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Effects of 1-Octyl-3-Methylimidazolium Bromide on the Growth, Photosynthetic Activity and Antioxidant Enzymes of *Chlorella pyrenoidosa*

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Abstract
In this present study, indoor experiments were performed to analyze and evaluate the effects of 1-octyl-3-methylimidazolium bromide ([C_8mim]Br) on the growth, photosynthetic activity and antioxidant enzymes of *Chlorella pyrenoidosa* using 96 h growth tests in a batch-culture system. Results showed that the growth of *C. pyrenoidosa* was significantly inhibited by [C_8mim]Br, which would destroy the photosynthetic system II (PSII), and hinder the photosynthetic electron transfer of *C. pyrenoidosa*. By changing the photosynthetic pigments, *C. pyrenoidosa* attempted to repair the destruction of PSII system and capture more light quanta for photosynthesis. Moreover, remarkable physiological and biochemical responses, especially for the antioxidant enzymes in *C. pyrenoidosa*, occurred in [C_8mim] Br treatments. [C_8mim]Br increased total soluble protein content and enhanced antioxidant enzymes activities at low concentrations, but inhibited them at high concentrations. These observations indicated that moderate [C_8mim]Br stress would stimulate the synthesis of proteins against stress and quenching of free radicals. And the general increase of MDA contents suggested that the physiological effects of [C_8mim]Br were probably exerted through free radical generation. Thus, we suggest that it is necessary to evaluate influences of ionic liquids before their release into the natural environment in order to predict their impacts, avoiding irreparable damages.

1. Introduction

Ionic liquids (ILs) are a novel class of organic salts consisting of a bulky organic cation, such as ammonium, imidazolium, pyridinium, piperidinium and pyrrolidinium, and organic or inorganic anions, such as dicyanamide ($[(CN)_2N]^-$), chlorine (Cl^-), tetrafluoroborate (BF_4^-), and bromide (Br^-) (Docherty and Kulpa 2005, Pernak et al. 2004, Ventura et al. 2010). Being composed entirely of ions, ILs have many special properties, such as extremely low vapor pressure, chemical and thermal stability, nonflammability, high ionic conductivity, wide electrochemical potential window and solvation ability (Cho et al. 2007, Patel and Lee 2012, Ventura et al. 2012). Therefore, ILs have been considered

as environmentally benign solvents and applied in many existing biological and chemical reactions to replace volatile organic solvents.

Imidazolium is one of the most popular cation used to form ILs, especially the 1-alkyl-3-methylimidazolium $[C_n mim]^+$ ions (Bailey et al. 2010). Imidazolium-derived ILs have been widely used in organic synthesis (Earle et al. 2008), catalysis (Zhang et al. 2011), biocatalysis (Moniruzzaman et al. 2010a, Moniruzzaman et al. 2010b, Muginova et al. 2010, Pinto et al. 2008), analytical chemistry (Sun and Armstrong 2010), electrochemistry (Shiddiky and Torriero 2011), nuclear industry (Rao et al. 2007), and food chemical science (Biswas et al. 2006), etc. The main advantage of imidazolium-derived ILs is their negligible vapor pressure, which could reduce the risk of air pollution; however, they have high solubility and inaccessible biodegradability in water (Bruzzone et al. 2011). Recently, some studies have documented that the release of imidazolium-derived ILs into aquatic environments may lead to water pollution and related potential risks (Cho et al. 2008, Latała et al. 2005, Ventura et al. 2010, Zhao et al. 2007), and the impacts of imidazolium-derived ILs on organisms or communities presented "alkyl side chain" effect (increase in antimicrobial activity with the elongation of alkyl chain) and "cut-off" effect (beyond a given chain length, the effects can not be increased any further) (Ventura et al. 2012). Thus, it is very essential to study the effects of imidazolium-derived ILs on aquatic organisms and ecosystems due to their commercial use (Cho et al. 2008, Swatloski et al. 2003).

As primary producers, microalgae play an essential role in nitrogen and phosphorus cycling to maintain the balance of aquatic ecosystem (Källqvist and Svenson 2003, Sabater and Carrasco 2001). If the growth and species of microalgae are affected by chemicals, the structure and function of whole aquatic ecosystem will change (Campanella et al. 2001, Verdisson et al. 2001, Wong 2000). Moreover, microalgae are known to be comparatively sensitive to chemicals, and many studies have utilized microalgae to assess aquatic toxicity (Real et al. 2003). In China, freshwater green microalgae *Chlorella pyrenoidosa* was recommended for toxicological tests as ecological indicators by the Chinese National Environmental Protection Agency (Chinese NEPA 1990).

Although more and more studies concerning toxicity of imidazolium-derived ILs to microalgae have been reported up to now (Ma et al. 2010), the toxic mechanism is still unclear. To our knowledge, this is the first report regarding toxic mechanism of imidazolium-based ILs to freshwater microalgae. In this study, 1-octyl-3-methylimidazolium bromide ($[C_8mim]Br$) was selected as the tested IL, which had been mentioned in some reports (Li et al. 2012, Luo et al. 2008, Ma et al. 2010, Yu et al. 2008). The aim of this study is to explore the toxic mechanism of $[C_8mim]Br$ to *C. pyrenoidosa*. The objectives of the study are as follows: 1) to evaluate the effects of $[C_8mim]Br$ on the growth of *C. pyrenoidosa*, and to determine its toxicity against *C. pyrenoidosa*; 2) to investigate the changes of photosynthetic activity of *C. pyrenoidosa* under the stress of $[C_8mim]Br$; 3) to estimate the responses of soluble protein contents, antioxidant enzyme activities, and lipid peroxidation degrees in *C. pyrenoidosa* under the $[C_8mim]Br$ stress.

2. Materials and Methods

2.1. Test Chemicals and Solutions

1-octyl-3-methylimidazolium bromide ($[C_8mim]Br$, CAS: 61545-99-1, purity>99.9%) was purchased from Chengjie Chemical Co. Ltd (Shanghai, China), and other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All of the chemicals were of analytical grade. The stock solutions were prepared in distilled water with a concentration of 10 g/L. Test solutions were then obtained by diluting the stock solution in BG11 medium (Stanier et al. 1971), and the following concentrations were used for the test solutions: 0, 45, 90, 135, 180, 225 mg/L.

2.2. Microorganism

Chlorella pyrenoidosa was purchased from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Sciences, and propagated photoautotrophically in 500 mL Erlenmeyer flasks containing 200 mL of BG11 medium. The inoculum was precultured aseptically in 250 mL Erlenmeyer flasks with 100 mL of BG11 medium. The flasks were placed in a 28 °C illuminated incubator (Jiangnan Instrument Factory, Ningbo, China) for 7 days under a 12 h light/12 h dark photoperiod and a light density of 40 $\mu E/m^2/s$.

2.3. Experimental Set Up

After precultivation, the algal inocula reached exponential growth phase. Five mL of the algal inoculum was collected using centrifugation (4,000 ×g, 4 °C, 15 min). The collected algal cells were washed twice with sterile distilled water, and then inoculated into the growth medium with an initial cell density of 2.5×10^6 cells/mL.

In the growth inhibition experiments, cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of modified BG11 medium with different concentrations of the test chemicals, each in triplicate. And the cultivation conditions were described above.

2.4. Microalgal Growth Analysis

Microalgal cell density was determined turbidometrically at 680 nm using a multi-mode microplate reader (SpectraMax M5, Molecular Devices Company, California, United States). The relationship between microalgal cell density (D, cells/mL) and optical density of the microalgal culture at 680 nm (OD_{680}) is shown in Eq. (1):

$$D = (5.0653 \times OD_{680} + 0.0499) \times 10^7, (R = 0.9851) \quad (1)$$

Furthermore, the specific growth rate (r_i, /h) was calculated according to the formula:

$$r_i\,(/\mathrm{h}) = \frac{\ln D_t - \ln D_0}{t} \quad (2)$$

where D_0 and D_t are the microalgal cell density at the beginning and at t h of inoculation, respectively.

2.5. Measurements of Photosynthetic Parameters

The photosynthetic parameters of *C. pyrenoidosa* exposed to different [C_8mim]Br concentrations for different times were measured with a pulse amplitude modulation fluorometry (Phyto-PAM, Heinz Walz GmbH, Effeltrich, Germany). Phyto-PAM is based on a fluorescence method described by Schreiber (1998), which measures fluorescence at four wavelength signals (470 nm, 520 nm, 645 nm and 665 nm) and therefore shows the contribution of various types of pigments. Prior to the measurement, samples were kept in the dark for 15 min, then, the photosynthetic parameters of *C. pyrenoidosa*, such as the maximal photochemical efficiency of PSII (F_v/F_m), the potential activity of PSII (F_v/F_0), the maximal relative electron transport rate ($rETR_{max}$), and the initial slope of the curve (α), were determined and calculated.

2.6. Photosynthetic Pigments Determination

Triplicate 10 ml of well-blended cultures were centrifuged at 4000 g for 15 min to discard the supernatants. And the pellets were homogenized with 5 mL 80% (V/V) acetone for pigments extraction. The mixtures were vigorously shaken using a vibrator, and placed in a refrigerator in the dark at 4 ºC for 24 h. Then the extracted samples were centrifuged at 10,000 g for 5 min to remove the pellets. Supernatants were transferred into 1×1 cm glass cuvettes, and measured for chlorophyll at 663 nm and 645 nm using a spectrophotometer (UV1800, Mapada Instruments Company, Shanghai, China). All absorbance values were corrected using the 80% acetone as control. The concentrations of chlorophyll a (Chl a) and chlorophyll b (Chl b) were calculated by the following equation (Hao et al. 2007) (Eqs. 3 and 4):

$$\text{Chl a (mg/L)}: C_a = 12.21 \times A_{663} - 2.81 \times A_{645} \quad (3)$$

$$\text{Chl b (mg/L)}: C_b = 20.13 \times A_{645} - 5.03 \times A_{663} \quad (4)$$

2.7. Biochemical Analysis

2.7.1. Enzyme Extraction and Protein Determination

After 96 h of [C_8mim]Br exposure, 40 mL of well-blended cultures were harvested by centrifugation at 4,000 × g for 15 min at 4 °C. The harvested microalgae were placed in 1.5 mL of extraction buffer containing 0.05 M sodium phosphate buffer (pH 7.8), and immediately lysed by sonication (Scientz Biotechnology Co. Ltd, Ningbo, China) for 10 min with a repeating duty cycle of 5 s in an ice bath. The cellular homogenate was centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatant liquid was stored at -70 °C for use in the enzyme assay.

Total soluble protein content was measured using the Bradford method with bovine serum albumin as the standard. The results are expressed as micrograms of protein per 10^7 cells (μg/10^7 cells).

2.7.2. Superoxide Dismutase (SOD), Catalase (CAT), Peroxidase (POD), and Malondialdehyde (MDA) Determination

The SOD, CAT, POD and MDA assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). According to the manufacturer's instructions, SOD, CAT, POD and MDA were extracted and determined from the supernatant liquids above. The results of enzymatic activities and MDA contents are given as units of enzyme activity per microgram of total soluble protein (U/μg protein) and nmol per 10^7 cells (nmol/10^7 cells), respectively.

2.8. Statistical Analysis

SPSS PASW Statistics 18 software was used in all statistical analyses. The mean values, confidence intervals, and standard deviation values of the triplicates for each treatment were calculated. The effects caused by [C_8mim]Br on the growth, photosynthetic activity and antioxidant enzymes were evaluated using one-way ANOVA at $P < 0.05$.

3. Results

3.1. Growth of *C. Pyrenoidosa*

Effects of [C_8mim]Br on the growth rate (/h) of *C. pyrenoidosa* were compared and analyzed. Fig. 1 shows that *C. pyrenoidosa* presented negative growth in all [C_8mim]Br treatments, and a minimum growth rate (-0.020/h) was obtained in 90 mg/L [C_8mim]Br treatment after 12 h exposure. Growth rate did not obviously changed with different exposure time when *C. pyrenoidosa* grew in culture with 45 mg/L [C_8mim]Br, however, the growth rate increased from 6 h to 12 h exposure and then decreased with exposure time in 90, 135, 180, and 225 mg/L [C_8mim]Br treatments. And the maximal growth inhibition was obtained in 90 mg/L [C_8mim]Br treatment for all exposure time. Treatment with high [C_8mim]Br (of > 90 mg/L) destroyed the morphology and membrane permeability of *C. pyrenoidosa* cells; transferring them to fresh, normal BG-11 medium did not revive the algal cells (data not shown). Therefore, it was demonstrated that [C_8mim]Br significantly inhibited the growth of *C. pyrenoidosa*.

3.2. Photosynthetic Activity of *C. Pyrenoidosa*

The photosynthetic parameters of *C. pyrenoidosa* exposed to [C_8mim]Br were determined and calculated (Fig. 2). As shown in Fig. 2A, the maximal photochemical efficiency of PSII (F_v/F_m) of *C. pyrenoidosa* decreased sharply with [C_8mim]Br concentrations after 6 h exposure, while there was a recovery of F_v/F_m values in all [C_8mim]Br treatments at 12 and 24 h exposure. And then, F_v/F_m values decreased again with exposure time and [C_8mim]Br concentrations. The

potential activity of PSII (F_v/F_0) of *C. pyrenoidosa* exhibited a similar change trend with F_v/F_m in all [C_8mim]Br treatments (Fig. 2B). However, Fig. 2C presented that the maximal relative electron transport rate ($rETR_{max}$) of *C. pyrenoidosa* was significantly inhibited by [C_8mim]Br relative to controls, especially at 6 h exposure. The initial slope of the curve (α), representing the light use efficiency of *C. pyrenoidosa*, decreased sharply within 6 h exposure, and then had a slight reduction from 6 h to 96 h exposure (Fig. 2D).

3.3. Changes of Photosynthetic Pigments and Soluble Protein Contents in *C. Pyrenoidosa*

The contents of Chl a and Chl b in *C. pyrenoidosa* under [C_8mim]Br treatments are illustrated in Fig. 3A. There was a similar change trend between Chl a and Chl b contents in *C. pyrenoidosa*, which increased first and decreased afterwards with increasing the concentrations of [C_8mim]Br. The maximal values of Chl a and Chl b contents, 0.91 and 1.46 μg/10^7 cells, respectively, were obtained in the cultures with 180 mg/L [C_8mim]Br. Moreover, with the increase of [C_8mim]Br concentrations, the ratio of Chl a:b raised from 0.619 to 0.634,which implied that there were more Chl a in treated microalgae exposing with [C_8mim]Br.

The changes in soluble protein contents exhibited a dose-dependency on the protein levels (Fig. 3B). After exposing to [C_8mim]Br, the soluble protein contents were significantly higher than that in the controls. [C_8mim]Br at 90 mg/L caused a maximal increase in soluble protein content of 0.47 μg/10^7 cells after 96 h exposure ($P < 0.05$), up to 2.16 times greater than that in the untreated group (0.22 μg/10^7 cells). Higher [C_8mim]Br concentrations (135 and 180 mg/L) decreased the protein contents significantly, while a slight increase of protein contents occurred at 225 mg/L [C_8mim]Br treatments.

3.4. Antioxidase Activities in *C. Pyrenoidosa*

Changes in the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are shown in Figs. 4. As illustrated in Fig. 4A, SOD activity in *C. pyrenoidosa* significantly increased ($P < 0.05$) with increasing [C_8mim]Br concentrations from 0 to 180 mg/L. A maximum SOD activity of 1.85 U/μg protein, was obtained from cultures exposed to 180 mg/L [C_8mim]Br, which was nearly 2.86 times higher than that in the untreated group. And then, SOD activity decreased when [C_8mim]Br concentrations were higher than 180 mg/L in the cultures.

CAT activity exhibited a similar change trend with SOD activity in *C. pyrenoidosa* (Fig. 4B). Lower [C_8mim]Br concentrations (<180 mg/L) caused a slight increase of CAT activity, which reached the maximum value (0.28 U/μg protein) at 180 mg/L [C_8mim]Br. However, it was also found that the CAT activity was inhibited by higher [C_8mim]Br concentrations (>180 mg/L) in *C. pyrenoidosa*.

Similarly, the [C_8mim]Br treatments resulted in a drastic increase of POD activity with respect to the untreated group (0.14 U/μg protein) ($P < 0.05$), and the maximum POD activity (0.75 U/μg protein) was observed in the 180 mg/L [C_8mim]Br cultures (Fig. 4C). And then, the POD activity decreased slightly in the 200 mg/L [C_8mim]Br cultures.

3.5. Lipid Peroxidation

The level of lipid peroxidation was determined in terms of MDA content. As presented in Fig. 5, an increase in the cellular MDA content was observed in all [C_8mim]Br treatments. The MDA content showed a significant increase ($P < 0.05$) with increasing the [C_8mim]Br concentrations. And a maximum MDA content (1.05 nmol/10^7 cells) were obtained at 225 mg/L [C_8mim]Br, which was 2.13 times higher than that of the untreated group (0.49 nmol/10^7 cells).

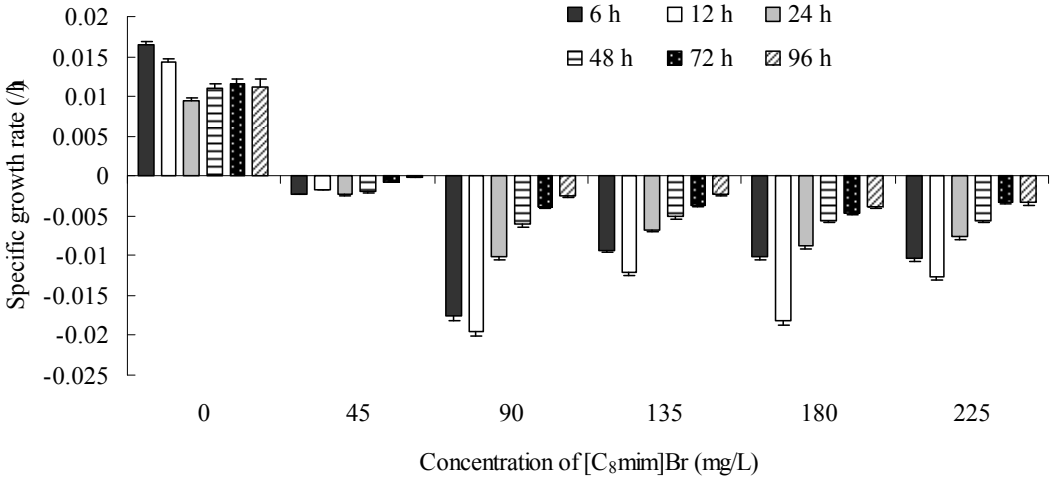


Fig. 1. *Growth rates of* Chlorella pyrenoidosa *under different [C_8mim]Br concentrations (mg/L) during 96 h exposure. The points represent the means of three replicates (n = 3); error bars represent standard deviations.*

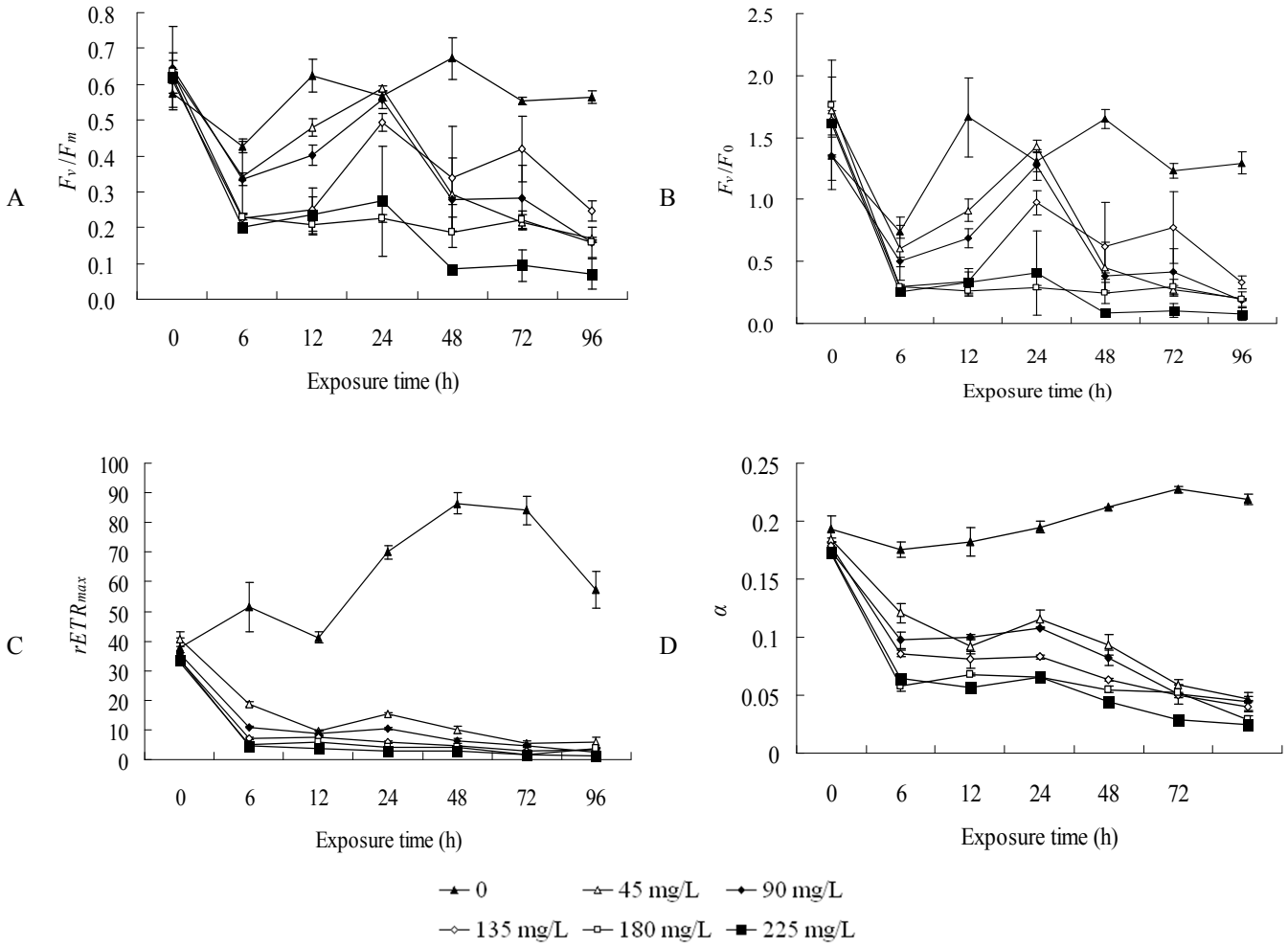
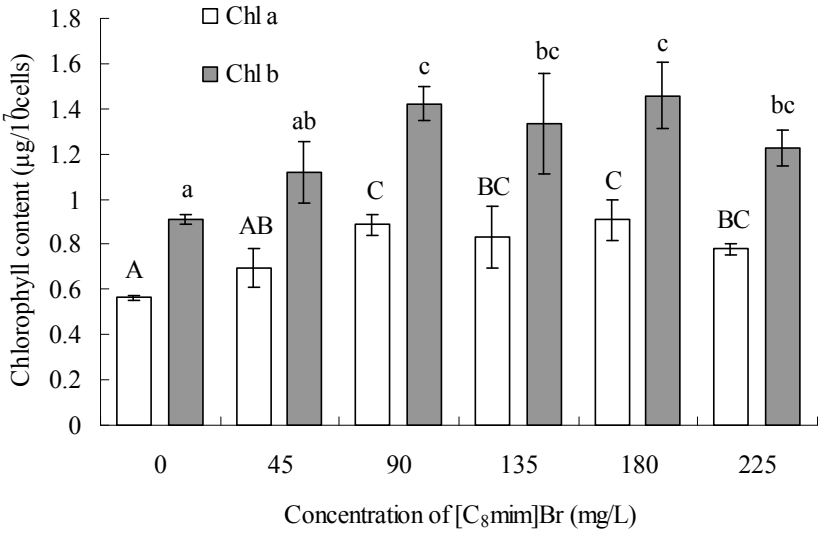
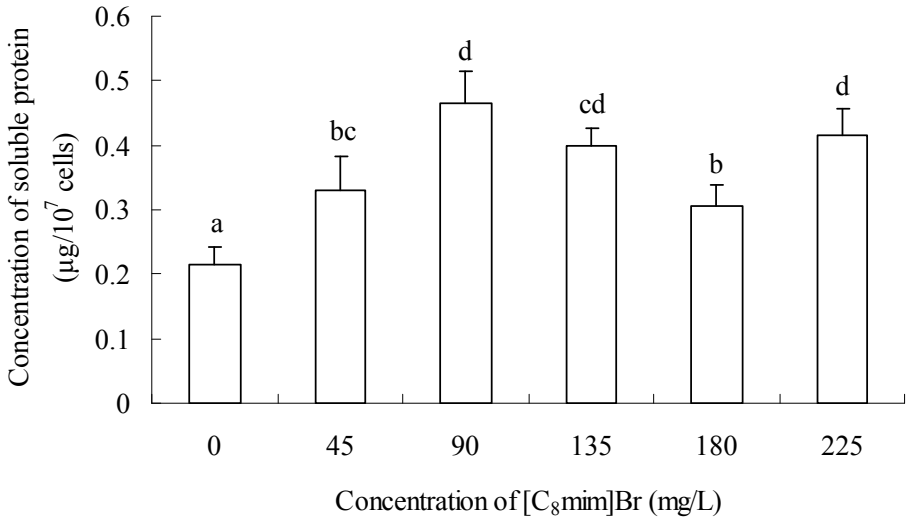


Fig. 2. *Photosynthetic activity changes of* Chlorella pyrenoidosa *under the stress of [C_8mim]Br during 96 h exposure. The points represent the means of three replicates (n = 3); error bars represent standard deviations.*

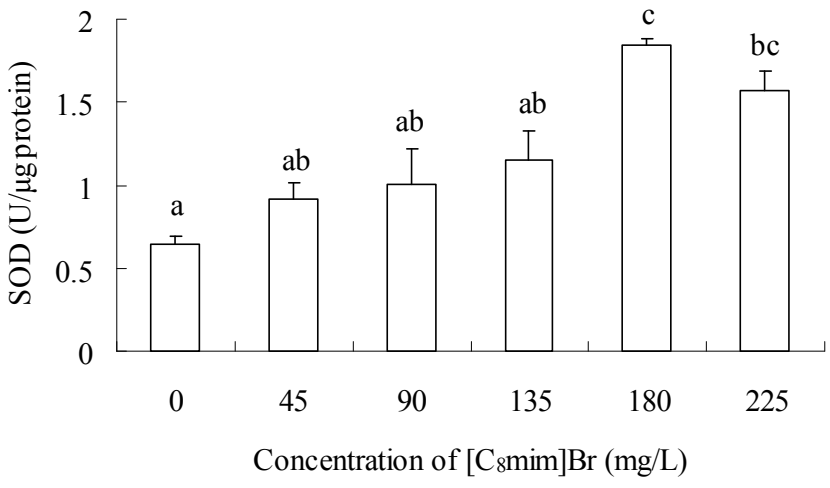


A

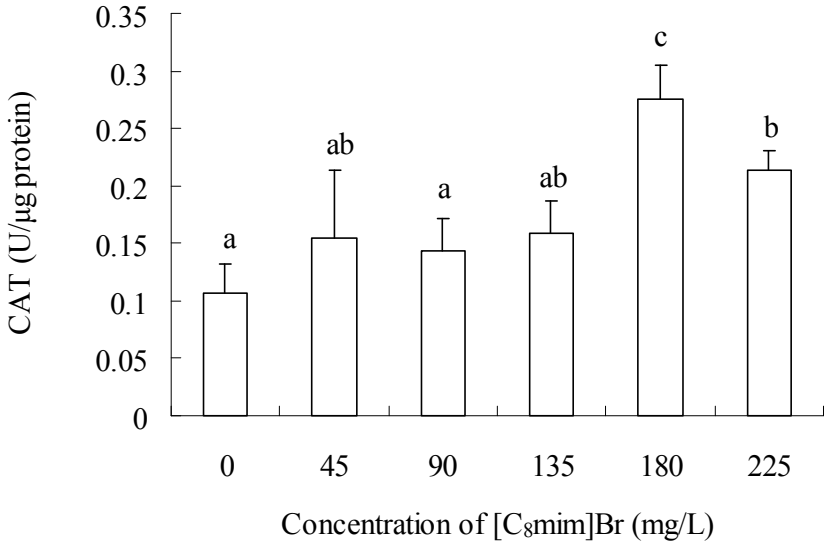


B

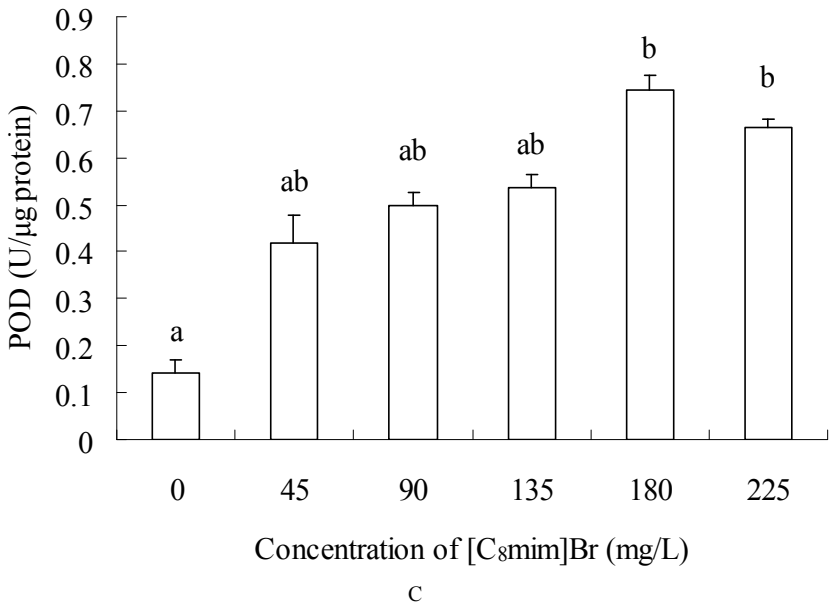
Fig. 3. *Effects of [C_8mim]Br on the chlorophyll contents (A) and total soluble protein contents (B) of* Chlorella pyrenoidosa *after 96 h of exposure. The points represent the means of three replicates (n = 3); error bars represent standard deviations. Data sets that are significantly different from each other are represented by different letters (P < 0.05).*



A



B



C

Fig. 4. *Responses of the superoxide dismutase (SOD) (A), catalase (CAT) (B), and peroxidase (POD) (C) activities in* Chlorella pyrenoidosa *to [C_8mim]Br after 96 h of exposure. Points represent means of three replicates (n = 3); error bars represent standard deviations. Data sets that are significantly different from each other are represented by different letters (P < 0.05).*

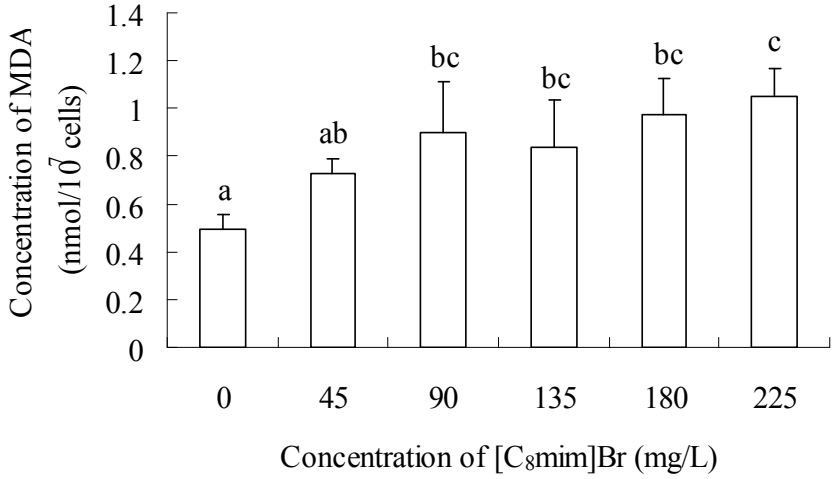


Fig. 5. *Changes of intracellular malondialdehyde (MDA) content in* Chlorella pyrenoidosa *under [C_8mim]Br stress after 96 h of exposure. Points represent means of three replicates (n = 3); error bars represent standard deviations. Data sets that are significantly different from each other are represented by different letters (P < 0.05).*

4. Discussion

With respect to traditional volatile organic solvents, ILs may avoid the loss of solvent to the atmosphere and decrease the worker exposure risk in virtue of their negligible vapor pressure, but this is not enough to consider ILs as "green solvents". Because ILs have high solubility and inaccessible biodegradability in water (Bruzzone et al. 2011), they may cause severe water pollution due to their potential influences on the aquatic organisms and communities, such as algae (Cho et al. 2008, Latała et al. 2009, 2010, Pretti et al. 2009), cladocerans (Bernot et al. 2005, Couling et al. 2006, Luo et al. 2008, Pretti et al. 2009, Ventura et al. 2010), mussel (Costello et al. 2009), and fish (Pretti et al. 2009, Pretti et al. 2006). However, their impacts on aquatic organisms and communities are largely unknown. In this present study, effects of an ionic liquid ([C_8mim]Br) were investigated on a freshwater microalga *C. pyrenoidosa*, since microalgae are considered as important primary producers in aquatic ecosystems and play important roles in nitrogen and phosphorus cycling.

Growth indices, such as cell density and growth rate, can reflect the growth status of microalgae. It was found that *C. pyrenoidosa* presented negative growth in all [C_8mim]Br treatments, and a minimum growth rate (-0.020/h) was obtained in 90 mg/L [C_8mim]Br treatment at 12 h exposure. Thus, [C_8mim]Br had a significant inhibition on the growth of *C. pyrenoidosa*, and its toxicity and potential risk should be evaluated before its release into the natural environment in order to avoid irreparable damages.

How did [C_8mim]Br affect the growth of *C. pyrenoidosa*?

As we all know, the growth of microalgae is based on the synthesis and accumulation of organic substances, which depend on the photosynthesis. Thus, the change in the photosynthesis of microalgae might be a key reason that environmetanl pollutants affect on the growth of microalgae. The photosynthetic parameters of *C. pyrenoidosa* exposed to [C_8mim]Br were determined using a Phyto-PAM. The F_v/F_m and F_v/F_0 values decreased sharply with [C_8mim]Br concentrations, while there was a recovery at 12 and 24 h exposure. It is suggested that the PSII system of *C. pyrenoidosa* was destroyed, which led to the decrease of the conversion efficiency of primary light energy. In addition, the $rETR_{max}$ of *C. pyrenoidosa* was significantly inhibited by [C_8mim]Br relative to controls, especially at 6 h exposure. The *rETR* mainly reflects the electron transport situation of PSII reaction center (Kitajima and Butler 1975). Thus, deduction of $rETR_{max}$ indicated that the photosynthetic electron transfer of *C. pyrenoidosa* was hindered by [C_8mim]Br. The α, representing the light use efficiency of *C. pyrenoidosa*, also decreased with [C_8mim]Br concentrations and exposure time. Therefore, it is concluded that [C_8mim]Br would destroy the PSII system, and hinder the photosynthetic electron transfer of *C. pyrenoidosa*, which caused the photosynthesis and metabolism of *C. pyrenoidosa* working disability and the death of microalgal cells.

On the other hand, how did *C. pyrenoidosa* respond to the stress of [C_8mim]Br? Firstly, chlorophyll is an index of photosynthesis, which allows microalgae to obtain energy from light (Kalaji and Guo 2008). Our results showed that Chl a and Chl b contents increased first and decreased afterwards with the increase of [C_8mim]Br concentrations, and Chl a contents were also increased. It is inferred that the increase of chlorophyll, especially of Chl a, would be a way to repair the destruction of the PSII system and capture more light quanta for photosynthesis. Under the stress [C_8mim]Br, the loss of photosynthesis in *C. pyrenoidosa* would be decreased via changing the contents and species of photosynthetic pigments.

Secondly, with increasing the concentrations of [C_8mim]Br, total soluble protein content changed significantly in *C. pyrenoidosa*, and lower [C_8mim]Br concentrations stimulated the synthesis of proteins. A maximum increase of soluble protein content (0.47 μg/10^7 cells) was recorded in 90 mg/L [C_8mim]Br cultures, which was up to 2.16 times greater than that in the untreated group (0.22 μg/10^7 cells). In the soluble protein, enzymes are important components, including those against abiotic stresses. Therefore, the increase of soluble protein content, including antioxidant and biotransformation enzymes, is an active defense mechanism to protect cells from [C_8mim]Br stress. It was demonstrated that many environmental pollutants, such as molinate (Yan et al. 1997), benthiocarb (Bhunia et al. 1991), bavistin, nimbicidin (Rajendran et al. 2007), and endosulfan (Kumar et al. 2008), would also induce the increase of protein content in microalgal cells. And a decrease in protein content was also observed when the concentrations of [C_8mim]Br was higher than 90 mg/L, which may be due to the increased reactive oxygen species (ROS) level (Leitao et al. 2003) or protease activity (Kumar et al. 2008) under high [C_8mim]Br stress.

Thirdly, the activities of antioxidant enzymes, such as SOD, CAT and POD, significantly increased ($P < 0.05$) with increasing [C_8mim]Br concentrations from 0 to 180 mg/L. When algae are exposed to various abiotic stresses, ROS production will be increased in the algal cells. ROS are highly reactive and toxic, which can cause damage to proteins, lipids, carbohydrates and DNA (Gill and Tuteja 2010). Meanwhile, algal cells can produce many antioxidant enzymes, such as SOD, CAT and POD, to protect against ROS. In organisms, SOD is the first important enzyme in ROS scavenging, which can catalyze the dismutation of highly reactive superoxide anion to H_2O_2. And then, CAT and POD can degrade H_2O_2 into H_2O and O_2 (Blokhina et al. 2003). It has been demonstrated that the activities of these antioxidant enzymes may be enhanced following exposure to ILs stresses in different organisms (Li et al. 2012, Yu et al. 2009, Zhang B. J. et al. 2012). However, the activities of antioxidant enzymes decreased when [C_8mim]Br concentrations were higher than 180 mg/L in the cultures, which indicated that antioxidant enzymes might be an important site of action for [C_8mim]Br in *C. pyrenoidosa*. The inactivation of antioxidant enzymes may result in high lipid peroxidation and low cellular inclusion content (e.g. photosynthetic pigments), thereby inhibiting algal cell growth.

Fourthly, lipid peroxidation often occurs in the microalgal cells when ROS is excessive, and an end product of lipid peroxidation (Malondialdehyde, MDA) will be detected (Apel and Hirt 2004). MDA may readily interact with several functional groups of molecules, such as proteins, lipoproteins, and DNA, and finally induce cellular damage (Maes et al. 2006). Thus, MDA content is often considered an indicator of lipid peroxidation status. In this study, the MDA content showed a significant increase ($P < 0.05$) with increasing the [C_8mim]Br concentrations. And a maximum MDA content (1.05 nmol/10^7 cells) were obtained at 225 mg/L [C_8mim]Br, which was 2.13 times higher than that of the untreated group (0.49 nmol/10^7 cells). It is indicated that free radical, like ROS, generated in the microalgal cells under [C_8mim]Br stress. And some studies also showed that free radical generated in other microalgal cells under the stresses of heavy metal stress, pesticides and other abiotic stresses, etc (Choudhary et al. 2007, Hong et al. 2009, Kumar et al. 2008, Sabatini et al. 2009). Moreover, the MDA content increased sharply with increasing [C_8mim]Br concentrations, which suggested that the effects of [C_8mim]Br are probably exerted through free radical generation because the increased MDA level is a dose-dependent effect of free radical generation.

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