Enhancement of COD, ammonia, phosphate and sulfide simultaneous removal by the anaerobic photosynthetic bacterium of *Ectothiorhodospira magna* in batch and sequencing batch culture

Xin Zhao, Xuejie Li, Nan Qi, Zhongtian Fu, Meng Chen, Binhui Jiang and Xiaomin Hu

**ABSTRACT**

An anaerobic photosynthetic bacterium, with chemical oxygen demand (COD), ammonia nitrogen (NH$_3$-N), total phosphorus (TP) and sulfide (S$^{2-}$) simultaneous removal ability, strain SU6, was isolated and identified as belonging to *Ectothiorhodospira magna*. Its removal efficiencies were simultaneously evaluated in batch culture and influenced in sequencing batch culture. The maximum COD, NH$_3$-N, TP and S$^{2-}$/C$^0$ removal rates of 93.04%, 86.70%, 37.55% and 99.99% were obtained in batch culture with an initial pH 8.0 at 35°C after 72 h. The simultaneous removal efficiency was enhanced in sequencing batch culture, and 789.27 mg/L COD, 68.91 mg/L NH$_3$-N, 70.20 mg/L S$^{2-}$ and 5.26 mg/L TP were removed by the end of the last cycle within 24 h. This was the first time of reporting contaminants’ simultaneous removal by a pure-cultured photosynthetic bacterium. The experimental results demonstrate that *E. magna* can efficiently serve as a good candidate in anaerobic wastewater contaminants’ simultaneous removal, and maybe as another model anaerobic photosynthetic microorganism for water purification investigations.

**Key words** | contaminants’ simultaneous removal, *Ectothiorhodospira magna*, isolation, mechanism analysis, photosynthetic bacterium

**INTRODUCTION**

The activated sludge process is considered to be one of the most effective methods for sewage and domestic wastewater treatment. Most of the BOD (biological oxygen demand), COD (chemical oxygen demand) and ammonia nitrogen are removed by this process to avoid serious water environmental alterations (Ahmad *et al.* 2015) such as eutrophication, which is caused by the discharge of these contaminants into the natural water (Mirbagheri *et al.* 2016). However, some new treatment processes should be developed to cope with the increasingly complex composition of sewage and some small-scale domestic wastewater treatment needs.

Photosynthetic bacteria (PSB) are widely distributed in natural environments like lakes, rivers, oceans, soil, sludge, high-temperature or low-temperature environments, where light can reach (Yang *et al.* 2017). They can utilize means of substrates as carbon and nitrogen sources for their metabolism and growth under the light conditions. In the 1970s, Japanese researchers Kobayashi & Tchan (1973) found that PSB has a great potential in purifying polluted water. Since then, PSB has been considered to investigate some kinds of wastewater treatment, for example cadmium wastewater, olive mill wastewater and sulfide containing wastewater (Myung *et al.* 2004; Lu *et al.* 2010; Anam *et al.* 2012; Chitapornpan *et al.* 2013). Madukasi *et al.* (2010) treated pharmaceutical wastewater with *Rhodobacter sphaeroides*, which result showed that 380 mg-COD/L/d was removed. In a treatment process of high ammonia concentration wastewater by using photosynthetic bacteria ISASWR2014, a much higher NH$_3$-N removal ability was obtained, and more than 1,000 mg NH$_3$-N/L/d and 333 mg-COD/L/d were removed (Zhou *et al.* 2015).

*Ectothiorhodospira*, a genus of photosynthetic bacteria, was found in 1936 by Skerman (Skerman *et al.* 1980). But,
only 12 genera have been found in the past 80 years. Researches on *Ectothiorhodospira* have mainly focused on light harvesting, synthesizing both nitrogen-containing and nitrogen-free compatible solutes, and measuring the photoactive yellow protein (Galinski & Herzog 1990; Hoff et al. 1994; Solov’ev et al. 2016). To our knowledge, there has rarely been a report on contaminants removal by *Ectothiorhodospira*. Only Yuan et al. (2010) reported their study on sulfide removal from livestock wastewater and fish wastewater by *Ectothiorhodospira* sp. PSB-1, which had the sulfide removal rate of 68.55% and 56.15%, respectively. However, similar to the other photosynthetic bacteria, *Ectothiorhodospira* may also have a contaminant removal ability, which may provide some new ideas for contaminant removal.

In the current work, an anaerobic photosynthetic bacterium belonging to *Ectothiorhodospira* was characteristically described and identified. To our best knowledge, this was the first time for investigating contaminants’ simultaneous removal by a pure-cultured anaerobic photosynthetic bacterium both in batch and sequencing batch culture. The culture temperature, pH, carbon source, NH$_3$-N and NO$_3$-N concentrations were investigated to determine the optimal conditions for various contaminants’ simultaneous removal.

**MATERIALS AND METHODS**

**Enrichment and isolation**

Strain SU6 was isolated from a river sediment sample, which was collected from Hunhe River in Shenyang, China. The enrichment was performed in a 100 ml anaerobic bottle containing 50 ml enrichment medium (1 liter contains NH$_4$Cl 0.5 g, KH$_2$PO$_4$ 0.5 g, MgCl$_2$ 0.2 g, NaCl 10 g, NaHCO$_3$ 10 g, Na$_2$CO$_3$ 5 g, yeast extract 0.1 g, sodium acetate 0.5 g, Na$_2$S·9H$_2$O 0.5 g, vitamin B$_{12}$ 20 μg, and trace element solution 1 ml) (Pfennig & Lippert 1966). The initial pH 9.0 was adjusted by 1 mol/l HCl or 1 mol/l NaOH solution. The medium was flushed with ultra-high-pure argon gas (99.99%) for 5 min, sealed with a rubber plug, and autoclaved at 121 °C for 15 min. About 1 g sediment sample suspension was transferred into the anaerobic bottle and anaerobically cultured in an air bath at 35 °C with a light intensity of 2,500 ± 500 lx for 72 h. The enrichments were transferred 4 to 5 times until a deep red was obtained. After the enrichment became red, isolation was performed by the Hungate roll-tube technique (Hungate 1969) with the above-mentioned enrichment medium agar (2% agar). Single colonies were transferred to the same culture medium and anaerobically incubated at 35 °C and 120 rpm, 2,500 ± 500 lx for 48 h. The pure strain, with several times repeating of the roll-tube procedure, was cultivated in the sealed anaerobic tube under a gaseous atmosphere of 99.99% Ar at 35 °C.

**Cell morphology and ultrastructure**

Gram staining was performed as previously described (Beveridge 2001). Morphological examinations were performed with a light microscope (Motic BA210, China) and a transmission electron microscope (TEM) (TECNAIG20, FEI). Cells were washed twice and re-suspended in phosphate-buffered saline (PBS, pH 7.4), and negative stained with phosphotungstic acid (2%) on a 200 mesh copper grid as previously described (Zhou et al. 2017).

**Identification and phylogenetic analysis**

The genomic DNA of strain SU6 was extracted using a Bacterial DNA Mini Kit (Solarbio, China). The 16S rDNA gene was amplified and sequenced as previously described (Zhao et al. 2017) using the universal primers of BSF8 and BSR1541 (forward 5′-AGA GTT TGA TCC TGG CTC AG-3′; reverse 5′-AAG GAG GTG ATC CAG CC-3′). The 16S rRNA gene sequence was aligned and identified against existing sequences in GenBank using the BLAST and CLUSTAL_W programs (Thompson et al. 1997).

**Simultaneous removal in batch culture**

In order to explore the optimal condition for growth and contaminant removal of strain SU6, the effects of different culturing conditions, including carbon source, ammonia and nitrate concentration, temperature and initial pH, were alternately varied and investigated. For experiments on carbon source, soluble starch, sodium acetate, sodium succinate, sodium glutamate and glucose were respectively employed as the sole carbon source to keep the C/N ratio of 10. The ammonia and nitrate removal ability determination set the initial concentration in the range of 50 mg/L to 250 mg/L. The culture temperature and initial pH were investigated in the range of 20–40 °C and pH 6–10, respectively.

When optimizing the culturing condition, the initial concentrations of the monitored variables were adjusted to 1,250 mg/L of COD, 108 mg/L of NH$_3$-N, 80 mg/L of...
and 50 mg/L of TP, respectively. Other components were consistent with the enrichment medium. At the logarithm phase, 5% (v/v) of the isolate was transferred into the medium in 100 ml anaerobic bottles. The experiment was set up under anaerobic conditions with shaking in an air bath at 35 °C and 120 rpm, with a light intensity of 2,500 ± 500 lx provided by incandescent illumination. Samples were withdrawn periodically every 24 hours and centrifuged to obtain the supernatant to test the amount of COD, NH₃-N, NO₃-N, S²⁻, and TP until they did not change, and the final removal efficiencies were obtained.

### Contaminants’ simultaneous removal in sequencing batch culture

In order to get insight into the simultaneous removal of contaminants by strain SU6, a sequencing batch anaerobic reaction system was established with 100 ml anaerobic bottles. Synthetic wastewater for the sequencing batch system had the same composition as the optimal medium. The initial concentrations of synthetic wastewater were 1,250 mg/L of COD, 108 mg/L of NH₃-N, 80 mg/L of S²⁻ and 50 mg/L of TP, respectively. Experiments were carried out for 4 cycles, with an initial hydraulic retention time of 72 h, and then decreased to 48 h, 36 h and 24 h. At the end of each cycle, the system was left standing for 4 hours for bacteria to settle naturally. 80 ml of supernatant was aspirated by a 50 ml syringe with a 10 cm stainless steel needle to maintain an anaerobic condition. Then, a similar volume of synthetic wastewater was injected into the system with another syringe and needle (both syringes and needles were sterilized). The continuous flow system was cultured in an air bath shaker at 35 °C, 120 rpm and 2,500 ± 500 lx. In the initial 72 h, the OD₆₀₀ value was measured every 12 h, and the concentrations of contaminants were measured every 24 h. In the second cycle, the OD₆₀₀ value and contaminant concentrations were measured every 24 h. In the last two cycles, the monitoring time interval was adjusted according to the corresponding cycle time between the 12th h and the 24th h.

### Analyses methods

The concentrations of COD, NH₃-N, NO₃-N, TP and S²⁻ were tested by APHA standard methods (APHA 2005). Biomass was measured as OD₆₀₀ according to a previously described method (David 2001) using a spectrophotometer (UV-1240, Shimadzu, Japan). Illuminance was measured with a light intensity (AR823, Smart, Hong Kong). The pH was measured using a pH tester (S220, Mettler-Toledo, Switzerland).

### RESULTS AND DISCUSSION

#### Isolation and identification

Strain SU6 is Gram-positive, rod, motile with several terminal flagella. The bacterial cell occurred singly and had a diameter of 0.5–1.0 μm and a length of 2.3–3.0 μm (Figure 1). A colony of strain SU6 was reddish brown in color, circular, smooth surface, opaque and slightly concave with a ring margin, and had a diameter of about 2.0 mm after cultivation on agar medium at 35 °C with a light intensity of 2,500 ± 500 lx for 48 h. Initial contaminant removal efficiencies of 67.50% COD, 80.12% NH₃-N, 13.86% NO₃-N, 16.11% TP and 83.19% S²⁻ were obtained at 96 h when cultured in enrichment culture medium containing 3,000 mg/L COD, 100 mg/L NH₃-N, 100 mg/L NO₃-N, 80 mg/L total phosphorus (TP) and 55 mg/L S²⁻.

The 16S rRNA gene fragment of 1,491 bp (GenBank accession No. KX619404) was amplified and showed 99.10% identity with Ectothiorhodospira magna B7-7ᵀ (HM149323) (Figure 2). The physiological and morphological characters were also consistent with Ectothiorhodospira magna. Basing on the physical biochemical characteristics and 16S rRNA gene analysis, strain SU6 was identified as a novel strain within the species, with the name of Ectothiorhodospira magna SU6.

![Transmission electron micrograph (TEM) of strain SU6.](image)
Denitrification of strain SU6

The ammonia and nitrate nitrogen removal capability of strain SU6 could be obtained from Figure S1 (available with the online version of this paper), and a remarkable ammonia nitrogen removal ability was observed. The removal efficiency of NH$_3$-N reached the peak value of 84.79% with the initial concentration of 50 mg/L. However, the nitrate nitrogen removal ability of strain SU6 was weak, with a removal rate below 18.17%, and nitrite was not detected during the whole process. It showed that the removal of ammonia may not occur by oxidation, especially for light-anaerobic conditions which had no aeration when the removal effect appeared. In order to analyze the nitrogen balance during the treatment, gas production was examined. After calculating the nitrogen removal balance, it showed about 50% of the removed ammonia was ultimately transformed into N$_2$, others were transformed into PSB biomass. Therefore, when nitrate nitrogen was used as the nitrogen source, the growth of the bacteria was not optimistic, cell biomass was fairly low. Ammonia nitrogen was more favorable than nitrite nitrogen for the growth of strain SU6.

Effect of carbon sources on contaminants’ simultaneous removal

Carbon compounds usually serve as energy and electron sources for photosynthetic bacteria. Experiments were conducted to determine the effects of soluble starch, sodium acetate (NaAc), sodium succinate (SS), sodium glutamate (SG) and glucose on the growth and contaminant removal efficiency of strain SU6 (Figure 3). NaAc was obtained as the most favorable carbon source for strain SU6, with the maximum COD, NH$_3$-N, TP, S$^{2-}$ removal rate of 84.19%, 89.74%, 30.94%, 93.95%, respectively, and the maximum OD$_{600}$ of 0.98. Although sodium succinate was also suitable for growing strain SU6, the COD, NH$_3$-N, and S$^{2-}$ removal rates were not as good as when cultured in NaAc, while a similar TP removal rate of 29.01% was observed. The growth of strain SU6 was also observed in the medium with sodium glutamate as the carbon source, but the color was lighter than that with NaAc and SS, and the contaminant removal efficiency was weak. Meanwhile, both soluble starch and glucose were negative for cell growth and contaminant removal. The growth of strain SU6 could not be observed. The removal rate or utilization of COD, NH$_3$-N, TP, and S$^{2-}$ was very low, with an OD$_{600}$ below 0.55.

Figure 2 | Phylogenetic tree showing the relationships of strain SU6 to closely related species.

Figure 3 | The effect of carbon source on contaminants’ simultaneous removal by strain SU6.
The results demonstrated that the type of organic carbon source significantly affected the growth and substrates utilization efficiency, which may be attributed to the natural characteristic of carbon sources and bacteria with a comprehensive consideration. It was proposed that the molecular weight, chemical structure and reducibility of the carbon source might influence the enzyme system and nitrogen utilization (Elefsiniotis et al. 2004; Zhao et al. 2010; Chen et al. 2013).

**Effect of culture temperature on contaminants’ simultaneous removal**

Growth and contaminants’ simultaneous removal by strain SU6 were observed at temperatures ranging from 20 °C to 40 °C with an optimal temperature of 35 °C (Figure 4). Cell growth was inhibited (OD600 below 0.75) when cultured at a temperature below 30 °C, and then increased obviously. The maximum cell biomass of OD600 = 0.96 was obtained when cultured at 35 °C, and then decreased above that temperature. The changes in contaminants’ simultaneous removal rates were similar to the cell growth. They were all inhibited at low temperature and influenced by culture temperature raising. However, the increasing trend of NH3-N removal rate was obtained more obviously; this exceeded that of COD with a culture temperature over 30 °C. The removal rates of NH3-N (74.59–90.35%) were higher than COD (65.14–85.54%) when cultured at between 30 and 40 °C. The results indicated that the contaminant removal abilities of strain SU6 were greatly affected by temperature, especially the removal effect of ammonia nitrogen. The reason may be that the growth of SU6 required relatively more nitrogen nutrients compared to carbon nutrients. The maximum COD, NH3-N, TP and S2− removal rates of 83.49%, 89.74%, 37.14% and 99.99% were obtained at 35 °C, respectively.

The amount of cell biomass had a direct effect on contaminant removal efficiency. Lower or higher temperatures inhibited the cell growth of strain SU6, which resulted in the inhibition of contaminants’ simultaneous removal. The raising of culture temperature has an obvious promoting effect on the ammonia nitrogen removal efficiency of *E. magna* SU6.

**Effect of initial pH on contaminants’ simultaneous removal**

The effect of initial pH (6–10) on cell growth and contaminant removal by strain SU6 is shown in Figure 5. Obviously, the biomass of strain SU6 was low (OD600 = 0.68) at initial pH 6.0, increased with initial pH raising, reached the maximum of OD600 = 0.98 at initial pH 8.0, and then decreased to OD600 = 0.83 at initial pH 10.0. The trends of COD, NH3-N and TP removal rates were similar to the biomass changes. The maximum removal rates of 84.35%, 92.56% and 37.78% were obtained, respectively, at initial pH 8.0. The S2− reduction rate reached 99.99% when cultured at initial pH 8.0, and kept at 99.99% with initial pH raising, which means a total 55 mg/L S2− was all reduced at an initial pH 8.0.

PH is one of the most important factors for the growth of microbes. A previous study showed that *Ectothiorhodospira magna* B7-7T could grow in a nutrient medium with initial pH between 7 and 12 (Bryantseva et al. 2010), which was consistent with the results of optimal pH 8.0 in
the present study. A higher pH adaptability obtained in strain SU6 will provide itself a better growth and contaminant removal capacity, efficiency and opportunity in alkaline environments.

**Contaminants’ simultaneous removal under optimal conditions**

Under the optimal experimental conditions with sodium acetate used as the sole carbon source, culture temperature at 55 °C, initial pH 8, a better cell growth and contaminant removal performance of strain SU6 was obtained (Figure S2, available online). At the 96th hour, the maximum removal efficiency of COD, NH$_3$-N, TP and S$^{2-}$ reached 86.70%, 93.04%, 37.55% and 99.99%, which were much higher than that before culture condition optimization. About 1,083.75 mg/L COD, 100.48 mg/L NH$_3$-N, 18.78 mg/L TP and 80 mg/L S$^{2-}$ were removed in 96 hours.

Phototrophic bacteria can produce enough adenosine triphosphate (ATP), so power is reduced during the process of anoxygenic photosynthesis (van Niel 1944). That means the energy of catabolism comes from light (Tim et al. 2014). They can assimilate the majority of organic matters directly into biomass (Azad et al. 2001). COD, nitrogen, and phosphorus are assimilated to biomass. Meanwhile, sulfide as electron donor together with organic acid of strain SU6, obtained energy from the light source, and relied on special photosynthetic pigments in the body to assimilate carbon dioxide for photosynthesis.

**Contaminants’ simultaneous removal enhancement in sequencing batch culture**

The experimental results of contaminants’ simultaneous removal by strain SU6 in a sequencing batch culture system are shown in Figure 6. During the first cycle, the cell biomass began to increase significantly after 36 h and reached a maximum OD$_{600}$ value of 1.04 at 72 h. At the same time, the contaminant removal arrived in better state with 73.66% of COD, 71.35% of NH$_3$-N, 99.99% of S$^{2-}$ and 23.78% of TP being removed. In the next 3 cycles, although about 60% of cell biomass was shifted out from the system with 80 ml supernatant in each time, vigorous growth and metabolic activity of strain SU6 was observed. The time for biomass reaching the maximum (OD$_{600}$ = 1.00–1.04) was substantially shortened, and was only 24 hours in the 4th cycle. With the rapid growth of cells, as well as the metabolic activity increasing, a mounts of substrate were consumed, and contaminants were removed. In the last cycle, the removal rates of COD, NH$_3$-N, S$^{2-}$ and TP reached 85.94%, 77.85% 92.98% and 11.34%, respectively, which means 789.27 mg/L COD, 68.91 mg/L NH$_3$-N, 70.2 mg/L S$^{2-}$ and 5.26 mg/L TP were removed by strain SU6 in 24 hours.

As far as we know, there has been no previous study conducted on five common contaminants’ (COD, NH$_3$-N, NO$_3$-N, TP and S$^{2-}$) simultaneous removal by pure-cultured anaerobic photosynthetic bacterium, only some researches focusing on one or two kinds of contaminants’ removal. In order to compare the contaminants removal efficiency in the sequencing batch culture of strain SU6 against previous studies, the removal percentages in previous studies were converted into the total contaminants’ average removal in 24 h for a parallel comparison (Table 1). Strain SU6 was found to have the highest COD removal efficiency of 789.27 mg/L in 24 hours, 200 mg/L higher than that of *Rhodopseudomonas palustris* (Wang et al. 2016). The NH$_3$-N removal efficacy was 68.91 mg/L in 24 h, which was about twice that of *Rhodopseudomonas capsulata* E1F1 (36.48 mg/L, Francisco et al. 1986). Compared to the TP and S$^{2-}$ removal efficacies (7.7 mg/L and 24.95 mg/L, respectively, Nagadomi et al. 2000) of a mix-cultured photosynthetic bacteria, the TP removal efficacy (5.26 mg/L in 24 h) was lower, but the S$^{2-}$ removal efficacy (70.20 mg/L) was about triple that.

Until now, there have been quite a few researches reporting on contaminants’ simultaneous removal by photosynthetic bacteria, where the capacity and efficiency were not impressive. This was the first time reporting several contaminants’ simultaneous removal by a pure-cultured photosynthetic bacterium. Generally, the performance of *Ectothiorhodospira magna* SU6 basically met the need of common contaminants’ simultaneous removal.

**Figure 6 |** Contaminants’ simultaneous removal by strain SU6 in sequencing batch culture.
CONCLUSIONS

The present study for the first time investigates the potential of *Ectothiorhodospira magna* SU6 to simultaneously remove contaminants under the optimal culture conditions of sodium acetate as the sole carbon source, culture temperature of 35°C and initial pH of 8.0. In this process, most organic matters of COD, nitrogen, and phosphorus were assimilated to the biomass. Sulfide, however, served as the electron donor for the photosynthesis of SU6. The contaminants’ removal efficiency was significantly enhanced in sequencing batch anaerobic culture compared to batch culture, in which 789.27 mg/L COD, 68.91 mg/L NH₃-N, 70.2 mg/L S²⁻, and 5.26 mg/L TP were simultaneously removed in 24 hours. The superior performance in contaminants’ simultaneous removal indicated that *E. magna* SU6 could be another model anaerobic photosynthetic microorganism for water purification investigations.

FUNDING

This research was supported by National Natural Science Foundation of China (No. 51408103), Natural Science Foundation of Liaoning Province, China (No. 2015020601), Fundamental Research Funds for Chinese Central Universities (No. N170104021), and Open Fund of State Key Laboratory of Silicate Materials for Architectures, China (No. SYSJJ2017-08).

ACKNOWLEDGEMENTS

We are grateful to the colleagues from Test Center of Northeastern University for their help during data monitoring.

REFERENCES


Zhao, B., He, Y. L., Huang, J., Taylor, S. & Hughes, J. 2010 Heterotrophic nitrogen removal by *Providencia rettgeri* strain YL. *Journal of Industrial Microbiology and Biotechnology* 37, 609–616.


First received 2 April 2018; accepted in revised form 17 July 2018. Available online 25 July 2018