Biotechnology and Bioprocess Engineering 17: 1232-1237 (2012)
DOI 10.1007/s12257-012-0210-3

RESEARCH PAPER

Selection of Extraction Solvent and Temperature Effect on Stability of the Algicidal Agent Prodigiosin

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Received: 2 April 2012 / Accepted: 5 June 2012

Abstract An organic solvent for extracting prodigiosin from culture broth was selected and a test to determine the long-term stability of prodigiosin was performed to develop prodigiosin as a biological control agent against *Chattonella antiqua*, a harmful alga that can cause red tides. Prodigiosin was extracted using nine solvents, and the extracts were analyzed by liquid chromatography-mass spectroscopy. Acetone was selected as the best organic solvent because of its high extraction efficiency and less processing time. Stability tests for prodigiosin were performed at various temperatures, and algicidal activity against *C. antiqua* was also tested. Ultimately, > 98% stability was sustained after 30 days at 4°C, whereas < 30% stability was maintained after 30 days at 37°C. Although prodigiosin was kept for 30 days in an optimum organic solvent, its stability was safely maintained and algicidal activity was sustained at 4°C. These results indicate that acetone is a very useful extraction and storage solvent for prodigiosin.

Keywords: acetone, algicidal activity, *Chattonella antique*, prodigiosin, red tide

1. Introduction

Red tides can be detected when the color of the ocean changes due to an excessive algae bloom. The occurrence of red tides caused by algae blooms has become a serious problem that affects the ocean ecosystem. In particular, contamination caused by red tides can damage seawater desalination facilities and may result in elevated organic content in source water and accelerated bio fouling of seawater reverse-osmosis installations [1]. As global warming continues, the occurrence of red tides has increased significantly. Harmful algae affect certain species of phytoplankton, causing damage to sea life and destroying fish populations. The red tide alga *Chattonella antiqua* is highly toxic to fish and is closely related to the disappearance of gill mucus, which leads to impaired osmoregulation [2]. Although physicochemical research on this species has been well documented [3,4], little is known about *in situ* outbreaks of *C. antiqua* red tides [5,6].

Prodigiosin is a red pigment produced by various microorganisms such as *Serratia marcescens* [7,8], *Vibrio psychroerythrus* [9], *Pseudomonas magnesiorubra* [10], and *Hahella chejuensis* [11]. Prodigiosin has a unique structure consisting of three pyrrole rings [12] and is a characteristic member of a group of compounds with a common pyrrolyl pyrromethene skeleton. Besides its algicidal activity, prodigiosin possesses immunosuppressive, antifungal, and antiproliferative properties [13-16], and studies have shown that prodigiosin induces apoptosis in various cancer cells [17,18]. Due to these characteristics, prodigiosin may have potential for medical applications. Because of its potential commercial value, there is a demand for high-throughput cost-effective bioprocesses for prodigiosin production.

Prodigiosin has been regarded as a biological control agent against harmful algae, including *Cochlodinium polykrikoides* [19] and *C. antiqua* [20]. In this study, we determined the most effective organic solvent for extracting prodigiosin from culture broth and confirmed that the stability and algicidal activity of prodigiosin against *C. antiqua* was safely maintained in the solvent.

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2. Materials and Methods

2.1. Seed cultures and fermentation

A single colony of *H. chejuensis* M3349 (KCTC 2396) was cultivated on sucrose-based marine agar medium (sucrose 10 g/L, peptone 8 g/L, yeast extract 2 g/L, NaCl 10 g/L, Na_2SO_4 12 g/L, $CaCl_2$ 1.8 g/L, $MgCl_2$ 0.7 g/L, H_3BO_3 22 mg/L, Na_2HPO_4 20 mg/L, Na_2SiO_3 8 mg/L, and agar 20 g/L) at 25°C for 3 days; this colony was then inoculated into 50 mL of Zobell medium (peptone 5 g/L, yeast extract 1 g/L, and seawater 750 mL/L) and cultivated at 25°C for 24 h. *H. chejuensis* M3349 cultivation for prodigiosin production was performed in a 5 L jar fermentor (Minifors, Infors HT, Bottmingen Switzerland) containing sucrose-based marine broth medium (sucrose 10 g/L, peptone 8 g/L, yeast extract 2 g/L, NaCl 10 g/L, Na_2SO_4 12 g/L, $CaCl_2$ 1.8 g/L, $MgCl_2$ 0.7 g/L, H_3BO_3 22 mg/L, Na_2HPO_4 20 mg/L, and Na_2SiO_3 8 mg/L) at 25°C for 5 days. The fermentation jar was agitated at 400 rpm with > 15% air saturation.

2.2. Prodigiosin solvent extraction

Nine solvents were assessed to select an optimal extraction solvent for prodigiosin produced by *H. chejuensis* M3349. One milliliter of culture broth containing 1.463 g/L of prodigiosin was transferred to 2 mL tubes and centrifuged at 13,000 rpm for 3 min. The supernatants were discarded, and one volume of each solvent was added to the pellet. After the tubes were shaken for 2 h, they were centrifuged at 13,000 rpm for 3 min. After each solvent was evaporated, pure acetonitrile of equal volume was added to each tube for liquid chromatography-mass spectroscopy (LC-MS) analysis.

2.3. Measurement of prodigiosin concentration

Prodigiosin concentration was measured using an LC-MS system (Agilent 6300 Series Ion Trap LC/MS system, Agilent Technologies, Santa Clara, CA, USA). A XDB-C18 (5 μm, 4.6 ×150 mm, Agilent Technologies) column was used for high-performance liquid chromatography analysis. Mobile phase A was mixed with water (95%) and acetonitrile (5%) containing formic acid (0.2%), and mobile phase B was mixed with water (5%) and acetonitrile (95%) containing formic acid (0.2%). The analysis was conducted using the following method: 10 ~ 100% B for 0 ~ 20 min, 100% B for 20 ~ 25 min, and 100 ~ 10% B for 25 ~ 30 min at a flow rate of 0.5 mL/min.

2.4. Determination of prodigiosin stability

The stability of prodigiosin extracted with acetone or ethanol was compared at 4, 25, and 37°C. The extracts were analyzed with an LC-MS system to measure the initial prodigiosin concentrations extracted with acetone and ethanol. After filtration, the extracts were diluted to 10% with pure acetonitrile for measuring the initial concentration. Two hundred microliters of prodigiosin extract was transferred to each of 30 micro-centrifuge tubes, and then 10 tubes each were maintained at 4, 25, and 37°C for 30 days in a dark chamber. Each tube was sealed with Parafilm to prevent solvent evaporation at the various temperatures. The stability of prodigiosin extracts preserved at various temperatures was confirmed by LC-MS every 3 days. Area values measured by LC-MS were converted to relative values. The relative amount of prodigiosin was expressed as mean±standard deviation (n = 3).

2.5. Algicidal activity assay

Algae were maintained and cultivated in 100 mL tissue culture dishes with Guillard's f/2 medium [21] at 25°C with a 16 h light/8 h dark illumination cycle under a light intensity of 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$. When the culture of *C. antiqua* reached a cell density of approximately 1.7 $\times 10^2$/mL, the algal culture was poured into another tissue culture dish. Acetone and ethanol extracts kept at 4, 25, and 37°C for 30 days were used. Pure prodigiosin produced by fermentation was extracted with acetone and ethanol for use as a control. Extracts were diluted with each solvent to adjust the concentration range to 0.1 ~ 100 mg/L, and then 10 μL aliquots of the solution were added to 90 μL of micro algal suspensions in 96 well plates and left for 1 h. Cell number was counted in 0.2% Lugol's solution-stained cells under a microscope after the 1 h incubation at room temperature. The following formula was used to calculate algicidal activity, and algicidal activity was expressed as a relative value.

$$\text{Algicidal activity (\%)} = \frac{\text{Number of initial cells} - \text{Number of cells that survived}}{\text{Number of initial cells}} \times 100$$

3. Results and Discussion

3.1. Prodigiosin production

The amount of prodigiosin produced by fermentation is shown in Fig. 1. The maximum production of prodigiosin was 2.687 g/L 48 h after inoculation; it then gradually decreased to 1.463 g/L. As shown in a previous study [22], prodigiosin production was limited by product inhibition in batch cultures, as secondary metabolites such as prodigiosin, cycloprodigiosin, and other prodigiosin analogues inhibit production [23]. Prodigiosin production in the bacterial cell *Serratia marcescens* is strongly affected by light and prodigiosin itself is degraded by light [24], suggesting that the amount of prodigiosin produced in a batch-type

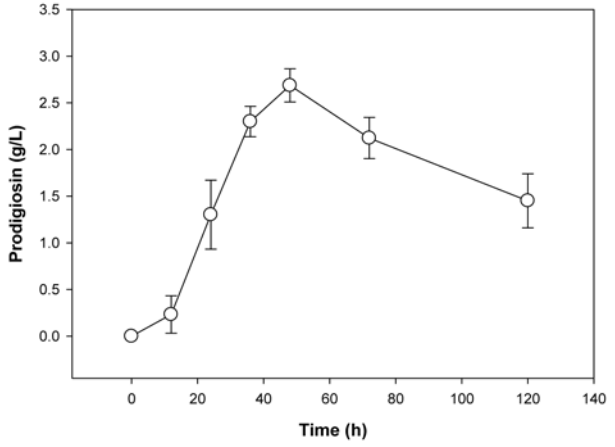

Fig. 1. Production of prodigiosin from *Hahella chejuensis*M3349. Cells were cultivated in a 5 L jar fermentor. Production of prodigiosin is shown with error bars, which represent the standard deviation of three samples.

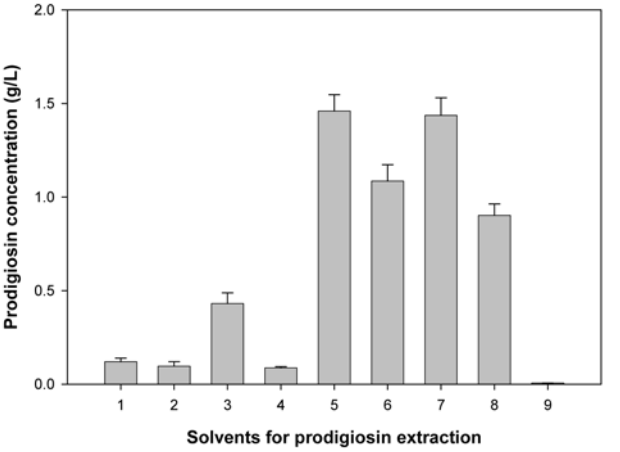

Fig. 2. Comparison of organic solvents for extracting prodigiosin. Nine solvents (1, hexane; 2, toluene; 3, chloroform; 4, ethyl acetate; 5, ethanol; 6, methanol; 7, acetone; 8, acetonitrile; 9, seawater) were tested.

fermentor might be reduced 60 h after fermentation for the same reason.

3.2. Selection of the optimal solvent for prodigiosin extraction

Ethanol was revealed as the best solvent in terms of extraction efficiency (Fig. 2). Acetone exhibited 98.5% extraction efficiency when compared to that of ethanol. Methanol and acetonitrile showed < 80 and 70% extraction efficiency, respectively. Chloroform, hexane, toluene, and ethyl acetate showed poor (<30%) extraction efficiency. Seawater exhibited the lowest extraction efficiency among the solvents used (Fig. 2). Overall, high-polarity solvents such as ethanol, methanol, acetone, and acetonitrile showed high prodigiosin extraction efficiency where as that of seawater was poor. As shown in Fig. 3, prodigiosin has a 2H-pyrrole ring and a pentyl chain [25]. The polarity and hydrophobicity of prodigiosin originates from amines on the pyrrole ring and pentyl chain, respectively. Taken together, it is likely that the extraction efficiency was correlated with the structural features of prodigiosin (Fig. 3).

Chloroform easily evaporates, because it has the second lowest boiling point among the organic solvents used in this study, but chloroform is toxic and showed poor relative extraction efficiency (30%), when compared with that of ethanol. Consequently, we determined that chloroform would be inadequate as an organic solvent for extracting prodigiosin. Acetone has good sensitivity and has frequently been used for extracting pigments, and it is considerably less toxic than chloroform and other solvents [26]. Finally, acetone was regarded as the optimal organic solvent for the extracting prodigiosin from culture broth. The amount of prodigiosin extracted with acetone was almost the same as that extracted with ethanol. Moreover, processing time was

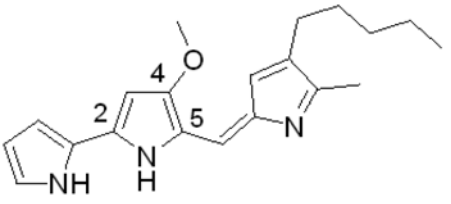

Fig. 3. Structure of prodigiosin ((*E*)-4-methoxy-5-((5-methyl-4-pentyl-2*H*-pyrrol-2-ylidene)methyl)-2,2'-bi(1*H*-pyrrole)).

reduced using acetone, because it has a lower boiling point than that of ethanol. Indeed, when 1 L of acetone was recycled five times, the process time was 140 min shorter than the process time when the same conditions were applied for ethanol extraction. Therefore, acetone was considered the optimum organic solvent for extracting prodigiosin from culture broth.

3.3. Effect of temperature on prodigiosin stability

Each prodigiosin extract was kept at three different temperatures for 30 days in a dark chamber to compare long-term temperature stability of the acetone-extracted and ethanol-extracted prodigiosin. After 30 days, the color of prodigiosin extracts maintained in acetone and ethanol changed from red to a deeper shade of red as temperature increased (Fig. 4), and the prodigiosin concentration decreased dramatically at all temperatures except 4°C; the concentration particularly decreased at 37°C (Fig. 5). In a previous study, a biological red pigment in acetone was quite stable even at 100°C for a period as short as 1 h [27]. In this study, prodigiosin was kept for 30 days at 37°C and was unstable. At a moderate temperature of 25°C, prodigiosin was quite stable, and > 80% prodigiosin remained stable for up to 25 days at this temperature (Fig. 5). However,

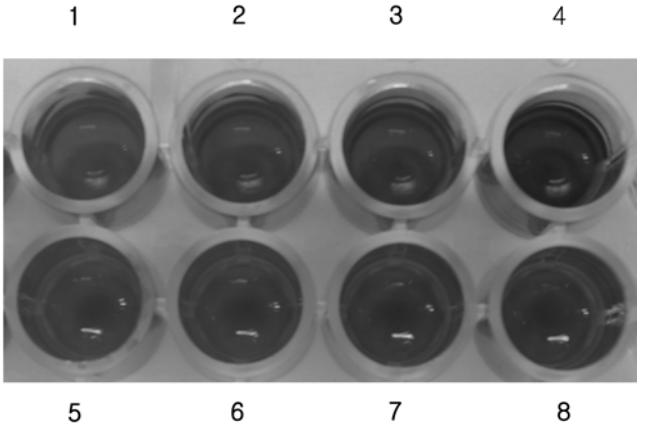

Fig. 4. Image of prodigiosin extracts kept at 4, 25, and 37°C for 30 days in acetone and ethanol. When prodigiosin broke down in the solvents, the color of the extracts changed from red to a deeper shade of red. (1, ethanol extract [control]; 2, ethanol extract preserved at 4°C; 3, ethanol extract preserved at 25°C; 4, ethanol extract preserved at 37°C; 5, acetone extract [control]; 6, acetone extract preserved at 4°C; 7, acetone extract preserved at 25°C; 8, acetone extract preserved at 37°C).

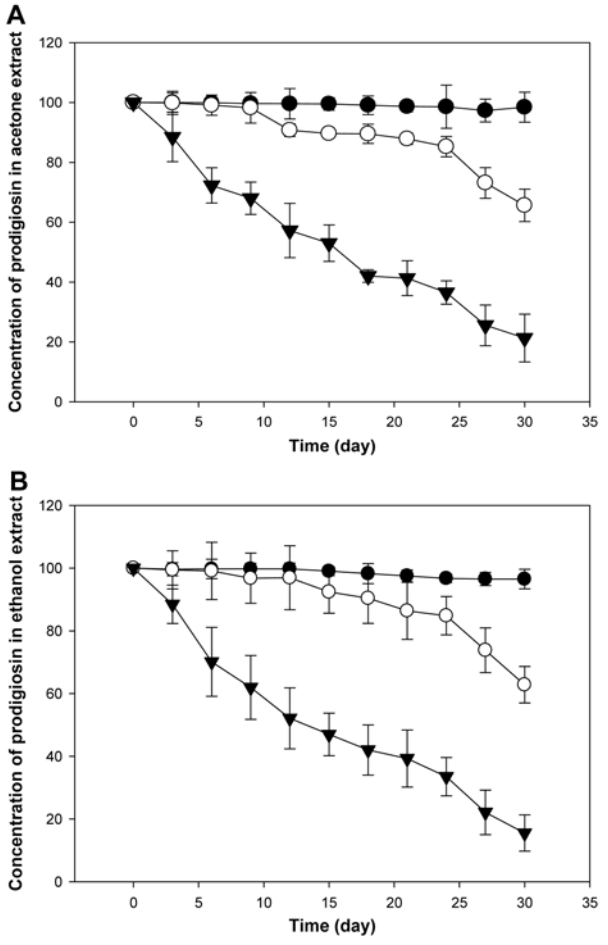

Fig. 5. Effect of temperature on prodigiosin stability in acetone (A) and ethanol (B). Prodigiosin extracted with acetone and ethanol was stored at 4°C (●), 25°C (○), and 37°C (▼) for 30 days.

prodigiosin concentration in acetone was maintained at > 98% even after 30 days at 4°C. The relative concentration of prodigiosin in ethanol was maintained at > 96% after 30 days at 4°C. Therefore, the stability of the acetone extracts was almost 2% higher than that of the ethanol extracts after 30 days at 4°C. Considering that prodigiosin is very unstable above 37°C, a low temperatures such as 4°C would enable prodigiosin to be stored safely in an optimum organic solvent for a long period of time.

3.4. Effect of storage temperature on prodigiosin algicidal activity

An assay was conducted to test prodigiosin as a candidate biological control agent against *C. antiqua* in the field. In this assay, changes in the shape and decay of *C. antiqua* cells that survived treatment with prodigiosin were

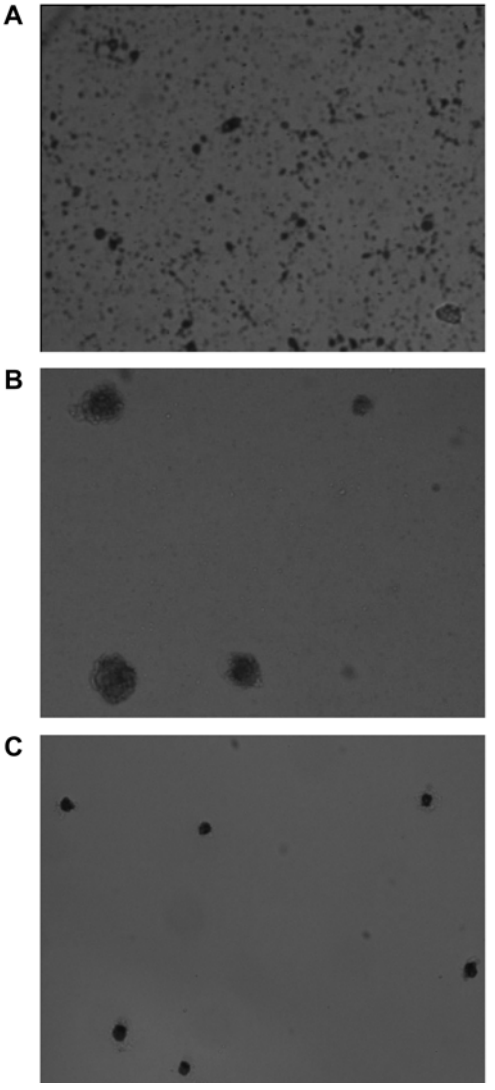

Fig. 6. Images of *Chattonella antiqua* killed by prodigiosin. (A), *C. antiqua* completely destroyed by prodigiosin; (B), *C. antiqua* just before lysis; (C), control.

observed. The *C. antiqua* cells that survived were compact, elliptical, or swollen, whereas the dead cells were completely broken (Fig. 6). Although all tested prodigiosin concentrations induced cellular damage, different activities were observed between the acetone and ethanol extracts. The higher storage temperature led to lower algicidal activity of both the acetone and ethanol extracts (Fig. 7). Prodigiosin extracts stored at a higher temperature were more degraded than those stored at a lower temperature. Particularly, algicidal activities of both the acetone and ethanol extracts decreased dramatically at all concentrations at 37ºC. The algicidal activity of the acetone extracts was higher than that of each ethanol extract at 4 and 37ºC. In all cases, algicidal activity was logarithmically correlated with prodigiosin concentration (Fig. 7). The algicidal activities of acetone and ethanol without prodigiosin were 1.5 ± 0.5 and 0.65 ± 0.1%, respectively. The LC_{50}values of prodigiosin in acetone and ethanol at 4ºC were 2.2×10^{-7} and 1.3×10^{-3} mg/L, respectively. These values were considerably lower than that of bacillamide from *Bacillus* sp. SY-1, which has an LC_{50} of 3.2 mg/L after 6 h [28]. Nakashima *et al*. reported that the prodigiosin-like pigment PG-L-1 shows a potent algicidal effect on *Heterosigma*, *Heterocapsa*, *Cochlodinium*, *Cymnodinium*, *Alexandrium*, and *Chattonella* sp., which are red tide phytoplankton [29,30]. The calculated LD_{50} values of PG-L-1 (5.0 ~ 12.5 mg/L) are higher than our values [29]. These results suggest that prodigiosin in acetone could be an efficient algicidal agent, although different and various targets exist.

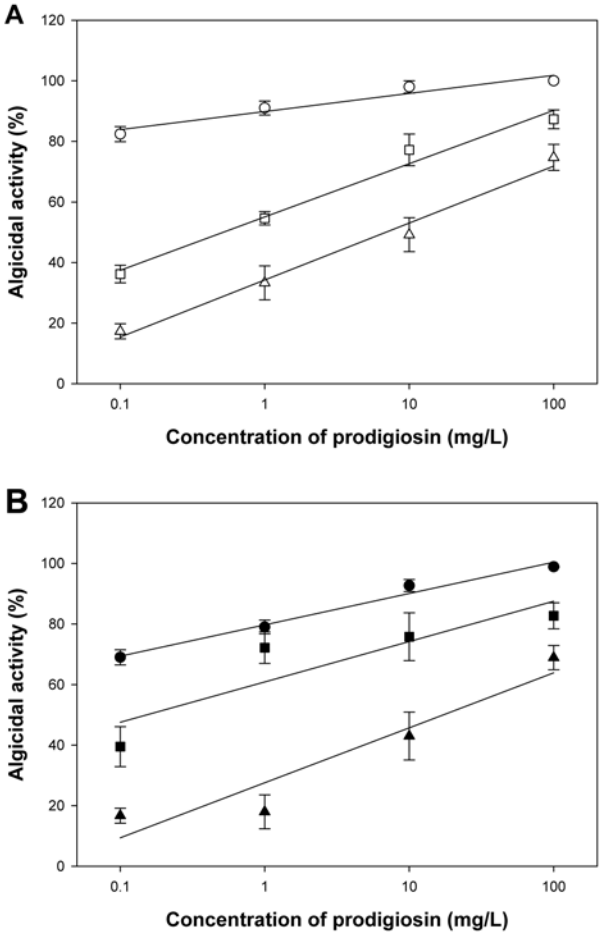

Fig. 7. Logarithmic correlation of algicidal activity and concentration of prodigiosin. (A) Acetone extracts preserved at each temperature (○, 4ºC; □, 25ºC; △, 37ºC); (B) ethanol extracts preserved at each temperature (●, 4ºC; ■, 25ºC; ▲, 37ºC).

More than 1 ~ 10% of the algicidal activity of prodigiosin extracted with acetone was sustained at 4ºC when compared with the prodigiosin extracted with ethanol. The acetone extracts showed relatively high algicidal activity because of the unique prodigiosin structure such as two 1H-pyrrole rings, a 2H-pyrrole ring, and a pentyl chain, which might be protected by the carbonyl group and two methyl groups of acetone at low temperature [25]. These results suggest that the algicidal activity of prodigiosin extracted with acetone was safely maintained at low temperatures such as 4ºC, and that acetone-extracted prodigiosin is potentially useful for controlling outbreaks of *C. antiqua* in the field.

4. Conclusion

Although prodigiosin has been analyzed by researchers in various fields of study, no published report is available on safe extraction and storage of prodigiosin. Therefore, we studied the optimum solvent for extracting prodigiosin from culture broth, the algicidal activity of prodigiosin extracts against the harmful algae *C. antiqua*, and the stability of prodigiosin extracts maintained at three different temperatures. The results showed that acetone was the best solvent in terms of extraction efficiency and processing time. Moreover, acetone extracts kept at low temperatures such as 4ºC showed strong algicidal activity against *C. antiqua* and stability was safely maintained.

In conclusion, the present study is the first to provide evidence for the remarkable algicidal activity of prodigiosin extracted with an optimum solvent and kept at low temperatures. Prodigiosin could be used as a biological control agent against the harmful algae *C. antiqua* in the field, although the practical aspect of using this agent needs to be assessed in more detail.

Acknowledgment

This study was supported by grants from the Korea Polar Research Institute (PE11060 and PN07020).

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