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The inhibition effect of recycled *Scenedesmus acuminatus* culture media: Influence of growth phase, inhibitor identification and removal

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ABSTRACT

Existing research shows that the presence of algogenic organic matter (AOM) in recycled media can inhibit the growth of algae. However, the characteristics and occurrence of the inhibitors are not well understood. In this study, changes in the algogenic organic matter present in culture media and the influence of these changes on the recultivation of *Scenedesmus acuminatus* were investigated. A hydrophobic humic substance was then extracted from the recycled medium, and its inhibitory effects on the growth of *Scenedesmus acuminatus* were assessed. The efficiency of the removal of the humic substance from the recycled media using granular activated carbon (GAC) treatment was evaluated. The results showed that the later the growth phase at which the recycled media was harvested, the stronger the growth inhibition was. In addition, it was found that the percentage of total carbohydrates, fatty acids, and proteins in the algogenic organic matter decreased with the prolongation of growth phase, while the percentage of the hydrophobic humic substance in algogenic organic matter increased as the algae gradually went into the declining phase. Furthermore, an inhibitory effect of the hydrophobic humic substances on the growth of *Scenedesmus acuminatus* was identified. The effectiveness of inhibitor removal using granular activated carbon column adsorption was confirmed. The results of this study can be applied to the sustainable utilization of water and nutrients in the mass production of microalgal biomass.

1. Introduction

Because algal cells are rich in lipid, protein, pigments, vitamins, polysaccharides and other bioactive substances, they are increasingly valued by the biofuels, drug, food, and cosmetics industries [1–5]. In addition, due to their highly-efficient absorption of nitrogen, phosphorus and heavy metals, algae are increasingly used in wastewater treatment and ecological restoration [6,7]. However, life-cycle analysis studies of microalgae biomass production have shown that, large amounts of water are required for the production of microalgal biomass. It has been reported that 1564 kg of water is required to produce 1 kg of algae through ponds [8]. In fact, the total cost of fresh water and wastewater treatment in open ponds and photobioreactors is significantly reduced if the algal culture media is recycled [9]. The recycling of algal culture media is considered essential to the sustainable development of the microalgae industry [10].

To date, many studies have shown that recycled media inhibits algae growth, compared with fresh media. Zhang et al. reported that recycled media from the stationary phase contained soluble algal products at concentrations ranging from 6.4 to 25.8 mg C L^{-1} that resulted in decreases of 50–80% and 35–70% in maximal cell number and maximal productivity of *Scenedesmus sp.* LX1, respectively, when compared with the use of fresh media [11]. Growth inhibition may gradually become evident as the number of times the culture media is recycled increases. Zhu et al. reported a decrease of 78% in dry weight with the use of recycled media obtained in the stationary phase after the third recultivation cycle of the wastewater culture of *Chlorella zofingiensis* [12]. Reduction in the biomass yield of algae may be one

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aspect of the negative effects of using recycled media. On the other hand, the lipid content, protein content and total fatty acid content of cells grown in the recycled media were reduced [13,14].

However, not all recycled media show inhibitory effects, and many studies have shown that the growth of algae in some recycled media is not affected. Das et al. found that recycled media obtained from the exponential phase had no significant impact on the growth of *Scenedesmus* sp. in a 1000-L raceway tank [15]. Similarly, Wu et al. harvested the *Scenedesmus* sp., *Chlorella vulgaris*, *Chlorococcum* sp., *Phaeodactylum tricornutum*, and *Nannochloropsis oculata* by high pH-induced flocculation in glass column photobioreactors. The results of recultivation showed that the use of recycled media did not affect the algal biomass [16]. Moreover, Xiong et al. used supernatants of *Dunaliella salina* obtained by the sand-enhanced electro-flocculation from the late exponential phase as a recycled media and found that the final cell number was not affected when the cells were grown in recycled media in a 2.5-L airlift bioreactor [17].

Some of the low-molecular-weight organic matter that is released into the media by algae can even promote algal growth. Sabia et al. found that the growth of *Neochloris oleoabundans* in 20-L capacity photobioreactor using recycled medium was enhanced compared with that of controls grown in fresh media, possibly due to the generation of the growth promoter polyamine (PA) [18]. Grabski & Tukaj suggested that a low-molecular-weight peptide or glycopeptide released by *Scenedesmus subspicatus* may produce growth stimulation in nutrient-supplemented recycled media [19]. Furthermore, recycled media from the exponential phase showed significant promotion of final biomass and algal growth when harvested by pretreatment methods, such as coagulation-flocculation (polyaluminum chlorosulfates such as chitosan at 80% and cationic polyacrylamide) and filtration (0.2 μm) [20,21].

These studies of culture media recycling indicate that the occurrence of inhibitory effects during recultivation requires certain conditions; the factors influencing the recultivation of algae may include the genera employed, the culture conditions, the harvesting phase, and the harvesting or pretreatment methods used. The study of Loftus & Johnson shows that the growth phase at which the recycled media is obtained may be an important factor influencing the recultivation of algae. Their research integrated data across 86 studies and showed that the effects of the use of recycled media on algae growth are often situation-specific and are not associated with environmental variables or harvesting methods; hence, some genera and growth phases appear to be more suitable for media recycling [22]. However, no systematic in-depth research on the mechanism through which growth phase influences recultivation in recycled media has been conducted. Algogenic organic matter (AOM) in recycled media has been identified as the major factor inhibiting in the growth of algae in many studies [11,13]. However, it is not clear how the algogenic organic matter (which includes proteins, lipids, carbohydrates, fatty acids, and humic substances) will affect recycling when obtaining media from different growth phases for recultivation in the media recycling process. The effect of AOM on cell growth in recycled media is a key to understanding the mechanism through which the growth phase at which the medium is harvested influences recultivation. Understanding AOM is also extremely challenging, because the composition and character of AOM are very complex and variable. In this study, *S. acuminatus* was cultivated to various growth phases and harvested by filtration through a 0.2-μm membrane. The concentrated filtrate, which had a total organic carbon (DOC) concentration distinct from that of fresh media, was spiked into the fresh BG11 media, and its effects on the growth of *S. acuminatus* were evaluated. The main objectives of this study included: 1) the characterization of the changes in the composition of AOM in media harvested from cultures at different growth phases to allow for growth inhibitor identification; and 2) an evaluation of the effectiveness of inhibitor removal using GAC in the media recycling process.

2. Materials and methods

2.1. Algae cultivation

Scenedesmus acuminatus strain GT-2 was obtained from the State Development and Investment Corporation (SDIC) microalgae biotechnology center. *S. acuminatus* GT-2 was cultured in 15-L indoor panels with a light path of 5 cm using modified BG11 media. In the modified media, the initial $NaNO_3$ concentration was adjusted to 187 mg L^{-1} [23]. The initial cell concentration was 1.6×10^9 per L (0.05 g L^{-1} in dry weight). Light of intensity 60 μmol·m^{-2}·s^{-1} (LI-1500, LI-COR, USA) was continuously supplied and the temperature was maintained at 25 ± 0.5 °C. Air containing 3% CO_2 was bubbled continuously into the reactors at a rate of 6 L min^{-1}. The pH was maintained at 6.5–7.5 by supplying CO_2.

The algal cell concentration was measured by cell counting using an optical microscope and the dry weight was measured using the gravity method every other day.

2.2. Characterization of algogenic organic matter (AOM)

At the end of the cultivation period, the algal suspension was harvested by centrifugation (3000 *g*, 10 min), prior to filtration through a 0.2-μm PES membrane. Harvesting was performed during the exponential phase (4th day), the stationary phase (14th day) and the declining phase (28th day) to obtain the recycled media.

The recycled media was concentrated from 1 L to 50 mL using a rotary evaporator (R215, BÜCHI Labortechnik AG, Flawil, Switzerland) at 25 °C and a pressure of 2 kPa. The total organic matter content of the recycled media was determined as dissolved organic carbon (DOC) using a total organic carbon analyzer (TOC-L CPH, Shimadzu Corp., Kyoto, Japan). The carbohydrates, proteins, fatty acids and humic substances contents of the recycled media obtained from different growth phases was further quantified using the methods described below.

The carbohydrate content of the recycled media was determined by ion chromatography. 5 mL of 2 M trifluoroacetic acid was added to the 5 mL 50 times concentrated recycled media, and the sample was hydrolyzed for 4 h in water of 100 °C. The hydrolysate was dried with a nitrogen blowing apparatus to remove trifluoroacetic acid, and then deionized water was added to re-dissolve the hydrolysate. After that the hydrolysate was then filtered by a 0.22-μm PES membrane. The carbohydrate content of the filtrate was analyzed by using ICS 5000+ ion chromatography (Dionex, Sunnyvale, CA, USA). Ten monosaccharides (sorbitol, mannitol, fucose, rhamnose, galactosamine, glucosamine, galactose, glucose, mannose, ribose) were used as standard samples and the content of total carbohydrates was calculated as the sum of the contents of the ten monosaccharides [24].

The protein content of the recycled media was measured by using Coomassie Brilliant Blue G-250 dye and a Hach Model DR 6000™ spectrophotometer (Hach, Loveland, CO, USA) [25]. Bovine serum albumin (BSA) concentrations of 0.5, 1, 2, 5, 10 mg L^{-1} were used as the standard samples to establish the standard curve.

To quantify the fatty acid, total lipids were extracted from 100 mL of recycled media using 10 mL chloroform/methanol solution (2/1, v/v) three times for 10 min in 200-mL glass tubes; the extracts were then combined and evaporated under nitrogen. The lipids were transesterified into fatty acid methyl esters (FAME) which were quantified by a gas chromatograph-mass spectrometer (GC–MS) (Agilent Technologies, Santa Clara, CA, USA) [26]. Sample was acidified to pH 2 and solved in the n-hexane for GC/MS analysis. The injector temperature was set at 250 °C. The oven temperature was initially maintained at 55 °C for 1 min and then increased at 25 °C min^{-1} to 200 °C, where it was held for 2 min and further increased at 5 °C min^{-1} to 260 °C. The MS quadrupole temperature was set at 150 °C and the ion source temperature at 250 °C.

2.3. Evaluation of recycled media on the growth of S. acuminatus

The residual nutrient ions in the recycled media were removed by dialysis against deionized water for 48 h using a membrane with a molecular weight cut-off (MWCO) of 100 Da (Spectrumlabs, USA). The AOM was concentrated and quantified using TOC analysis. To evaluate the effects of AOM on the growth of *S. acuminatus*, fresh BG11 medium was spiked with known amounts of AOM to obtain the final concentrations of 30 and 100 mg L^{-1}. The growth of *S. acuminatus* was then evaluated using a photobioreactors array consisting of 30-ml glass tubes (Φ3 × 12 cm) [27]. Air containing 3% CO_2 was bubbled continuously into the reactors at a rate of 50 mL min^{-1}. Other cultivation conditions were the same as those described in Section 2.1. Algae cultivated in BG11 media was used as the control group. The medium used for the experimental group that received spiked AOM was replenished to contain the same amount of nutrients present in BG11.

The dry weight of *S. acuminatus* was measured every other day. The F_V/F_M ratio, which is found by dividing the variable fluorescence (Fv) by the maximum fluorescence (Fm) to determine the maximal quantum yield of photosystem II (PS II) and indicates the activity of PS II [28]. The F_V/F_M ratio of the cells was measured at room temperature every other day by using a Dual-PAM-100 portable fluorimeter (Walz, Effeltrich, Germany). The algal cells were placed in a quartz cube and maintained in the dark for 15 min prior to measurement of F_V/F_M.

2.4. Characterization of the hydrophobic humic substance and performance of the growth inhibitory test using S. acuminatus

The hydrophobic humic substance in the recycled media obtained from the declining phase was fractionated by using Φ3 × 20 cm column filled with the XAD-8 resin (Sigma) [29], which preferentially adsorbs humic substances. The humic substance was then eluted from the resin using 0.1 M NaOH and then quantified as DOC.

The humic substance in the recycled media was analyzed by using three-dimensional (3D) fluorescence excitation-emission matrix (FEEM) spectra. FEEM measurements were conducted using a Model G9800A Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Selango, Malaysia) that which has a maximal emission intensity of 1000 arbitrary units (AU). The excitation and emission slits were set to a 10-nm bandpass. Excitation wavelengths between 200 nm and 450 nm were incrementally increased by 5 nm. Emissions at longer wavelengths were detected in 2-nm increments for each excitation wavelength. To limit second-order Rayleigh scattering, a 290-nm cutoff was used for all samples [13].

To evaluate the inhibitory effect of humic substances on the growth of *S. acuminatus*, different amounts of the humic substance were spiked into the culture media to yield final concentrations of 5, 20, 35, and 50 mg C L^{-1}. The pH of the culture was adjusted to 7.0, and the media was replenished with nutrients based on BG11. *S. acuminatus* was cultured in the using a 30-mL photobioreactors array (Φ3 × 12 cm), and the dry weight of the culture was measured every other day.

2.5. Removal of humic substance from recycled media using GAC and evaluation of the removal of algal growth inhibitory effect

Approximately 80 g of granular activated carbon (Norit-1240, Netherlands), the matrix that demonstrated the highest removal efficiency of AOM from the culture media (data not shown), was placed in a column with a length of 70 cm and a diameter of 2 cm. The column was washed with deionized (DI) water until the DOC of the effluent water was < 0.5 mg L^{-1}. The empty bed contact time (EBCT) of the column was 6 min. Recycled media obtained in the stationary phase was then treated by GAC filter used a predetermined flow rate of 2.4 L h^{-1}. The GAC-treated effluent was collected and characterized by FEEM. The DOC and the extracted humic fraction were further quantified using a TOC analyzer.

The effect of the GAC-treated recycled media on algal growth was evaluated using a 30-mL glass column photobioreactors array; the culture conditions were the same as those described in section 2.3. The experiment used fresh BG11 media as the control group and recycled medium and GAC-treated recycled media as the experimental groups. The experimental groups were replenished with nutrients based on BG11. The dry weight was measured every other day, and the results were used to evaluate the inhibitory effect of recycled media on the growth of *S. acuminatus*.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with a least significant difference (LSD) post hoc test was used to evaluate the statistical significance of the differences between the control and experimental groups. In all data analyses, a *P*-value of 0.05 was considered statistically significant. All experiments were repeated three times. The variability of data presented in the figures and tables are shown as ± standard deviation.

3. Results and discussion

3.1. Cultivation of S. acuminatus in the panel photobioreactor

In this research, *S. acuminatus* was cultivated in BG11 media for 28 days in a 15-L photobioreactor. The observed changes in the cell number and dry weight of the cultured *S. acuminatus* are shown in Fig. 1. The maximal cell density of *S. acuminatus* reached 2.48 × 10^{10} cells per L cultivated at 8 days of cultivation and decreased gradually after 22 days of cultivation. The maximal cell number of *S. acuminatus* observed in this study is comparable to the results obtained in a previous study by Zhang et al., in which a maximal *Scenedesmus sp*. LX1 cell density 7.2 × 10^9 cells L^{-1} was reported when the cells were cultured under a light intensity of 60 μmol·m^{-2}·s^{-1} for 15 days [11]. The maximal dry weight observed in this study is in close agreement with the findings of Castrillo et al., who reported a maximal dry weight of *Scenedesmus obliquus* of approximately 1.4 g L^{-1} when the cells were cultured for 12 days in fresh media in 70-L photobioreactors [30]. Dry weight continued to increase, while cell number gradually declined after 16th day. This may be due to the increasing size of individual cells, even if the cell starts to die [31]. According to the study of Barofsky et al., the culture period was divided into an exponential phase (Days 1 to 8), a stationary phase (Days 8 to 22) and a declining phase (Days 22 to 28) based on the cell density [32]. This observation is consistent with the results reported by Oukarroum, who grew *S.*

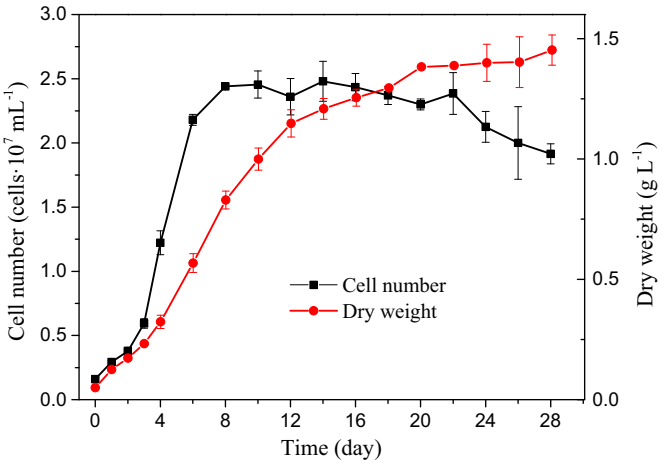

Fig. 1. Changes in dry weight and cell number of *Scenedesmus acuminatus* during the culture period. Values are means ± standard deviation (n = 3).

Table 1
Concentration of organic fractions in recycled media obtained from three growth phases, expressed as the average ± standard deviation of triplicate measurements (n = 3).

	Exponential phase	Stationary phase	Declining phase
Dissolved organic matter (mg C L^{-1})	3.58 ± 0.19	10.58 ± 0.08	14.24 ± 0.05
Protein (mg L^{-1})	0.21 ± 0.01	0.27 ± 0.02	0.46 ± 0.04
Total carbohydrate (mg L^{-1})	2.81 ± 0.14	6.19 ± 0.25	5.41 ± 0.32
Fatty acid (μg L^{-1})	43.4 ± 1.7	62.9 ± 5	77.5 ± 4.6
Humic substance (mg C L^{-1})	0.49 ± 0.02	2.26 ± 0.12	4.28 ± 0.25

obliquus exponentially for 10 days to stationary phase under a continuous illumination of 100 μmol·m^{-2}·s^{-1} [28].

3.2. Characteristics of algogenic organic matters (AOM) obtained from different growth phases

The total organic matter, total carbohydrate, fatty acid, protein and humic substance content in the recycled media obtained from different growth phases is summarized in Table 1. The total protein, fatty acid and humic substance content increased as the growth phase increased, whereas the total carbohydrate content was maximal in the stationary phase, but decreased during the declining phase. Although the total protein, carbohydrate and fatty acid content in the recycled media obtained from the stationary phase and declining phase was higher than that obtained from the exponential phase, the rate at which the content of these substances increased was much lower than the rate at which the total organic matter increased during the growth phase. In other words, the percentage composition of the total organic matter decreased with the growth phase increased. The concentration increased by 195.6% and 297.9% for total organic matter, by 28.5% and 119% for proteins, by 120.3% and 92.4% for carbohydrates, and by 44.9% and 78.5% for fatty acids in the stationary and declining phases, respectively, compared to the exponential phase. In contrast, the percentage composition of humic substances in the total organic matter increased with the growth phase. The concentration of humic substances increased by 361.2% and 773.4% in the stationary and declining phases, respectively, compared to the exponential phase. (Fig. 2).

The results presented in this section show that the percentage composition of many metabolites released by *S. acuminatus* differs in different growth phases. Differences in the metabolite composition of the media in different growth phases were also shown in research on *Chlorella vulgaris*, *Microcystis aeruginosa*, *Asterionella formosa* and *Melosira* sp. [33]. This finding is a key to an understanding of the mechanism through which growth phase influences recultivation.

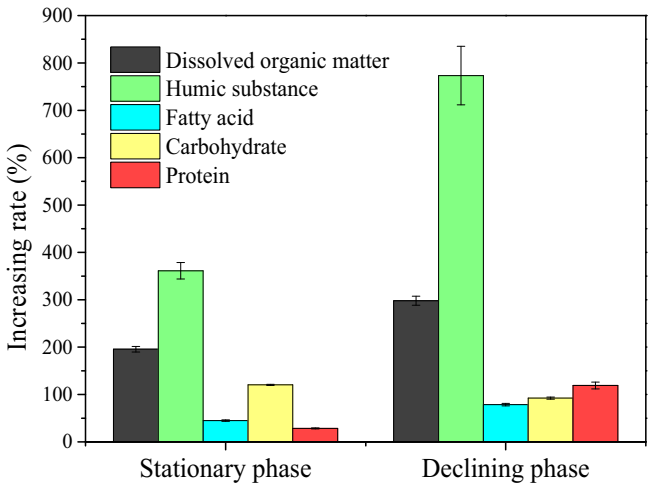

Fig. 2. The increasing rates of organic fractions in recycled media obtained from the stationary and declining phases compared with that obtained during the exponential phase. Values are means ± standard deviation (n = 3).

3.3. Growth of S. acuminatus in the recycled media obtained from different growth phases

Obvious differences in the dry weight of the cultured *S. acuminatus* after 12 days were found when recycled media obtained from different growth phases, with initial AOM concentrations of 30 and 100 mg C L^{-1}, were used (Fig. 3a). When *S. acuminatus* was cultured in fresh media and in recycled media that had an initial AOM concentrations of 30 mg C L^{-1}, the final dry weight achieved using recycled media obtained from the exponential phase was higher than that obtained using fresh media. However, the final dry weights achieved using recycled media obtained from the stationary and declining phases were lower than the final dry weight achieved when fresh media was used. When the recycled media contained 100 mg C L^{-1} AOM, the final dry weight obtained using media from the exponential phase was the same as that obtained using fresh media. However, the final dry weight of cells grown in recycled media obtained from the stationary phase was lower than that of cells grown in fresh media, and the final dry weight of cells grown in the recycled media obtained from the declining phase was the lowest observed.

A 7.9% increase in productivity was observed when recycled media obtained in the exponential phase with an initial AOM concentration of 30 mg C L^{-1} was used compared with fresh media. The culture media harvested in the stationary and declining phases showed inhibitory effects that resulted in 12.3% and 14.6% decreases in productivity, respectively ($p < 0.05$ in both cases relative to fresh media). When the recycled media contained 100 mg C L^{-1} AOM, both the recycled media obtained from the stationary phase and declining phase reduced the growth rate of *S. acuminatus*, and the inhibitory effect increased from 16.9% to 41.9% as the stage from which the recycled media was obtained progressed from the stationary phase to the declining phase ($p < 0.05$ in both cases relative to fresh media) (Fig. 3b). Statistical analysis showed that the use of the recycled media obtained in the stationary or declining phases significantly inhibited of the biomass productivity of *S. acuminatus* ($P < 0.05$). The results in this section show that recycled media obtained in the different growth phases had significantly different impacts on the regrowth of *S. acuminatus*. Harvesting algae in the exponential phase may produce the most suitable media for reuse, whereas media obtained at later phases of growth may be more likely to cause inhibition.

Many studies have indicated that recycled media obtained from the exponential phase has slight stimulatory effects on the growth of algae, while recycled media obtained from the stationary phase and declining phase have significant inhibitory effects on the growth of algae in the culture media recycling process [12,14,34]. Loftus & Johnson integrated data across 86 studies and suggested that the use of recycled media obtained from exponential phase cultures resulted in significantly greater stimulatory probability to biomass concentration than the use of media obtained from stationary-phase cultures. The use of recycled media from earlier growth phases may favor *S. acuminatus* growth because the recycled media contain metabolites that favor growth [22]. Grabski & Tukaj suggested that a low-molecular-weight peptide or glycopeptide released by rapidly dividing cells of

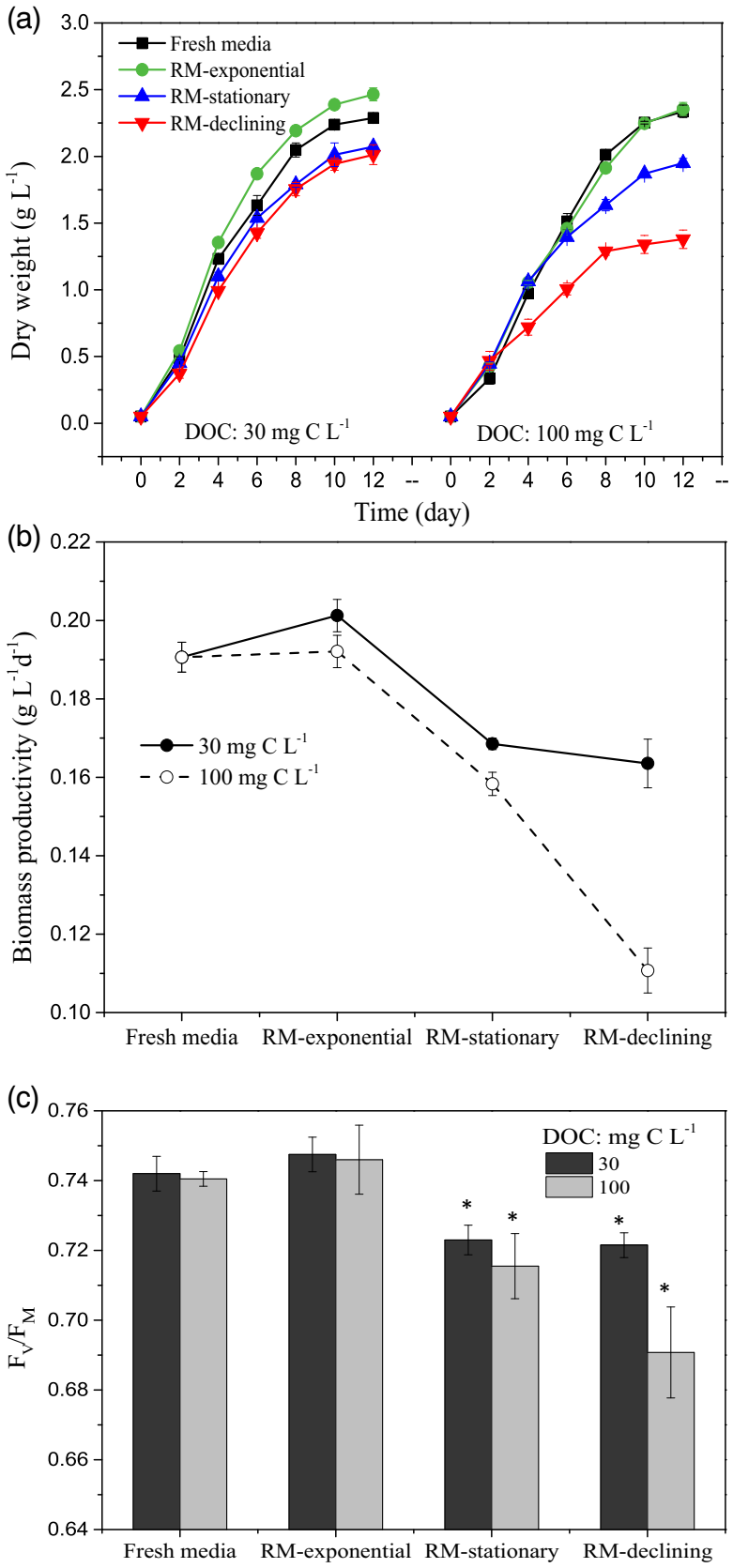

Fig. 3. Changes in the dry weight (a), biomass productivity (b) and F_V/F_M (c) of the *Scenedesmus acuminatus* cultured in recycled media obtained from different growth phases spiked with the initial AOM concentrations of 30 and 100 mg C L^{-1}, respectively. (Note: RM = Recycled media; DOC = Dissolved organic carbon; Values are means ± standard deviation (n = 3); * means $p < 0.05$ relative to fresh media).

Scenedesmus subspicatus may produce growth stimulation in nutrient-supplemented recycled media, and the levels of this peptide were found to be considerably higher in culture filtrates derived from the exponential phase than in filtrates derived from stationary-phase media [19]. Another previous study found that the productivity of *S. acuminatus* in recycled media obtained from the stationary phase was 13.3% lower than that of *S. acuminatus* cultured in fresh media [27]. In this study, the different effects were first revealed and the harvesting phase of the recycled media shown to be a very important factor that was responsible for the controversial effect.

F_V/F_M reflects the ability of algal cells to absorb, transmit and dissipate light energy during the process of photosynthesis and is a good indicator of the physiological state and growth rate of algae [28,35]. Obvious changes in the F_V/F_M ratio of the *S. acuminatus* cultured after eight days were found when the cells were grown in recycled media obtained at different growth phases and spiked with initial AOM concentrations of 30 and 100 mg C L^{-1} (Fig. 3c). At concentrations of AOM of both 30 and 100 mg C L^{-1}, the F_V/F_M ratio of algae grown in recycled media obtained during the exponential phase was the same as the F_V/F_M ratio of *S. acuminatus* samples grown in fresh media. However, the algae grown in recycled media obtained during the stationary and declining phases had a lower F_V/F_M ratio than the *S. acuminatus* samples grown in fresh media. The above results show that growth in recycled media obtained from stationary-phase and declining-phase cultures had a significant inhibitory effect on the F_V/F_M ratio of *S. acuminatus* ($P < 0.05$). When the recycled media contained 100 mg C L^{-1} AOM, the later the harvesting phase, the lower the F_V/F_M ratio was.

Depraetere et al. reported that the recycled media obtained from the stationary phase had organic matter concentrations of up to 104 mg C L^{-1} that resulted in a significant decrease in the F_V/F_M ratio of *Arthrospira platensis* compared with growth in fresh media [35]. Similarly, a significant decrease in the chlorophyll content of cells after day 8 was also found by Liu et al. during the recultivation of *Parietochloris incisa* in recycled media obtained from the declining phase [14]. Thus, the inhibitory effect of recycled media on the photosynthesis by *S. acuminatus* may be due to the production and accumulation of inhibitors produced during the late growth phase.

3.4. Characteristics of the hydrophobic humic substance extracted from the recycled media

As shown in Fig. 2, the percentage composition of humic substances in total organic matter increased as the growth phase increased. According to the above results, humic substances may potentially be related to the growth inhibitory factor. Three-dimensional (3D) fluorescence excitation-emission matrix (FEEM) spectrometry, which has been widely used to analyze the composition of algal organic material (AOM), was applied in this study to characterize the humic substance [13,36]. The FEEM spectra were divided into five regions according to Chen et al. Regions I (Ex/Em: 220–270 nm/280–330 nm) and II (Ex/Em: 220–270 nm/330–380 nm) correspond to the fluorescence response of aromatic proteins (AP) (those containing tyrosine and tryptophan). Regions III (Ex/Em: 220–270 nm/380–550 nm), IV (Ex/Em: 270–440 nm/280–380 nm) and V (Ex/Em: 270–440 nm/ 380–550 nm) are associated with fulvic acid (FA)-like materials, soluble microbial products (SMPs, e.g., proteins and polysaccharide-like materials) and humic acid (HA)-like materials, respectively [37].

The FEEM spectra of the humic substance are shown in Fig. 4. The fluorescent signal of the humic substance, revealed two components and was distributed among excitation wavelengths (EX) of 220–240 nm and 380–410 nm and emission wavelengths (EM) of 400–470 nm; these regions correspond to fulvic acid-like organics and humic acid-like materials, respectively [37]. The structures of the humic substance compounds contain several phenol hydroxyl groups, amino groups, quinone groups, carbonyl groups and methoxy groups; these moieties can affect and control the migration and transformation of various

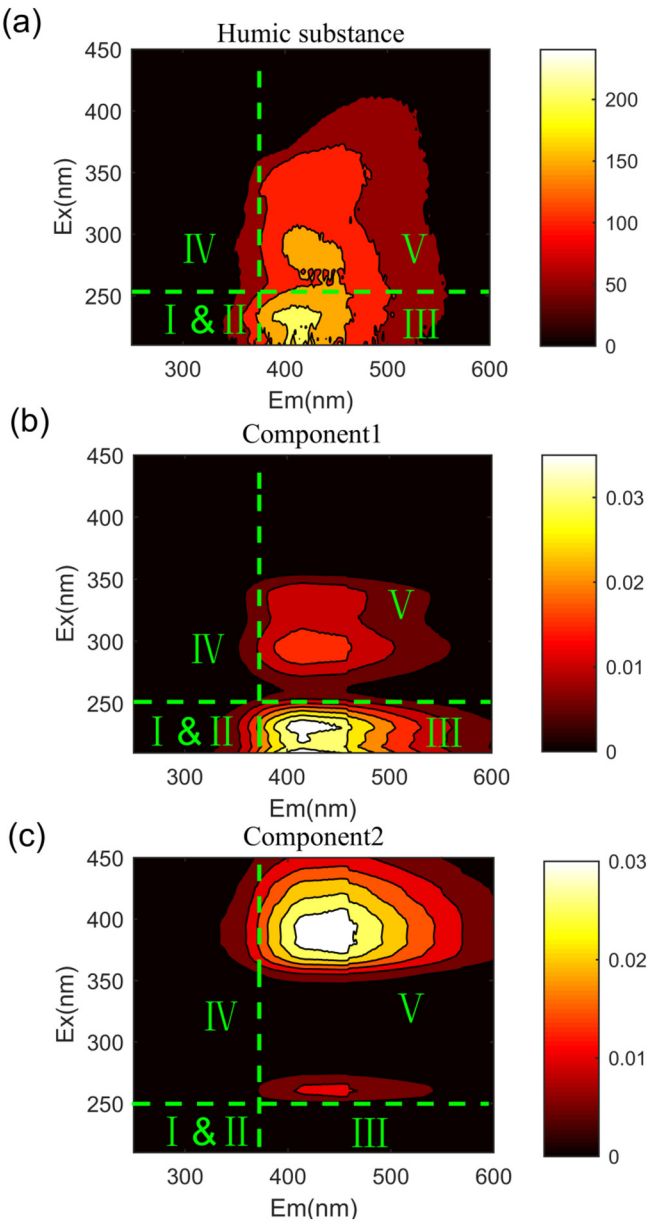

Fig. 4. Three-dimensional (3D) fluorescence excitation-emission matrix (FEEM) spectra of humic substance (a), component 1 (b), and component 2 (c).

organic substances as well as heavy metals in water, which including chemical degradation of organics, photolysis, biological absorption, migration and volatilization [38–40]. Because the humic substance was found to be potentially related to the growth inhibitory factor, further characterization and evaluation of cytotoxicity were performed to identify the effect of the humic substance on the growth of *S. acuminatus*.

3.5. Growth of S. acuminatus exposed to humic substances

Obvious changes in the dry weight of the cultured *S. acuminatus* after 12 days were revealed in our research on the effects of humic substances (Fig. 5a). When the algae were cultured in fresh BG-11 media, to which the humic substance was added at initial concentrations of 0, 5, 20, 35, and 50 mg C L^{-1}, the final dry weights were 2.425 ± 0.075, 2.325 ± 0.072, 2.045 ± 0.063, 1.525 ± 0.097 and 0.755 ± 0.136 g L^{-1} respectively (Fig. 5a). As the concentration of humic substance increased, the final dry weight of *S. acuminatus*

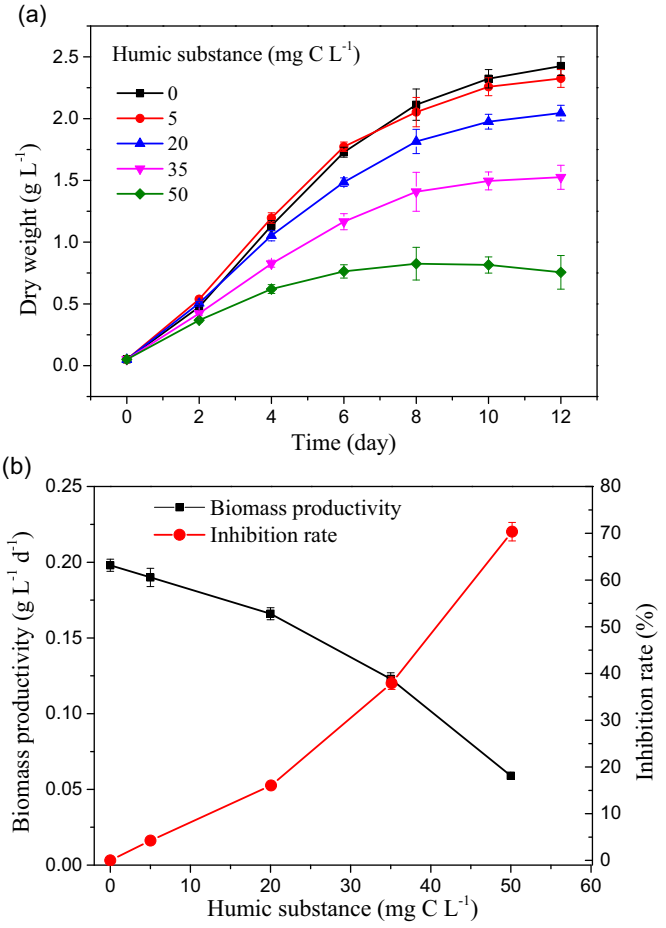

Fig. 5. Changes in the dry weight (a), biomass productivity and inhibition rate (b) of *Scenedesmus acuminatus* exposed to humic substance at different concentrations. Values are means ± standard deviation (n = 3).

gradually decreased. Similarly, the biomass productivity exposed to high humic substance concentrations was much lower (Fig. 5b). The biomass productivity was 0.198, 0.19, 0.166, 0.123 and 0.059 g L^{-1}·d^{-1} at initial humic substance concentrations of 0, 5, 20, 35, and 50 mg C L^{-1}, respectively. Decreases of 4.3%, 16%, 37.9% and 70.3% in biomass productivity were observed in cultures with initial humic substance concentrations of 5, 20, 35, and 50 mg C L^{-1}, respectively ($p < 0.05$ in these cases relative to fresh media) (Fig. 5b). These results show that the presence of the humic substance at certain concentrations has a significant inhibitory effect on the growth of *S. acuminatus* ($P < 0.05$). Notably, the correspondence between the dose of the humic substance and the effect on the growth of *S. acuminatus* were similar to the results obtained when algae grown in recycled media for the same predetermined time periods were tested. This result suggests that the humic substance is a major inhibitory factor in recycled media.

A previous study supported this conclusion and found that the content of humic substances in recycled media decreased by > 50% after advanced oxidation processes (UV/peroxydisulfate and UV/H_2O_2) were used. At the same time, the inhibitory effect of the recycled media on *S. acuminatus* growth disappeared [27]. The results are consistent with the conclusion reached by Sun et al. [41]. Their research reported that the maximal cell concentration of cyanobacterium *Anabaena circinalis* cultured in media containing humic acid (HA) concentrations of 0.1 and 0.3 mg C L^{-1} was approximately 71% and 30%, respectively, of the concentration observed when the cell was grown in the absence of HA [41]. Notably, cyanobacteria are more sensitive than eukaryotic algae to exposure to humic substances, probably due to their simpler cell structure [42]. In addition, significant reductions in dry weight and in maximum PS II quantum yield of Microcystis PCC7806 were also found by Bährs and Steinberg when the concentration of HS1500 was increased from 0.17 to 4.17 mM DOC [43].

3.6. Removal of inhibitors represented by the hydrophobic humic substance in recycled media using GAC and the regrowth of S. acuminatus

Existing studies have proven that adsorption by activated carbon is an effective method for the removal of dissolved organic matters from water and wastewater [44]. After treatment with GAC Norit-1240 at a flow rate of 2.4 L h^{-1}, the DOC in recycled media decreased by 71.4%, from 31.2 ± 1.7 to 8.91 ± 0.54 mg C L^{-1}. The FEEM spectra of the organic matter present in recycled media before and after GAC treatment are presented in Fig. 6a. After GAC treatment, the fluorescence intensity of fulvic acid-like and humic acid-like materials in the recycled media were dramatically reduced. According to the TOC analysis of the humic substance fraction, its concentration in recycled media decreased by 73.2%, from 6.8 ± 0.4 to 1.82 ± 0.11 mg C L^{-1}, after GAC treatment. The results show that GAC filter adsorption is very effective for the removal of growth inhibitors, represented by humic substances, in recycled media. It is reported that GAC filters generally effectively remove natural organic matter (NOM) fractions in the molecular weight range of 500–4000 g mol^{-1}; these fractions contain most of the humic substances [45].

Fig. 6b shows the growth curves of *S. acuminatus* cultured in untreated recycled media, in GAC-treated recycled media and in fresh media. The untreated recycled media showed a significant inhibitory effect, resulting in a 14.3 ± 0.43% decrease in the final dry weight obtained on day 12, from 2.38 ± 0.06 g L^{-1} to 2.04 ± 0.05 g L^{-1} ($p < 0.05$). However, the GAC treatment significantly increased the final dry weight to 2.33 ± 0.04 g L^{-1}, which was almost the same as the dry weight obtained after growth in fresh media ($p > 0.05$). The calculated yield of *S. acuminatus* reached 194 ± 3.7, 190 ± 5 and 166 ± 4 mg L^{-1}·d^{-1} when the cells were cultured in fresh media, recycled media and treated recycled media, respectively, again showing the effectiveness of GAC treatment.

Humic substances can bind Fe ions, which play an important role in photosynthesis. However, Sun et al. indicated that the potential mechanism of the inhibitory effect of cyanobacterium *Anabaena circinalis* was not reduction of Fe bioavailability, but rather the oxidative damage to cells through the inhibition of peroxidase mediated by humic substances [46]. Increasing evidence has been presented that appeared dissolved humic substances can directly interact with some macrophytes and algae through its diverse functional groups and thereby interfere with their photosynthesis and growth. Because of the low molecular masses of their building blocks, these substances easily pass through cytomembranes. Once internalized, quinones in the humic substances are thought to interfere with photosynthetic electron transport [42,47]. In fact, the toxic effects of quinones on the growth and photosynthesis of *Scenedesmus* strains have been confirmed [48]. The specific mechanism through which of humic substances inhibits the growth of *S. acuminatus* should be further studied because it is great significance not only for the sustainable utilization of water and nutrients in the microalgal industry but also for our understanding of the impact of allelochemicals on microalgae in the aquatic environment.

4. Conclusions

In this study, the percentage of total carbohydrates, fatty acids, and proteins in the AOM all decreased as the growth phase increased, while the percentage of humic substances in that increased. In addition, the growth phase at which the recycled media was obtained was found to have great impact on the growth of *S. acuminatus*. The later the growth phase at which the recycled media was obtained, the stronger the growth inhibition. Furthermore, humic substances, at concentrations

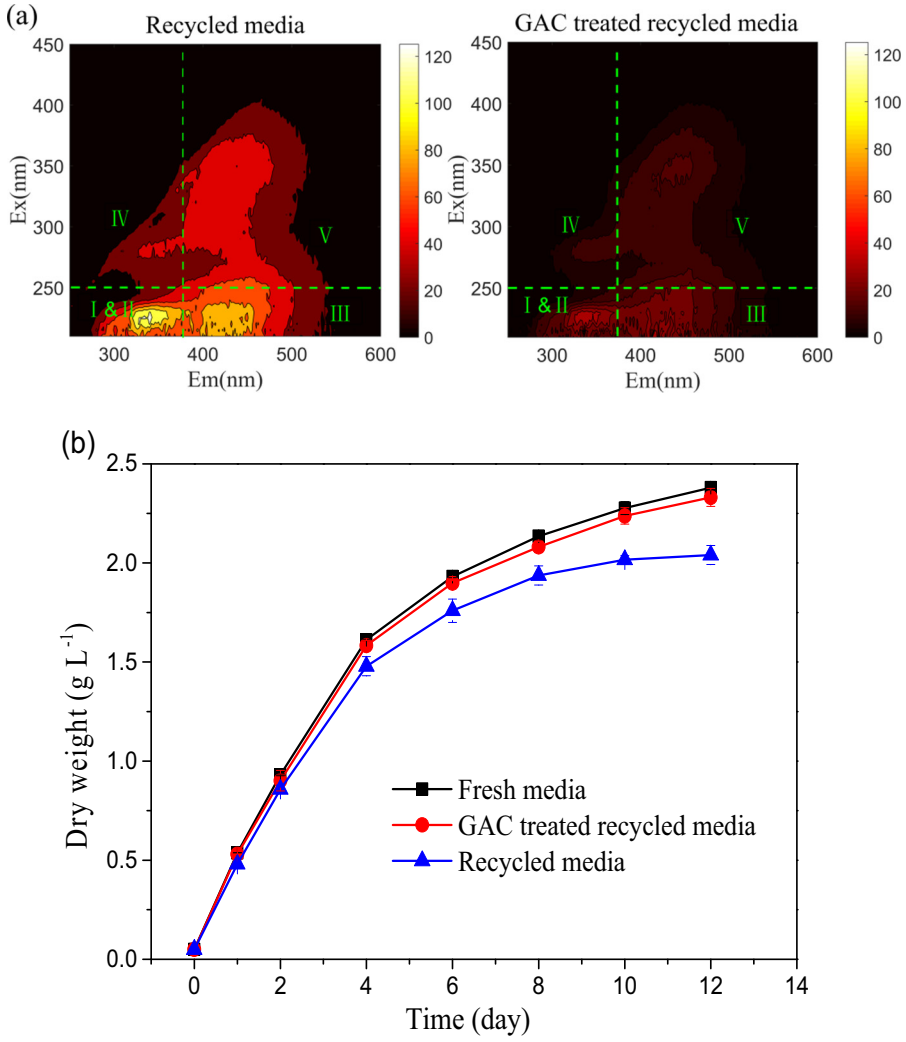

Fig. 6. (a) FEEM spectra of the GAC-treated and untreated recycled media; (b) Growth of *Scenedesmus acuminatus* in fresh media, recycled media and GAC-treated recycled media. Values are means ± standard deviation (n = 3) (Note: GAC = granular activated carbon).

higher than 20 mg C L^{-1}, had a significant inhibitory effect on the growth of *S. acuminatus*, showing that it is a major growth-inhibiting factor in recycled media. The effectiveness of inhibitor removal using GAC filter adsorption was confirmed.

In the future, with the increasing use of algae in the fields of biofuels, food and medicine production, water consumption will be greatly increased. Because water is a precious and limited resource, it is essential to recycle the water used in these activities. The results of this study not only contribute to the sustainable utilization of nutrients and water in the microalgal industry but also help us understand the impact of humic substances on the occurrence and growth of microalgae in the aquatic environment.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

YSC and XZZ were responsible for the design and funding of the study. JS and ZYL completed the most of the experiments and drafted the article. In the experiments, JY and GHW determined the contents of total dissolved organic matter, protein and polysaccharide in the recycled medium. YSC, XZZ and QH assisted the results interpretation and critical reviewed the manuscript.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101612.

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