Bioresource Technology 219 (2016) 439–444

Contents lists available at ScienceDirect

Bioresource Technology

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

Metagenomic analysis of microbiota structure evolution in phytoremediation of a swine lagoon wastewater

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HIGHLIGHTS

- Swine lagoon wastewater was treated by introducing an exogenous *Chlorella* strain.
- Two phytoremediation modes (shaking flask and CO_2-air bubbling) were applied.
- Metagenomic approach was performed to reveal the diverse native bacterial profiles.
- The native microbiota structure was altered by algae addition in different modes.

ARTICLE INFO

Article history:
Received 2 June 2016
Received in revised form 2 August 2016
Accepted 4 August 2016
Available online 6 August 2016

Keywords:
Chlorella
Swine wastewater
Nutrient removal
Metagenomics
Microbiota structure

ABSTRACT

Pytoremediation was studied in this project to treat swine manure lagoon wastewater characteristic of high concentrations of organic carbon, ammonium (N) and phosphorus (P). The impacts of introducing exogenous microalgae *Chlorella* into the lagoon wastewater on the removal of major nutrients and the transformation of the native wastewater microbiota structure were explored under two phytoremediation modes (shake flask and CO_2-air bubbling). The results showed that the inoculation of microalgae could significantly enhance N and P removal. Metagenomic analysis of the native microbiota composition in the wastewater affected by algae inoculation revealed that a substantial population of algicidal bacteria was developed in the shake flask system, while in the CO_2-air bubbling system, a niche for more mutualistic bacteria was created, which benefited the maximal algal growth with the simultaneous optimal N and P removal. To our knowledge, this study presents, the first reported case of applying metagenomic approach to a phytoremediation system treating real swine lagoon wastewater.

1. Introduction

Wastewater generated in the confined swine production is characterized by high concentrations of chemical oxygen demand (COD), ammonium and phosphorus, which would cause serious environmental problems if not properly managed or treated before discharge. Currently, the wastewater is handled in three ways, i.e., land application as cropland fertilizer (Choudhary et al., 1996), natural stabilization in lagoons (Ro et al., 2009), and biological (anaerobic/aerobic) treatment to removal nutrients (Luo et al., 2002), all of which are not very efficient in dealing with the significant amount of nitrogen and phosphorus in the wastewater, the two typical chemical species seriously threatening the surface water quality.

Phytoremediation by microalgae has obtained wide applications in treatment of various wastewaters, such as, urban wastewater (Mennaa et al., 2015), aquaculture wastewater (Nasir et al., 2015), textile wastewater (El-Kassas and Mohamed, 2014), dairy wastewater (Wang et al., 2010) and so on. The advantage of phytoremediation rests with the superior capability of microalgae for nitrogen and phosphorus removal, which the native microflora is unable to accomplish. The efficacy of phytoremediation hinges on many biotic and abiotic factors, e.g., microalgae species (Arbib et al., 2014), operating mode (Matamoros and Rodríguez, 2016), pH control (González et al., 2008), light regime (Lee and Lee, 2001), etc. Naturally, most microalgae are found in association with other microorganisms (Subashchandrabose et al., 2011), and past researchers reported that cooperative symbiosis demon-

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http://dx.doi.org/10.1016/j.biortech.2016.08.013
0960-8524/

strated significant enhancement of microalgal growth and benefits of more efficient detoxification of pollutants (Abed, 2010; Kumar et al., 2010; Subashchandrabose et al., 2011).

While considering introduction of an exogenous microalgae strain in treating a real wastewater, the impact of the algae introduction on the nutrient removal efficiency needs to be clearly evaluated. Among many biotic and abiotic factors influencing the nutrient removal efficiency, the interaction between the introduced microalgae and the native microbiota has always been neglected, which may deserve special attention. Meanwhile, although the superiority of phytoremediation in nitrogen and phosphorus removal was witnessed by many researches (González et al., 2008; de Godos et al., 2009; Wang et al., 2010), the potential influence of the exogenous microalgae's introduction on the transformation of the native wastewater microbiota structure was rarely reported.

In this study, the effects of the exogenous microalgae *Chlorella*'s introduction on the removal of major nutrients and the transformation on the native wastewater microbiota structure were explored under two phytoremediation modes, i.e., shake flask and CO_2-air bubbling, incubated under continuous lighting condition. Metagenomic analysis of the native microbiota composition in the wastewater as affected by algal inoculation was performed by high-throughput sequencing via the Illumina Miseq platform followed by taxonomy analysis. This study presents, to our knowledge, the first reported case of applying metagenomic approach to a phytoremediation system in treating real swine manure lagoon wastewater by introducing an exogenous Chlorella strain.

2. Methods

2.1. Wastewater characterization

The wastewater used in this study was collected from a swine manure lagoon on a small-scale swine farm in Chongming, Shanghai, China. After collection, the wastewater was pre-settled overnight to remove large particles, while the indigenous microflora was retained without any disinfection treatment. The characteristics of the wastewater were determined following the Hach DR 2800 Spectrophotometer Manual as follows: pH, 7.8; COD, 323 ± 21 mg/L; ammonia nitrogen (NH_3–N), 49.1 ± 3.0 mg/L; nitrate nitrogen (NO_3–N), 2.4 ± 1.1 mg/L; and total phosphorus (TP), 4.5 ± 1.2 mg/L.

2.2. Microalgae inoculum

One of the wild-type *Chlorella* sp. species was used in this study, the characteristics of which were described in the previous study (Wang et al., 2016). The strain was preserved in standard BG-11 media and the seed was maintained at constant temperature (25 ± 2 °C) under cool white fluorescent lamps with around 100 μmol photon m^{-2} s^{-1} to provide continuous lighting and kept at the exponential growth phase by periodic plating and transferring.

2.3. Wastewater treatment experiment set-ups and water sample analysis

The three experimental set-ups for lagoon wastewater treatment were: (1) control with no inoculation of exogenous microorganism in the shaking flasks, (2) Inoculation of *Chlorella* sp. in the wastewater in the shaking flasks, (3) Inoculation of *Chlorella* sp. in the wastewater in the CO_2-air lift columns, with the two treatments receiving algal biomass inoculation at a level of around 0.1 g/L. All experiments lasted 3 days, which was found to be sufficient for the maximal removal of all the major nutrients. 20 ml Mixed liquid samples for all the treatments were taken at the end point and subject to nutrient analysis, while 30 ml samples for metagenomics analysis were taken on a daily basis. The total volume of the flask was 500 ml with a working volume of 300 ml. The air-lift column had a total volume of 400 ml with a working volume of 300 ml, which was bubbled with air containing 1% CO_2 (v/v) and the air flow rate is 0.2 vvm. The rotation speed of the shaking flasks was 150 rpm. The light intensity applied was around 100 μmol photon m^{-2} s^{-1} continuous lighting and all groups were cultured at 25 ± 2 °C in triplicates. COD, NH_4-N and TP of the centrifuged water samples were measured according to the methods described by Wang et al. (2016).

2.4. Metagenomic DNA sequencing via Illumina Miseq platform

In order to assess the native wastewater microbial community evolution under different experimental conditions, polymerase chain reaction (PCR) followed by pyrosequencing was performed on the DNA extracts of the unfiltered water samples of the original wastewater, and the treated wastewaters from the three different experimental set-ups. DNA was extracted by using the PowerWater DNA isolation kit (MoBio Laboratories Inc., CA, US) according to the manufacturer's protocol. The V4–V5 region of bacterial 16S-rDNA genes were amplified using the universal primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT). The PCR analysis was performed in the following sequence: initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The Illumina Nextera XT Index kit (Illumina Inc., CA, US) with dual 8-base barcodes were used for multiplexing. Eight cycles PCR reactions were used to incorporate two unique barcodes to either end of the 16S amplicons. Cycling conditions consisted of one cycle at 94 °C for 3 min, followed by eight cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, followed by a final extension cycle at 72 °C for 5 min. Prior to library pooling, the barcoded PCR products were purified using a DNA gel extraction kit (Axygen, China) and quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, CA, US). The libraries were sequenced by 2 × 300 bp paired-end sequencing on the MiSeq platform using MiSeq v3 Reagent Kit (Illumina Inc., CA, US).

2.5. Bioinformatics analysis

Sequences preprocessing was performed mainly upon software of mothur (version 1.35.1) (Schloss et al., 2009), following the MiSeq analysis pipeline outlined at http://www.mothur.org/wiki/MiSeq_SOP. Sequences were filtered to remove ambiguous reads and those shorter than 200 bp or longer than 600 bp. The remaining sequences were simplified using the 'unique.seqs' command to generate a unique set of sequences, then aligned with the SILVA databases, version 119 (Pruesse et al., 2007). Any sequence aligned with eukaryotes, chloroplasts or mitochondria, as well as the unknown ones, were discarded. Finally, the sequences were clustered at the genus level with 16S rDNA sequences identity scores of 95–97%, at the class level with 16S rDNA sequences identity scores of 80–85% and at the phylum level with 16S rDNA sequences identity scores of 77–80%. The majority consensus taxonomy for each operational taxonomic unit (OTU) was obtained by the classify. otu command with default parameters. The OTUs that reached a 97% similarity level were used for calculating the Shannon diversity index using the Mothur program. The percentage of bacterial identification was analyzed for each sample to provide the relative abundance information of individual samples based upon the number of sequences affiliated with that particular