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# Ability of marine cyanobacterium *Synechococcus* sp. VDW to remove ammonium from brackish aquaculture wastewater

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ABSTRACT

Nitrogen-rich wastewater is a major problem for the aquaculture industry. To investigate whether algae could be used to remove ammonium from brackish shrimp aquaculture wastewater, marine cyanobacterium *Synechococcus* sp. VDW was cultured in BG11 medium supplemented with Turks Island salt solution and different concentrations of $NH_4Cl$ (1.0–40.0 mg $L^{-1}$) for 18 days. The cell density of the *Synechococcus* sp. VDW cultures increased in medium containing between 1 and 10 mg $L^{-1}$ of $NH_4Cl$, while ammonium concentrations greater than 20.0 mg $L^{-1}$ had a negative effect on growth. Glutamine synthetase and glutamate synthase activities were also examined, and were found to increase with cell density. Meanwhile, glutamate dehydrogenase activity increased in response to high $NH_4Cl$ concentrations (20.0–40.0 mg $L^{-1}$). The cellular response to ammonium excess was confirmed by measuring gene expression levels using quantitative PCR. Expression of both *glnA* and *gltB* was down-regulated compared with the control, while that of *gdhA* was up-regulated. At an initial concentration of 1–10 mg $L^{-1}$, 98–100% of the ammonium was removed by day 6 of cultivation. Therefore, these findings suggest that *Synechococcus* sp. VDW can remove ammonium from contaminated brackish water, and may be helpful for improving the quality of aquaculture wastewater.

## 1. Introduction

Thailand is one of the top 15 producers in the aquaculture industry worldwide, and is the largest exporter of crustaceans in South-East Asia (FAO, 2014). A significant problem associated with the growing aquaculture industry is the high quantity of nitrogenous waste that is produced during protein metabolism by the animals, and from the decomposition of nutrients in the aquaculture ponds. Ammonium levels in aquaculture effluent can vary widely, even in aquaculture source waters. However, levels are generally in the range of approximately 1–10 mg $L^{-1}$ (Chiu-Mei et al., 2016; Gustavo et al., 2006). Ammonium contamination from untreatedwater causes both environmental and health issues, including eutrophication and toxic effects on aquatic life (Li et al., 2007). Coastal shrimp farming, producing brackish water effluent, often contributes to the eutrophication of receiving waters (Dierberg and Kiattisimkul, 1996; Paez-Osuna et al., 1998). Elevated concentrations of environmental ammonia have been reported to affect growth, molting, oxygen consumption, and reproduction of *Penaeus* (prawn) species (Chen et al., 1988; Chen and Kou, 1992; Chen and Lin, 1992).

Wastewater treatment is essential for enhancing the sustainable aquaculture industry and reducing marine pollution. Various efforts, such as air stripping, chemical precipitation, adsorption, and biological treatment, have been applied to remove ammonium from different types of wastewater (Sarioglu, 2005). Currently, one established tool used in public sewage treatment facilities is biological treatment, which is used for activated sludge. However, this technique requires mechanical ventilation providing a significant volume of oxygen. It also costs 45–75% of the total energy requisite for a plant (Oilgae, 2010). Besides, waste-activated sludge matching the quantity of wastewater treated is the main derivative of this approach. Thus, considerable

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energy and a sizeable area of land are needed for the treatment and removal of waste-activated sludge (Metcalf and Eddy, 2003). On the whole, limitation factor for the development of the aquaculture industry in numerous regions is the cost-effective removal of nitrogen (N) from aquaculture farm waste, which likewise fails to supplement any value to the process other than the elimination of polluting nutrients from the discharge. Therefore, biological treatment using algae has gained a lot of attention. Algae are very efficient at removing nutrients from wastewater, and the accumulated biomass can be used in the production of high-value products including biofuel and natural compounds (Wang et al., 2010; Ramaraj et al., 2015). Many microalgae can utilize various forms of inorganic nitrogen, including $NH_4^+$, $NO_3^-$, and $NO_2^-$, for growth, and depending on the species and other environmental conditions, can survive in the presence of high nitrogen concentrations. They are fairly ubiquitous, and are found in both fresh and salt water, as well as municipal wastewaters. Several previous studies have successfully used algal cultures to remove ammonium from various types of wastewater (Arbib et al., 2014; Van den Hende et al., 2014). Lim et al. (2010) found that *Chlorella vulgaris* UMACC 001 could reduce $NH_4$-N concentrations in textile wastewater by 44.4–45.1% (initial concentration = 6.5 mg $L^{-1}$), while Feng et al. (2011) reported that 97% of $NH_4$-N was removed by *C. vulgaris* cultivated in artificial wastewater (initial concentration = 20 mg $L^{-1}$).

Marine cyanobacteria belonging to the genus *Synechococcus* are some of the most abundant picoplanktonic photoautotrophs in the world's oceans, and are capable of fulfilling their nitrogen requirements using nitrate, ammonia, or in some cases, urea. *Synechococcus* species also have the ability to adapt to a variety of nutrient concentrations and temperatures (Ting et al., 2002; Mella-Flores et al., 2012). Recently, *Synechococcus* species have been used as a model for synthetic biology studies, with numerous biotechnological applications including production of biofuels (Anne, 2014; McNeely et al., 2010) and various bioactive compounds (Alka and Jayashree, 2010).

Although biological wastewater treatment using algae has been studied for over 50 years, most algal strains investigated have been from freshwater environments, and thus there is limited information available regarding the ammonium tolerance of algae in saline/brackish water. Theoretically, marine algal strains also have the ability to use inorganic nitrogen for growth. Therefore, this study aimed to examine the effects of different ammonium concentrations on the growth of *Synechococcus* sp. VDW under simulated shrimp aquaculture wastewater conditions. We also investigated the activity of several key enzymes involved in ammonium assimilation, including glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.1.13), commonly known as the GS-GOGAT pathway. The direct amination of 2-oxoglutarate, catalyzed by glutamate dehydrogenase (GDH; 1.4.1.4), was also examined. In addition, quantitative PCR (qPCR) was used to measure the expression of *glnA*, *gltB*, and *gdhA*, encoding glutamine synthetase, glutamate synthase, and glutamate dehydrogenase, respectively, following exposure to various ammonium concentrations. Finally, the ammonium removal efficiency of *Synechococcus* sp. VDW from synthetic medium was also determined.

## 2. Materials and methods

### 2.1. Algal strain and culture conditions

*Synechococcus* sp. VDW (accession number MH393765), originally isolated from natural seawater in Thailand (Tinpranee et al., 2018), was provided by Bioenergy Research Unit, King Mongkut's Institute of Technology Ladkrabang, Thailand. This strain was cultured in 250-mL Erlenmeyer flasks containing 100 mL of BG11 medium supplemented with Turks Island salt solution (pH 7.5) at 30 ± 2°C (Incharoensakdi and Karnchanatat, 2003). Aeration was provided by regular shaking at 150 rpm, and cultures were grown under a fluorescent light irradiance of 30 μmol photon $m^{-2}$ $s^{-1}$ with a 12 h light/12 h dark cycle. Each treatment was performed in triplicate, and cultures were uniformly prepared with an initial optical density at 730 nm ($OD_{730}$) of approximately 0.1. Absence of microbial contamination was verified by cultivation on agar medium (Allen and Gorham, 1981). The specific growth rate (μ) was determined from the increase in algal cell density during the exponential growth phase compared with the initial cell density, and calculated using the following equation:

$$\mu = \frac{\ln \quad X_t - X_0}{T}, \tag{1}$$

where, $X_t$ is the cell density at time t, $X_0$ is the cell density in the initial logarithmic growth phase, and t is the period between $X_0$ and $X_t$.

### 2.2. Estimation of biologically relevant ammonium concentrations from shrimp farm effluent

To estimate the concentration of ammonium in standard shrimp farm wastewater, samples of shrimp farm effluent were collected from five shrimp farms in Chantaburi Province, one of the largest shrimp farming areas in Thailand. The samples were collected after shrimp harvesting. Sampling procedures and analyses were conducted in accordance with the Standard Methods for the Examination of Water and Wastewater of the American Public Health Association (APHA, 2005). Physicochemical parameters of the wastewater, including biochemical oxygen demand ($BOD_5$), pH, ammonia ($NH_3$-N) concentration, total phosphorus (TP) concentration, total Kjeldahl nitrogen (TKN) concentration, and nitrite ($NO_2$-N) concentration were measured in all samples

### 2.3. Experimental set up

To determine the limiting concentration of nitrogen from ammonia on algal growth and enzyme activity, a shake flask growth assay was performed. Ammonium chloride ($NH_4Cl$) was added to the culture medium to a final concentration of 10, 20, 30, or 40 mg $L^{-1}$ ($NH_4$-N), covering the range of concentrations identified in the shrimp farm effluent samples. Three replicate flasks, each containing 100 mL of culture medium, were prepared for each concentration, along with a control containing no $NH_4Cl$. These experiments were performed at the same incubation temperature and under the same illumination conditions as for standard culturing. Triplicate flasks for each concentration were collected at six time point. During the experimental period, one milliliter of culture was harvested and the $OD_{730}$ was measured using a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Japan). The remaining culture was harvested by centrifugation at 12,000 × *g* for 15 min. The cells were washed twice with sterile saline solution (0.85% NaCl) and used for enzyme activity assays. The supernatant was used to measure ammonium concentration (as described in section 2.7). Samples were measured every 3 days over the 18-day experimental period, including day 0.

For experiments addressing the changes in gene expression induced by high ammonium concentrations, algal cells were cultured under the conditions described in section 2.1. Total cells at mid-logarithmic phase were harvested by centrifugation at 12,000 × *g* for 15 min, resuspended in 5 mL of sterile saline solution, and then transferred into 100 mL culture medium containing 20 or 40 mg $L^{-1}$ $NH_4Cl$. Culture medium without $NH_4Cl$ was used as the control. Samples were then collected at the indicated time points and prepared as described in section 2.5.

### 2.4. Enzyme assays

#### 2.4.1. Preparation of cell-free extracts for enzymatic activity assays

The cell pellet was washed twice with 50 mM Tris−HCl buffer (pH 8.0) and then centrifuged at 12,000 × *g* for 15 min at 4°C. The pellet was then resuspended in 3 mL of Tris−HCl buffer and sonicated at 4°C