The potential application of plasma-activated water for growth of *Chlorella* sp.

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Abstract: A microalgal *Chlorella* sp., cultivated in plasma-activated water (PAW) was investigated. The contents of chemical oxygen demand, nitrate nitrogen, and ammonia nitrogen in PAW were significantly increased by 11.2-, 2.4- and 2.1-folds higher than that of original water. By controlling the initial pH and adding 25% to 100% nutrients in PAW, the microalgal biomass productivity were 11.8- to 19.7-fold higher than without adding nutrients. The results show that there is a potential application for *Chlorella* sp. growth using PAW.

Keywords: *Chlorella* sp., plasma-activated water, biomass productivity.

1. Introduction

Atmospheric-pressure plasma treatment is an emerging non-thermal energy disinfection and surface modification technology, which is environmentally friendly without using chemicals, it has been applied in the fields of biology, medicine and agriculture [1,2]. Plasma treatment of water, known as plasma-activated water (PAW), creates an acidic environment, leading to changes in redox potential, electrical conductivity, and the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can react with bacterial structural components and following organelles, leading to death [3,4]. ROS and RNS are the main sources of active substances in tissue engineering, including sterilization, microbial adhesion and culture. ROS and biological cells are two-sided. On the one hand, ROS can oxidize any type of biological compound, producing deleterious oxidative damage, which are commonly explored in studies of the bactericidal effects of atmospheric plasmas [2,5]. On the other hand, they act as cell signalling molecules, triggering important physiological responses such as carotene production in green microalgae [6,7].

In addition, nitrogen is one of the key components that affect the growth of microalgal biomass, and is an important component of structural and functional proteins [8]. Common nitrogen sources added in microalgal cultivation are nitrate, nitrite, urea and so on. However, few studies have studied the usage of PAW as the nitrogen source in microalgal cultivation. To explore the feasibility of using tap water treated with atmospheric pressure plasma for microalgae cultivation in this study. The microalgal biomass can be further applied as the feedstock of biodiesel and extracted the functional components, such as lutein, zeaxanthin, protein, to produce the high economic value products [9].

2. Materials and Methods

2.1 Microalgal cultures, media and chemicals

The freshwater microalga *Chlorella* sp. was obtained from the Bioresource Collection and Research Center in Taiwan and was used in the study. A modified freshwater medium was used as the microalgal culture medium and was composed of the following (per liter): 0.5 g KNO_3, 0.5 g $NaNO_3$, 0.5 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 60 mg $ZnSO_4{\cdot}7H_2O$, 50 mg H_3BO_3, 40 mg $CaCl_2{\cdot}2H_2O$, 20 mg $Fe_2(NH_4)_3(C_6H_5O_7)_3$, 15 mg $CuSO_4{\cdot}5H_2O$, 10 mg $MnCl_2{\cdot}4H_2O$, 5 mg Na_2MoO_4 and 5 mg $CoCl_2{\cdot}6H_2O$. The initial pH media were made by NaOH addition.

2.2 Preparation of plasma-activated water (PAW)

PAW was made by tap water with atmospheric-pressure plasma treatment. Each time 40 mL tap water was treated with atmospheric-pressure plasma jet (100 W, 5 slm Argon) for 20 min. The pH of tap water after atmospheric-pressure plasma treatment was approximately 3.5.

2.3 Preparation of the inoculum

For inoculation, a stock culture of *Chlorella* sp. was incubated in a 250-mL flask containing 1 L working volume of the modified freshwater medium. The culture was maintained at a temperature of 28 ± 1 ℃ with a light intensity of 4,300 lux. The logarithmic phase of *Chlorella* sp. was used as the initial inoculum, which was diluted in the fresh medium to achieve the biomass concentration of 5 mg/L for further experiments.

2.4 General culture conditions

The microalgal cells were cultured in the 250-mL flask with 50 mL of working volume under approximately 4,300 lux of surface light intensity provided by continuous cool-white fluorescent lights. The microalgal cultures were shaken at 130 rpm at 28 ± 1°C. The initial microalgal biomass concentration in fresh cultures was approximately 5 mg/L. The microalgal cells in each culture were sampled every 48 h to determine the biomass concentration.

2.5 Batch cultivations

To evaluate the potential of plasma-activated water for growth of *Chlorella* sp., the microalgal cells were cultured in PAW with 0%, 25%, 50%, 75%, 100% modified medium addition without controlling initial pH for 4 days. To investigate the microalgal growth of the *Chlorella* sp., the microalgal cells were cultured in PAW with 0%, 25%, 50%, 75%, 100% modified medium addition and controlling initial pH 7 for 12 days. Compared with the growth profile of the *Chlorella* sp. cultured in the modified freshwater medium without atmospheric-pressure plasma treatment.

2.6 Determination of microalgal cell biomass

According to a previously reported method [10], a calibration equation of optical density versus biomass concentration (dry weight per liter) was established for *Chlorella* sp. by measuring their optical density at a wavelength of 680nm (A_{682nm}) in the absorbance range of 0.1–1.0, as follows in equation (1).

$$Biomass\ conc.(g/L) = 0.2852 \times A_{680nm} - 0.0041 \quad (1)$$

The dry weight per liter of microalgal biomass concentration of *Chlorella* sp. were precisely calculated ($R^2 = 0.997$; $p < 0.001$). The cultured sample of microalgae was adequately diluted to detect its absorbance.

The biomass productivity (g/L/d) of *Chlorella* sp. was calculated as follows, as shown in equation (2).

$$Biomass\ productivity\ (g/L/d) = \frac{(W_2 - W_1)}{(t_2 - t_1)} \quad (2)$$

where W_2 and W_1 are the biomass concentrations (g L^{-1}) on t_2 and t_1 (days), respectively.

2.7 Determination of chlorophyll a, chlorophyll b, and total chlorophyll concentrations in microalgal cells

According to the methods of chlorophylls by UV-Vis spectroscopy [11], the chlorophyll a and chlorophyll b of *Chlorella* sp., which was extracted by pure methanol, was determined by measuring their optical density at a wavelength of 665.2nm ($A_{665.2nm}$) and 652.4nm ($A_{652.4nm}$) in the absorbance range of 0.1–1.0, as follows in equation (3) and equation (4).

$$Chlorophyll\ a\ (\mu g/mL) = 16.72 \cdot A_{665.2nm} - 9.16 \cdot A_{652.4nm} \quad (3)$$
$$Chlorophyll\ b\ (\mu g/mL) = 34.09 \cdot A_{652.4nm} - 15.28 \cdot A_{665.2nm} \quad (4)$$

The total chlorophyll productivity (μg/g/day) was calculated as follows in equation (5).

$$Total\ chlorophyll\ productivity\ (\mu g/g/day) = (Chlorophyll\ a + Chlorophyll\ b)/biomass\ productivity \quad (5)$$

2.8 Components analysis in plasma-activated water

The resulting plasma-activated water was analyzed for chemical oxygen demand (COD), ammonia-N (NH_4^+-N), and nitrate-N (NO_3^--N) according to the corresponding analytic reagents and manual of Lovibond MultiDirect Photometer (Tintometer GmbH, Dortmund, Germany).

2.9 Statistics

All values were expressed as the mean standard deviation (±SD). The data were compared using the one-way analysis of variance (ANOVA) test to evaluate the differences between multiple groups. The differences were considered statistically significant when $p<0.05$. Statistical analysis was performed using statistical software (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1 Tap water treated with atmospheric plasma

PAW was produced from tap water with atmospheric plasma treatment, and the COD, NO_3^--N, and NH_4^+-N contents of PAW were 280, 24, and 2.1 mg/L, respectively, as shown in **Table 1**. Compared with tap water without atmospheric plasma treatment, the COD, NO_3^--N, and NH_4^+-N contents of PAW were increased 11.2, 2.4, and 21-fold, respectively. The higher COD contents mean that the higher chemically oxidized-organic matters in water bodies could be used for microalgal growth, and the same is true for NO_3^--N, and NH_4^+-N. Therefore, the studies about a decrease of COD, NO_3^--N, and NH_4^+-N contents of wastewater were also investigated by microalgae [9,12,13,14].

Table 1. Nutrient analysis in PAW and tap water.

Components	PAW (mg/L)	Tap water (mg/L)
COD	280	25
NO_3^--N	24	10
NH_4^+-N	2.1	0.1

3.2 *Chlorella* sp. growth without controlling initial pH

To investigate the potential application of PAW for microalgal cultivation, *Chlorella* sp. was cultured in PAW with 0% (C1), 25% (C2), 50% (C3), 75% (C4), 100% (C5) modified medium addition. As shown in **Fig. 1**, compared with the growth profile of the *Chlorella* sp. cultured in the modified freshwater medium, the growth of *Chlorella* sp. cultured in PAW with modified medium addition was ineffective. For each group containing PAW, the *Chlorella* sp. stopped growing after 1 day of culture. This probably occurred because pH is too low to inhibit the microalgal growth, although the pH was increased with modified medium addition (**Fig. 2**). Each microalgal species has an optimal pH range for growth, and an acceptable range for most microalgal species is pH 7–9 [15,16,17].

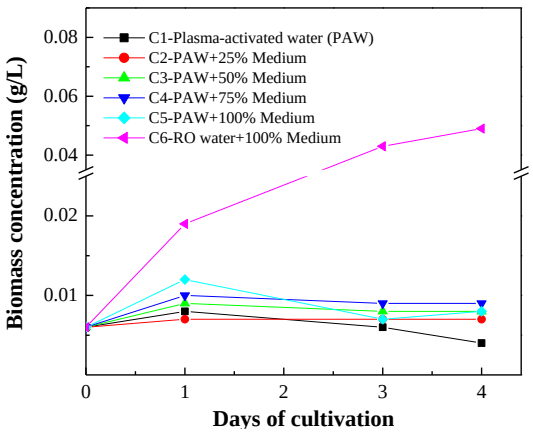

Fig. 1. Growth profiles of *Chlorella* sp. cultured in PAW with 0% (C1), 25% (C2), 50% (C3), 75% (C4), 100% (C5) modified medium addition, and cultured in the modified freshwater medium (C6). The microalga was cultured in 4300 lux light intensity, 130 rpm, and 28 ± 1 °C for 4 days.

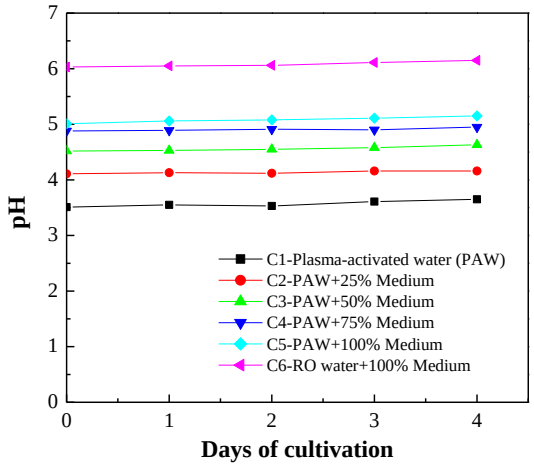

Fig. 2. The pH profiles of *Chlorella* sp. cultured in PAW with 0% (C1), 25% (C2), 50% (C3), 75% (C4), 100% (C5) modified medium addition, and cultured in the modified freshwater medium (C6). The microalga was cultured in 4300 lux light intensity, 130 rpm, and 28 ± 1 °C for 4 days.

3.3 *Chlorella* sp. growth with controlling initial pH 7

It is probably caused the growth inhibition of microalgae because the pH of PAW is too low to 3.5. Therefore, when using PAW as aquaculture water, the growth of *Chlorella* sp. cultured in PAW with 0% (A1), 25% (A2), 50% (A3), 75% (A4), 100% (A5) modified medium addition with controlling the condition of initial pH=7 is investigated. As shown in **Fig. 3**, from the results of group A1 without adding medium in PAW, the growth of *Chlorella* sp. was inhibited because of the low content of nutrient sources. However, when the proportion of nutrient sources in the PAW gradually increased, the growth of *Chlorella* sp. in groups A2, A3, and A4 increased significantly after the 6 days of cultivation. The possible reason was that the forming highly reactive free radicals (H^-, HO^-, and HOO^-) in the PAW caused the slow growth of microalgae despite controlling initial pH 7 [18,19]. When the nutrient source is enough, it helps to reduce the inhibition of microalgae growth by free radicals in PAW. There is no difference in the growth of group A5 using PAW and group A6 using RO water under 100% medium addition, and the microalgae have no hysteresis in the initial stage of culture. This result also indicates that the free radicals in the PAW can be eliminated by the addition of the modified medium, which will not inhibit the growth of *Chlorella* sp., and the PAW can be used in the cultivation of *Chlorella* sp. The pH of microalgal culture will gradually rise when the medium addition in PAW was less than 50%. Hence, the overall microalgal culture is neutral when higher contents of medium addition, as shown in **Fig. 4**. It is speculated that the compositions of the medium could help to maintain the pH stability of the medium. The total concentration of chlorophyll increases with the increase of the contents of medium added in PAW, mainly chlorophyll a (**Fig. 5**). Among the total chlorophyll, chlorophyll a is the primary photosynthetic pigment, and chlorophyll b is the accessory pigment, which are responsible for absorbing light energy and transferring it to chlorophyll a for photosynthesis [20]. The results of this study show that the ratio of chlorophyll a to total chlorophyll in microalgae produced in PAW is 74~82% slightly higher than that in RO water is 64%. Therefore, in the future, it is possible to further study whether PAW has the effect of increasing the specific functional components in *Chlorella* sp. It can be clearly shown in **Table 2** that the use of PAW with controlling initial pH 7 could improve the growth of *Chlorella* sp. With controlling initial pH 7 in PAW, the biomass productivity of *Chlorella* sp. was increased with the total chlorophyll productivity. The results mean that the usage of PAW is not only without inhibition of microalgal growth but also without inhibition of pigment synthesis.

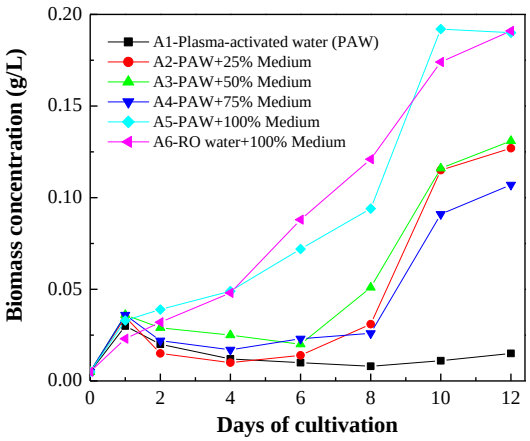

Fig. 3. Growth profiles of *Chlorella* sp. cultured in PAW with 0% (A1), 25% (A2), 50% (A3), 75% (A4), 100% (A5) modified medium addition, and cultured in the modified freshwater medium (A6). All microalgal cultivation was controlled initial pH 7. The microalga was cultured in 4300 lux light intensity, 130 rpm, and 28 ± 1 °C for 12 days.

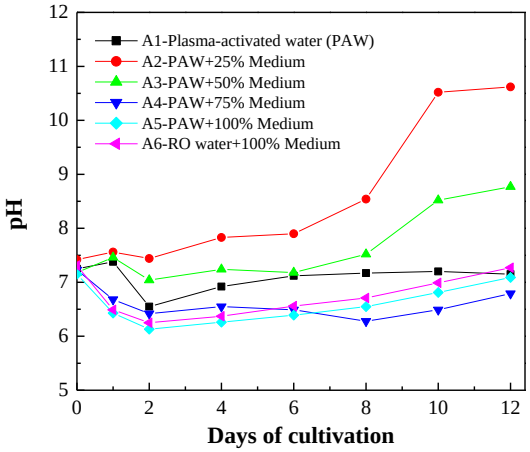

Fig. 4. The pH profiles of *Chlorella* sp. cultured in PAW with 0% (A1), 25% (A2), 50% (A3), 75% (A4), 100% (A5) modified medium addition, and cultured in the modified freshwater medium (A6). All microalgal cultivation was controlled initial pH 7. The microalga was cultured in 4300 lux light intensity, 130 rpm, and 28 ± 1 °C for 12 days.

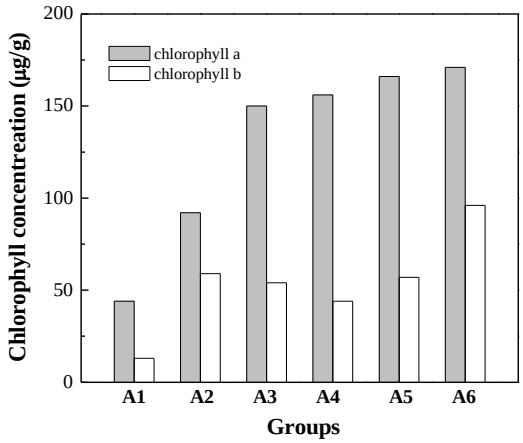

Fig. 5. The chlorophyll a concentration and chlorophyll b concentration of *Chlorella* sp. cultured in PAW with 0% (A1), 25% (A2), 50% (A3), 75% (A4), 100% (A5) modified medium addition, and cultured in the modified freshwater medium (A6) at day 12. All microalgal cultivation was controlled initial pH 7. The microalga was cultured in 4300 lux light intensity, 130 rpm, and 28 ± 1 °C.

Table 2. Biomass productivity and total chlorophyll productivity of *Chlorella* sp. biomass cultured without controlling initial pH and with controlling initial pH 7.

Groups	Biomass productivity (mg/L/day)	Total chlorophyll productivity (μg/g/day)
Without controlling initial pH		
PAW	0.08 ± 0.11	ND
PAW+25% medium	0.22 ± 0.14	ND
PAW+50% medium	0.45 ± 0.15	ND
PAW+75% medium	0.64 ± 0.15	ND
PAW+100% medium	0.41 ± 0.08	ND
RO water +100% medium	8.91 ± 0.38	10.1 ± 0.8
With controlling initial pH 7		
PAW	0.9 ± 0.3	4.4 ± 0.3
PAW+25% medium	10.1 ± 0.4	12.7 ± 0.7
PAW+50% medium	10.9 ± 0.4	16.6 ± 0.4
PAW+75% medium	9.3 ± 0.9	17.1 ± 0.5
PAW+100% medium	17.1 ± 1.5	18.7 ± 0.5
ROW+100% medium	16.1 ± 0.6	22.2 ± 0.7

ND: Non-detected.

4. Conclusions

After the tap water was treated by atmospheric-pressure plasma, the COD, NO_3^--N, and NH_4^+-N of PAW are significantly increased by 2 to 10-folds compared with that without atmospheric-pressure plasma treatment, and an main increase is COD concentration. When *Chlorella* sp. was cultivation in PAW with 100% medium addition and initial pH 7, it has no hysteresis in the initial stage of cultivation, and without inhibition microalgal growth. In addition, the synthesis of total microalgal chlorophyll was no affected, the chlorophyll a was mainly increased during microalgal cultivation.

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