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Toxicity of algicidal extracts from *Mangrovimonas yunxiaonensis* strain LY01 on a HAB causing *Alexandrium tamarense*

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HIGHLIGHTS

- Stable and eco-environmental algicidal extracts were used to HABs-control.
- Algicidal extracts induced cell-death and nuclear damage in *Alexandrium tamarense*.
- Algicidal process and nuclear damage were confirmed by TEM and CLSM.
- The transcription of *rbc*S, *hsp* and PCNA genes were influenced by algicidal extracts.

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ABSTRACT

Toxicity of algicidal extracts from *Mangrovimonas yunxiaonensis* strain LY01 on *Alexandrium tamarense* were measured through studying the algicidal procedure, nuclear damage and transcription of related genes. Medium components were optimized to improve algicidal activity, and characteristics of algicidal extracts were determined. Transmission electron microscope analysis revealed that the cell structure was broken. Cell membrane integrity destruction and nuclear structure degradation were monitored using confocal laser scanning microscope, and the *rbc*S, *hsp* and proliferating cell nuclear antigen (PCNA) gene expressions were studied. Results showed that 1.0% tryptone, 0.4% glucose and 0.8% $MgCl_2$ were the optimal nutrient sources. The algicidal extracts were heat and pH stable, non-protein and less than 1 kD. Cell membrane and nuclear structure integrity were lost, and the transcription of the *rbc*S and PCNA genes were significantly inhibited and there was up-regulation of *hsp* gene expression during the exposure procedure. The algicidal extracts destroyed the cell membrane and nuclear structure integrity, inhibited related gene expression and, eventually, lead to the inhibition of algal growth. All the results may elaborate firstly the cell death process and nuclear damage in *A. tamarense* which was induced by algicidal extracts, and the algicidal extracts could be potentially used as bacterial control of HABs in future.

1. Introduction

Harmful algal blooms (HABs) caused by rapid propagation of algae cells under certain conditions, pose a great threat to aquatic environment and human health [1–3]. In recent years, HABs breakout frequently with increasing pollution deterioration and climate change [4], and have brought about huge economic losses [5,6] and ecological damage [7,8]. Harmful algae often produce toxins to the environment, and the toxic products jeopardize marine organisms and destroy marine ecosystem balance [9,10], also endangering the safety of human life through the food chain [11,12]. *Alexandrium tamarense* is a marine toxic dinoflagellate which causes paralytic

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shellfish poisoning (PSP) of both shellfish and fish [13–15]. PSP is one of the most widely distributed and damaging HAB toxins [16–18] and, because of its huge impacts on marine fisheries and people's health, it has become a research hotspot [19–21].

To control HABs and relieve the damage, many methods have been proposed to regulate algal bloom development [22–25]. Among the factors that regulate HAB dynamics, algal–bacterial interactions are increasingly cited as potential regulators [26], and this has led to the suggestion that algicidal bacteria may play a major role in regulating HAB dynamics [27–30]. Bacteria can have an effect on the cell membrane [31], chloroplasts [32] and the nucleus [33] and thus inhibit algal growth. Bacteria can contact with the algal cell wall, and break the cell membrane, this algicidal effect is direct [34]. The other algicidal effect is indirect, since without contact with the algal cells, bacteria can produce an algicidal compound which causes the cell to lose its membrane integrity, destroys chloroplasts and results in nuclear degradation. Although the effects of algicidal extracts on the chloroplast and nuclear function in algal cells have been reported, the bacterial toxicity on carbon dioxide fixation (*rbc*S), heat shock protein (*hsp*) and proliferating cell nuclear antigen (PCNA)-related gene expressions have not been studied in *A. tamarense*. Therefore, we studied the *rbc*S, *hsp* and PCNA-related gene expressions in *A. tamarense* under the toxicity of algicidal extracts from marine bacterium.

Our previous research has studied the algicidal activity and mode of algicidal bacterium and demonstrated that *Mangrovimonas* sp. LY01 showed high but indirect algicidal activity on *A. tamarense* [35]. Here, the medium components and nutrient concentrations were optimized, and the heat stability, pH tolerance, molecular weight range and aqueous solubility of the algicidal extracts were determined. The algicidal procedure was observed using transmission electron microscopy (TEM), cell membrane integrity and nuclear structure degradation were monitored using a confocal laser scanning microscope (CLSM), and the *rbc*S, *hsp* and PCNA gene expressions were studied. The objectives of our study were to improve bacterial biomass and algicidal activity, characterize the algicidal extracts, and monitor the algicidal procedure, membrane integrity, nuclear structure degradation and the change of gene expression under the toxicity of the algicidal extracts.

2. Materials and methods

2.1. Algal cultures and algicidal activity of the algicidal bacterium

A. tamarense ATGD98-006 was supplied by the Algal Culture Collection, Institute of Hydrobiology, Jinan University (Guangzhou, China). All cultures were maintained in f/2 medium [36] (prepared with 0.45 μm of filtered seawater) at 20 ± 1 °C under a 12-h light/12-h dark cycle with a light intensity of 50 μmol photons m^{-2} s^{-1}.

Mangrovimonas yunxiaonensis strain LY01 (GenBank No. JQ937283) was isolated from a surface sediment sample collected in November 2011 at a depth of 20 cm from the Yunxiao Mangrove National Nature Reserve (23°55′N 117°24′E), Fujian Province of China [28], and the strain was deposited in the BCCM/LMG (Ghent) Bacteria Collection, which accession number is LMG 27142. Bacteria were cultured using 2216E medium (peptone 5 g, yeast extract 1 g, ferric phosphorous acid 0.1 g, agar 10 g, pH 7.6–7.8, in 1 L natural sea water) followed by incubation for 24 h at 28 °C with shaking at 120 rpm.

In order to investigate the algicidal activity of strain LY01, the algicidal activity was determined according to [35].

The algicidal rate was calculated using the formula:

$$\text{algicidal rate}\,(\%) = \frac{N_C - N_T}{N_C} \times 100$$

where N_C represents the number of algal cells in the control group, and N_T represents the number of algal cells in the treatment group. Algal cells were counted under microscope after fixed with Lugol's iodine reagent. Only intact algal cells were counted. All experiments were repeated in three biological replicates.

2.2. Optimization of medium components and nutrient concentrations

Beef extract, tryptone, yeast extract, soybean peptone and bacto peptone were added to the basal medium (ferric phosphorous acid 0.1 g, pH 7.6–7.8, in 1 L natural sea water) to determine the optimum nitrogen source; glucose, sucrose and soluble starch were used as different carbon sources, and added to the basal medium with the determined optimum nitrogen source in order to determine the optimum carbon source. To investigate the effects of K_2HPO_4, $FeSO_4{\cdot}7H_2O$, $MgCl_2$ and $CaCl_2$ on the growth and algicidal effect of strain LY01, we added different minerals into the basal medium. Six samples were sampled every 24 h. Three samples were used to measure the absorbance value at wavelength of 600 nm, to indicate the growth rate of the bacterium. Another were used to investigate the algicidal rate.

Tryptone with concentrations of 0.7, 1.0, 1.5, 2.0 and 3.0% was added into the basal medium; glucose with concentrations of 0.1, 0.4, 0.8 and 1.2% were added into the basal medium with the tryptone; and $MgCl_2$ with concentrations of 0.4, 0.8, 1.2 and 2.0% were added into the basal medium with tryptone. The bacterial growth rate and algicidal activity were measured every 24 h as described above.

2.3. Characterization of the algicidal extracts

LY01 cultures were grown in the optimum medium at the optimum concentration (as determined above) at 28 °C for 24 h and then centrifuged at 6000 × *g* for 10 min to collect the supernatant. The supernatant was then incubated at −80, 40, 80 and 120 °C for 1 h to test the effects of temperatures on algicidal activity. The pH of the supernatant was adjusted and maintained at 3, 9 and 11 for 1 h, and then adjusted to the initial pH to test the pH tolerance of the algicidal extracts. To make sure whether the algicidal extracts was protein, 150 mL ethanol was added into 50 mL supernatant, and centrifuged at 6000 × *g* for 10 min after standing under normal temperature, and the supernatant was condensed to repeat the above steps twice. The supernatant was loaded into dialysis bags with molecular intercept values of 1 kD and dialyzed in sterile seawater for 48 h to test the molecular weight range of the algicidal extracts; the seawater was replaced every 12 h, after which the algicidal activity of the dialyzed supernatant was evaluated after being adjusted to the original volume with sterile seawater. The supernatant of LY01 was mixed with an equal volume of organic solvent (hexane, methylene chloride, butanol, and ethyl acetate) with vigorous shaking in a separation funnel for 30 min. The mixture was allowed to stand for 30 min to collect the organic phase. The extraction was repeated three times. The organic phases were combined and evaporated to dryness and weighed to obtain extracts. All extracts were dissolved in dimethyl sulfoxide (DMSO) to test the algicidal activity, and fresh algal cells, with the same volume of DMSO added, acted as a negative control.

2.4. Algicidal procedure with the extracts from strain LY01

Algal cells were treated with algicidal extracts for 24 h, and were then prepared for TEM. Samples were fixed overnight at 4 °C in 0.1 M PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, in 1 L distilled water, 50 mM, pH 7.4) containing 2.5% glutaraldehyde

(v/v) and then postfixed in 1% OsO_4 in the same buffer for 2 h. After being washed twice with PBS, samples were embedded in araldite resin. Sections (60–80 nm), obtained with an ultramicrotome, were stained in 3% acetic acid uranium-citric acid and viewed using TEM (model JEM-2100HC; JEOL).

2.5. Cell membrane integrity and nuclear structure degradation

After treatment with algicidal extracts, 5 mL of algal cells were collected at 3000 × *g* for 5 min followed by washing with 1 mL of PBS (50 mM, pH 7.4). Next, 800 μL PBS with 100 μL acridine orange (Beyotime, China) was added together with 100 μL propidium iodide (Beyotime, China) and they were incubated in the dark for 20 min. After centrifugation at 3000 × *g* for 5 min to remove supernatant, 1 mL PBS was added to resuspend the algal cells. DAPI nuclear staining was operated after the algal cells were collected as described above, by adding 200 μL DAPI (Beyotime, China) to cover the samples. They were incubated at room temperature in the dark for 5 min, the algal cells were resuspended in 1 mL of PBS after washing twice with PBS. A small droplet of algal suspension was placed on a pre-cleaned glass slide and trapped under a coverslip. The algae cells were imaged using a CLSM (Zeiss LSM 780) through a ×401.2 N.A. water immersion objective. Acridine orange fluorescence was observed through a 530 nm band pass filter; propidium iodide fluorescence was observed through a 617 nm band pass filter; DAPI fluorescence was observed through a 435–485 nm band pass filter and chlorophyll fluorescence through a 650–710 nm bandpass filter. More than 100 cells from each treatment were examined under a ×40 objective and representative pictures were taken.

2.6. RNA extraction, reverse transcription and real-time analysis

After treatment with algicidal extracts, 30 mL of algal culture was centrifuged at 3000 × *g* at 4 °C for 5 min. RNA was extracted as soon as possible using the RNAiso kit (TaKaRa Company, Dalian, China) following the manufacturer's instructions. For reverse transcription, 1 μg of total RNA was reverse transcripted following the instructions of the PrimeScript RT reagent kit (TaKaRa Company). Real-time PCR was carried out using an SYBR Premix EX TaqTM II kit (TaKaRa Company). The PCR program was: one denaturation step at 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s, then increasing from 60 to 95 °C by 0.5 °C each 5 s. 18S rRNA was used as a housekeeping gene to normalize the expression changes. The relative gene expression among the treatment groups was quantified using the $2^{-\Delta\Delta Ct}$ method [37].

2.7. Statistics

All data were presented as means ± standard error of the mean and were evaluated using one-way analysis of variance followed by the least significant difference test, with $p < 0.01$ and $p < 0.05$ (Origin 8.5 for Windows).

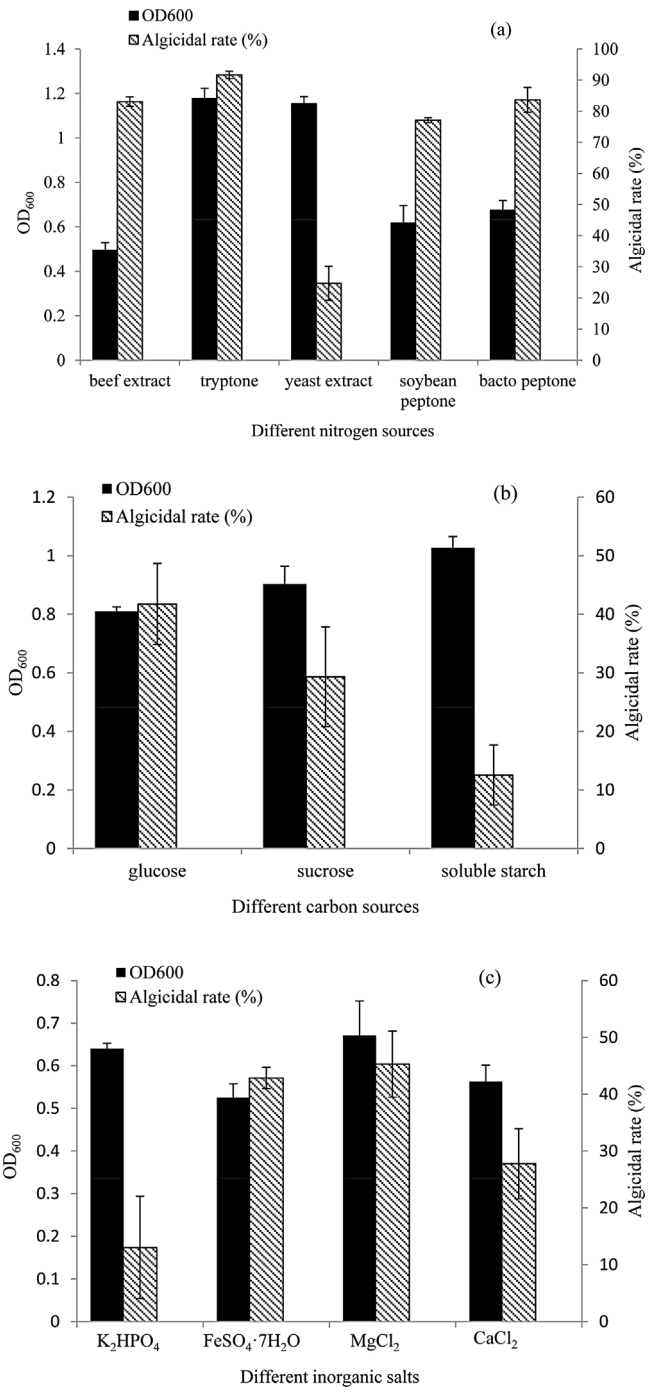

Fig. 1. Medium component optimization of strain LY01. The effect of different nitrogen sources (a), carbon sources (b) and inorganic salts (c) on the bacterial growth rate and algicidal activity.

3. Results

3.1. Optimization of medium components and nutrient concentrations for strain LY01

The results of optimization for the bacterial growth rate and algicidal activity using different medium components and nutrient concentrations are shown in Figs. 1 and 2. Different nitrogen sources had significantly different effects on the growth and algicidal activity of strain LY01 (Fig. 1a). When the bacterium was cultured with beef extract, soybean peptone and bacto peptone, the algicidal activities were all high; while the bacterial growth rates were low. The bacterial growth rate was high when yeast extract was supplied but, at the same time, the algicidal activity was low. Among the five nitrogen sources we examined, tryptone led to the highest biomass and algicidal activity. Among the carbon sources evaluated, soluble starch and sucrose promoted bacterial growth, while the algicidal activity did not increase greatly compared to the glucose (Fig. 1b). Mineral is an integral component in the cell growth and metabolism of microorganisms, and Fig. 1c showed that K_2HPO_4 and $CaCl_2$ had no promoting effect on biomass or algicidal activity, while $FeSO_4{\cdot}7H_2O$ and $MgCl_2$ could induce a significant

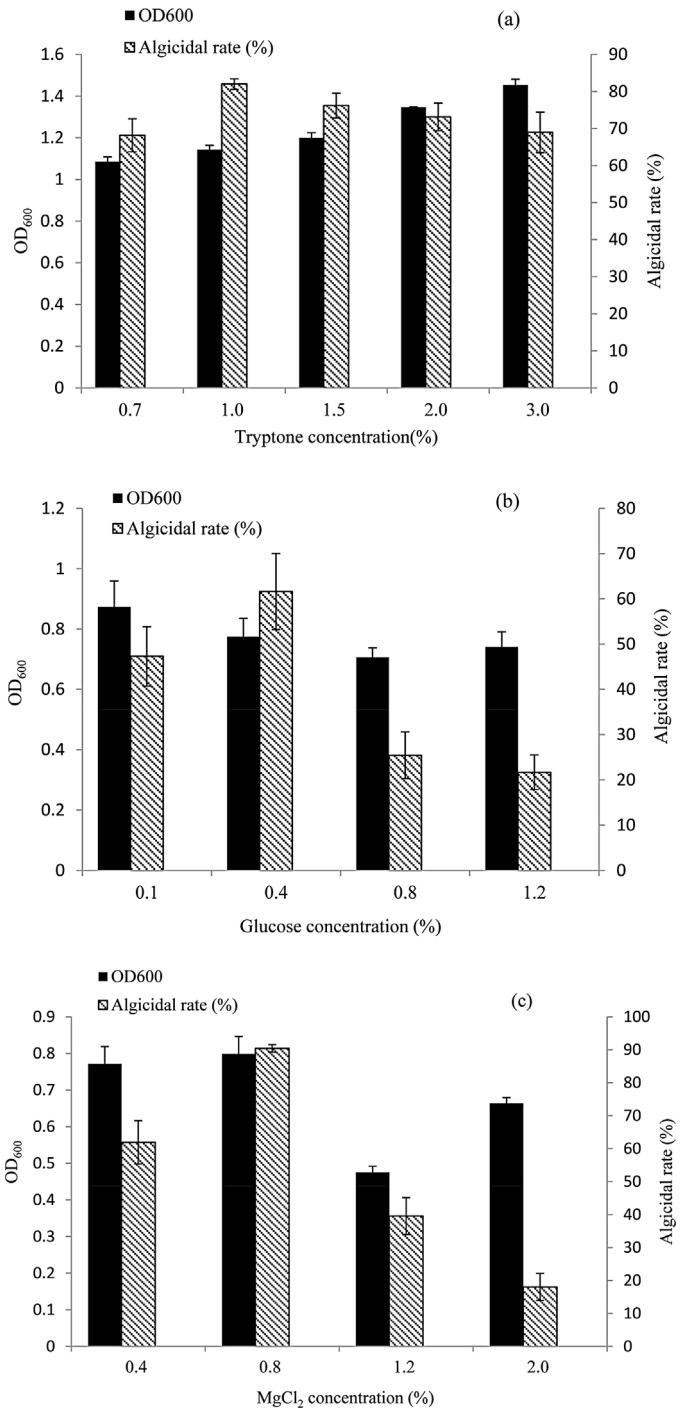

Fig. 2. Medium concentration optimization of strain LY01. Effect of different tryptone concentration (a), glucose concentration (b) and $MgCl_2$ concentration (c) on the bacterial growth rate and algicidal activity.

increase in the bacterial growth rate and algicidal activity, and $MgCl_2$ had an even better effect.

In terms of the optimal nutrient concentrations for bacterial growth, the results in Fig. 2a indicated that bacterial growth was kept relatively stable with different concentrations of tryptone, but when the concentration was 1.0%, the algicidal rate reached its maximum level. The growth rate increased with increasing tryptone concentration. The algicidal activity increased when glucose concentrations increased from 0.1 to 0.4%, but decreased with increasing glucose concentration, whereas the growth rate decreased continuously with increasing glucose concentration

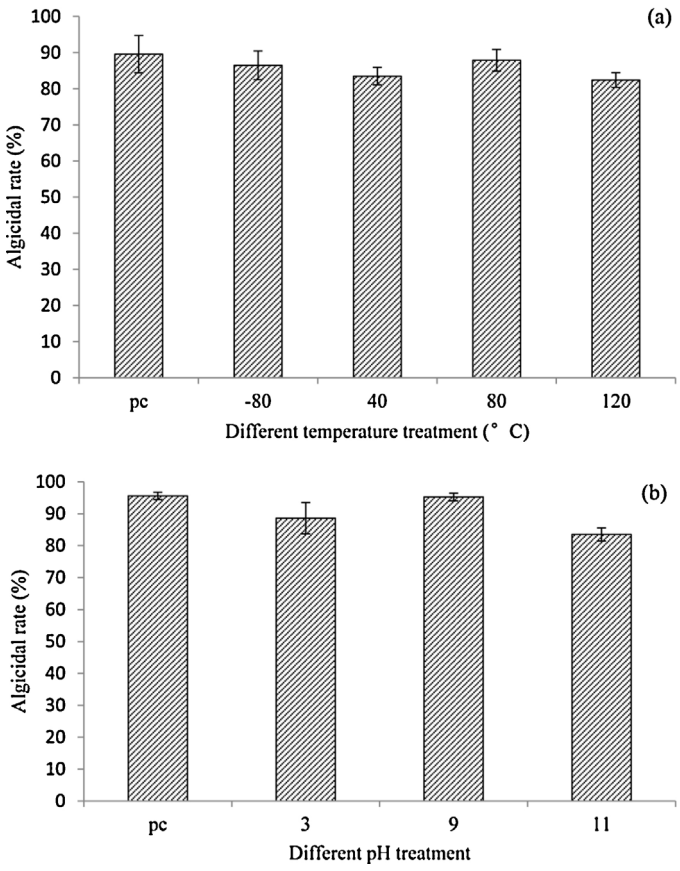

Fig. 3. Algicidal rate of supernatant treated with different temperatures (a) and pH (b); pc represents the positive control where the bacterium was cultured without any treatment.

(Fig. 2b). The bacterial growth rate and algicidal activity increased with an increased $MgCl_2$ concentration from 0.4 to 0.8%, but showed a decrease when $MgCl_2$ concentration increased above 0.8%.

3.2. Characterization of the algicidal extracts

Different temperature and pH treatments did not have a significant influence on the algicidal activity of the LY01 supernatant, compared with the positive control (Fig. 3a and b), and all values were similar to or slightly lower than the controls.

When ethanol was used to change the isoelectric point of the LY01 supernatant, the algicidal rate in the precipitates was significantly lower, whereas the algicidal rate of the algicidal extracts in the dissolved phase did not change obviously compared with the positive control (Fig. 4a).

From the results (Fig. 4b), the extracts outside the dialysis bag showed a high algicidal rate with a similar effect to the positive control, while the extracts inside the dialysis bag did not have any significant algicidal effect on the algal cultures. Among the four organic solvents used (hexane, methylene chloride, butanol and ethyl acetate), ethyl acetate extracts showed the strongest algicidal activity (83.6%) (Fig. 4c), which was similar with the positive control (89.2%). However, the other three organic solvents showed almost no algicidal activity.

3.3. Effect of algicidal extracts on subcellular structure

TEM analysis showed obvious alterations in the ultrastructure of *A. tamarense* under the effects of the algicidal extracts, with

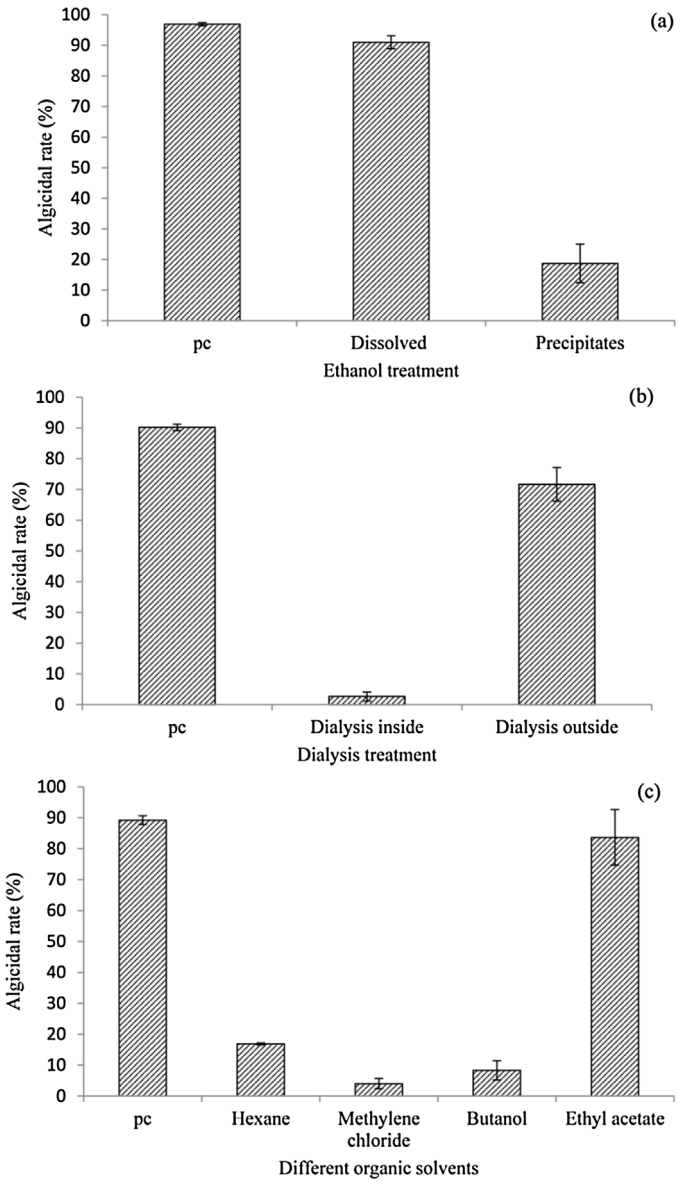

Fig. 4. The influence of ethanol precipitates (a), different dialysis fractions (b) and different organic solvents (c) on algicidal activity (pc represents the positive control; dialysis bag diameter is 1 kD).

loss of the organelles integrity (Fig. 5). In the control, the cell wall and membrane remained intact, and the cytoplasm was dense and intact and normal organelles including chloroplast and mitochondrion could be clearly seen (Fig. 5a and b). After treatment with the algicidal extracts for several hours, obvious plasmolysis could be observed in the algal cells and, at the same time, a large number of vacuoles appeared, and the structure and morphology of the cell wall was significantly affected (Fig. 5c and d). After a prolonged treatment time, the cell membrane was broken, and the intracellular substances severely spilled out (Fig. 5e), the chloroplasts became sparse, there was disorganization and deformation of the chloroplasts and the membrane structures of the mitochondria were obscured (Fig. 5e and f).

3.4. Cell membrane integrity analysis

Acridine orange can pass through the normal cell membrane, and stain the nucleus with green fluorescence, while propidium iodide can only pass through a broken cell membrane and emitted red fluorescence after embedded in the double-stranded DNA, and since the algal cells in the control group only showed red fluorescence as a result of both stains (Fig. 6a), this meant that the propidium iodide did not pass through the cell membrane because the cell membrane was intact. However, exposed to the algicidal extracts for 24 h, the algal cells were gradually stained with red fluorescence, and the red fluorescence intensity increased and the green one decreased with time (Fig. 6b–d). At the late treatment time, the green fluorescence intensity had almost disappeared and the whole algal cells were stained with red fluorescence, indicated complete breakdown of the cell integrity (Fig. 6e and f).

3.5. Degradation of nuclear structure

After DAPI nuclear staining, CLSM revealed that normal cells in the control group (Fig. 7a) showed homogeneous DAPI staining, which was confined to the nuclear area. After exposed to the algicidal extracts, the cells presented irregular DAPI staining and a slight degree of chromatin clumping could be observed (Fig. 7b). Fig. 7c shows that chromatin aggregation occurred in the whole cell, the DAPI fluorescence was obscure and weak, at the same time, the cell wall was broken. The chloroplast fluorescence also decreased greatly during the treatment procedure, and the nuclear structure was significantly damaged (Fig. 7d and e). After cleavage of the cell membrane, the cellular inclusions including nucleus and chloroplast flowed out of the algal cells (Fig. 7f).

3.6. Effect of algicidal extracts on gene expression

The *rbc*S, *hsp* and PCNA gene expressions were significantly influenced by different concentrations of algicidal extracts (Fig. 8). Within 6 h exposure, the transcription of *rbc*S was inhibited by 0.70 ($p < 0.05$), 0.09 ($p < 0.01$) and 0.57-fold ($p < 0.01$) compared to the control in the 10, 25 and 50 μg/mL treatment groups, and the transcription of *rbc*S was also inhibited by higher concentration treatment groups (25 and 50 μg/mL) in the 24 h treatment time. However, the 10 μg/mL treatment group did not influence the gene expression within the 24 h exposure (Fig. 8a). The transcription of *hsp* was inhibited in the 10 μg/mL treatment group within 6 h exposure, but showed no influence to gene expression in the 24 h exposure; it was increased by 1.34-fold ($p < 0.01$) compared to the control in the 25 μg/mL treatment group, but there was no effect on gene expression within the 24 h treatment; and the 50 μg/mL treatment group showed no effects within 6 h exposure, but after 24 h, the transcription of *hsp* was significantly increased by 2.09-fold ($p < 0.01$) compared to the control in the 50 μg/mL treatment group (Fig. 8b). The transcription of PCNA was significantly inhibited by 0.34 ($p < 0.01$) and 0.43-fold ($p < 0.01$) compared to the control in the 25 and 50 μg/mL treatment groups within 6 h exposure, and also inhibited by 0.11 ($p < 0.01$) and 0.34-fold ($p < 0.01$) compared to the control in the 25 and 50 μg/mL treatment groups within 24 h exposure, but the 10 μg/mL treatment group did not show any effect on gene expression during the whole exposure procedure (Fig. 8c).

4. Discussion

To improve the algicidal activity of strain LY01, we optimized the medium components and nutrient concentrations (Figs. 1 and 2). Nitrogen is an important nutrient factor for marine bacteria, and heterotrophic bacteria must obtain nitrogen from additional sources for normal growth [38,39]. Different nitrogen sources can influence bacterial growth [40], and Fig. 2a shows the algicidal activity and growth rate of strain LY01 cultured under different nitrogen sources in our studies. Compared with other nitrogen sources, tryptone was the optimal nitrogen source for bacterial

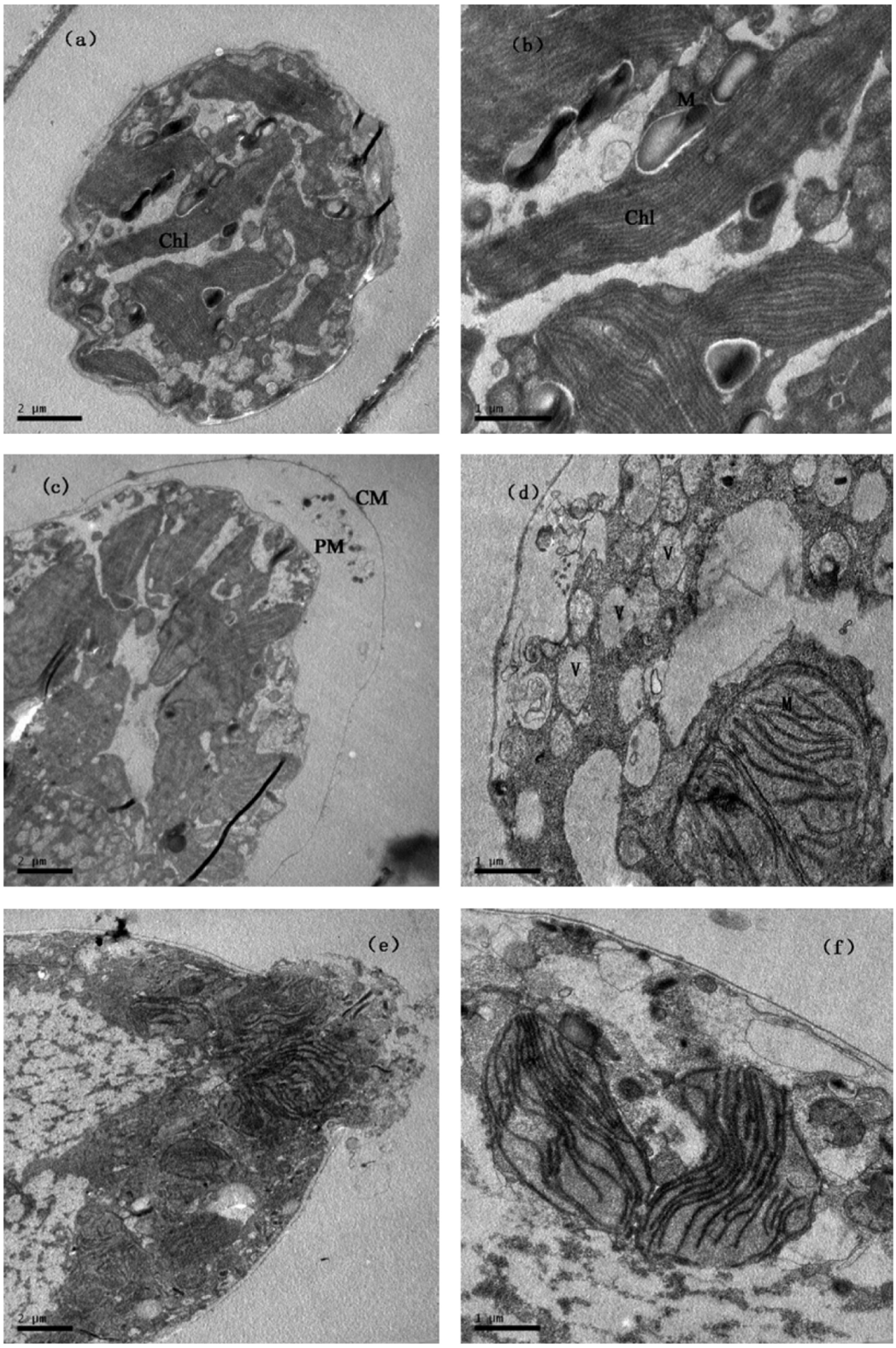

Fig. 5. Ultrastructure of *A. tamarense* after exposure to LY01 supernatant with a concentration of 50 μg/mL for 24 h. (a) overview of control cell; (b) detailed view of control cell; (c)–(f) details of treated cells. Abbreviations: CW: cell wall; CM: cell membrane; Chl: chloroplast; M: mitochondrion; V: vacuole; N: nucleus. Bars (a), (c) and (e) 2 μm; (b), (d) and (f) 1 μm.

biomass and algicidal activity. Wilson et al. and Kientz et al. also isolated and cultured marine bacteria with tryptone as the nitrogen source in their bacterial growth medium [41,42]. Carbon source and minerals are also essential to marine bacteria [43,44] and bacterial cells maintain an osmotic pressure essential for growth and division, using organic compatible solutes and inorganic ions. We studied the bacterial biomass and algicidal activity with different carbon sources (Fig. 1b), bacterial biomass maintained a high level when cultured in all three carbon sources (glucose, sucrose and soluble starch), however the algicidal activity only reached

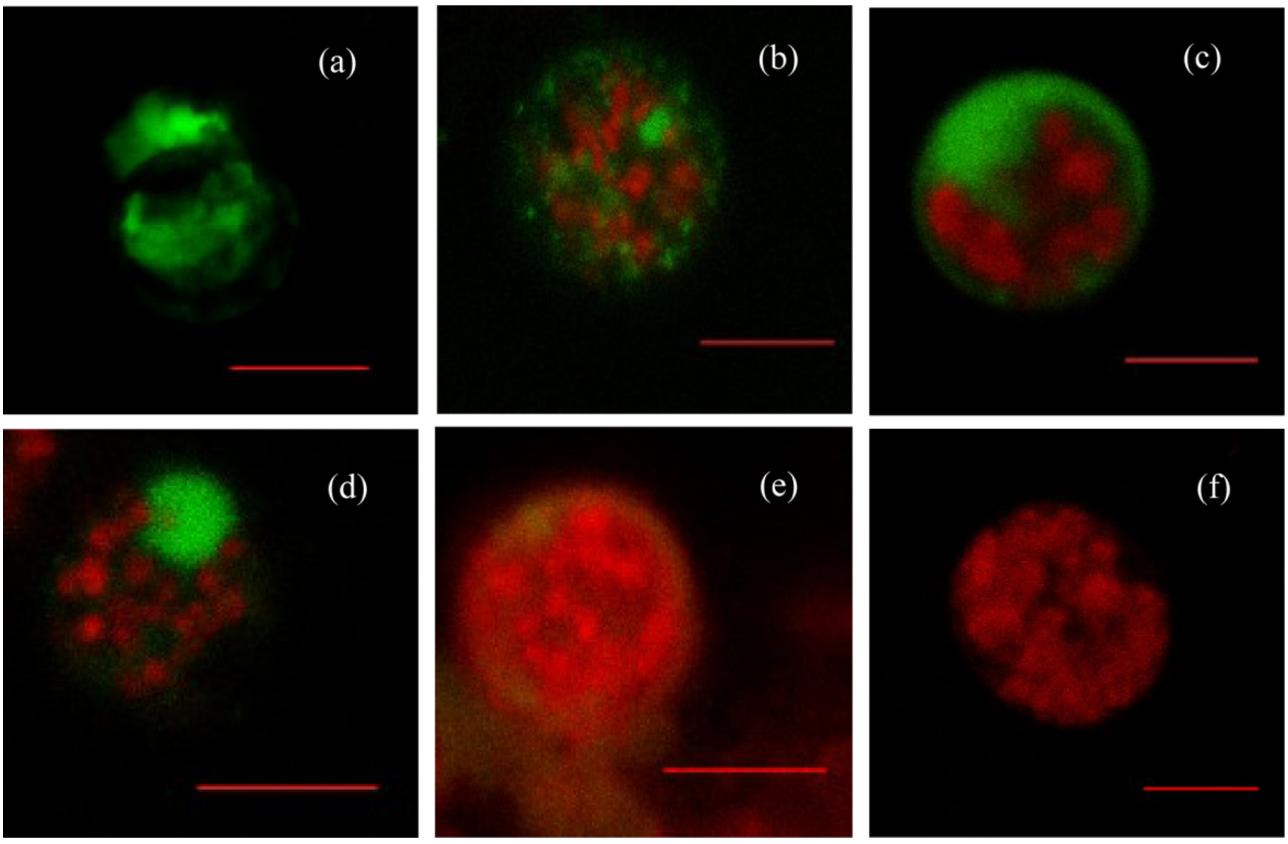

Fig. 6. Cell membrane integrity of *A. tamarense* after exposure to LY01 supernatant extracts (50 μg/mL) for 24 h. (a): control cells with green fluorescence staining; ((b)–(f)): cell membrane integrity sabotage when cells with red fluorescence staining appear after exposure to the extracts for 48 h. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

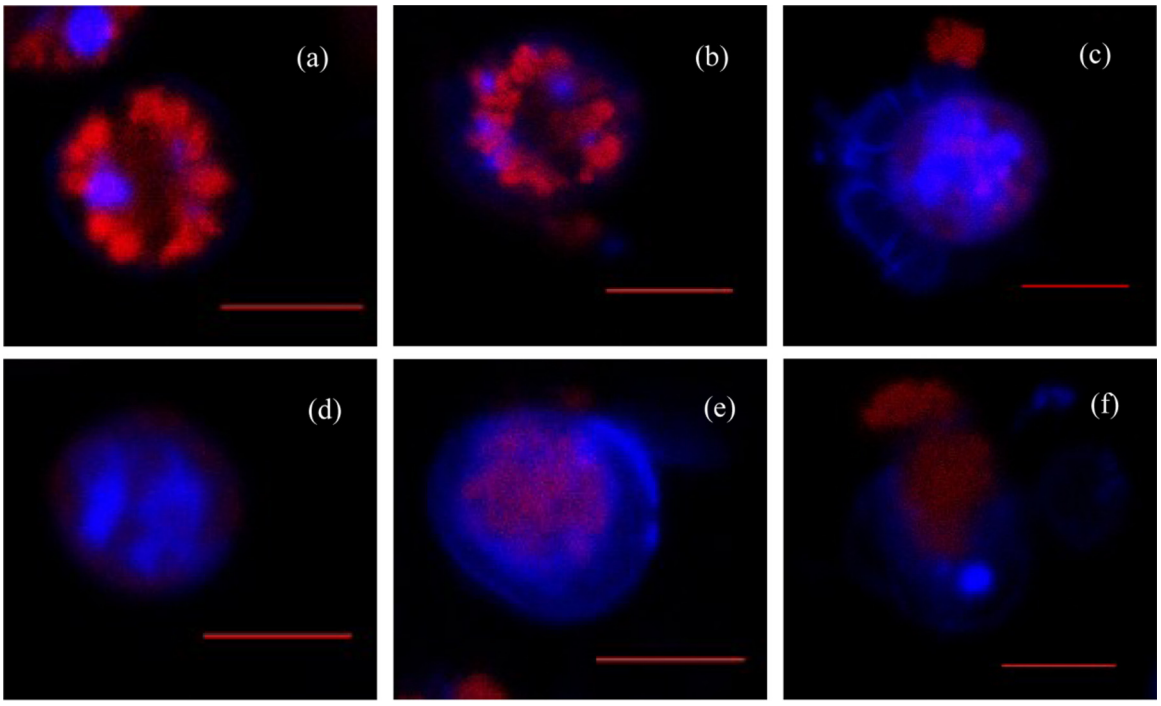

Fig. 7. Degradation of the nuclear structure of *A. tamarense* after exposure to LY01 supernatant extracts (50 μg/mL) for 24 h. (a): control cells; ((b)–(f)): degradation of nuclear structure of algal cells after exposure to extracts for 48 h. Red fluorescence was algal chloroplast fluorescence, which represents the strength of algal vitality; blue fluorescence represents the nuclear area. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

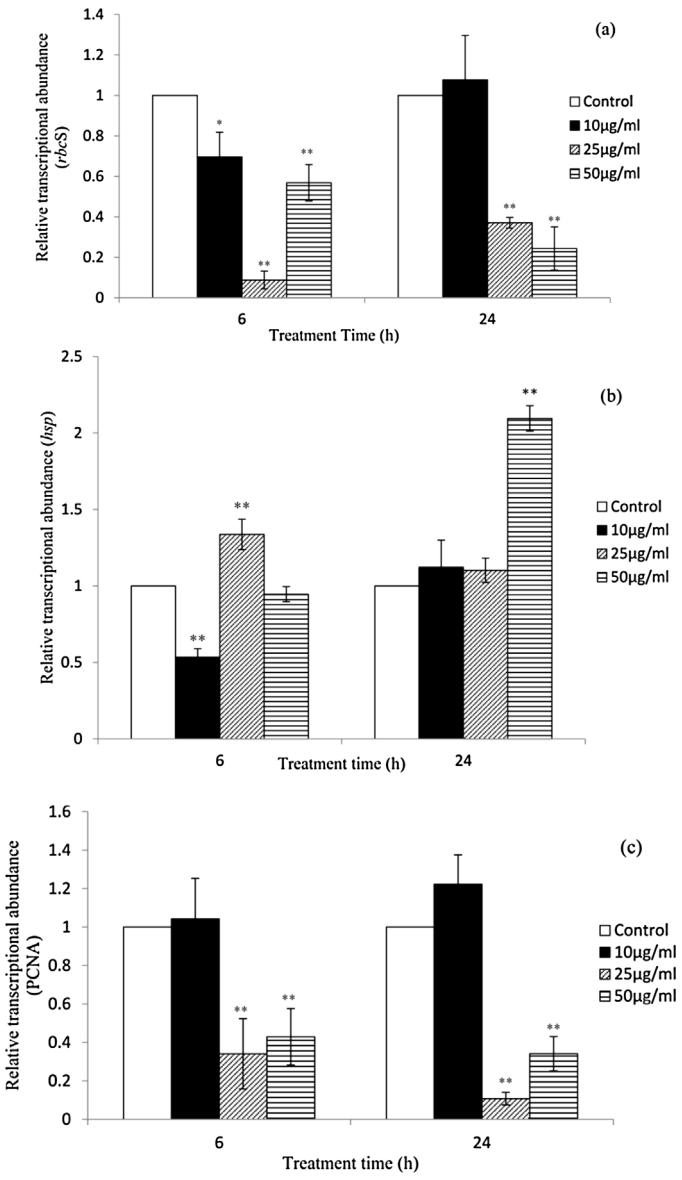

Fig. 8. Effect of algicidal extracts on the transcription of *rbc*S, *hsp* and PCNA genes in *A. tamarense*. All error bars indicate the SE of the three biological replicates. * Represents a statistically significant difference of $p < 0.05$ when compared to the control; ** represents a statistically significant difference of $p < 0.01$.

a high rate when strain LY01 was cultured in glucose, and so we were sure that glucose was the carbon source which strain LY01 needed. Zhang et al. investigated the response of bacterioplankton to a gradient of carbon (glucose) addition [45], and found that differences in the extent of stimulation suggested different bacterial life strategies under different nutrient conditions and showed clear and gradual changes in bacterial community structure along the gradients of glucose concentrations. Since minerals affect cell growth and bioactive agent production of bacteria, we determined the most suitable inorganic salt for strain LY01, by comparing four inorganic salts (K_2HPO_4, $FeSO_4{\cdot}7H_2O$, $MgCl_2$ and $CaCl_2$). Of these, only $MgCl_2$ could promote bacterial growth and produce more algicidal extracts. Heldal et al. [43] determined that Mg^{2+} can replace organic compatible solutes when growing in carbon-limited condition; Mg^{2+} has a major role as osmolyte in marine bacteria; and the Mg^{2+}/Na^{2+} ratio is related to its physiological condition and nutritional status. In summary, tryptone, glucose and $MgCl_2$ were the optimal nutrient sources for bacterial growth and algicidal activity. Nutrient concentrations can influence bacterial growth and metabolite productivity [46], and low nutrient concentration limits bacterial growth and keeps the bacterial cells in a starvation situation [47]. High nutrient concentration can result in waste of resources and production of useless metabolites. Fig. 3 shows the bacterial biomass and algicidal activity of strain LY01 with different concentrations of the nutrients. The algicidal rate reached its maximum level when the tryptone concentration reached 1.0%, thus implying that higher concentration may be useless in terms of algicidal extracts production (Fig. 2a). The algicidal rate reached its maximum level at a glucose concentration of 0.4% with the decreasing of the bacterial biomass (Fig. 2b). Fig. 3c showed that bacterial biomass and algicidal activity both reached their highest level when the $MgCl_2$ concentration was 0.8%. After optimization of the medium components and the nutrient concentrations, the algicidal extracts production and algicidal rate were higher than before (data not shown).

To determine the characteristics of the algicidal extracts of strain LY01, we studied the influence of different temperature and pH, ethanol treatment, dialysis treatment and aqueous solubility on this algicidal extracts (Figs 3 and 4). From the results in Fig. 3, the algicidal activity was almost unchanged after treatment under different temperatures and pH, and this implied that the algicidal extracts was very stable. After treatment with ethanol, the dissolved part also maintained high algicidal activity, and the precipitates lost most of their algicidal activity (Fig. 4a). This showed that the algicidal extracts was not a protein, which could be aggregated to the precipitates by ethanol [48]. The algicidal activity of the extracts outside the dialysis bag showed high algicidal activity, but there showed no algicidal activity inside the dialysis bag. This confirmed that the algicidal extracts could pass through dialysis, and that the molecular weight of the algicidal extracts was less than 1 kD. To make sure of the aqueous solubility of the algicidal extracts, we compared the different aqueous solubility effects, and the ethyl acetate extracts showed the highest algicidal rate compared to the other organic solvents, and therefore the active extracts was more soluble in ethyl acetate, and so this was used as the extraction agent to extract the algicidal extracts from the supernatant. Through characterization of the algicidal extracts, we make sure that the algicidal extracts were stable and eco-environmental (Table S1).

The cell membrane acts as a barrier to prevent extracellular material passing freely through algal cells, and thus ensures a relatively stable environment within the cell, so that the orderly operation of the various biochemical reactions can be implemented [49]. It is reported that algicidal substances could change the membrane permeability and broke cell membrane integrity, inducing the algal cells to lose intracellular substances [50,51]. Zhang et al. studied the algicidal supernatant from the marine algicidal actinomycete BS01 in terms of its action on the algal cell membrane [30]. The indicator of lipid peroxidation (MDA) increased greatly after the algal cells were treated by supernatant for 8 h, and this implied that cell membrane lipid peroxidation is caused by an algicidal compound, and cell membrane integrity is lost. In our study, we observed that the cell membrane was broken after treatment by algicidal extracts for 24 h (Fig. 6e). The intracellular contents (including chloroplast and mitochondrion) left the algal cell, and large numbers of vacuoles appeared inside the cell. The nucleus of active algal cells could be stained with green fluorescence by arcidine orange, however, dead cells could not [52,53]. On the other hand, propidium iodide stains algal cells with red fluorescence only after the cell membrane is broken [54]. Therefore we stained the algal cells with both acridine orange and propidium iodide, to observe the procedure of cell membrane breakage. In Fig. 6a, algal cells are stained with green fluorescence without any red fluorescence, which showed that normal algal cells could not combine

with propidium iodide, and the cell membrane remained intact. After treatment with the algicide for a period of time, the green fluorescence began to decrease and, at the same time, the red fluorescence could be observed in the algal cells (Fig. 6b–d). At the last treatment time, the green fluorescence could almost not be found, and the whole algal cells were stained with red fluorescence, which implied that the algal cell membranes were broken, so propidium iodide could pass into the cells (Fig. 6e and f). Simultaneously, acridine orange could not combine with the dead cells, and the algal cells lost their activity. Liang et al. also reported that the green alga *Chlorella vulgaris* responded to the stress of cetyltrimethyl ammonium bromide (CTAB) and, after algal cells were stained with FDA and PI, the fluorescence photographs were obtained using CLSM [55]. At the end of a six-day test, the result of staining the algal cells with FDA/PI revealed a decrease of viable cell percentage with an increase of CTAB concentration. From the above results, we concluded that cell membrane integrity was lost after treatment with algicidal extracts from strain LY01.

Nuclei can maintain the integrity of genes and influence cell activity through the regulation of gene expression, and play an important role in cell metabolism, growth and differentiation [56]. To study whether the nuclear structure was influenced by algicidal extract, we observed the change of the nuclear structure by staining with DAPI after different treatments (Fig. 7). In the initial treatment time, the nucleus was stained by DAPI with blue fluorescence, the nuclear structure was confined to a certain area, and the blue fluorescence was powerful. After exposed to the algicidal extracts, the blue fluorescence began to spread, and the fluorescence intensity decreased, implying that the nuclear membrane was perhaps broken, and the nuclear material dispersed in the algal cells. Thus, the nucleus was influenced by algicidal extracts and the nuclear structure was damaged. At the same time, the algal chloroplast fluorescence intensity became weaker, and could not be seen at the final treatment time (Fig. 7e), which implied that the chloroplast structure had also been destroyed by the algicidal extracts.

To study whether the algicidal extracts from strain LY01 could influence carbon dioxide fixation, heat shock protein and proliferating cell nuclear antigen gene expressions, the *rbc*S, *hsp* and PCNA genes were chosen to characterize the related-gene expressions (Fig. 8). The family of *rbc*S nuclear genes encodes the rubisco small subunits, which can also influence the carboxylation catalytic efficiency and CO_2/O_2 specificity of the enzyme. The small subunits also contain the structural elements responsible for targeting rubisco to the algal pyrenoid, which is the site where CO_2 is concentrated for optimal photosynthesis [57]. Our results showed that *rbc*S was significantly inhibited ($p < 0.01$) by high concentrations of algicidal extracts (25 and 50 μg/mL) over the whole treatment procedure (Fig. 8a). The 10 μg/mL treatment group inhibited gene expression only in the 6 h treatment time ($p < 0.05$), and showed no influence on gene expression in the 24 h treatment time. This may be the function of algal self-repair, since Roegner et al. report that self-reproduction and self-repair capacity is especially required for algal cells. In the 6 h treatment time, the low concentration could inhibit gene expression, at the same time, self-repair could be induced in the algal cells, so that the inhibitory effect of gene expression would be alleviated in 24 h of treatment [58]. However, the higher concentration treatment groups could produce more inhibition activity than self-repair effect and, therefore, the gene expression was always inhibited in the high concentration treatment groups. The *hsp* gene is a key component contributed to survival in the abiotic or biotic stress response, and the *hsp* gene can be overexpressed under stress [59,60]. To determine whether the algicidal extracts could pose a threat to algal growth, we studied *hsp* gene expression while the alga was under the effects of the algicidal extracts (Fig. 8b). In the initial treatment time (6 h), the *hsp* gene expression was only up-regulated in the 25 μg/mL treatment group ($p < 0.01$), and the 50 μg/mL treatment group did not influence gene expression, while the 10 μg/mL treatment group inhibited *hsp* gene expression. This implied that only the 25 μg/mL treatment group could induce algal cells to respond. After the 24 h treatment time, *hsp* gene expression was up-regulated in the 50 μg/mL treatment group ($p < 0.01$), but the 10 and 25 μg/mL treatment groups did not influence gene expression. Thus, high concentration treatment groups could induce an algal response, and pose a threat to algal growth. PCNA is a common gene in all eukaryotes as a potential cell cycle marker for algal growth rate studies [61], and has a close relationship with DNA synthesis, plays an important role in cell proliferation, and is a good indicator to reflection of cell proliferation [62]. Fig. 8c shows that the PCNA expression was significantly inhibited ($p < 0.01$) by the 25 and 50 μg/mL treatment groups in both the 6 and 24 h treatment times, however, the 10 μg/mL treatment group did not influence PCNA expression over the whole treatment procedure. This implied that the nuclear function could not be exerted normally under high concentrations of algicidal extracts. In summary, the transcription of *rbc*S, *hsp* and PCNA genes were influenced by the algicidal extracts.

5. Conclusions

In conclusion, 1.0% tryptone, 0.4% glucose and 0.8% $MgCl_2$ were the optimal nutrient sources for growth and algicidal effect of *Mangrovimonas yunxiaonensis* strain LY01. The algicidal extracts were heat and pH stable, non-protein and less than 1 kD. Ethyl acetate extracts of the algicidal extracts destroyed cell membrane integrity, damaged nuclear structure, inhibited *rbc*S and PCNA gene expression, and induced up-regulation of *hsp* gene expression. After understanding the algicidal procedure, mechanism and optimizing the algicidal activity, the stable and eco-environmental algicidal extracts could be used to control HABs for field application in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.06.032.

References

[1] D.M. Anderson, Approaches to monitoring, control and management of harmful algal blooms (HABs), Ocean Coastal Manage. 52 (2009) 342–347.
[2] T.I. McLean, G.A. Sinclair, Harmful Algal Blooms, Environmental Toxicology, Springer Press, Berlin, 2013.
[3] L. Medlin, Molecular tools for monitoring harmful algal blooms, Environ. Sci. Pollut. Res. (2013), http://dx.doi.org/10.1007/s11356-012-1195-3.
[4] A. Handå, T.A. McClimans, K.I. Reitan, Ø. Knutsen, K. Tangen, Y. Olsen, Artificial upwelling to stimulate growth of non-toxic algae in a habitat for mussel farming, Aquacult. Res. (2013), http://dx.doi.org/10.1111/are.12127.
[5] E.M. Bertrand, Niche of harmful alga *Aureococcus anophagefferens* revealed through ecogenomics, Proc. Nat. Acad. Sci. U.S.A. 108 (11) (2013) 4352–4357.
[6] B.M. Hickey, V.L. Trainer, K.P. Michael, N.G. Adams, T.P. Connolly, N.B. Kachel, S.L. Geier, A springtime source of toxic *Pseudo-nitzschia* cells on razor clam beaches in the Pacific Northwest, Harmful Algae 25 (2013) 1–14.
[7] L.H. Pettersson, D. Pozdnyakov, Biology and ecology of harmful algal species, in: Monitoring of Harmful Algal Blooms, Springer, 2013, pp. 25–47.

[8] B.M. Riegl, A.W. Bruckner, K. Samimi-Namin, S.J. Purkis, Diseases, Harmful algae blooms (HABs) and their effects on gulf coral populations and communities, in: Coral Reefs of the Gulf, Springer Press, Berlin, 2012.
[9] P. Glibert, L. Bouwman, Land-based nutrient pollution and the relationship to harmful algal blooms in coastal marine systems, Loicz Newsletter Inprint 2 (2012) 5–7.
[10] E.S. Reichwaldt, H. Song, A. Ghadouani, Effects of the distribution of a toxic microcystis bloom on the small scale patchiness of zooplankton, PLoS One 8 (2013) e66674.
[11] A. Agrawal, K. Gopal, Toxic Cyanobacteria in water and their public health consequences, in: Biomonitoring of Water and Waste Water, Springer Press, Berlin, 2013.
[12] V. Mulvenna, K. Dale, B. Priestly, U. Mueller, A. Humpage, G. Shaw, I. Falconer, Health risk assessment for cyanobacterial toxins in seafood, Int. J. Environ. Res. Public Health 9 (2012) 807–820.
[13] M. Asakawa, K. Miyazawa, H. Takayama, T. Noguchi, Dinoflagellate *Alexandrium tamarense* as the source of paralytic shellfish poison (PSP) contained in bivalves from Hiroshima Bay, Hiroshima Prefecture, Japan, Toxicon 33 (1995) 691–697.
[14] A. Cembella, M. Quilliam, N. Lewis, A. Bauder, C. Dell'Aversano, K. Thomas, The toxigenic marine dinoflagellate *Alexandrium tamarense* as the probable cause of mortality of caged salmon in Nova Scotia, Harmful Algae 1 (2002) 313–325.
[15] S. Gracia, S. Roy, M. Starr, Spatial distribution and viability of *Alexandrium tamarense* resting cysts in surface sediments from the St. Lawrence Estuary, Eastern Canada, Estuarine Coastal Shelf Sci. 121–122 (2013) 20–32.
[16] D.M. Anderson, D.M. Kulis, Y.Z. Qi, L. Zheng, S. Lu, Y.T. Lin, Paralytic shellfish poisoning in Southern China, Toxicon 34 (1996) 579–590.
[17] M. Kodama, S. Sato, S. Sakamoto, T. Ogata, Occurrence of tetrodotoxin in *Alexandrium tamarense*, a causative dinoflagellate of paralytic shellfish poisoning, Toxicon 34 (1996) 1101–1105.
[18] E.L. Lilly, K.M. Halanych, D.M. Anderson, Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae), J. Phycol. 43 (2007) 1329–1338.
[19] K. Ichimi, T. Suzuki, A. Ito, Variety of PSP toxin profiles in various culture strains of *Alexandrium tamarense* and change of toxin profile in natural *A. tamarense* population, J. Exp. Mar. Biol. Ecol. 273 (2002) 51–60.
[20] A. Murata, Y. Nagashima, S. Taguchi, N. P ratios controlling the growth of the marine dinoflagellate *Alexandrium tamarense*: content and composition of paralytic shellfish poison, Harmful Algae 20 (2012) 11–18.
[21] T. Suzuki, K. Ichimi, Y. Oshima, T. Kamiyama, Paralytic shellfish poisoning (PSP) toxin profiles and short-term detoxification kinetics in mussels *Mytilus galloprovincialis* fed with the toxic dinoflagellate *Alexandrium tamarense*, Harmful Algae 2 (2003) 201–206.
[22] H. Kim, Mitigation and controls of HABs, in: Ecology of Harmful Algae, Springer Press, Berlin, 2006.
[23] M. Sengco, D.M. Anderson, Controlling harmful algal blooms through clay flocculation, J. Eukaryot. Microbiol. 51 (2004) 169–172.
[24] W. Yang, X. Zhang, J. Liu, J. Gao, P. Zhang, Inhibitory effect and sinking behaviour of wood meals from China fir on *Alexandrium tamarense* in cultures, Acta Hydrobiol. Sin. 29 (2005) 215–218.
[25] L. Zhou, X. Chen, T. Zheng, Study on the ecological safety of algacides: a comprehensive strategy for their screening, J. Appl. Phycol. 22 (2010) 803–811.
[26] C. Yang, Y. Li, Y. Zhou, W. Zheng, Y. Tian, T. Zheng, Bacterial community dynamics during a bloom caused by *Akashiwo sanguinea* in the Xiamen Sea Area, China, Harmful Algae 20 (2012) 132–141.
[27] S.J. Bai, L.P. Huang, J.Q. Su, Y. Tian, T.L. Zheng, Algicidal effects of a novel marine actinomycete on the toxic dinoflagellate *Alexandrium tamarense*, Curr. Microbiol. 62 (2011) 1774–1781.
[28] Y. Li, S. Bai, C. Yang, Q. Lai, H. Zhang, Z. Chen, J. Wei, W. Zheng, Y. Tian, T. Zheng, *Mangrovimonas yunxiaonensis* gen. nov., sp. nov., isolated from mangrove sediment, Int. J. Syst. Evol. Microbiol. 63 (2013) 2043–2048.
[29] B. Wang, X. Yang, J. Lu, Y. Zhou, J. Su, Y. Tian, T. Zheng, A marine bacterium producing protein with algicidal activity against *Alexandrium tamarense*, Harmful Algae 13 (2012) 83–88.
[30] H. Zhang, X. An, Y. Zhou, B. Zhang, S. Zhang, D. Li, Z. Chen, Y. Li, S. Bai, J. Lv, W. Zheng, Y. Tian, T. Zheng, Effect of oxidative stress induced by *Brevibacterium* sp. BS01 on a HAB causing species-*Alexandrium tamarense*, PLoS One 8 (2013) e63018.
[31] Y. Xu, F. Ge, N. Wang, R. Zhu, N. Tao, Selective algicidal activity of surfactant and its mechanism 1, J. Hazard., Toxic, Radioact. Waste 15 (2010) 21–25.
[32] S.C. Park, J.K. Lee, S.W. Kim, Y. Park, Selective algicidal action of peptides against harmful algal bloom species, PLoS One 6 (2011) e26733.
[33] J.Y. Cho, Algicidal activity of marine *Alteromonas* sp. KNS-16 and isolation of active compounds, Biosci. Biotechnol., Biochem. 76 (2012) 1452–1458.
[34] I. Imai, Algicidal ranges in killer bacteria of direct attack type for marine phytoplankton, Bull. Plankton Soc. Jpn. 44, 1997, pp. 3–9.
[35] Y. Li, H. Zhu, C. Guan, H. Zhang, J. Guo, Z. Chen, G. Cai, X. Lei, W. Zheng, Y. Tian, X. Xiong, T. Zheng, Towards molecular, physiological, and biochemical understanding of photosynthetic inhibition and oxidative stress in the toxic *Alexandrium tamarense* induced by a marine bacterium, Appl. Microbiol. Biotechnol. 98 (10) (2014) 4637–4652.
[36] R.R.L. Guillard, Culture of phytoplankton for feeding marine invertebrates, in: W.L. Smith, M.H. Canley (Eds.), Culture of Marine Invertebrate Animals, Plenum Press, New York, NY, 1975.
[37] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (4) (2001) 402–408.
[38] U.L. Zweifel, B. Norrman, A. Hagstrom, Consumption of dissolved organic carbon by marine bacteria and demand for inorganic nutrients, Mar. Ecol.: Prog. Ser. 101 (1993) 23–32.
[39] P.A. Wheeler, D.L. Kirchman, Utilization of inorganic and organic nitrogen by bacteria in marine systems, Limnol. Oceanogr. 31 (5) (1986) 998–1009.
[40] J.C. Goldman, M.R. Dennett, Growth of marine bacteria in batch and continuous culture under carbon and nitrogen limitation, Limnol. Oceanogr. 45 (2000) 789–800.
[41] G.S. Wilson, D.A. Raftos, S.L. Corrigan, S.V. Nair, Diversity and antimicrobial activities of surface-attached marine bacteria from Sydney Harbour, Australia, Microbiol. Res. 165 (2010) 300–311.
[42] B. Kientz, H. Agogué, C. Lavergne, P. Marié, E. Rosenfeld, Isolation and distribution of iridescent *Cellulophaga* and other iridescent marine bacteria from the Charente-Maritime coast, French Atlantic, Syst. Appl. Microbiol. 36 (2013) 244–251.
[43] M. Heldal, S. Norland, E.S. Erichsen, R.A. Sandaa, A. Larsen, F. Thingstad, G. Bratbak, Mg^{2+} as an indicator of nutritional status in marine bacteria, ISME J. 6 (2011) 524–530.
[44] S.K. Schmidt, M. Alexander, Effects of dissolved organic carbon and second substrates on the biodegradation of organic compounds at low concentrations, Appl. Environ. Microbiol. 49 (1985) 822–827.
[45] R. Zhang, M.G. Weinbauer, Y.K. Tam, P.Y. Qian, Response of bacterioplankton to a glucose gradient in the absence of lysis and grazing, FEMS Microbiol. Ecol. 85 (2013) 443–451.
[46] A.L. Shanks, D. Trent Jonathan, Marine snow: microscale nutrient patched, Limnol. Oceanogr. 24 (1979) 8504354.
[47] L. Gómez-Consarnau, N. Akram, K. Lindell, A. Pedersen, R. Neutze, D.L. Milton, J.M. González, J. Pinhassi, Proteorhodopsin phototrophy promotes survival of marine bacteria during starvation, PLoS Biol. 8 (2010) e1000358.
[48] S.M. Singh, J. Cabello-Villegas, R.L. Hutchings, K.M. Mallela, Role of partial protein unfolding in alcohol-induced protein aggregation, Proteins: Struct., Funct., Bioinf. 78 (2010) 2625–2637.
[49] M.J. Veldhuis, G.W. Kraay, K.R. Timmermans, Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth, Eur. J. Phycol. 36 (2001) 167–177.
[50] M.A. Alamsjah, S. Hirao, F. Ishibashi, Y. Fujita, Isolation and structure determination of algicidal compounds from Ulva fasciata, Biosci. Biotechnol., Biochem. 69 (2005) 2186–2192.
[51] X. Wang, L. Gong, S. Liang, X. Han, C. Zhu, Y. Li, Algicidal activity of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*, Harmful Algae 4 (2005) 433–443.
[52] S. Aravindan, C.R. Delma, S.S. Thirugnanasambandan, T.S. Herman, N. Aravindan, Anti-pancreatic cancer deliverables from sea: first-hand evidence on the efficacy, molecular targets and mode of action for multifarious polyphenols from five different brown-algae, PLoS One 8 (2013) e61977.
[53] J. Kováčik, B. Klejdus, P. Babula, Oxidative stress, uptake and bioconversion of 5-fluorouracil in algae, Chemosphere 100 (2013) 116–123.
[54] M. Pereira, N. Raposo, R. Brayner, E. Teixeira, V. Oliveira, C. Quintao, L. Camargo, L. Mattoso, H. Brandao, Cytotoxicity and expression of genes involved in the cellular stress response and apoptosis in mammalian fibroblast exposed to cotton cellulose nanofibers, Nanotechnology 24 (2013) 075103.
[55] Z. Liang, F. Ge, H. Zeng, Y. Xu, F. Peng, M. Wong, Influence of cetyltrimethyl ammonium bromide on nutrient uptake and cell responses of *Chlorella vulgaris*, Aquat. Toxicol. 138–139 (2013) 81–87.
[56] Y. Kobayashi, S. Imamura, M. Hanaoka, K. Tanaka, A tetrapyrrole-regulated ubiquitin ligase controls algal nuclear DNA replication, Nat. Cell Biol. 13 (2011) 483–487.
[57] T. Genkov, M. Meyer, H. Griffiths, R.J. Spreitzer, Functional hybrid rubisco enzymes with plant small subunits and algal large subunits engineered *rbc*S cDNA for expression in *Chlamydomonas*, J. Biol. Chem. 285 (2010) 19833–19841.
[58] M. Roegner, Metabolic engineering of cyanobacteria for the production of hydrogen from water, Biochem. Soc. Trans. 41 (2013) 1254–1259.
[59] S. Choi, Identification and analysis of HSP70 gene family in marine red algae, *Pyropia seriata*, in: Plant and Animal Genome XXII Conference, Plant and Animal Genome, 2014.
[60] F. Liu, X. Sun, W. Wang, Z. Liang, F. Wang, De novo transcriptome analysis-gained insights into physiological and metabolic characteristics of *Sargassum thunbergii* (Fucales, Phaeophyceae), J. Appl. Phycol. 26 (3) (2014) 1519–1526.
[61] Y. Hou, H. Zhang, L. Miranda, S. Lin, Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: microalgal pcna as the model gene, PLoS One 5 (2010) e9545.
[62] J. Huang, S. Liang, Z. Sui, Y. Mao, H. Guo, Cloning and characterization of proliferating cell nuclear antigen gene of *Alexandrium catenella* (Dinoflagellate) with respect to cell growth, Acta Oceanolog. Sin. 29 (2010) 90–96.