Research on the granular sludge domestication of phenol wastewater treatment under low temperature and analysis of microbial community structure

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Abstract. Due to low efficiency of phenol wastewater treatment in cold areas of northern China, there is an urgent need to search novel strategies for phenolic wastewater treatment. The experiment used phenolic wastewater as the object, performed in self-made experimental equipment with a gradient of increasing phenol-containing raw water under low temperature, further evaluating the removal efficiency of modified high-rate anaerobic apparatus. The PCR amplification was used to separate and identify dominant bacteria in sludge. The results showed that the removal of COD was up to 50% for pure phenol wastewater (844.5 mg·L⁻¹) with the temperature ranging from 10°C to 15°C, meanwhile B/C value increased from 0.08 to 0.21 after domestication, with a rise of 162.5%. The analysis of genetic indicated that the Trichococcus sp. was the dominant bacteria, accounting for 68% of the total bacterial.

1. Introduction

The waste water from gas, coking, petrochemical, oil refining and other plants mostly contains phenol pollutants. Besides, phenol is widely used in industrial production. Phenol, as a protoplasmic toxin, which is volatile and highly biologically toxic, can solidify protein and cause a variety of diseases. It is one of the most important pollutants to control in China, the United States and other countries. Due to phenol has “three causes” (carcinogenic, mutagenic and teratogenic) and inhibits microorganism growth, biological treatment of phenol wastewater has been the problem of environmental scientific research[1].

At present, there are many treatment methods of phenol wastewater, extraction, liquid membrane separation are commonly used with high concentration (> 500 mg·L⁻¹) phenol waste water[2]; The method of active sludge and biological filter method are often used in the wastewater containing medium concentration (5 ~ 500 mg·L⁻¹); Low concentration (< 5 mg·L⁻¹) phenol wastewater is suitable for the use of activated carbon adsorption, resin exchange and other methods[3]. In practical application, due to the high cost of chemicals, membrane and adsorbent, and possibility of secondary pollution, more effective and no secondary pollution methods has been more used, such as biological processing system to deal with the waste water containing phenol. But, the temperature of biological filter can fall to 12 °C in some cold areas of northern China. As a result, the microbial degradation declines sharply
and phenol concentration in effluent is impermissibly high, which increases the difficulty for normal operation of wastewater treatment plant during winter\cite{4}. This study aims to provide theoretical based technical support for large scale application of high-rate anaerobic apparatus on treatment of phenol wastewater in consideration of the actual environmental conditions of winter temperatures in northern areas. Modified expanded granular sludge bed reactor (A spoiler diversion device and a flexible fiber filler biofilm is installed in traditional EGSB reactor sludge bed area) was used to treat phenolic wastewater. Additionally, PCR amplification was used for separation and identification of dominant bacteria\cite{5}.

2. Materials and methods

The influent is prepared according to the following values throughout the duration of the experiment: chemical oxygen demand (COD): 2000 mg·L\(^{-1}\). PH: 6.8～7.2. Phenol concentration of the influent increased from 100 mg·L\(^{-1}\) to 844.5 mg·L\(^{-1}\).

The scheme of the experimental setup is shown in figure 1. The self-made experimental setup is upflow forced circulation of external circular reactor. A spoiler diversion device is added to the reaction zone of reactor, which is helpful to control the expansion degree of granular sludge bed and the mixing intensity\cite{6}. Meanwhile, due to the addition of a flexible fiber filler biofilm in the reaction zone, thereupon the amount of biomass greatly increased.

2.1. The operation of up-flow Anaerobic Sludge Bed.

The experiment simulated a low temperature environment from 10 to 15 °C. The granular sludge, which is in good condition and stabilized remove effect, is about 2～4mm. The mean settling rate of sludge average is about 42 m·h\(^{-1}\), and the integrity coefficient of the average mechanical strength of granular sludge is 38.44%. Experimental water is self-made phenol wastewater. The granular sludge in EGSB taking from Harbin High-Tech (GroupC0.LTD) is used as the experimental seed sludge. The determination COD and BOD refer to the Method of Examining and Analyzing the Water and Waste Water. Effluent phenol concentrations were ascertained by Gas Chromatography-Mass Spectrometer (GC-MS)\cite{7}.
2.2. Molecular biological analysis of dominant bacteria

PCR amplification of the 16S rRNA gene: Total DNA is extracted from activated sludge collected from reactor using DNA extraction kit (Shanghai Huashun Bioengineering Corporation). Primers used for amplification of the 16S rDNA fragments are as follows:

BSF 8/27: 5’-AGAGTTTGATCCTGGCTCAG-3’;
BSR1525/1541: 5’-AAGGAGGTGATCCAGCC-3’.

2.2.1. Clone library constructs. PCR products of 16S rDNA are purified and cloned into pMD19-T vector. Recombinant pMD19-T were electroporated into E. coli DH5a. Two hours after electroporation and incubation at 37 °C, cells are then spread on LB agar plate. Candidate colonies are randomly selected and identified by ABI 3730 automatic sequencer. 50 positive transformants are picked from each sample respectively.

2.2.2. Statistical analysis. Sequence alignment of 16S rDNA is carried out via the method of BLASTN. Phylip-4.0 software is used to realize the construction of the evolutionary tree. Phylogenetic tree was drawn by using Neighbor-Joining method following Jukes-Cantor algorithm. A bootstrap analysis with 1,000 replicates was carried out to check the robustness of the tree [8].

3. Results and analysis

3.1. The efficiency COD in adapting process

In order to fortify the adaption of granular sludge and screen out the phenol degradation bacterizes, measurement of gradually increasing concentration of phenol in inflow is utilized.

![Figure 2. Influent and effluent COD concentration and COD removal efficiency](image)

The process involved three steps respectively: firstly, increasing the ratio of phenol wastewater in inflow (gradient of 5%), with a constant total COD. And secondly, mix sucrose- and phenol-containing wastewater in 1:1 in the inflow. Eventually, a total phenol-containing wastewater is used as the raw water. Figure 2 shows the COD concentration of influent and effluent and COD removal: during the adapting process, COD removal reduced from 85% to 50% gradually, with a constant concentration of COD (2000mg/L, approximately). Firstly, the removal reduced from 85% to 76%, a decrease of 9%, about 25.7% of the total removal decline, falling by an average
1.64% per day; In the third stage, gas product smoothly, and COD removal reduced from 53% to about 50%, with a drawdown speed of COD removal about an average 0.37% per day. The effluent COD maintained in the vicinity of 1000 mg·L⁻¹, and COD removal stabilize at about 50%. Due to toxicity of phenol to granular sludge, and the dose-dependent manner of phenol toxicity, a higher COD removal occurred in the first step, then decreased gradually, eventually stabilized around 50%, which signified the adapting process of granular sludge to phenol has been accomplished and the dominant bacteria has already developed.

3.2. The impact of Phenol concentration on phenol removal
Figure 3 shows in the first six days, phenol removal keeps at around 10%, relatively low and the COD removal keeps at around 80%, resulting from the long-term high glucose conditions making the granular sludge almost have no ability to degrade phenol.

![Phenol removal rate ascends gradually on the eighth day, suggesting that the granular sludge gradually had the capacity to degrade phenol. In the second stage, phenol removal increased sharply rising to 45% in twenty-eighth days, while COD removal rate drop from 76% to 53%, demonstrating that removal capacity and adaptability to phenol of granular sludge have been enhanced significantly and phenol degrading bacteria started to multiply. Passing the process of adaptation, phenol concentration of influent reach the maximum value (844.5 mg·L⁻¹), and both the phenol removal and COD removal stabilised at 50% respectively, which implies that anaerobic granular sludge was acclimated successfully.

3.3. Biodegradability analysis of effluent
BOD⁵/COD (B/C) is widely used to measure the biodegradability of wastewater. It is considered to hardly biodegrade, when the BOD⁵/COD value is lower than 0.2 B/C value of experimental phenol wastewater used in this study was 0.08, meaning the wastewater is non-biodegradable.

| Categories | Prior to domestication | After domestication | Cumulative gas production(mL) |
|------------|------------------------|---------------------|-------------------------------|
|            | COD | BOD  | B/C | COD | BOD  | B/C |                      |
| Reactor    | 2120 | 169.6 | 0.08 | 1484 | 318.8 | 0.21 | 134.3 |
| Note: COD, BOD units are mg/L.
It is difficult to guarantee removal effect of phenol wastewater for the conventional wastewater treatment process, and the effluent can hardly meet the prescribed standard. In this study, we modifies the conventional EGSB, to enhance the hydraulic circulatory of the system of the EGSB and anaerobic digestion of microorganisms significantly. In principle, the modified EGSB can decompose nonbiodegradable macromolecular organic matters into biodegradable small molecules, and then improves the biodegradability of effluent. As shown in Table 1, the B/C value increased from 0.08 to 0.21 after acclimation, with increasing of 162.5%. The results indicates that this type of sludge microbes is not only adaptive to phenol, but also help to improve biodegradation and transformation.

3.4. selection and identification of dominant bacteria
The phylogenetic tree (Figure 5) drawn by Neighbor-Joining method revealed 34 strains like b01 and b11 shared high homology of 16S rDNA gene sequence with *Trichococcus sp.*, reaching 99%. The homology of 16S rDNA gene sequence of b07 strain with *Propionigenium sp.* is up to 89%. The homology of 16S rDNA gene sequence of b23, b47 strains with *Clostridium sp.* is up to 99%. The homology of 16S rDNA gene sequence of b28 strain with *Citrobacter sp.* is up to 99%. The homology of 16S rDNA gene sequence of b38, b44 strains with *Bacteroidetes sp.* is up to 86%, 97%. The homology of 16S rDNA gene sequence of b43 strain with *Endophytic bacterium sp.* is up to 95%. The homology of 16S rDNA gene sequence of b49 strain with *Syntrophobacter sp.* is up to 88%. Basically these strains can be determined that these species.

![Figure 4](image-url)

As shown in Figure 4, *Trichococcus sp.* which is the most important microorganisms in phenol-resistant activated sludge systems accounted for 68 % of the total bacterial culture. *Trichococcus sp.* is gram-positive bacteria, no exercise and no born spores, Faculative anaerobic. Heterotrophic, fermented, and slow growth[9]. In addition, there are five strains(4 species) acid bacteria after resistant phenolic experiments: *Syntrophobacter sp.*, *Propionigenium sp.*, *Clostridium sp.*, *Citrobacter sp.*. However the number of them is relatively few, but particularly concerned, with their own capable of withstanding phenolic poisoning, co-metabolism with *Trichococcus sp.* and plenty of other Uncultured *Bacteroidetes sp.* under the effect of inducible enzyme at low temperatures. These acid-producing bacteria could turn phenols methyl, methyl aldehyde, and hydroxyl groups into organic acids, cracked phenolic benzene ring structure to mitigate the toxicity and degradation of organic matter.
Figure 5. Phylogenetic tree derived from 16S rDNA gene clone libraries.
4. Conclusions
Under low temperature, through improving the EGSB process to enhance the removal of phenol, we draw the following conclusions:

1. When the influent phenol concentration is 100mg/L, COD removal rate reached 85%. But increasing concentration of phenol, COD removal decreases to about 50%; After bioacclimation B/C value increased from 0.08 to 0.21, with an increase of 162.5%, greatly improves biodegradability.

2. Increasing the concentration of phenol gradually, granular sludge in the phenol wastewater is able to adapt to the change, and the phenol removal increased. When the phenol concentration is 844.5mg / L, the removal rate stabilizes about 50%.

3. Phylogenetic classification identified that the dominant microflora is the *Trichococcus sp.* after bioacclimation, accounting for 68% of the total bacterial culture, different from the literatures.

4. Through this study, dominant bacteria of phenol have been selected and identified under low temperature, which can help to improve the efficiency of northern phenol wastewater treatment. Thence, developing phenol microbial inoculum, not only has theoretical significance, but also has great application value and broad market prospects for the treatment of phenol wastewater.

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References
[1] Lettinga G. Advanced anaerobic wastewater treatment in the near future[J]. Wat Sci. Tech, 1997, 35(10):5-12.
[2] Lettinga G, Rebac S, Zeeman G. Challenge of psychrophilic anaerobic wastewater treatment[J]. Trends in Biotech, 2001, 19(9):363-370.
[3] Schellinkhout A, Collazos C J. Full-scale application of the UASB technology for sewage treatment[J]. Wat Sci Tech, 1992, 25:159-166.
[4] R.I. Amann, B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, D.A. Stahl, Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations, Appl. Environ. Microbiol. 56 (1990) 1919–1925.
[5] National Environment Protect Bureau. Method of Examining and Analyzing the Water and Waste Water. 4th ed. Beijing: China Environmental Science Press, 2002
[6] V. Saravanan, T.R. Sreekrishnan, Modelling anaerobic biofilm reactors – a review, J. Environ. Manag. 81 (2006) 1–18.
[7] J. Field, R. Sierra-Alvarez, Microbial degradation of chlorinated phenols, Rev. Environ. Sci. Biotechnol. 7 (2008) 211–241.
[8] M. Farhadian, D. Duchez, C. Vachelard, C. Larroche, Monoaromatics removal from polluted water through bioreactors—a review, Water Res. 42 (2008).
[9] Collins, G., Woods, A., McHugh, S., Carton, M.W., O’Flaherty, V., 2003. Microbial community structure and methanogenic activity during start-up of psychrophilic anaerobic digesters treating synthetic industrial wastewaters. FEMS Microbiol. Ecol. 46, 159–170.