academicJournals

Vol. 7(44), pp. 5119-5125, 7 November, 2013
DOI: 10.5897/AJMR2012.2289
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation and characterization of a *Bacillus* strain for alkaline wastewater treatment

Kun Chen[1, 2], Jing Yang[1, 3] and Hua Zhao[1, 3]*

[1]Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, College of Bioengineering, Tianjin University of Science and Technology, Tianjin, China.
[2]School of Pharmaceutical, Liaocheng University, Shandong, China.
[3]Tianjin Key Laboratory of Industrial Microbiology, Tianjin, China.

Accepted 8 October, 2013

Biological treatment is one of the considerable choices for removing of organic pollutants present in petrochemical wastewaters. In this study, five strains, named as BS1, BS2, BS3, BS4 and BS5, were isolated from sludge close to a petroleum smelter. BS5, the isolate with the highest chemical oxygen demand (COD) removal rate, was identified as *Bacillus flexus*, based on 16S rDNA sequences. Subsequently, the optimized COD removal conditions of BS5 were investigated. It was indicated that the optimal conditions were 0.5% corn starch, 1% corn steep liquor, 35°C pH 7.5, and 10% (v:v) inoculation size. Under such circumstance, the removal rate of COD can reach 81.04%. The isolation of *Bacillus flexus* strain BS5 provided an alternative for the bioremediation of alkaline wastewater. Lastly, the study showed that consecutive disposal process may help to reducing COD of wastewater effectively.

Key words: Alkaline wastewater, *Bacillus flexus*, chemical oxygen demand (COD).

INTRODUCTION

Petrochemical production technology had became more sophisticated in recent years, in turns, organic substances in the sewage tends to be prosperous, which caused a great burden to traditional treatment process (Rao et al., 2007). Sewage in petroleum processing industry was mainly resulted from liquid hydrocarbon alkali refining, diesel alkaline cleaning, and ethane pyrolysis gas alkaline cleaning process and so on (Garcia et al., 2000). Such sewage contained neutral oil, volatile phenol, sulfide and other toxic and detrimental organic substance in abundance (Xie et al., 2007), which endowed it with a high chemical oxygen demand (COD), high total dissolved solids, high levels of volatile phenol and sulfide, and high alkalinity (Ma et al., 2006).

The sewage discharged contaminates the environment gravely and also has seriously impacts on the subsequent disposal process (Prisciandaro et al., 2005).

Nowadays, most of sewage disposal techniques based on the recovery of the naphthenic acid and phenol (Choe et al., 2005), while the alkaline residue wastewater should be processed after the recovery (David and Peter, 1995).

Modern biotechnology, especially molecular techniques, to select and cultivate the dominant strains so to enhance the biological systems removal capability to refractory organics (Richard, 1983). Comparing with other methods, this method has many advantages, such as lower cost, higher efficiency, easier to be handled and without secondary pollution (Loh and Liu, 2001). All those characteristics attracted a great attention from all over the world (Leal et al., 1998). Many relevant studies had been carried out, including printing and dyeing, chemical waste water and removal characteristics (García Becerra et al., 2010).

In this study, bacteria with high efficiency of degrading alkaline wastewater had been screened from sludge

*Corresponding author. E-mail: zhaohua@tust.edu.cn. Tel.: +86 22 60601369. Fax: +86 22 60601369.

Table 1. Composition of the sublayer liquid.

Property	pH	COD (g/L)	Sulfide (mg/L)	Phenol (mg/L)
Value	6.73	58	996	41.39

nearby a petroleum smelter. Furthermore, a strain with the highest COD removal rate was identified and characterized for the possible applications for environmental management.

MATERIALS AND METHODS

Samples

Sludge samples were collected from a petroleum smelter.

Bacteria screening culture medium

Screening culture medium was LB medium which containing a different proportion of pretreated alkaline wastewater.

Alkaline wastewater obtained from the oil refinery should be pretreated. Firstly, 5.5% sulfate acids was added. 24 h later, the sublayer liquid was collected and used as experimental material. The main composition of the sublayer liquid obtained showed as Table 1.

Degrading medium

The degrading medium was consisted of pretreated alkaline wastewater, necessary salts for growth, extra carbon and nitrogen sources. The pH value was adjusted to 7.0. The medium was sterilized at 121°C for 20 min.

Isolation of strains degrading alkaline wastewater

Enrichment and isolation of bacteria degrading alkaline wastewater were done using bacteria screening culture medium.

Identification of the bacteria isolated

DNA extraction was adapted from the protocol previously (Hoisington et al., 1997). The 16S rDNA was amplified by PCR using the following universal primers pairs, 5'-AGTTTGATCCTGGCTCA-3' and 5'-ACGGCTACCTTGTTACGACTT GCA-3' (David and Peter, 1995). The protocol consisted of 30–33 cycles of incubation at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by extension for 5 min at 72°C. The 16S rDNA amplified was sequenced and blast before it was submitted to NCBI GenBank.

Method of chemical oxygen demand (COD) determination

The COD was determined by potassium dichromate method (Rocenkery, 1993). Wash culture tubes and caps with 20% H_2SO_4 before using to prevent contamination. Place sample in culture tube or ampule and add digestion solution. Carefully run sulfuric acid reagent down inside of vessel so an acid layer is formed under the sample-digestion solution layer and tightly cap tubes or seal ampules, and invert each several times to mix completely. Place tubes or ampules in block digester preheated to 150°C and reflux for 2 h behind a protective shield. Cool to room temperature and place vessels in test tube rack. Some mercuric sulfate may precipitate out but this will not affect the analysis. Remove culture tube caps and add small TFE-covered magnetic stirring bar. Add 0.05 to 0.10 mL (1 to 2 drops) ferroin indicator and stir rapidly on magnetic stirrer while titrating with standardized 0.10 M FAS (ferrous ammonium sulfate titrant). The end point is a sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample.

Determination of the optimized alkaline-degrading condition

The effects of different carbon sources, nitrogen sources, pH, temperature, inoculum concentration and consecutive treatment process on COD removal rate were investigated.

RESULTS AND DISCUSSION

Isolation of strains with high alkaline wastewater degrading activity

Five bacteria exhibiting high alkaline wastewater degrading activity were isolated after serial enrichment, which called BS1, BS2, BS3, BS4 and BS5 respectively.

The COD removal rate of the five optimized strains was compared as Figure 1. It was showed that BS5 had the highest COD removal rate, which was up to 73.42%.

Identification of BS5

The genome DNA of BS5 was extracted and its 16S rDNA was amplified by PCR. The 1067 bp product was sequenced and analyzed. It was identified as *Bacillus flexus*. The 16S rDNA sequence was submitted to NCBI GenBank and the accession number was JX677863.

Effects of nutrient substances on the chemical oxygen demand (COD) removal rate of BS5

In the processes of alkaline wastewater treatments, bacteria make use of all kinds of organic pollutant as nutrition for their growth and proliferation. But these nutrients in industry wastewater cannot fully meet the need of these demands. So, some extra nutrition should be added.

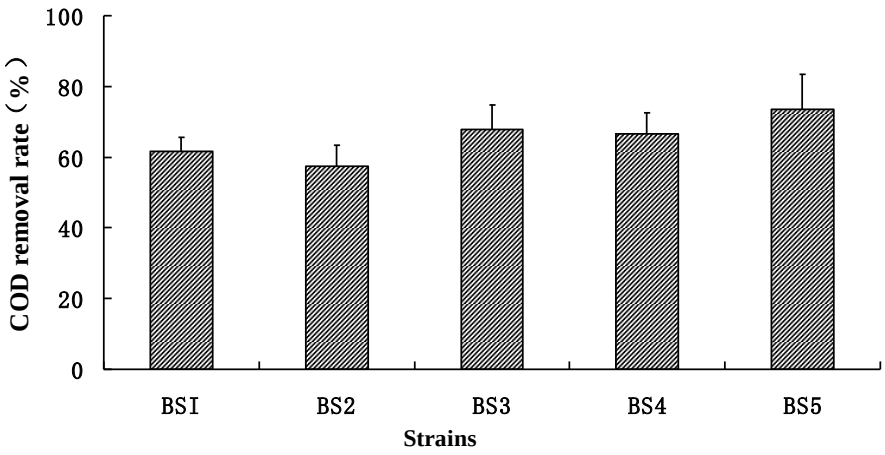

Figure 1. COD removal rate of five strains. The strains were inoculated into pretreated alkaline wastewater, respectively, and then cultured at 35°C at 150 r/min, the pH was control at 7.0. Fifty hours later, the COD value was measured.

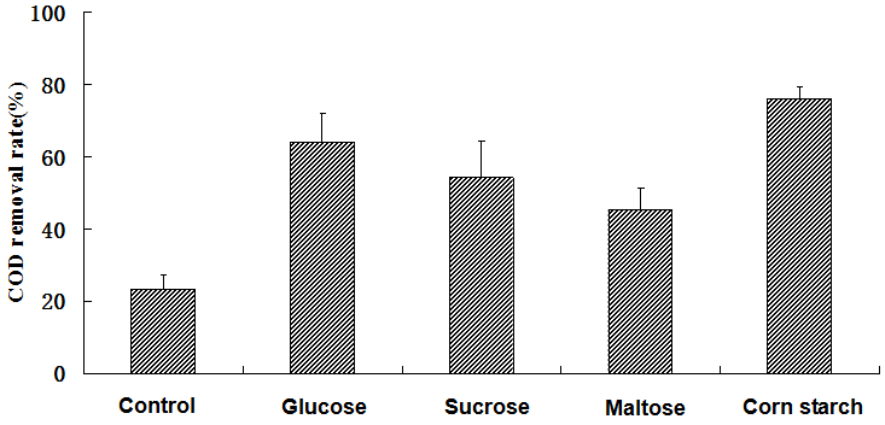

Figure 2. Effects of different carbon sources on the COD removal rate. BS5 was inoculated into degrading medium. 0.5% (mm) glucose, sucrose, maltose and corn starch were added respectively, inoculum size 10%, then cultured at 35°C at 150 r/min, the pH was control at 7.0. Fifty hours later, the COD value was measured.

Effects of different carbon sources on the COD removal rate

Glucose, sucrose, maltose and corn starch were chosen as carbon source, respectively. The group without extra carbon source was used as control. As shown in Figure 2, the addition of carbon source remarkably increased the COD removal rate of the alkaline wastewater. It was proved that the best carbon source was corn starch.

Effects of different nitrogen sources on the chemical oxygen demand (COD) removal rate

Ammonium nitrate, ammonium sulfate, bran, yeast powder and corn steep liquor was selected as extra nitrogen source, respectively. The group without additional nitrogen source was used as control. It was showed that these nitrogen sources can increase the COD removal rate dramatically (Figure 3). Corn steep liquor was performed as the optimal nitrogen source.

Besides nutrition, the physical culture conditions can also have dramatically influences on the wastewater disposal process.

Effects of temperature on the chemical oxygen demand (COD) removal rate

The effects of temperature on the COD removal rate of

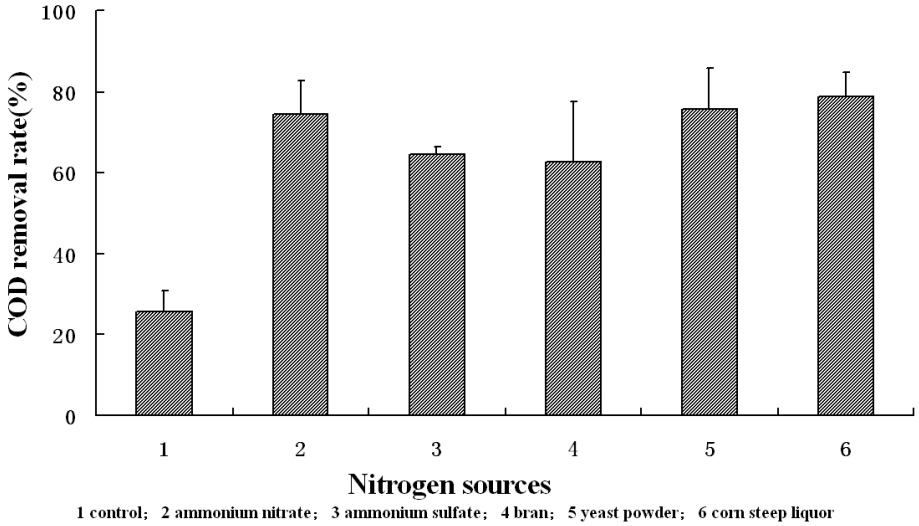

Figure 3. Effects of different nitrogen sources on the COD removal rate. BS5 was inoculated into degrading medium. 1% (m:m) ammonium nitrate, ammonium sulfate, bran, yeast powder and corn steep liquor was added, respectively. Corn starch (0.5%) was used as carbon source. The culture condition were inoculum size 10%, 35°C, pH 7.0, and agitation rate 150 r/min. Fifty hours later, the COD value was measured.

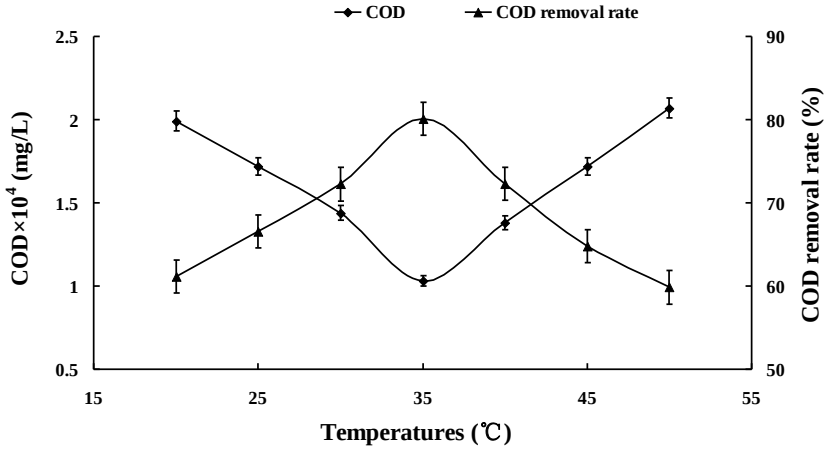

Figure 4. Effects of temperature on the COD removal rate. BS5 was inoculated into degrading medium and cultured at temperatures ranging from 20°C to 50℃. The culture condition were 0.5% corn starch, 1% corn steep liquor, inoculum size 10%, pH 7.0, and agitation rate 150 r/min. Fifty hours later, the COD value was measured.

BS5 in degrading medium were studied. It was showed that the optimum growth temperature for higher degrading rate was 35°C. When the temperature was less than 25°C or more than 40°C, the COD removal rate decreased significantly (Figure 4).

Effects of pH on the chemical oxygen demand (COD) removal rate

The effects of pH on the COD removal rate were tested. It was obviously presented that the optimum pH value for COD removal was 7.5 (Figure 5). When pH < 6.0, COD removal rate was less than 50%. If pH was higher than 6.0, the COD removal rate increased rapidly while pH increased. Until pH was 7.5, COD removal rate reached its peak. If pH>7.5, the COD removal rate decreased dramatically.

Effects of inoculum size on the chemical oxygen demand (COD) removal rate

The influence of inoculum size on the COD removal rate

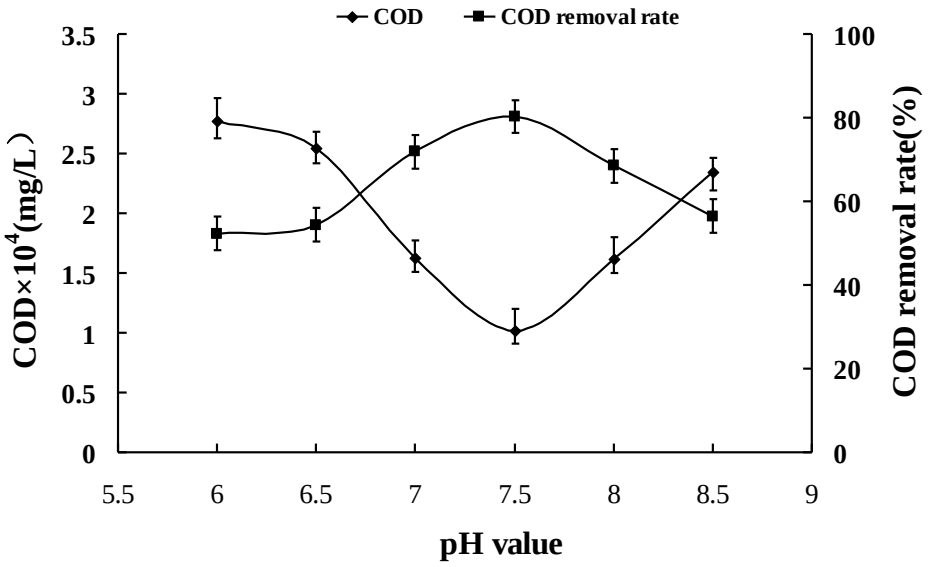

Figure 5. Effects of pH on the COD removal rate. BS5 was inoculated into degrading medium at pH 6.0, 6.5, 7.0, 7.5, 8, and 8.5, respectively. The condition of culture was as following, the volume of the alkaline wastewater was 30 mL/250mL, 0.5% corn starch, 1% corn steep liquor, 10% inoculum size, then cultured at 35°C at 150 r/min. Fifty hours later, the COD value was measured.

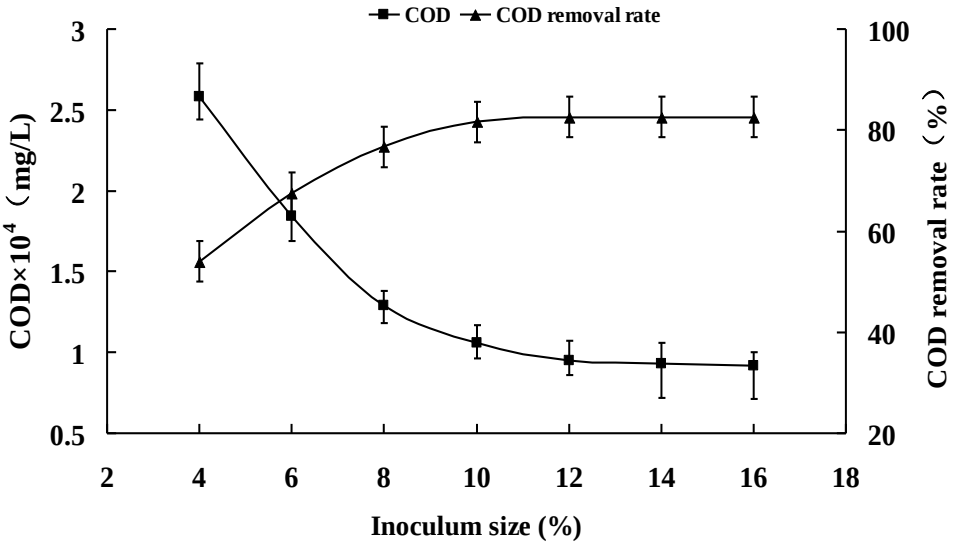

Figure 6. Effects of inoculum size on the COD removal rate. BS5 was inoculated into degrading medium at six different inoculum size (4, 6, 8, 10, 12, 14 and 16%). The condition of culture was as following, the volume of the alkaline wastewater 30 mL/250mL, 0.5% corn starch, 1% corn steep liquor, then cultured at 35°C, pH 7.5, at 150 r/min. Fifty hours later, the COD value was measured.

was investigated. Six different inoculum sizes (4, 6, 8, 10, 12, 14 and 16%) were test. It could be concluded from the results presented in Figure 6 that inoculum size would have a major influence on bacteria breeding and COD removal rate. When inoculum size was low, the degradation was lower obviously. When the inoculum size increased, the COD removal rate increased gradually. The COD removal rate reached 81.04% while the inoculum size was 10%. But the increment of COD removal rate was not obvious if inoculum size continued to increase.

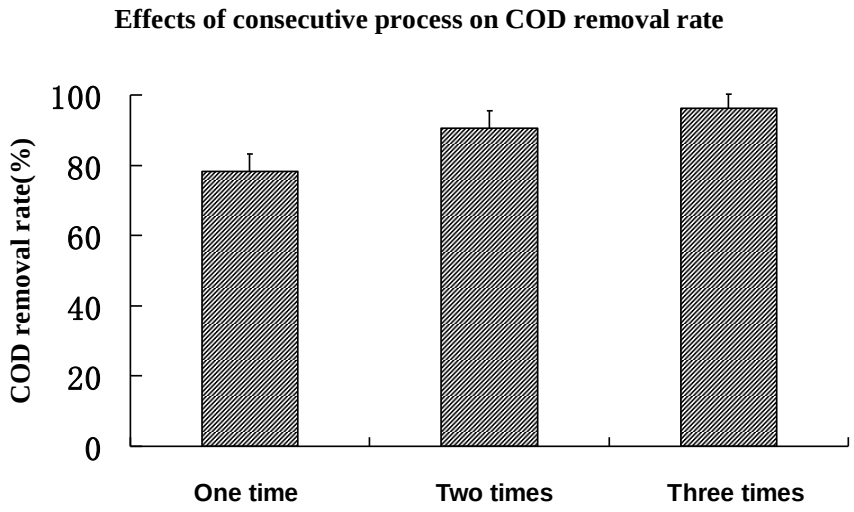

Figure 7. Effects of consecutive process on COD removal rate. Firstly, treated alkaline wastewater at the speed 4000r/min, 10 min, and take 30 mL clear liquid to 250 mL flask. The culture condition were 0.5% corn mill, 1% corn steep liquor, 35°C, pH 7.5, 10% inoculum size and agitation rate 150 r/min. Fifty hours later, the COD value was measured. The wastewater disposed by one, two, three times process, and they correspond COD removal rate was showed.

So the optimum inoculum size was set as 10%.

Effects of consecutive treatment on the COD removal rate

The performance of optimized strain BS5 was stable by taming.TheCODofalkalinewastewaterobviouslydecreased under the optimized condition after 50 h treatment with the bacteria. But when the initial COD of wastewater was very high, the COD of treated wastewater cannot satisfy the demand of discharge or utilization standards. Take it into account, consecutive treatment processes may be necessary.

The wastewater disposed for one, two or three times process, and the corresponding COD removal rate was showed. According to the data presented in Figure 7, the aim of consecutive treatment on the COD removal rate was achieved. After the first round treatment, the COD removal rate was up to 78. 3%. Whereas, the second round treatment culture enabled the COD removal rate reached 90.5%. Through a third disposal process, the COD removal rate was 96.2%. It was considered that consecutive disposal process may help to reducing COD of wastewater effectively.

Conclusion

The organic pollutants in petrochemical and oil refining which are resistant to degradation can be dreadfully hazardous to human health. As they persist in the environment, they are capable of long range transportation, bioaccumulation in human and animal tissue and biomagnification in food chain (Robles et al., 2000). Thus treatment of alkaline wastewaters is necessary and biological methods are the most appropriate techniques due to mineralization of toxic organic compounds and inexpensiveness (Prieto et al., 2002).

The use of microbial catalysts in the biodegradation of organic compounds has advanced significantly during the past days. It has been found that large numbers of microbes co-exist in almost all natural environments. Identification of effective microbial species is considered as one of the important priorities for production of the biomass in order to achieve desirable kinetic of biological reactions (Liu et al., 2002).

At the same time, several external factors can limit the rate of biodegradation of organic compounds. These factors may include temperature, pH, oxygen content and availability, substrate concentration and physical properties of contaminants. Each of these factors should be optimized for the selected organism for the maximum degradation of the organic compound of choice.

In this study, strains degrading alkaline wastewater were isolated from the sludge nearby petroleum smelter and enriched, among which BS5 performed the highest degradation ability. Furthermore, BS5 was identified as *Bacillus flexus* through 16S r DNA and some other data (not showed in this paper).

Data presented in this study demonstrated that the strain's optimum disposal condition should be 0.5% corn starch, 1% corn steep liquor, temperature 35°C, initial pH 7.5, 10% inoculation size. In such conditions, the removal

rate of COD can be up to 81.04% and almost 10.38% higher than before. Meanwhile, successive process was developed to enhance the degradation efficiency.

In conclusion, a *Bacillus flexus* strain characterized stable performance and inexpensive cost in alkaline wastewater treatment was isolated and identified, which has prospective application values in this area.

ACKNOWLEDGEMENTS

This work was supported by the Tanggu Technical Development Foundation Grant of Tianjin in China.

REFERENCES

Choe EK, Son EJ, Lee BS, Jeong SH, Shin HC, Choi JS (2005). NF process for the recovery of caustic soda and concentration of disodium terephthalate from alkaline wastewater from polyester fabrics. Desalination, 186(1-3):29-37.

David AB, Peter RR (1995). Sequence and analysis of the O antigen gene (*rfb*) cluster of *Escherichia coli 0111*. J. Gene, 164(1):17-23.

García Becerra FY, Acosta EJ, Allen DG (2010). Alkaline extraction of wastewater activated sludge biosolids. Bioresour. Technol., 101(18): 6972-6980.

Garcia GI, Pena PRJ, Venceslada B, Martin AA, Santos MAM, Gomes ER (2000). Removal of phenolic compounds from olive mill wastewater using *Phanerochaete chrysosporium, Aspergillus terreus* and *Geotrichum candidum*. Proc. Biochem., 35(8):751-758.

Leal K, Chacin E, Behling E, Gutierez E, Fernandeza N, Forstef CF (1998). A mesophilic digestion of brewery wastewater in an unheated anaerobic flter. Bioresour. Technol., 65(1-2):51-55.

Liu WT, Chan OC, Fang HH (2002). Characterization of microbial community in granular sludge treating brewery wastewater. Water Res. 36(7):1767-1775.

Loh KC, Liu J (2001). External loop inversed fluidized bed aircraft bioreactor (EIFBAB) for treating high strength phenolic waste water. Chem. Eng. Sci., 56:6171-6176.

Ma FR, Mu T, Bai B, Zhao LQ, Zhang XH, Cui YL (2006). Application of High-concentration Sewage Disposal Technology in Treatment of Diesel Alkaline Residue. J. Environ. Protect. Oil Gas Fields, 16(2):19-21.

Prieto MB, Hidalgo A, Rodriguez FC, Serra JL, Llama MJ (2002). Biodegradation of phenol in synthetic and industrial wastewater by *Rhodococcus erythropitics*. UPV-1 immobilized in air-stirred reactor with clarifier. Appl. Microbiol. Biotechnol. 58: 583-859.

Prisciandaro M, Mazziotti di Celso G, Vegliò F (2005). Development of a reliable alkaline wastewater treatment process: optimization of the pre-treatment step. Water Res. 39(20):5055-5063.

Rao AG, Reddy TS, Prakash SS, Vanajakshi J, Joseph J, Sarma PN (2007). pH regulation of alkaline wastewater with carbon dioxide: A case study of treatment of brewery wastewater in UASB reactor coupled with absorber. Bioresour. Technol., 98(11):2131-2136.

Richard ES (1983). Anaerobic biotechnology for industrial wastewater treatment. J. Environ. Sci. Technol. 17(9):416-427.

Robles A, Lucas R, Cienfueges AD, Galvez A (2000). Phenol oxidase activity in strains of the hyphomycete Chalara paradoxa isolated from Olive mill wastewater disposal ponds. Enzyme Microbial Technol. 26(7):484-490.

Rocenkery M (1993). The fast method of the COD determination. Anal. Lett., 26 (9)：2025-2030.

Xie WY, Tan GQ, Zhong L (2007). Research and application of treatment technology for alkaline waste from oil refinery. J. M. Chem. Ind., 27(6):10-14.