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Toxic effect on the membrane system and cell proliferation of *Prorocentrum donghaiense* caused by the novel algicidal fungus *Talaromyces purpurogenus* YL13

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Abstract A fungal strain YL13 with algicidal activity against the dinoflagellate *Prorocentrum donghaiense* was isolated from Wild Fox Island, Zhuhai, China, and identified as *Talaromyces purpurogenus* YL13 on the basis of 18S ribosomal RNA (rRNA) sequence. Strain YL13 exhibited algicidal activity through the mechanism of indirect attack, and its algicidal activity was improved from 80.3 to 96.8 % by optimization of environmental and nutrient factors with response surface methodology (RSM). Effects of strain YL13 on the membrane system and cell proliferating of *P. donghaiense* were investigated to elucidate the algicidal mechanism. The increase in both ATPase activities and malondialdehyde (MDA) contents suggested that the membrane in algal cells was damaged. Damage was observed in atomic force microscopy (AFM) images and the surface morphology of cells. Real-time PCR assay showed changes in the transcript abundance of proliferating cell nuclear antigen (PCNA) gene. The release of nucleic acids and changes of PCNA gene expression indicated that DNA synthesis was affected, and this influenced cell proliferation and the membrane system of *P. donghaiense*. The fungal supernatant might be potentially used as a bioagent for controlling harmful algae. Strain YL13 is the first record of a *T. purpurogenus* being algicidal to the harmful dinoflagellate *P. donghaiense*, and this is the first report to explore the mechanism of toxic effects on membrane system and cell proliferation of the marine *T. purpurogenus* against harmful *P. donghaiense*.

Keywords *Prorocentrum donghaiense* · Algicidal fungus · Culture optimization · Membrane damage · Gene expression

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Introduction

Harmful algal blooms (HABs) and their negative impacts on aquatic ecosystems and human health have been reported all over the world (Hallegraeff 1993; Mcleod et al. 2012). HABs cause enormous economic losses in aquaculture industries, mass death of marine organisms, threats to human health, and pollution of coastal areas (Deeds et al. 2002; Fogg 2002; Stoecker et al. 2008). *Prorocentrum donghaiense*, a notorious bloom-forming dinoflagellate, has continuously caused large-scale HABs in the Changjiang River Estuary of China and its adjacent sea in recent years (Lu and Goebel 2001; Wang and Huang 2003). Therefore, there is an imperative and urgent need to develop effective and environmentally friendly ways to manage this species.

Currently, many researchers have focused their attention on biological control techniques, including the use of bacteria (Nakashima et al. 2006; Paul and Pohnert 2013; Tilney et al.

2014), fungi (Jia et al. 2010), viruses (Sheik et al. 2014), protozoa (Jeong et al. 2008), and seaweeds (Oh et al. 2010; Yang et al. 2015a, b) for harmful algae control. Studies have shown marine-derived fungi to be a rich source of structurally unique and biologically active secondary metabolites (Jia et al. 2010), and these metabolites have been found to play an important role in the termination of HABs. Although some algicidal fungi have been isolated and exploited, such as *Bjerkandera* (Han et al. 2011), *Trichoderma* (Mohamed et al. 2014), and *Phoma* (Hussain et al. 2014), most studies have focused mainly on their ability to control harmful *Microcystis aeruginosa*, whereas the search for fungi with algicidal activity on *P. donghaiense* has not been given much attention.

The algicidal activity is associated with the growth of the target fungi and can be affected individually and collectively by various environmental and nutrient factors (Xu et al. 2013). It has been difficult to consider the interactions between the various factors, unless the relationship between these factors can be systematically manipulated. The response surface methodology (RSM) is a useful tool for evaluating such multiple factor interactions based on quantitative data (Li et al. 2011). To date, little attention has been paid to the optimization of environmental and nutrient factors with RSM that could improve algicidal activity.

Membrane systems and cell proliferation play an important role in the growth of cells and in the stabilization of metabolism and influence cell activity through the regulation of gene expression and osmotic balance (Kobayashi et al. 2011). Although some reports about membrane morphology of microorganisms inhibiting algal growth are available, the precise inhibition mechanisms and the effects on membrane properties and functions remain largely unknown. The activities of the Na^+-K^+ ATPase (Na^+ pump) and Ca^{2+}-Mg^{2+} ATPase (Ca^{2+} pump) are affected by membrane integrity and involved in osmotic balance (Malfatti and Azam 2009). Zhou et al. (2011) also have reported that the change of Ca^{2+} and Na^+ pumps and malondialdehyde (MDA) levels induced by phthalic acid esters (PAEs) might be an important signal of developmental toxicity in membrane integrity and permeability. Atomic force microscopy (AFM) enables imaging of the surface of algal cells, which could directly provide important information about cell membrane integrity and its response to the microniche environment. The proliferating cell nuclear antigen (PCNA) gene is the sliding clamp for DNA polymerases δ and ε in cells and is required for DNA synthesis, replication, and repair (Prelich et al. 1987; Kelman 1997). Algal cell nucleotide levels and PCNA-related gene expressions are good indicator of the state of cell proliferation. Recently, Li et al. (2014) studied the PCNA gene, and the results showed that the algicidal bacterium *Mangrovimonas yunxiaonensis* LY01 supernatant inhibited its expression in *Alexandrium tamarense*. However, there has been no report on AFM images, ATPase activity, and PCNA gene expression during the cell death phase of *P. donghaiense*.

In this study, a strain of fungus (YL13) with indirect algicidal activity against the *P. donghaiense* was isolated from Wild Fox Island, Zhuhai, China, and was identified as *Talaromyces purpurogenus* on the basis of physiological characteristics and the 18S ribosomal RNA (rRNA) sequence. The environmental and nutrient factors for growth of strain YL13 were optimized using Box-Behnken design (BBD) and RSM in order to obtain a high algicidal activity. In addition, the potential inhibition mechanisms of oxidative stress, ATPase activities, membrane integrity, release of nucleic acids, and PCNA gene expression on *P. donghaiense* were examined during the algicidal process. To the best of our knowledge, strain YL13 is the first record of a *T. purpurogenus* being algicidal to the harmful dinoflagellate *P. donghaiense*. This is also the first report exploring the mechanism of toxic effects on the membrane system and cell proliferation of a marine *T. purpurogenus* against the harmful dinoflagellate *P. donghaiense*.

Materials and methods

Algal cultures and treatment

The culture of *P. donghaiense* (strain PD02) was supplied by the Algal Culture Collection, Institute of Hydrobiology, Jinan University (Guangzhou, China). Before being used in the experiments, algal cells were cultured in f/2 medium (Yang et al. 2015a, b) under an irradiance of 80 μmol photons m^{-2} s^{-1} in a 12/12 h light-dark cycle at 20 ± 1 °C (light incubator GXZ-0328), using artificial seawater with a salinity of 25‰ instead of natural seawater (Guillard 1975). The cell numbers were counted under a light microscope. A new test was started when the concentration of algae reached approximately 10^7 cells mL^{-1}. Throughout the experiments, the incubation conditions of the algal species after adding the YL13 supernatant were kept the same as for the algal cultures.

In order to assay the algicidal activities, total chlorophyll *a* was extracted with 90 % acetone and quantified using the method of Lichtenthaler and Wellburn (1982) The chlorophyll *a* concentration was calculated by using the formula: $\text{Chlorophyll a (mg L}^{-1}) = 11.6 \times A_{665} - 1.31 \times A_{645} - 0.14A_{630}$. The algicidal activity was calculated by using the following equation: $\text{Algicidal percentage (\%)} = (1 - N_t / N_c) \times 100$, where N_t and N_c are the chlorophyll *a* content of the treated groups with the strain YL13 supernatant and the control groups, respectively.

Isolation and identification of algicidal fungus

The algal-lysing fungus (strain YL13) was isolated from approximately 25 fungal isolates from the Wild Fox Island (22°

28′ N 113° 58′ E), Zhuhai, China, and was cultured with Czapek-Dox medium (30 g sucrose, 1 g K_2HPO_4, 1 g KCl, 2 g $NaNO_3$, 0.5 g $MgSO_4{\cdot}7H_2O$, and 0.01 g $FeSO_4{\cdot}7H_2O$ in 1 L of distilled water, pH adjusted to 5.0–5.5 and autoclaved at 121 °C for 30 min). Strain YL13 was identified as *T. purpurogenus* on the basis of physiological characteristics and the 18S rRNA sequence (KR809559 in GenBank). The fungal genomic DNA was sequenced and assembled by ABI-3730 and vector NTI suite 8, respectively. The sequences were checked against BLAST and then deposited in the NCBI GenBank.

Determination of algicidal mode

Fungal cells were collected using centrifugation (6000×*g*, 10 min), washed three times using sterile f/2 medium, and re-suspended in sterile f/2 medium. The supernatant was filtered through a 0.22-μm Millipore membrane filter and stored at −4 °C. To determine the algicidal mode of strain YL13, 1.5 % (*v*/*v*) cell suspensions, supernatant and sterilized medium were inoculated into exponential growth phase *P. donghaiense* cultures, and a no addition control was also used in the experiments. Algicidal mode was illustrated by the algicidal percentage, calculated after co-culture for 5 days, and microscopically observed.

Optimization of environmental and nutrient factors

Various environmental and nutrient factors were supplemented in Czapek-Dox medium to find the ideal culture conditions and nutrients. The effects of different concentration of these nutrients were determined to find out the optimal combination to obtain a higher algicidal activity. The experimental design was performed by using Box-Behnken design (BBD), and RSM analysis was done by using Design-Expert 8.0.5. Table 1 shows the three factors and their levels used in the experiments. This design included 27 experiments to investigate the effects of factors and their interactions, and the design matrix is listed in Table 2.

Table 1 Evaluated factors, factor notation, and their levels in response surface methodology (Box-Behnken design)

Factor	Notation	Level		
		−1	0	1
Sucrose (mg mL^{-1})	A	15	30	45
$(NH_4)_2SO_4$ (mg mL^{-1})	B	0.5	2	3.5
KH_2PO_4 (mg mL^{-1})	C	0.4	1.0	1.6

Table 2 Real values of variables in response surface methodology (Box-Behnken design) and experimental data of algicidal percentage

Run	A (g L^{-1})	B (g L^{-1})	C (g L^{-1})	Algicidal percentage (%)
1	45.0	0.5	1.0	79.3 (±1.7)
2	30.0	0.5	1.6	90.7 (±2.9)
3	15.0	2.0	0.4	41.8 (±0.6)
4	15.0	3.5	1.0	47.5 (±1.1)
5	15.0	0.5	1.0	38.9 (±0.8)
6	45.0	2.0	0.4	88.4 (±2.3)
7	30.0	3.5	1.6	89.3 (±1.9)
8	30.0	2.0	1.0	94.5 (±2.6)
9	45.0	2.0	1.6	89.7 (±1.3)
10	45.0	3.5	1.0	87.6 (±1.5)
11	15.0	2.0	1.6	46.4 (±0.5)
12	30.0	2.0	1.0	92.9 (±2.4)
13	30.0	2.0	1.0	94.1 (±2.7)
14	30.0	3.5	0.4	91.5 (±1.6)
15	30.0	2.0	1.0	94.6 (±2.3)
16	30.0	0.5	0.4	89.8 (±1.2)
17	30.0	2.0	1.0	94.4 (±1.8)

Determination of protein and MDA contents, total antioxidant capacity, and ATPase activity

Algal cells were harvested by centrifugation (3000×*g*, 5 min), resuspended in 1 mL phosphate-buffered saline (PBS, 50 mM, pH 7.8), disrupted with ultrasound (80 W, 5:5 s, 40 times), and then centrifugated (10,000×*g*, 5 min). One milliliter of the supernatant was used to assay protein content using bovine serum albumin as the standard (Bradford 1976). The rest of the supernatant was stored at −80 °C until it was used to analyze the alteration of MDA and the total antioxidant capacity (T-AOC). The Na^+-K^+ ATPase (Na^+ pump) and Ca^{2+}-Mg^{2+} ATPase (Ca^{2+} pump) activities were determined by quantifying the release of inorganic phosphorus from adenosine triphosphate. Specific activity was expressed as the concentration of adenosine diphosphate liberated per unit time and standardized to protein content. All the assay methods were carried out by following the kit's operation manual (Nanjing Jiancheng Bioengineering Institute, China; Zhang et al. 2011).

Atomic force microscopy analysis

AFM was used to observe the cellular morphology after adding strain YL13 supernatant (1.5 %, *v*/*v*). Samples were first fixed with glutaraldehyde (2.5 %) overnight and then washed by 0.1 M phosphate buffer solution (pH 7.2) three times and deionized water twice. Fifty milliliter of sample was spotted on freshly cleaved mica, allowed to dry completely. With sterile scissors, a piece of filter was cut out and

attached to a glass slide with double-sided sticky tape. The roughness (Rq) was analyzed by NanoScope Analysis. Images were acquired in ScanAsyst mode. The scanning AFM probe was Tap150Al-G (BudgetSensors) with the scan rates of 0.51–1 Hz ($k = 5$ N m^{-1}).

Nucleotide levels and PCNA gene expression

The amount of the DNA and RNA released from the cytoplasm was estimated through the detection of absorbance at 260 nm (Chen and Cooper 2002). One milliliter of YL13 supernatant was added to 250 mL each conical flasks containing 100 mL algal culture. After co-culture for 1, 2, 3, 4, and 5 days the supernatant was obtained by centrifugation (3500× g, 5 min). The optical density (OD) of the supernatant was measured at 260 nm. The OD_{260} ratio between treated groups and the control was used to evaluate the level of nucleotides released.

The total RNA was extracted following the manufacturer's recommendations of RNAiso (TaKaRa, China), and algal cultures were centrifuged at 2500× g for 5 min to collect precipitates. Electrophoresis was used to check RNA integrity, and the absorbances at 260 and 280 nm were measured to determine the RNA concentration and purity (Zhang et al. 2014a, b). Reverse transcription step was carried out in strict accordance with the TaKaRa PrimeScript RT reagent Kit (Perfect Real Time) instructions. Quantitative real-time PCR (RT-qPCR) was performed by using CFX96 Real-Time PCR System (Bio-Rad, USA) and TaKaRa SYBR Premix Ex Taq II (Tli RnaseH Plus). The reaction conditions were run 15 min at 37 °C and 5 s at 85 °C. Primer pairs are listed in Table 3. PCR were run as follows: 95 °C for 30 s, 45 cycles with 15 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C.

Statistical analysis

The RT-qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All data are expressed as mean ± standard deviation of triplicate experiments ($n = 3$) and were evaluated by using two-way ANOVA, with $p < 0.01$ and $p < 0.05$ (Origin 8.0 for Windows).

Table 3 Sequences of primer pairs used in real-time PCR

	Sequence (5′–3′)
18S rRNA (192 bp)	Forward: CATTTGCCAGGGATGTTTTC
	Reverse: AAGTTTCAGCCTTGCGACC
PCNA (135 bp)	Forward: GAGTGTCACGGTCCACGAAC
	Reverse: TGATACTTCACGAGCAGAGGC

Results

Fungal isolation, identification, and algicidal mode

Among different fungal strains isolated from Wild Fox Island, strain YL13 showed a strongest algicidal activity against *P. donghaiense*. Its colonies were found to be round, swollen, neat edged, and radial growth of white cells. Cells secreted a yellow pigment which caused the back of the colonies to be yellow. The 18S rRNA gene sequence of this fungus (GenBank accession number KR809559) was compared with the sequence of ITS region in GenBank database by means of BLAST search and was found to be highly homologous to *T. purpurogenus*, with 99 % sequence similarity, and the genetic distance was the smallest (Fig. 1).

As shown in Fig. 2, the algicidal percentage of *P. donghaiense* exposure to 1.5 % supernatant of strain YL13 was 80.3 ± 3 % after 5 days of treatment, whereas the cell suspensions and sterilized medium did not show any obvious algicidal activity compared with the no addition control.

Optimization of environmental and nutrient factors

Sets of experiments were carried out to obtain the optimal values of culture conditions and nutrient contents (data not shown here but given supplementary materials Figs. S1, S2, S3, S4, S5, S6, S7, and S8). These values were culture time of 4.5 days (Fig. S1), temperature 28 °C (Fig. S2), pH 5.5 (Fig. S3), salinity 30‰ (Fig. S4), rotation speed of 160 rpm (Fig. S5), sucrose (Fig. S6), $(NH_4)_2SO_4$ (Fig. S7), and KH_2PO_4 (Fig. S8). BBD and RSM analysis were then carried out with the parameters as given in Tables 1 and 2 to optimize the nutrient concentrations for fungal algicidal activity. An equation of the initial response surface model was generated by the Design-Expert 8.0.5 software as follows:

$$Y = -73.02083 + 8.78833\,A + 8.63889\,B + 6.88889\,C - 0.00033333\,AB - 0.091667\,AC - 0.86111\,BC - 0.12117\,A^2 - 1.56111\,B^2 - 0.72917\,C^2$$

where Y, A, B, and C represent the algicidal activity of the strain YL13, sucrose, $(NH_4)_2SO_4$, and KH_2PO_4 contents of culture medium, respectively. The 3D surface plots between every two independent variables on the basis of the equation are shown in Fig. 3a–c. Figure 3a shows the effects of sucrose and $(NH_4)_2SO_4$ contents on the fungal algicidal activity. When the $(NH_4)_2SO_4$ contents were fixed, the algicidal activity of strain YL13 increased with increasing sucrose content until reaching a maximum and then slowly decreased. $(NH_4)_2SO_4$ content showed a similar pattern to sucrose content. Figure 3b shows the effects of sucrose and KH_2PO_4 contents on the fungal algicidal activity. It can be seen that the KH_2PO_4 contents had no obvious effect on

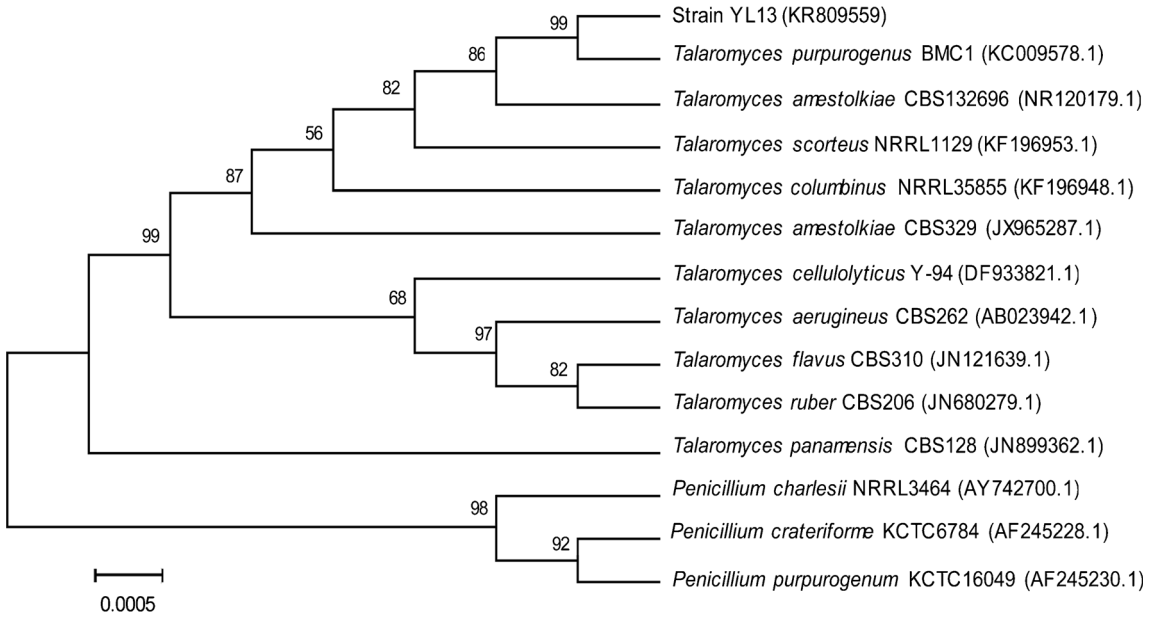

Fig. 1 Phylogenetic tree based on 18S rRNA gene sequences showing the relationship between strain YL13 and related genera. The *scale bar* represents 0.0005 substitutions per nucleotide position. Accession numbers for sequences are as shown in the phylogenetic tree

the algicidal activity of the strain YL13 at a given sucrose content. When the KH_2PO_4 content was fixed, the algicidal activity of the fungus increased and then decreased. As shown in Fig. 3c, the effect of $(NH_4)_2SO_4$ content on the fungal algicidal activity showed a similar pattern to that in Fig. 3a. Moreover, when the $(NH_4)_2SO_4$ content was kept at a certain value, the algicidal activity of strain YL13 increased rapidly with increasing KH_2PO_4. This trend was reversed when the fixed $(NH_4)2SO_4$ value was greater than 2.68 g L^{-1}.

Levels of protein, MDA, and total antioxidant capacity

As is shown in Fig. 4a, cell protein in the algae decreased with increased treatment concentrations of the supernatant. After 5 days of treatment with 0.5, 1.0, and 1.5 % concentrations of the supernatant, cell protein was reduced to 43.9 ± 5 % ($p < 0.05$), 42.7 ± 6 % ($p < 0.05$), and 24.7 ± 2 % ($p < 0.01$) of the control, respectively. In contrast, 0.5, 1.0, and 1.5 % (*v*/*v*) of strain YL13 supernatant resulted in respective MDA contents increasing 1.91 ± 0.04, 1.93 ± 0.06, and 1.97 ± 0.03 times ($p < 0.05$) at 1 day (Fig. 4b). MDA contents after 4 days of exposure were the highest and then decreased at each concentration. The effects of YL13 supernatant on T-AOC are shown in Fig. 4c. After 1-day exposure, T-AOC showed a slight increase compared to that of the controls. As exposure time increased, T-AOC reduced significantly at day 4. Maximum reduction rates (compared to the controls) of 36.8 ± 3 % ($p < 0.01$), 45.2 ± 4 % ($p < 0.05$), and 47.5 ± 7 % ($p < 0.05$), when treated with 0.5, 1.0, and 1.5 % (*v*/*v*) of YL13 supernatant, respectively, were observed.

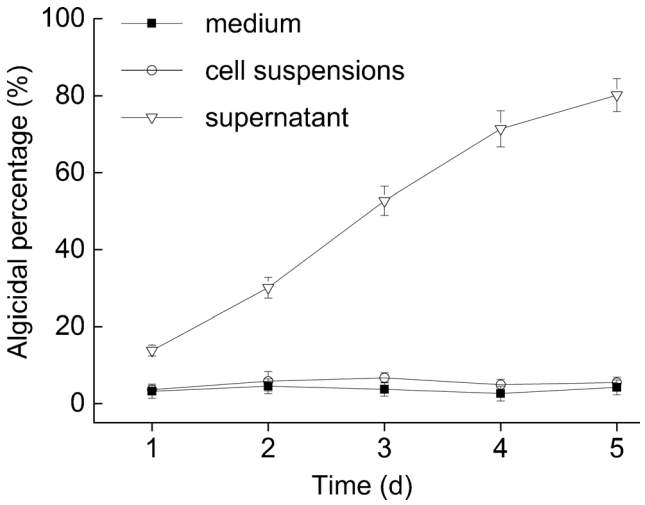

Fig. 2 The algicidal percentage of different parts of fungal cultures (1.5 %, *v*/*v*). All *error bars* indicate SE of three replicates ($n = 3$)

Na^+ pump and Ca^{2+} pump

The activities of the Na^+ pump and the Ca^{2+} pump were examined, because they are involved in membrane integrity and osmotic balance. The results showed that enzyme activities were dose dependent (Fig. 4d, e). The level of the Na^+ pump was significantly increased in the treated groups compared to the controls, while the Ca^{2+} pump declined at first and then significantly increased with the prolongation of treatment time. Within 5 days of treatment, Na^+ pump levels were approximately 2.65 ± 0.02, 3.29 ± 0.07, and 3.87 ± 0.04 times those of the control and Ca^{2+} pump activities were 2.22 ± 0.03, 3.18 ± 0.12, and 3.57 ± 0.08 times those of the control after exposure to 0.5, 1.0, and 1.5 % (*v*/*v*) concentrations of the supernatant, respectively.

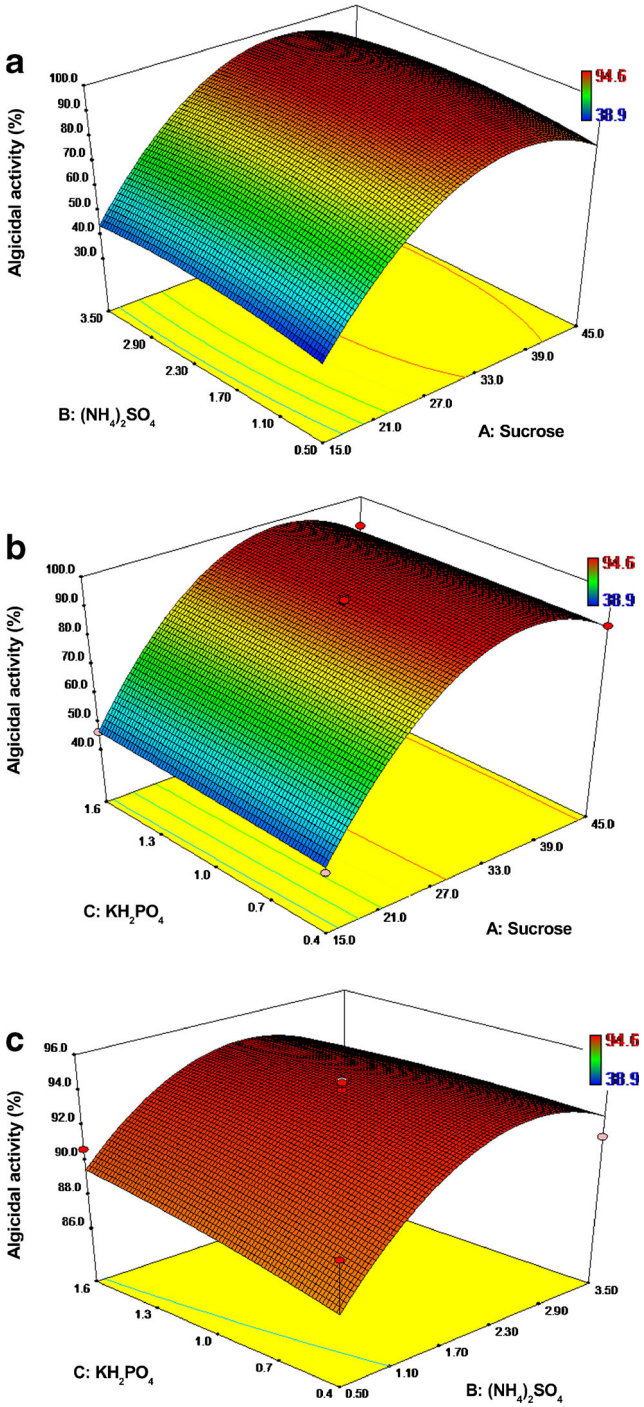

Fig. 3 Three-dimensional contour plots showing the experimental factors and their mutual interactions: **a** effect of sucrose and $(NH_4)_2SO_4$ on the algicidal activity of strain YL13, **b** effect of sucrose and KH_2PO_4 on the algicidal activity of strain YL13, and **c** effect of $(NH_4)_2SO_4$ and KH_2PO_4 on the algicidal percentage of strain YL13

Morphological changes of cell membrane

The morphological changes of cell membranes were observed with AFM captures. The images of biofilm morphology of *P. donghaiense* are shown in Fig. 5. Strain YL13 supernatant increased the surface roughness of algal cells. In the AFM images, cells in control groups were predominantly plump and scattered with a roughness (Rq) value of 28.9 nm (Fig. 5a). On the other hand, those cells treated for 3 days were clustered after exposure to 1.5 % YL13 supernatant (Fig. 5b). Cell membranes in the AFM observations showed wrinkles and breaks on their surfaces, and there were many fragments around the cells. The roughness (Rq) value of the surface in treated cells was 121 nm.

Nucleotide levels and PCNA gene expression

In order to find out the effects of active substances on the cell proliferation system, the nucleotide levels and PCNA gene expression were determined. The OD_{260} ratio of all the different concentrations of treatments was increased remarkably (Fig. 6a). Within 4 days of treatment, the maximum ratios were found to be 5.79 ± 0.24, 6.38 ± 0.18, and 6.76 ± 0.22 after exposure to 0.5, 1.0, and 1.5 % (*v*/*v*) supernatant, respectively. Figure 6b shows PCNA gene expression of *P. donghaiense* under strain YL13 supernatant (1.5 %, *v*/*v*) treatment. The relative transcript abundance of PCNA gene in treated algae was 1.38 ± 0.03 times that of the control at the first day. After 3 days of exposure, PCNA gene expression decreased to 0.47 ± 0.01 times that of the control and then below detection limit from 6 days of treatment onwards.

Discussion

The dinoflagellate *P. donghaiense* has continuously caused large-scale blooms along the Chinese coast in recent years. These blooms have brought tremendous loss to the local economy and serious impacts to the local environment (Wang and Huang 2003). There is an urgent need to seek effective methods to control the developments of *P. donghaiense* species. Some studies have shown that some fungi are algal parasites, which can be an important factor in controlling seasonal phytoplankton succession (Van Donk 1989), whereas others have reported that fungi can produce antibiotics to lyse cyanobacteria (Redhead and Wright 1980). To date, there are few reports on the effects of fungi on harmful blooms, and most of them only describe the relationship between freshwater algae and fungi. Almost no work of the interaction between fungi and *P. donghaiense* species has been reported.

In the current study, algicidal fungus YL13 was isolated with algicidal activity against *P. donghaiense*, and phylogenetic analysis revealed that strain YL13 belonged to the genus *Talaromyces* (Fig. 1). The algicidal percentage was not significantly affected by the fungal cell suspension nor by sterilized medium, but was affected by the supernatant of YL13 cultures (Fig. 2), suggesting that strain YL13 lysed *P. donghaiense* cells through indirect attack, by excreting active substances

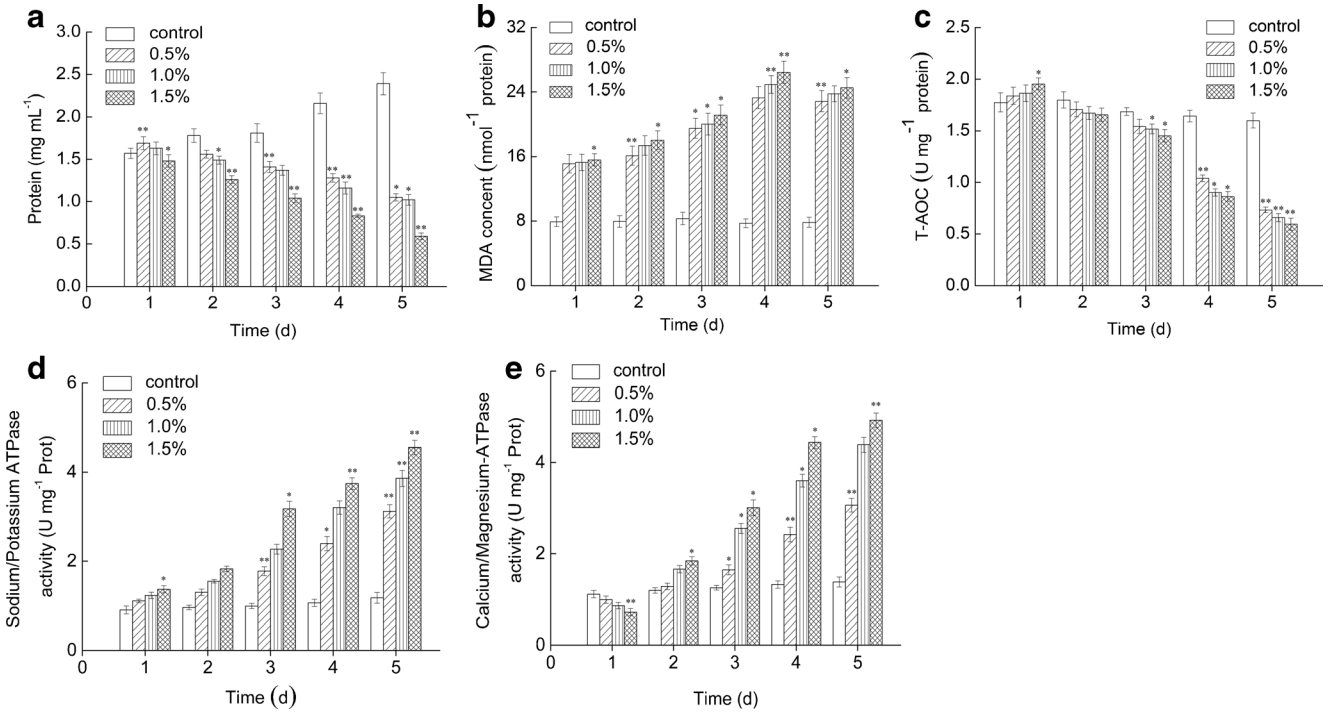

Fig. 4 The protein content (**a**), MDA content (**b**), T-AOC level (**c**), Na^+-K^+ ATPase activity (**d**), and Ca^{2+}-Mg^{2+} ATPase activity (**e**) in *P. donghaiense* exposed to different concentrations of YL13 supernatant. All *error bars* indicate SE of three replicates ($n = 3$). *Asterisks* represent a statistically significant difference of $p < 0.05$ when compared to the control; *double asterisks* represent a statistically significant difference of $p < 0.01$

into the medium. The fungal cultures were optimized to improve the algicidal activity. Previous studies showed that RSM was found to be effective in increasing the cell density of the bacterium NP23 (Liao and Liu 2014) and that the bacterium LY01 cultured with different nutrient concentrations exerted different algicidal activities against its host algae (Li et al. 2014). Therefore, using the appropriate concentration of nutrients to culture the fungus would increase the algicidal activity. After experimentally obtaining the ideal conditions and nutrients (data not shown here), BBD and RSM analyses were carried out (Tables 1 and 2) to optimize nutrient concentrations for fungal algicidal activity. The algicidal activity of the fungus was determined to be 96.8 ± 2 % (Fig. 4a), which indicated that the algicidal ability was significantly enhanced under the following concentration of nutrients: sucrose 35.84 g L^{-1}, $(NH_4)_2SO_4$ 2.45 g L^{-1}, and KH_2PO_4 1.03 g L^{-1}.

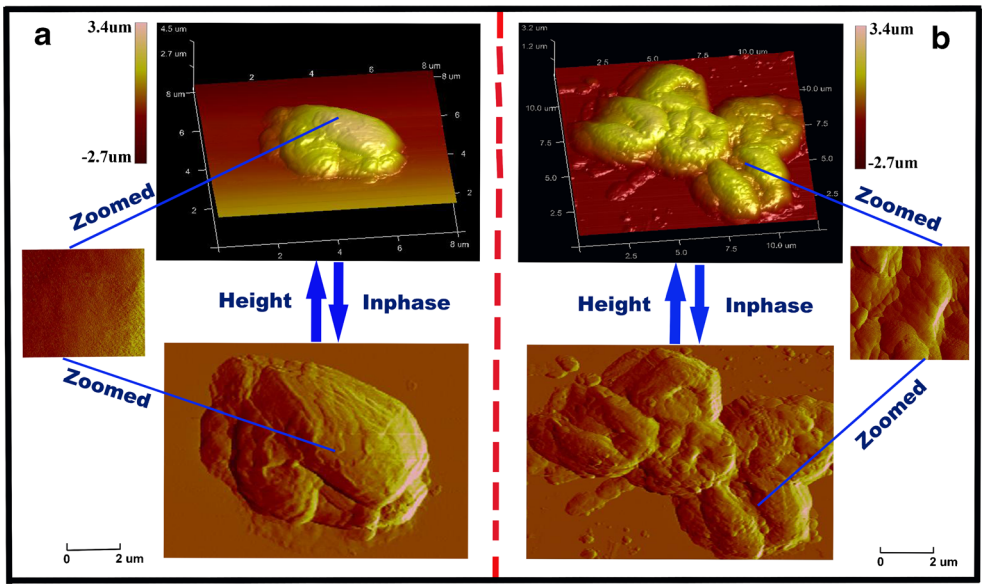

Fig. 5 AFM topographic images of *P. donghaiense* before (**a**) and after strain YL13 supernatant (1.5 %, *v*/*v*) treatment for 3 days (**b**). *Color bar* indicates the *Z*-range (μm)

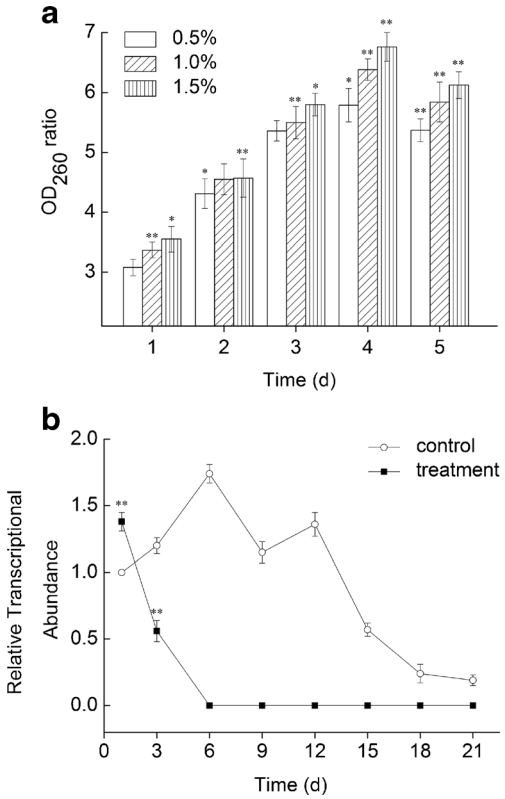

Fig. 6 Effects of strain YL13 supernatant on **a** OD_{260} and **b** PCNA gene expression of *P. donghaiense* with different addition concentrations. All *error bars* indicate SE of three replicates ($n = 3$). *Asterisks* represent a statistically significant difference of $p < 0.05$ when compared to the control; *double asterisks* represent a statistically significant difference of $p < 0.01$

To further investigate the interaction between strain YL13 supernatant and *P. donghaiense*, experiments were conducted to explore the inhibition mechanisms. Cellular-soluble protein is one of the basic indicators to reflect the physiological state of cells. The protein contents of algal cells at low concentrations showed a slight increase when compared to that of the control after 1 day of exposure (Fig. 4a). Organic compounds at a low level can accelerate the synthesis of DNA, RNA, and protein (Wang et al. 2002). The significant decrease of protein contents as exposure time increased and supernatant concentration increased may imply that the protein synthesis process was inhibited by YL13 supernatant which led to disruption of normal physiological metabolic functions of the algal cells.

MDA, an end product of lipid peroxidation, is usually used as a biomarker for lipid peroxidation to indicate cellular membrane system damage and oxidation degree (Bailly et al. 1996). With increased concentrations of strain YL13 supernatant and increased duration of exposure, the MDA content was increased (Fig. 4b) indicating that algal cells experienced serious oxidation damage. T-AOC is an integrated index, which can reflect a comprehensive situation of the defense system (Sun et al. 2009). Our results showed that T-AOC induced by YL13 supernatant decreased in a time-dependent and concentration-dependent manner (Fig. 4c). This indicated that the decrease of antioxidant capacity led to more serious oxidative damage, which further resulted in the increase of MDA content in order to maintain the normal function of membranes. This observation was consistent with those reported in previous studies (Zhang et al. 2011, 2014a, b).

The cell membrane acts as a barrier to ensure a relatively stable environment within the cell (Veldhuis et al. 2001). The indicator of lipid peroxidation (MDA) increased after exposure to strain YL13 supernatant, which suggested that membrane lipid peroxidation was caused by strain YL13 supernatant. To directly observe damage to the cellular membrane system, AFM analysis was carried out to observe morphological alterations of the cell surface treated with 1 % (*v*/*v*) YL13 supernatant (Fig. 5). The clustering cells and increased roughness of the surface caused by YL13 supernatant would lead to the eventual disintegration of algal cells because of the disruption of normal physiological metabolism. Apart from the morphological changes, the activities of the Ca^{2+} and Na^{+} pumps were significantly enhanced under YL13 supernatant treatment and appeared to be positively correlated with the morphological results (Fig. 4d, e). These physiological responses indicated that algicidal substances induced membrane ionic channel changes in the algal cells. Zhou et al. (2011) also reported that the alteration of Ca^{2+} and Na^{+} pumps induced by PAEs might be an important signal of developmental toxicity in membrane integrity or permeability. The slight decrease in Ca^{2+} pumps in algal cells during the algicidal process and the clustering cells might point to a thecae protection hypothesis.

Cell proliferation plays an important role in the growth and reproduction of the organism and influences cell activity through the regulation of gene expression (Kobayashi et al. 2011). To explore whether cell proliferation was interdicted by strain YL13 supernatant, the nucleotide levels in algal cell and PCNA gene expression were determined as they are good indicators of the state of cell proliferation (Huang et al. 2010). This is the first report to show long-term changes of PCNA gene expressions during the algicidal process, although previous study has shown similar changes from *A. tamarense* at two specific time points (Li et al. 2014). The values of OD_{260} in the treated groups were more than three times those of the controls (Fig. 6a), and this implies that the release of extracellular nucleic acids was considerable under the stress of strain YL13 supernatant. The reason that PCNA gene in controls obviously changed (Fig. 6b) was that the expression levels varied along with the growth phase of *P. donghaiense* (Zhao et al. 2009). In the initial treatment time, the PCNA gene expression was increased when compared to that of the control and then significantly decreased to below detection. This phenomenon was in accordance with the release of extracellular nucleic acids and indicated that strain YL13 supernatant induced the algal cells to respond. However, the

function of nucleotide could not be exerted normally and that eventually destroyed the DNA replication and repair processes of algal cells at long exposure periods (6 days).

In summary, the algicidal activity of *T. purpurogenus* YL13 supernatant was improved from 80.3 to 96.8 % through optimization of environmental and nutrient factors using RSM. Strain YL13 supernatant caused membrane lipid peroxidation, destroyed the antioxidant capacity, changed the membrane ionic channels, damaged cell membrane integrity, destroyed DNA synthesis and replication, caused nucleic acids leakage, and eventually induced algal cell death. Based on the results of this study, the algicidal fungal strain *T. purpurogenus* YL13 has potential for use to control HABs. However, structure determination of the algicidal compounds from the fungus is required to better understand the algicidal mechanism. Moreover, it is also important to carry out detailed investigations on the risk of using an algae-lysing fungus on the ecosystem prior to its application in large bodies of water.

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