Effect of Fe$^{2+}$ and Mn$^{2+}$ addition on growth and β-carotene production of Dunaliella salina

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Abstract. Dunaliella salina is a unicellular chlorophyte, which could grow in a wide range of harsh environmental condition. Under growth-limitation conditions, D. salina was known as famous for a high β-carotene producer with up to 10% of its dry cell weights, therefore it could be a β-carotene supplement. The research aimed to find out the effect of Iron (Fe$^{2+}$) and Manganese (Mn$^{2+}$) towards β-carotene productivity as result of oxidative stress from the second photosystem (PS II). The analysis method was carried out by the ultrasonic extraction for short and cheap lyses of phytoplankton biomasses; infra-red (IR) to find out the interaction metal ion, and UV/VIS spectrophotometer to determine the β-carotene concentration from phytoplankton the crude extract. The result showed that the interaction occurred between metal ions and M-N, -O-M and M←OH-C groups in the amino acid of phytoplankton. The highest impact was indicated on 0.3 ppm Fe$^{2+}$ to D. Salina. The highest β-carotene concentration was 13.08 µg/g DW for 0.3 ppm Fe$^{2+}$ and 8.08 µg/g DW for 0.8 ppm Mn$^{2+}$. The dry weight concentrations of β-carotene indicated that D. salina with 0.6 ppm Fe$^{2+}$ addition had more potential as a β-carotene supplement.

1. Introduction
Carotenoid was a natural product with antioxidant activity. Phytoplankton was known widely had many antioxidants. Species of phytoplankton had been used in many countries in the world [1]. Oxidative stress plays an important role in the pathogenesis of various diseases such as atherosclerosis, alcoholic liver cirrhosis, cancer, etc. Oxidative stress was initiated by free radicals, especially reactive oxygen species (ROS). Oxidative stress occurs when the generation of free radicals or reactive oxygen species (ROS) exceeds the antioxidant capacity of a biological [2,3,4,5].

Dunaliella, Chlorella, and Spirulina are three types of phytoplankton that had commercial potential that had been used to produce fats, proteins, and pigments [6,7].

Several studies had shown that the use of metal ions play an active role in the process of photosynthesis and oxidative stress. As known oxidative processes were intimately associated with the production of antioxidant compounds such as carotenoids. Research had been done on production astaxanthin [8], using Haematococcus pluvialis with the addition of Fe$^{2+}$-EDTA and Fe$^{3+}$-EDTA. The addition of Fe$^{2+}$-EDTA was known to stimulate the production of more effective astaxanthin, whereas Fe$^{3+}$-EDTA was more effective in improving cell growth. The Fe$^{2+}$ ion is a catalyst that can cause decomposition of H$_2$O$_2$ in the Haber-Weiss reaction and Fenton oxidative reactions. Both of these reactions produce hydroxyl radical (HO•), which could stimulate production of astaxanthin on
microalgae *Haematococcus pluvialis*. Other studies have also shown that induction of oxidative stress occurs with the addition of high concentrations of Co$^{2+}$ and Mn$^{2+}$ on microalgae *Pavlopa viridis*. In this case, the addition of metal Co$^{2+}$ and Mn$^{2+}$ were known to stimulate the activity of catalase (CAT) and glutathione (GSH) [9].

Antioxidant activity had shown in the methanol crude extract from *Isochrysis aff galbana, Chlorella vulgaris, Nannochloropsis oculata, Tetraselmis* and *Chaetoceros calcitrans* [10]. Cell damage caused by free radicals was believed due to the aging process and degenerative nature of the disease. β-carotene supplementation was one of the preferred sources of antioxidants that could be consumed. However, β-carotene supplements contain only synthetic product isomer trans-β-carotene (all-trans-β-carotene) a pro vitamin A [11,12] that would be converted into vitamin A. While the 9-cis isomer-β-carotene (9-cis-β-carotene), which had a higher antioxidant activity than the trans-β-carotene (all-trans-β-carotene) in natural products only. So that should be pursued natural product which is maximal a 9-cis-β-carotene produced.

*Dunaliella bardawil* and *D. salina* showed potential as a source of β-carotene [7, 12-17] (Pisal and Lele, 2005; Oren, 2005; Tafreshi and Shariati, 2009; Abusara, 2011). El-Baky et al obtained β-carotene levels of 54.08% of the total carotenoid, at a condition of 16% NaCl [6]. Interclinical Laboratories data provide information that β-carotene of Dunaliella salina is higher than that of carrots. The amount of β-carotene of *D. salina* is 1,100-2,100 mg/100 g while in carrots it was only 5.8 mg/100 g [12].

This study has investigated that the addition of metal Fe$^{2+}$ and Mn$^{2+}$ can determine their effect on the productivity of β-carotene in *D. salina*. The addition of metal ions Fe$^{2+}$ is expected to stimulate oxidative stress that could induce productivity of β-carotene which was a natural product predominantly consists of the isomer trans-β-carotene (all-trans-β-carotene) and 9-cis-β-carotene (9-cis-β-carotene). Infrared spectrophotometer (IR) was used to determine the initial data of functional groups that interact with metal ions Fe$^{2+}$ on the mechanism of formation of β-carotene productivity by these species.

2. Material and Method

Materials used were pure culture of *D. salina* (the phytoplankton cultivation of Jepara, Yogyakarta, Indonesia), acetone pa, FeSO$_4$7H$_2$O, MnSO$_4$H$_2$O; Stock A medium (FeCl$_3$.6H$_2$O; MnCl$_2$.4H$_2$O; H$_2$BO$_3$; Na$_2$EDTA; Na$_2$PO$_4$.2H$_2$O; NaNO$_3$); Stock B (ZnCl$_2$; CoCl$_2$.6H$_2$O; (NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O; CuSO$_4$.5H$_2$O); Stock C (Vitamin B$_12$ dan Vitamin B$_1$); β-carotene powder and distilled water.

Equipment used was commonly used glassware, microscopes, haemocytometer (Neubauer-improved), Hand counter, Salinometer, SPNISOSFD oven, digital balance Ohaus NO AP 110, FT-IR Shimadzu 820 1PC, centrifugation, Ultrasonic, and UV-VIS spectrophotometer.

2.1 Procedures

2.1.1 Preparation of Medium Conwy. Medium conwy were three stocks, namely A, B and C. The A nutrient solution was 1000 mL which were added 2 mL to the B stock. The mixture was added to a solution of Conwy in sterile sea water in the ratio of 2 mL per 1000 mL of sea water that has been sterilized. Furthermore, a mixture of stocks A and B added 1 drop of stock C solution of vitamin.

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2.1.2. *Phytoplankton growth*. Determination phytoplankton growth pattern had been done by counting the number of cells per milliliter of medium every day. Sample was taken with a Pasteur pipette, dropped about 1-2 mL on a haemocytometer, and then observed through a microscope. The cell density was calculated using equation (1).

\[
\frac{\text{cell}}{\text{mL}} = \frac{\text{number of cells in 4 blocks}}{\text{number of blocks (}=4)} \times 10^4
\]  

(1)

2.1.3. *Identification by FT IR*. Identification of functional groups in phytoplankton that could be potential to bind Fe$^{2+}$. Phytoplanktons were harvested, dried, crushed and screened. Biomass without and with exposure to metal ions were analysed using an infrared spectrophotometer (IR).

2.1.4. *β-carotene extraction by sonication method*. Working performed in a room with low intensity lighting and at a cold room. Phytoplankton biomass obtained was put into a centrifuge tube and mixed with acetone as much as 3 mL, with a sealed tube, the mixture was sonicated for 50 min at 40°C. Samples were centrifuged for 20 min at 5000 rpm and the supernatant was taken for analysis of β-carotene.

3. Result and Discussion

3.1. *Phytoplankton Cell Growth of D. salina*

This research was used phytoplankton species *D. salina*. The pattern of growth without the addition of metal ions is shown in figure 1.

![Figure 1. Cell Density of Phytoplankton D. salina.](image)

It is clear that *D. salina* requires two days for adaptation to the culture medium Conwy. This was relatively short period. Significant increase observes on the third day, which means optimal cell division begins. This figure also shows the growth pattern of the phytoplankton. The nutrient requirement was highly correlated with the cell size and the cell movement rate, where the cell size was 9-11 μm [18]. It had a flagellum using nutrients in addition to the process of cell growth and division, nutrients were also required for movement. The end of cultivation was stopped at fourteenth days.

3.2 *Determination of Fe$^{3+}$ and Mn$^{2+}$ concentration to Tolerable by Phytoplankton D. salina*

The growth pattern of *D. salina* with and without the addition of Fe$^{2+}$ and Mn$^{2+}$ is given in figure 2. The concentration of Fe$^{2+}$ and Mn$^{2+}$ ion having the highest and the lowest cell density can be seen in table 1.
Figure 2. Growth Pattern of *D. salina* (a) without and with the addition of \( \text{Fe}^{2+} \) and (b) without and with the addition of \( \text{Mn}^{2+} \) at various concentrations.

Table 1. The Concentration of \( \text{Fe}^{2+} \) and \( \text{Mn}^{2+} \) with the highest and the lowest cell density

| Density              | [Fe\(^{2+}\)] (ppm) | Cell Density (x10\(^4\) sel/mL) | [Mn\(^{2+}\)] (ppm) | Cell Density (x10\(^4\) sel/mL) |
|----------------------|----------------------|----------------------------------|----------------------|----------------------------------|
| Highest Growth Density | 0,3                  | 203                              | 1,5                  | 158,5                            |
| Lowest Growth Density | 0,6                  | 43,5                             | 0,6                  | 35,5                             |

It is clear that the concentration of \( \text{Fe}^{2+} \) and \( \text{Mn}^{2+} \) with the highest cell density is different. This occurred because there was differences in morphological properties (cell wall, cell size, movement of phytoplankton) and metal ion functions in metabolic processes. In microalgae, Fe metal ions played a very important role in the regulation of cell metabolism as an essential element, in addition to nutrients and in the process of photosynthesis [19-21].
High growth is shown in the high concentration of Mn\(^{2+}\) to the phytoplankton of *D. salina* (table 1). While low growth was in a low concentration of the ion, this suggests that photosynthesis requires more Mn metal ions to produce O\(_2\) located in the process of photosystem II (PS II). The important role of the Mn metal ion in the photosynthesis process lies in the water-solving system of Photosystem II, which prepares electrons for the electron transfer of photosynthesis. Groups of four Mn associated with the oxygen complex (OEC~Oxygen Evolving Complex) were bound to the photosystem II (PSII) protein [22].

The table also showed that flagged phytoplankton require in a higher concentrations of Mn\(^{2+}\) for cell division and development. It was suitable with [23] and [21] that Manganese ions had functions in photosynthesis process and cell structure.

![Figure 3. IR Spectrum IR of *D. salina* (DS), (a) DS ; (b) DS + Fe\(^{2+}\).](image-url)
Figure 4. IR Spectrum IR of *D. salina* (DS), a) DS ; b)DS + Mn$^{2+}$.

The obtained Infra-Red data showed in Fig 3 and 4 that Fe$^{2+}$ and Mn$^{2+}$ generally had an effect on the M-N functional region giving M-N interaction, M-S functional group with possible M-S interaction, then the signal for the M←OH-C complex compound was also detected. The interaction of ions metal indicates as M-N, -O-M and M←OH-C complex in the phytoplankton amino acid.

3.3 Data of β-Carotene Analysis on D. salina, Fe$^{2+}$ and Mn$^{2+}$

Biomass of each species was extracted, either by the addition of Fe$^{2+}$ and Mn$^{2+}$ at various concentrations. The extraction process was done by a sonication method to streamline the time and get more extract and use less solvent volume. The method utilized ultrasonic waves that can destroy the cell to accelerate the process of mass transfer of compounds from the cell into the solvent. Figure 3 shows β-Carotene analysis on *D. salina* after the addition of Fe$^{2+}$ and Mn$^{2+}$ ions.
The highest amount of β-carotene is obtained after the addition of Fe\(^{2+}\) with the concentration of 0.6 ppm. The amount of β-carotene produced after the addition of 0.6 ppm Fe\(^{2+}\) is 13.08 μg/g dried weight which is three times higher than produced by the D. salina control. This indicates that the addition of 0.6 ppm Fe\(^{2+}\) is highly potential to produce β-carotene. From table 1, it is shown that the addition of Fe\(^{2+}\) with the same concentration gives low growth of D. salina. A low growth in carotenoid productivity was also expressed by Hadi et al. [23].

The low growth followed by a decline in the amount of D. salina biomass was also reported on Haemotococcus pluvialis with Fe\(^{2+}\)-EDTA [8]. This was possibly due to the action of Fe\(^{3+}\) as an effective catalyst in the breakdown of H\(_2\)O\(_2\) [24,25], which was produced from superoxide anion radicals by the action of the enzyme superoxide dismutase, the catalase enzyme that broke hydrogen peroxide to produce O\(_2\) and H\(_2\)O. However, the presence of Fe\(^{2+}\) as the catalyst will produce hydroxyl radicals (OH \(^*\)) given the Haber-Weiss reaction [24] as follows:

\[
O_2^*\ +\ H_2O_2\ +\ Fe^{2+} \rightarrow Fe^{3+}\ +\ O_2\ +\ OH^-\ +\ HO^*.,
\]

and the Fenton reaction:

\[
H_2O_2\ +\ Fe^{2+} \rightarrow Fe^{3+}\ +\ OH^-\ +\ HO^*.
\]

Therefore, the increase in the productivity of β-carotene as a type of antioxidant in the protection of cells from radical compounds will be observed.

The dry weight concentrations of β-carotene was generally not significantly affected by the addition of Mn\(^{2+}\). It is possible because the Mn metal ion has a small reduction potential compared to the Fe metal ion. In addition, Mn\(^{2+}\) has a dominant role as a cofactor of catalase, peroxidase and superoxidase (SOD) that play a protective role against radical pressure [24].

![Graph](image)

**Figure 5.** Analysis of β-carotene in D. salina after the addition of Fe\(^{2+}\) or Mn\(^{2+}\) at various concentrations.
4. Conclusion
Based on the results presented above, it can be summarized that the addition of Fe$^{2+}$ in general has a greater influence on the growth of *D. salina*. The highest amount of β-carotene, *i.e.* 13.08 and 8.08 µg/g dried weight, was obtained after the addition of 0.6 ppm Fe$^{2+}$ and 0.3 ppm Mn$^{2+}$, respectively. The interaction occurred between ions metal indicates and M-N, -O-M and Me-<OH>-C complex of amino acid in the phytoplankton. *D. salina* with the addition of 0.6 ppm Fe$^{2+}$ addition was potential to be used as the β-carotene supplement.

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