# Screening Predominant Bacteria and Construction of Efficient Microflora for Treatment of Papermaking White Water

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Three strains of bacteria were isolated and purified from activated sludge for white water treatment in the laboratory. These strains were identified as *Bacillus subtilis, Bacillus cereus,* and *Virgibacillus pantothenticus* through a morphological analysis, the MIDI Sherlock automatic microbial identification system, and 16S rRNA methods. The results of the construction of efficient microflora for white water showed that a mass percentage ratio of *B. subtilis*, *B. cereus,* and *V. pantothenticus* of 50%:35%:15% achieved an optimal treatment effect. Analysis by gas chromatograph-mass spectrometer (GC-MS) established that the content of characteristic pollutants in white water decreased notably after treatment with the efficient microflora, and detected the intermediate products of short chain fatty acids, alcohols, and other compounds. Moreover, through measuring the removal rate of chemical oxygen demand (COD), electrical conductivity, and cationic demand (CD), the optimal retention time for white water treatment with the efficient microflora was 4 h to 6 h, and when the removal rate of COD reached approximately 90%, the electrical conductivity and the cationic demand were reduced to lower values.

*Keywords: Papermaking white water; Efficient bacteria; Purification; Construction*

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## INTRODUCTION

The closed cycle utilization of papermaking white water could save many water resources. However, the soluble colloidal substances and inorganic salts contained in white water are continuously accumulated. Among them, the concentration of anionic trash and lipophilic extract have increased, which makes various cationic additives to the papermaking process lose their effectiveness. So the retention of cellulosic fines and fillers in paper becomes inefficient, and paper properties can be negatively affected (Wang and Zhang 2013). Therefore, high levels of colloidal substances and salts have a negative effect on the quality of the paper such as decreased surface performance (smoothness, glossiness, surface strength and so on) and paper strength (Xiao *et al.* 2017). In addition, with the dissolved substances the conductivity and the concentration of anionic trash are increasing in the piping, resulting in pipes and parts of equipment becoming clogged and corroded (Ricard and Reid 2004; Wu *et al.* 2014). The dissolved and colloidal substances in the process water can also make it more difficult to achieve targets of hydrophobic sizing,

increased levels of slime in the system, and a higher frequency of paper web breakage and the operational failures of the paper machine (Liang *et al.* 2011; Mänttäri *et al.* 2015). When white water cycling is closed, the content of lignin compounds and surface modifiers accumulate, producing a large amount of foam (Karikallio *et al.* 2011), which affects the transportation and storage of white water. To overcome such problems, the white water must be treated such that it can be used repeatedly within the papermaking system.

Currently, the super effect shallow air flotation method, adsorption, and emerging membrane separation technologies are commonly used for white water reuse in China and abroad. The super effect shallow air flotation is an advanced type of the dissolved air flotation, which has been applied as an option to purge contaminants from white water. Besides, this method has the advantages of easy operation and large treatment capacity (Benalycherif and Girault 2010), but it can only be taken as a pretreatment process in which the pollutants are simply transferred from the liquid phase to the solid phase without being removed completely, causing secondary pollution. Adsorption technology has been one of the most effective methods for wastewater treatment. Recently, a fluidized bed reactor has been proposed for adsorption of white water within a paper machine. The fluidization promoted the contact between contaminants and cationic solid adsorbents, thus maximizing the mass transfer between liquid pollutants and a solid adsorbent (Loranger *et al.* 2010a,b). The technology has been effectively applied in the catalytic process of the petrochemical industry, but it remains challenging for white water treatment because valuable cellulose compounds (fibers and fines) can lead to significant interference for the bed expansion behavior in the fluidization process.

Membrane separation technology can completely remove the colloidal and some dissolved substances in papermaking white water (Servaes *et al.* 2016), but its application is limited in the strong viscosity and poor fluidity of white water (Mänttäri *et al.* 2015). In recent years, enzymes have been used to improve the performance of white water (Idris and Bukhari 2012). However, the defects of the enzymatic method should not be neglected, *i.e.*, the higher treatment cost, the narrow application condition, and the specificity of enzymes reactions. In fact, the biodegradability of white water is weak, containing various organic substances that are poisonous and difficult to degrade. The commonly used activated sludge process is ineffective in degrading such substances because the amount of the predominant bacteria in the system is low.

Today, the research and use of dominant bacteria has become more extensive. The predominant bacteria screened by specific pollutants has had good degradation effects on the pollutants. The predominant bacteria for coconut juice processing wastewater has been screened (Lertsriwong *et al.* 2017). It was found that these predominant bacteria can degrade the pollutants to ethanol, butanol, acetone, 2,3-butanediol, and other low mass molecules of organic matter. Jebelli *et al.* (2017) screened out the dominant bacteria that degrade arsenic from the wastewater. They found that these predominant strains could significantly improve the transformation of arsenite to arsenate, which was successfully used in bioremediation of arsenic contaminated soil. Efficient microbial communities rapidly removed pollutants during their growth and formed a stable ecosystem for co-metabolism. Therefore, its function was complete, and the treatment effect was obvious compared to a single dominant bacterium. The application of dominant bacteria to the aerobic treatment reduced the sludge production, shortened the aeration time, enhanced the treatment effect, and stabilized the effluent quality (Su *et al.* 2017). Li *et al.* (2014) studied the effect of temperature on the treatment of the wastewater containing p-phthalic acid (PTA) by microorganisms. The results showed that there were all kinds of dominant

bacteria with the temperature, and the removal rate of specific pollutants was higher with the presence of dominant bacteria. In this study, an efficient flora was constructed for the white water of paper making to provide an economical and effective method for the reuse of white water.

## EXPERIMENTAL

### Materials

White water was obtained from the alkaline hydrogen peroxide mechanical pulp in the tray below the paper machine forming fabric in a paper mill of Qingdao, Shandong Province, China. The chemical oxygen demand (COD) of the white water was about 1,320 mg/L, the cationic demand (CD) was around 510 μeq/L, the electrical conductivity was around 1894 μs/cm, the pH was 7.64, and the total solid content and ash content were 0.01% and 0.001%, respectively. Strains were isolated and purified from aerobic activated sludge domesticated in the authors' laboratory in Qingdao, China. The aerobic activated sludge comes from the secondary settling tank of a sewage treatment station in the paper mill; it was then domesticated with white water in a laboratory.

### Methods

*Analysis methods*

The purified colonies were identified by use of the MIDI Sherlock® Microbial Identification System (MIS) (Biolog, Tampa, FL, USA). The library of the system included TSBA6, CLIN6, BHIBLA3, and ACTIN1. For the 16S rRNA sequencing measurement, genomic DNA was extracted from the purified colonies using a bacterial DNA extraction kit (Shanghai Sangon Biotech Ltd., Shanghai, China). The purification and sequencing of polymerase chain reaction (PCR) products were carried out by Qingdao Qing Ke Zhi Xi Biological Technology Co., Hangzhou, China. The 16S rRNA gene fragments were amplified using universal primers of 27F (5 'AGAGTTTGGATCCTGGCTCAG3') and 1492R (5 'CGGTTACCTTGTTACGACTT3').

The concentration of $COD_{Cr}$ was determined by a DR2700 type portable water quality analyzer (HACH, Loveland, USA). The conductivity was measured by a DDS-11C type conductivity meter (Shanghai Shengke Instrument Equipment Co., Ltd., Shanghai, China). The absorbance was obtained using a TU-1810 type ultraviolet visible spectrophotometer (Puxi, Beijing, China) at different times and was used to indicate the number of bacteria.

Cationic demand (CD) was measured using the Particle Charge Detector (PCD-03; BTG Ltd., Eclépens, Switzerland). The blank and white water samples (10 mL) were diluted 10 times with deionized water and then titrated with 0.001N poly-DADMAC (Luyue Shandong Chemical Co., Ltd., Taian, China).

The components of white water were analyzed by GC-MS (Shimadzu, Kyoto, Japan) instrument. Water samples needed to be treated before GC-MS analysis, and the specific method was the following: 1000 μL water sample was removed with a 1000-μL transfer liquid gun and added to a 2-mL bottle. Then, 1000 μL MTBE (methyl tert butyl ether) was used to join them with violent oscillation for 5 min. Next, was the careful removal of the upper extract and its placement into the 2-mL GC sample bottle after standing and layering. The GC-MS analysis conditions were a HP-5 capillary column with the size of 0.25 μm ×

0.32 mm × 30 m. The initial temperature was kept at 60 °C for 1 min and then programmed to be heated to 300 °C at a speed of 10 °C/min and kept at this temperature for 5 min. The inlet temperature was 280 °C. High purity helium was used as the carrier gas. The injection sample was 1 μL with the flow rate of 1 mL/min without shunt. The conditions of mass spectrometry were an electron bombardment voltage of 70 eV, the temperature of the ion source was 200 °C, the mass gain was 254 times, the emission voltage was 1388 V, and the spectra library was NIST08.LIB (Gaithersburg, MD, USA).

*Isolation and purification of bacteria*

The formula for the medium was as follows: Beef extract was 5 g/L, peptone was 10 g/L, and sodium chloride was 5 g/L. Inoculation loops were used, with an aseptic technique to dip into the mixture, which was aerobic activated sludge from domestic wastewater treatment. The mixed colonies were isolated by four zone marking method and cultured in LRH-150 incubator for 24 hours at 30 °C. Single colonies grew in the fourth district. Then, several typical colonies were selected and cultured respectively.

### Construction of Dominant Flora

Three kinds of pure bacteria powder were taken into a 250-mL conical bottle, and the dosage of each pure bacteria powder was 20 mg. Then, 200 mL white water was taken into the conical bottle, and the pH was controlled at 6 to 7. The conical bottle was placed in a constant temperature water bath of 30 °C, and then it was aerated with a cycle (hydraulic retention time) of 12 h. The COD concentration and removal rate were measured each cycle.

### Optimum Mixture Ratio of Dominant Microflora

According to the above method, it was necessary to take 1 mL of the bacterial solution after 10 cycles of domestication. Then the bacterial solution was diluted 10, $10^2$, $10^3$, $10^4$, and $10^5$ times according to the gradient. Then 0.2 mL of bacterial solution was taken by transfer liquid gun from the $10^4$ and $10^5$ test tubes and placed in the plates. Volumes in the range 15 to 20 mL of media, which had been cooled to 50 °C, were poured into the plates. Then the plates were rotated in order to mix the bacterial solution and media evenly. The plates were cultured in an LRH-150 incubator for 48 hours at 37 °C. Finally, the numbers of bacteria were counted.

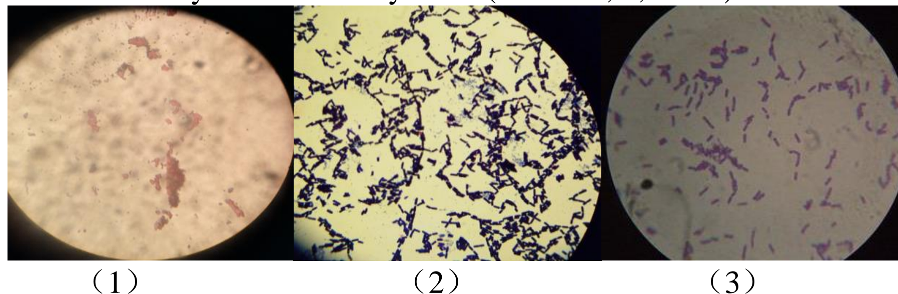
## RESULTS AND DISCUSSION

### Identification of Dominant Bacteria

Three colonies of pure bacteria were obtained from the acclimated sludge. The gram stain results of each dominant bacterium were shown in Fig. 1. The colonies are described Table 1.

The three dominant bacteria were stained according to the Gram procedure (Moussavi and Behrouzi 2004). The results showed that the bacterium of Colony 1 was rod-shaped with uniform red color observed by the microscope, indicating it was gram negative bacterium. The bacterium of Colony 2 was observed as rod-shaped with deep purple color, showing a Gram-positive result. The bacterium of Colony3 also showed a

Gram-positive result, but with ellipsoidal and columnar shape. Colony 1, Colony 2, and Colony 3 were identified by microbial fatty acids (Tables 2, 3, and 4).



（1） （2） （3）

（1）Virgibacillus pantothenticus（2）Bacillus cereus strain（3）Bacillus subtilis strain

**Fig. 1.** Gram stain results of dominant bacteria

**Table 1.** Descriptions of the Bacterial Colony

| Bacterial Colony | Form | Wetting Degree | Altitude | Pellucidity | Pigment | Border |
|---|---|---|---|---|---|---|
| 1 | Suborbicular | Drier | Apophysis | Opacification | Creamy-white | Irregular |
| 2 | Circular | Moist | Flat | Translucent | Creamy-white | Regular |
| 3 | Circular | Dry | Middle concave convex | Opacification | Ivory | Irregular |

For Colony 1, three similar bacteria were found in the RTSBA6 library, namely *Virgibacillus pantothenticus* (*Bacillus*), *Bacillus atrophaeus*, and *Bacillus circulans* GC subgroup B, with the similarity indices of 0.683, 0.421, and 0.269. For Colony 2, *Bacillus cereus* GC subgroup A, and *Bacillus cereus* GC subgroup B were identified in the RTSBA6 library with the similarity indices of 0.732 and 0.398. For Colony 3, there were two similar bacteria being tested in the RTSBA6 library, which were *Bacillus subtilis* strain and *Pediococcus pentosaceus* with the similarity indices of 0.577 and 0.409. For the identification system of microbial fatty acids, the identification results can be used when the similarity index is greater than 0.5. Therefore, the results showed that the Colony 1 was *Mycobacterium pantothenate*, the Colony 2 was *Bacillus cereus*, and the Colony 3 was *Bacillus subtilis*.

**Table 2.** Matches of Colony 1

| Library | Sim Index | Entry Name |
|---|---|---|
| TSBA6 6.21 | 0.697 | *Virgibacillus-pantothenticus* (Bacillus) |
| | 0.421 | *Bacillus-atrophaeus* |
| | 0.269 | *Bacillus-circulans*-GC subgroup B |

**Table 3.** Matches of Colony 2

| Library | Sim Index | Entry Name |
|---|---|---|
| RTSBA6 6.21 | 0.732 | *Bacillus-cereus*-GC subgroup A |
| | 0.398 | *Bacillus-cereus*-GC subgroup B |

**Table 4.** Matches of Colony 3

| Library | Sim Index | Entry Name |
|---|---|---|
| RTSBA6 6.21 | 0.577 | Bacillus subtilis strain |
| | 0.409 | *Pediococcus-pentosaceus.* (TSBA) |

An analysis of 16S rRNA sequences of the three strains was performed. The results of the 16S rRNA sequence analysis of the three dominant bacteria are shown in Figs. 2, 3, and 4 and Tables 5, 6, and 7.

**Table 5.** The 16S rRNA sequence alignment results of Colony 1

| Accession | comparison | Ident |
|---|---|---|
| KJ139434.1 | *Virgibacillus pantothenticus* ATCC 14576 16S ribosomal RNA gene, partial sequence | 99% |
| KR780430.1 | *Virgibacillus pantothenticus* ATCC 23355 16S ribosomal RNA gene, partial sequence | 99% |

**Table 6.** The 16S rRNA sequence alignment results of Colony 2

| Accession | comparison | Ident |
|---|---|---|
| KX036611.1 | *Bacillus cereus* strain SIIA_Pb_E3 16S ribosomal RNA gene, partial sequence | 99% |
| KF863832.1 | *Bacillus cereus* strain ATCC 14579 16S ribosomal RNA gene, partial sequence | 99% |
| KC248215.1 | *Bacillus cereus* strain ATCC 14579 16S ribosomal RNA gene, partial sequence | 99% |

**Table 7.** The 16S rRNA sequence alignment results of Colony 3

| Accession | comparison | Ident |
|---|---|---|
| KJ139434.1 | *Bacillus subtilis* strain G-13 16S ribosomal RNA gene, partial sequence | 99% |
| KR780430.1 | *Bacillus subtilis* strain CR26 16S ribosomal RNA gene, partial sequence | 99% |
| KR029823.1 | *Bacillus subtilis* strain FY99 16S ribosomal RNA gene, partial sequence | 99% |

CCGACTTCGGGTGTTAAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTC
ACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCGGCGGTGGCTCTAAAGGTTACCTC
ACTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACC
TCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCAT
GATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACT
GAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATT
GTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCG
CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCT
TAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACC
AGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATT
CCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTT
TCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCA
CCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGT
ACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCAT
CACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGT
AGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTT
GCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCG
AAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGG
AGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGA
GCAAGCTCCCATCTTCCGCTCGACTGCAG

**Fig. 2.** 16S rRNA gene sequence of colony 1

TTAGGCGGTGGCTCAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGG
CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCA
GCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGATTAGCTCCACC
TCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCAT
GATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTT
AATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTT
TCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
TTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTT
AATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGT
GGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACC
AGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTC
CACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTT
CACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCAC
CTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTG
CCAGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATC
ACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTA
GGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTG
CCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGCCGA
AGCCGCCTTTCAATTTCGAACCATGCGGTTCAAAATGTTATCCGGTATTAGCCCCGGTTTCCCGGA
GTTATCCCAGTCTTATGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACTTCATAAGA
GCAAGCTCTTAATCCATCGCTCGACTGCA

**Fig. 3.** 16S rRNA gene sequence of colony 2

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CTTCGGCGGTGGCTCTAAAGGTTACCTCACCGACTTCGGGTGTTAAAACTCTCGTGGTGTGACGGG
CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCA
GCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACC
TCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCAT
GATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACT
GAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC
GAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATT
GTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCG
CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCT
TAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACC
AGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATT
CCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTT
TCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCA
CCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGT
ACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCAT
CACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGT
AGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTT
GCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCG
AAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGG
AGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGA
GCAAGCTCCCATCTTCCGCTCGACTGCAG
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**Fig. 4.** 16S rRNA gene sequence of colony 3

As shown in Fig. 2 and Table 5, the species was identified as *Virgibacillus pantothenticus* ATCC 14576 or *Virgibacillus pantothenticus* ATCC 23355; both of the matching indices were as high as 99%. The two strains belonged to different strains of *Virgibacillus pantothenticus*, so the results showed that Colony 1 was *Mycobacterium pantothenate*, which was consistent with the results of the MIDI Sherlock® Microbial Identification System.

As shown in Fig. 3 and Table 6, it was identified as either the *B. cereus* strain SIIA-Pb-E3, *B. cereus* strain ATCC 14579, or *B. cereus* strain LH8 with the matching index of 99%. However, the three strains belonged to the different strains of the *B. cereus* strain, so the results showed that Colony 2 was *B. cereus*, which was consistent with the results of the MIDI Sherlock automated microbial identification system.

As shown in Fig. 4 and Table 7, the species was identified as either *B. subtilis* strain G-13, *B. subtilis* strain CR26, or *B. subtilis* strain FY99 with the matching index of 99%. However, the three strains belonged to the different strains of the *B. subtilis* strain, so the results showed that Colony 3 was *Bacillus subtilis*, which was consistent with the results of the MIDI Sherlock automated microbial identification system.

The results of the above analysis methods showed that Colony 1 was *Mycobacterium pantothenate*, Colony 2 was *Bacillus cereus*, and Colony 3 was *Bacillus subtilis*. Thus, the morphological observations of the three dominant bacteria under light microscope and the results of gram staining were consistent with those described in the Berger's Manual of bacterial identification (Buchanan and Gibbons 1984).

## Construction of Dominant Flora

The COD concentration and removal rate were measured each cycle (hydraulic retention time), and the results are shown in Fig. 5.

As shown in Fig. 5, when the white water was treated by the mixed dominant bacteria, the removal rate of COD increased with the treatment cycle. After 8 cycles, the COD removal rate reached the maximum value and remained constant, which indicated that the three dominant bacteria had formed a stable microbial community with a specific proportion.

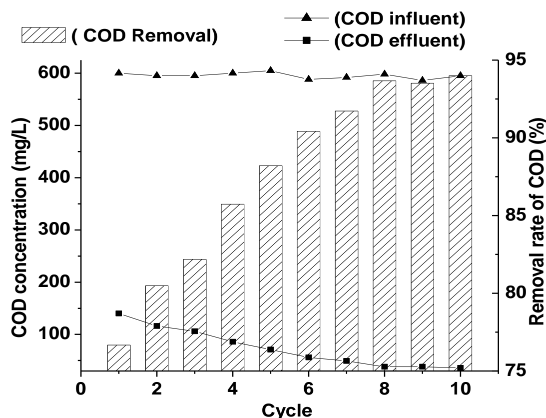


**Fig. 5.** Change of COD with the treatment time

## Optimum Mixture Ratio of Dominant Microflora

The relative proportions of the three dominant bacteria were determined by the plate count method. The count results are shown in Tables 8 and 9.

**Table 8.** Results of Colony Count

| Dilution Factor / Bacteria Strain | *Bacillus cereus* | *Bacillus subtilis* | *Mycobacterium pantothenate* |
|---|---|---|---|
| $10^4$ | 317 | 211 | 85 |
| $10^5$ | 30 | 21 | 9 |

**Table 9.** Data Analysis

| Duplicate Samples | *Bacillus cereus* | *Bacillus subtilis* | *Mycobacterium pantothenate* |
|---|---|---|---|
| 1 mL Stoste ($10^4$) | 3170000 | 2110000 | 850000 |
| 1 mL Stoste ($10^5$) | 3000000 | 2100000 | 900000 |
| Average Value | 3085000 | 2105000 | 875000 |
| Relative Standard Deviation (%) | 3.89 | 0.34 | 4.04 |
| Percentage (%) | 50 | 35 | 15 |

The results showed that the relative standard deviations of the parallel samples of the three dominant bacteria were less than 5%, which meant that the experimental results were reliable. When the white water treatment efficiency reached a stable level, the proportions of *B. cereus*, *B. subtili*s, and *M. pantothenate* were 50%:35%:15%. The effect of dominant flora for white water treatment increased considerably compared to the single dominant bacteria. In other words, the removal rate of COD was approximately 94% after 12 h, while the value by the single dominant bacteria was less than 80% (data obtained in the laboratory). At the same time, the results also showed that through the co-metabolism and synergistic action of the flora, the purification ability of the mixed dominant bacteria groups was clearly enhanced. Sarkar *et al.* (2017) discovered that *Burkholderia*, *Kocuria*, *Enterobacter*, and *Pandoraea* strains had the ability of versatile metabolic. This study established that catabolically efficient bacteria resides naturally in complex petroleum refinery wastes and those can be useful for bioaugmentation based bioremediation.

## Composition Changes of Wastewater

The compositions of the wastewater were determined by GC-MS, and the results are shown in Tables 10 and 11.

**Table 10.** Main Components of Wastewater Before Treatment

| Peak Labeling | Retention Time | Peak Area | Percentage of Peak Area (%) | Substance Name | Constitutional Formula |
|---|---|---|---|---|---|
| 1 | 3.54 | 92863 | 0.76 | Oxime-, methoxy-phenyl- | |
| 2 | 6.677 | 741975 | 6.03 | 3-methoxy-4-hydroxybenzaldehyde | |
| 3 | 12.08 | 319540 | 2.6 | Phenol, 2,4-bis(1,1-dimethylethyl)- | |
| 4 | 14.495 | 103610 | 0.84 | Heneicosane | |
| 5 | 19.845 | 1822460 | 14.82 | Eicosyl acetate | |
| 6 | 20.1 | 245649 | 2 | Hexadecanoic acid, 2-hydroxyethyl ester | |
| 7 | 20.24 | 178851 | 1.45 | 2-tert-Butyl-4-methyl-6-(1-methyl-1-phenylethyl) phenol | |
| 8 | 20.665 | 216011 | 1.76 | 3-Trifluoromethylbenzoic acid, 4-pentadecyl ester | |
| 9 | 21.715 | 6998524 | 56.92 | Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-phenol | |

Whether or not wastewater was sampled before or after treatment, the benzene-group-containing compounds 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl]-phenol, hexadecanoic acid, and 2-hydroxyethyl ester were detected. These were understood to be the product of lignin depolymerization and the most important pollutant in white water. Additionally, a higher concentration of 3-methoxy-4-hydroxybenzaldehyde was found in raw water, commonly known as methyl vanillin, which was a derivative of lignin. After treated by the dominant bacteria, the peak areas of 2,2'-methylenebis[6-(1,1-dimethylethyl) -4-methyl]-phenol, hexadecanoic acid, and 2-hydroxyethyl ester decreased substantially, while the peak of 3-methoxy-4-hydroxybenzaldehyde did not appear, and thus the content of long fatty acids and lignin derivative compounds was remarkably reduced. Furthermore, acetic acid and glycerol accounted for a very large proportion in the treated water samples, but they did not occur in the raw water, indicating that some of the macromolecular organics were degraded by the flora into small molecules. Relevant research supports this conclusion. The biochemical method of lignin degradation products was determined using liquid chromatography and formic acid, acetic acid, glycerol, furfural, p-hydroxybenzoic acid, vanillin, guaiacol, and syringaldazine (Tian *et al.* 2017). In this study, compared with the wastewater, the total amount of aromatic compounds containing benzene rings in the effluent decreased considerably, and the total amount of pollutants was also notably

reduced. This was because some of the short fatty acids and other low molecular substances were gradually degraded into $CO_2$ and $H_2O$ by the microflora.

**Table 11.** Main Components of Wastewater After Treatment

| Peak Labeling | Retention Time | Peak Area | Percentage of Peak Area (%) | Substance Name | Constitutional Formula |
|---|---|---|---|---|---|
| 1 | 6.732 | 1023697 | 10.23 | Acetate | |
| 2 | 9.975 | 139678 | 1.62 | Glycerin | |
| 3 | 10.305 | 424510 | 4.91 | Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester | |
| 4 | 12.085 | 345962 | 4 | Phenol, 2,4-bis(1,1-dimethylethyl)- | |
| 5 | 14.5 | 107755 | 1.25 | Heneicosane | |
| 6 | 19.835 | 1185137 | 13.71 | Eicosyl acetate | |
| 7 | 20.1 | 193276 | 2.24 | Hexadecanoic acid, 2-hydroxyethyl ester | |
| 8 | 20.24 | 131809 | 1.52 | 2,6-Di-tert-butyl-4-(2,4,6-trimethylbenzyl) phenol | |
| 9 | 20.665 | 192340 | 2.22 | Acetic acid, 3-(6,6-dimethyl-2-methylenecyclohex-3-enylidene)-1-methylbutyl ester | |
| 10 | 21.71 | 3417874 | 39.51 | Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-phenol | |

## Determination of the Treatment Cycle of the Microflora

To meet the needs of practical applications, it was necessary to determine the cycle of white water treatment by the microflora. The variation of the COD removal rate, the conductivity, and the cationic demand with time for the $10^{th}$ cycle were researched, and the results are shown in Figs. 6, 7, and 8.

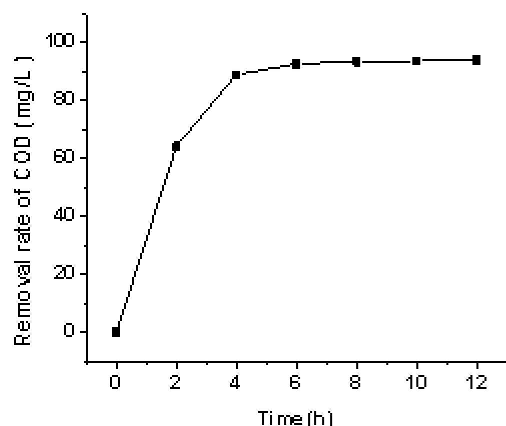


**Fig. 6.** Variation of COD removal rate with time

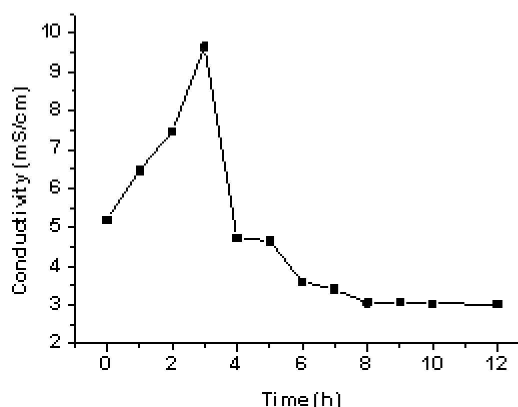


**Fig. 7.** Variation of conductivity with time

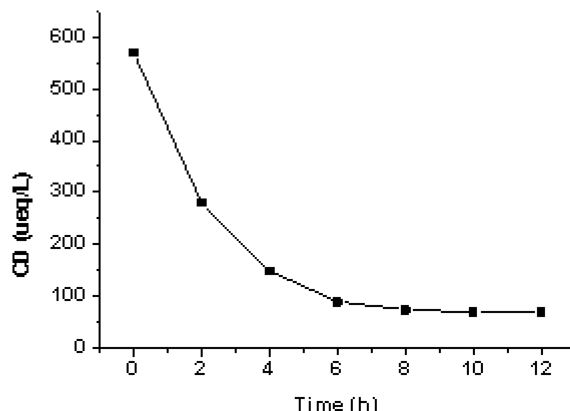


**Fig. 8.** Variation of cationic demand with time

As shown in Figs. 6 through 8, when the white water was treated for 6 h by the microflora, it achieved a better treatment effect. The removal rate of COD reached 92.6%, the conductivity was reduced to 3.60 mS/cm, and the CD was reduced to 70 μeq/L. Additionally, when the treatment time was 4 h, the exhibited treatment effect was better. In laboratory tests, the treatment effect of single dominant bacteria was obviously lower than that of mixed dominant flora. Specifically, the results showed that the optimum metabolic temperature and pH of all the three dominant bacteria were 30 °C and 6, under which COD removal rates were 75.4%, 77%, and 67.7% by *Bacillus subtilis*, *Bacillus cereus*, and *Virgibacillus pantothenticus*, respectively, for 14 h treatment. The CD values were reduced to 140 μeq/L, 90 μeq/L, and 175 μeq/L. The conductivity values were reduced to 4.72 mS/cm, 4.14 mS/cm, and 4.14 mS/cm, respectively. In summary, better removal efficiencies were achieved by the mixed dominant flora than that of single dominant bacteria. The main contributors of COD were degradation products and derivatives of lignin that accounted for 60%, while carbohydrates, sterols, and triglyceride compounds accounted for 20%. However, most of the substances were degraded by the dominant bacteria, leading to the decrease of the COD concentration. Moreover, *B. cereus*, *B. subtilis*, and *M. pantothenate* can convert compounds of polycyclic aromatic molecules and other long chain organic molecules into small molecular substances (Ai *et al.* 2014). Some lignin derivatives were decomposed into small molecule acids, phenols, and alcohols and these degradation products could be ionized to hydrogen ions and the negative ions, increasing the conductivity of wastewater. Consequently, the conductivity steadily climbed. With increased processing time, a part of small molecule intermediates was assimilated by the dominant bacteria. The other part was completely decomposed into water and $CO_2$ to provide energy for the microbial growth and catabolism. Furthermore, $CO_2$ was released from the system and into the air. As a result, the conductivity descended. Therefore, the increase of conductivity reflected the degradation efficiency of organic matter in the white water, and the declining degree of subsequent conductivity reflected the mineralization efficiency of small molecules. In the process, colloids in the white water with a negative charge were decomposed into small molecular substances, making the anionic trash decrease, and thus the cationic demand decreased. However, after 6 h, the nutrient matrix in the wastewater had been consumed substantially, and as a result, the treatment effect was not changed. In conclusion, the most suitable treatment cycle for white water by the micro flora was 4 h to 6 h.

## CONCLUSIONS

1. Through a traditional morphological analysis, the MIDI Sherlock automatic identification system, and the 16S rRNA sequence alignment, the three dominant bacteria isolated from activated sludge were identified as *Mycobacterium pantothenate*, *Bacillus cereu*s, and *Bacillus subtilis*.
2. When the treatment effect of white water reached a stable level, the proportion ratio of *B. cereus*, *B. subtilis*, and *M. pantothenate* was 50%:35%:15%. The white water treatment effect had increased notably with 90% of the COD removal rate at the proper processing time of 4 h to 6 h.
3. Through the results of GC-MS analysis, resin acid, fatty acid, vanillin, and other lignin derivatives in the white water were greatly reduced after treatment by the efficient microflora, and the contents of small molecules, such as glycerol, acetic acid, acetone, and so on, were clearly increased. The total amount of pollutants was obviously reduced after being treated by the efficient microflora.

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