Diversity of Eukaryotic Algae in Propylene Oxide Saponification Wastewater Activated Sludge

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Abstract Eukaryotic algae play an important role to maintain the energy cycle and provide oxygen in the ecosystem of water environment, such as rivers, lakes, and wastewater activated sludge. As one of the important microorganisms in the activated sludge, the community structure of eukaryotic algae in the activated sludge is still not clear. Activated sludge process had been successfully used to dispose the propylene oxide (PO) saponification wastewater, which was contaminated with high chemical oxygen demand (1200–1500 mg/L) and high chlorine concentration (22000–26,000 mg/L). In this study, we focused on the diversity of eukaryotic algae in the PO saponification wastewater and municipal wastewater treatment plant. Based on Illumina Miseq high-throughput sequencing, the algae could be divided into nine different groups for the municipal wastewater activated sludge. By contrast, the algae in the PO saponification wastewater activated sludge could be grouped into two unidentified groups, which were quite different from the municipal sludge. In order to further analyze the diversity of eukaryotic algae in the PO saponification wastewater activated sludge, 18S rDNA clone library of eukaryotic algae was constructed, and 31 clones were obtained. On account of BLASTn analysis, one of them was identified as *Desmodesmus intermedius*, while others were considered as uncultured eukaryotic algae. Moreover, *D. intermedius*, which had the potential biodiesel-producing capacity, was successfully isolated from the PO saponification wastewater activated sludge. This study would open an avenue to understand the eukaryotic algae diversity and utilize the

biodiesel-producing eukaryotic algae in the PO saponification wastewater activated sludge.

Keywords Illumina Miseq high-throughput sequencing · 18S rDNA · Eukaryotic algae · Phylogeny · Activated sludge

Introduction

Activated sludge process has been widely applied to dispose wastewater since 1913 [1]. As the dominant component for the treatment of organic pollutants in the aeration tank, the activated sludge fundamentally determines the treatment capacity of sewage treatment plants [2]. The biological activity of activated sludge microorganisms mainly depends on the structure and function of microbial community [3]. In general, bacteria, archaea, fungi, protists, and eukaryotic algae are involved in the activated sludge, which could degrade various pollutants, including organic compounds, toxicants, and xenobiotics [4, 5].

As one of the important microorganisms in the activated sludge, eukaryotic algae is a kind of algae which has chlorophyll as its primary photosynthetic pigment and lacks a sterile covering of cells around those reproductive cells [6]. Eukaryotic algae play an important role in the flow of energy and nitrogen fixation in the activated sludge [7]. Furthermore, eukaryotic algae could act as primary producers in the ecosystem of the lake and take an important role in the entire food web energy cycle of water environment [8]. Cong Li reported that phagotrophic algae had the unique ability to survive by ingesting insoluble organic particles including microbial cells, and could recover organic matter of residual activated sludge into the biomass of phagotrophic algae [9]. Analysis of eukaryotic algae community in the activated

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sludge would provide us a better understanding of this useful microorganism.

The saponification wastewater from the process of PO production is contaminated with high chemical oxygen demand (1200–1500 mg/L) and chlorine concentration (22000–26,000 mg/L), and the activated sludge process had been successfully used to dispose the saponification wastewater [10]. High-throughput sequencing technology is an efficient tool to reveal the community structures of the activated sludge. Based on Illumina Miseq high-throughput sequencing, the bacterial community composition of PO wastewater activated sludge was proved to be quite different from the municipal wastewater activated sludge (Results will be published in another paper).

In this research, Illumina Miseq high-throughput sequencing, and construction of 18S rDNA clone library were used to analyze the eukaryotic algae communities in the PO saponification wastewater activated sludge. Moreover, the potential biodiesel-producing alga, *Desmodesmus intermedius*, was successfully isolated from the activated sludge. This study would offer a reference to understand the eukaryotic alga diversity and utilize the biodiesel-producing eukaryotic alga in the PO saponification wastewater activated sludge.

Material and Methods

Activated Sludge Sampling

Activated sludge samples used in this research were acquired from the aeration tank of a PO saponification wastewater treatment plant in Befar Group Co., Ltd., Shandong, China, and a municipal wastewater treatment plant in Jinan, China, respectively. Samples were taken in sterile polyethylene bottles, transported to the laboratory on ice, and stored at 4 °C in the refrigerator for the following experiments.

DNA Extraction, PCR, and Illumina Miseq High-Throughput Sequencing (HTS)

Activated sludge samples were centrifuged at 6000g for 10 min at 4 °C to collect the sediments, and the supernatants were discarded. Genomic DNA of samples (concentration ≥ 10 ng/ μ L, total amount of DNA ≥ 500 ng, OD_{260/280} > 1.80) was extracted by E.Z.N.A.TM Soil DNA Kit of OMEGA. PCR was performed using 18S universal primers: 18S-F (5'-ACCTGGTTGATCCTGCCAGT-3') and 18S-R (5'-TCACCTACGGAAACCTTGT-3'). PCR mixtures (50 μ L) were prepared in duplicate and each contained 2 μ L of DNA template, 5 μ L of 10 $\times$ PCR buffer (50 mM KCl, 10 mM Tris-HCl and 1.5 mM MgCl₂), 200 mM of dNTP, 0.2 mM of each primer, and 2.5 U Taq polymerase (Promega, Madison, WI, USA). The PCR thermal regime consisted of an initial denaturation of 6 min at 94 °C, followed by 30 cycles of 30 s

at 94 °C, 30 s at 55 °C, 150 s at 72 °C, and a final cycle of 10 min at 72 °C [11]. PCR products were pooled and purified employing 0.8 volume of the AMPure XP Beads (Beckman). Finally, the DNA library was constructed and run on the Illumina Miseq at Shanghai Hanyu Biotech Co., Ltd.

The low-quality sequences and chimera were detected and removed by Mothur (version 1.32.01) and UCHIME (version 4.2.40), respectively. Operational taxonomic units (OTUs) were generated by Mothur (version 1.32.01) at the 97% sequence similarity threshold. The taxonomic assignment was performed using the Ribosomal Database Project Classifier program with a confidence cutoff of 80%. The eukaryotic algae taxa at the phylum, class, family, and genus level were analyzed, respectively.

PCR, 18S rDNA Library Cloning and Sequencing

Near-full-length eukaryotic 18S rDNA were amplified using the 968F (5'-AAAGTTAGGGGATCG-3') and 1643R (5'-CATTCAATCGGTAGG-3') specific eukaryotic primer set [12]. PCRs were performed with the following parameters: denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 53.5 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 7 min [12]. PCR products were then electrophoresed in 1% agarose at 100 V for 30 min. The buffer was 1 $\times$ Tris-acetate-EDTA, and pH was adjusted to 8.0.

PCR products were purified with the Gel Extraction kit (OMEGA BIO-TEK) and cloned with the pGM-T Ligation Kit (TIANGEN). The resulting recombinant vectors were then transformed into the competent cells of *Escherichia coli* JM109 (Promega). Thirty-one positive clones (white colonies) from each library were picked randomly. Plasmid DNAs of these clones were extracted by plasmid miniprep kit (BIOMIGA). At last, these clones were sequenced in a commercial company (BIOSUNE, Shanghai, China).

Phylogenetic Analysis

The sequences were subjected to BLASTn searching (<http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi>). A rooted neighbor-joining tree was achieved by MEGA (version 4.0) with 1,000-fold bootstrap support, and an unrooted phylogenetic tree was drawn using the interactive Tree of Life online program based on the distance matrix data calculated by MEGA 4.0 [13].

Isolation and Purification of Microalgae

Artificial seawater medium (F/2 medium) was used to incubate the eukaryotic algae. Agar plates and micropipettes were employed to isolate the algae. Each purified strain was initially cultured in a test tube (30 ml) at 25 $\pm$ 1 °C under continuous illumination of white fluorescent light at an intensity of

70 $\mu\text{mol s}^{-1} \text{m}^{-2}$ for 1 to 3 weeks. The algae were then incubated into a 100 ml flask with sterile air at $25 \pm 1^\circ\text{C}$ under an intensity of 80 $\mu\text{mol s}^{-1} \text{m}^{-2}$ for 10 days [14].

Results and Discussion

Illumina Miseq High-Throughput Sequencing Analysis of Algae Community in Two Kinds of Activated Sludge

In order to study the role of eukaryotic algae and community structure in the activated sludge, two kinds of activated sludge were acquired from PO saponification wastewater and municipal wastewater treatment plant, respectively. The metagenomics were extracted from two different activated sludge and then 18S rDNA were sequenced through Illumina Miseq high-throughput sequencing. After removal of low-quality sequences and chimeras, the average number of Clean Reads for the PO saponification wastewater activated sludge genome was 22,101, while the municipal wastewater activated sludge genome was 21,116. The results of OTU

cluster analysis using mothur were 21,969 OTUs, and 38 species belonged to eukaryotic algae. Phylogenetic tree analysis of 38 OTUs cluster revealed that the eukaryotic algae in the municipal wastewater activated sludge were divided into nine different groups (Fig. 1). Five of these groups were assigned to *Ostreococcus*, *Symbiodinium*, *Anurofeca*, *Lotharella*, and *Thalassiosira*, and the other four groups were unidentified eukaryotic algae. By contrast, the algae in the PO saponification wastewater activated sludge could be grouped into two unidentified groups, which were quite different from the municipal sludge. The sequencing data were deposited in NCBI, and the ID of the sequence was PRJNA361045 (SRP098945).

Phylogeny of 18S rDNA Clones

To further analyzed the diversity of eukaryotic algae in the activated sludge of PO saponification wastewater, a pair of primers based on 18S rDNA hypervariable regions were selected, which could not be used for high throughput sequencing. So 18S rDNA clone library technology was used for the following steps.

Fig. 1 Phylogenetic analysis of 38 OTUs eukaryotic algae in the activated sludge

Fig. 2 The cloning of eukaryotic algae 18S rDNA fragments. **a** Gel electrophoresis of PCR-amplified 18S rDNA fragments from activated sludge. **b** Gel electrophoresis of PCR-amplified 18S rDNA fragments from plasmids. AS 1–31 represents PCR-amplified 18S rDNA fragments from different plasmids

In this study, a pair of primers named 968F and 1643R were employed, which was specifically used for eukaryotic algae community [12]. The metagenomic DNA extracted from the activated sludge was taken as template for PCR amplification. The 700 bp DNA bands were exhibited in agarose gel electrophoresis (Fig. 2a). The result is consistent with a previous study [12], demonstrating the existence of eukaryotic algae in the activated sludge. Furthermore, we constructed clone libraries of the purified PCR products by inserting the

purified DNA into the pGM-T plasmid vector and transforming into the competent cells of *Escherichia coli* JM109 cells. Altogether, 31 positive clones were selected and were amplified with the primers 968F and 1643R. DNA bands around 700 bp were appeared in all clones (Fig. 2b), which illustrated that target fragments were contained in all positive clones.

Afterwards, the sequenced 18S rDNA were searched using BLASTn program in the National Center for Biotechnology

Fig. 3 Neighbor-joining phylogenetic tree of 18S rDNA sequences obtained in this study. ★ represent the eukaryotic algae which was isolated from PO saponification wastewater treatment activated sludge

Fig. 4 Morphology of eukaryotic algae isolates of the *Desmodesmus intermedius*. Scale bars, 10 μ m

Information (NCBI) database, and the neighbor-joining tree was then constructed (Fig. 3). All of 31 sequences are compared to 18S rDNA of eukaryotic algae. Thirty of them were considered as uncultured eukaryotic algae. Only one sequence was identified as *D. intermedius* (Fig. 3).

In addition, the phylogenetic tree also showed that uncultured eukaryotic algae belonged to two different species at least. In summary, the algae in the activated sludge of propylene oxide saponification wastewater were different from that in the municipal wastewater. There were two reasons contributed to this situation. One reason was that PO saponification wastewater was produced from the production process of propylene oxide in the chemical plant, and the components of this wastewater were stable and simple, which caused that the

diversity of eukaryotic algae is low. In contrast, municipal wastewater has complex components and higher species diversity. The other reason was that most of the eukaryotic algae were annotated as unidentified eukaryotic algae in PO saponification wastewater activated sludge. There were many uncertainties of eukaryotic algae diversity.

Isolation of Eukaryotic Algae

An eukaryotic algae named JY001 was successfully isolated from PO saponification wastewater activated sludge using microorganism separation techniques (Fig. 4). Through 18S rDNA sequencing, the alga was identified as *D. intermedius*. *D. intermedius* was reported with the characteristics of thermo-tolerance, high growth rate, and high lipid content, and was often employed to produce biodiesel [14–16]. Through observed using optical microscope, *D. intermedius* JY001 was spherical, and the diameter of this alga was no more than 10 μ m.

It was worth noting that we did not separate other eukaryotic algae except for *D. intermedius*. Most of the eukaryotic algae in the PO saponification wastewater activated sludge were uncultured and could not grow in the artificial culture. However, *D. intermedius* could adapt to the artificial culture environment and was isolated successfully. In order to isolate more uncultured eukaryotic algae, it is necessary to improve the medium and the culture conditions.

Diversity of Eukaryotic Algae in Different Ecosystems

The diversity of eukaryotic algae in the PO saponification wastewater activated sludge and the municipal activated sludge were compared with several different ecosystems (Table 1). In all of them, the highest abundance of algae was found in Calcifying Biofilms of Two Karstic Streams in Germany. The identified species could be divided into three classes, which were *Trebouxioophyceae*, *Chlorophyceae*, and

Table 1 Diversity of eukaryotic algae in different ecosystems

Ecosystems	Total	Uncultured algae	Identified algae
Zoige Alpine Wetland [17]	23	18	5 <i>Chlorella</i> , <i>Planktosphaeria</i> , <i>Scenedesmus abundans</i> , <i>Scenedesmus bijuga</i> , <i>Bacillariophyta</i>
Lake taihu [18]	14	12	2 <i>Cryptophyta</i> , <i>Bacillariophyta</i>
Calcifying Biofilms of Two Karstic Streams in Germany [19]	58	24	34 Three classes, <i>Trebouxioophyceae</i> , <i>Chlorophyceae</i> , <i>Ulvophyceae</i>
Propylene Oxide Saponification Wastewater Activated Sludge (this research)	31	30	1 <i>Desmodesmus intermedius</i>
Municipal Wastewater Treatment Plant Activated Sludge (this research)	9	4	5 <i>Ostreococcus</i> , <i>Symbiodinium</i> , <i>Anurofeca</i> , <i>Lotharella</i> , <i>Thalassiosira</i>

Ulvophyceae, respectively. Similar to Zoige Alpine Wetland and Lake taihu, uncultured algae dominated the majority of eukaryotic algae in the PO saponification wastewater activated sludge in our research. It was interesting that *D. intermedius* was found and successfully isolated from PO activated sludge, which was not found in other ecosystems. Further research will be carried out in order to isolate the uncultured eukaryotic algae in this activated sludge, which would be helpful to reveal the role of eukaryotic algae in the activated sludge ecosystem.

Conclusions

In this study, the eukaryotic algae communities in the activated sludge from PO saponification wastewater and municipal wastewater treatment plant were analyzed respectively. In municipal wastewater activated sludge, the algae could be divided into nine different groups. By contrast, the algae in the PO saponification wastewater activated sludge could be grouped into two unidentified groups. In order to further analyze the diversity of eukaryotic algae in the PO saponification wastewater activated sludge, 18S rDNA clone library of eukaryotic algae was constructed, and 31 clones were obtained. The phylogenetic tree also showed that eukaryotic algae from PO saponification wastewater activated sludge belonged to two different species. Based on BLASTn analysis, one of them was identified as *D. intermedius*, while others were considered to uncultured eukaryotic algae. In addition, *D. intermedius* was successfully isolated. This study would be helpful to understand the eukaryotic algae diversity and utilize the biodiesel-producing eukaryotic algae in PO saponification wastewater activated sludge.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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