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Evaluation of Active Oxygen Effect on Photosynthesis of *Chlorella vulgaris*

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The relationship between O_2 and an active oxygen scavenging system in *Chlorella vulgaris* var.*vulgaris* (IAM C-534) was investigated. When *Chlorella vulgaris* was exposed to 2% O_2, only traces of active oxygen scavenging enzymes were found. When the *Chlorella vulgaris* was treated with 20% or 50% O_2, it was shown that the level of enzyme activity increased as the O_2 concentration increased. An increase in enzyme activity was not found in any specific enzyme but in all of the enzymes, but the level of glutathione and ascorbate remained the same in all the cases. In addition, the photosynthetic efficiency also decreased as the concentration of O_2 was increased. These results suggest that an O_2 enriched environment can lead to an increase in the production of active oxygen species such as $O^{\bullet}_2$ and H_2O_2 and to a decrease in the photosynthetic efficiency in *Chlorella vulgaris*. The hydroxyl radical (•OH) was detected directly in the *Chlorella vulgaris* suspension with a spin trapping reagent. It was also clear that the increase in the •OH intensity as the visible light intensity increased was unrelated to the O_2 concentration. It was suggested that the conditions for producing •OH and the other active oxygen species were different, and that two types of oxygen stress should exist in the *Chlorella vulgaris*.

Keywords: Free radical, hydroxyl radical, antioxidative enzyme, visible light, microalgae, photosynthetic efficiency

INTRODUCTION

To achieve large-scale algal production for new energy and chemical sources, the productivity of CO_2 mitigation through microalgal photosynthesis needs to be improved. However, it was reported that O_2, a product of photosynthesis and a main accumulation product during cultivation, reaches concentration levels in the microalgal culture under severe light conditions that tend to be higher than that under air saturation conditions. In this high level of oxygen, it was thought that the formation of toxic oxygen species such as $O^{\bullet}_2$ and H_2O_2 is a potential threat to cellular components in the microalgal cultivation. Hence, we focused on the relationship between the effect on photosynthetic efficiency and active oxygen that is supposed to cause harmful effects on photosynthesis under both high light intensity and high oxygen levels. On the other hand, to prevent oxidative damage microalgal cells already are equipped with scav-

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enging systems such as low molecular weight antioxidants and antioxidative enzymes that remove active oxygens.

Antioxidative enzymes can be induced or activated in response to changes in specific environmental factors. It is expected that the levels of enzymes, associated with O_2 metabolism, would be regulated to accommodate elevated O_2 concentrations. The O_2-dependent induction of superoxide dismutase with a parallel increase in O_2 tolerance has been observed in blue-green algae[1,2] and green algae.[3,4]

In the present study, we have evaluated the effect of increased atmospheric O_2 concentration on photosynthetic efficiency and levels of certain enzyme activity, particularly antioxidative enzymes and antioxidative components, in microalgae *Chlorella vulgaris*.

MATERIALS AND METHODS

Reagents

5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Labotec Co. Ltd. (Japan). All other reagents were of the highest grade commercially available.

O_2 Evolution Rate

The O_2 evolution rate in the *Chlorella vulgaris* solution was measured using an O_2 electrode at 25°C.

Preparation of Algae

Chlorella vulgaris var. *vulgaris* (IAM C-534) was obtained from the culture collection of the Institute of Applied Microbiology, University of Tokyo.

The cultivation of the *Chlorella vulgaris* was done in an experimental vessel with 180 mm height, 150 mm width, and 50 mm depth. The culture was exposed to solar simulated light using a xenon lamp covered with a UV cut-off filter. The medium content was as follows; KNO_3, 5g; KH_2PO_4, 1.25g; K_2HPO_4, 0.1g; $MgSO_4 \cdot 7H_2O$, 2.5g; NaCl, 1.8g; $FeSO_4 \cdot 7H_2O$, 2.8mg; trace-metal mixture A_5, 1ml in 1L of deionized water. Trace-metal mixture A_5 contained the following components: H_3BO_3, 2.86g; $MnCl_2$, 1.81g; $ZnSO_4 \cdot 7H_2O$, 0.22g; $CuSO_4 \cdot 7H_2O$, 0.08g; Na_2MoO_4, 0.021g; concentrated H_2SO_4, 1 drop in 1L of deionized water. The algae were grown under controlled conditions (light/dark regimen 12/12 h at 25° C) with light intensities ranging from 100 to 800 mW/cm^2 corresponding to 10,000 lux to 80,000 lux. The algal cultures were continuously sparged with air containing 5% CO_2 at a flow rate of 75 ml/min at 25 °C for 1 week and then the cell concentrations remained constant for 3 or 4 weeks. The culture concentrations were kept at 800 mg/l by drawing off part of the culture. The concentrations of the *Chlorella vulgaris* were determined by OD_{750}.

Equipment

Hydroxyl radicals were analyzed using an ESR spectrometer, JEOL Model RE-3X, having an aqueous quartz flat cell (inner size 60mm × 10mm × 0.31 mm) with the spin trapping agent 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (Makino *et al.* 1990). The Mn^{2+} cation fixed in the ESR cavity was used as an internal standard to calculate relative amounts of ESR signal intensity. The *g* values of the peaks were 2.0334 and 1.9810 at the resonance frequency of 9450.0 MHz. The ESR conditions were as follows; microwave power: 6 mW, modulation frequency: 100 kHz, modulation amplitude: 0.1 mT, response time: 0.03 sec, gain: × 200 ~ 790 and sweep time: 2.5 mT/min.

Measurement of Enzymes

For the measurement of enzymes, the Chlorella cells were collected and then washed twice with 50 mM Na, K-phosphate buffer (pH 7.0) containing 1 mM EDTA and 2% sorbitol by centrifuging twice at 2000 × g for 10 min. The washed pellets

were resuspended in the washing solution. The slurry was disrupted with ultrasonication at 0 °C and centrifuged at 18,000 × g for 30 min. Part of the clear supernatant solution was used for assaying catalase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase. The remaining part of the supernatant was dialyzed against 20 mM Na, K-phosphate buffer (pH 7.8) at 4 °C and centrifuged at 18,000 × g for 30 min. The supernatant was used for assaying guaiacol peroxidase, pyrogallol peroxidase and superoxide dismutase (SOD).

The activity of ascorbate peroxidase (AsA por) was measured according to Nakano and Asada[5] by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM • cm^{-1}). The reaction mixture contained 50mM Na, K-phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM H_2O_2, 0.5 mM ascorbate (AsA), and the enzyme aliquot at 25 °C. No change in absorption was observed in the absence of AsA in the test tube.

The guaiacol peroxidase activity was assayed according to the method of Nakano and Asada[5] by following the increase in absorbance at 470 nm due to guaiacol oxidation (ε = 26.6 mM • cm^{-1}). The reaction mixture contained 50 mM Na, K-phosphate buffer (pH 7.0), 10 mM guaiacol, 0.1 mM H_2O_2 and the enzyme aliquot.

For the measurement of the pyrogallol peroxidase activity, the reaction mixture contained 50 mM Na, K-phosphate buffer (pH 7.0), 18 mM pyrogallol, 0.1 mM H_2O_2 and the enzyme aliquot. The activity was determined from the decrease in absorbance at 430 nm due to purpurogallin.

The superoxide dismutase (SOD) activity was measured according to McCord and Fridovich[6] with a slight modification based on the inhibition of the reduction of Cyt C with $O^{\bullet}_2^-$, which was generated by the xanthine-xanthine oxidase system. The assay mixture for SOD contained 50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 0.1 mM xanthine, 0.27 mM cytochrome c, an enzyme aliquot as 1 ~ 2 units of SOD, and 2.9 × 10^{-3} units of xanthine oxidase in a final volume of 1 ml. One unit of SOD is defined as the amount of sample that inhibits the rate of control with $O^{\bullet}_2^-$ by 50%.

Dehydroascorbate reductase (DAsA Red) was assayed according to the method of Nakano and Asada by following the decrease in absorbance at 265 nm due to AsA formation (ε = 14 mM • cm^{-1}).[5] The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 2.5 mM reduced glutathione (GSH), 0.1 mM EDTA, and the enzyme aliquot at 25°C. The reaction rate was corrected for non-enzymatic reduction of dehydroascorbate by GSH.

Monodehydroascorbate reductase was assayed spectrophotometrically by following the decrease in absorbance at 340 nm due to NADH oxidation (ε = 6.2 mM • cm^{-1}).[7]

The assay of glutathione reductase was carried out by measuring the NADPH oxidation at 340 nm (ε = 6.2 mM • cm^{-1}).[8] The reaction mixture contained 40 mM Tricine-NaOH (pH 7.8), 0.1 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and the enzyme aliquot at 25°C.

The catalase activity was measured in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), and enzyme aliquot at 25°C.[9] The reaction was started by adding 8.8 mM H_2O_2 and the activity was determined from the decrease in absorbance at 240 nm (ε = 39.4 mM • cm^{-1}) due to H_2O_2 decomposition.

Determination of Glutathione and Ascorbate

For the measurement of glutathione and ascorbate, the Chlorella cells were collected and then washed twice with 50 mM Na, K-phosphate buffer (pH 7.0) containing 1 mM EDTA and 2% sorbitol by centrifuging twice at 2000 × g for 10 min. The washed pellets were resuspended in the washing solution. The slurry was homogenized with ultrasonication at 0°C and centrifuged at 18,000 × g for 30 min. Part of the clear supernatant solution was used for assaying glutathione and ascorbate. Glutathione was determined using glutathione reductase by the method of Law,[10] and

ascorbate was determined by the color development with Fe^{3+} and α, α'-dipyridyl according to Okamura.[11]

Protein Concentration

The concentration of protein was measured by the method of Bradford,[12] with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Photosynthetic Efficiency of Chlorella Vulgaris

The effect of light intensity on the photosynthetic efficiency and the photosynthetic oxygen evolution rate of the *Chlorella vulgaris* was investigated. Figure 1 shows that the photosynthetic efficiency of the *Chlorella vulgaris* decreased as the intensity of irradiated light increased; whereas, the photosynthetic oxygen evolution rate increased until 60 mW/cm^2 and then it remained constant. These data indicate that the light saturation phenomenon in the *Chlorella vulgaris* started at severe light conditions greater than 60 mW/cm^2 or about 60,000 lux.

In addition, the effect of oxygen on the photosynthetic efficiency of the *Chlorella vulgaris* was studied under severe light conditions. The photosynthetic efficiency of the *Chlorella vulgaris*, which were pre-cultivated in 20 or 50% O_2 for 7 days, was measured under the oxygen conditions such as 2 or 20 or 50% O_2 respectively. Figure 2 shows the results of the oxygen effect on the photosynthetic efficiency of the *Chlorella vulgaris*. It is clear that the *Chlorella vulgaris* that were pre-cultivated under the higher oxygen condition, indicate a higher photosynthetic efficiency in the lower oxygen condition. From these results, it was expected that pre-cultivation under higher oxygen conditions was effective in producing a high yield of biomass of *Chlorella vulgaris*.

Relationship Between Photosynthetic Efficiency and Active Oxygen Scavenging Enzymes and Antioxidative Compounds

The levels of glutathione and ascorbate did not change (data not shown), but the activity of

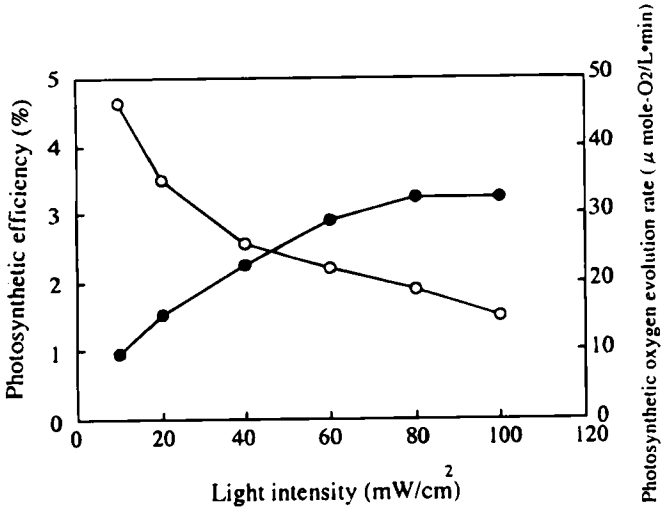

FIGURE 1 Effect of light intensity on the photosynthetic efficiency of *Chlorella vulgaris*. The data were measured under the following conditions, Temperature, 25°C; pH,7; Algal concentration, 800mg/L; Gas condition, 5% CO_2 + 20%O_2. ●, photosynthetic oxygen evolution rate; ○, photosynthetic efficiency.

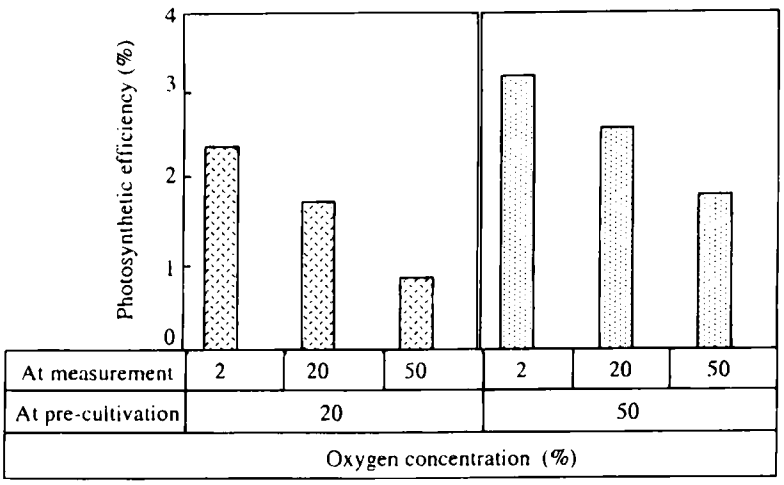

FIGURE 2 Effect of oxygen on the photosynthetic efficiency of the *Chlorella vulgaris*. The data were measured under the following conditions, Temperature, 25°C; pH,7; Algal concentration, 800mg/L; Gas condition, CO_2 5% + 2% or 20% or 50% O_2.

active oxygen scavenging enzymes (antioxidative enzymes) in the *Chlorella vulgaris* responded to changing oxygen concentrations; therefore, the activities of active oxygen scavenging enzymes were adopted as a guideline for oxygen stress analysis. The *Chlorella vulgaris* were grown under various controlled oxygen conditions with 5% CO_2. The effects of the O_2 concentration on the antioxidative enzyme activities and the photosynthetic efficiency are shown in Figure 3. When the *Chlorella vulgaris* was exposed to 2% O_2, the activity of the active oxygen scavenging enzyme was barely evident, and the photosynthetic efficiency achieved 2.5% of the maximum value, which corresponded to a productivity of 30 $g/m^2 \cdot d$. When the *Chlorella vulgaris* was treated with the 20% O_2, the level of enzyme activity increased 4-fold compared to the 2% O_2 level. The increase in the enzyme activity was not found in a specific enzyme but was found in all of the enzymes. When the *Chlorella vulgaris* was exposed to 50% O_2, the level of enzyme activity increased 6-fold compared to the 2% O_2 level. The increase in the enzyme activity was also not found in any specific enzyme but was found in all of the enzymes. As the activity of antioxidative enzyme using glutathione and ascorbate as substrate also increased parallel to oxygen concentration, it was thought that the levels of glutathione and ascorbate under various O_2 conditions was constant. Specifically, the level of activity of active oxygen scavenging enzymes was not detectable when *Chlorella vulgaris* was exposed to 2% O_2, which indirectively means that active oxygen must not be present in *Chlorella vulgaris* under the condition of 2% O_2 at 5% CO_2. On the contrary, it was thought that active oxygens are easily produced from the intracellular of *Chlorella vulgaris* at higher O_2 conditions from these data.

When the *Chlorella vulgaris* cells were grown in 20 or 50% O_2, the photosynthetic efficiency was lower. As mentioned above, the level of the activity of the active oxygen scavenging enzyme was not detectable when *Chlorella vulgaris* was exposed to 2% O_2, it was estimated that the decrease in the photosynthetic efficiency in the 20 or 50% O_2 was caused by active oxygens in the *Chlorella vulgaris* cells.

On the other hand, as mentioned above, the *Chlorella vulgaris* achieved a high photosynthetic efficiency at lower oxygen conditions after precultivation in higher oxygen conditions. From these results, it was also thought that the high photosynthetic efficiency of the *Chlorella vulgaris*

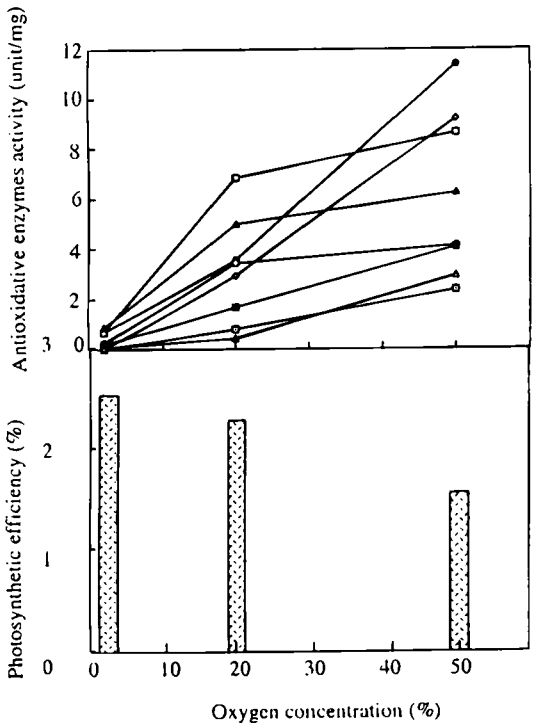

FIGURE 3 The relationship between the photosynthetic efficiency and enzyme activity of scavenging active oxygen of *Chlorella vulgaris*.
The data of activity of scavenging active oxygen were expressed using the same order value. ●, ascorbate peroxidase (×10); ○, superoxide dismutase (× 10^{-1}); □, catalase; ■, glutathione reductase (×10); △, monodehydroascorbate reductase (×10); ▲, dehydroascorbate reductase (×10); ⊡, guaiacol peroxidase (× 10^4); ◇, pyrogallol peroxidase (× 10^2).

was due to obtaining the anti-oxidative mechanisms such as active oxygen scavenging enzymes. Hence, it was also expected that the addition of anti-oxidative reagents, for instance paraquat, might lead to a high photosynthetic efficiency or a high yield of biomass of *Chlorella vulgaris*.

Detection of Active Oxygen with the Spin Trapping Method

To directly examine the effects of active oxygen on *Chlorella vulgaris*, the detection of active oxygen was attempted with a spin trapping reagent. As a result of this test, the DMPO-OH adduct, which is assigned as the product of the hydroxyl radical (DMPO-OH; aN = 1.49 mT, aH = 1.49 mT) was observed (Fig. 4[13]), and its signal height increased as the visible light intensity in the *Chlorella vulgaris* suspension increased.[14] Since the inhibition of the DMPO-OH signal from hydroxyl radical scavengers was detected, it was clear that the DMPO-OH adduct in the *Chlorella vulgaris* suspension was the consequence of hydroxyl radicals that were free from artifacts.[15] In order to investigate in detail the condition of the •OH production in the *Chlorella vulgaris* suspension, the •OH intensity was measured at various oxygen concentrations with 5% CO_2 and compared with an air atmosphere that was 0.035% CO_2 with 20% O_2 before the ESR measurement. Figure 5 compares the •OH intensity when the ESR measuring conditions were constant and all sample turbidities were adjusted to OD_{750} equals 6.1 under some of the conditions. It is clear that •OH is produced more easily in 0.035% CO_2 with 20% O_2 than under 5% CO_2 conditions with 2. 20 or 50% O_2, and •OH in the 5% CO_2 at various O_2 conditions was rarely detected. In addition, •OH was detected more readily in the shorter bubbling time of 5% CO_2. Hence, •OH production appears not to be related to the higher O_2 concentration at sufficient CO_2 level of 5%, but to be related to just insufficient levels of CO_2 at severe light conditions.

In contrast, as previously mentioned, it was clear that the antioxidative enzyme activities

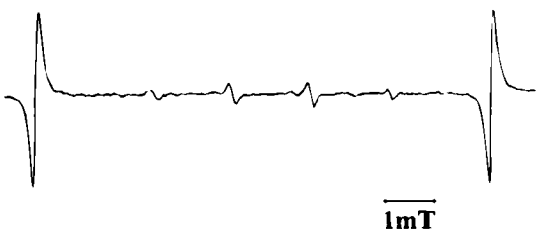

FIGURE 4 Detection of hydroxyl radical by ESR in the *Chlorella vulgaris* suspension with the light intensity at 80,000lux.

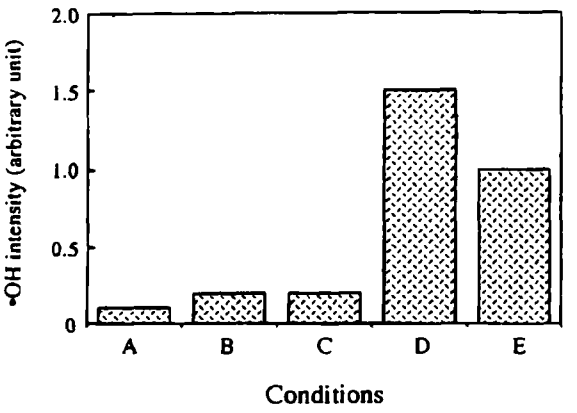

FIGURE 5 Comparison of the •OH intensity under various conditions of *Chlorella vulgaris*. The ESR measuring conditions were constant and all of the sample turbidities were adjusted to OD_{750} equal to 6.1 under the various conditions. To determine the production factor of the •OH, the ESR measuring samples were changed to various CO_2 and O_2 levels conditions. The samples were continuously sparged with the various levels of CO_2 and O_2 for more than 20 min (except in (E)) until just before the ESR measurement and then a light level of 80,000 lux was irradiated into the ESR cell during the ESR measurement. A, 2% O_2 + 5% CO_2; B, 20% O_2 + 5% CO_2; C, 50% O_2 + 5%CO_2; D, Air(20% O_2 + 0.035% CO_2); E, shorter bubbling time (2min) of 20% O_2 + 5% CO_2.

increased with increasing O_2 concentration at a CO_2 level of 5%. The behavior of the antioxidative enzyme activity appears to be related to intracellular active oxygen species such as O_2 and H_2O_2 and not related to •OH. Based on these points, we theorized that there are two types of oxygen stress in the *Chlorella vulgaris* cultivation. One type of oxygen stress is considered a higher O_2 stress in a sufficient level of CO_2, which is protected by active oxygen scavenging enzymes without producing the •OH. The other type of stress is the light intensity but just at insufficient levels of CO_2, and under these conditions it is possible to produce •OH. •OH is known as an extremely reactive oxygen species.[16] We also reported that superoxide dismutase is inactivated by •OH using a model system.[17] Therefore, the detection of •OH in the *Chlorella vulgaris* suspension suggests that some damage might be produced by free hydroxyl radicals especially at insufficient CO_2 levels under severe light conditions.

As mentioned above, it seems that high photosynthetic efficiency of the *Chlorella vulgaris* is due to producing anti-oxidative species such as active oxygen scavenging enzymes. Hence, the enhancement of anti-oxidative mechanisms by various reagents, or the supply of sufficient CO_2 for suppression of the •OH is considered to be important for obtaining better microalgal biomass for new energy and chemical sources obtained of under severe light conditions in actual cultivation of microalgae.

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