container, added with 2 mL of Conwy medium and unialgal strain of N. salina with the initial populations of about 30 x 10\(^4\) cells/mL. The volume of the mixture was adjusted with sterile water to be 1 L. The solution was mixed, connected to an aerator with the following culture conditions: continuous irradiation (≈ 4.000 lux), aeration and a room temperature of 20 °C [1]. The growth of N. salina was observed with a haemocytometer every day until the maximum growth was obtained. The population number based on the 4 fields observation A, B, C, and D on the haemocytometer was calculated using equation (1).

\[\sum \text{cell} = \frac{A + B + C + D}{4} \times 10^4 \text{ cells/mL} \] (1)

2.3.2. Exposure of metal ions into the culture of N. salina. Sterile freshwater or sterile seawater was put into 4 containers of 1 L, solutions of Co\(^{2+}\) with a concentration of 2, 4, and 8 ppm were separately put into 3 containers. The other container was used as a control. A Conwy medium solution (2 mL) and the unialgal strain of N. salina (4 mL) with the initial population of 30 x 10\(^4\) cells/mL was added into each container, and the volume was adjusted into 1 L using sterile freshwater or sterile seawater. The solution was aerated and the growth of N. salina was observed every day.

2.3.3. Determination of chlorophyll a. 10 mL of sample was filtered using an aspirator and a vacuum pump, equipped with cellulose paper. Cellulose filter paper was prepared and folded and then put into a testing jar containing 7.5 mL of 90% acetone solution. The mixture was then kept in the freezer for 24 hours, centrifuged at a speed of 3500 rpm for 10 minutes and left for 2 hours. The clear liquid was poured into a cuvette and its absorbance was measured using a visible spectrophotometer at wavelengths of 750; 664; 647; and 630 nm. Acetone was used as a blank. Absorbance was recorded and chlorophyll
concentration calculated using Eq. (2), where $V_s$ is the sample volume, $V_1$ is the volume of acetone extract. The OD664 (absorbance 664 minus absorbance 750), OD647 (absorbance 647 minus absorbance 750), OD630 (absorbance 630 minus absorbance 750).

\[
\text{Chlorophyll } - a \left(\frac{mg}{m^3}\right) = \left(911.85 \times OD_{664}\right) - \left(1.54 \times OD_{647}\right) - \left(0.08 \times OD_{630}\right) \times \frac{V_s}{V_1}
\]

(2)

3. Results and Discussion

3.1. Growth Rate of N. salina

The growth rate of N. salina in the control and Co$^{2+}$ exposed sample in freshwater with a salinity of 5‰ is shown in Figure 1.

![Figure 1. The growth rate of N. salina in control and Co$^{2+}$ exposed medium with a salinity of 5‰.](image)

It is clear that the optimum growing time of N. salina in the control medium was on day-15, with the cell population of 1170 x10$^4$ cells/mL. It also occurs in the medium containing Co$^{2+}$ with concentrations of 2 and 4 ppm. The optimum growing time was on day-15 with the population of 622 and 533.75 x10$^4$ cells/mL, respectively. On the other hand, the optimum growing time of N. salina sample with the Co$^{2+}$ exposure of 8 ppm is on day-11 with the population of 311.25 x10$^4$ cells/mL. N. salina in the control medium was observed to have slow growth rate until day-6 as it needs time to adapt to new environment. This stage was called adaptation phase. In this stage, N. salina is more sensitive to nutrients, temperature, and other parameters which differ from its former condition [8]. N. salina will be at the growing phase after that, where it experiences rapid growth until day-15. N. salina constantly carries out cell division, and the increase of population is well-balanced with the nutrients.

After day-16 to day-25, it is observed that the population of N. salina decreases. At this stage, N. Salina experienced death phase. The decrease in the population was caused by limited nutrients in the medium, corresponding with the increase of population. At this stage, death cells were counted more than the live ones. The dead cells of microalgae which sedimented to the bottom of the medium becoming a new competitor to the live N. salina. The nutrients were getting more limited, and the deposition of toxic organic compounds increased [1].

N. salina in the Co$^{2+}$exposed medium with a concentration of 2 and 4 ppm also experiences adaptation phase until day-6. Then the growth increases rapidly until day-15. Day-16 to day-20 is the death phase for this variance. In contrast with the other variances, the optimum growing time for N. salina with Co$^{2+}$exposure of 8 ppm occurs on day-11, while day-12 to day-18 is the death phase. It is considerably earlier than the variance of control, 2 ppm exposure and 4 ppm exposure. This was because
of the high extent of heavy metal exposed to the cells. Cobalts is one of the essential heavy metals needed in trace amount. However, at excessive concentration, cobalts can be very toxic, due to its ability to inhibit chlorophyll biosynthesis caused by magnesium insertion to the protoporphyrin ring [9]. The population decrement of *N. salina* in the environment was affected by the metals concentration in the medium. The higher the concentration of metals in the medium, the higher the decrement in population *N. salina*. This shows the reverse correlation between metals concentration in the medium to the growth of *N. salina*. Bark [10] reported that *N. salina* grows very well in the low salinity environment (2.5‰), with the optimum growing time reached on day 14-15, while Fakhri et. al. [4] reported that the optimum growth and chlorophyll production in *Nannochloropsis* sp. occurred in a salinity of 15‰.

![Figure 2](image-url)

Figure 2. The growth of *N. salina* in control medium and Co\(^{2+}\) exposed medium in a salinity of 25‰.

In a salinity 25‰, the growth of *N. salina* in the control and Co\(^{2+}\) exposed medium is shown in Figure 2. The highest population of *N. salina* in the control medium is observed on day-8 with the population of 301.75\(\times10^4\) cells/mL. The optimum growth in this variance was obtained earlier than that in a salinity of 5‰ with the population of 4 times lower than that in a salinity of 5‰. The same phenomena is also observed in the growth of *N. salina* in the Co\(^{2+}\) exposed medium with a concentration of 2, 4 and 8 ppm. The optimum growth time is on day-8 with the population of 70.25; 63.00 dan 45.5 (\(\times10^4\) cells/mL), respectively. The population is 8 times less than the population in 5‰ salinity. On day-9 to day-11 the population of *N. salina* in both control and Co\(^{2+}\) exposed medium experienced decrement.

The population decrement was also caused by the accumulation of organic compound from dead biomass which sedimented at the bottom of the medium. This biomass will be the competitor for living *N. salina* to the use of dissolved oxygen in culture medium [11]. There is a gap between the population of *N. salina* in control sample and Co\(^{2+}\) exposed sample. The higher the concentration of Co\(^{2+}\) the lower the population of *N. salina*. The growth inhibition was caused by Co\(^{2+}\) metals which is essential but relatively toxic.

### 3.2 Effect of Co\(^{2+}\) to the chlorophyll a concentration in *N. Salina*

The Uv-Vis spectrophotometer determination to detect changes in chlorophyll *a* concentration after exposure of Co\(^{2+}\) compared to the control is shown in Table 1. In a salinity of 5‰, the concentration of chlorophyll *a* in the control was 446.37 mg/m\(^3\) while its concentration in the Co\(^{2+}\) exposed medium with a concentration of 2, 4 and 8 ppm was 507.40; 432.15; and 400.13 mg/m\(^3\) respectively. The exposure of 2 ppm Co\(^{2+}\) increases the concentration of chlorophyll *a* in *N. salina* around 61.03 mg/m\(^3\). This was because, in a low concentration of Co\(^{2+}\) exposure, the biosynthesis rate of chlorophyll is sped up. Cobalt is needed to synthesize vitamin B\(_{12}\) as cobalamin, where this vitamin is one of the micronutrients needed
by microalgae [12]. On the other hand, the exposure of Co\(^{2+}\) with a concentration of 4 and 8 ppm decrease the chlorophyll \(\text{a}\) concentration in \(N. \text{salina}\) of 14.22 mg/m\(^3\) dan 46.24 mg/m\(^3\) respectively. It was because the high concentration of cobalt inhibit the biosynthesis of chlorophyll \(\text{a}\) and the inhibition effect was stronger in the higher concentration [13].

**Table 1.** Chlorophyll \(\text{a}\) concentration in \(N. \text{salina}\) in control and Co\(^{2+}\) exposed medium at a salinity of 5\% and 25\%.

| Salinity (‰) | Chlorophyll \(\text{a}\) conc. (mg/m\(^3\)) | \(\Delta\) Chlorophyll \(\text{a}\) conc. with control (mg/m\(^3\)) | \(\Delta\) Chlorophyll \(\text{a}\) conc. with control (%) |
|--------------|------------------------------------------|-------------------------------------------------|-------------------------------------------------|
|              | Control 2 ppm | Co\(^{2+}\) 2 ppm | Co\(^{2+}\) 4 ppm | Co\(^{2+}\) 8 ppm | Control 2 ppm | Co\(^{2+}\) 2 ppm | Co\(^{2+}\) 4 ppm | Co\(^{2+}\) 8 ppm | Control 2 ppm | Co\(^{2+}\) 2 ppm | Co\(^{2+}\) 4 ppm | Co\(^{2+}\) 8 ppm |
| 5            | 446.37        | 507.4             | 432.15           | 400.13           | 61.03         | -14.22           | -46.24           | 13.67            | -2.80           | -10.70          |
| 25           | 176.12        | 198.45            | 189.46           | 171.91           | 22.33         | 13.34            | -4.21            | 12.68            | 6.72            | -2.22           |

\(\Delta = \) different of sample concentration with exposure of Co\(^{2+}\) with control

At a salinity of 25\%, the concentration of chlorophyll \(\text{a}\) of \(N. \text{salina}\) in the control is 176.12 mg/m\(^3\), while in the Co\(^{2+}\)-exposed medium with a concentration of 2, 4 and 8 ppm is 198.45; 189.46 and 171.91 mg/m\(^3\) respectively. The chlorophyll \(\text{a}\) concentration in the control and 2 ppm Co\(^{2+}\)-exposed medium is 3 times lower than in the sample at 5\% salinity, while the 4 ppm and 8 ppm Co\(^{2+}\)-exposed medium are 2 times lower than that at 5\% salinity. The consequence of increasing the concentration of Co\(^{2+}\) is an increment of chlorophyll \(\text{a}\) concentration in \(N. \text{salina}\). It was also proven in the data that the addition of metal ions Co\(^{2+}\) increase the chlorophyll \(\text{a}\) concentration produced by \(N. \text{salina}\), which is shown in 2 ppm and 4 ppm variances. However, at higher concentration of 8 ppm Co\(^{2+}\), the decrement of chlorophyll \(\text{a}\) concentration was observed far less than the control. The similar result was also reported by El-Sheekh et al. [12], that at a lower concentration of Co\(^{2+}\) (0.5 and 1.5 ppm), the chlorophyll and carotenoid concentration increase significantly in \(N. \text{perminuta}\). On the other hand, at higher concentration (2.5; 3.5; and 5 ppm) Co\(^{2+}\) caused the decrease in pigment concentration progressively. It was also observed that at 3.5 and 5 ppm, the inhibition effect to chlorophyll \(\text{a}\) was much stronger than to carotenoid. This inhibition occurred as the result of magnesium displacement mechanism in chlorophyll molecules, causing the accumulation of protoporphyrin and blockage of chlorophyll synthesis.

**Figure 3.** The difference between chlorophyll \(\text{a}\) concentration in \(N. \text{salina}\) in the control and Co\(^{2+}\)-exposed medium.
It is obvious that the chlorophyll $a$ concentration of $N. salina$ in freshwater with a salinity of 5‰ is relatively higher than that of $N. salina$ in seawater with a salinity of 25‰, contrary to Ak et al. [14] report that chlorophyll $a$ content of $Dunaliella viridis$ increased with increasing salinity.

4. Conclusions
The growth of $N. salina$ and the concentration of chlorophyll $a$ in cultivating medium with a salinity of 5‰ is better than in the medium with a salinity of 25‰. The optimum growing time was obtained on day-15 for a medium with a salinity of 5‰ and day-8 for a medium with a salinity of 25‰. The contribution of $\text{Co}^{2+}$ to increase chlorophyll $a$ concentration in $N. salina$ in both cultivating medium occurred at a concentration of $\text{Co}^{2+}$ 2 ppm, while at 8 ppm inhibition was observed. For a medium with a salinity of 5‰, $\text{Co}^{2+}$ with a concentration of 4 ppm was also observed causing increment to the concentration of chlorophyll $a$. It can be concluded that at low concentrations, $\text{Co}^{2+}$ has an increasing effect of chlorophyll $a$ on $N. salina$.

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