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Effects of *Ascophyllum nodosum* extract on growth and antioxidant defense systems of two freshwater microalgae

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Abstract Microalgae in genus *Chlorella* and *Scenedesmus* are common in aquatic ecosystems and are widely used for various studies on algal growth and applications. Macroalgae may play an important role for control of microalgal growth, attributable to their rich content of bioactive compounds. In this study, the brown seaweed *Ascophyllum nodosum* was extracted with 70% acetone and the extract was used to treat the green microalgae, *Chlorella vulgaris* and *Scenedesmus* sp. Cell density and chlorophyll *a* concentration were used as growth indexes to evaluate the effects of *A. nodosum* extract (ANE) on the microalgae. The ANE with concentrations > 1% exhibited significant capability of inhibition of the growth of microalgae by over 80%. On the contrary, 1% ANE caused varying degrees of acceleration of cell proliferation and chlorophyll *a* synthesis in *C. vulgaris* and *Scenedesmus* sp., respectively. Analysis of antioxidant activities of the enzymes superoxide dismutase (SOD) and catalase (CAT) revealed the impact of ANE on the antioxidant defense system of the microalgae. The SOD and CAT activities were significantly depressed by high concentrations (> 2%) ANE, while a slight increase of the enzyme activities was observed with 1% ANE at the early period, which could be correlated to the growth response. Therefore, the mechanism of microalgae control could be related to the interaction between the ANE and the antioxidant defense systems. Phlorotannins are proposed as the principal algistatic components in the ANE which could be utilized in controlling microalgae growth.

Keywords *Ascophyllum nodosum* extract · Microalgae growth · Antioxidant enzyme activities · Phlorotannins

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Introduction

The microalgae *Chlorella* and *Scenedesmus* have been widely used as indicators for environmental monitoring and studies on the relationship between the algal growth and environmental stress (Gao et al. 2017). They are dominant in algal blooms (Auer et al. 1996) arising from water eutrophication, having detrimental influences on biological environment, public health, and economies (Anderson et al. 2002; Davidson et al. 2012). On the other hand, the economic value of both microalgae has been realized in the fields of biofuel, health care products, pigments, and aquaculture. These applications of the microalgae are attributable to the abundance of lipids, chlorophyll, proteins, and other nutrients (Rosenberg et al. 2014; Eustance et al. 2015;). More recently, heavy metal adsorption, and biogas and biofuel production by these microalgae have also been investigated (Mirghaffari et al. 2015; Jia et al. 2016; Mahdy et al. 2016; Senturk and Yildiz 2016).

Methods to control the growth of these microalgae have been widely studied. Physical (Anderson 2004) and chemical (Paerl 2008) methods have long been used to control the growth of microalgae. Low-dose-rate gamma irradiation has been reported to induce the enhancement of biomass and lipid content of marine microalgae for biofuel production (Jeong et al. 2017). Sun et al. (2014) also found that lower Se concentrations positively promoted the growth of *Chlorella vulgaris*, but the effect was opposite in the treatments with higher Se concentrations. Biological approaches feature higher efficiency, lower cost, and lower toxicity than physical and chemical methods (Oh et al. 2010; Wang et al. 2017). Aquatic plants (Marshall and Orr 1948) and algicidal bacteria (Salomon and Imai 2006) can directly attack the harmful algae or release algicidal compounds into water (Anderson 2009). Allelochemicals excreted by or isolated from aquatic plants has drawn much attention for algae-bloom control (Della Greca et al. 1998; Gross 2003; Hong et al. 2008a, b). A negative correlation of the abundance between microalgae and macroalgae has been reported (Crawford 1979; Lee and Olsen 1985; Fong et al. 1993). In view of the rapid effect, inhibitory compounds excreted by macroalgae instead of nutrient or sunlight competition have been proposed to influence the growth of microalgae (Fletcher 1975; Borowitzka 2016). Macroalgae of the Chlorophyta and Rhodophyta can affect the growth of microalgae and this has been attributed to compounds such as terpenoids (Konig et al. 1999), bromoperoxidase (Ohsawa et al. 2001), and polyunsaturated fatty acids (Chiang et al. 2004; Alamsjah et al. 2008; Oh et al. 2010).

Phlorotannins isolated from the brown alga *Ecklonia kurome* significantly decreased the swimming cell density of three red tide microalgae species within 30 min of application (Nagayama et al. 2003). Phlorotannins are also well known to have diverse effects on biological systems, such as anti-viral (Ahn et al. 2004), anti-cancer (Parys et al. 2010), antibacterial (Lopes et al. 2012), and antioxidant activity (Abu et al. 2013; Queguineur et al. 2013). They are usually extracted from brown algae using various solvents (Gall et al. 2015), e.g., 70% acetone is the most effective solvent for phlorotannin extraction from *Fucus vesiculosus* (Koivikko et al. 2005). The brown alga *Ascophyllum nodosum* is rich in phlorotannins (Zubia et al. 2009) with approximately 6.5% of the dry weight (Breton et al. 2011; Queguineur et al. 2013). However, *A. nodosum* extract (ANE) containing phlorotannins has not been studied for controlling freshwater microalgal growth. Moreover, the effect of phlorotannins on cell physiology of microalgae is not well understood.

Environmental stresses such as herbicides (Qian et al. 2008) and allelopathic effect of macrophytes (He et al. 2008) cause oxidative damage in microalgae by triggering the increase in reactive oxygen species (ROS) such as superoxide radical ($O_2{\bullet}^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) (Mittler 2002). For protecting cells against the potential damage by ROS, the activity of antioxidant enzymes is generally stimulated (Gao et al. 2017). These enzyme activities have been used to assess the toxicity to microalgae (Huang et al. 2012; Pereira et al. 2014; Sun et al. 2014). In this study, *C. vulgaris* and *Scenedesmus* sp. were selected for investigation of the effects of and *A. nodosum* extract (ANE) containing phlorotannins on the growth and antioxidant defense systems of the freshwater microalgae.

Material and methods

Freshwater microalgae

The unialgal inoculants of *Chlorella vulgaris* (PKU AC176) and *Scenedesmus* sp. (PKU AC158) were obtained from Peking University Algal Collection (PKU AC) and cultured in sterilized BG11 medium (Rippka et al. 1979). The pH value was adjusted to 7.1 using 1 M NaOH or HCl. The stationary microalgal cultivation was under an irradiance of 40–60 mmol photons m^{-2} s^{-1} and a photoperiod of 12 h light: 12 h dark at 24 °C. Microalgal cells in the logarithmic phase were used throughout the experiments.

Preparation of ANE

Dry powder of *A. nodosum* was purchased from Starwest Botanicals Inc., Canada. Portions (3 g) of milled powder (100 mesh) were extracted using 100 mL of 70% acetone. Three-hour ultrasonic extraction (ultrasonic time: 3 s; rest time: 9 s) was done at 400 W by an ultrasonic cell disintegrator (JY92-IIN, Ningbo Xinzhi Instruments, Inc., China) in an ice-water bath. The supernatant after centrifugation (12,857×*g* for 10 min at 4 °C) was concentrated at 30 °C under reduced pressure and then freeze-dried to obtain the crude extract (green-brown powder).

Microalgal assays with ANE

The crude extract (about 0.6 g) was re-suspended in 20 mL deionized water, and added to the suspensions of *C. vulgaris* and *Scenedesmus* sp. in BG11 medium (100 mL for each culture). Three separate cultures were prepared for each experiment. The concentration of extract in the suspensions varied from 1 to 4% (*v*/*v*). Before and after the cultivation for 1 to 7 days under the conditions stated in Section 2.1, cells densities were measured with Countstar Automated Cell Counter (Inno-Alliance Biotech, Inc., USA). The percent inhibition was calculated using the following formula: $Inhibition\ (\%) = 100 - \frac{\text{cell densities in treatments with extract}}{\text{cell densities in control treatment}} \times 100$. The concentration of chlorophyll *a* was determined every other day by hot-ethanol extraction method (Jespersen and Christoffersen 1987) with ultrasonic cell disintegration. The effective concentration of

the ANE that inhibits 50% of the microalgae population (EC_{50}) during the growth period was calculated using Probit analysis of transformed chemical concentration by plotting the natural logarithm values versus percentage of inhibition in MINITAB software (Release 15.1., Minitab Inc., 2007).

The soluble protein and antioxidant enzyme activity assays

Assay kits for measuring total protein, superoxide dismutase (SOD), and catalase (CAT) activities were purchased from Nanjing Jiancheng Bioengineering Institute, China. On each day from day 0 to day 4, microalgal cells were collected by centrifugation (12,857×*g*) at 4 °C for 10 min. The cell pellets were washed by phosphate-buffered saline (PBS) solution (50 mM, pH 7.0) twice and re-suspended in 1 mL PBS solution. Cell homogenization was conducted by ultrasonic cell disintegration at 400 W for 4 cycles (ultrasonic time: 3 s; rest time: 9 s) in an ice bath. The supernatant was used for soluble protein content and antioxidant enzyme activity measurements. Total protein was measured by colorimetric method based on BCA (bicinchoninic acid) at 562 nm (Smith et al. 1985) using the Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., USA). Bovine serum albumin was used as standard. SOD activity was measured by using WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H–tetrazolium, monosodium salt) to produce a water-soluble formazan with $O_2^{\cdot-}$ and determining the inhibitory ability of SOD to the rate of the reduction of WST-1 with $O_2^{\cdot-}$. CAT activity was measured by the decrease of the absorbance of H_2O_2 at 240 nm, as CAT could catalytically decompose H_2O_2 (Aebi 1974).

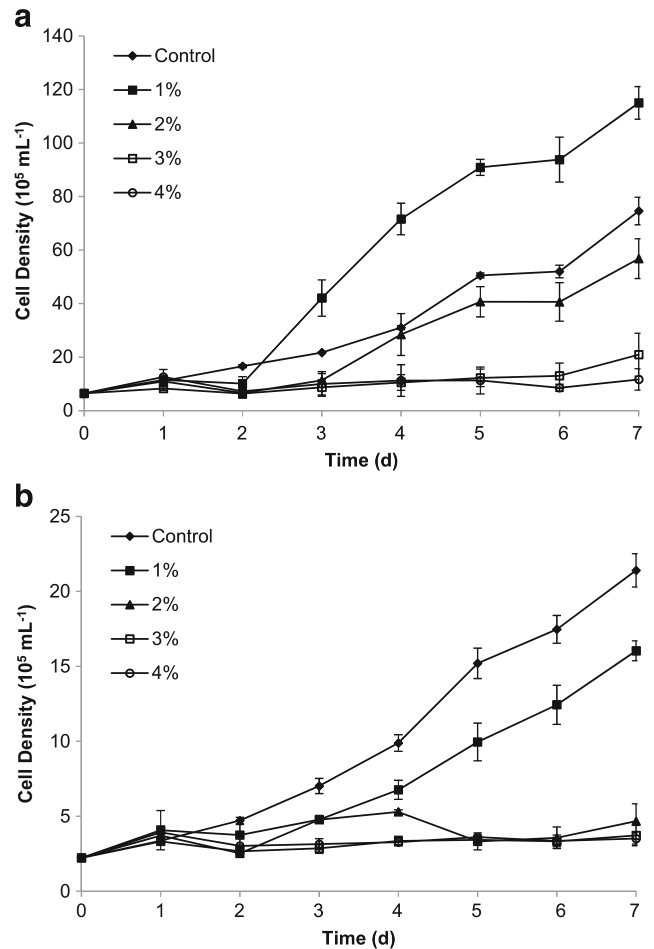

Fig. 1 Density of microalgal cells of *Chlorella vulgaris* (**a**) and *Scenedesmus* sp. (**b**) cultured with *A. nodosum* extract (1–4%). Data represent average values (n = 3) with corresponding error bars (2SE)

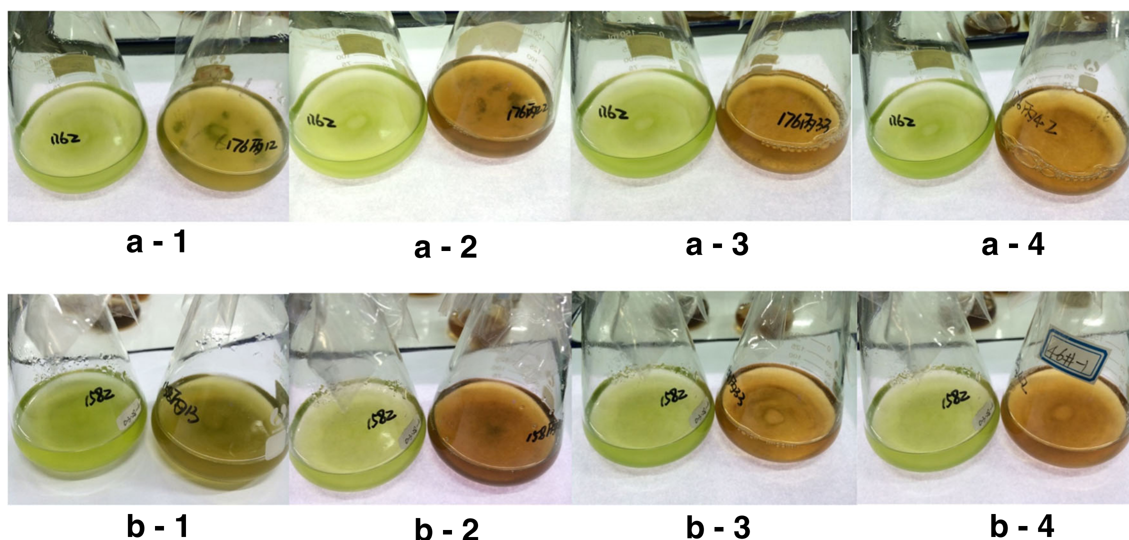

Fig. 2 Photographs of cultured suspensions of *C. vulgaris* (**a**) and *Scenedesmus* sp. (**b**) with four concentrations (1–4%) of ANE on day 5. In each photograph, control is on the left and treatment is on the right

Table 1 The EC_{50} values (%) of *A. nodosum* extract for two microalgae during the growth period. Data represent average values (n = 3) with corresponding error bars (2SE)

Microalgae	2d-EC_{50}	3d-EC_{50}	4d-EC_{50}	5d-EC_{50}	6d-EC_{50}	7d-EC_{50}
Chlorella vulgaris	2.544 ± 0.016	2.742 ± 0.015	2.861 ± 0.014	2.592 ± 0.010	2.489 ± 0.009	2.490 ± 0.008
Scenedesmus sp.	3.350 ± 0.075	2.873 ± 0.052	2.195 ± 0.033	1.330 ± 0.018	1.280 ± 0.017	1.282 ± 0.015

Analysis of algistatic compositions in the ANE

The crude extract was re-suspended in acetone and further extracted for five times until the residue became whitish. The acetone-soluble fraction was concentrated, vacuum dried and re-suspended in 20 mL deionized water. This solid–iquor mixture (1 mL) was added to the cultures of *C. vulgaris* and *Scenedesmus* sp. in sterilized BG11 medium (30 mL for each culture with three replicates), in parallel with the crude extract for comparison and sterile water as control.

Total phenolic compounds (PC) in different fractions were analyzed by the Folin–Ciocalteu method adapted from Audibert et al. (2010). ANE, Folin–Ciocalteu reagent and sodium carbonate were mixed and incubated at room temperature for 1 h. The optical absorbance of the solution was measured at 765 nm. The content of PC shown as gram of gallic acid equivalents (GAE) per gram of ANE (dry weight) was calculated according to a calibration curve obtained with gallic acid.

Statistical analyses

The mean and standard deviation of triplicates were calculated for each treatment and the control at each sampling time of both microalgae. The statistical significance of differences between treatments and the control were tested by independent-samples *t* tests (SPSS Version 19.0. SPSS Inc., USA).

Results

The effects on the growth of microalgae

The ANE was successfully prepared with 70% acetone (ESM_1). Without the ANE treatment, the cell density of both species increased rapidly in 7 days (Fig. 1). The ANE (> 1%) significantly ($p < 0.01$) inhibited the cell proliferation in both species after day 2. The algistatic effect was enhanced with increasing concentration, and the inhibitory ratios for *C. vulgaris*

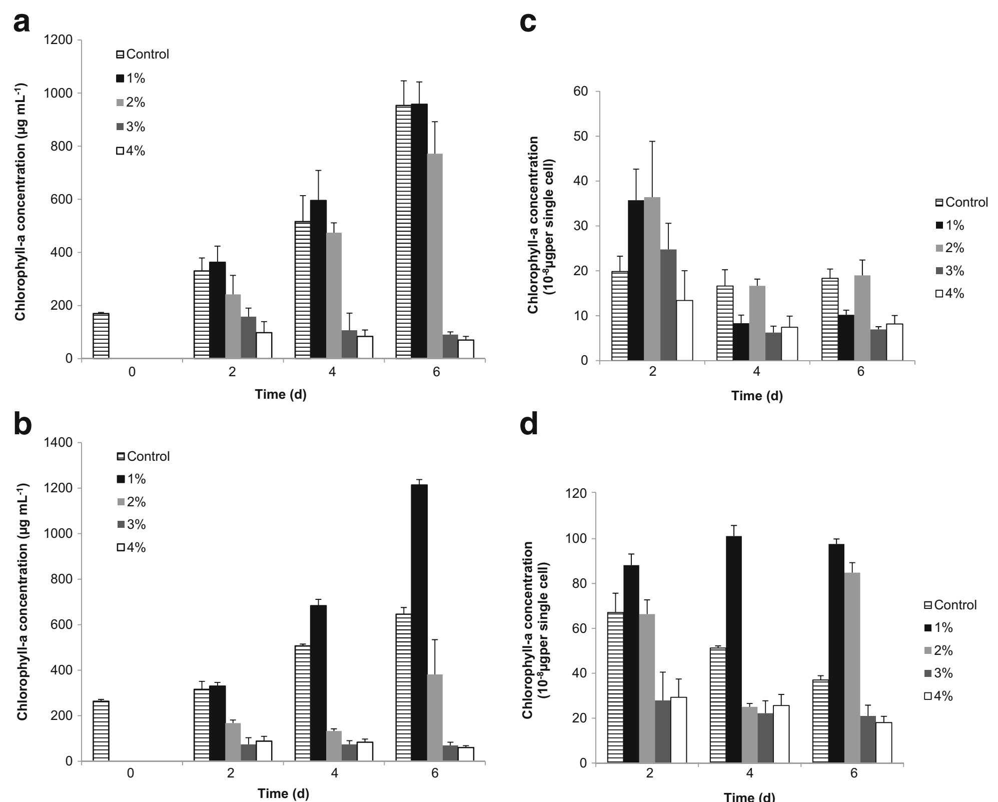

Fig. 3 Effects of *A.nodosum* extract (1–4%) on the concentration of chlorophyll *a* in total (**a**, **b**) and in individual cells (**c**, **d**) of *Chlorella vulgaris* (**a, c**) and *Scenedesmus* sp. (**b**, **d**). Data represent average values (*n* = 3) with corresponding error bars (2SE)

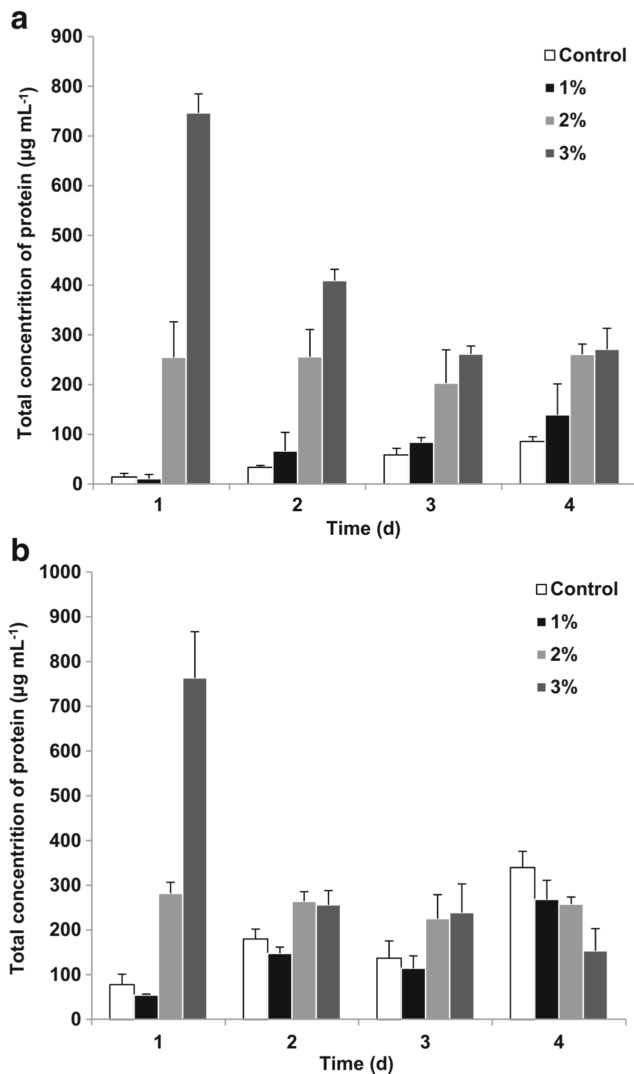

Fig. 4 Effects of different contents of *A. nodosum* extract (1–3%) on the total concentration of protein in cells of *Chlorella vulgaris* (**a**) and *Scenedesmus* sp. (**b**) during the growth period. Data represent average values ($n = 3$) with corresponding error bars (2SE)

and *Scenedesmus* sp. reached 84.3 and 83.6%, respectively. Obvious difference between the two species was observed for 1% ANE. It reduced the cell density of *Scenedesmus* sp., but surprisingly promoted the growth of *C. vulgaris* ($p < 0.01$) after day 3. Flocculation was visible in the cultivation of *C. vulgaris* with 1 and 2% extract (Fig. 2a(1, 2)). The EC_{50} values of *C. vulgaris* remained in the range of 2.49 to 2.86%, while the EC_{50} for *Scenedesmus* sp. decreased steadily from 3.35 to 1.28% in 7 days (Table 1). This implies the dose-dependent and time-dependent inhibitory effects of the ANE on *C. vulgaris* and *Scenedesmus* sp., respectively.

Effects on photosynthesis of microalgae

Chlorophyll *a* content was also influenced by the ANE in a concentration-dependent manner (Fig. 3). Total chlorophyll *a* concentrations of both species significantly decreased after treated with 3 and 4% ANE ($p < 0.01$). The decrease in chlorophyll *a* concentration turned to be less significant for lower concentrations of extract. The 1% extract could even increase the total content of chlorophyll *a* in *Scenedesmus* sp. By comparing the chlorophyll *a* concentration per cell, the 3 and 4% ANE hindered the synthesis of chlorophyll *a* in both species. The dilute (1 and 2%) ANE significantly enhanced the synthesis of chlorophyll *a* in cells of *Scenedesmus* sp. ($p < 0.01$), but the effects on *C. vulgaris* were negligible (Fig. 3c, d).

Effects on soluble protein and antioxidant enzyme activities

Before the cultivation, the initial concentrations of soluble protein were 8.47 μg mL^{-1} for *C. vulgaris* and 67.40 μg mL^{-1} for *Scenedesmus* sp. The total concentration of soluble protein in the microalgal cells cultured without ANE increased steadily over time (Fig. 4). Application of extracts above 1% significantly stimulated the synthesis of soluble protein in both microalgal species within 1 day. The highest concentrations of total protein in cells of *C. vulgaris* and *Scenedesmus* sp. cultured with 3% ANE were 47 and 17 fold that of the control, respectively. After 2 days, the stimulation became less significant.

High activities of SOD and CAT in *C. vulgaris* were observed during the first day, which suggested antioxidant enzymes of *C. vulgaris* were more sensitive to the environmental changes. The 2 and 3% extracts severely inhibited the SOD activities in both microalgae for the entire study period ($p < 0.05$) (Fig. 5a, b). Instead of being inhibited, the SOD activity of both microalgae significantly increased ($p < 0.05$) by 24-h exposure to the 1% extract and then decreased to the control level 1 day later. The inhibitory effect on CAT activity of *C. vulgaris* was highly significant in the 3% ANE, and became less effective for lower concentrations, while for *Scenedesmus* sp., the inhibitory effect was clear for all treatments on day 1, but the CAT activities for all treatments were not significantly different from control thereafter (Fig. 5d).

Algistatic component analysis

The content of PC in the crude extract (about 0.6 g) was determined to be 24.33%, corresponding to 5.76% of the dry weight of *A. nodosum*. The acetone-soluble fraction was equivalent to 38.48% of the crude extract, but contained 73.86% of the total PC extracted from *A. nodosum*. The acetone-soluble fraction containing 46.7% PC demonstrated strong inhibition of both *C. vulgaris* and *Scenedesmus* sp. with percentage inhibition of 73.8 and 83.7%, respectively, which were comparable with the crude extract with equivalent contents of PC. This consistency implies that PC would have played the important role of algistatic effects. The PC content contributing to the

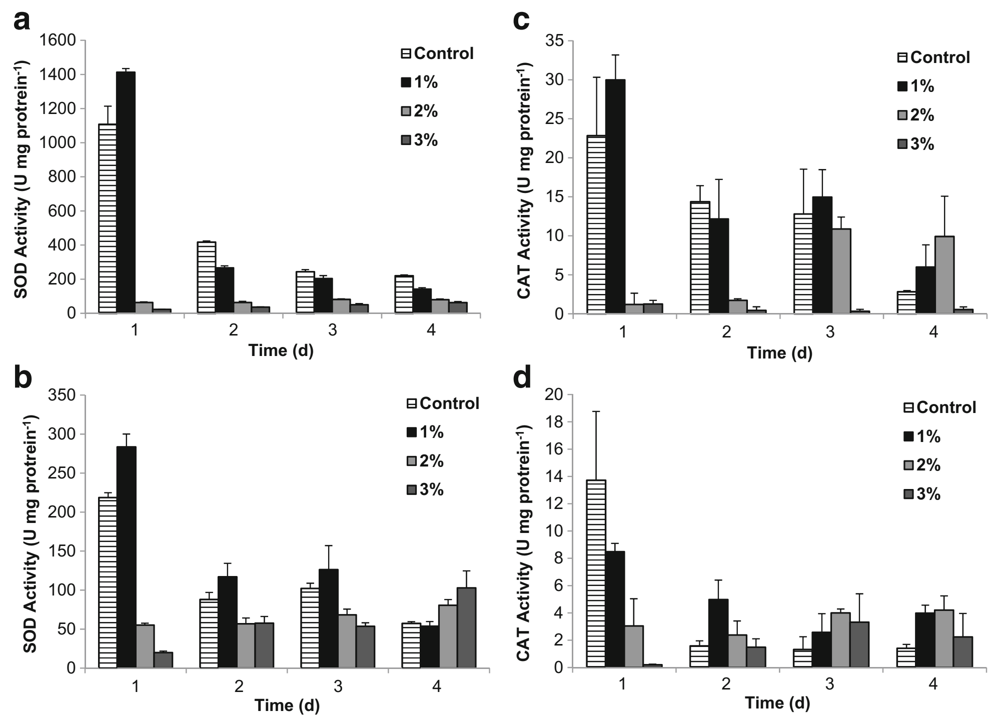

Fig. 5 Effects of different contents of *A. nodosum* extract (0–3%) on SOD (**a**, **b**) and CAT (**c**, **d**) activities in cells of *Chlorella vulgaris* (**a**, **c**) and *Scenedesmus* sp. (**b**, **d**) during the growth period. Data represent average values ($n = 3$) with corresponding error bars (2SE)

96 h EC_{50} of ANE for *C. vulgaris* and *Scenedesmus* sp. was determined to be 60.14 and 46.14 mg L^{-1}, respectively.

Discussion

The cell density and chlorophyll *a* concentration are commonly reported for toxicity indexes of algicidal components (Zhang et al. 2015). With low concentration of the ANE, the stimulated cell division in *C. vulgaris* was similar to its general response to other slight stresses (Sun et al. 2014). For chlorophyll *a* content, the different trend between total and per cell concentration might be due to the inconsistent rate of cell division and chlorophyll *a* synthesis. The enhanced chlorophyll *a* synthesis in individual cells of *Scenedesmus* sp. was similar with the previous study on the toxicity of wastewater against *Scenedesmus obliquus* (Zhang et al. 2015), indicating that chlorophyll *a* synthesis in *Scenedesmus* sp. was more sensitive than biomass production. Intensive photosynthesis may be one of the steps in cellular recovery by increasing the rate of the light utilization and synthesizing more carbohydrates (Rym 2012). Therefore, low dosage of the ANE may be applied for acceleration of the microalgal cultivation.

Flocculation of *C. vulgaris* can be induced by environmental stresses such as high pH, Zn^{2+} and Cd^{2+} in the medium (Vandamme et al. 2012; Nguyen et al. 2014; Alam et al. 2015). Alam et al. (2015) indicated the flocculating microalgae were more tolerant to Zn^{2+} and Cd^{2+} stress in contrast with non-flocculating microalgae. Flocculation ability was also correlated to the inhibitor tolerance of other microorganisms such as yeast *Saccharomyces cerevisiae* (Westman et al. 2014). Therefore, flocculation may be one of the responses for *C. vulgaris* to improve the tolerance to the stress of *A. nodosum* extract.

The enhancement of protein synthesis could be one of the responses towards the stress induced by exposure to ANE. Protein can be synthesized by plants under stressful conditions, such as salinity stress, osmotic stress, extreme temperature, anaerobiosis, infection with pathogens, gaseous pollutants, UV radiation, and so on (Dubey 1999). Soluble protein synthesis was found to be enhanced or induced by UV radiation in the algae *Chlamydomonas reinhardtii* (Nicholson and Howe 1989). These stress-induced proteins have been reported to be osmosis pressure adjustors (Cheng et al. 2016), regulator of chlorophyll synthesis (Tzvetkova-Chevolleau et al. 2007) or enzymes with antioxidant activities. Nagayama et al. (2003) reported algicidal activity of phlorotannins due to the interaction between enzyme and the channel proteins in cell membranes. Moreover, acceleration of protein synthesis could damage the cells due to changes in osmosis pressure.

Analysis of superoxide dismutase (SOD) and catalase (CAT) activities within the cells of these two microalgae provided an understanding of the different physiological response to the ANE. The cellular antioxidant defense system containing SOD and CAT for oxidative stress (Mager and Dekruijff 1995) is considered to be the first defense line against ROS damage with capability to convert $O_2\bullet^-$ to H_2O_2 and O_2, and further convert H_2O_2 to H_2O (Ken et al. 2005; Ballesteros et al. 2009). This system also plays an important role in radical scavenging and intracellular protective mechanism. The occurrence of non-inhibition on the cell division and the

chlorophyll *a* synthesis by ANE of low concentration may be correlated with a rise in enzyme activity and the recovery ability in the microalgal cells. Increasing SOD activities of both microalgae in the early stage indicated that SOD contributed to the scavenging of $O_2•^-$ produced as a result of the ANE. There was no significant difference of CAT activities in both microalgae between low-dose treatment and control. That suggests that CAT did not play a role in scavenging H_2O_2 in this study and other antioxidant enzymes may function as H_2O_2 scavengers produced from the dismutation of superoxide catalyzed by SOD or direct production via photorespiration. Both microalgae showed low concentration tolerance to the stress induced by ANE and the fast recovery in cell division and chlorophyll *a* synthesis, which is in accordance with the behavior by the exposure to nonylphenol (Gao et al. 2017). However, the exposure to the ANE at high concentration could depress the activity of antioxidant enzymes despite enhanced protein synthesis. Inactive SOD and CAT failed to remove more $O_2•^-$ and H_2O_2 induced by high dosage of ANE, which might destroy the defensive system and cause the inhibition of microalgal growth.

In conclusion, the ANE at concentrations higher than 1% had a consistent inhibitory effect on the growth of *C. vulgaris* and *Scenedesmus* sp. with a maximum percentage inhibition of over 80%. ANE at 1% significantly stimulated the cell proliferation and chlorophyll *a* synthesis of *C. vulgaris* and *Scenedesmus* sp., respectively. The analysis of antioxidant enzyme activity implied that the inhibitory effects on the activities of SOD and CAT within the antioxidant defense system could be one of the algistatic mechanisms. Phlorotannins are proposed to be the key algistatic compounds with active concentrations at the ppm level. Due to the abundance of phlorotannins, the ANE has the potential for controlling microalgae growth.

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