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Effects of SO_2 and NO_2 in Flue Gas on CO_2 Sequestration and Intracellular Microstructures Analysis of *Chlorella* sp.

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Research Article

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ABSTRACT

This study investigated the effects of CO_2, NO_2 and SO_2 on the growth of algae strain (*C. vulgaris*) and intracellular energetic substance accumulation. The algae can grow well with high concentration of CO_2-air mixture gases (10%, 15% (v/v)) and reached the maximal biomass when growing with 15% CO_2-air mixture gases by a week, but lipid appear easier to be accumulated in the algae cultivated with 5% CO_2-air mixture gases due to the increase of pH in the medium. NO_2 and SO_2 with moderate amount (NO_2: 4.4 mg/d; SO_2: 0.159 mg/d) have no obvious effect of inhibition on the algae growing but induce the energetic substance accumulation in alga cells, such as carbohydrate and lipid, which were accordant with the results of microstructure observations as well as the Rubisco activities analysis. Consequently, NO_2 and SO_2 with moderate amount sometimes can beneficial for energetic microalgae production with high concentration of CO_2.

INTRODUCTION

Flue gases in the atmosphere resulting from the combustion of fossil fuels contain NO_2 and SO_2 and CO_2 components, among of them CO_2 is a valuable resource for bio-utilization of microalgae. Biological fixation and storage of CO_2 via microalgae are essentially photosynthesis, which convert water and CO_2 into organic compounds without additional energy consumption and secondary pollution. In addition, microalgae CO_2 fixation has several advantages, such as high photosynthesis rate [1], easy controlled working environment to cultivate microalgae in photo bioreactor [2,3], CO_2 fixation by incorporated into carbohydrates and lipids renewable energy substances which resulting no CO_2 net emission to atmosphere layer [4,5]. However, direct utilization of flue gas for large-scale algal cultivation could cause obvious inhibition of algal growth because of some quantities of NO_2 and SO_2 in flue gases [6-8]. High CO_2 fixation ability of microalgae from the environment is related to the activity of the enzyme ribulose-1,5-biosphosphate carboxylase oxygenase (Rubisco) which fixes CO_2 in the Calvin cycle, converting it into organic carbon. When Rubisco is fully saturated with CO_2, the photosynthetic efficiency reaches the maximum. However, actual photosynthesis efficiency is much lower than that in ideal condition. At moderate CO_2 levels, Rubisco can function at only 25% of its catalytic capacity because the concentration of substrate CO_2 is lower than that of highest photosynthesis. The majority of dissolved inorganic carbon levels in alga are dissolved CO_2 and HCO_3^- for photosynthesis, but CO_2 makes up only a small fraction of available inorganic carbon. Carbon anhydrase (CA) plays a vital role in catalyzing the inter-conversion of HCO_3^- and CO_2 in order to maintain the normal photosynthetic activity [9-13]. Besides, high concentration of O_2 competes with CO_2 as a substrate for Rubisco [12]. In this paper, we will discuss the effect of components of flue gas, such as high concentration of CO_2, moderate quantities of NO_2, and SO_2 on the growth of algae and energetic substance accumulation. In more details, activities of Rubisco associated with CO_2 bio-fixation by *Chlorella* sp. and the intracellular microstructures of algae cultivated with moderate SO_2 and NO_2 were investigated. Through the analysis of experimental results, the relative suitable conditions for cultivation will be discussed.

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MATERIALS AND METHODS

Strain and Culture Medium

The alga *C. vulgaris* (FACHB-1227) seed suspension was purchased from Chinese Academy of Sciences Hydrobiology Institute (Wu Han, China) and later was maintained by transferring the seed suspension to BG11 medium. The BG11 medium had the following compositions (in mg/l): $NaNO_3$ 1500; K_2HPO_4 40; $MgSO_4 \cdot 7H_2O$ 75; $CaCl_2 \cdot 2H_2O$ 36; Citric acid 36; Ammonium ferric citrate 4; $EDTANa_2$ 1; Na_2CO_3 20; and 1 ml A5 solution (in mg/l) H_3BO_3 2860, $MnCl_2 \cdot 4H_2O$ 1810, $ZnSO_4 \cdot 7H_2O$ 222, $CuSO_4 \cdot 5H_2O$ 79, $Na_2MoO_4 \cdot 2H_2O$ 21, $Co(NO_3)_2 \cdot 6H_2O$ 500.

Composition Analysis

The dried biomass was analyzed for lipid, carbohydrate, protein. Lipid was determined by colorimetric sulfo-phospho-vanillin (SPV) method [14]. While phenol-sulfuric method was used for total carbohydrate analysis [15]. Lowry method combined with the method described by Pruvost et al. was used for protein determination [16,17].

Total lipid Analysis

The total lipid content was determined by the colorimetric SPV method with some modifications [14]. The standard lipid stocks were prepared by dissolving 10 mg corn oil (Sigma-Aldrich) in 10 ml chloroform. Different amounts of standard stocks containing 50-500 µg lipids were added to the bottom of 2 ml tube. The solvent was dried under a nitrogen flow to avoid oxidation. Subsequently, 100 µl deionized water was added to the 2 ml tube mixed with a known amount of algal biomass and some amounts of standard stocks. 1.5 ml of concentrated sulfuric acid was added and mixed well by vortexing, then the mixture were heated for 10 min at 100°C cooled in ice bath for 5 min and mixed again. 100 µl of the mixture was pipetted into the bottom of microplate wells. Background absorbance at 530 nm is measured. 100 µl of vanillin-phosphoric acid reagent (1.2 mg vanillin per ml 68% phosphoric acid) was added to each well for color development for 20 min and the absorbance was measured at 530 nm.

Total Carbohydrate Analysis

The known amount of algae biomass was used for carbohydrate measurement by phenol-sulfuric acid method [15]. 1.8 ml 1 M H_2SO_4 was added to the algae sample, mixed well by vortexing, heated in a water bath at 95°C for 2 h, cooled to room temperature and centrifuged at 14000 rpm for 10 min. After that, 0.4 ml of the supernatant or standard glucose solution was added to 0.2 ml of 5% phenol solution in a 2 ml tube. Subsequently, 1 ml of concentrated sulfuric acid was added rapidly and mixed well by vortexing immediately. After cooling the tube for 30 min at room temperature for color development, the absorbance was measured at 490 nm.

Total Protein Analysis

The protein solution of alga sample was prepared as described by Pruvost et al. [17], with some modification. 1 ml 1 N NaOH was added to the sample, mixed well by vortexing, and heated in a water bath at 95°C for 10 min. After alkaline lyses with NaOH, the mixture was neutralized by 0.5 ml 1.6 N HCl. Then the sample was cooled to room temperature and centrifuged at 14000 rpm for 10 min. The supernatant was used for protein measurement by the Lowry method [16].

Analysis of Ribulose-1,5-biosphosphate carboxylase oxygenase (Rubisco) in Algae Cell

Extraction of rubisco from algal sample: The same amounts of cell samples collected from different cultivated conditions were prepared by centrifuging the same concentrations and volumes of fresh algal cultures at 5000rpm for 5 min. After pouring the supernatant out, the wet samples were added 1 ml pre-cooled crude enzyme extraction solutions (50 mM Tris-HCl, pH 8.0, 1 ml M EDTA, 50 mM $NaHCO_3$, 2 mM DTT, 10 mM $MgCl_2$) and were disrupted under 160 W ultrasonic for 3 min respectively. Then, the supernatants were collected for subsequent determination of Rubisco activities after the disrupted cell suspensions were centrifuged again at 3000 g for 5 min [18].

Determination of Rubisco activities: Rubisco activities were determined by microplate absorbance reader associated with Rubisco test kit. Rubisco test kit was purchased from Shanghai based industry Co., Ltd. The experiment is based on the double antibody sandwich method for analyzing the levels of Rubisco. Pure Rubisco antibody is fixed on 48 microplate to prepare insolubilized antibody, after that the standard Rubisco solutions and sample solution were added accordingly into the 48 microplate for 30 minutes at 37°C to produce antibody-antigen complex. After washing 5 times by washing solutions, the HRP (Horseradish peroxidase) labeled Rubisco antibody solutions were added to form antibody-antigen-labeled enzyme complex, which were also washed 5 times by washing solutions before reacting 30 minutes at 37°C before adding substrate tetramethyl benzidine (TMB) to develop blue and subsequently develop yellow in acidic conditions. At the end, stop buffer were added to inactivate the relative enzymatic activities. After that, the optical density (OD) values were read in 15 minutes by microplae absorbance reader. Therefore, Rubisco activities were quantified by calculating the equation associated with absorbance at 450 nm.

Statistical Analysis

The ANOVA analysis and multiple comparisons on the experimental data were applied. The significance of individual factors

including NO_2, SO_2 and CO_2 and their interactions on lipid, carbohydrate, protein as well as Rubisco activities, was identified using ANOVA analysis.

Microstructure Analysis

Sample preparation for SEM observation: The harvested fresh algal cells were centrifuged and washed by distilled water for three times to ensure the cells clean. Then, the sufficient amounts of samples (about 1 cm high deposit in 2 ml tube) were gradually dehydrated with 15, 50 and the 100% (v/v) ethanol using centrifugation (3 min each time). The ethanol gradient used for dehydration enable us to significantly shorten the processing time. Part of the supernatant was removed, and the remainder was used as a protective layer. Dehydrated cells in the residual 100% ethanol were oscillated for 1 min use a vortex. The samples were quickly dispersed onto pieces of aluminum foils. In order to evaporate the ethanol quickly, the cell-containing foil pieces were dried at 90°C in a drying oven for less than 10 min. However, drying samples for too long would have disrupted the attachment of algal cells to the foils; the dried pieces of cell-containing aluminum foil were cut into circle-shaped samples with diameters of 2 cm, which are then mounted on microscopy stubs using carbon sticky tape. The cell morphologies were imaged using a Hitachi (Tokyo, Japan) S-4800 field emission scanning electron voltage of 0.8-1.0 kV and current of 10.0 μA.

Sample preparation for TEM observation: The samples were harvested and prepared as above methods. Then, the cell deposits in 2 ml tube were washed by phosphate buffer solution (0.1 mol/L) for three times, the obtained samples was fixed in 0.1 mol/L OsO_4^- phosphate buffer solution for 3 hours at 4°C, and were dehydrated gradually by ethanol with 10% increasing concentration. Then, the sample were embedded in epoxide resin and cut into slices by ultramicrotome (LKB-NOVA). The slices were dyed by lead citrate for observation under transmission electron microscope (HITACHIH-7000).

Alga cultivation in photo-bioreactor: The algae cell was incubated in 250 ml flask containing 200 ml modified BG11 medium. The flasks were placed on an orbital shaker at 25 ± 1°C. The light intensity was approximate 72 μmol/m^2/s at the surface of the flask under continuous cool white fluorescent light. Filtered ambient air mixed with CO_2 by a self-made device **(Figure 1)** to give different concentrations of CO_2 in mixed flow (v/v). SO_2 are produced in the flask by injecting concentrated sulfuric acid solution into Na_2SO_3 powder **(Figure 2a).** The aeration time of SO_2 just began at the outlet gas of flask made Fuchsine solution faded. The flow velocity (bubbles/min) of SO_2 at the outlet of gas generator was monitored by adjusting the injection speed of concentrated SO_2 solution to Na_2SO_3 powder. NO_2 was prepared in the suction flask by dropping concentrated HNO_3 solution to Cu powder **(Figure 2b).** The aerated time was recorded when the color of gas turned brown at outlet of NO_2 generator and made the pH test paper turned red. Culture solutions in the flask were aerated with the CO_2-air mixture gas continuously at a certain gas flow velocity (0.25 vvm). Besides, SO_2 and NO_2 gases produced from generators were transferred into algal solution through silica gel at certain flow velocities and the residual exhaust gases were absorbed by NaOH solution when required. Moderate alga solution was sampled daily from the flask to determine the algal growth, which was monitored by cell counting. The pH of culture was directly determined by using pH meter (PHS-3C). At the end of cultivation, the algal samples were collected for biochemical analysis and microstructure.

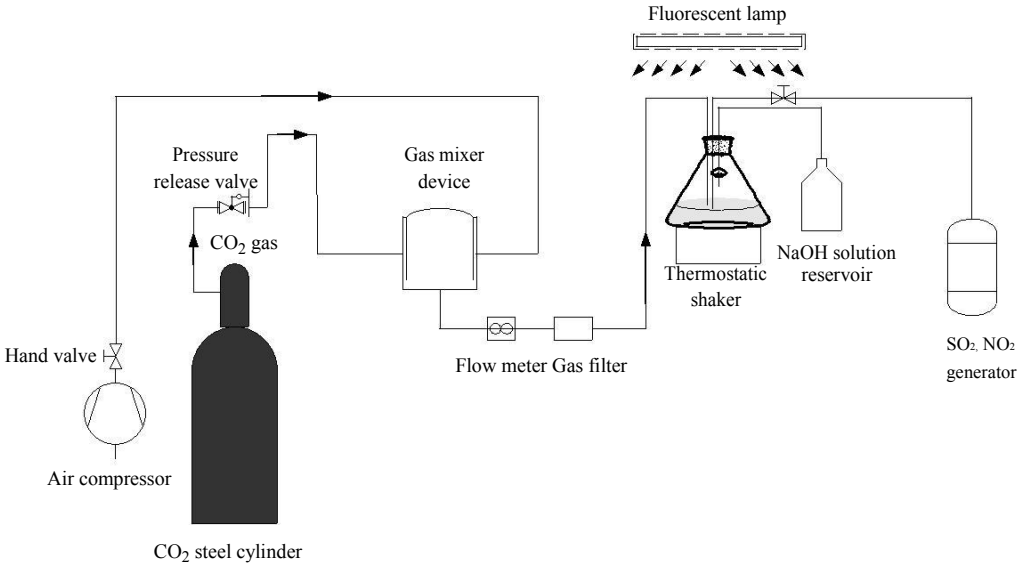

Figure 1. Schematic diagram of microalgal cultivation experiments with high concentration of CO_2 and moderate NO_2 and SO_2. The mixture gases with different CO_2 concentrations were adjusted by gas mixer device. External illumination of light intensity was provided with 72 μmol/m^2/s by continuous, cool white fluorescent light.

Quantities and relative calculation of SO_2 and NO_2 gases in the form of bubbles

The SO_2 and NO_2 can be produced by self-installation devices **(Figure 2).** NO_2 generator was designed as **Figure 2b** and the chemical reaction equation: Cu HNO Cu NO NO H O 3 3 2 2 2 • 4 •( ) •2 ••2

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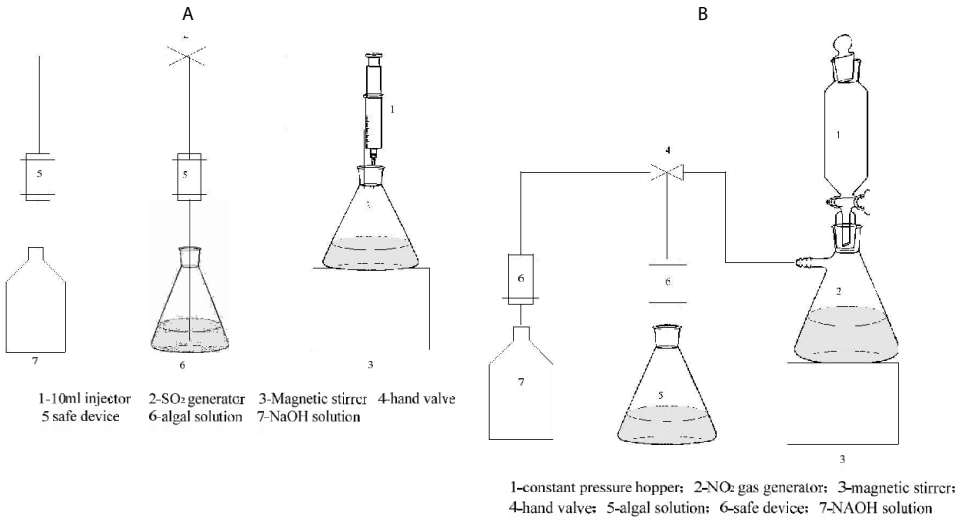

Figure 2. The schematic diagram of SO_2 (a) and NO_2 (b) generator. The amounts of SO_2 and NO_2 gases generated from reactors were controlled by hand valves for one bubble per second.

The experiment has the high sensitivity to produce pure NO_2 and the beginning of reaction can be easily judged by the changes of brown color of gas. SO_2 generator was designed as **Figure 2a** and the chemical reaction equation: Na SO H SO Na SO SO H O $_{23242422}$ • 4 •( ) •2 ••2

The amounts of SO_2 and NO_2 were calculated by the numbers of bubble produced by the NO_2 and SO_2 dissolved in culture solutions. The rates of gases were controlled as one bubble per second. In order to know about the exact mass of a bubble, the volume of a bubble should be measured according to the following steps. First, 5 ml air was sucked by an injection exactly and the needle was immerged into the water before pushing piston of the injection carefully. Second, the bubbles formed in the water were counted until the air in the injection was drained completely. Third, the above process was performed in triplicates for average volume of a bubble. It was measured that the volume of one bubble is approximate 8.427 × 10^{-2} ml. Assuming atmosphere (101.325 × 10^3 Pa) and the temperature (298 K) and according to the ideal gas equation, the mass per bubble of SO_2 was about 0.22 mg and 0.159 mg for NO_2.

RESULTS AND DISCUSSION

The Cultivation of Alga with Different Concentration of CO_2-air Mixture Gases (v/v)

It can be seen from **Figure 3a**, the growth of alga with 5% (v/v) CO_2-air mixture gases has no adaptation at the initial cell density (4 × 10^6/ml) and quickly attained to the steady-growing state, which means the 5% CO_2-air mixture gases has little restrained effect on the growth of algae. However, the algae have the three day's adaptation periods before entering into the fast-growing state in the other two growth conditions (10% and 15%). Finally, the cell densities in these three cultivated conditions reached 11.8 (10^6/ml), 26.72 (10^6/ml), 28.25 (10^6/ml) within a week respectively. The pH changes in the three cultivated conditions were observed in **Figure 3b.** The pH values in the culture with 5% CO_2-air mixture gas has always increased in the whole growth process. Especially, there was a jumped increase happened on pH after three days, which reflects the supply of CO_2 was not sufficient for the fast growth of algae which result in the decease of dissolution of CO_2 in the culture. However, there were obvious drops in pH value happened on the cultures with 10% and 15% CO_2-air mixture gases at the first two days, before bouncing to above pH 7.0. The pH changes were conformed to growing tendency of the algae, which indicated that both of the initial growth was restrained by the high concentration of CO_2 mixture gases at the beginning and results to abundant CO_2 was dissolved in the culture to form ion H^+. When the algae overcame the restrained effect and start to strong growth, the dissolved CO_2 was assimilated by algae and caused the obvious increase of pH in the medium consequently.

The Cultivation of Alga with CO_2-air, CO_2-air-SO_2 , CO_2-air-NO_2 Mixed Gases (v/v)

In order to investigate the effects of quantities of trace gases on the growth of algae, exact quantity of SO_2 and NO_2 were controlled by bubble injection experiments (section 2.6). NO_2 and SO_2 were prepared by injection of 20 bubbles (20 × 0.22 mg=4.4 mg) and 1 bubble (1 × 0.159 mg=0.159 mg) once a day (excessive SO_2 and NO_2 have caused the death of algae in pre-experiment), with the rate of a bubble per second in the continuous cultivation with 10% CO_2-air mixture gases. As we can see from **Figure 4a**, the algae growing with 10% CO_2-air-NO2 and 10% CO_2-air-SO_2 mixture gases has more obvious growth inhibition compared to 10% CO_2-air at the first five days. The periods of exponential growth phase in the first two mixture gases were longer until the ninth day. However, which of that in latter mixture gas was finished in a week. Besides, the pHs also had a little fall in fourth and fifth days before going up to the maximum **(Figure 4b).** The concentration of NO_3-had a quick rebound when cultivated with moderate NO_2 gas at the fifth day. It would be explained that the NO_2 was dissolved in the medium and participate the chemical reaction to produce some NO_3-in the aqueous liquid. 3NO • H O • 2NO •• NO • 2H • $_{223}$.

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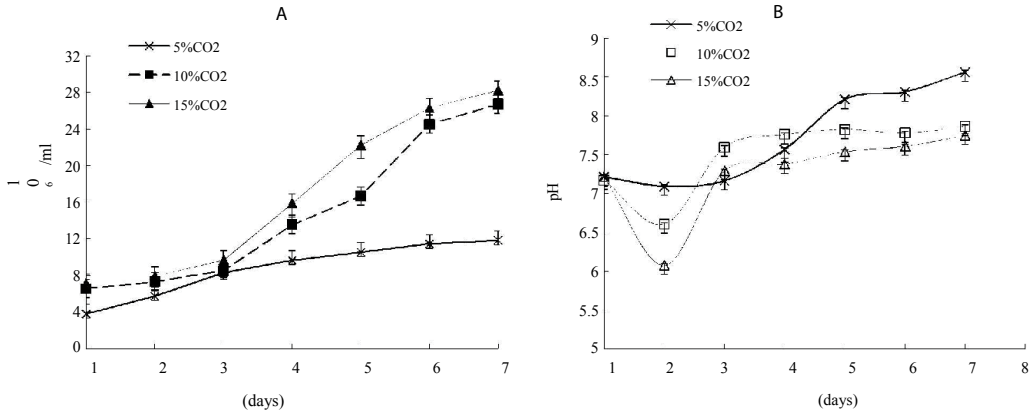

Figure 3. The growth of algae with different concentration of CO_2 -air mixture gases (a), the changes of pH in the cultures with different concentration of CO_2-air mixture gases (b).

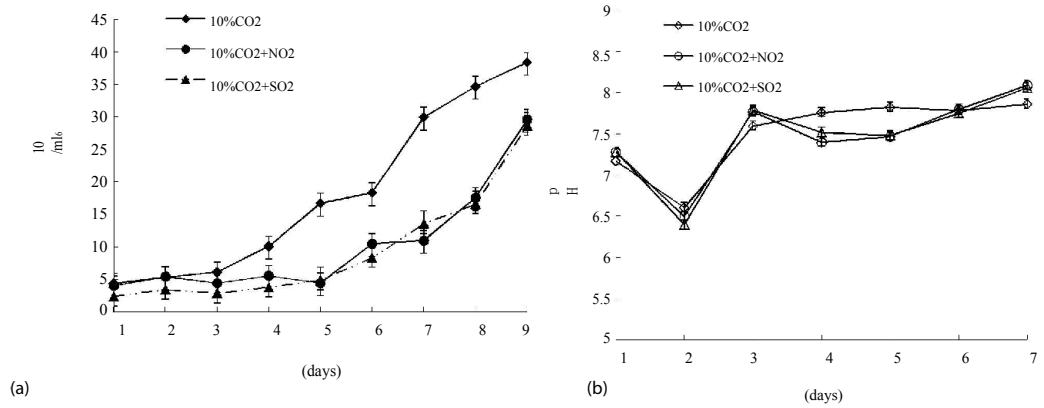

Figure 4. The growth of *Chlorella vulgaris* with 10% CO_2-air, 10% CO_2-air-SO_2, 10% CO_2-air-NO_2 mixture gases (c); the changes of pH in the cultures with 10%CO_2-air, 10% CO_2-air-SO_2, 10% CO_2-air-NO_2 mixed gases (d)

One assumption maybe described as follows: First, the alga assimilated the NO_{3-} in the medium at the first four days. After a period of NO_2 aeration, the amount of increasing NO_{3-} in the medium exceeded the requirement of algae and resulted to the rebound of the concentration of NO_{3-} at the fifth day, and the concentration of NO_{3-} of that had a sharp decrease as the number of algal cells doubled. Another explanation could be following. At the beginning, the alga assimilated the NO_{3-} in the medium at the first four days as well as the NO produced by NO_2 reacted with H_2O of the medium. After a period of NO_2 adaptation, the algae prefer utilizing the NO to leaving the NO_3-in the medium which results to the rebound of the concentration of NO_{3-} at the fifth day, and the consumption of NO_{3-} of that had an increase when the number of algal cells also doubled. The second explanation was concluded according to Nagase et al. **(Figure 5)** [19].

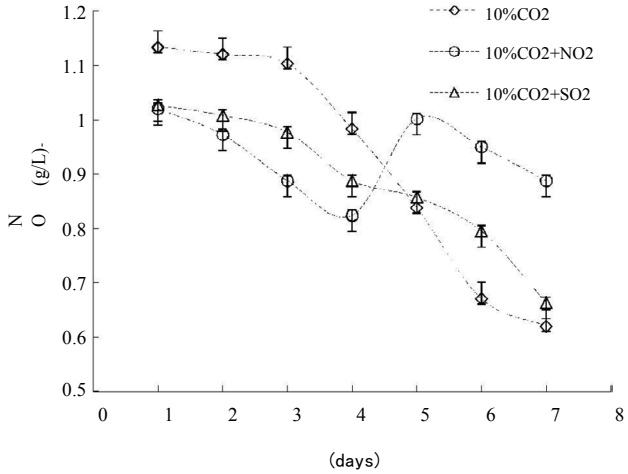

Figure 5. The changes of NO_3^- in the cultures with 10% CO_2-air, 10% CO_2-air-SO_2, 10% CO_2-air-NO_2 mixed gases.

Enzymatic Activities of Rubisco Influenced by CO_2-air, CO_2-SO_2 , CO_2-NO_2 Mixed Gases

The bar graph **(Figure 6)** indicated that algae cultivated with 10% CO_2-air-SO_2 mixture gas and 10% CO_2-air-NO_2 mixture gas at the end of ninth day had the higher intracellular rubisco activities as to that only with 10%-CO_2-air mixture gas, which means

that algae have the stronger CO_2 fixation with the help of trace gases after a period of cultivation. Moreover, the outcomes were accordant with that of the composition analysis and microstructure analysis (section 3.4, 3.5). Therefore, moderate concentrations of SO_2 and NO_2 gases may contribute to CO_2 fixation by algae.

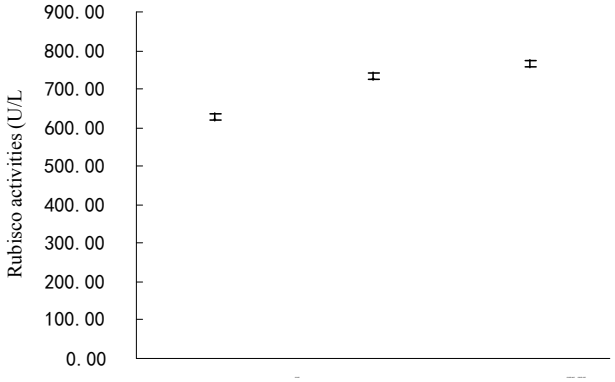

Figure 6. Activities of rubisco of *C. vulgaris* cultivated with 10% CO_2-air, 10% CO_2-air-SO_2, 10% CO_2-air-NO_2 mixture gases.

Composites Analysis

It was indicated from **Figure 7a** that the algae growing with 10% CO_2.air mixture gas has the highest content of the carbohydrate, followed with 15% and 5% CO_2-air mixture gases. But the lipid content of the algae cultivated with 5% CO_2-air mixture gas had a little increase because of the pH in this culture increased to 8.5, which inhibited the growth of algae and caused the lipid accumulation. 10% and 15% CO_2 were beneficial for growth of algae **(Figure 3a).** Furthermore, 10% CO_2 was a better choice for carbohydrate and protein accumulation. It was showed in bar graphs **(Figure 7b)** that NO_2 and SO_2 had contributed to the lipid and carbohydrate accumulation after a period of cultivation.

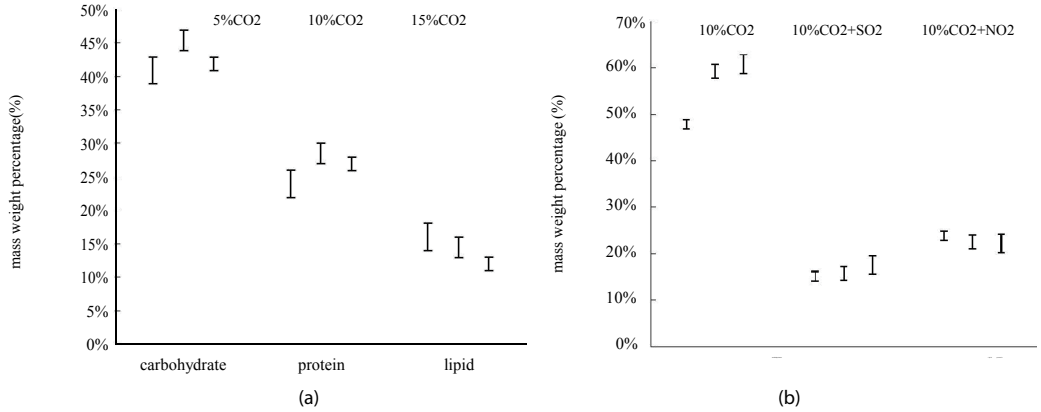

Figure 7. The comparison on mass weight percentages of bio-products in algal cell with different of concentrations of CO_2-air mixture gases (a) and also with addition of SO_2, NO_2 gases (b)

Microstructure Analysis

SEM images **(Figure 8)** of algae growing with 10% CO_2-air (a1, a2), 10% CO_2-air-SO_2 (b1, b2), 10% CO_2-air-NO_2 (c1, c2) mixture gases are presented in **Figure 8.** The surface of algal cells (b2 and c2) were more smooth and fine compared to that of the alga cells (a2), and the morphologies of the algal cells in b1 and c1 appear to be full and round which would be explained that the walls of algae were induced to be thicker and denser to adapt the new circumstance. For example: low pH and the effects caused by SO_2, NO_2 compared with images a1, b1 and c1, the sizes of the algal cells from the two images (b1 and c1) were a little bigger than that from the image a1. It was found that the images of pyrenoids in cells cultivated with 10% CO_2-air mixture gas were almost vanished in TEM images **(Figure 9)** of A1, A2, but the images of pyrenoids in the algal cells cultivated with 10% CO_2-air-SO_2 and 10% CO_2-air-NO_2 mixture gas mixture gas were still distinguished in TEM images of B1, B2 and C1, C2. The above phenomenon maybe explained that pyrenoid is sub-cellular micro-compartments found in chloroplasts of *chlorella sp.* which are associated with the operation of carbon-concentrating mechanism (CCM). High concentration of CO_2 circumstance would fulfill the requirement of algal growth and need not depend on CCM for providing more CO_2 around Rubisco. Therefore, the pyrenoid would disappear and thylakoid become loose under the condition of high concentration of CO_2 [20-22]. Consequently, the pictures A1 and A2 were accorded with the outcome of above conclusion. However, the clearer pyrenoids appeared in B1, B2, C1, C2 might be interpreted that the sharp decrease of pH in the medium caused by NO_2 and SO_2 also reduced the aquatic CO_2 dissolution which would induce the CCM to get sufficient CO_2 from acidic medium. In addition, there are abundant starch particles and liposome can be observed in the cells from the image C1 and the thylakoid became denser and order.

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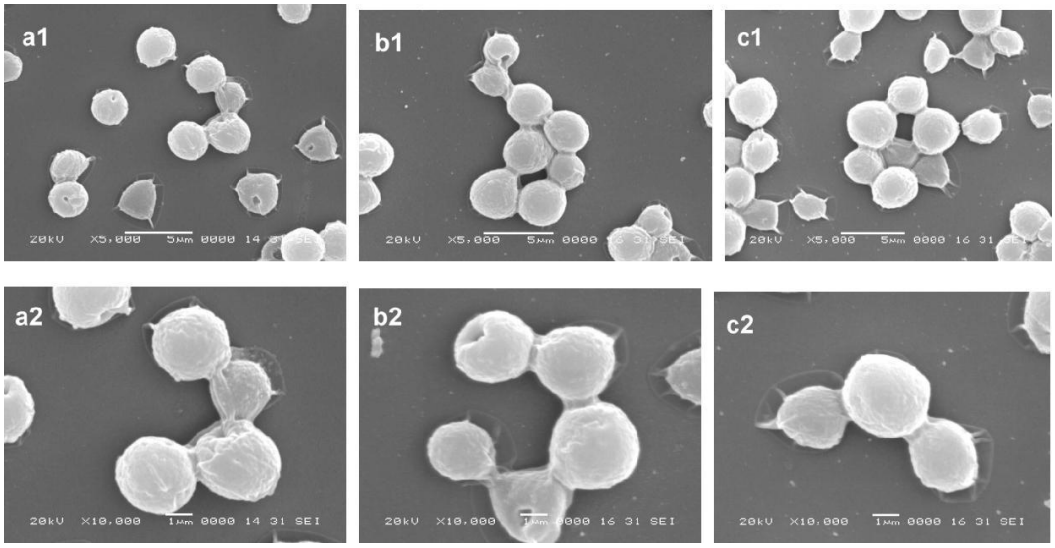

Figure 8. SEM images of C. vulgaris samples prepared with different growth conditions: (10% CO_2-air): a1 (5000×), a2 (10000×); 10% CO_2-air-NO_2: b1 (5000×), b2: (10000×); 10% CO_2-air-SO_2: c1 (5000×), c2 (10000×)

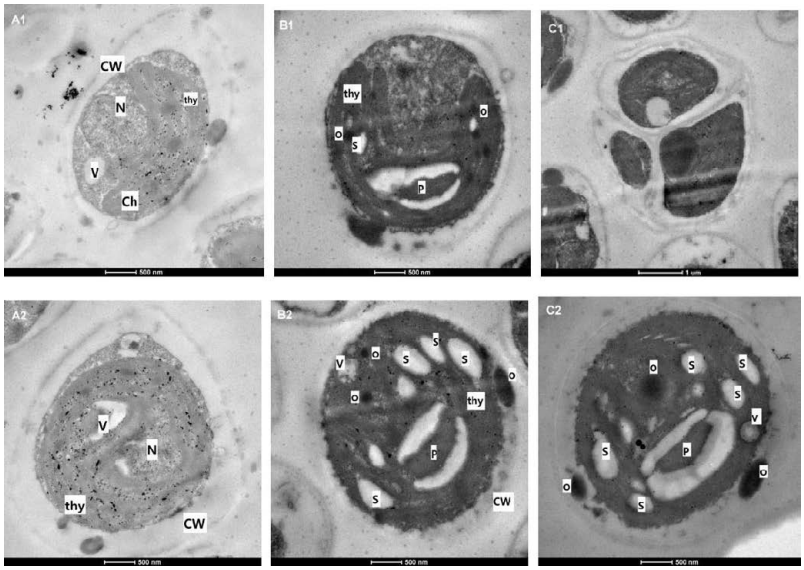

Figure 9. TEM images of *C. vulgaris* samples prepared with different growth conditions: (10% CO_2-air):A1, A2; 10% CO_2-air-NO_2 B1, B2; 10% CO_2-air-SO_2 **C1, C2** (CW: Cell Wall; N: Nucleus; V: Vacuole; CH: Chloroplast; thy: Thylakoid; S: Starch; P: Pyrenoid; O: liposome)

CONCLUSION

Excessive NO_2 and SO_2 could restrain the growth of the algae, which was witnessed by the work of the Tatsan et al. [23], who reported that *Synechococcus sp.* and *Chlorella* sp. could not tolerate higher exposure of NO_2 and SO_2 unless addition of triacontanol and sodium bicarbonate to the culture medium. Rapid decrease of pH in the culture medium caused by NO_2 and SO_2 is unavoidable because of excessive supplement. Jiang et al. [24] reported that *S. dimorphus* could tolerate high concentrations of CO_2 and NO by addition of $CaCO_3$ associated with intermittent sparing method to adjust the pH at about 7.0. In this work, we found that moderate NO_2 and SO_2 present had a positive effect on the CO_2 sequestration process after the algae had adapted the circumstance of acid gases. That's because the pH bounced to around 7.5 at the third day when the algae adapted the acidic circumstance and the whole process could adjust the pH by itself without other consideration for culture optimization. Moreover, moderate NO_2 and SO_2 contributed to energetic substance accumulation. Therefore, if low exposure levels of NO_2 and SO_2 were accurately controlled in the process, the algae could tolerate NO_2 and SO_2 at the beginning and began to grow fast and later higher levels of NO_2 and SO_2 could be considered which is the potential characteristic to be the energy material for cultivation with industrial flue gas.

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