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Biological activity of a red-tide alga—*A. tamarense* under co-cultured condition with bacteria

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Abstract: The relationship between *Alexandrium tamarense* (Lebour) Balech, one of red-tide alga, and two strains of marine bacteria, *Bacillius megaterium* (S_7) and *B. halmapulus* (S_{10}) isolated from Xiamen Western Sea, was investigated by evaluating the growth state of *A. tamarense* and the variation of β-glucosidase activity in co-culture system. The results showed the growth and multiplication of the alga were related with the concentration, genus speciality of the bacteria, and growth stage of the alga itself. The growth of *A. tamarense* was obviously inhibited by S_7 and S_{10} at high concentration. Either inhibition or promotion contributed much more clearly in earlier than in later stage of the growth of the alga. Furthermore, there was a roughly similar variation trend of the activity of extra-cellular enzyme, β-glucosidase, in the water of the separately co-cultured bacteria S_7 and S_{10} with the alga. The β-glucosidase activity (β-GlcA) rapidly increased during the later algal growth accompanying the increase of the lysis of the alga cells. The obvious inhibition of *A. tamarense* by marine bacteria at high concentration and evident increase of β-GlcA in co-colture system would help us in better understanding the relationship between red-tide alga and bacteria, and also enlightened us the possible use of bacteria in the bio-control of red-tide.

Keywords: *Alexandrium tamarense*; β-glucosidase; bacteria

Introduction

The occurrence of red-tide has caused severe world-wide ecological problems. There have recently been discussions concerning the important roles of algal-bacterial interaction in algal bloom dynamics, such interactions range from beneficial trophic relationships to more negative impacts of algal growth inhibition by bacteria (Daft, 1975; Docucette, 1995). Great efforts have been made to investigate the relationships between marine bacteria and red-tide micro-algae in recent years for understanding the both outbreak and termination mechanisms of red-tide. It has also been reported that several bacteria isolated in association with various HABs, including dinoflagellates and raphidophytes, were able to inhibit the growth of red-tide algae (Fukami, 1992; Dakhama, 1993; Imai, 1995; Docucette, 1999).

These bacteria capable of inhibiting various HAB species are leading to the suggestion that these microbes may affect phytoplankton species succession. Such bacteria that inhibit algal growth take effects through direct (physical contact with algal cells) and indirect attack (excrete active compounds in the surrounding water) (Imai, 1993; Lovejoy, 1998). Reports showed that the activity of extracellular enzyme produced by bacteria increased evidently accompanying the development of algal blooms (Middelboe, 1995). Enzymatic catalysis plays an important role in the flow of material and energy in marine ecosystem (Münster, 1991). High β-glucosidase means high hydrolyzing rate to relevant substance, it can release more available dissolved matter from high molecular weight organic matters (Chróst, 1989). The study on the β-glucosidase would be helpful to have a better understanding of the role of heterotrophic bacteria in the carbon cycle of algal bloom dynamic. But until now, few studies on this field were carried out.

Evaluating the role of inhibitory bacteria in HAB dynamic and their possible use as microbial control of red-tide require further understanding of the algal-bacteria relationship. In this study, two strains of bacteria isolated from Xiamen western sea of Fujian Province were co-cultured with the *Alexandrium tamarense* to study the effects of marine bacteria on the alga growth and the activity of β-glucosidase in the co-culture media.

1 Materials and methods

1.1 Algal culture

The alga *A. tamarense* provided by the Institute of Aquatic Biology, Jinan University, was cultured in f/2 without silicate enriched seawater (Guillard, 1975). Cultures were maintained at 20 ± 1℃ with a 12 h light and 12 h dark cycle.

1.2 Bacterial strains

Two strains of bacteria previously isolated from Xiamen western sea area and maintained in 2216E media were identified to be *Bacillius megaterium* (S_7) and *B. halmapalus* (S_{10}), respectively (Zheng, 2003).

1.3 Counting of the alga

The density of *Alexandrium tamarense* cells was monitored by direct microscope count after fixation with Lugol's iodine.

1.4 Counting of the bacteria

After incubation in 2216E for 48 h at 25 ± 1℃, the bacteria was collected after centrifuged at 5000 r/min for 5 min and washed twice by sterile seawater, then the cell pellet was re-suspended in sterile seawater. Then the initial concentration of bacteria was measured by colony forming units (CFU) count.

1.5 Determination of β-glucosidase activity (β-GlcA)

10 mmol/L working solution of fluorescence indicator MUF-β-glucoside (Sigma Co.) was prepared and kept in cool for the determination of the activity of β-GlcA.

The determination of β-GlcA was conducted by using fluorogenetic model substance (Hoppe, 1993). The β-GlcA was measured as an increase in fluorescence intensity when the nonfluorescent 4-methylumbellifery substrates were

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enzymatically hydrolyzed with a release of the highly fluorescent product 4-methylumbelliferone (MUF). The rate of MUF production was equivalent to the rate of substrate hydrolyzed.

The enzyme reaction was carried out by adding 50 μl MUF-β-d-glucosidase working solution as substrate to the test sample, for each sample, aliquot 2 ml subsample was distributed to six flasks (three for blanks and three for tests). Immediately 50 μl 2 mmol/L $HgCl_2$ solution was introduced to blanks to stop reaction. Then all the subsamples were incubated in dark for 3 h. After incubation 50 μl 2 mmol/L $HgCl_2$ was added to stop the enzymatic reaction. Then the fluorescent values were measured with a fluorescent spectrometer (Ex = 353 nm, Em = 450 nm, scanning speed = 60 s^{-1}; Hitachi 860, Japan). The β-glucosidase activity was calculated according to the following Eq.(1):

$$V = (F - F_b)/(T \times S). \quad (1)$$

Where V (μmol/(L·h) is the hydrolysis rate β-glucosidase to substrate; F is the fluorescence intensity of the parallels; F_b is the fluorescent intensity of the blanks; T is the culture time(h), and S is the fluorescence intensity of standard fluorogenic substance per μmol.

1.6 Experimental design

The two strains of bacteria (both at three final concentration, 2×10^{10}, 2×10^{9}, 2×10^{8} cells/ml, labeled as C_1, C_2, C_3 respectively) were added separately to the alga in lag phase and in exponential phase during the culture of alga to investigate the effects of the bacteria on the growth of the alga and the activity of β-glucosidase, in the water of co-culture media. The control of the alga, the bacteria, and the co-culture of the alga and bacteria were in triplicate, and arithmetic average were used to express the experiment results,

2 Results

2.1 Effect of bacteria on the multiplication of the alga in lag phase

The effect of the bacteria with different concentrations added in lag phase of the alga on the multiplication of the alga *A. tamarense* varied according to the co-culturing time. In the co-culture treatment, high concentration C_1 (2×10^{10} cells/ml) of strain S_7 showed an inhibition trend in the earlier growth stage(the day 10) of the alga, about 38% of the cell density of the control C_0, as shown in Fig.1. However, this inhibition markedly decreased in the later stage(the day 18) of the alga growth, about 84% of the cell density of the control. The statistic analysis showed a highly inhibitory function of higher concentration treatment C_1 compared to the control C_0. The regression equation was $Y_{S_7}(A + C_1) = 22.430 + 0.955x$, $r^2 = 0.917$. On the contrary, lower concentration treatments C_2 and C_3 (2×10^{9} cells/ml and 2×10^{8} cells/ml, respectively) showed promotion effect to the growth of the alga to some extent. The alga cell density of the treatments C_2 and C_3 was about 164% and 154% of the control on earlier the day 10, and about 110% and 167% on the day 18, respectively.

At the same time, high concentration C_1 (2×10^{10} cells/ml) of strain S_{10} also showed an inhibition trend in the earlier growth stage(the day 6) of the alga, about 54% of the cell density of the control C_0, but 233% and 254% of the control for the treatments of C_2 and C_3, respectively. This inhibition trend varied in later growth stage of the co-cultured alga *A. tamarense* as demonstrated in Fig. 2. The cell density of the treatments C_1, C_2, and C_3 were about 64%, 298% and 232% compared to the control on the day 10, and changed to be 10%, 316%, and 167% of the control (the day 18), respectively. This showed that the bacterium S_{10} in high concentration strongly inhibited the growth of the alga, but promoted the growth of the alga in lower concentrations. The statistic analysis tended to show a high correlation between the treatments C_1 ($Y_{S_{10}(A+C1)} = 39.386 + 0.086x$, $r^2 = 0.901$) and the control, also for the treatments C_2 ($Y_{S_{10}(A+C2)} = 0.156 + 3.201x$, $r^2 = 0.979$), and C_3 ($Y_{S_{10}(A+C3)} = 44.757 + 1.555x$, $r^2 = 0.996$) and the control, respectively.

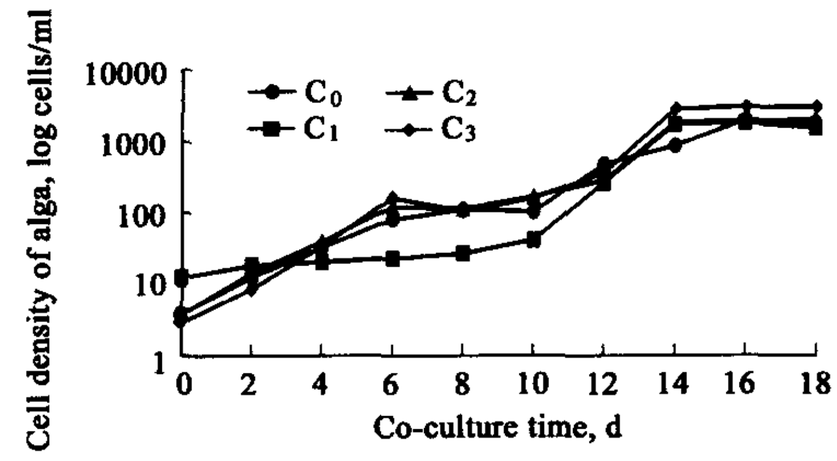

Fig.1 Effect of bacterium S_7 in different concentrations on the growth of *A. tamarense* in lag phase

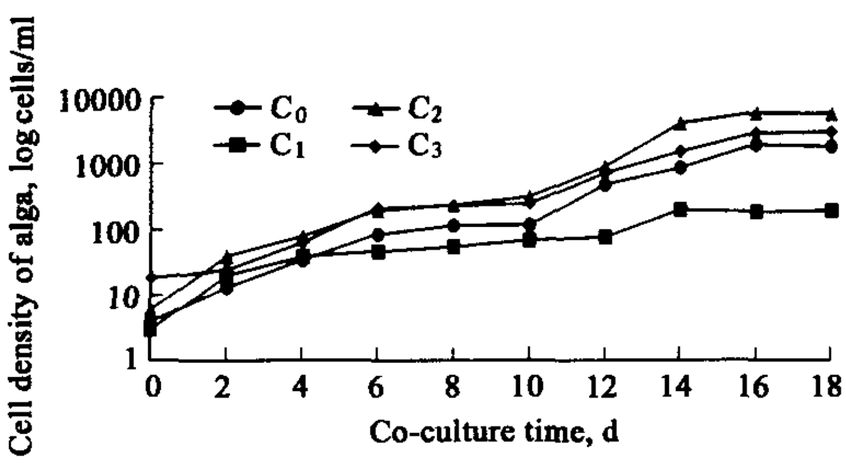

Fig.2 Effect of bacterium S_{10} in different concentrations on the growth of *A. tamarense* in lag phase

2.2 Effect of bacteria on the multiplication of the alga in exponential phase

As shown in Fig.3, in the earlier growth stage(10 d) of the alga there was no obvious effect of the bacteria S_7 or S_{10} on the multiplication of the alga *A. tamarense* in exponential phase; however, it tended gradually to promote the growth of the alga in the later stage(the day 20). For example, the alga cells co-cultured with the bacteria S_7 was about 112% on the day 14, or S_{10} about 126% on the day 18, of the control at their peak values, respectively. The regression equations between the treatment A + S_7 and the control, the treatment A + S_{10} and the control were statistically calculated as follows:

$$Y_{(A+S_7)} = 1.018x - 0.933, \ r^2 = 0.98;$$

$$\text{and } Y_{(A+S_{10})} = 1.217x - 83.537, \ r^2 = 0.99 .$$

2.3 Effect of the co-cultured alga and bacteria on the activity of β-GlcA in water

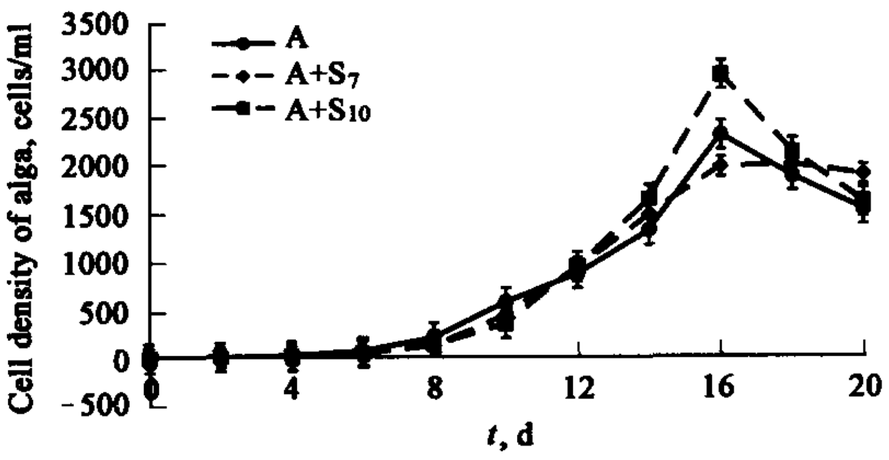

Fig.3 The growth of *A. tamarense* co-cultured with bacteria S_7 and S_{10} in exponential phase

The effect of the co-cultured bacterium S_7 and alga *A. tamarense* on the β-GlcA is shown in Fig.4. The β-GlcA of the control(bacterium S_7 only) was 0.133 ± 0.003 μmol/(L·h) on the day 8, and 0.048 ± 0.011 μmol/(L·h) on the day 20. However, the β-GlcA in the co-cultured bacteria and alga (A + S_7) obviously was larger than that of S_7, statistically could be described in a regression equation of $Y_{(A+S_7)} = 0.083 + 1.186x$, $r^2 = 0.92$. On the other hand, the β-GlcA of the control (alga A only) maintained at relatively lower level until the day 16, it increased gradually (0.104 ± 0.02)μmol/(L·h) and reached maximum(0.385 ± 0.023 μmol)/(L·h) on 20 d.

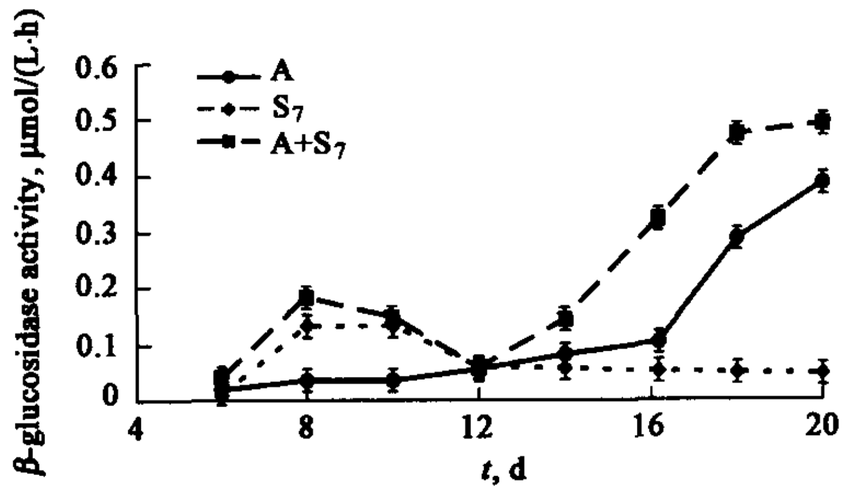

Fig.4 β-Glucosidase activity in the water of the co-cultured bacterium S_7 and *A. tamarense*

Meanwhile, the β-GlcA of the control (bacterium S_{10} only) was comparatively low, roughly varied from 0.03 to 0.07 μmol/(L·h) in the duration of experiments, but the β-GlcA of the co-cultured S_{10} and alga showed a similar trend of the co-cultured S_7 and alga, as shown in Fig.5. The β-GlcA of the co-cultured S_{10} and alga decreased from 0.482 ± 0.005 μmol/(L·h) on the day 8 to 0.111 ± 0.01 μmol/(L·h) on the day 12, then increased again to be 0.49 ± 0.013 μmol/(L·h) on the day 16. There was no significant correlation between the treatment A + S_{10} and the control(alga A only), although a similar trend was shown between the treatment A + S_{10} and A + S_7.

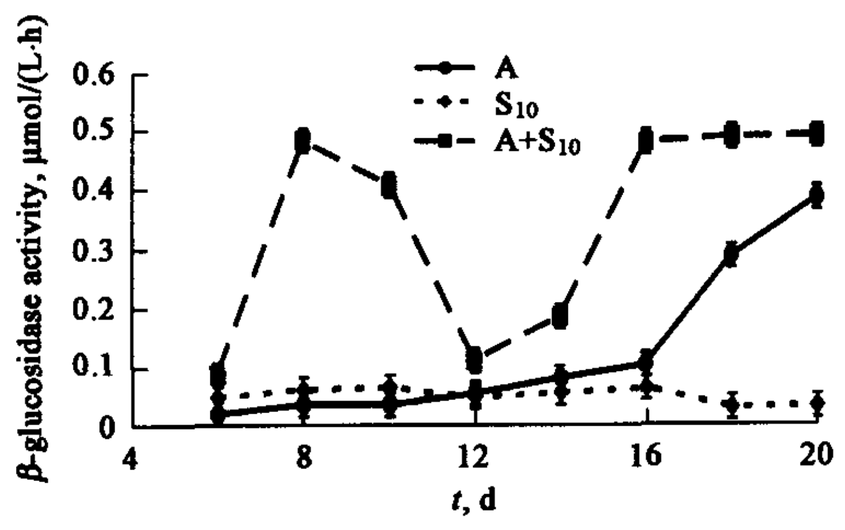

Fig.5 β-Glucosidase activity in the water of the co-cultured bacterium S_{10} and *A. tamarense*

3 Discussion

Our recent study using scanning-electron microscopy revealed that bacteria often co-occur with algae and that these bacteria tended to invade the algal cells and remain in their cytoplasm(Wang Y L, unpublished observations). When the bacteria reached a sufficiently high density, the interior structure of algae began to disintegrate. It was also found that the decaying algal cells tended to contain large numbers of bacteria, suggesting the possibility that the bacteria could have multiplied within the algal cells. Ms. Wang suggested that the bacteria which have invaded the algal cells could cause either morphological or structural damage to the cells, leading to an accelerated aging and earlier death (Wang, 1999). This may be one of the reasons why high concentrations of bacteria had a greater inhibition on the growth of the algae.

Another possible explanation for the inhibition of the growth of the algae by bacteria may be nutrition competition between the algae and the bacteria. In addition, some bacteria can secrete substances that can specifically inhibit the growth of algae (Dakhama, 1993; Fukami, 1992; Hayashida, 1991). However, up to now, the interactions between marine bacteria and marine algae have not been fully elucidated, and the mechanism of inhibition on the growth of the algae by the bacteria has remained unknown.

There are some reports stating that synthesis of extra-cellular enzyme were inhibited in the initial stage of red tide, but synthesized in large quantity in declination stage of red tide(Li, 1996; Middelboe, 1995). Those enzymes play important role in the conversion and circulation of organic substances in aquatic ecological environment. Some reports showed remarkable increase of extra-cellular enzymes in aquatic environment when blooms developed in lakes or ponds, thus possibly inhibit the growth of dinoflagellate and diatom(Fukami, 1991; Shilo, 1970; Zheng, 2003; Wang, 1999). Among the extra-cellular enzymes, β-glucosidase widely exists in aquatic environment. It is usually considered to be an important indicator to expatiate on the relationship between organisms and heterotrophic microbes in marine environment. Therefore, β-glucosidase activity (β-GlcA) was use in this experiment to indicate the effect of the bacteria on the co-cultured alga *A. tamarense*.

It is commonly known that it exists in symbiosis, competition, and antagonist relationship between the marine bacteria and red tide algae in aquatic ecological environment. Marine microbes mainly supply algae with nutrient salts in the formation process of red tide, and decompose organic substance in decay of the alga. The value of β-GlcA for A + S_7 is roughly equal to the sum of the control A and S_7 in earlier growth stage of the alga, the alga *A. tamarense* cells seem no promotion on the increase of the β-GlcA; but the value of β-GlcA for A + S_{10} is larger than that of the control A and S_{10}, suggesting that the alga cells of *A. tamarense* promote the increase of the β-GlcA for the bacterium S_{10}. The phenomenon might also be figured to related to the genus speciality of the bacterium, and nutrient competition between the bacterium and alga (Dakhama, 1989; Lin, 2001;

Münster, 1990). The result also showed relatively lower β-GlcA in interval growth stage of the alga, this maybe the inhibition caused by the fast multiplication of the alga. There was a marked increase of β-GlcA in the later growth stage of the alga, this maybe the result of synthesis in quantity of β-GlcA induced by existence of large amount of macromolecules in the decay of the algae.

4 Conclusions

In aquatic ecosystems, the relationship between algae and bacteria has drawn more attention in recent years. Bacteria could provide the algae with nutrition and some necessary growth factors and, on the other hand, bacteria could also inhibit the growth of algae or possibly even lyse the algal cells and thus kill the algae. It has caused people to consider the importance of microbes in the study of the mechanisms regulating the occurrence, development and senescence of algal blooms.

In this study, the obvious inhibition of *A. tamarense* by marine bacteria *Bacillius megaterium* (S_7) and *B. halmapulus* (S_{10}) at high concentration and evident increase of β-GlcA in co-colture system would help us in better understanding the relationship between red-tide alga and bacteria, and also enlightened us the possible use of bacteria in the bio-control of red-tide.

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