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Biocidal effect of thymol and carvacrol on aquatic organisms: Possible application in ballast water management systems

Hyunwoo Kim[a,1], Sungmin Lee[a,1], Beomseok Son[a], Jaewan Jeon[a], Daehoon Kim[b], wonku Lee[c], HyeSook Youn[d], Jae-Myung Lee[e], BuHyun Youn[a,f,*]

[a] Department of Integrated Biological Science, Pusan National University, Busan 46241, Republic of Korea
[b] Nuclear Science Research Institute, Pusan National University, Busan, Republic of Korea
[c] QuantomBio. Co., Ltd, Busan 46241, Republic of Korea
[d] Department of Integrative Bioscience and Biotechnology, Sejong University, Seoul 05006, Republic of Korea
[e] Department of Naval Architecture and Ocean Engineering, Pusan National University, Busan 46241, Republic of Korea
[f] Department of Biological Sciences, Pusan National University, Busan 46241, Republic of Korea

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ABSTRACT

Ballast water is essential for maintaining the balance and integrity of a ship. However, exchanging ballast water resulted in discharging water of different origins in vessel recipient ports, and this may have caused ecosystem disturbance or aquatic pollution. The ballast water management (BWM) system is essential for the purification and disinfection of the ballast water that is taken up. Because current BWM systems widely use biocides for the treatment of aquatic organisms, the biocides may result in unintended toxicity of the discharged ballast water. In this study, we suggested thymol and carvacrol as chemical biocides for BWM systems and investigated their effectiveness using *Artemia salina* and *Escherichia coli*. Thymol and carvacrol showed biocidal effects in our study. A combination of these substances showed a synergistic increase in the biocidal effects. Moreover, carvacrol naturally degrades after disinfection, which indicates that natural substances may be promising candidates to increase the efficacy and reduce unwanted side effects of the BWM system.

1. Introduction

Ballast water regulates the roll of a ship during loading or unloading of cargo by maintaining the stability, balance, and structural integrity of the ship (Hua and Liu, 2007). As the weight of moving ballast water has to correspond to that of cargo, the annual amount of ballast water moved is approximately 3.1 billion tons (David and Gollasch, 2015). Ballast water is taken up by a ship from the sea adjacent to the departing harbor and discharged to the sea adjacent to the arriving harbor. This process results in mixing of seawater from different seas, which sometimes leads to ecosystem disturbance or marine pollution (Werschkun et al., 2014). Therefore, the International Maritime Organization (IMO) developed regulations for ballast water management (BWM) systems and required minimal functions of such systems. The regulations focus not only on the capacity to eliminate aquatic organisms but also on the environmental safety of the discharged ballast water, which concerns the protection of aquatic environments (Čulin and Mustać, 2015; Tsolaki and Diamadopoulos, 2010).

Currently, BWM systems are of two types. One type employs physical methods of high energy, such as electricity and irradiation, to filter solid particles from seawater and eliminate aquatic organisms (Tsolaki and Diamadopoulos, 2010). However, the filters in such systems need to be changed after damage and deterioration during the filtering step, and the high operational cost of these filters lowers the disinfection efficacy of electricity and irradiation systems (Nanayakkara et al., 2011). The other type employs chemical methods to eliminate aquatic organisms, namely, treatment with a chemical biocide. Although various chemical compounds with high reactivity are known to show cytotoxicity at a low concentration, the excessive toxicity of these chemical compounds causes secondary problems when ballast water is discharged to the sea (Cañizares et al., 2009). Although recent studies have focused on the natural degradation of active substances and preventing secondary cytotoxicity, optimization of the concentration of the biocide and the duration of the biocidal effect remains to be investigated (La Carbona et al., 2010; Perrins et al., 2006; Werschkun et al., 2014).

“Natural product” is the general term for derivatives from natural organisms including plants, fungi, and animals. Natural products

* Corresponding author at: Department of Biological Sciences, Pusan National University, Busandaehak-ro 63 beon-gil, Geumjeong-gu, Busan 46241, Republic of Korea.
E-mail address: bhyoun72@pusan.ac.kr (B. Youn).

[1] H. Kim and S. Lee contributed equally to this work.

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usually show remarkable biological effects through their high activity and selectivity (Wright et al., 2007). The other outstanding characteristic of natural products is that they are easily degraded in the natural environment because of their eco-friendly structure, derived from biosynthesis processes (Nicolaou and Snyder, 2005). Recently, many biological and engineering studies have suggested the usefulness of natural products as novel compounds for the control of biological events and disease (Bauer and Bronstrup, 2014; Koehn and Carter, 2005). Thymol and carvacrol are phenolic monoterpenes of the essential oil from thyme and oregano, respectively (Castillo et al., 2014). Although they have a simple molecular structure and few reactive groups, the antimicrobial, antifungal, and antiviral effects of these compounds have been reported (Mechergui et al., 2016). However, the application of thymol and carvacrol in ballast water disinfection has never been attempted. Investigation of these products has great potential for revealing useful novel compounds.

Escherichia coli is widely known as a colonic bacillus, gram-negative microorganism, and commensal in the human intestine (Baker, 2014). *E. coli* can survive in various environments, and it induces pathogenicity by decomposing substances that surround it, including food, living organisms, and even seawater. Because of its pathogenicity, *E. coli* is classified as a microorganism that must be disinfected by BWM systems. *Artemia salina* is a plankton commonly found in the saline environment, and its size satisfies that suggested by IMO BWM guidelines (MEPC.279(70)). These two species were chosen in previous studies as models for validating the efficiency of BWM systems (Chen et al., 2016; Wright et al., 2009). Following those studies, we utilized the two species for validating the biocidal effects of thymol and carvacrol in this research.

In this study, we searched biocidal natural product pools for materials with high potential to be used in BWM systems. As a result, we proposed novel natural products, and their high biocidal effects were shown through biological investigations using plankton and an aquatic microorganism. Moreover, the potential for utilizing these substances in BWM systems was evaluated.

2. Materials and methods

2.1. Measurement of seawater characteristics

To analyze the chemical characteristics of natural seawater and synthetic seawater, we collected natural seawater samples from adjacent sea around Songdo Beach, Busan, Republic of Korea, and prepared synthetic seawater by mixing sea salt (Sigma-Aldrich, St. Louis, MO) to distilled water (DW). The large particles in natural seawater samples were eliminated by filtering with a 0.45-μm polyethersulfone (PES) membrane filter. We compared samples of the two types of seawater to analyze four characteristics including dissolved oxygen (DO), salinity, total dissolved solids (TDS), and conductivity. DO was calibrated and measured using a DO-300 L DO meter (Istek, Seoul, Republic of Korea). Salinity, TDS, and conductivity were assessed using a YSI Pro30 conductivity meter (YSI Inc., Yellow Springs, OH).

2.2. Microorganism cultivation and treatment

For investigating the biocidal effect of natural products on aquatic microorganisms, *E. coli* was used for experiments. The DH5α strain of *E. coli* was purchased from Bethesda Research Laboratories Inc. (Rockville, MD). The stock solution of *E. coli* was made by adding glycerol (20% of v/v) to the medium with *E. coli,* and this was stored in deep freezer (−70 °C) until use in experiments. The cells were thawed on ice for 10 min, transferred to fresh lysogeny broth (LB) media, and incubated at 37 °C in a shaking incubator (Vision Scientific Co. Ltd., Daejeon, Republic of Korea). To mimic the aquatic growth environment, LB medium with 3.5% salinity was formulated (3.5% NaCl, 1% Bacto Tryptone, and 1% Bacto Yeast extract in DW). For investigating the biocidal effect of thymol and carvacrol, 1×10^5 cells were inoculated to fresh LB media and treated with thymol or carvacrol. Thymol and carvacrol were purchased from Sigma-Aldrich.

2.3. Plankton cultivation and treatment

For investigating the biocidal effect of natural products in planktons, *A. salina* was used for experiments. The eggs of *A. salina* were purchased from Artemia International LLC. (Houston, TX) and were incubated in seawater at 25 °C. The eggs hatched after 3 days, and *A. salina* larvae of length 1–3 mm were used for further studies. The *A. salina* larvae were treated with thymol or carvacrol in a 100-mL beaker.

2.4. Measurement of the survival of E. coli and A. salina

To measure the number of the *E. coli* grown in LB media treated with natural products, a UV spectrophotometer (UV-1800, Shimadzu Corp., Tokyo, Japan) was used. One milliliter of the LB medium containing *E. coli* was sampled, and the absorbance at a 600-nm wavelength was analyzed every 1 h. To measure the survival of *A. salina* upon treatment of natural products, approximately 50 planktons were sampled from the seawater treated with natural products, and the survival of planktons was checked through microscopic observation with an Olympus IX71 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) every 2 h.

2.5. High-performance liquid chromatography (HPLC)

To assess the amount of carvacrol in LB media, a Waters 1525 Binary HPLC pump (Waters, Milford, MA) and a Waters 2489 UV/Visible detector (Waters) were used for analysis by reversed-phase high-performance liquid chromatography. Ten microliters from an aliquot of LB medium were injected and separated on a Sunfire C18 column (4.6 × 250 mm; Waters). The mobile phase was an isocratic combination of acetonitrile:H_2O (50:50) with a flow rate of 1 mL/min. The effect of carvacrol was verified by measuring the absorbance at a 274-nm wavelength. The amount of carvacrol was calculated with Breeze™ HPLC software (Waters) by quantifying the area of the carvacrol peak.

3. Results

3.1. Selection of the natural biocide for BWM systems

For the development of a BWM system with natural products, we first selected natural products to be utilized in the system. Because large aquatic organisms including fish, shells, and aquatic plants are easily filtered by the initial management system, the objective of the research was to eliminate aquatic organisms of size approximately 50 μm. We used plant-derived monocyclic monoterpenes for analysis, which previously were suggested to have high antibacterial functions (Koziol et al., 2014). Among the monoterpenes with similar molecular structures, we noticed that thymol and carvacrol have hydroxyl groups in a monocyclic structure, which plays a crucial role in their bioactivity (Fig. 1A and B) (Veldhuizen et al., 2006). Despite the previously reported biocidal effects of these two natural products, their utilization in the treatment of ballast water has not yet been reported; therefore, we selected thymol and carvacrol for further investigation (Botelho et al., 2007; Kordali et al., 2008).

Further, we established an experimental system for verification of the biocidal effect of the natural products in the BWM system. The IMO set the standards for BWM systems regarding planktons and microorganisms according to their size (MEPC.279(70)). In addition, *E. coli* and toxic *Vibrio cholerae* were selected as specific target species for BWM. *A. salina* and *E. coli* also were widely used in studies covering BWM (Holm et al., 2008; Tsolaki and Diamadopoulos, 2010; Tsolaki et al., 2010). According to the IMO regulations and previous studies, we