Bioresource Technology 250 (2018) 290–298

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

The influence of complex fermentation broth on denitrification of saline sewage in constructed wetlands by heterotrophic nitrifying/aerobic denitrifying bacterial communities

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ARTICLE INFO

Keywords:
Constructed wetland
Salinity
Denitrification
Heterotrophic nitrifying–aerobic denitrifying bacteria

ABSTRACT

An experimental vertical-flow constructed wetland (CW) was tested to treat salt-containing sewage. CW clogging deposits and withered *Pontederia cordata* L. were collected into a complex fermentation broth to serve as the carbon source and its effects on the denitrification capacity and microbial composition of the CW were examined. Addition of the complex fermentation broth into the CW influent (1.8% salinity) led to high removal efficiencies of NH_4^+-N > 99.82 ± 0.00% and TN > 90.39 ± 0.05%. Heterotrophic nitrifiers and aerobic denitrifiers were entirely dominant in the middle and upper layers of the CW, where obligate halophilic, aerobic denitrifiers *Zobellella* occurred. The CW successfully cultivated and enriched heterotrophic nitrifying–aerobic denitrifying bacteria, overcoming the effects of salinity and insufficient organic carbon sources on the denitrification capacity of CW. This type of complex carbon sources can also facilitate the utilization of waste resources, such as CW clogging deposits and withered wetland plants.

1. Introduction

Constructed wetland (CW) is a sewage treatment technique that imitates the development of a natural wetland ecosystem and has the benefits of comparatively low operating costs and simple operational maintenance (Liu et al., 2015). CWs can effectively remove nitrogen from low-salinity sewage. However, high salinity condition during the treatment of salt-containing sewage changes the osmotic pressure in microbial cells, leading to swelling of bacterial cell or even lysis, as well as denaturation of cellular enzymes, which in turn affects microbial physiological activities and inhibits the conventional nitrification-denitrification pathways. The removal efficiencies of NH_4^+-N and TN from salt-containing sewage are 21.2–49.8% and 47.5–62.8%, respectively, in constructed wetlands (Su et al., 2011; Leung et al., 2016). Therefore, it is difficult to effectively remove nitrogen from salt-containing sewage (Wang et al., 2017a).

In recent years, heterotrophic nitrifiers have been found in offshore sediments and farming sewage (Tsukuda et al., 2015; Jin et al., 2017). Heterotrophic nitrifiers have higher growth rates, organic loading capacities, and halotolerant capacities than autotrophic nitrifiers in conventional nitrification-denitrification systems (Chen et al., 2016). Chen et al. (2015a) employed a sequencing batch reactor (SBR) for the biological treatment of high-salinity organic sewage and found that the SBR was dominated by heterotrophic *Flavobacterium phragmitis* (genus Flavobacterium) and *Paracoccus denitrificans* due to salinity stress. *P. denitrificans* is not only capable of nitrification and large-scale removal of organic matter, but also of aerobic denitrification, which helps to shorten the duration required for denitrification, thus providing a new route for denitrification under high salinity. However, there are no published reports on whether this type of highly efficient, halotolerant denitrifiers could be enriched to achieve high denitrification efficiencies in a CW.

Previous studies showed that halotolerant, heterotrophic nitrifying–aerobic denitrifying bacteria are mostly heterotrophic bacteria that require organic carbon sources at large scales to provide nutrients and electrons for growth and denitrification (Duan et al., 2015; Wang et al., 2015). Residues in CWs (e.g., clogging deposits and withered plant material) contain high levels of organic matter (Fu et al., 2013). Therefore, these two types of residues could be fermented to release macromolecular organic matter to serve as a carbon source for denitrifying microbes. The residues alleviate the clogging of wetlands and improve the denitrification efficiency of CWs.

High-throughput sequencing (HTS) is an effective approach for studying microbial community distributions in environmental samples

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https://doi.org/10.1016/j.biortech.2017.11.057
Received 21 September 2017; Received in revised form 17 November 2017; Accepted 18 November 2017
Available online 21 November 2017
0960-8524/

(Shendure and Ji, 2008; Ye et al., 2011) and has been applied to study microbial diversity in lakes, oceans, wetlands, and sewage treatment systems (Li et al., 2013). Illumina high-throughput sequencing and quantitative real-time polymerase chain reaction (qPCR) can more accurately reveal the microbial community structure and diversity of purifying microorganisms in CW matrices. In this study, clogging deposits and withered wetland plants were fermented as organic carbon sources in an attempt to cultivate and enrich heterotrophic bacteria in a CW system. The acquisition of obligate, highly efficient, halotolerant, heterotrophic nitrifying–aerobic denitrifying bacteria using qPCR and HTS methods is also evaluated to assess the efficiency of nitrogen removal from salt-containing sewage.

2. Materials and methods

2.1. Acquisition of clogging deposits and preparation of complex fermentation broth

The clogging deposits consisted of organic matter accumulated in the long-operated Wenshan Lake CW on the Shenzhen University campus. The clogging deposits were dug and collected from the CW during the resting period. Large-particle impurities, such as stones and plant residues, were removed. The pH, moisture content, volatile solids/total solids (VS/TS), soluble chemical oxygen demand ($SCOD_{Cr}$), and total chemical oxygen demand ($TCOD_{Cr}$) of clogging deposits were 5.57 ± 0.35, 71.77 ± 0.02%, 0.32 ± 0.03, 253.66 ± 118.79 mg/L, and 2,358.33 ± 18,160.85 mg/L, respectively.

P. cordata L. is a common wetland plant in the Wenshan Lake CW. The withered plants were cut into 1–3 cm pieces and were dried at 50–65 °C to a constant weight prior to storage. Alkaline condition can promote the release of carbon sources from activated sludge and biomass (Krishania et al., 2013); thus, an alkaline fermentation method was adopted in this study. Briefly, 164.77 g of recovered CW clogging deposits and 37.5 g of *P. cordata* L. were added to 1.5 L of ultra-pure water in a 2-L conical flask. The mixture was adjusted to pH 12 with 10 M sodium hydroxide and then sealed for a 5-d fermentation period at 28 °C and 120 rpm/min agitation. Next, the fermented mixture was centrifuged at 8000 rpm to harvest its supernatant as the complex fermentation broth, which was subsequently analyzed for soluble chemical oxygen demand ($SCOD_{Cr}$), total nitrogen (TN), and volatile fatty acids (VFAs).

2.2. Construction and operating conditions of CW

Four vertical-flow experimental CW systems were constructed in a greenhouse room (27 ± 2 °C) on the parterre of Shenzhen University. Each experimental system contained a polyvinyl butyral (PVB) column (diameter: 30 cm; height: 50 cm). Both top and bottom layers of the system were filled with limestone (particle size: 1 cm; porosity: 0.44; layer thickness: 5 cm) while the middle layer was filled with sand (particle size: 0–1 mm; porosity: 0.37; layer thickness: 35 cm). Sand and excess sludge (collected from Nanshan Sewage Treatment Plant, Shenzhen) in the middle layer were mixed at a volume ratio of 3.3:1 for the inoculation of microbes (Fu et al., 2016). The experimental system was cultivated with *Kandelia candel,* while five sampling points were assigned at the inlet, the matrix layer (10 cm, 25 cm, and 40 cm depths from the top), and the outlet of the small-scale experimental system.

Artificial sewage containing only 15 mg/L of NH_4^+-N was used as influent for the experimental vertical-flow CW system (Table 1). The experiment was separated into two stages and the complex fermentation broth was not added into the influent until the second stage to adjust the influent to a C/N (carbon/nitrogen) ratio of 6 (Fu et al., 2016). Each stage was operated for three months and was further separated into freshwater (C-CW) and saltwater (S-CW) experimental groups with two replicates each. The salinity of the S-CW group was adjusted to 1.8% in accordance with that of Shenzhen coastal seawater.

Table 1
Operating conditions of vertical-flow constructed wetlands.

	Phase I*		Phase II*	
	C-CW	S-CW	C-CW	S-CW
Salinity	0%	0%	1.8%	1.8%
NH_4^+-N (mg/L)	15	15	15	15
COD_{Cr} (mg/L)	30	30	90	90
C/N	2	2	6	6
Hydraulic retention time (day)	0.92			
Hydraulic surface loading (m^3/m^2·d)	0.19			

* Phase I: Artificial sewage without complex fermentation broth; Phase II: Artificial sewage with complex fermentation broth.

The influent was supplied intermittently for 10 min every 4 h, equivalent to 6 L of influent within 24 h. The influent was supplied in a vertical direction from the surface of the matrix layer to the bottom. The hydraulic retention time (HRT) of each device was 0.92 d while the surface hydraulic load was 0.19 m^3/m^2·d.

2.3. Collection and analysis of water samples

COD_{Cr} in the clogging deposit complex fermentation broth was determined using a Hach COD kit (Hach, USA). The VFA content of the fermentation broth was measured using a gas chromatography (GC) system (Agilent 7890, USA). NH_4^+-N, NO_2^--N, NO_3^--N, and total nitrogen (TN) contents of the fermentation broth and the effluent from each outlet of the CW system were measured via a CleverChem 380 analyzer (DeChem-Tech, Germany).

2.4. Matrix collection, total DNA extraction, and HTS

Following the completion of the experiments described in Section 2.3, the sewage in both the C-CW and S-CW systems was fully drained. Then, 70 g of the 10 cm upper-layer (C1 and S1), 25 cm middle layer (C2 and S2), and 40 cm bottom layer (C3 and S3) matrices were collected from the system for elution and centrifugation. This enabled the extraction of biofilm, which was freeze-dried at −80 °C for 24 h and then weighed. Total DNA was extracted using the EZNA Soil DNA Kit (Omega, USA) and the concentration of the extracted DNA was measured using a NanoDrop 2000 spectrophotometer (Thermo, USA). The DNA extracts were then stored at −20 °C. The DNA extract was aliquoted for qPCR assay to determine the changes in the abundance of denitrification genes (*amoA*, *nxrA*, and *nirS*) and 16S rRNA genes before and after the addition of fermentation broth. The remaining DNA extract was sent for HTS (Genewiz, Suzhou, China).

The qPCR assay was performed using a ViiA-7 real-time qPCR detection system (Applied Biosystems, Inc., USA). All specific primers for target genes were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Each 20 μL reaction system consisted of 10 μL of SYBR Premix (Takara, China), 2 μL of DNA template or total DNA, 0.8 μL each of forward and reverse primers, 0.4 μL of ROX reference dye (Takara, China), and 6 μL of sterile water. Forward and reverse primers for target genes and the reaction protocol are shown in Table 2. All plasmids containing the target genes were provided by Sangon Biotech Co., Ltd. (Shanghai, China) and subjected to 10× serial dilution to serve as DNA templates for the construction of standard curves ($R^2 > 0.999$). The data for these genes were normalized to the number of copies per gram substrate sample (copies/g).

For the 16S rDNA HTS, the concentration of DNA samples was measured using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) while the sequencing libraries were constructed from 30–50 ng of DNA as a template using a MetaVx library construction kit (Genewiz, Inc., South Plainfield, NJ, USA). Briefly, the V3 and V4 regions of bacterial 16S rRNA genes were amplified by the forward primer CCT