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Title

Combination of heterotrophic nitrifying bacterium and duckweed (*Lemna gibba* L.) enhances ammonium nitrogen removal efficiency in aquaculture water via mutual growth promotion

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Running title

NH_4^+-N removal from aquaculture water

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Abstract

We created a combined system using duckweed and bacteria to enhance the efficiency of ammonium nitrogen (NH_4^+-N) and total nitrogen (TN) removal from aquaculture wastewater. Heterotrophic nitrifying bacterium was isolated from a sediment sample at an intensive land-based aquaculture farm. It was identified as *Acinetobacter* sp. strain A6 based on 16S rRNA gene sequence (accession number MF767879). The NH_4^+-N removal efficiency of the strain and duckweed in culture media and sampled aquaculture wastewater at 15°C was over 99% without any accumulation of nitrite or nitrate. This was significantly higher than strain A6 or duckweed alone. Interestingly, the presence of NO_3^- increased NH_4^+-N removal rate by 35.17%. Strain A6 and duckweed had mutual growth promoting-effects despite the presence of heavy metals and antibiotics stresses. In addition, strain A6 colonized abundantly and possibly formed biofilms in the inner leaves of duckweed, and possessed indoleacetic acid (IAA)- and siderophore-producing characteristics. The mutual growth promotion between strain A6 and duckweed may be the reason for their synergistic action of N removal.

Keywords: *Acinetobacter* sp., ammonium nitrogen, aquaculture wastewater, duckweed, removal

Introduction

To meet the requirements of aquatic products in China, over 6,000 kha of freshwater is required (a datum collected from the State Statistics Bureau, see http://www.chyxx.com/). To obtain high aquaculture output, up to 6,500 of fish or 10,000 shrimp are needed per 667 m^2 based on our investigations. As a result, high-protein feeds are needed in these aquatic systems. Urea, liquid cow manure, or even pig manure and chicken manure with high N content are often supplemented during this process (Lin and Yi 2003; Moav et al. 1977; Soletto et al. 2005; Zoccarato et al. 1995). Budget-wise, about 87% of N comes from feed, while only 1% is released by denitrification (Acosta-Nassar et al. 2010). This results in the generation of substantial amounts of polluted effluent containing unconsumed feed and feces, and thus, leads to an increase in environmental pollution (Crab et al. 2007; Read and Fernandes 2003). In these kinds of aquatic systems, levels of ammonia-N (NH_3-N), nitrite, and dissolved oxygen (DO) drastically affect aquaculture production (Crab et al. 2007; Zoccarato et al. 1995). Of these factors, NH_3-N is a critical concern; as it leads to an increase in nitrite and a decrease in DO due to the nitrification (Grommen et al. 2002; Kim et al. 2008; Ruiz et al. 2003). In addition, it is toxic for aquatic organisms (Romano and Zeng 2013; Thompson et al. 2002). The presence of NH_3-N is inevitable, especially during intensive aquaculture, as they are generated from feed residues and manure supplements. Thus, there has been a lot of research trying to develop integrated pond systems using duckweed (Steen et al. 1999; Zimmo et al. 2003) or combined systems with other aquatic organisms such as algae (van der Steen et al. 1998), and cyanobacteria (Duong and Tiedje 1985). Using the duckweed treatment system, not only NH_3-N, but also bacterial pathogens (El-Shafai et al. 2007; Steen et al. 1999), some antibiotics (Iatrou et al.

2017), and chemical contaminants (Gatidou et al. 2017; Türker et al. 2017; Wang et al. 2017) could be removed to increase water quality.

Despite the advantages of using duckweed for the removal of NH_3-N from aquacultures, the growth of duckweed is inhibited to a certain extent under high concentrations of NH_4^+ and NH_3-N, as well as salt (Caicedo et al. 2000; Liu et al. 2017). Thus, it is necessary to find aquatic organisms that can promote duckweed growth and/or increase their resistance to these environmental stresses. To date, only a few studies have reported on this topic. Stout et al. (2010) reported that certain bacteria had roles in promoting *Lemna minor* plant growth by enhancing root growth, with minor effects on enhancing plant cadmium uptake. Hence, isolating and identifying bacteria that are capable of promoting duckweed growth and eliminating NH_3-N may be a feasible way to overcome the present concerns for aquaculture.

Duckweed is intolerant to high concentrations of NH_3 and NO_2^-, low DO and pH beyond its optimal range (Crab et al. 2007). We isolated a heterotrophic nitrifying bacterium that had the ability to remove NH_4^+-N and tested its synergistic effects on NH_4^+-N removal with *Lemna gibba*. Co-culture had a mutual growth promotion activity, which may be the possible mechanism for their optimal efficiency in removing NH_4^+-N. In addition, we provide an aquatic safety assessment to aquatic fish in this study.

Materials and methods

Isolation and identification of heterotrophic nitrifying bacteria

During a periodic cleanup of sediment at an intensive land-based aquaculture in Dongfanglvzhou, Dafeng, Jiangsu Province in Feb., 2016, we took five sediment samples from different ponds and mixed them into one. The aquaculture farm had operated for four years continuously without any sediment cleaning.

In the laboratory, 10 g of sediment was added to 90 mL of enrichment medium (pH 7.2) containing 0.05 g of $(NH_4)_2SO_4$, 0.07 g of KH_2PO4, 0.05 g of $MgSO_4·7H_2O$, 0.05 g of $CaCl_2·2H_2O$ and 0.1 mL of a trace mineral solution (Huang et al. 2013; Yang et al. 2011). The culture solution was incubated at 15°C (a relatively low temperature of aquaculture water in Jiangsu) on a rotary shaker at 160 rotations per minute (rpm). Every 7 days, 1 mL of the enrichment culture was transferred to a fresh enrichment medium and this process was repeated four times. Afterwards, 0.1 mL of culture solution was spread onto an agar plate containing 0.77 g of NH_4Cl, 1.0 g of CH_3CH_2ONa, 0.05 g of $MgSO_4·7H_2O$, 0.2 g of K_2HPO_4, 0.12 g of NaCl, 0.01 g of $MnSO_4$ and 0.01 g of $FeSO_4$ (per liter) (Huang et al. 2013). Purified isolates were obtained by repeated streaking on agar plates. A total of 24 isolates were separately inoculated in the abovementioned media without agar and incubated at 15°C. Their ability to remove NH_4-N (initial concentration of 200 mg/L) was measured using the Nessler's reagent colorimetric method (He et al. 2016). NO_2^- and total nitrogen (TN) was measured using the ultraviolet spectrophotometric method (He et al. 2016) and the potassium persulfate digestion ultraviolet spectrophotometric method (HJ 535-2009). After screening, bacteria capable of eliminating NH_4^+-N rapidly without nitrite residues were selected for further study. The bacterial strain, named A6, was suspended in 20% glycerol solution and placed at −80°C for long-term storage.

The cell morphology of strain A6 was obtained using a scanning electron microscope (SEM) (Quanta200, Holland). Briefly, after an overnight culture of strain A6 in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 28°C on a rotary shaker at 160 rpm, cells were harvested by centrifugation, and washed 3 times and resuspended in sterile distilled water. Twenty microliters of suspension was spread onto a microscope slide and air dried. Afterwards, the sample was coated with gold under vacuum followed by microscopic examinations using SEM at 15 kV.

The physiological and biochemical characteristics of strain A6 were analyzed based on the method described in Dong and Cai. (2001). Genomic DNA of strain A6 was extracted using the DNA extraction kit (Tiangen, China). An almost full-length 16S rRNA gene was then amplified using universal primer pairs, forward primer 27f (5'-AGAGTTTGATCATGGCTCAG-3') and reverse primer 1492r (5'-TACGGTTACCTTGTTACGACTT-3') (Heuer et al. 1997). The amplified product was submitted to Sangon (Shanghai, China) for sequencing, and was performed using the automated sequencer ABI3730xl DNA Analyzer (Applied Biosystems). The sequence was compared with reference sequences in GenBank using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence was deposited in Genbank with an accession number MF767879. A phylogenetic tree was constructed using MEGA 5 using the neighbor joining method (Tamura et al. 2011).

Effect of strain A6 on nitrogen removal using different nitrogen sources

To assess if strain A6 has the capacity for both nitrification and denitrification, NH_4^+, NO_3^-, and NH_4^++ NO_3^- were selected as the initial nitrogen sources, and their reduction over time were measured (He et al. 2016). A 500 mL conical flask containing 200 mL of culture medium was autoclaved at 121°C for 20 min. There were three replicates for each treatment. Strain A6 that was previously cultured in LB at 15°C in a shaker at 160 rpm for 18 h was centrifuged at 5,000 rpm at 4°C. Cells were then washed with sterile double distilled water (ddH_2O) three times and re-suspended in sterile ddH_2O at a final concentration of 10^7 cfu/mL. The 2% seed inoculum was then added into each flask of culture media containing the different N sources. Each flask was incubated at 15°C in a shaker at 160 rpm. Every 24 h, samples were taken and the following were measured; cell density, NH_4^+, NO_3^-, and TN concentrations. All treatments and determinations were performed in triplicate. In addition, to further prove the ability of nitrification and denitrification, *amo*A, *hao*, *nxr*A, *nar*G, *nap*A, *nir*K, *nir*S, *nrf*A, *nor*B, and *nos*Z were amplified and sequenced. The gene specific primer pairs are shown in Table S1.

Collection and disinfection of duckweed (*Lemna gibba*)

Duckweeds were originally collected from a pond in Yancheng Teachers University, Jiangsu Province. In the laboratory, duckweed was surface-sterilized with 5% sodium hypochlorite for 5 min. Following treatment, the duckweed was rinsed with sterile ddH_2O at least five times. The duckweed was identified as *Lemna gibba* based on its morphology as determined by Prof. Yanqiu Yu from the Yancheng Teachers University (Les et al. 2002).

Synergistic effect of strain A6 and duckweed on NH_4-N removal from aquaculture wastewater

Duckweed with, or without, strain A6 was cultured in sterile aquaculture wastewater collected from Dongfanglvzhou, Dafeng, Jiangsu Province. Since high ammonium concentrations (>20 mg/L NH_4-N) have a negative impact on the growth rate of duckweed (Caicedo et al. 2000), NH_4-N was added and adjusted to 10 mg/L with ammonium chloride based on a previous study (Grommen et al. 2002). Four treatments groups consisting of the control (neither duckweed nor strain A6), strain A6 only (initial concentration 10^3 cfu/mL, see the below-mentioned experiment), duckweed only (initial abundance around 160 frond numbers), and duckweed + strain A6, were used to assess the efficiency of ammonia removal from aquaculture wastewater. The experiment was conducted with glass fish tanks (40 cm length × 30 cm width × 30 cm height). Each tank contained 20 L of aquaculture wastewater. The inoculation method was similar to the above-mentioned process. The fish tanks were maintained indoors under the following conditions; 16/8 h light/dark cycle at 15°C. Each fish tank had a rotor that worked at a rate of 30 min every 6 h. Water samples were taken at one-day intervals and NH_4^+, NO_3^-, and TN concentrations were measured for time course analysis. All treatments and determinations were performed in triplicate.

The mutual growth-promoting effects of strain A6 and duckweed

To determine if strain A6 could enhance the tolerance of duckweed against heavy metals and antibiotics in aquaculture wastewater, 1000 μM of Pb^{2+}, 340 μM of Cr^{6+}, 780 μM of Cu^{2+}, 0.05 mg/L of oxytetracycline, and 0.05 mg/L of gentamicin (the median lethal concentration for strain A6) were added to the abovementioned aquaculture wastewater in fish tanks. Strain A6 was inoculated into the wastewaters at an initial concentration of 10^3 cfu/mL. Duckweed was added to half the tanks with the culture conditions being similar to the previous experiments. Water samples were taken at regular intervals of 24 h and bacterial cell growth was determined spectrophotometrically by measuring the $OD_{600\ nm}$. After 96 h of incubation at 15°C, duckweed were harvested and placed on absorbent paper to remove surface water. Afterwards, the duckweed was immediately weighed to determine the fresh weights.

Next, we investigated if strain A6 had growth-promoting effects on duckweed and we determined the optimal inoculum dose of strain A6. Serial inoculum doses of strain A6 of 0 (blank control), 10^2, 10^3, 10^4, and 10^5 cfu/mL, were selected for the experiments. The initial concentration of duckweed in each tank was around 160 frond numbers, and subsequent frond numbers were counted and recorded every day.

Effect of duckweed extract on biofilm formation of strain A6

After disinfection, 10 g of duckweed were mixed with 10 mL of 0.1 M phosphate buffer (pH 7.2) in a sterile grinding bag and placed in an ice box, followed by grinding using a wooden dowel. The extracts were then centrifuged at 5,000 rpm for 10 min at 4°C, and the supernatants were further filtered using a 0.22-μm-filter membrane. Half of the extracts were then autoclaved at 121°C for 15 min. The two duckweed extracts were referred to as "filtration" and "autoclaving" and were used in the following amounts; 0% (control), 5%, 10%, and 20% for biofilm formation of strain A6. The crystal violet staining method was used for measuring biofilm formation (Kang et al. 2014; O'Toole and Kolter 1998).

Observation of biofilm formation of strain A6 on duckweed

Twenty milliliters of sterile aquaculture wastewater were poured into two Petri dishes (Φ 90 cm). One of which was mixed with strain A6 cell solution (10^7 cfu/mL) at a final concentration of 10^3 cfu/mL. Afterwards, wastewaters were covered with 50 individual duckweeds, and then incubated at room temperature for 24 h under a natural light-dark cycle.

The duckweeds were then harvested and placed on sterile Whatman filter paper to remove surface water. Afterwards, they were fixed with 2.5% of glutaraldehyde, followed by washing with a 0.1 M phosphate buffer for 15 min (total of 3 washes). Samples were then dehydrated sequentially using 50%, 70%, 80% of ethanol solution, ethanol and amyl acetate (2:1, v/v), ethanol and amyl acetate (1:1, v/v), and amyl ester for 30 min each. Afterwards, the inner and outer surfaces of the roots and leaves were examined using a scanning electron microscopy (Quanta200, Holland) at 25 kV. A total of three independent experiments were set up and only one representative picture is shown in the corresponding results.

Characteristics related to duckweed growth promotion

Production of indole acetic acid (IAA) and siderophores, possibly related to duckweed growth promotion, were determined based on the methods developed by Glickmann and Dessaux (1995) and Schwyn and Neilands (1987), respectively. For IAA measurement, strain A6 was incubated in LB containing 0.5 g/L L-tryptophan at 25°C for 48 h. Two milliliters of culture solution was then centrifuged at 10,000 rpm for 15 min, and the supernatant was mixed with 2 mL of Salkowski reagent (4.5 g $FeCl_3$ in 1 L of 10.8 M H_2SO_4). After color development for 30 min at room temperature in the dark, the optical density was measured at 530 nm. IAA production was calculated based on a standard curve using serial concentrations of IAA. For siderophore measurement, strain A6 was inoculated on a chrome azurol S agar plate (Schwyn and Neilands 1987) and cultured at 25°C for 48-72 h. Strain A6 was capable of producing siderophores if bacterial colonies were surrounded by green-yellow haloes.

Data analysis

Raw data were analyzed using SPSS Statistics for Windows Version 24.0 (SPSS, IBM, Somers, NY, USA) to calculate means, standard errors (SE), as well as differences between treatments using Duncan's multiple range tests. The significance level was set at a *p*-value of 0.05. The figures presented were produced using Sigma Plot for Windows Version 10.0 (Systat Sofware, San Jose, CA, USA).

Results and discussion

Isolation and identification of a heterotrophic nitrifying bacterium

A total of 24 bacterial strains were isolated from sediment samples by an enrichment process. Their ability to remove NH_4^+-N was tested. One isolate, named strain A6, showed the highest efficacy and was selected for identification and later study. Strain A6 was Gram-negative, non-spore-forming, catalase-positive, indole-negative, oxidase-negative, no flagellum and non-motile, and nitrate reduction-negative. The SEM image of strain A6 (Fig. 1A) indicated that it was cocci or a short rod with a width of approximately 1.2 μm.

The partial 16S rRNA gene (1306 bp) of strain A6 was amplified and sequenced. Using BLAST, strain A6 was identified as being closely related to members of the genus *Acinetobacter*, of which *Acinetobacter johnsonii* strain EPS-11 (KY848819) had the highest similarity (100%). The resulting phylogenetic tree consisted of a partial 16S rRNA gene sequence of strain A6 and some members of *Acinetobacter* (Fig. 1B), which further revealed that strain A6 was clustered with species from *Acinetobacter*. Consequently, strain A6 was identified to be an *Acinetobacter* species. To date, several isolates belonging to *Acinetobacter* sp. have been reported to be capable of eliminating ammonia from both aquaculture wastewater and industrial effluents (Fan et al. 2015; Huang et al. 2013; Sarioglu et al. 2012; Zhao et al. 2010a), demonstrating the potential future use of this isolate for wastewater treatment.

Ammonia elimination by strain A6 from three different nitrogen sources

At 15°C, about 70% of NH_4^+-N was eliminated from the media containing NH_4^+-N after 72 hrs, which was substantially faster compared with *A. calcoaceticus* STB1 isolated by Sarioglu et al. (Sarioglu et al. 2012). At 120 h, most of the NH_4^+-N was eliminated by strain A6 with no accumulation of NO_2^--N (not shown in Fig. 2) and NO_3^--N (Fig. 2A), which was consistent with that of *Microbacterium* sp. strain SFA13 (Zhang et al. 2013) and *Pseudomonas tolaasii* Y-11 (He et al. 2016). This indicated that strain A6 could be used as an inoculant for removing ammonia without any negative impacts for aquaculture. The ammonium elimination was mainly due to bacterial assimilation (Zhao et al. 2010a). The loss of TN suggests that some ammonium may be converted to gaseous nitrogen during the nitrification process. The nitrification rate of strain A6 at 15°C was 1.45±0.18 mg NH_4^+-N/L/h, which was lower compared with *Bacillus methylotrophicus* L7 (2.14 mg NH_4^+-N/L/h) (Zhang et al. 2012) and *P. tolaasii* Y-11 (2.04 mg NH_4^+-N/L/h) (He et al. 2016), but similar to that of *P. alcaligenes* AS-1 (1.15 mg NH_4^+-N/L/h) (Su et al. 2006) and *Pesudomonas* sp. ADN-42 (1.38 mg NH_4^+-N/L/h) (Jin et al. 2015), and higher than *Bacillus* sp. LY (0.43 mg NH_4^+-N/L/h) (Zhao et al. 2010b) and *Acinetobacter* sp. Y16 (0.092±0.006 mg NH_4^+-N/L/h) (Huang et al. 2013).

When NO_3^--N was the sole nitrogen source, the exponential growth phase began at 48 h (Fig. 2B), demonstrating a slower growth rate compared with the media with NH_4^+-N only or a mixture of NH_4^+-N and NO_3^--N (Fig. 2C). This indicated that i) strain A6 could perform aerobic denitrification with nitrate nitrogen, and ii) strain A6 utilized NH_4^+-N preferentially compared with NO_3^--N. This became more evident when strain A6 was cultured with a mixture of NH_4^+-N and NO_3^--N. Strain A6 preferred to use NH_4^+-N first, and then use NO_3^--N when NH_4^+-N was exhausted after 96 h (Fig. 2C). Within 120 h, 93.04% of NO_3^--N could be removed by strain A6. The nitrate removal rate of strain A6 at 15°C was 1.45±0.10 mg NO_3^--N/L/h, which was almost equal to the ammonium removal rate. The nitrate removal rate was higher compared with *Rhodococcus* sp. CPZ24 (0.93 mg NO_3^--N/L/h at 30°C) (Chen et al. 2012), but lower than that of *P. tolaasii* Y-11 (1.99 mg NO_3^--N/L/h) (He et al. 2016). The total loss of TN with NO_3^--N was similar to that of NH_4^+-N, suggesting that an equivalent amount of gaseous nitrogen was released during the nitrification and denitrification processes. No nitrite was detected during the measurement period, while NH_4^+-N increased gradually to 19.46 mg at 168 h, which is similar to several previous reports (He et al. 2016; Jin et al. 2015). Ammonium originates from death cells containing organic nitrogen, and may contribute to NH_4^+-N accumulation during the later growth phases. However, whether strain A6 can conduct dissimilatory nitrate reduction to the ammonium process under possibly a micro-anaerobic environment (referring to the later growth phase) is still unknown and needs to be determined.

Simultaneous nitrification and denitrification (SND) accomplished by one particular strain of bacterium highlights its advantages in nitrogen polluted wastewater (Jin et al. 2015) compared to the traditional SND process performed by several different bacterial strains (Xia et al. 2008). Strain A6 seemed to be capable of performing simultaneous heterotrophic nitrification and aerobic denitrification, which was reflected in the loss of NH_4^+-N and NO_3^--N within 7 days (Fig. 2C). However, the processes of nitrification and denitrification are not totally simultaneous. Strain A6 preferred to use NH_4^+-N first, and then use NO_3^--N when NH_4^+-N was exhausted at 96 h, which was similar to that observed in *P. tolaasii* Y-11 (He et al. 2016). The situation of exhausting NH_4^+-N and having a stationary phase at 96 h with a lower DO may contribute to the use of NO_3^--N. From our transcriptome experiments (data is not shown because they are not related), we found that the prior use of NH_4^+-N by strain A6 was not affected by the nitrate reductase gene, but may be possibly related to the up-regulation of the carbonic anhydrase gene in the medium containing NH_4^+-N. NO_3^--N suppress the activity of carbonic anhydrase (Glass and Silverstein 1998) and transcriptional activity of the encoded gene (data not shown), which suggests that the carbonic anhydrase gene is of relevance. The nitrification rate of strain A6 with both NH_4^+-N and NO_3^--N was 1.96±0.02 mg NH_4^+-N/L/h, which was higher compared with NH_4^+-N only. Comparatively, the nitrification rate of A6 was similar to that of *P. tolaasii* Y-11 (He et al. 2016) but lower compared with *P. versutus* LYM (Zhang et al. 2015). This may be due to the possible activation of NH_4^+-N assimilation related genes by NO_3^--N. The nitrate removal rate of strain A6 in this medium was 3.55±1.51 mg NO_3^--N/L/h from 96 h to 120 h. This stagnation in the rate may be due to the accumulation of NO_3^--N converted from NH_4^+-N during the latter phases. At the initial TN of 480.01 mg/L, the removal efficiency was only 23.65±2.47%, suggesting that gaseous nitrogen was possibly released during the latter phases in the medium with NH_4^+-N and NO_3^--N.

We qualitatively identified several genes that are involved in the heterotrophic nitrification–aerobic denitrification process. The results showed that *amo*A, *hao*, *nxr*A, *nap*A, and *nir*S were found to be positive (Fig. S1). This further proved that strain A6 was capable of performing nitrification and denitrification. There are still key experiments that are needed to determine accurately the pathway of nitrogen metabolism by strain A6; however, this is beyond the current scope of this study.

Rate of ammonium removal by the combination of strain A6 and duckweeds

Several studies have suggested the importance of bacteria for duckweed growth and ammonium removal (Duong and Tiedje 1985; Körner and Vermaat 1998; Stout et al. 2010; Xu and Shen 2011). However, an intensive study using a specific bacteria combined with duckweed is lacking. To better understand and reinforce the ammonium elimination performance of strain A6, duckweed was used as the supporting material to conduct experiments on aquaculture wastewater. We found that both strain A6 and duckweed could significantly remove NH_4^+-N, NO_3^--N, and TN (Fig. 3). The efficiency of ammonium removal by duckweed plus strain A6 was 99.18±0.22% at Day 10, which was compared to duckweed (83.84±5.51%) and strain A6 (70.94±10.03%) alone. Most of the TN (98%) in swine-waste-polluted duckweed ponds is removed once every year (Mohedano et al. 2012). Residual ammonia was 0.41 mg N/L with removal efficiencies of 98% (El-Shafai et al. 2007). Using the combined system containing strain A6 and duckweed, we obtained a comparable result within 10 days compared with the previous studies. Grommen et al. (2002) demonstrated that using nitrifying bacteria can shorten the start-up period of a bio-filter, which was confirmed in this study.

The levels of NO_3^--N in the control treatment group increased with time (Fig. 3B), and was opposite to the time course for NH_4^+-N. This may be attributed to the nitrification process. In addition, it was found that there was ~20% of TN loss in the control treatment group on Day 10 (Fig. 3C), suggesting that the nitrification process still occurred and that some N was released as gaseous nitrogen (likely NO, see Fig. S1). For the strain A6 treatment group, an obvious change in NO_3^--N levels were observed with time, indicating that from day 6 some denitrifying bacteria may function in DO-decreasing conditions. The elimination rate of NO_3^--N by duckweed was much slower compared with NH_4^+-N. This suggested that duckweeds may utilize NH_4^+-N preferentially compared with NO_3^--N.

On Day 10, the TN elimination efficacies of the control, strain A6, duckweed, and strain A6 plus duckweed, treatment groups were 31.65%, 68.64%, 57.07%, and 96.31%, respectively (Fig. 3C). It has been demonstrated that 80% of N removal was through plant uptake, 5% by sedimentation and 15% by unknown factors (El-Shafai et al. 2007). In another study, it was found that in duckweed-based ponds, nitrification/denitrification by microorganisms was the major mechanism for N removal (Zimmo et al. 2003). An earlier study indicated that duckweed was directly responsible for 30–47% of the total N-loss through the uptake of ammonium (Körner and Vermaat 1998). Our results showed that nitrifying bacteria had a stronger effect on TN removal compared with duckweed, which may be due to the much larger specific surface-area of strain A6 compared with duckweed, and thus could assimilate more nutrients, including ammonium. The differences in the studies mentioned above could be explained by distinct pond systems and water conditions. Differences in environmental conditions and treatment efficiencies have been observed in algae-based ponds and duckweed-based pond systems (Zimmo et al. 2002).

Mutual growth-promoting effects between strain A6 and duckweed

To understand the factors that may be responsible for the enhanced ammonium and TN removal efficiencies of the combined system with strain A6 and duckweed, the mutual effects of strain A6 and duckweed under stressed conditions were determined. Results showed that heavy metals, such as Pb, Cr(VI), and Cu, and antibiotics including oxytetracycline and gentamicin, could significantly inhibit the propagation strain A6 (Fig. 4A), and the co-culture of duckweed could mitigate the repressive effects of these heavy metals except for Cu (Fig. 4B). Stout et al. (2010) demonstrated that even in the presence of cadmium-tolerant bacteria, they could not enhance duckweed uptake of cadmium. Organic acids and phytochelatins released by plants could help chelate heavy metals and reduce the detrimental effects for the growth of bacterial strain (Ghosh and Singh 2005). In addition, duckweed have the ability to degrade antibiotics (Iatrou et al. 2017), which may be a reason for the growth promotion observed in strain A6. Moreover, some heat-sensitive substances from duckweed could significantly promote the biofilm formation of strain A6 (Fig. 5), which could be a factor responsible for the enhanced growth promotion observed even in the presence of heavy metals and antibiotics stressed conditions (Harrison et al. 2004; Teitzel and Parsek 2003). In addition, the attached biofilm may have nitrogen removal capability (Körner et al. 2003). Strain A6 had growth-promoting effects on duckweed at a concentration of 10^3 cfu/mL (Fig. 4C). At this dose, strain A6 also relieved the negative impact of several heavy metals and antibiotics on duckweed growth (Fig. 4D). In addition, production of IAA and siderophores, possibly involved in duckweed growth promotion were examined. Our results demonstrated that strain A6 could produce both IAA (9.47 μg/mL) and siderophores (Fig. 6). This was consistent with several other bacterial isolates belonging to *Acinetobacter* sp. (Dorsey et al. 2004; Gulati et al. 2009; Srivastava and Singh 2014; Yamamoto and Sakakibara 1994). At 15°C, strain A6 also produced IAA (7.26 μg/mL) and siderophores (data not shown), indicating that the strain is functional in real environmental conditions. Because of the water-soluble nature of IAA (Arancon et al. 2006) and siderophores (Baret et al. 1995), it was inferred that strain A6 could exert growth-promoting effects more noticeably in water compared to soil. Several publications have shown that pathogens like *E. coli* could be removed by duckweed (Awuah et al. 2001; Steen et al. 1999). It is known that siderophore-producing rhizobacteria can promote plant growth by providing available iron to plants (Ghavami et al. 2016) and also by depriving iron from iron-dependent pathogens (Miethke and Marahiel 2007).

Using SEM technology, we observed the colonization of strain A6 on/in duckweed (Fig. 7). Strain A6 colonized in the inner leaves compared to the roots or surfaces. Strain A6 possibly formed biofilm in the inner leaf and thus exerted more growth-promoting effects on leaf proliferation (Fig. 4D) compared to root elongation (data not shown). Interestingly, strain A6 lacks flagella (Fig. 1A) which is important for biofilm formation (O'Toole and Kolter 1998). We inferred that strain A6 may be assimilated and transported into the inner leaves via root flow, and then, like other *Acinetobacter* sp., exhibit twitching motility (Bitrian et al. 2013) for biofilm formation.

Conclusions

To increase the efficiencies of ammonium and TN elimination in aquaculture wastewater, a heterotrophic nitrifying bacterium, identified as *Acinetobacter* sp., was isolated and used in a co-culture system with duckweed. The ammonium removal efficiency in culture media and sampled aquaculture wastewater at 15°C was over 99%, with no accumulation of nitrite and nitrates. This was significantly higher compared with bacterium or duckweed alone. *Acinetobacter* sp. strain A6 and duckweed had mutual growth-promoting effects under chemical stress conditions. Strain A6 possibly colonized in the inner duckweed leaves, and displayed IAA- and siderophore-producing characteristics. This may be the mechanism of their synergistic efficiency regarding N removal.

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Figure Legends.

Figure 1. Cell morphology observed by scanning electron microscopy (A) and phylogenetic tree of strain A6 (B).

Figure 2. Time course of nitrogen removal in culture media containing ammonium-N only (A), nitrate-N only (B), and ammonium-N + nitrate-N (C) at 15°C. The dashed line in Fig. C indicates the timepoint when strain A6 starts to use nitrate.

Figure 3. Time course of the elimination efficiencies of ammonium-N (A), nitrate-N (B), and total-N (C) at 15°C with sampled aquaculture wastewater.

Figure 4. Mutual growth-promoting effects of strain A6 and duckweed. Growth of strain A6 in the absence (A) and presence of duckweed (B); Effect of different inoculation doses of strain A6 on duckweed growth (C); Effect of strain A6 on the growth of duckweed in the presence of chemical stresses (D); different alphabets between treatments denotes significant differences (ANOVA; $p < 0.05$, Duncan's test).

Figure 5. Effects of duckweed extracts obtained by filtration with 0.22-µm-membrane filter (A) or autoclaving (B) on biofilm formation of strain A6. Different alphabets between treatments denote significant differences (ANOVA; $p < 0.05$, Duncan's test).

Figure 6. Cell morphologies of strain A6 observed on the chrome azurol S agar plates after 72

h and 96 h incubation at 25°C (A) and 15°C (B), respectively. The green-yellow haloes surrounding bacterial colonies denote siderophore-producing positive.

Figure 7. Colonization of strain A6 in/on duckweed observed by scanning electron microscopy.

Figure S1. The putative pathway for heterotrophic nitrification–aerobic denitrification process of strain A6. Arrows with a solid line indicate positive results by PCR; arrows with a dashed line indicate negative results by PCR.

Fig. 1

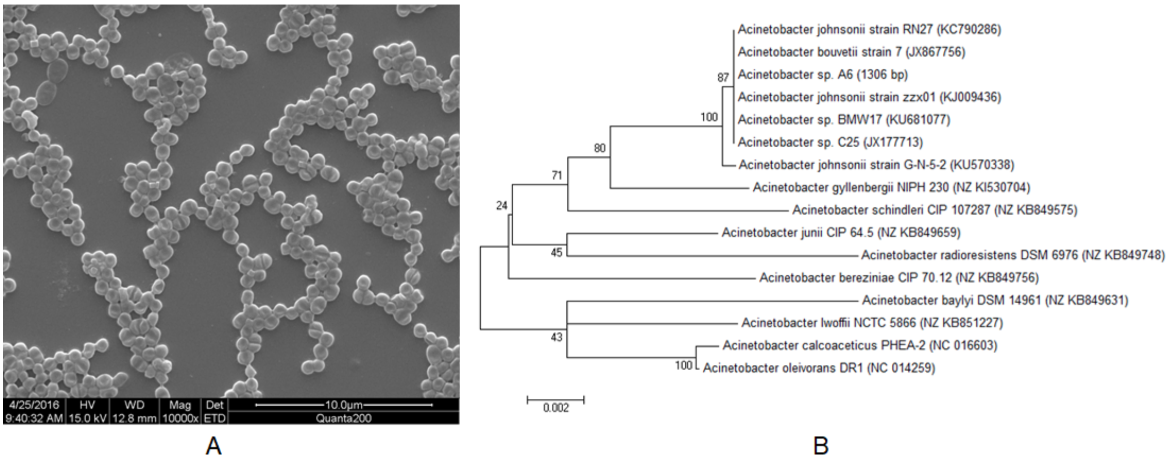


Fig. 2

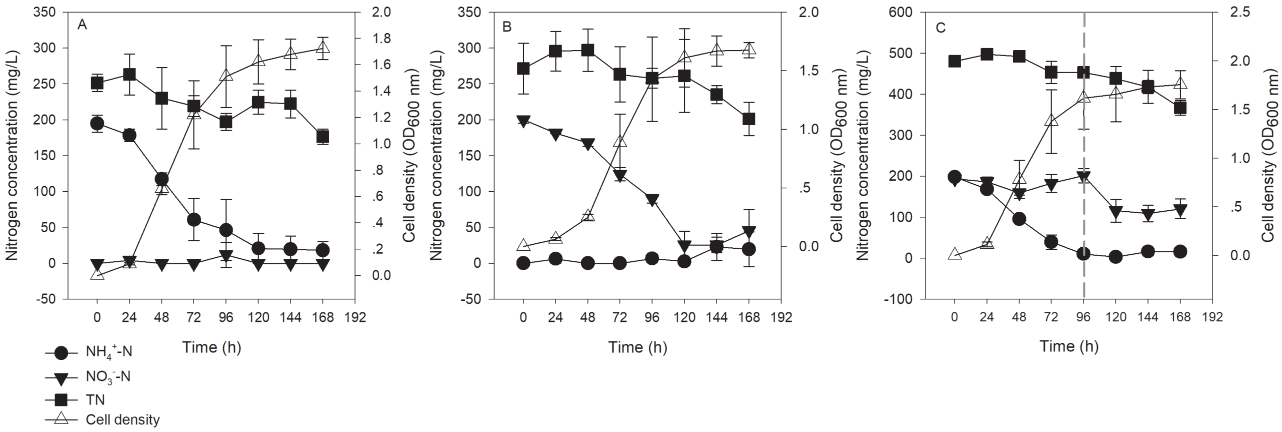


Fig. 3

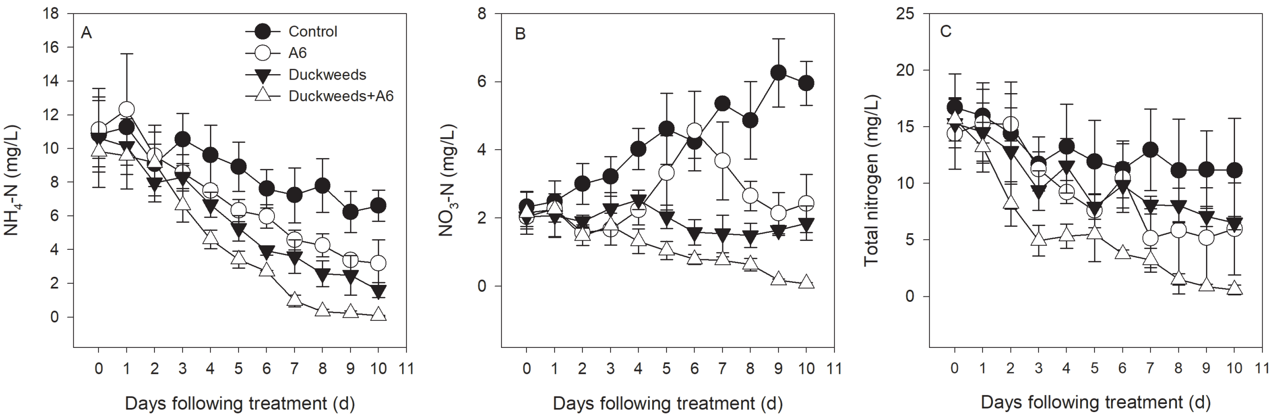
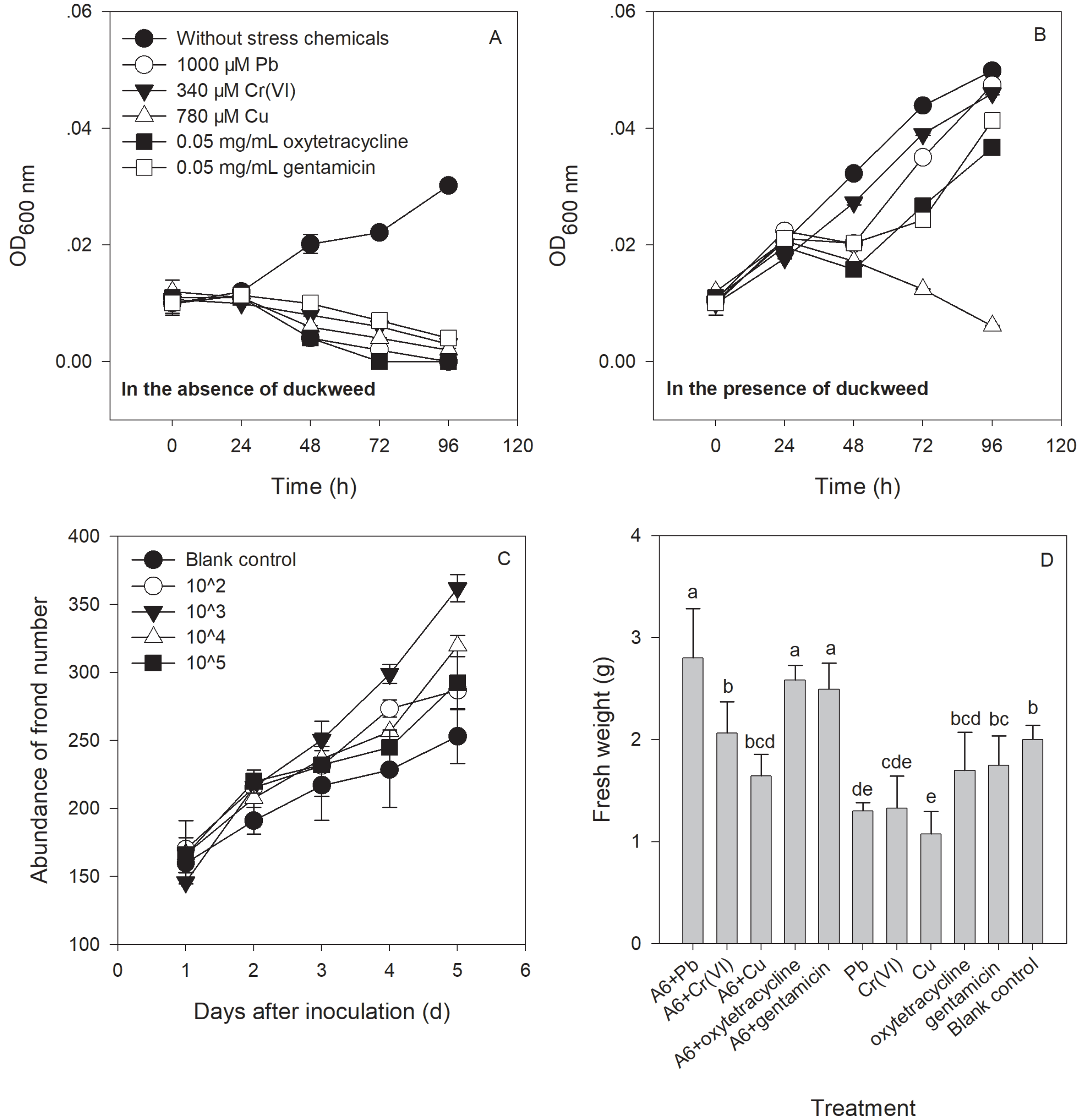
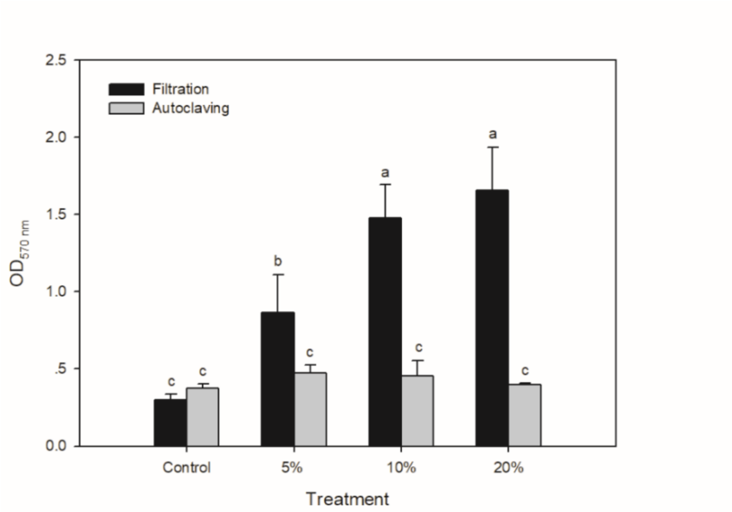


Fig. 4



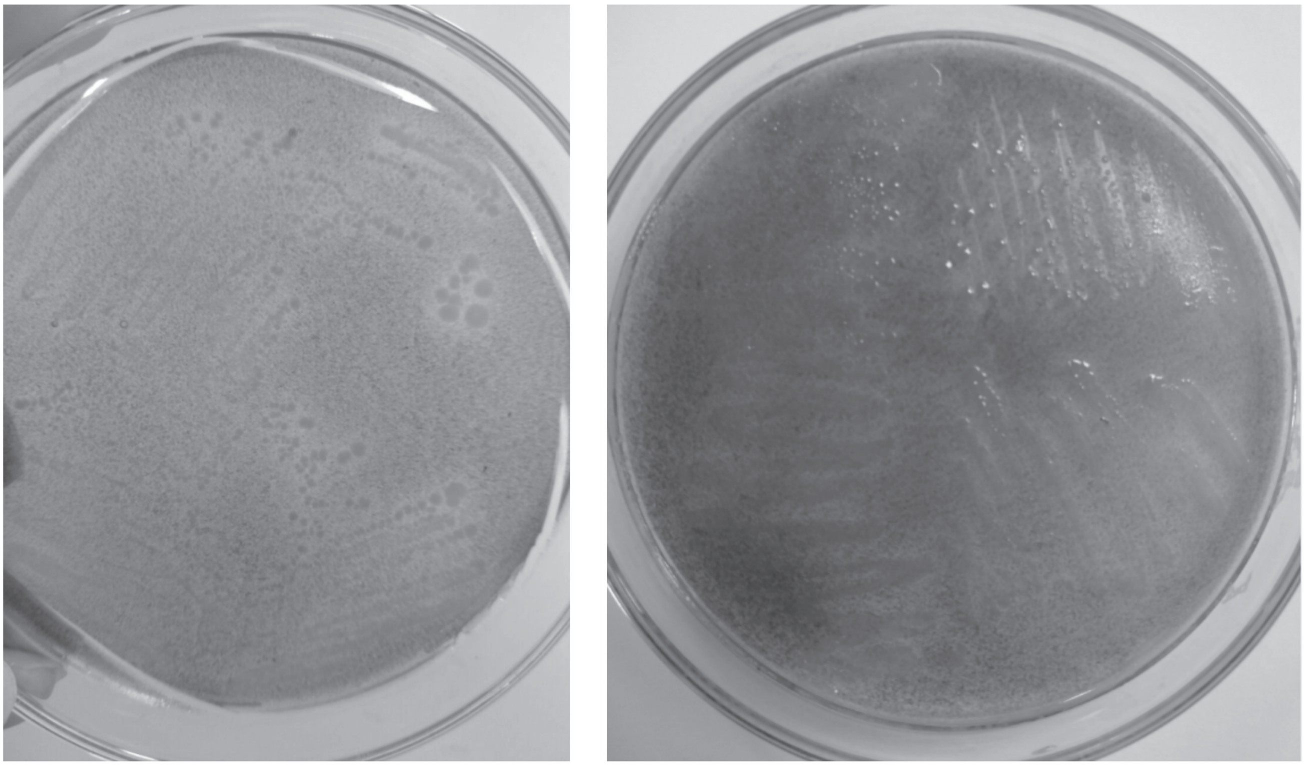
A
B
C
D
.06
.04
.02
0.00
OD600 nm
Without stress chemicals
1000 µM Pb
340 µM Cr(VI)
780 µM Cu
0.05 mg/mL oxytetracycline
0.05 mg/mL gentamicin
In the absence of duckweed
In the presence of duckweed
0
24
48
72
96
120
Time (h)
400
350
300
250
200
150
100
Abundance of frond number
Blank control
10^2
10^3
10^4
10^5
0
1
2
3
4
5
6
Days after inoculation (d)
4
3
2
1
0
Fresh weight (g)
a
b
bcd
a
a
de
cde
e
bcd
bc
b
A6+Pb
A6+Cr(VI)
A6+Cu
A6+oxytetracycline
A6+gentamicin
Pb
Cr(VI)
Cu
oxytetracycline
gentamicin
Blank control
Treatment

Fig. 5



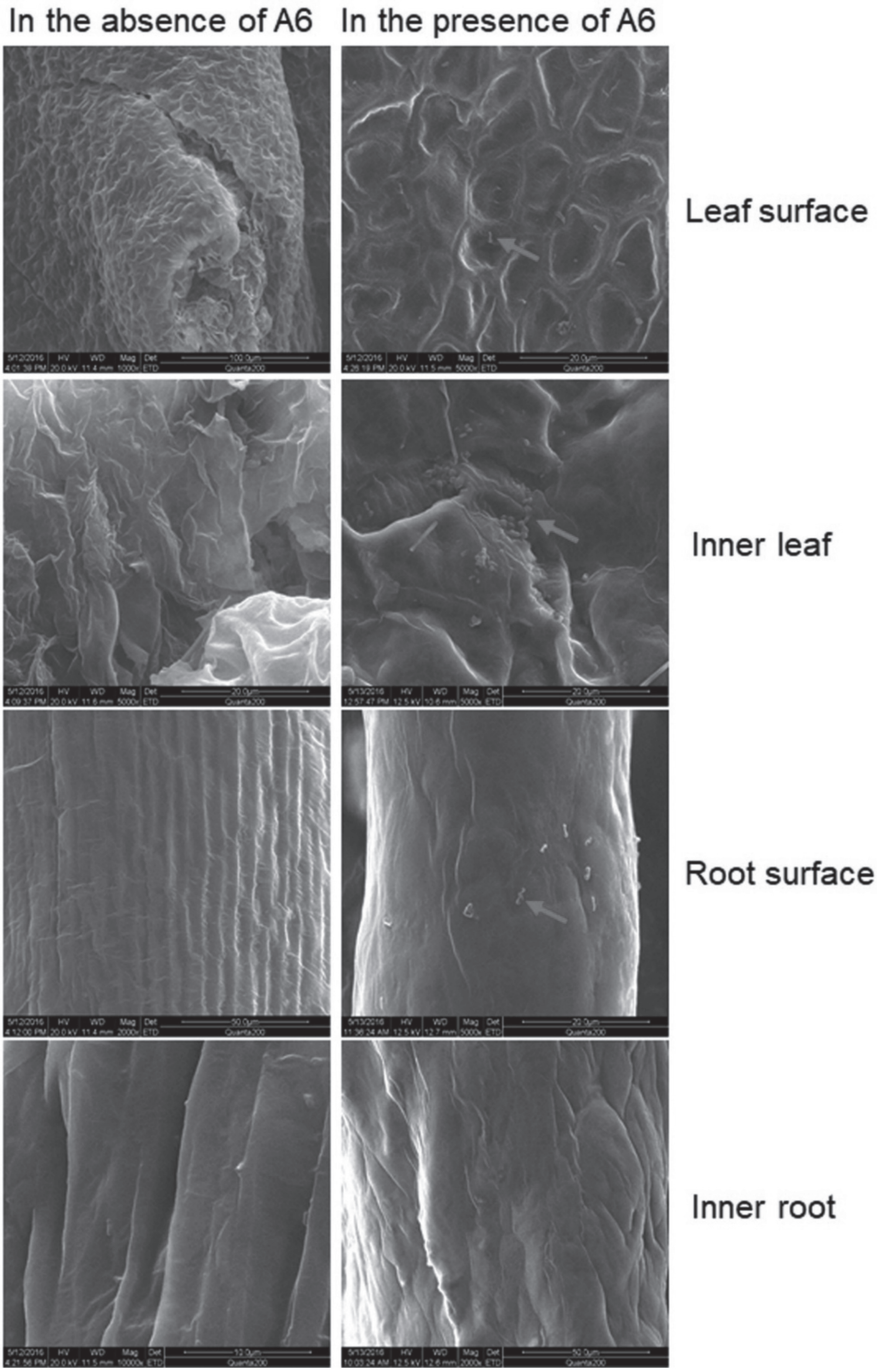
Filtration
Autoclaving
OD570 nm
2.5
2.0
1.5
1.0
.5
0.0
a
a
b
c
c
c
c
c
c
Control
5%
10%
20%
Treatment

Fig. 6



A B

Fig. 7


In the absence of A6
In the presence of A6
Leaf surface
Inner leaf
Root surface
Inner root