Chemical oxygen demand removal in Na2SO4 saturated wastewater by cultivable halo-tolerant bacterial community

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Chemical oxygen demand removal in Na_2SO_4 saturated wastewater by cultivable halo-tolerant bacterial community

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Running title: COD removal in saline wastewater by CHBC.

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Abbreviations: A_{600}, absorbance measured at 600nm wavelength; BOD_5, biochemical oxygen demand after 5 days; COD, chemical oxygen demand; CHBC, cultivable halo-tolerant bacterial community; DO, dissolved oxygen; MLSS, mixed liquor suspended solid; TDS, total dissolved solids.

The GenBank/EMBL/DDBJ accession numbers for the nearly complete 16S rRNA gene sequences of strains CB03, MN3-2, NH7-4, BY4, XM15, BYXT, AXY1, T211, HM4, PY3-1, YY1 are KF956044, KF956045, KF956046, KF956047, KF956048, KF956049, KF956050, KF956051, KF956052, KF956053, KF956054 respectively.

Abstract

Industrial wastewater with high Na_2SO_4 concentration is characterized by high osmotic pressure and salinity, and requires pretreatment before discharging into central treatment facilities. To date, few studies have addressed the COD removal in biotreatment of Na_2SO_4 wastewater. Here, a novel aerobic system for treating Na_2SO_4 wastewater was developed based on screening of halo-tolerant bacterial strains. The system has maintained a stable chemical oxygen demand (COD) removal at > 90% in saturated concentration (varied from 5% to 40% depends on temperature) of Na_2SO_4 for five years. Activated sludge was initially constructed by cultured strains of *Hyphomicrobium sp.* CB03, *Dietzia sp.* XM15, *Staphylococcus sp.* T211, *Flavobacterium sp.* AXY1, *Ochrobactrum sp.* BY4, *Bacillus sp.* BYXT, *Sphingobacterium sp.*YY1, *Rhodococcus sp.* NH7-4, *Stappia sp.* HM4, *Microbacterium sp.* PY3-1 and *Pseudomonas sp.* MN3-2. Uncultured phylum TM7 was detected in sludge after acclimatization. The present aerobic cultivable halo-tolerant bacterial community (CHBC) was successfully applied to large scale removal of organic pollutants in saline wastewater.

Keywords: COD, cultivable halo-tolerant bacteria, microbial community, saline wastewater, sulfate.

1. Introduction

Sulfate-rich wastewater is generated by industrial processes that used sulfuric acid, sulfite and sulfide. The high concentration of sulfate leads to increased osmotic pressure and high salinity of the wastewater. Under such condition, the acclimatization of activated sludge used for biotreatment becomes severely inhibited. We postulate that the high osmolarity is directly responsible for the failure to acclimatize activate sludge; however, we cannot rule out other factors at this point. Moreover, sulfate is reduced to sulfide in the anaerobic bioreactor, which increases the toxicity of the wastewater hence further undermining the COD removal rate [1]. As such, biotreatment of sulfate-rich wastewater remains a distinct challenge and underutilized despite its great potential. Here, we describe a novel system that leverages halotolerant bacteria to treat high salinity wastewaters.

Halo-tolerant microorganisms and halophiles from archaea, bacteria and eukaryote domains, can survive in extremely hypertonic environment. Much research has revealed the mechanisms underlying their halo-tolerance [2, 3]. Some halo-tolerant microbes not only thrive but also utilize organic compounds at an impressive rate under high osmotic pressure [4], making them valuable candidates for the bio-treatment of saline waste. However, few studies to date have investigated the performance of aerobic biotreatment method under high Na_2SO_4 concentration level.

In this study, a cultivable halo-tolerant bacterial community (CHBC) was developed to treat an extreme sulfate-rich wastewater : Na_2SO_4 saturated effluent (Na_2SO_4 concentration up to 400000 $mg \cdot l^{-1}$, COD ≈ 20000 $mg \cdot l^{-1}$, $BOD_5/COD = 0.44 \sim 0.51$) yielded by a typical fine chemical factory. The factory mainly produces aliphatic esters from aliphatic acids and excess alcohols, using concentrated sulfuric acid as catalyst and dehydrator in the condensation reaction (Supplemental Material, Fig. S1, a).

The result is extremely high level of sulfuric acid in the sewage. In addition, the effluent contains alcohols, aliphatic acids, esters, sulfuric acid, sodium chloride and other undetermined pollutions. After neutralization by NaOH, the sulfuric acid turns into crystallized Na_2SO_4 (Supplemental Material, Fig. S1, b). Approximately 40 tons of this sewage is yielded per day. According to wastewater regulations in China [5], wastewater of COD > 500 $mg \cdot l^{-1}$ is not allowed to be discharged into the local sewage treatment center. Therefore, pre-treatment is necessary. Prior to this study, a conventional treatment system (Supplemental Material, Fig. S1, c) had been built, but it failed to meet the law restriction of COD < 500 $mg \cdot l^{-1}$, consequence of the strong inhibitory effect of high level osmotic pressure on regular sludge [6].

2. Materials and methods

2.1. Effluent

The effluent samples were collected from Haiyan Fine Chemical Factory (Jiaxing, Zhejiang, China, 30°36'04" N, 121°02'21" E). To investigate the impact of Na2SO4 concentration, neutralized sewage incubated at 4, 10, 20, 30 °C were filtered after crystallization to make different final concentrations of Na_2SO_4.

2.2. Strains

To obtain effective halo-tolerant microbial community to treat the sulfate-rich wastewater, a massive screening of strains was conducted. Over the past decade, hundreds of halo-tolerant prokaryotic species have been isolated from different salty samples (e.g. sea water, mirabilite mine, salt lake) in our laboratory, and preserved permanently in freeze-dried ampules in our own collection (availability:

http://www.zjubiolab.zju.edu.cn/wumin). All strains had been classified by 16S rRNA gene sequence as previously described [7, 8]. 0.1% KH_2PO_4 was used as selective medium to screen strains for adaptability and A_{600} of the cultured medium was measured (spectrophotometer, DR3900, HACH) to determine the activities of strains. Filtered (0.22μm, at 20 °C) sewage was supplemented with 0.1% (w/v) urea.

2.3. Bioreactor

For laboratory simulation, plexiglass simulators (Supplemental Material, Fig. S2, a) were used to simulate actual treatment system at Haiyan Fine Chemical Factory. The selected strains were mixed and acclimatized in it to form sludge. The power of aerator was fixed to make DO range from 3 to 4 $mg \cdot l^{-1}$. The sewage continuously flowed in and out of the reactor at a fixed rate of 2 liters per day. Hydraulic retention time (HRT) was 48 hours. COD loading rate of aerated reactors was from 3.0 to 4.3 $g \cdot l^{-1} \cdot day^{-1}$ and MLSS was about 1.0 $g \cdot l^{-1}$. Pure cultured sewages of selected strains were mixed equally in simulator for acclimation. Influent sewage was stored at 20 °C and simulator worked in thermostat room at 24°C. The solubility of Na_2SO_4 varies with temperature, and variable osmotic pressure could damage sludge in some cases [6]. Based on the simulator mentioned above, the impact of Na_2SO_4 concentration on sludge viability was investigated. To obtain sewages of different Na_2SO_4 concentrations, original sewages were neutralized and kept at 4, 10, 20, 30 °C overnight and the upper clear liquors were independently decanted to simulators for treatment at 37 °C with other parameters same of the above.

For actual application in factory setting, the existing facilities in Haiyan Fine Chemical Factory were adapted to the new system developed in this study (Supplemental Material, Fig. S2, b). Instead of plexiglass, the tanks were concrete, sulfate

corrosion proof structures. The sludge was bred in oxidation tank until sufficient sludge was acquired. The sewage was de-greased first, and then neutralized by NaOH. After the removal of sediments, the upper sewage flowed continuously into oxidation tank containing the sludge. HRT = 48 h, DO = 3.0 - 4.0 $mg \cdot l^{-1}$, COD loading rate was from 3.2 to 4.0 $kg \cdot m^{-3} \cdot day^{-1}$ and MLSS was from 0.9 to 1.3 $g \cdot l^{-1}$.). The treated effluent was discharged after precipitating the sludge. 21.9 mg urea and 1.4 mg KH_2PO_4 per 1000 mg COD were added to sewage as supplements for both laboratory simulation and actual implementation.

2.4. Analysis

2.4.1. Interaction

Some bacteria secrete antibiotics and acids to inhibit other species, which may be destructive for maintaining a stable and effective bacterial community [9]. To understand if any strains studied in this research could potentially inhibit the others, cells of all well-grown strains (Sewage $A_{600} > 0.5$ after cultivation for a week) were collected and pairs ofstrains were mixed equally and cultured in sewage for a week at 25 °C in flasks. Every possible pairing combination was investigated. The cultured sewages were filtered (0.22μm) to determine residual COD every day during cultivation. The lowest detected COD value of each flask was recorded in Table 1.

2.4.2. Morphology

The sludge morphology reflects the microbial diversity and influences the activity of sludge [10]. Sludge was observed directly by optical microscopy (BX40F4, Olympus). After critical point drying treatment [11], the microbes in sludge was observed

by scanning electron microscopy (SEM) (S-3000N, Hitachi).

2.4.3. Microbial diversity

To investigate the microbial diversity, the total DNA in the sludge was extracted by Fast DNA Spin Kit for Soil (MP biomedicals). After extraction, archeal 16S rRNA genes were amplified by forward primer W17 (5′-ATTCYGGTTGATCCYGSCRG-3′) and reverse primer W02 (5′-GNTACCTTGTTACGACTT-3′) [12]. Eukaryotic 18S rRNA genes were amplified by forward primer W99 (5′-CGGTAATTCCAGCTCC-3′) [13] and W02. PCR reactions were performed as described (Lefebvre et al. [14]) . Bacterial diversity based on 16S rRNA gene were sequenced and analyzed by Illumina MiSeq Platform as previously described [15].

2.4.4. Other analyses

COD, BOD_5, MLSS, $N\text{-}NH_3$ and $P\text{-}PO_4^{3-}$ were analyzed according to Standard Methods [16]. DO, pH and temperature were determined by multi-parameter handheld meter (Multi 350i; WTW). The amount of SO_4^{2-} was determined by $BaCl_2$ [17].

3. **Results and discussion**

3.1. Effluent quality

Several batches of sewage were collected for laboratory use Their COD varied from 18600 to 25500 $mg \cdot l^{-1}$; $N\text{-}NH_3^+$ from 2.1 to 4.3 $mg \cdot l^{-1}$; $P\text{-}PO_4^{3-}$ from 0 to 0.2 $mg \cdot l^{-1}$; $pH < 1$; appeared light yellow and exuded acrid smell. After neutralization and filtration, the samples displayed COD ranging from 6100 to 8500 $mg \cdot l^{-1}$; $N\text{-}NH_3^+$ from 1.2 to 3.8 $mg \cdot l^{-1}$; $P\text{-}PO_4^{3-}$ from 0 to 0.2 $mg \cdot l^{-1}$; $pH = 7.2$ and appeared colorless.

3.2. Selected strains

37 of 475 strains survived (A_{600} > 0.1) in sewage and 15 of them grew to A_{600} > 0.5. The compatibility tests among the 15 strains were conducted by mixed cultivation as Table 1. The result showed that *Hyphomicrobium sp.* CB03, *Dietzia sp.* XM15, *Staphylococcus sp.* T211, *Flavobacterium sp.* AXY1, *Ochrobactrum sp.* BY4, *Bacillus sp.* BYXT, *Sphingobacterium sp.*YY1, *Rhodococcus sp.* NH7-4, *Stappia sp.* HM4, *Microbacterium sp.* PY3-1 and *Pseudomonas sp.* MN3-2 are compatible , while *Pseudomonas sp.* GE2, *Halobacterium sp.* AE4-1, *Natrinema sp.* XJ5 and *Actinopolyspora sp.* DH9-1 should be eliminated from the mixture. Some species of *Natrinema* and *Actinopolyspora* were reported to produce antibiotics which might be the reason of inhibition to other strains [18, 19]; however, further research is needed to confirm this speculation. The chosen strains belonged to *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes* families according to systematic analysis by MEGA 5.0 software [20] (Fig. 1). As the Na_2SO_4 concentration of the sewage was over 15%, all of the chosen strains exceeded expectation in halo-tolerance given that they were originally isolated from sea water, ocean sediment and saline wastewaters with TDS (mainly NaCl) less than 4%, which suggested that CHBC might be less sensitive to Na_2SO_4 concentration.

3.3. Bioreactor performance

Effluent COD in the simulator bioreactor was maintained at < 500 $mg \cdot l^{-1}$ and its removal rate > 90% after running for 23 days. Although the influent quality varied, the COD removal rate was maintained at > 90% (Fig. 2). Residual $N\text{-}NH_3^+$ and $P\text{-}PO_4^{3+}$ was maintained below 5 $mg \cdot l^{-1}$ and 0.5 $mg \cdot l^{-1}$ respectively all through the test.

Influent pH started out at 6.8, but was raised to 7.6-8.2 in 24 hours. The success of simulator indicated that, with CHBC, organic pollution removal at Na_2SO_4 saturated concentration was feasible.

3.4. Impact of Na_2SO_4 concentration

The Na_2SO_4 concentrations of sewages at 4, 10, 20, 30 °C were stable at 64.2±1.8 $g \cdot l^{-1}$, 89.9±3.3 $g \cdot l^{-1}$, 186.5±7.2 $g \cdot l^{-1}$ and 383.0±6.5 $g \cdot l^{-1}$ respectively. Then the simulation was conducted at 37 °C to study the evolution of COD removal rate (Fig. 3) of sewages with four different Na_2SO_4 concentrations mentioned above. COD removal was enhanced by reduced Na_2SO_4, albeit the effluent COD in highest Na_2SO_4 level still maintained the < 500 $mg \cdot l^{-1}$ threshold. The result indicated that the increasing Na_2SO_4 reduced COD removal rate of CHBC significantly (*t*-test, α=0.05), which was similar to the effect of NaCl seen in previous studies [6]. Despite reduced efficiency, the treatment system with CHBC achieved COD < 500 $mg \cdot l^{-1}$ threshold even under the extreme constraint of highest Na_2SO_4 level.

Traditionally, sulfate is first removed from the wastewater before being treated in an anaerobic anoxic treatment system. Sulfate removal reduces osmolarity and avoids production of sulfide, thus making the wastewater amendable to common anaerobic biotreatment methods. However, sulfate removal often produces hazardous solid wastes, which causes secondary pollution and incur extra treatment costs. Here, our system circumvents the step of sulfate removal and directly applies the activated sludge comprised of selected halo-tolerant bacterial strains to Na_2SO_4 saturated wastewaters. Our assumption is that Na_2SO_4 in itself does not constitute additional environmental threats beyond the fact that it increases the osmolarity of the wastewater . Although we do not have direct evidence of this assumption, our result

indicated that increased osmolarity was the main effect of sulfate. Based on the hypothesis, we developed a halo-tolerant bacterial community, used only aerobic reactors to avoid production of sulfide in anaerobic process, and achieved COD removal without removing the sulfate. Thus, our system represents a novel method for treating sulfate rich wastewater. This method is suitable for pretreatment of wastewater containing sulfate.

3.5. Application performance

CHBC was applied in the Haiyan Fine Chemical Factory. The residual COD of discharged effluent had been monitored for more than five years (Fig. 4, a). The result showed that the new system meets the law restriction of COD < 500 $mg \cdot l^{-1}$. During the processing, an accident caused a set of aerators to shutdown in December 2009, which reduced the DO in oxidation tank to 0.2 $mg \cdot l^{-1}$. Anaerobes produced copious amount of sulfides that made the sewage black and putrid. The effluent COD rose higher than 500 $mg \cdot l^{-1}$ until the aerators were repaired. It took several months to rejuvenate the activity of sludge; and while low temperature during the winter decreased the activity of bacteria, but also reduced Na_2SO_4 concentration. The net effect of the low temperature and low Na_2SO_4 concentration was that residual COD became slightly higher in winter but still remained below 500 $mg \cdot l^{-1}$. Compared with the effect of regular sludge (acquired from local wastewater treatment center) used in previous systems, CHBC maintained higher and more stable COD removal rate (Fig. 4, b) in actual application.

3.6. Microbial community

After acclimation in the laboratory setting, the sludge was transplanted to the ac-

tual oxidation tank in the factory wastewater processing center. Under an open system, the sludge was exposed to external microbes and consequently, the composition of original strains mixture was altered. Nevertheless, the high osmotic pressure created by the hypersaline environment prevented the establishment of most exogenous microbes: neither archaeal 16S rRNA gene nor eukaryotic 18S rRNA gene was detected by PCR amplification, and no protist was observed by microscope. The MiSeq analysis of bacterial community revealed that exogenous uncultured phylum TM7 occupied approximately 15% in relative abundance. Meanwhile, *Proteobacteira*, *Actinobacetira* and *Bacteroidetes* families comprised approximately 75% relative abundance as the dominant phyla in the sludge (Supplemental Material, Fig. S3). Short partial 16S rRNA gene (about 300 bps) sequences read by MiSeq Platform were consistent with GenBank KF956044 – 956054, indicating that the original 11 strains settled in the sludge and formed dominant species except *Staphylococcus sp.* T211 and *Bacillus sp.* BYXT. Furthermore, confirming the result of PCR, the SEM graphic showed abundance of bacteria (Supplemental Material, Fig. S4) and absence of eukaryotic cells. While similar bacterial distribution in saline sludge was discovered in previous research [14], the CHBC revealed a very simple distribution of cultivable strains which made it easier to analyze, control and rebuild.

4. Conclusion

High salinity in sewage inhibits bioactivity by raising the osmotic pressure to lethal levels for cells [21, 22], making biotreatment of sulfate-rich wastewater a distinct challenge. This study circumvents this challenge with the use of CHBC, and demonstrates that CHBC can be effectively applied to treat sulfate-rich wastewater with variable Na_2SO_4 concentration (6.4% - 38.3%) at wide temperature range (4°C - 37°C).

Proteobacteira, *Actinobacetira* and *Bacteroidetes* were the main classes in this case. Application of CHBC revealed consistently high COD removal rate of > 90% for years. This method proved suitable for removing bio-degradable organic pollutants with high salinity.

5. Acknowledgement

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6. Conflict of interest

The authors have declared no conflict of interest.

References

[1] L. W. Hulshoff Pol, P. N. L. Lens, A. J. M. Stams, G. Lettinga, Anaerobic treatment of sulphate-rich wastewaters. *Biodegradation*. **1998**, *9, 213-224.*

[2] A. Oren, Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Syst*. **2008**, *4:2*.

[3] R. H. Vreeland, Mechanisms of Halotolerance in Microorganisms. *Crit Rev Microbiol*. **1987**, *14, 311-356*.

[4] C. Hinteregger, F. Streichsbier, *Halomonas sp.*, a moderately halophilic strain, for biotreatment of saline phenolic waste-water. *Biotechnol Lett*. **1997**, *19, 1099-1102*.

[5] MEPC, *Integrated wastewater discharge standard, GB 8978-1996*. Ministry of Environmental Protection of China, Beijing, **1996**.

[6] D. F. Kincannon, A. F. Gaudy Jr., Some effects of high salt concentrations on activated sludge. *J. Water Pollut. Control Fed.* **1966,** *38, 1148–1159.*

[7] X. W. Xu, S. J. Liu, D. Tohty, A. Oren, M. Wu, P. J. Zhou, *Haloterrigena saccharevitans* sp. nov., an extremely halophilic archaeon from Xin-Jiang, China. *Int J Syst Evol Microbio*l. **2005**, *55, 2539–2542.*

[8] X. W. Xu, Y. H. Wu, Z. Zhou, C. S. Wang, Y. G. Zhou, H. B. Zhang, Y. Wang, et al., *Halomonas saccharevitans* sp. nov., *Halomonas arcis* sp. nov.and *Halomonas subterranean* sp. nov., halophilic bacteria isolated from hypersaline environments of China. *Int J Syst Evol Microbiol.* **2007**, *57, 1619–1624.*

[9] P. Ilke, R. Ateequr, U. Emine, J. Daniel, O. Derin, Pyrosequencing reveals the inhibitory impact of chronic exposure to erythromycin on activated sludge bacterial community structure. *Biochem Eng J*.**2014**,*90,195-205.*

[10] J. C. Williams, F. L. de los Reyes, Microbial community structure of activated sludge during aerobic granulation in an annular gap bioreactor. *Water Sci Technol*.

2006, *54, 139-146.*

[11] J. H. Carr, R. L. Anderson, M. S. Favero, Comparison of chemical dehydration and critical point drying for the stabilization and visualization of aging biofilm present on interior surfaces of PVC distribution pipe. *J Appl Bacteriol*. **2008**, *80, 225-232*.

[12] J. J. Godon, E. Zumstein, P. Dabert, F. Habouzit, R. Moletta, Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol.* **1997**, *63:2802–22813.*

[13] V. P. Edgcomb, D. T. Kysela, A. Teske, A. de Vera Gomez, M. L. Sogin, Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. *Proc Natl Acad Sci USA.* **2002**, *99:7658–7662.*

[14]O. Lefebvre, N. Vasudevan, K. Thanasekaran, R. Moletta, J. J. Godon, Microbial diversity in hypersaline wastewater: the example of tanneries. *Extremophiles.* **2006,** *10,505-13.*

[15] J. G. Caporaso, C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, et al., Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **2012**, *6, 1621-1624.*

[16] APHA, *Standard Methods for the Examination of Water and Wastewater, 20th ed.* American Public Health Association, Washington, D.C., **1998**.

[17] MEPC, *Water quality, determination of sulfate, gravimetric method, GB 11899-89.* Ministry of Environmental Protection of China, Beijing, **1989**.

[18] P. Karthikeyan, S. G. Bhat, M. Chandrasekaran, Halocin SH10 production by an extreme haloarchaeon *Natrinema sp.* BTSH10 isolated from salt pans of South India. *Saudi J Biol Sci*. **2013**, *20,205-212.*

[19] C. R. Kokare, K.R. Mahadik, S.S. Kadam, B.A. Chopade, Isolation, characterization and antimicrobial activity of marine halophilic *Actinopolyspora* species AH1

from the west coast of India. *Curr Sci*.**2004**, 86:593–597.

[20] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* **2011,** *28, 2731–2739.*

[21] A. Ventosa, M. C. Marquez, M. J. Garabito, D. R. Arahal, Moderately halophilic gram-positive bacterial diversity in hypersaline environment. *Extremophiles.* **1998,** *2, 297-304.*

[22] A. Ventosa, J. J. Nieto, A. Oren, The biology of aerobic moderately halophilic bacteria. *Microbiol Mol Biol Rev.* **1998,** *62, 504-544.*

Legends to Figures:

Fig. 1. Neighbor-joining tree based on the 16S rRNA gene sequences of selected strains. Bootstrap percentages are based on 1000 replicated datasets; bar, 0.05 substitutions per nucleotide position.

Fig. 2. Evolution of COD and its removal rate in simulators during acclimatization.

Fig. 3. Evolution of COD in simulators at different Na_2SO_4 concentrations. The data were collected after simulators were kept running for 5 days. Height of blue background indicated the influent sewage COD of three batches used in this test.

Fig. 4. Application effect of regular sludge and CHBC. a, the evolution of effluent COD after treatment; current system replaced the old one in Jun 2009; red short lines represent the summer days, blue lines for winter; the curve was drawn according to the average COD values per 30 days. Increasing red indicates higher COD value. b, comparison of the COD data of the old system used regular sludge and current system used CHBC; data for statistical analysis was collected from Aug 23, 2008 to Apr 25, 2009 and from Mar 10, 2010 to Mar 29, 2013 for previous and current system respectively; inf., influent; eff., effluent; st.dev., standard deviation.

Legends to Table

Table 1. The interaction of the strains. Increasing red indicates higher residual COD. COD_n (n=1-15) was calculated as average of the 14 residual COD values of cultured sewage mixed with strain *n* and the other 14 strains in pairs. The $COD_{1, 6, 10, 11}$ were significantly higher than the average level of COD_n (Welch's test, α=0.05), which indicated that *Pseudomonas sp.* GE2, *Halobacterium sp.* AE4-1, *Natrinemapallidum sp.* XJ5 and *Actinopolyspora sp.* DH9-1 reduced the COD removal effect when mixed with other strains

Strain	No.														
Halobacterium sp. AE4-1	1	628													
Hyphomicrobium sp. CB03	2	774	500												
Dietzia sp. XM15	3	1160	366	580											
Staphylococcus sp. T211	4	1010	216	337	438										
Flavobacterium sp. AXY1	5	1260	205	600	186	622									
Natrinema sp. XJ5	6	1320	1150	1220	1100	1290	580								
Ochrobactrum sp. BY4	7	764	438	235	568	528	1250	825							
Bacillus sp. BYXT	8	970	502	224	198	415	730	590	758						
*Sphingobacterium sp.*YY1	9	1080	254	535	530	466	1040	548	590	410					
*Actinopolyspora sp.*DH9-1	10	732	1240	815	955	880	1290	706	784	1090	778				
Pseudomonas sp. GE2	11	880	738	1160	830	970	850	1080	1340	1020	800	574			
Rhodococcus sp. NH7-4	12	1330	419	554	200	276	920	347	518	568	778	704	624		
Stappia sp. HM4	13	1310	310	225	216	229	950	371	475	544	822	1020	590	502	
Microbacterium sp. PY3-1	14	1190	395	526	494	194	1090	279	475	344	868	1230	472	292	311
Pseudomonas sp. MN3-2	15	1300	406	344	432	406	1170	455	256	222	1310	980	460	454	556	504
COD_n		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		1077	530	593	519	565	1098	583	576	631	934	972	581	558	600	625

Residual COD ($mg \cdot l^{-1}$): 1400, 1200, 1000, 800, 600, 400, 200, 0

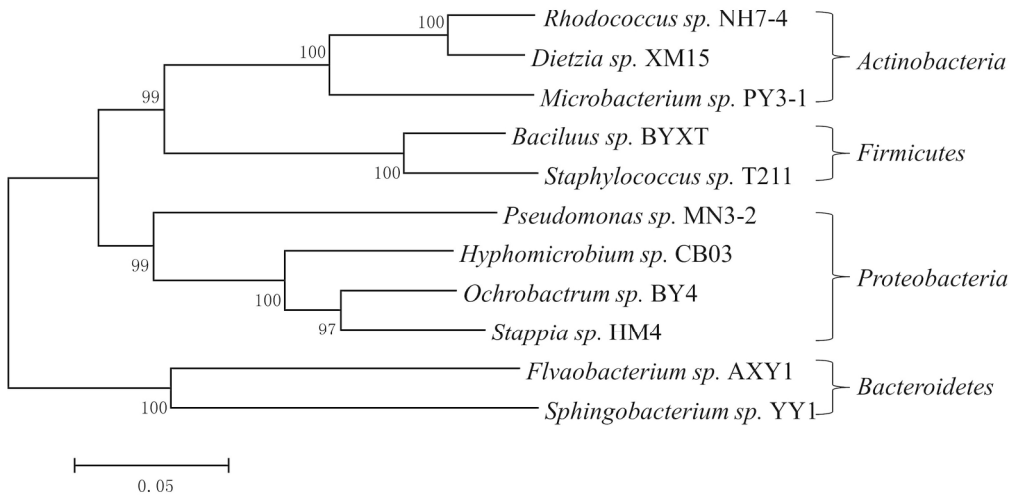

Fig. 1. Neighbor-joining tree based on the 16S rRNA gene sequences of selected strains. Bootstrap percentages are based on 1000 replicated datasets; bar, 0.05 substi-tutions per nucleotide position.
190x142mm (300 x 300 DPI)

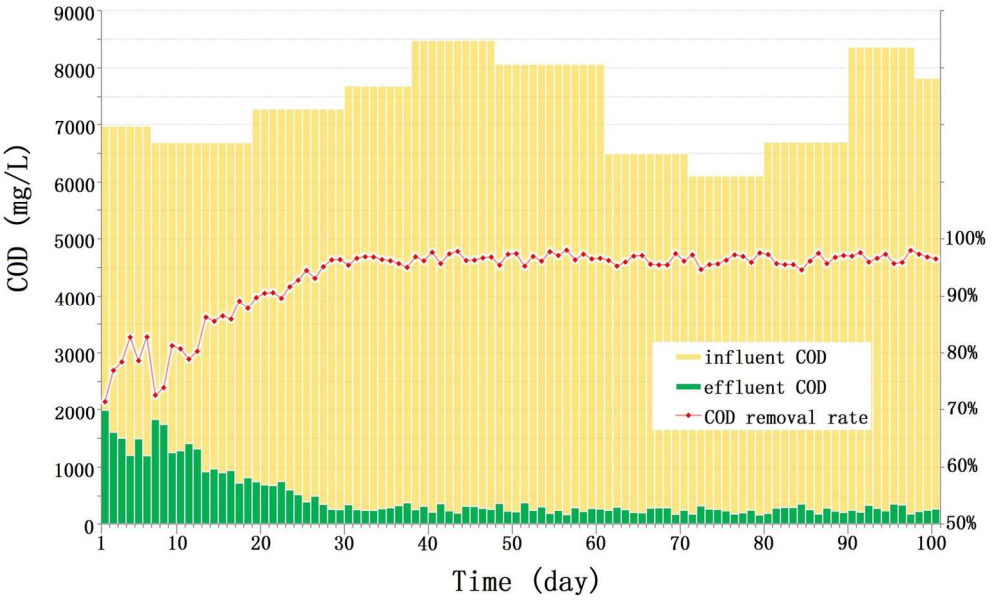

Fig. 2. Evolution of COD and its removal rate in simulators during acclimatization.
164x101mm (300 x 300 DPI)

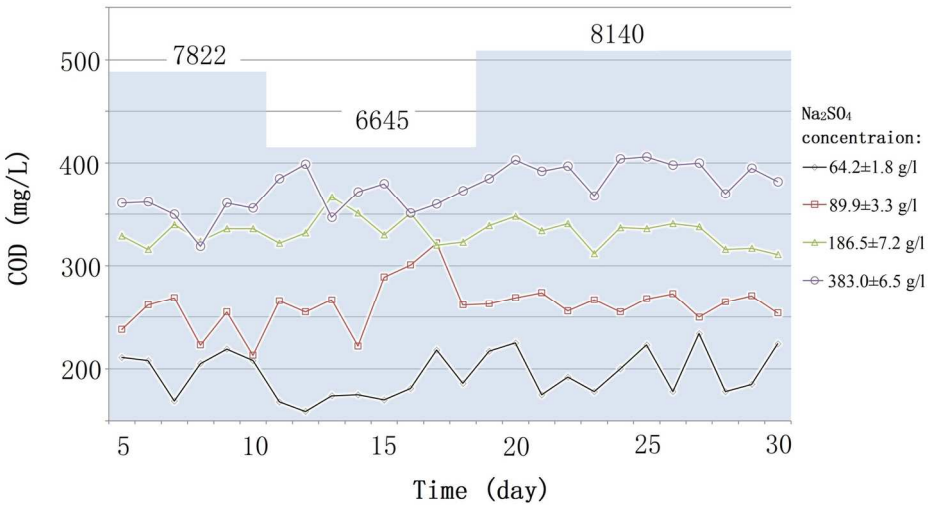

Fig. 3. Evolution of COD in simulators at different Na2SO4 concentrations. The data were collected after simulators were kept running for 5 days. Height of blue back-ground indicated the influent sewage COD of three batches used in this test.
151x79mm (300 x 300 DPI)

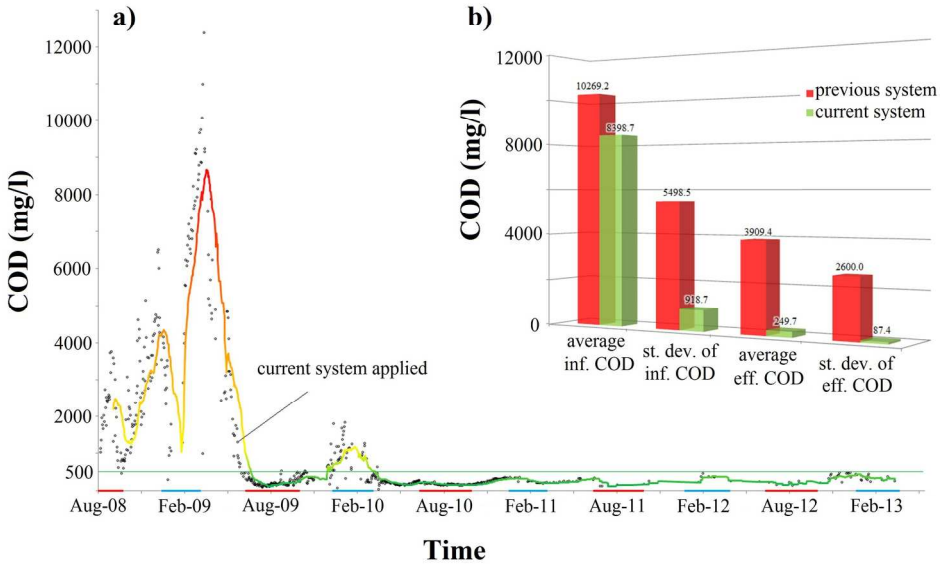

Fig. 4. Application effect of regular sludge and CHBC. a, the evolution of effluent COD after treatment; current system replaced the old one in Jun 2009; red short lines represent the summer days, blue lines for winter; the curve was drawn according to the average COD values per 30 days. Increasing red indicates higher COD value. b, comparison of the COD data of the old system used regular sludge and current system used CHBC; data for statistical analysis was collected from Aug 23, 2008 to Apr 25, 2009 and from Mar 10, 2010 to Mar 29, 2013 for previous and current system respec-tively; inf., influent; eff., effluent; st.dev., standard deviation.
183x120mm (300 x 300 DPI)

Supplementary

Fig. S1. The chemical reaction yielding Na_2SO_4 ; diagram of the treatment system used prior to this study. a, the main chemical reaction of Na_2SO_4 production; b, the $Na_2SO_4 \cdot 10H_2O$ crystal formed in the sewage after neutralization; c, the treatment system operated before the study. R and R', hydrocarbon chain; UASB, Up-flow anaerobic sludge bed.

Fig. S2. Simulator and treatment system applied in this study. a, the simulator with cells of 5 liters; b, the treatment system converted from the simulator to treat large scale industrial wastewater.

Fig. S3. Relative abundance of phyla detected in sludge by MiSeq.

Fig. S4. SEM graphic of sludge formed by CHBC.

Fig.S1.

a)

$$\text{R-COOH} + \text{R'-OH} \xrightarrow[\Delta]{H_2SO_4} \text{R-COO-R'} + H_2O$$

b)

c)

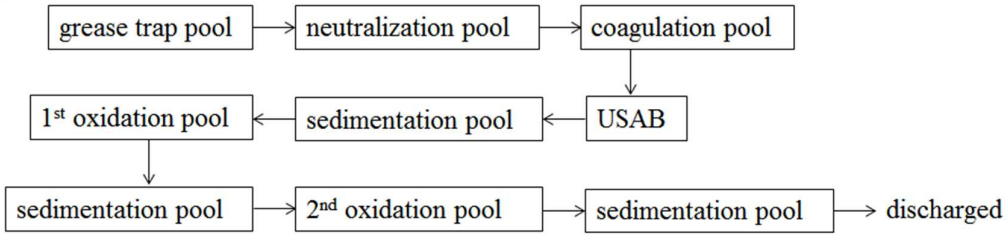

Fig. S2.

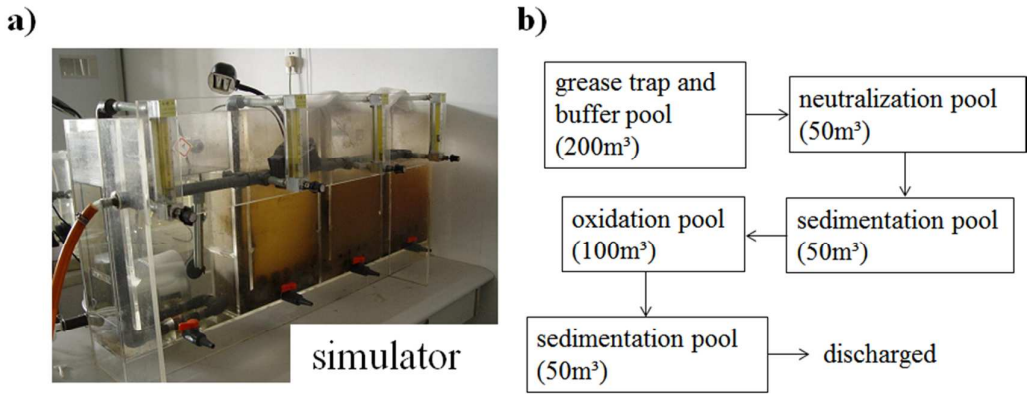

Fig. S3.

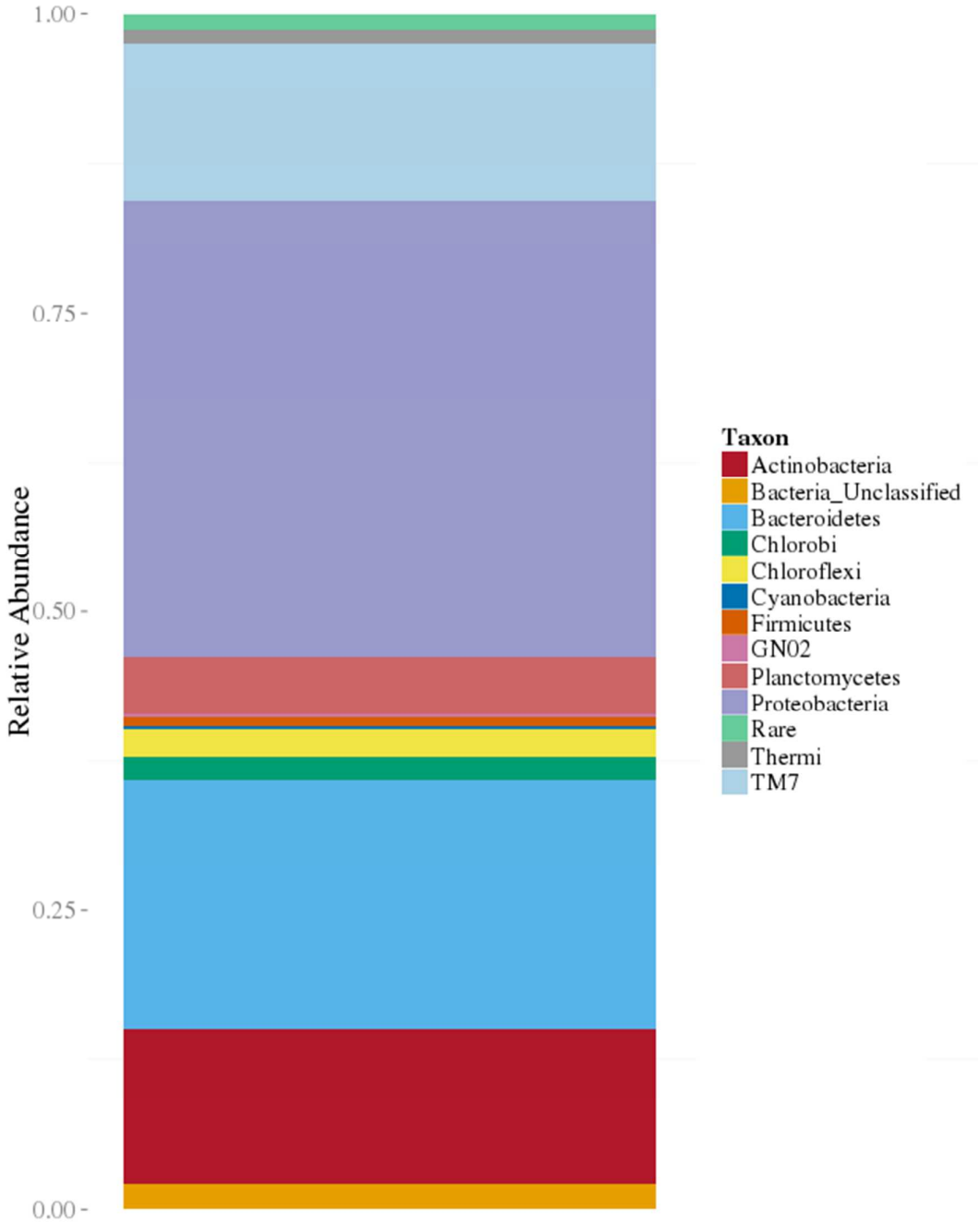
1.00
0.75
0.50
0.25
0.00
Relative Abundance
Taxon
Actinobacteria
Bacteria_Unclassified
Bacteroidetes
Chlorobi
Chloroflexi
Cyanobacteria
Firmicutes
GN02
Planctomycetes
Proteobacteria
Rare
Thermi
TM7

Fig. S4