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Microalgal-Based Carbon Sequestration by Converting LNG-Fired Waste CO_2 into Red Gold Astaxanthin: The Potential Applicability

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Abstract: The combinatorial approach of anthropogenic activities and CO_2 sequestration is becoming a global research trend to alleviate the average global temperature. Although microalgae have been widely used to capture CO_2 from industrial flue gas, the application of bioproducts was limited to bioenergy due to the controversy over the quality and safety of the products in the food and feed industry. Herein, the waste CO_2 emitted from large point sources was directly captured using astaxanthin-hyperproducing microalgae *Haematococcus pluvialis*. Astaxanthin production was successfully carried out using the hypochlorous acid water-based axenic culture process under highly contamination-prone outdoor conditions. Consequently, after 36 days of autotrophic induction, the productivity of biomass and astaxanthin of *H. pluvialis* (the mutant) reached 0.127 g L^{-1} day^{-1} and 5.47 mg L^{-1} day^{-1} under high summer temperatures, respectively, which was 38% and 48% higher than that of wild type cell. After grinding the wet astaxanthin-enriched biomass, the extract was successfully approved by compliance validation testing from Korea Food and Drug Administration. The assorted feed improved an immune system of the poultry without causing any side effects. The flue gas-based bioproducts could certainly be used for health functional food for animals in the future.

Keywords: LNG-fired waste CO_2, microalgal CO_2 sequestration; *Haematococcus pluvialis*; astaxanthin; HClO-based pure culture process; compliance validation testing; poultry feed test

1. Introduction

The energy generated by the combustion of traditional energy sources is the driving force of a nation's development, however the world is facing the urgent environmental issue of global warming due to the increased carbon dioxide (CO_2) emissions, which mostly result from burning excessive fossil fuel forms, like coal, oil and natural gas [1,2]. Therefore, it would be an inevitable demand that power generation must be combined with CO_2 sequestration process. Although CO_2 capture and storage (CCS) technology can reduce a considerable amount of CO_2 at once, it is not a perfect technology yet because there are many demerits, such as difficulty of choosing a location for stable CO_2 storage, the risk of leakage, the high cost for installation of infrastructures including pipeline and facilities of injection, and large scale storage [3]. On the contrary, CO_2 capture and utilization (CCU) technology would be an alternative solution for CO_2 sequestration, which brings the striking and instant utilization of stored CO_2 and effectively solves the challenges of leakage and space for long-term storage.

In recent years, microalgae cultivation system is considered as an eco-friendly CCU technology and one of potentially viable models for capturing waste CO_2 emitted from large point sources [4–9].

Microalgae efficiently convert inorganic CO_2 into organic biomass via photosynthesis and the biomass contains various value-added biochemicals, such as high energy density biofuels and high value pharmaceutical ingredients [10–13], which could reduce the overall cost of CCU-hybrid system. Astaxanthin is a high value keto-carotenoid pigment ($7000 kg^{-1} USD) [14] and a super antioxidant because its antioxidant activity surpasses that of other carotenoids and antioxidants in the market. Astaxanthin has a 550-times more powerful activity than vitamin E [15]. Among the various microalgal astaxanthin producers, *Haematococcus pluvialis* is the richest source of natural astaxanthin (3–5%, *w*/*w*) [16]. Above all, the *H. pluvialis* extracts predominantly consist of 3S, 3'S stereoisomer which is the most effective isomer for human and animal application as compared to the other optical isomer forms (i.e., 3R, 3'R and 3R, 3'S) [17].

During *Haematococcus* cultivation, separation of the first stage ("green" stage) for cellular multiplication (vegetative growth) and the second stage ("red" stage) for encystment (inductive growth) and carotenogenesis (astaxanthin accumulation) are necessary steps to achieve the high level of biomass production and astaxanthin yield [14,18], particularly for an industrial scale production [14,18–20]. In the green stage, the number of green motile flagellates increases under low-stress conditions, whereas in the red stage, cell proliferation is stopped due to exposure to high stress, but the single cell begins to increase in mass and color via morphological transformation from immature green cells to mature red cysts (aplanospore) to accumulate astaxanthin.

In the present study, *H. pluvialis* was cultured via employing a two-stage culture strategy under autotrophic conditions using natural sunlight and waste CO_2 (3–5%, *v*/*v*) resulting from the combustion of liquefied natural gas (LNG) in combined heat and power plants. Consequently, 3S, 3'S-astaxanthin was produced from *H. pluvialis* under tough or harsh outdoor conditions, lipid (bio-oil), which can be used for renewable energy generation, was also co-produced. Moreover, a remarkable increase in CO_2 removal rate, as well as biomass and astaxanthin productivity, were confirmed in outdoors conditions using previously developed highly photosensitive and photoinducible *Haematococcus* mutant (PP-PS 160), by adopting red cyst inoculation and iron (II) supplementation methods.

In the microalgae industry, the constant and stable supply of the waste CO_2 emitted from large point sources is regarded as a critical issue for the economical production of microalgae biomass [18,21]. Although the waste CO_2 from flue gas has been broadly used for microalgae research, the produced biomass was mainly applied in the field of biofuel production, such as bio-diesel or solid fuels [4–6,22–28], not used in human food or animal feed, due to safety concerns over the waste CO_2. The main focus of this study is to identify the potential application of the biomass derived from industrial flue gas in the animal feed industry. The *H. pluvialis* biomass containing valuable astaxanthin was successfully produced in an outdoor facility using natural sunlight and LNG-fired flue gas as gaseous carbon feedstock, which is relatively clean compared to other coal combustion flue gases. The astaxanthin extract was validated and approved by Korea Food and Drug Administration (KFDA), to meet KFDA-recognized consensus standards for application to the animal feed industry. After that, physiological studies were carried out to monitor the health benefits of the astaxanthin extract powder on the poultry feeding. The assorted feed improved the immune system of the poultry without unintended side effects.

2. Materials and Methods

2.1. Establishment of Outdoor Microalgal Culture System Using LNG-Fired Flue Gas

For effective conversion of waste CO_2 from industrial flue gas into high value products, such as bio-oil (omega-3 fatty acids) or astaxanthin, microalgal culture process was installed near the LNG combustion stack of the Korea District Heating Corporation (KDHC) located at Baekhyeon-dong, Bundang-gu, Seongnam-si, Gyeonggi-do, in South Korea [7–9]. In the system, *H. pluvialis* cells were cultured autotrophically by using a transparent polymeric thin-film (polyethylene-nylon hybrid) 100 L-PBR [9] with the technical specifications of 120 cm diameter (D) and 180 cm height (H), which is

consisted of serially connected six bubble columns (D of 15 cm, H/D ratio of 9:1), for outdoor mass production of the biomass packed with astaxanthin. In the PBR, biomass production was carried out using solar energy (heat and light) and LNG-fired flue gas at a flow rate of 0.1 vvm (vessel volumes per min). Composition of flue gas was 3.5 ± 0.5% (*v*/*v*) CO_2, 3 ± 1.15 ppm CO, 10.15 ± 0.71% O_2, and 21.57 ± 3.48 ppm NOx [7–9].

2.2. Pure Culture System of *H. pluvialis* Without Microbial Contamination

The slightly acidic hypochlorous acid (HClO) water-based axenic culture method was developed to cultivate contamination-free *H. pluvialis* under highly contamination-prone outdoor conditions, as *Haematococcus* cells are highly sensitive to the biotic stress, thus likely to contaminate easily (Figure 1). In this study, the pH of the culture medium was maintained within a constant range via a dissolved inorganic carbon (DIC) buffer system using 10 mM KOH and waste CO_2 from the LNG-fired flue gas during long periods of outdoor autotrophic cultivation. In addition, to minimize the biotic contamination during medium transfer, the nutrient medium (NIES-C or NIES-N) was directly injected in the PBR in the form of a concentrated stock solution (500×).

The entire microalgae culture process for contamination control can be described as follows: (1) Manufacturing polymeric thin-film PBR, (2) washing and filling the PBR using HClO water, (3) injecting flue gas in the PBR to degas Cl_2 for 2–3 days (HClO water → pure water), (4) injecting waste CO_2 containing flue gas in the PBR filled with 10 mM KOH to facilitate improved DIC level as well as buffered the solution (bicarbonate system), (5) supply of appropriate concentration of nutrients NIES-C medium stock solution (500×) in the PBR and inoculation of microalgal seed for biomass cultivation.

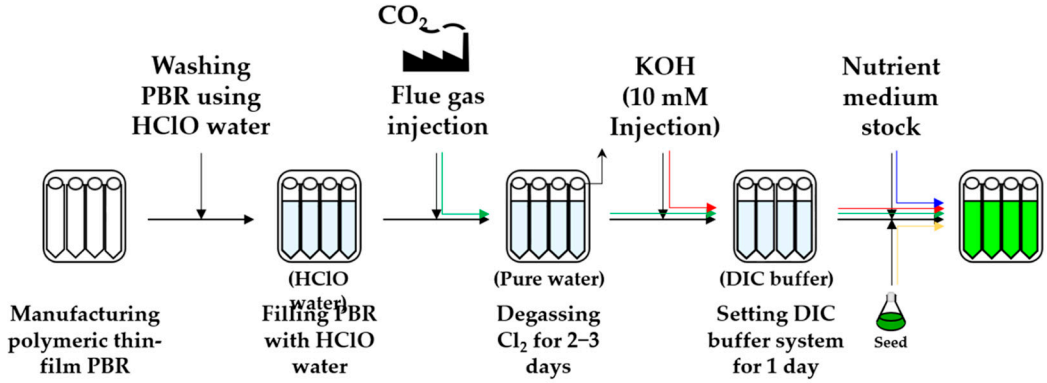

Figure 1. Overall process scheme for mass cultivation of microalgae without microbial contamination.

2.3. Algal Strains and Culture Conditions

The *H. pluvialis* NIES-144 (wild type) was purchased from the National Institute for Environmental Studies (NIES) in Tsukuba, Japan. The partially PSII-deficient and photosensitive (PP-PS) *Haematococcus* mutant PP-PS 160, which was derived from *H. pluvialis* NIES-144, was obtained from the Nanobiotechnology Laboratory at Korea University in Seoul, South Korea. The two *H. pluvialis* strains were cultured in two media of NIES-C (nitrogen-replete) and NIES-N (nitrogen-deplete) medium for cell proliferation and astaxanthin induction, respectively [29].

NIES-C medium is comprised of 0.15 g L^{-1} $Ca(NO_3)_2$, 0.10 g L^{-1} KNO_3, 0.05 g L^{-1} β-glycerophosphoric acid disodium salt pentahydrate, 0.04 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.50 g L^{-1} Tris-aminomethane, 0.01 mg L^{-1} thiamine, 0.10 µg L^{-1} biotin, 0.10 µg L^{-1} vitamin B12, and 3.00 mL L^{-1} PIV metal solution, which consisted of 1.0 g L^{-1} Na_2EDTA, 0.196 g L^{-1} $FeCl_3{\cdot}6H_2O$ and (in mg L^{-1}) 36.0 $MnCl_2{\cdot}4H_2O$, 22.0 $ZnSO_4{\cdot}7H_2O$, 4.0 $CoCl_2{\cdot}6H_2O$, and 2.5 $Na_2MoO_4{\cdot}2H_2O$. The NIES-N was prepared by excluding an N source from the NIES-C medium, by substituting $CaCl_2{\cdot}2H_2O$ 0.13 g L^{-1} and KCl 0.07 g L^{-1} for $Ca(NO_3)_2$ and KNO_3, respectively, for a supplement of calcium and potassium ions.

A two-stage strategy was introduced to improve photoautotrophic astaxanthin production from *H. pluvialis* using waste CO_2 under outdoor spring and summer conditions (Figure 2). In the green stage, the two strains were firstly grown for 15–20 days in nitrogen-replete NIES-C medium at 25–45 µmol photons m^{-2} s^{-1} of limiting light conditions. The initial cell density of all cultures was approximately 0.12–0.15 g L^{-1}.

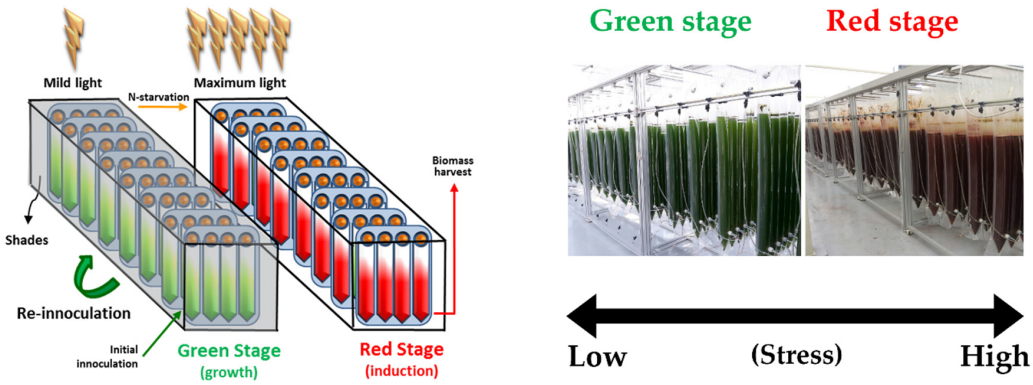

Figure 2. Schematic illustration and actual photographs of two-stage strategy.

In particular, during summer experiments, the two methods of cyst inoculation (germination) and Iron(II) (Fe^{2+}) supplementation were consecutively employed for vegetative growth in the green stage and astaxanthin production in the red stage, respectively, to cope with harsh high-temperature environments [7,30]. Nitrogen deplete NIES-N medium stock solution (500×) and 50 µM Fe^{2+} were additionally injected into the photobioreactor (PBR) at the end of green stage (initial red stage), to accelerate nitrogen starvation and heat stress-driven Haber–Weiss reaction, respectively, thereby enhancing astaxanthin production under high temperature conditions [7,30].

Cells were cultured at high solar irradiance of 305–360 µmol photons m^{-2} s^{-1} and 315–380 µmol photons m^{-2} s^{-1} in spring and summer, respectively. During the outdoor spring and summer seasons, all cultivations were conducted in almost clear sunny days, thereby ensuring the intensity of solar light radiation. Nonetheless, natural light does not meet the requirements of constant and continuous strength of light intensity during daytime. Accordingly, irradiance was roughly controlled by using synthetic shading sheet, but not for the culture medium temperatures.

2.4. Analytical Methods

2.4.1. Analysis of Cell Growth

The cell density was determined by measuring DCW using pre-weighed filter paper via filtering aliquots of samples. Then, the cell suspensions (10 mL) followed by DI water (as flow through) were filtered using GF/F glass microfiber filters (Whatman, Cambridge, UK), and dried at 105 °C overnight. Then, the weight of dried cells was determined by the difference between the mass of the biomass-containing filter paper and that of pre-weighed filter paper [17].

2.4.2. Analysis of Astaxanthin

In order to assay intracellular astaxanthin, cell suspensions (5–10 mL) were collected by centrifuging the cell culture fluid at 5590 g, and 4 °C, for 5 min, decanting the supernatant, and then rinsing the cell pellet with pre-chilled TE buffer (pH 7.5). The pellet was dissolved in pre-chilled 100% methanol with glass beads to extract pigments and homogenized with Tissue Lyser II (Qiagen, Valencia, CA, USA) using pre-chilled Tissue Lyser adaptors. The extraction procedure was repeated until the cell debris was colorless [30]. The homogenized lysate was centrifuged at 5590 g, at 4 °C, for 10 min to separate the supernatant from the cell debris, and the fresh extracts were collected and saponified using the alkali-method [30,31]. For saponification of esterified astaxanthin from *H. pluvialis*, 0.01 M

KOH was used for 12 h to convert the esterified forms (10–50 mg L^{-1}) into the free form. Under the conditions, the selective separation of free astaxanthin by alkaline hydrolysis of astaxanthin-esters can be efficiently performed in methanol by adding hydroxyl groups (OH^-).

After saponification, the astaxanthin concentration of each sample was determined by HPLC equipped with Quaternary Pump VL G7111A (Agilent, Santa Clara, CA, USA) and 1260 Infinity Variable Wavelength Detector (Agilent, USA). The extracts were separated using a 250 × 4.6 mm YMC Carotenoid C30 column (YMC, Kyoto Japan). The mobile phase consisted of solvents A (dichloromethane:methanol:acetonitrile:water, 5.0:85.0:5.5:4.5, v v^{-1}) and solvent B (di-chloromethane:methanol:acetonitrile:water, 22.0:28.0:45.5:4.5, v v^{-1}). Linear gradient system was used for the effective separation of astaxanthin: 0% B for 8 min, a linear gradient from 0 to 100% B for 12 min and 100% B for 50 min. The flow rate was set at 1.0 mL min^{-1} and the peaks were measured at 480 nm [29,30].

2.4.3. Analysis of Lipid

Total cell lipid was determined using the modified Bligh and Dyer method [32]. The mixed solution of methanol and chloroform (2:1, v v^{-1}) was directly used for cell lysis and extraction of lipid molecules from the wet cells without using the dried cells. After that, the extracted wet lipid molecules were dried and then the total lipid was examined by measuring the weight.

2.4.4. Analysis of Carbohydrate

Total cell carbohydrate was measured via a colorimetric assay with an anthrone reagent [33]. To analyze the intracellular quantity of carbohydrate, cell suspensions (3–5 mL) were harvested by centrifuging the culture fluid at 5600 g for 4 min at 4 °C, discarding the supernatant, and rinsing the cell pellet with pre-chilled TE buffer (pH 7.5). The pellet was homogenized with Tissue Lyser II (Qiagen, Valencia, CA, USA) in 1 mL of pre-chilled 100% methanol with glass beads in the pre-chilled Tissue Lyser adaptors. After evaporation of methanol in drying oven, 1 mL of 72% perchloric acid was added to the samples, which were subsequently reacted for 6 h at 4 °C. After that, each sample was mixed with 2 mL of 67% sulfuric acid by vortexing for 30 min and subsequently reacted with anthrone reagent at 100 °C in the water bath for 5 min. The quantitative amount of carbohydrate of each sample was measured at 630 nm.

2.4.5. Analysis of Protein

Total cell protein was examined by the Bradford method [34]. To assay intracellular protein quantity, cell suspensions (3–5 mL) were collected by centrifuging the culture fluid at 5600 g for 5 min at 4 °C, discarding the supernatant, and then rinsing the cell pellet with pre-chilled PBS buffer (pH 7.5). The pellet was homogenized with Tissue Lyser II (Qiagen, Valencia, CA, USA) in 2 mL of pre-chilled PBS buffer (pH 7.5) with glass beads in the pre-chilled Tissue Lyser adaptors, the supernatant was colored by mixing with Bradford reagent (Sigma Chemical Co., St. Louis, MO, USA), and The quantitative amount of protein of each sample was measured at 595 nm.

2.4.6. Ball Milling Method to Rupture *H. pluvialis* Red Cysts and KFDA Approval of the Extracts

After centrifugation of the suspensions containing astaxanthin-enriched *H. pluvialis* biomass, the harvested red cell cake (150–200 g L^{-1} of red cell density) was subsequently grinded by ball mill device containing zirconium oxide milling ball (Zirconia, 10 mm, Tencan) for 24 h and the extracts were sent to KFDA for compliance testing.

2.4.7. Physiological Studies of *H. pluvialis* Biomass on Poultry Feeding

After drying the mixture of ruptured *H. pluvialis* biomass and astaxanthin extract, resulting in the powder, the biomass-astaxanthin extracts powder were directly used to feed poultry. A total of

270 number of egg-laying hens (eight days old) were employed in this trial. Dietary treatments in this experiment consisted of feeding a corn-soybean meal control diet alone or supplemented with 0, 0.1, and 0.2% ruptured *H. pluvialis* powder for a period of seven weeks. Three replicate groups of 30 hens were randomly dosed to each of the three treatments.

3. Results

3.1. Outdoor Biomass and Astaxanthin Production Using LNG-fired Flue Gas During Spring and Summer Seasons

Wild type and highly photosensitive *Haematococcus* mutant PP-PS 160 were compared for the production of biomass and astaxanthin under outdoor transient culture conditions of light intensity and temperature using the flue gas from the combustion of LNG during spring and summer seasons. Consequently, the CO_2 removal rate of the mutant was 36.8% and 37.3% in spring and summer, respectively, compared to that of wild type cell (spring: 0.125 g L^{-1} day^{-1}, summer: 0.171 g L^{-1} day^{-1}). Output details are summarized in Table 1.

In spring season, as shown in Figure 3, the production of biomass and astaxanthin from wild type cell reached to 3.989 g L^{-1} and 134.04 mg L^{-1} after 72 days of autotrophic induction (two-stage) respectively. The productivity of biomass and astaxanthin was 0.055 g L^{-1} day^{-1} and 1.861 mg L^{-1} day^{-1} in the wild type cell, respectively. Remarkably, the highly photosensitive mutant showed enhanced biomass and astaxanthin production in spring compared to that of wild type cell. After 57 days of autotrophic induction (two-stage), the production yield of biomass and astaxanthin of the mutant reached up to 4.310 g L^{-1} and 158.52 mg L^{-1}, respectively. Overall, the productivity of biomass and astaxanthin by the mutant was 36% and 49% higher than that of wild type cell.

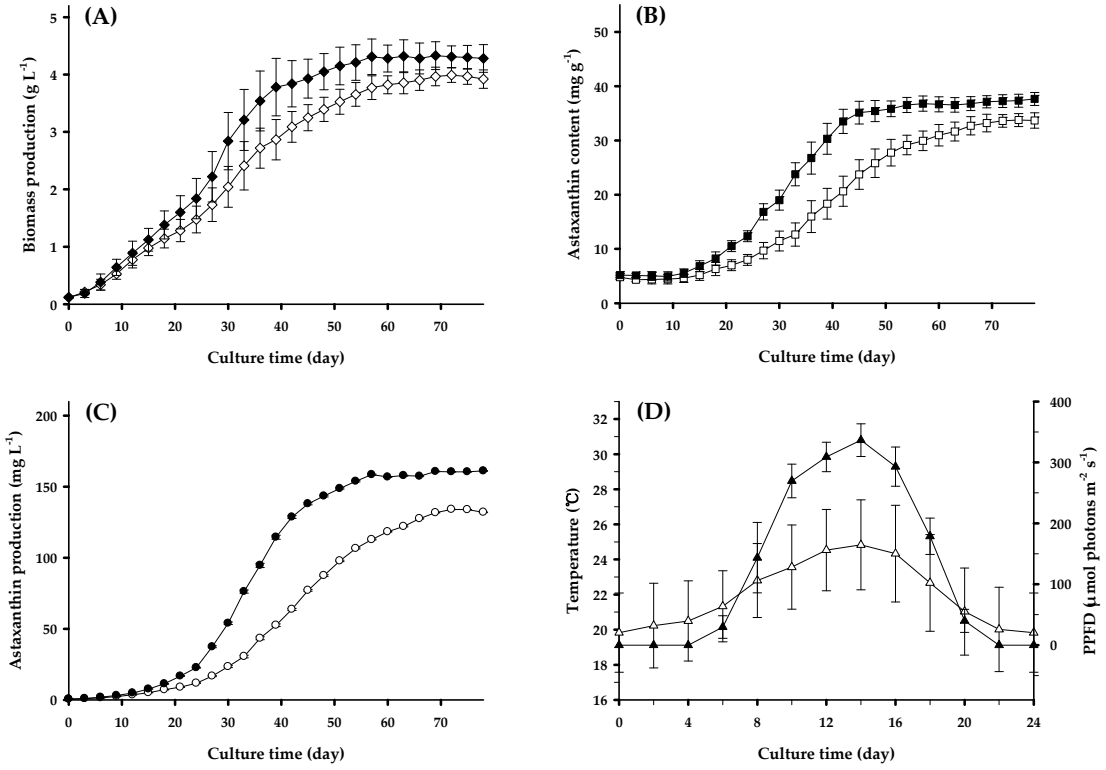

Figure 3. Comparison of (**A**) biomass production (diamond), (**B**) astaxanthin content (square), and (**C**) astaxanthin concentration (circle) from *H. pluvialis* cells of wild type (white) and the mutant PP-PS 160 (black) under moderate temperature conditions in spring. (**D**) Photosynthetic photon flux density (PPFD) (black triangle) and medium temperature (white triangle) during the daylight period in spring (March–May). Each value is mean ± standard deviation of three replicates.

Table 1. Comparison of biomass production, astaxanthin production, and CO_2 removal rate between *H. pluvialis* cells of wild type and mutant under outdoor spring and summer conditions.

	Spring		Summer	
	Wild Type (72 Days)	**Mutant (57 Days)**	**Wild Type (45 Days)**	**Mutant (36 Days)**
Biomass yield (g L^{-1})	3.989	4.31	4.161	4.564
Biomass productivity (g L^{-1} day^{-1})	0.055	0.075	0.092	0.127
Astaxanthin content (mg g^{-1})	33.60	36.78	40.02	44.02
Astaxanthin accumulation rate (mg g^{-1} day^{-1})	0.466	0.645	0.889	1.223
Astaxanthin yield (mg L^{-1})	134.04	158.52	166.43	196.88
Astaxanthin productivity (mg L^{-1} day^{-1})	1.861	2.781	3.698	5.469
CO_2 removal rate (g L^{-1} day^{-1}) [1]	0.125	0.171	0.209	0.287

[1] CO_2 removal rate of biomass was calculated as follows: CO_2 removal rate (biomass) (g L^{-1} day^{-1}) = [Cell density (g L^{-1}) × Carbon content in biomass (61.8%, *H. pluvialis*) × 44/12 (conversion factor: CO_2/C)]/culture period (day).

When we compared the yield of biomass and astaxanthin within the wild type strain, the summer season was more productive as shown in Figure 4, the yield of biomass and astaxanthin was higher than that of spring season, reaching to 4.161 g L^{-1} and 166.43 mg L^{-1} after 45 days of autotrophic induction (two-stage), respectively, the productivity was 0.092 g L^{-1} day^{-1} and 3.698 mg L^{-1} day^{-1} respectively. However, the highly photosensitive mutant exhibited improved performance on the biomass and astaxanthin production compared to that of wild type cell during both seasons, in particular summer was a better yielding season. After 36 days of autotrophic induction (two-stage), the production yield of biomass and astaxanthin of the mutant reached to 4.564 g L^{-1} and 196.88 mg L^{-1}, respectively. Consequently, the productivity of biomass and astaxanthin in the mutant was 38% and 48% higher than that of wild type cell.

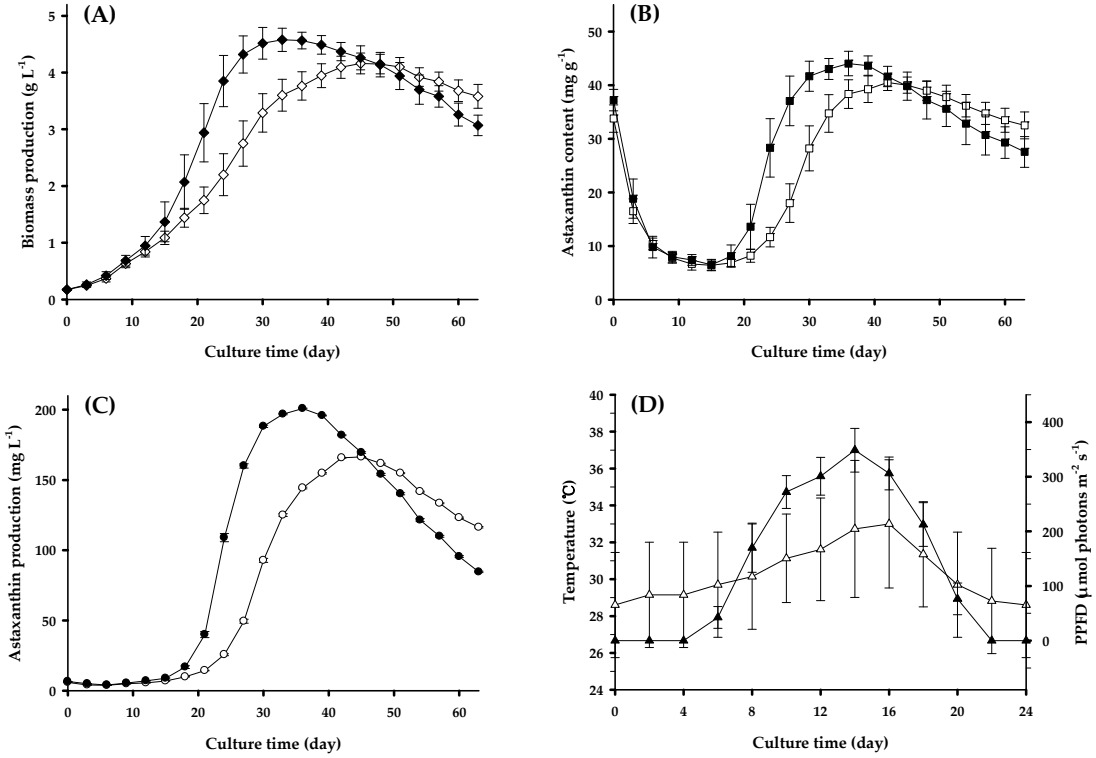

Figure 4. Comparison of (**A**) biomass production (diamond), (**B**) astaxanthin content (square), and (**C**) astaxanthin concentration (circle) from *H. pluvialis* cells of wild type (white) and the mutant PP-PS 160 (black) under high-temperature conditions in summer. (**D**) Photosynthetic photon flux density (PPFD) (black triangle) and medium temperature (white triangle) during the daylight period in spring (June–August). Each value is mean ± standard deviation of three replicates.

3.2. Outdoor Lipid and Carbohydrate Production Using LNG-Fired Flue Gas During Spring and Summer Seasons

The intracellular contents of protein, carbohydrate, and lipid of the two *H. pluvialis* cells were also measured at the end of each stage during outdoor cultivation in spring and summer. As a result, under the moderate temperature conditions in spring, the protein content decreased by half, but carbohydrate and lipid contents were increased in wild type cell at the end of the induction stage (Figure 5). Although the change in patterns for the contents of the mutant was similar to the wild type cell, the increase in the lipid content of the mutant was much higher than that of wild type cell. The phenomenon of high lipid content at the end of the induction stage was more prominent under the high temperature conditions in summer.

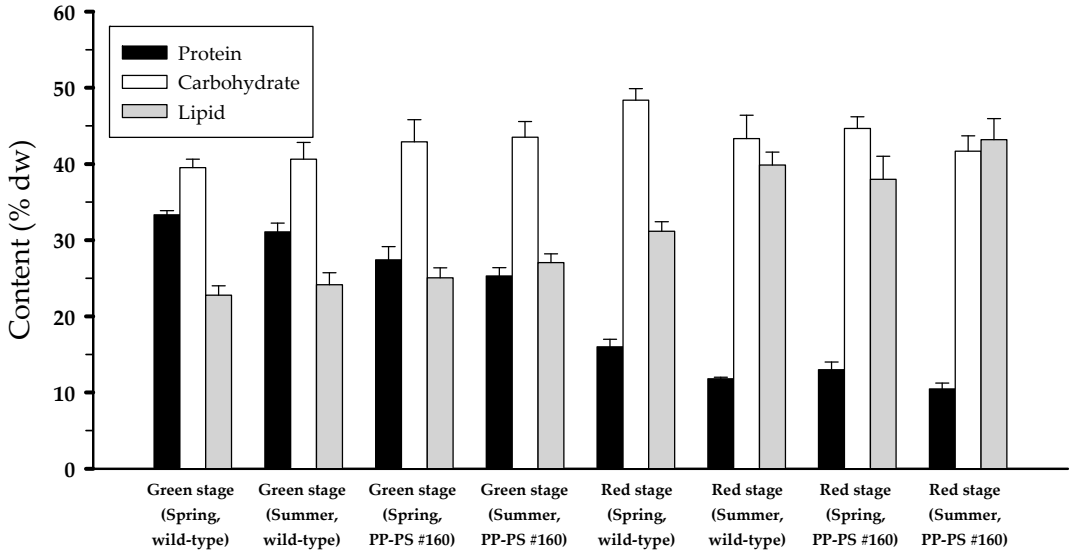

Figure 5. Comparison of the contents of protein, carbohydrate, and lipid from *H. pluvialis* cells of wild type and the mutant PP-PS 160 at the end of each stage of green and red during spring and summer conditions. Each value is mean ± standard deviation of three replicates.

3.3. KFDA Approval and Physiological Studies of H. pluvialis Extracts

The astaxanthin-enriched wild type cells were harvested by centrifugation and the red cyst biomass cake was collected and ground by ball mill with zirconia beads. As a result, the cell wall of wet *H. pluvialis* was easily ruptured by treatment of ball mill for one day (Figure 6), thereafter astaxanthin was extracted without the use of organic solvent. As shown in Table 2, the *H. pluvialis* biomass and astaxanthin-mixed extract derived from the LNG-fired flue gas was approved by KFDA (Supplementary Materials), and thereafter used for successful physiological studies on poultry. The characteristic of the extract was a dark reddish brown viscous fluid without unpleasant flavor and odor. The astaxanthin content of the extract was closely meet the standards. As the microalgal cells were derived from flue gas, thus heavy metal contamination was expected in the final cell biomass. All the contents of the heavy metals such as Pb, Cd, Hg, and As were tested and determined as lower than the detection limits suggested by KFDA. Moreover, microbial contaminants, as well as organic solvents, were not detected as expected.

In order to confirm the toxicity and other side effects of the extracts, the mixed powder of homogenized and dried *H. pluvialis* biomass and astaxanthin extract was tested by feeding to poultry. As a result, the immune system of poultry was boosted by feeding the mixture powder of ruptured *H. pluvialis* biomass and astaxanthin extract without specific side effects (Table 3). Accordingly, the mortality rate of the egg-laying hens fed with 0.1% and 0.2% ruptured *H. pluvialis* powder was decreased by 17% (8.3%) and 15% (8.5%) compared to that of the egg-laying hens fed without the astaxanthin powder (10%), respectively.

Table 2. Korea Food and Drug Administration (KFDA) approval and compliance testing for the *H. pluvialis* extracts derived from the LNG-fired flue gas.

Items	Standard Amount	Result	Decision
Characteristic	It should have an inherent color and glossy, lacking unpleasant flavor and odor	It is a dark reddish-brown viscous fluid lacking unpleasant flavor and odor	Accepted
Astaxanthin content	50 mg g^{-1}	54.39 mg g^{-1}	Accepted
Heavy metal	Total Pb (<1 mg kg)	0.0181 mg kg^{-1}	Accepted
	Total Cd (<1 mg kg)	0.0083 mg kg^{-1}	Accepted
	Total Hg (<1 mg kg)	0.0070 mg kg^{-1}	Accepted
	Total As (<1 mg kg)	0.1175 mg kg^{-1}	Accepted
Contaminant	Bacteria negative	Negative	Accepted
Solvent remnant	Acetone (<30 mg kg^{-1})	Negative	Accepted

Table 3. Effect of dried *H. pluvialis* extracts on the physiological activity and immune system of poultry.

Parameters [1]	Unit	Regular Range	0% Biomass	0.1% Biomass	0.2% Biomass	Remarks [2]	Contents
RBC	M μL^{-1}	2.0–6.0	3.61	3.72	3.99	A	O_2 carrier function
WBC	K μL^{-1}	10.0–30.0	13.99	13.83	18.58	A	Immune function
Lymphocytes	%	50–100	73.35	68.68	68.47	A	Immune function
Monocytes	%	5–15	9.11	10.25	10.35	A	Immune function
Eosinophils	%	0–3	1.81	2.08	2.41	A	Defense function (parasite)
Basophils	%	0–1	0.48	0.50	0.60	A	Defense function (parasite)
Neutrophils	%	10–30	17.64	19.19	21.97	A	Phagocytosis function
Hemoglobin	g dL^{-1}	10–20	12.20	12.90	13.48	A	O_2 carrier function
Hematocrit	%	30–50	35.22	36.18	39.54	A	Red cell amount (RBC volume B $volume^{-1}$)
MCV	fL	50–150	97.86	99.14	99.28	A	Average RBC size
MCH	pg	20–40	33.98	34.72	33.80	A	Hemoglobin amount per RBC
MCHC	g dL^{-1}	25–50	34.74	37.70	34.48	A	Hemoglobin amount relative to the size of the cell per red blood cell
Platelet	K μL^{-1}	800–1200	993.0	992.0	1010.8	A	Congelation of blood
ALP	U L^{-1}	800–1600	1200.8	1112.6	1145.4	B	Liver function index (bile excretion)
TBIL	mg dL^{-1}	1.5–3.5	2.52	2.38	2.20	B	Liver function index (Jaundice)
BUN	mg dL^{-1}	3.0–6.0	4.64	4.30	4.22	B	Kidney function index (Urea concentration in blood)

[1] Abbreviations for parameters are as follows: RBC, Red blood cell; WBC, White blood cell; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; ALP, Alkaline phosphatase; TBIL, Total bilirubin; BUN, Blood urea nitrogen (urea). [2] A: The values higher than the regular range indicate that the function was more activated, B: The values lower than the regular range indicate that the function was more activated.

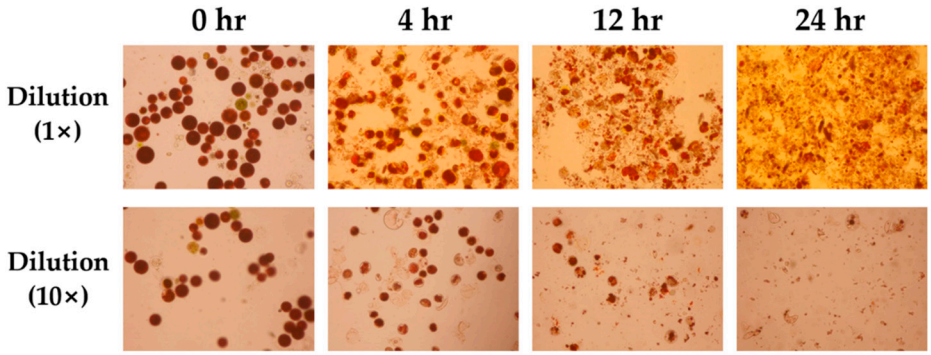

Figure 6. Morphological change of *H. pluvialis* cysts after mechanical pretreatment of ball mill for 12 h.

4. Discussion

In order to capture waste CO_2 from large point sources in outdoor cultivation, the freshwater microalgae *Chlorella* sp., *Scenedesmus acutus* and *Desmodesmus abundans,* have been commonly used for flue gas mitigation because they often exhibit an outstanding tolerance to biotic and abiotic stresses [4–6] compared to the other microalgae species. Astaxanthin production can be the most attractive option from a corporate standpoint to maximize the viability of CO_2-emiting companies, even situated in a space-limited urban area.

Although astaxanthin-producing microalgae *Chlorella zofingiensis* was commonly an interesting host in the microalgal industry, the intracellular content of astaxanthin is still low and the individual carotenoids pool includes high levels of canthaxanthin [35], which could be harmful to the human cells, compared to *H. pluvialis*. Undoubtedly, the green microalga *H. pluvialis* is regarded as the richest source of natural astaxanthin in nature and cultivated on an industrial scale [14,18–20].

However, the production of *H. pluvialis* biomass enriched with astaxanthin often fails in outdoor cultivations due to the microbial contamination [36,37]. Typically, four species of contaminants, such as the chytrid zoosporic, *Paraphysoderma sedebokerensis* (fungus), *Poterioochromonas malhamensis* (golden algae), *Scenedesmus* sp. (microalga), and the *Bacillus* sp. (bacteria), have primarily led *Haematococcus* culture failure in our process. The *Bacillus* sp. attack to eat the encysted *H. pluvialis* cells by persistent drilling of the rigid cell walls. The *Poterioochromonas malhamensis* produces dangerous toxins and covers the encysted *H. pluvialis* cells to be starved and died. The *Paraphysoderma sedebokerensis* is an extremely detrimental predator of *H. pluvialis*. Once the PBR is contaminated by the fungus, all the encysted *H. pluvialis* cells are unmercifully destroyed in one to three days resulting in a color change of the PBR from red to white or yellow-brown. Although the microalga *Scenedesmus* sp. does not eat or kill *H. pluvialis*, there is no way to separate the two microalgal cells produced, thereby losing the quality of products. Therefore, the HClO water-based pure culture system was designed for the stable production of astaxanthin from *H. pluvialis* in this study. The slightly acidic electrolyzed water was harmless for the human but swiftly fatal for the contaminants. The concentration of Cl_2 in the HClO water was 30 ppm, which was an effective concentration to kill the contaminants. Intriguingly, the HClO water can be simply converted into pure water by bubbling to degas dissolved Cl_2 as the column reactor was efficient for Cl_2 degassing (data not shown).

The buffer system is necessary for sustaining the required pH, particularly at neutral or mildly alkaline pH (7.5–8.0), in the microalgal culture medium because high or low pH is detrimental for the vegetative growth as well as the production of value-added products by microalgae [38,39]. In general, Tris and HEPES have been widely used as a buffer for microalgae cultivation [12,29], which are very costly. It was previously reported that DIC buffer system using sodium hydroxide (NaOH) and CO_2 has remarkable buffer potential for microalgal cultivation [40,41]. In addition, our previous study showed that the productivity of biomass and astaxanthin by *H. pluvialis* was slightly enhanced by DIC buffer system under the optimal conditions of 10 mM of KOH and LNG-fired flue gas containing 3–5% waste CO_2 [8]. It is noteworthy that an aqueous environment is normally a CO_2-limited condition [42],

hence the improved yield from such environment can be partially attributed to the increased uptake of DIC (bicarbonate, HCO_3^-), which is converted to CO_2 by intracellular carbonic anhydrase and then can be used for biomass and astaxanthin production [8]. In this study, it was also revealed that under outdoor moderate (spring) or high (summer) temperature conditions the DIC buffer system based on NaOH and LNG-fired flue gas successfully improved biomass and astaxanthin production from both *H. pluvialis* cells of the highly photosensitive mutant PP-PS 160 as well as the wild type NIES-144.

Consistent with many other studies [7,16,29,30], which were conducted under nutrient stress, *H. pluvialis* cells were exposed to starvation, which rapidly stopped cellular multiplication but exhibited a considerable increase in volumetric growth (mass per single cell) in this study. Therefore, N-deficient medium was additionally used for rapid consumption of intracellular nitrogen remnant, thereby enhancing photoautotrophic induction in *H. pluvialis*. Although it is worth measuring the minimum inhibitory concentration (MIC) of intracellular nitrogen source to affect astaxanthin production in *H. pluvialis*, the rapid switching effect of transcription pools for carotenoid biosynthesis by the addition of N-deplete medium might be more contributable to the astaxanthin induction than the amount of intracellular nitrogen source. In *Chlorella pyrenoidosa*, under N- and P-deplete conditions, the biomass increased by as much as 112% compared to an initial cell density [43]. In *Neochloris oleoabundans*, under N-deplete conditions, the biomass increased by as much as 127% compared to an initial cell density [44].

In microalgae, carotenogenesis is mainly regulated by biotic or abiotic stressors, such as nutrient deprivation, high irradiance, salinity, drought and moderate temperature [45,46]. Reactive oxygen species (ROS) are mediators between abiotic stress and astaxanthin synthesis, whenever there is excess reducing power for photosynthesis [30]. Therefore, the second stage of a two-stage strategy is highly dependent on the light intensity in *H. pluvialis*, [7,16,29,30,45,46]. The more biomass increases, the more intensive light is needed for rapid induction. Therefore, the selection of photosensitive mutant would be one of the most important issues for economic astaxanthin production [16,17]. In our previous study, we found highly photo-inducible *H. pluvilais* mutants derived from increased photo-sensitivity and proposed the mutant library to improve the output of the total astaxanthin production under moderate light conditions [16,17], which is limiting for efficient astaxanthin accumulation in the wild type cell. In the mutant, partial photosystem II (PSII) deficiency primarily contributed to the extreme generation of ROS, thereby accelerating both encystment for inductive growth and carotenogenesis for astaxanthin accumulation.

Although it has been previously reported that the *H. pluvialis* mutant, which is less sensitive to ROS, also showed enhanced astaxanthin production under high-light condition (250 μmol photons m^{-2} s^{-1}) [47], the main downside of the mutant might be the difficulty of the efficient induction under the conditions of high cell density cultivation. In this regard, the highly photosensitive astaxanthin producer could be more useful when performing high cell density culture for achieving high astaxanthin concentration because it showed outstanding astaxanthin-accumulating performance under moderate light condition. Above all, under outdoor thermal conditions in summer, in which variations of the diurnal temperature occur, the mutant PP-PS 160 showed incredible inducibility of high-titer astaxanthin production. It clearly supports that in the PP-PS mutant, the enhanced photo-inducibility, which was arisen by a partially defective PSII, was related with the high-light and high-temperature stresses, hence accelerating an intracellular ROS generation.

In our system, the temperature in the culture medium was not controlled during outdoor spring or summer cultivation because the temperature control process is usually costly and energy consuming in a microalgal industry. The cost might be reduced using heat-utilizing technology. It has been reported that excessive heat stress negatively affects the autotrophic astaxanthin production in *H. pluvialis* [7,30,48,49], particularly during outdoor cultivation in the summer [7,30]. Under the summer conditions, the vegetative growth was severely interrupted in the green stage and the inductive growth and inducibility for the biosynthesis of astaxanthin were inhibited in the red stage. Our previous study solved this problem by inoculating the red cyst cells to maintain relatively moderate inner

carotenoid levels during the green stage and supplementing 50 μM Fe^{2+} to induce the heat stress-driven Haber–Weiss reaction by converting less reactive oxygen species ($O_2^{\bullet-}$ and H_2O_2) into more reactive oxygen species ($^1O_2$ and OH•) via the iron (II)-catalyzed Haber–Weiss reaction during the red stage to promote lipid peroxidation, thereby enhancing astaxanthin production under photoautotrophic high-temperature conditions [7,30].

Although many types of PBR, such as flat panel, tubular reactor, and bubble columns, have been extensively proposed as outdoor closed PBR system in the field of microalgal industry, only bubble columns and tubular PBRs have proven to be capable of scaling up to high volumes [50]. In the present study, the plastic film bubble column PBR, which is made from cheap materials, is cost-effective, easy to manufacture, and could be recycled several times if it is not contaminated yet. However, the scalability of the thin-film PBR could not be higher than the other bubble column PBRs made from more robust plastic or glass materials because the height of the reactor was normally limited to a relatively low level due to the low durability. The leakage of culture medium often occurred when the height of the reactor was higher than 180 cm. The height limit of the PBR might lead to an increase in the land lease fee, thereby restricting scalability of the microalgal process.

Although optimal autotrophic conditions for microalgal cultivation using the simulated gas containing CO_2 have been thoroughly studied, articles about the actual application of industrial flue gas to microalgal cultivation are still scarce, paticularly about photobiroeactor for maximizing the mass transfer of CO_2 to cells in an aqueous environment, as shown in Table 4. Various types of photobireactors, such as airlift, column, and the thin-film column, have been proposed to mitigate CO_2 from flue gas [4–6,8,22–28,51]. The variation in the unit working volume of each PBR was 1.5 to 1000 L for airlift PBR, 0.18 to 50 L for column PBR, and 5 L to 100 L thin-film column PBR. As mentioned previously, the majority of targeted products based on the flue gas strategy was biofuels, such as biodiesel, because microalgae could easily absorb and adsorb toxic molecules, such as heavy metals derived from the flue gas. In this study, the LNG-fired flue gas was directly used without further purification in this study. Nonetheless, the critical amount of heavy metals detrimental to health was not detected from the *Haematococcus* extracts (Table 2).

Although various combined physical/chemical methods have been proposed to extract astaxanthin from *H. pluvialis* due to the unique structure of robust cell wall and rigid cell membrane in cyst cells [52,53], the cells were easily homogenized by ball mill treatment using zirconia beads without an organic solvent. Therefore, acetone could not be detected by compliance testing from KFDA. Above all, microbial contaminants also could not be detected. It is implied that the HClO water-based contamination control technology is highly efficient for quality control of the outdoor astaxanthin production process of *H. pluvialis*.

The mixture powder of ruptured *H. pluvialis* biomass and astaxanthin extract, which was derived from the LNG-fired flue gas, successfully improved the immune system of poultry without exhibiting any side effects in this study. It has been previously reported that when the egg-laying hens uptake the microalgal biomass, omega-3 fatty acids (ALA, EPA, and DHA) as well as astaxanthin, they are not easily digested, but accumulate in the yolk of an egg laid by the poultry [54–58]. The recommended daily intake of astaxanthin is about 5 mg day^{-1} for human. The astaxanthin and omega-3 containing eggs could be a popular choice for everyone at dining tables, thereby increasing a probable profit of the related corporations with large industrial point sources emitting considerable waste CO_2.

Table 4. Comparision of the performance of the bimass production and CO_2 removal rate from microalgae using industrial flue gas under different stragegies.

PBR Type	Culture Volume	Microalgae	Targeted Products	Biomass Productivity ($g\ L^{-1}\ day^{-1}$)	CO_2 Removal Rate [1]	Flue Gas Composition (Autotrophic Condition)	References
Open thin-layer PBR	330 L	*Chlorella vulgaris* P12	-	22.8 $g\ m^{-2}\ day^{-1}$	42.8 (Calculated)	7% CO_2, 9% O_2, 27 ppm NOx, 2 ppm CO (LNG-fired heat and power plant)	[22]
Airlift PBR	5.5 L	*Chlorella emersonii*	Biodiesel	0.14	0.23 (Presented)	15% CO_2 (cement plant, coal-fired)	[23]
Air-lift PBR	100 L	*Scendenesmus obliquus* (mutant WUST4)	Biodiesel	0.11	0.21 (Calculated)	18% CO_2, 2% O_2, 200 ppm Sox, 150 ppm NOx	[24]
Airlift PBR	1000 L	*Scenedesmus acutus* UTEX B72	Biodiesel	23 $g\ m^{-2}\ day^{-1}$	43.24 (Calculated)	9% CO_2, 53 ppm NOx, 28 ppm Sox (coal-fire power plant)	[5]
Column PBR	0.18 L	*Chlorella vulgaris* P12	Biodiesel	2.5	4.4 (Presented)	13% CO_2, 10% O_2 (waste-fired power plant)	[25]
Column PBR	0.8 L	*Chlorella* sp. (mutant MB-9)	Biodiesel	0.21	0.39 (Calculated)	20% CO_2, 70% CH_4, 50 ppm or below H_2S (desulfied biogas)	[4]
Column PBR	3 L	*Chlorella* sp. C2	Biodiesel	0.093	0.17 (Calculated)	15% CO_2, 300 ppm NO (caprolactam production plant)	[26]
Column PBR	30 L	*Dunaliella tertiolecta* (UTEX LB999)	-	2.4×10^9 cells $L^{-1}\ day^{-1}$	-	8% CO_2, 20 ppm NOx (cogeneration power plant)	[27]
Column PBR	50 L	*Chlorella* sp. MTF-15	Biodiesel	0.515	0.968 (Calculated)	23% CO_2, 4% O_2, 78 ppm NOx, 87 ppm Sox (coke oven in steel corporatioin)	[28]
Customized PBR	1 L	*Desmodesmus abundans* UTEX2976	Biodiesel	0.227	0.416 (Presented)	25% CO_2, 800 ppm NOx, 200 ppm SOx (cement plant)	[6]
PET PBR	15 L	*Spirulina* sp. J774A.1	Polysaccharide, C-phycocyanin	0.118	0.22 (Calculated)	12% CO_2 (coal-fired power plant)	[51]
Thin-film column PBR	5 L	*Haematococcus pluvialis* NIES-144	Astaxanthin	0.065	0.147 (Calculated)	3.5% CO_2, 10% O_2, 20 ppm NOx, 3 ppm CO (LNG-fired heat and power plant)	[8]
Thin-film column PBR	100 L	*Haematococcus pluvialis* (mutant M160)	Astaxanthin	0.127	0.187 (Calculated	3.5% CO_2, 10% O_2, 20 ppm NOx, 3 ppm CO (LNG-fired heat and power plant)	This study

[1] The CO_2 removal rate was calculated as follows: Biomass productivity ($g\ L^{-1}\ day^{-1}$) × Biomass conversion factor (1.88) [59].

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Author Contributions: M.E.H. and W.S.C. have equally contributed to developing the concept and drafted the manuscript. A.K.P. provided academic feedback on the study and revised the manuscript. M.S.O. and J.J.L. provided the raw data obtained by performing outdoor *H. pluvialis* cultivation using LNG-fired flue gas. S.J.S. directed the research process and supervised the overall work. All authors read and approved the final manuscript.

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