



Enhanced astaxanthin production by oxidative stress using methyl viologen as a reactive oxygen species (ROS) reagent in green microalgae *Coelastrum* sp.

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ABSTRACT Microalgae are known to be a potential resource of high-value metabolites that can be used in the growing field of biotechnology. These metabolites constitute valuable compounds with a wide range of applications that strongly enhance a bio-based economy. Among these metabolites, astaxanthin is considered the most important secondary metabolite, having superior antioxidant properties. For commercial feasibility, microalgae with enhanced astaxanthin production need to be developed. In this study, the tropical green microalgae strain, *Coelastrum* sp., isolated from the environment in Malaysia, was incubated with methyl viologen, a reactive oxygen species (ROS) reagent that generates superoxide anion radicals (O_2^-) as an enhancer to improve the accumulation of astaxanthin. The effect of different concentrations of methyl viologen on astaxanthin accumulation was investigated. The results suggested that the supplementation of methyl viologen at low concentration (0.001 mM) was successfully used as a ROS reagent in facilitating and thereby increasing the production of astaxanthin in *Coelastrum* sp. at a rate 1.3 times higher than in the control.

KEYWORDS Astaxanthin; *Coelastrum* sp.; methyl viologen; reactive oxygen species

1. Introduction

The ketocarotenoid astaxanthin (3,3'-dihydroxy-4,4'-diketo- β -carotene) is a secondary carotenoid from the same family as β -carotene, canthaxanthin, zeaxanthin, lycopene and lutein (Lorenz and Cysewski 2000). The carotenoids, astaxanthin is the highest value carotenoid since its potent antioxidative activity and more effective in scavenging free radicals (Dragoş et al. 2010). Its superior antioxidant property possesses a broad range of applications in food supplements, nutraceutical, and pharmaceutical industries as well as act as pigmentation sources for fish aquaculture (Guerin et al. 2003; Ambati et al. 2014; Fraser and Bramley 2004). Astaxanthin biosynthesis has been observed in a limited number of organisms, including bacteria, yeast *Phaffia rhodozyma*, fungi and in some green microalgae (Orosa et al. 2001). Among the carotenoid producing organisms, green microalgae are a potential resource of high-value metabolites

with the potential of producing astaxanthin (Liu et al. 2014).

However, the low productivity of these products in the native microalgae requires to be overcome (Clarens et al. 2010). Microalgae with improved growth rate and enhanced carotenoid accumulation will generate the commercial production of astaxanthin more feasible. Presently, green microalgae that have potential in accumulating astaxanthin has received tremendous attention because of its high cost and the possibility of health benefits (Nakano et al. 1995). Numerous attempts have been made to improve strain with a high yield of astaxanthin. To obtain the production process more feasible, optimization of cultivation and genetically modified strains have been applied for the past few decades but yet have not been fully satisfied (Kilian et al. 2011). As an alternative method, chemicals as enhancers have been proposed to initiate the production and accumulation of astaxanthin. The application of chemical enhancers could be a valuable approach

in addressing the low productivity of astaxanthin (Asada 1994).

Environmental oxidative stresses can enhance the massive accumulation of astaxanthin by green microalgae under the condition of high illumination, nitrogen starvation, salt stress or temperature stress (Lee and Soh 1991). The effects of these unfavorable conditions have been attributed to the formation of reactive oxygen species (ROS). The excessive ROS may damage the ability of the cells to detoxify the reactive intermediates and leading to oxidative stress conditions. These highly reactive ROS can react with lipid membranes, proteins and nucleic acids, and ultimately cause oxidative damage resulting in cell death (Lafarga et al. 2020). Therefore, ROS will be used as signal molecules in microalgae to trigger the accumulation of astaxanthin and protect an oxidative stress damage (Apel and Hirt 2004). It might function as an effective antioxidant with a primary line of defense against oxidative damage in scavenging free radicals (Hu et al. 2018).

Biosynthesis of astaxanthin in the cell of microalgae can be enhanced by the addition of ROS reagent (Kobayashi 2003). A study from Li et al. (2010) shown that the tolerance to excessive ROS is higher in astaxanthin-rich cells with the capacity to detoxify superoxide anion radical. In addition of the iron into the culture medium, the excess levels of ROS in *Haematococcus pluvialis* increased ROS level. Thereby, the synthesis of fatty acids and astaxanthin was observed in the cell for protecting their lipid vesicles (Hong et al. 2015). Induction of oxidative stress using ROS reagent in *Chlorella zofingensis*, was also effective to increase the carotenoid accumulation (Hu et al. 2018). It is known that the addition of various ROS reagents into the culture medium was able to improve the carotenoid synthesis in microalgae (Chokshi et al. 2017).

Previously, ROS generating the methyl viologen (MV) and iron ion (Fe^{2+}) compounds can lead to the formation of the superoxide anion radical and hydroxyl radical, respectively (Kobayashi et al. 1997). The most common source of ROS reagents for astaxanthin synthesis was hydrogen peroxide (H_2O_2), methylene blue (MB), and methyl viologen (MV) for hydroxyl radicals, singlet oxygen, and superoxide anion radicals, respectively (Ma and Chen 2001). The appropriate concentration of ROS reagent was important to enhance astaxanthin formation in microalgae. Hydrogen peroxide (H_2O_2) was used by Ip and Chen (2005) to generate hydroxyl radical for astaxanthin production in the heterotrophic culture of *C. zofingensis*. The production of astaxanthin was increased by the addition of 0.1 mM H_2O_2 due to the formation of hydroxyl radicals (Ip and Chen 2005). The H_2O_2 (0.1 mM) and MV (0.01 mM) was found to be the best ROS reagent in inducing carotenogenesis in *Chlorococcum* sp. where astaxanthin content increased almost 80% (Ma and Chen 2001). Similar results have been reported in *H. pluvialis*, the superoxide anion radical generated from MV is the most effective ROS reagent involved in astaxanthin accumulation (Kobayashi et al. 1993).

Currently, a green microalgae species of *Coelastrum* sp. has proved to be a potential producer of astaxanthin. Tharek et al. (2020a) identified that *Coelastrum* sp. as a viable strain with the capability in producing astaxanthin from a natural source under high light intensity and nitrogen starvation in mixotrophic culture. Besides that, studies on *Coelastrum* sp. HA-1 showed that nitrogen limitation in the culture medium of this species enhances the production of astaxanthin (Liu et al. 2013). Also, *Coelastrum* cf. *pseudomicroporum* culture in municipal wastewater and salinity stress can increase carotenoid production (Úbeda et al. 2017). However, further research is required to improve the astaxanthin content in this species for commercial astaxanthin production. Therefore, the present study aimed to enhance astaxanthin yield using reactive oxygen species (ROS) reagent by investigating the effect of oxidative stress generated by ROS reagent of methyl viologen towards growth and astaxanthin synthesis in tropical green microalgae strain isolated from the environment in Malaysia, *Coelastrum* sp. The selection strategy was focused on driving *Coelastrum* sp. into a high yield and cost-effective production of astaxanthin.

2. Materials and Methods

2.1. Cultivation of *Coelastrum* sp.

The green microalgae *Coelastrum* sp. isolated from a sampling site at Hulu Langat river, Kuala Selangor, Malaysia was cultured in AF-6 medium comprising NaNO_3 , NH_4NO_3 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Fe-citrate, Citric acid, KH_2PO_4 , K_2HPO_4 , trace metal solution ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) and a mixture of vitamins (Biotin, Pyridoxine, Thiamine) according to media recipe available in the Microbial Culture Collection National Institute for Environmental Studies (NIES-collection), Japan. The cultures were grown under normal conditions at 25 ± 1 °C with continuous aeration and enriched with 1% CO_2 . It was illuminated at a continuous light intensity with fluorescence light at normal photon flux densities (PFD) of $70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ until *Coelastrum* sp. cultures reach exponential growth phase for a period of 5 d. Cell growth was observed by measuring absorbance at 750 nm using a spectrophotometer.

2.2. Supplementation culture for stress induction

In order to induce the astaxanthin biosynthesis, the biomass of *Coelastrum* sp. was harvested and various supplements were added according to optimize conditions in accumulating astaxanthin in *Coelastrum* sp. with details described in our previous work (Tharek et al. 2020b). Sodium acetate, sodium chloride and sodium nitrate were used at a final concentration of 0.5 g/L, 3 g/L and 0.1 g/L, respectively. *Coelastrum* sp. then was subsequently exposed under continuous illumination of high photon flux densities (PFDs) of $250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. The cells were then subjected to extraction of astaxanthin and all the

experiments were carried out in triplicates.

2.3. Exposure of reactive oxygen species generating reagent

Methyl viologen (MV) is a reactive oxygen species reagent that can produce superoxide anion radical (O_2^-) (Rabinowitch et al. 1987). The tolerance of the cells of *Coelastrum* sp. towards MV was identified by the addition of 10% isolate to cultivation medium supplemented with various concentrations of MV (0–1.0 mM). To examine a possible effect of ROS reagent on astaxanthin synthesis, the ROS generating reagent of MV was added to *Coelastrum* sp. cultures grown under stress condition for superoxide anion radical (O_2^-). MV solution was filtered through a membrane filter of 0.25 μm pore size and different concentrations of MV solution (0–1.0 mM) were added to 5-day supplementation cultures of *Coelastrum* sp. The cultures were then incubated for 7 d at 25 ± 1 °C enriched with 1% CO_2 under continuous illumination ($250 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) for induction of astaxanthin production. All cultures were incubated in triplicates.

2.4. Determination of chlorophyll

Microalgae culture at the end of the exponential phase (5-day culture) was subjected for analysis. The 200 μL of microalgae culture was treated with 80% acetone. The mixture was vortexed for 30 s and centrifuged at maximum speed for 5 min. The absorbance of extracted chlorophyll was read at 663.6 nm and 646.6 nm. The concentration of chlorophyll a, b and total chlorophyll were calculated by the Lichtenthaler equation and expressed in mg/L content (Lichtenthaler 1987).

2.5. Determination of astaxanthin

To measure the astaxanthin content, a known volume of microalgae culture was taken and centrifuged at $2000 \times g$ for 10 min. The pellet was then lyophilized using a freeze dryer (Lyphlock 6; Labconco, USA). Then, the carotenoids were extracted using solvent extraction by homogenized the cells with acetone and kept in a water bath at 70 °C for 10 min followed by vortexing for few min-

utes. The mixture was centrifuged at $2000 \times g$ for 10 min and the supernatant was collected. Supernatant collections were conducted repeatedly until the cells were faded. The astaxanthin concentration was then measured by the spectrophotometric method and calculated with the equation, $c \text{ (mg/L)} = 4.5 \times A_{480} \times (V_a / V_b) \times f$. Where c is the astaxanthin concentration, V_a (mL) is the volume of solvent, V_b (mL) is the volume of algal sample, and f is the dilution ratio. The absorption peak of astaxanthin is at 480 nm and thus, A_{480} was determined by measuring the absorbance at 480 nm. Acetone was used as blank for the measurement.

2.6. Statistical analysis

The experiment was carried out with replication from three separate cultures. All values shown in the figures are expressed as mean SD. Student's t test was used to determine significant differences.

3. Results and Discussion

In general, there are two crucial roles of carotenoids in photosynthetic organisms. First, they act as light-harvesting pigments by trapping light energy and passing it to chlorophylls. Second, and more importantly, carotenoids can quench singlet oxygen (1O_2) by protecting the photosynthetic apparatus from unfavorable conditions (Young 1991). Shaish et al. (1993), have reported that massive amount of carotenoid accumulated in green algae cells are involved in triggering β -carotene biosynthesis to protect the photosynthetic cell against oxidative stress. Under unfavorable conditions, such as high light, salt stress or nutrient deprivation, the reactive oxygen species (ROS) was generated in the chloroplast when the photosynthetic process and CO_2 fixation were perturbed (Mittler 2002). The ROS will be produced whenever there is excessive reducing power in photosynthesis and will then be used as signal molecules to initiate the production and accumulation of many bioproducts (Asada 1994).

Only few studies have focused on the involvement of oxidative stress using ROS reagent in carotenoid synthesis (Lafarga et al. 2020). In the present study, the addition of

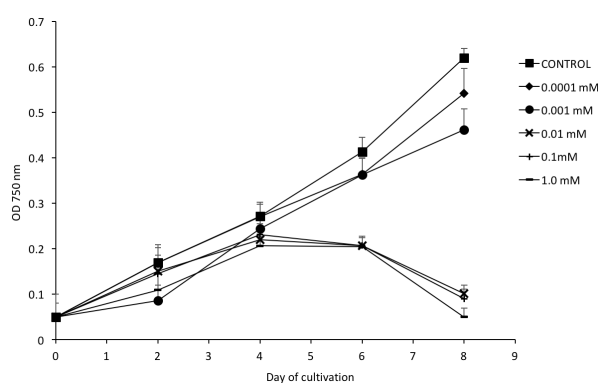


FIGURE 1 Tolerance of *Coelastrum* sp. cells towards Methyl Viologen (MV). Data represent an average of 3 replications and error bars indicate mean \pm SD.

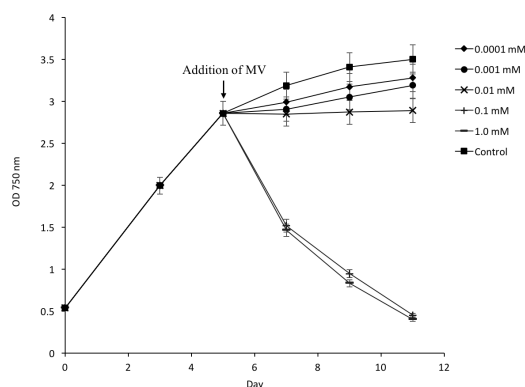


FIGURE 2 Effects of cell growth after incubation of *Coelastrum* sp. with Methyl viologen (MV). Data represent an average of 3 replications and error bars indicate mean \pm SD.

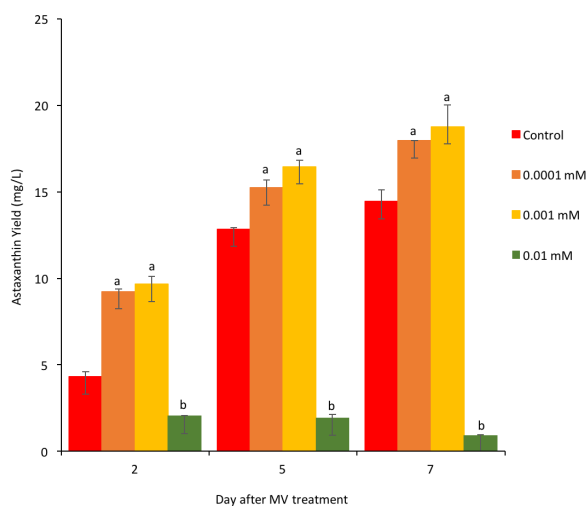


FIGURE 3 Astaxanthin production in *Coelastrum* sp. under different concentrations of Methyl viologen (MV). All data represent an average of 3 replications and error bars indicate mean \pm SD. Statistical analyses were conducted using student's t-test. Different small letter represents significant different among control and different treatments. Small letters a and b above the bar graph indicates significant increases and decreases respectively, between control and treatments groups ($P < 0.05$). *a for groups that higher than control; and b for groups that lower than control

reactive oxygen species (ROS) reagent, which was methyl viologen (MV), can rapidly auto oxidizes to produce superoxide anion radical (O_2^-). To investigate the tolerance of *Coelastrum* sp. cells towards ROS reagent, different concentrations of MV were tested by growing 10% inoculums of *Coelastrum* sp. in the presence of MV and shaken manually daily. Based on the results obtained, the growth of cultures was markedly decreased after the 4th day in 0.01 mM, 0.1 mM and 1.0 mM of MV, as shown in Figure 1. Besides, the microalgae cultures in these conditions turn to white depicting the death of cells. The growth of culture in 0.0001 and 0.001 mM of MV was shown to increase even after the 4th day of incubation, indicating the ability of the cells to survive in this range of MV concentration.

To investigate further the effect of superoxide anion radical (O_2^-) towards astaxanthin synthesis, ROS reagent (MV) was added to *Coelastrum* sp. culture during the exponential growth phase (5-day culture) where a rapid utilization of the substrate and cell division occurred at this stage. The same cell density was applied for all cultures supplemented with MV. The parameters included in the study to examine the effect of MV were the growth of *Coelastrum* sp., Chlorophyll content and astaxanthin content. Figure 2 shows the growth of *Coelastrum* sp. grown after incubated under different concentrations of MV enriched with 1% of CO_2 enrichment.

The growth of *Coelastrum* sp. without the addition of MV (Control) was observed to be higher compared to cultures supplemented with MV. In contrast, at higher concentration (0.1 mM and 1.0 mM), the growth of *Coelastrum* sp. were significantly decreased; therefore, the accumulation of astaxanthin was inhibited (Figure 3). The

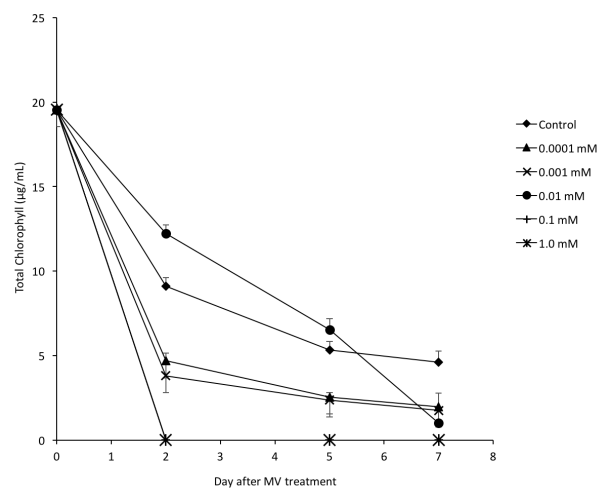


FIGURE 4 Effects of chlorophyll content after adding different concentrations of Methyl viologen (MV) to the 5-day culture of *Coelastrum* sp. Data represent an average of 3 replications and error bars indicate mean \pm SD.

result shown in Figure 3 showed that the astaxanthin content of *Coelastrum* sp. was significantly varied ($P < 0.05$) at different concentrations of MV. With an excessive addition of MV (1.0 mM and 0.1 mM), the chlorophyll content as depicted in Figure 4 was drastically reduced from 19.55 mg/L. This result is illustrated in Figure 5 after different concentration of Methyl Viologen (MV) were added to *Coelastrum* sp. during 5-day culture (exponential growth phase) by which the color of the cells turns to white even after the first day of incubation neither to the addition of MV at 1.0 mM nor 0.1 mM MV suggesting the death of the cells.

At the beginning of culture (day 0), the algal cells were in the green color relatively because of high chlorophyll content and low carotenoid content. With the addition of MV, the astaxanthin production proceeded markedly with a reduction of chlorophyll content, as shown in Figures 3 and 4. Superoxide anion radical generates by MV was found to be more effective for astaxanthin production at an extremely low concentration of 0.001 mM and 0.0001 mM. The results obtained in Figure 3 showed that MV at 0.001 mM increased astaxanthin content with 1.3 times higher than control after seventh day of incubation depicting the highest astaxanthin content. While the color of *Coelastrum* sp. changes to orangish color after 7 d of incubation under the lower concentration of MV as depicted in Figure 5 indicating the faster accumulation of carotenoids. However, the production of astaxanthin did not proceed at 0.01 mM MV and the astaxanthin content was found to decreased about 50% after 7 d of incubation, as shown in Figure 3 suggesting the low astaxanthin accumulation may be due to the free radicals being scavenged (Raman and Ravi 2011). At high concentration of 0.1 mM and 1.0 mM MV, the growth of microalgae was reduced and inhibited astaxanthin accumulation.

In corroboration of this findings showed that astaxanthin rich cells are more effective to the concentration that

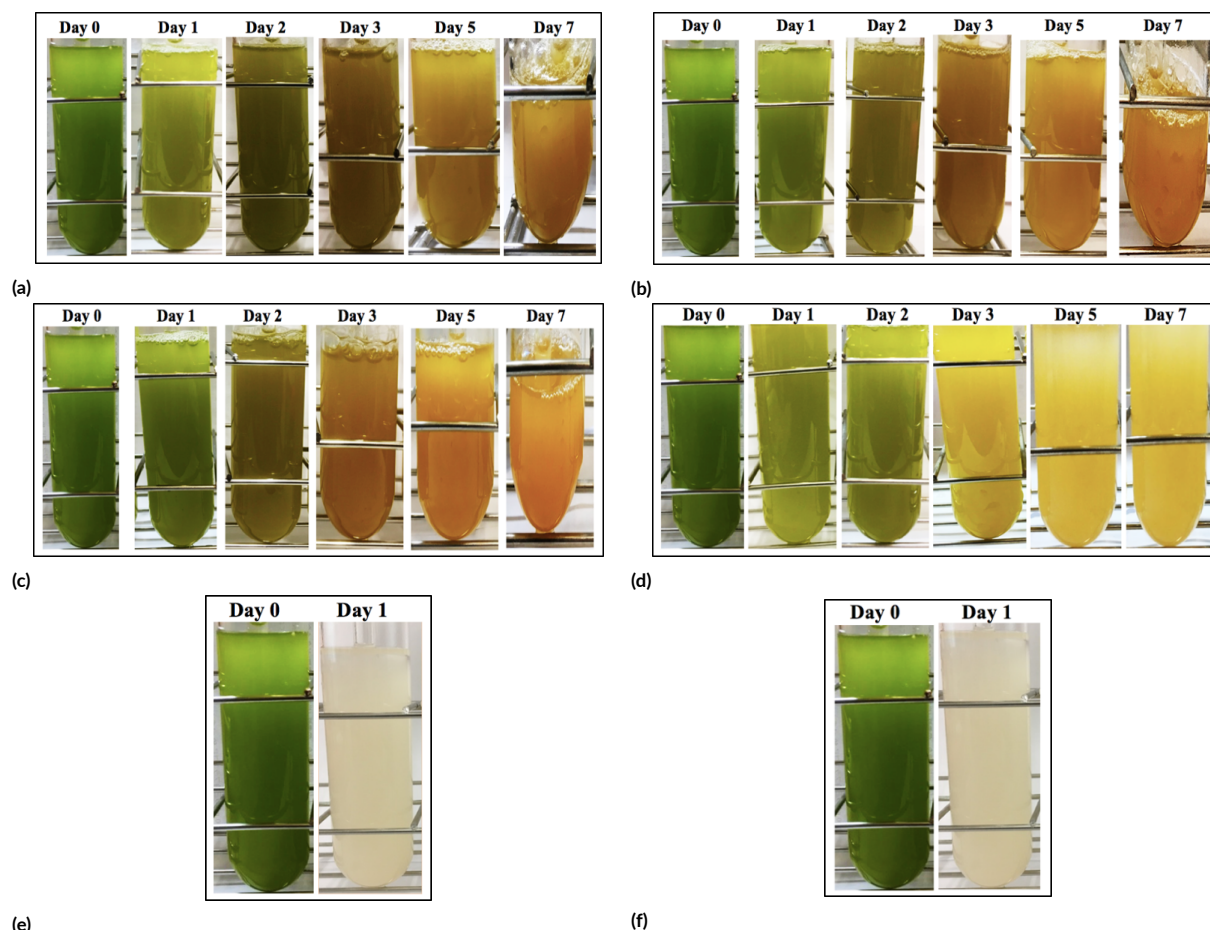


FIGURE 5 Illustration culture of *Coelastrum* sp. after different concentration of Methyl Viologen (MV) were added to *Coelastrum* sp. culture during their exponential growth phase (5-day culture); (a) Control; (b) 0.0001 mM MV; (c) 0.001 mM MV; (d) 0.01 mM MV; (e) 0.1 mM MV; (f) 1.0 mM MV.

can tolerate with the cells of microalgae. Methyl viologen, which generated superoxide anion radical (O_2^-), was capable to trigger the astaxanthin synthesis in *Coelastrum* sp. and effective at low concentration of MV. This radical might enhance carotenoid formation in microalgae cyst cells by participating directly in the carotenogenic enzyme reactions as an oxidizer (Kobayashi et al. 1993). Therefore, the accumulation of carotenoid acts as a protective agent against oxidative stress damage (Shaish et al. 1993). However, excessive addition of MV could cause massive cell death in the end and drastically reduced astaxanthin formation. Astaxanthin plays a vital role in protecting the algal cells against oxidative damage of reactive oxygen species. Consequently, the cell of microalgae has developed an efficient defense system to help it to survive under unfavorable condition.

4. Conclusions

To produce bio-products in an economically feasible way, the low productivity of microalgae needs to be addressed. Therefore, methyl viologen as reactive oxygen species (ROS) reagent has been applied as an enhancer to improve the accumulation of high yield of astaxanthin from *Coelas-*

trum sp. In this study, we concluded that the methyl viologen reacts as ROS reagent by generating superoxide anion radical at low concentration of MV (0.001 mM) and consequently lead to highest astaxanthin production with 1.3 times higher than control.

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Authors' contributions

AT carried out the experimental work, analysed the data and prepared the manuscript. All authors contributed intellectually to the presented work and critically revised the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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