Desalination and Water Treatment
www.deswater.com

doi:10.5004/dwt.2018.22237

111 (2018) 236–240
April

Bio degradation of phenol by halophilic *Pseudomonas* sp. strain SL-1 and evaluation of its resistance of heavy metals

Yangyang Feng*, Xian Xu, Lei Zhang, Chenyang Zhang

School of Pharmaceutical Sciences, Nanjing Tech University, No.30 Puzhu South Road, Nanjing, 211800, China, Tel. +86 25 83313960, email: yangyangfeng@njtech.edu.cn (Y. Feng), xianxu@njtech.edu.cn (X. Xu) zhanglei@njtech.edu.cn (L. Zhang), cyzhang@njtech.edu.cn (C. Zhang)

Received 26 October 2017; Accepted 24 March 2018

ABSTRACT

Phenol is one typical pollutant in many kinds of industrial wastewater, and some of them frequently existed in saline condition and coexistence with heavy metals. These factors lead to more difficulties of phenol biodegradation. In this study, an efficient phenol-degrading halophilics train SL-1 was isolated from petroleum-contaminated soil and identified as *Pseudomonas* sp. Strain SL-1 was able to completely degrade up to 800 mg/L phenol less than 40 h but biodegradation was inhibited at higher phenol concentrations. The considerable pH condition for strain SL-1 was slightly acidic condition in a range between 5.0 and 7.0, and the optimal pH value was 7.0. Meanwhile, strain SL-1 was able to biodegrade phenol efficiently in high NaCl concentration up to 20%. Furthermore, strain SL-1 showed a good tolerance of heavy metals. It maintained high phenol removal efficiency in the presence of 10 mM of Zn(II), Cu(II), Mn(II) and Pb (II). In additional, functional genes encoding phenol hydroxylase and catechol 2,3-dioxygenase were present in strain SL-1 as determined. This indicated that phenol biodegraded by strain SL-1 via meta-cleavage pathway. The excellent biodegradation performance showed that strain SL-1 possesses a broad application prospect in phenol bioremediation under complex environmental conditions.

Keywords: Degradation; Functional genes; Halophilic; Heavy metals; Phenol; *Pseudomonas* sp.

1. Introduction

With the rapid economic and social development, phenol as an important organic raw material is widely used in petroleum refining, coking, pesticide, and medicine synthesis industries [1–3]. Meanwhile, an increasing amount of phenol-rich wastewater has been discharged to environment with the expansion of industrial scale, and in some kinds of industrial effluents phenol concentrations are pretty high, which has resulted in accumulation in water body and soil since phenolic compounds have recalcitrant structures which makes it hard to be decomposed. The phenolic pollutants can destroy the ecological balance and has deleterious effect on human health due to the toxicity, mutagenicity, and carcinogenicity [4,5]. Because of the high toxicity and recalcitrance, the phenol discharge has strong potential to create serious environmental pollution problems [6–9]; methods for improving the removal of phenol are needed.

Meanwhile, heavy metals are some of the toxic inorganic pollutants coexist in industry wastewater. Heavy metals can be absorbed by plants and can leach into groundwater resulting in poisonous and harmful effects [10,11]. Nickel, zinc, copper, lead, manganese and cadmium are some of the most widely used industrially [12,13].

Compared with the traditional physicochemical treatment methods such as zonation, adsorption, and solvent extraction, biological processes presented several advantages such as economic, eco-friendly and no secondary pollution [14–16]. Therefore, biological processes are widely accepted as commercial methods particularly for degradation recalcitrance organic pollutants [17–20]. However, the common biologic treatment methods cannot be applied

*Corresponding author.

in the high salinity condition. Halophilic microbes can be successfully degradation organic pollutants from high-salinity condition with out dilution the salt concentration in advanced.

In previous studies, majority attention was paid to the phenol biodegradation by non-halophilic bacteria. Few studies focused on the phenol biodegrading ability in halophilic condition. In the present study, a salt tolerant strain SL-1 utilizing phenol as sole carbon source was isolated from the petroleum contaminated soil and its phenol degradation ability was test. Certain factors such as initial substrate concentration, pH, salinity and heavy metals were also examined for the effects on the phenol biodegradation. These advantages suggested that strain SL-1 is able to tolerate a wide range of environmental variations might be promising in bioremediation of halophilic phenol-rich environment.

2. Materials and methods

2.1. Isolation of phenol-degrading bacterial strain SL-1

Petroleum-contaminated soil was obtained from Shengli oil field (Dongying, China). 5 g soil sample was inoculated into 100 ml of minimal salt medium (MSM) which contained (g/L)$(NH_4)_2$ SO_4 2.0, KH_2PO_4 1.5, $MgSO_4{\cdot}7H_2O$ 0.2 , $FeSO_4{\cdot}7H_2O$ 0.001 , $CaCl_2{\cdot}2H_2O$ 0.01, $Na_2HPO_4{\cdot}12H_2O$ 1.5, and amended with 100 mg/L phenol and 5% NaCl (W/V). The mixture was in cubated at 30°C and shaking at 150 rpm for five days. Fresh MSM with 100 mg/L phenol was inoculated with 10 ml previous culture and cultivated under the same conditions. After four transfers, serial dilutions of the cultures were prepared and spread-plated on mineral medium agar plates supplemented with phenol and incubated at 30°C for 3 d. Colonies were harvested from dilution plates based on distinct colony morphology, and transferred to mineral medium agar plates several times to ensure culture purity.

2.2. Characterization of the strain

Genomic DNA of strain SL-1was extracted by the DNA extraction kit (Sangon Biotech, Shanghai, China) under the manufacture's instruction. The 16S r DNA was amplified by PCR under standard reaction condition with the universal primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1 (5′-CCATGCAGCACCTGTGTCTG-3′). The PCR product was sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The sequence was compared to the Gen Bank database using the BLAST program.

2.3. Phenol biodegradation

A series 250 mL flasks supplemented 100 MSM with varying phenol concentrations increasing from 100 to 1500 mg/L (100, 200, 400, 800, 1200, and 1500 mg/L, respectively) with 5% NaCl were used to test the bio degradation ability. Strain SL-1 (5.6×10^{10} cfu) were inoculated into each flask, and incubated at 150 rpm and 30°C for 36 h. The residual phenol concentrations of each flask were analyzed at intervals of 4 h.

2.4. Factors of pH, salinity and heavy metals affecting phenol biodegradation

In order to investigate the effects of certain factors on phenol biodegradation, strain SL-1 was cultivated in MSM amended with 100 mg/L phenol at 150 rpm and 30°C.

The effect of pH on phenol biodegradation was assessed by adjusting medium pH value in to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 individually. The pH value was adjusted by 1 M NaOH or 1 M HCl solution. The effect of salinity on phenol biodegradation was evaluated by different NaCl concentrations increasing from 0% to 20% (w/v).

The tolerance of heavy metals was assessed by adding 10 mM of different heavy metal ions, such as Zn(II), Cu(II), Mn(II), Pb(II) and Ni(II) in 100 mL MSM amended with 100 mg/L phenol. In additional, another MSM sample containing 100 mg/L phenol and 5% NaCl without any extra heavy metals was used as contrast.

2.4. Carbon source utilization of strain SL-1

The ability of strain SL-1utilizing different aromatic compounds as sole carbon source was discussed. The purified strain SL-1 was inoculated into MSM containing 100 mg/L one of the following compounds: 3-nitrophenol, 4-nitrophenol, 4-chlorophenol, benzene, toluene, xylene, naphthalene and catechol. Growth was measured by the OD_{600} value of the culture.

2.5. Amplification of phenol degradation functional genes

In order to analyze the functional genes of strain SL-1, the primers sets used for PCR amplification of phenol hydroxylase (Lph), catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase(C23O) were as follow: Lphf (5′-CGCCAGAACCAT TTATCGATC-3′), Lphr (5′-AGGCATCAAGATCACCGACTG-3′) (Xu et al., 2001); C12Of (5′-ACCATCGARGGYCCSCTSTAY-3′), C12Or (50-GTTRATCTGGGTG GTSAG-3′); and C23Of (5′-GARCTSTAYGCSGAYAAGGAR-3′), C23Or (5′-RCCGCTSGGRTCGAAGAARTA-3′) [21,22].

2.6. Analytical methods

Samples were collected from each flask and centrifuged for 2 min at 12000 rpm. Residual phenol concentration was measurement by the 4-aminoantipyrine spectrophotometric method. The measurement was carried out according to standard method [23].

3. Results and discussion

3.1. Isolation and identification of strain SL-1

After more than 20 d enrichment and isolation, one halophilic strain with efficient phenol-degrading ability was obtained, which was named as SL-1. Strain SL-1 showed faint yellow, transparent, smooth and moist colonies on LB agar plate.

The result of sequencing and homology analysis by BLAST indicated that strain SL-1 (Gen Bank accession numberMF143796) was a member of the genus *Pseudomonas* and

exhibited 100% sequence similarity with the 16S rRNA of *Pseudomonas aeruginosa* strain DSM 50071 (Gen Bank accession number NR17678).

3.2. Phenol biodegrading study

In order to investigate the effect of substrate concentration on phenol biodegrading efficiency, the biodegradation experiments were carried out with initial phenol concentrations increasing from 100 to 1500 mg/L. The degradation performances are shown in Fig. 1. Phenol could be completely degraded by SL-1 in 40 h when initial concentration was below 800 mg/L. The time required for completely degradation was longer when initial phenol concentration increased. The necessary time to completely degrade were 16 h, 20 h, 28 h and 40 h corresponding to the initial phenol concentration values of 100, 200, 400 and 800 mg/L. The degradation efficiency was obviously decreased to 50% when the initial phenol concentration increased to 1200 mg/L. At a higher phenol concentration value of 1500 mg/L, the phenol degradation efficiency decreased to only 17.6%.

These results suggested that phenol degradation efficiency was declined sharply when the initial concentration up to 1200 mg/L. This may due to the high phenol concentration was inhibited the biodegradation activity of strain SL-1. At the highest concentration of 1500 mg/L, the concentration value was decreased rarely. It suggested that the phenol tolerance of strain SL-1 was 1500 mg/L.

3.3. Effect of certain factors on phenol degradation

A series of degradation experiments were carried out at varying pH from 4.0 to 10.0, with phenol concentration, temperature, and rotating rate at values of 400 mg/L, 30°C, and 150 rpm, respectively.

The results of pH test (shown as Fig. 2) were indicated that phenol could be completely removed by strain SL-1 in 32 h with the pH value in the range of 5.0–7.0. Compared with the removal efficiencies at other pH values, the degradation efficiencies with the pH value of 7.0 were best. The phenol removal rates in 8 h, 16 h and 24 h reached 23%, 64% and 97%, respectively. According to the results, the considerable pH condition for strain SL-1 was slightly acidic condition in a range between 5.0 and 7.0, and the optimal pH value was 7.0.

The effect of salinity on the degradation efficiency was evaluated for NaCl concentrations varying from 0% to 20% (w/v), with substrate concentration, pH, temperature, and rotating rate at values of 400 mg/L, 7.0, 30°C, and 150 rpm, respectively. Strain SL-1 presented a high salinity tolerant ability showed as Fig. 3. Strain SL-1 could remove phenol completely in 36 h when NaCl concentration below 10%, the removal rates with 15% and 20% of salinity were also reached to 88% and 82.3% in 36 h. However, the degradation efficiency was decreased to 55.5% when the salinity increased to 25%.The results indicated that strain SL-1 possesses well salinity tolerance, and the best degrading efficiency was at 5% NaCl. It indicated that low salinity concentration could promote the activity of strain SL-1.

Previous studies have already reported a few phenol-degrading strains can grow well at low salt concentrations. However, to our best knowledge, such halophilic bacterial

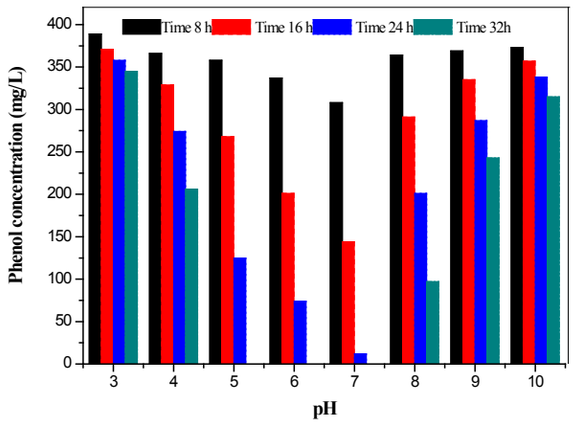

Fig. 2. The effect of different pH values on phenol degradation by strain SL-1.

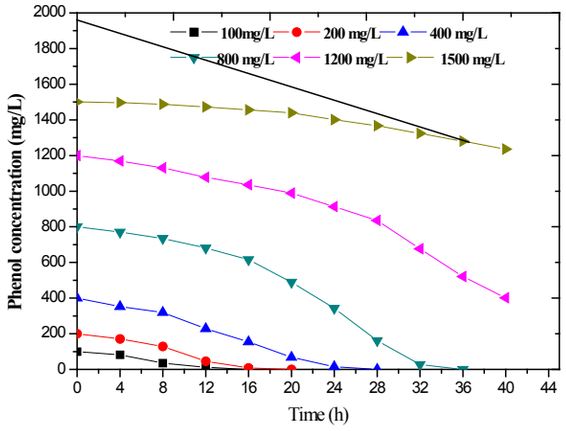

Fig. 1. The effect of different phenol concentration on the degradation efficiency of strain SL-1.

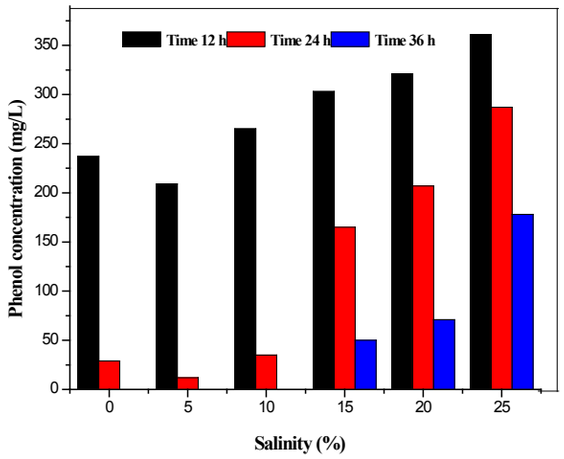

Fig. 3. The effect of different salinity concentrations on phenol degradation by strain SL-1.

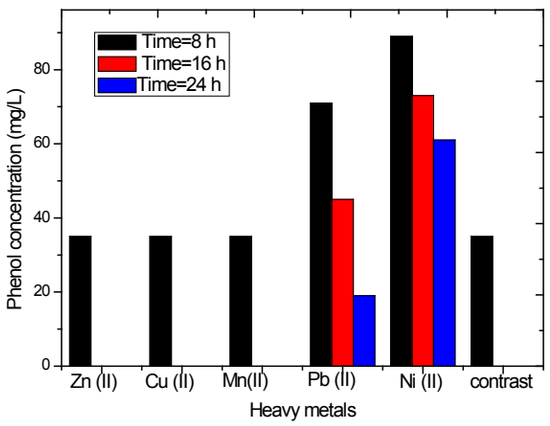

Fig. 4. The effect of heavy metals on phenol degradation efficiency by strain SL-1.

strain can biodegrade phenol efficiently in high NaCl concentration up to 20% was few reported.

3.4. Tolerance of heavy metals

Phenolic wastes are often occurred together with heavy metals. Heavy metals usually inhibited the biomass activity and harnessed for degradation efficiency. Thus, phenol degradation activity of strain SL-1 under different heavy metals condition was investigated. The results (shown as Fig. 6) indicated that strain SL-1 was able to resist certain heavy metals stress. The phenol removal efficiency varied greatly with five different heavy metals. Zn (II), Cu (II) and Mn (II) were no affect phenol removal efficiency of strain SL-1. Strain SL-1 also exhibited a good resistance to Pb (II). The phenol removal efficiency was maintained above 70% in the presence of Pb (II). However, Ni(II) inhibited the biodegradation activity of strain SL-1 significantly. Phenol removal efficiency was decreased sharply to 40% in the presence of Ni(II). The good heavy metals tolerance indicated that strain SL-1 possesses a broad application prospect in bioremediation of phenol and heavy metals multi-polluted sites.

3.5. Utilization range of substrate by strain SL-1

In order to investigate the ability of strain SL-1utilizing different a romatic compounds, eight benzene series were used as sole carbon and energy source, the utilization ability was evaluated by the increased value of OD_{600} after 7 d incubation. As shown in Table 1, strain SL-1 could use 3-nitrophenol, 4-nitrophenol, 4-chlorophenol, benzene, toluene, xylene, and catechol as the sole carbon source, but not naphthalene (showed as Table 1). The results suggested that strain SL-1 could utilize a very broad substrate as sole carbon source, and indicating its degrading diversity.

3.6. Analyses of phenol degradation functional genes

In order to detect three key phenol-degrading functional genes, three primers were used for amplify the functional genes encoding Lph, C12O and C23O. Two gene sequence fragments were obtained by PCR amplification. These two sequences exhibited 99% and 100% similarity to Lph and C23O genes, respectively (showed as Table 2). Identification of two PCR products revealed that strain SL-1 possessed function algenes encoding L ph and C23O. Lph and C23O are the two key enzymes in phenol meta-cleavage pathway. This indicated that phenol biodegraded by strain SL-1 via meta-cleavage pathway.

Table 1
Utilization of different carbon sources by strain SL-1

Substrate	Strain SL-1
3-nitrophenol,	+
4-nitrophenol	+
naphthalene	–
4-chlorophenol	+
benzene	++
toluene	++
xylene	+
catechol	++

(++) Good growth: $OD_{600} > 0.1$; (+) growth: $0.05 < OD_{600} < 0.1$; (–) no growth $OD_{600} < 0.01$.

Table 2
The BLAST results of functional gene sequences

Primer	GenBank Accession No.	The most relatives sequences	Similarity
Lph	MF186710	*Diaphorobacter* sp. J5-51 phenol hydroxylase gene (GU017973)	99%
C23O	MF186711	*Pseudomonas aeruginosa* catechol 2,3-dioxygenase (D83042)	100%

4. Conclusions

In this study, a halophilic phenol-degrading strain SL-1 was isolated from Petroleum-contaminated soil obtained from Shengli oil field. It was identified as *Pseudomonas* genus. Strain SL-1 could completely degrade less than 800 mg/L of phenol in 40 h, but higher substrate concentrations inhibited the biodegradation activity distinctly. Strain SL-1 could degrade 400 mg/L phenol completely less than 32 h with the pH value at the range from 5.0 to 7.0, and the optimal pH value was 7.0. The phenol was degraded by strain SL-1 via the meta-cleavage pathway catalyzed by the key enzymes of phenol hydroxy lase and catechol 2, 3-dioxygenase. Strain SL-1 could also utilize a very broad benzene series substrate as sole carbon source. Compared with previous studies, strain SL-1 showed a better salinity tolerance capability. It can degrade phenol efficiently in a high NaCl concentration up to 20%.Furthermore, strain SL-1 showed a good tolerance of heavy metals. The excellent performance under different environmental conditions illustrated that

strain SL-1 might be promising in remediating different kinds of phenol-polluted sites containing heavy metals in saline conditions.

Acknowledgments

The present work was supported by the National Natural Science Foundation of China (No. 51608261).

References

[1] B. Basak, B. Bhunia, S. Dutta, S. Chakraborty, A. Dey, Kinetics of phenol biodegradation at high concentration by a metabolically versatile isolated yeast Candida tropicalis PHB5, Environ. Sci. Pollut. R., 21 (2014) 1444–1454.
[2] M.R.L. Bonfa, M.J. Grossman, F. Piubeli, E. Mellado, L.R. Durrant, Phenol degradation by halophilic bacteria isolated from hyper saline environments, Biodegradation, 24 (2013) 699–709.
[3] M. Karimi, M. Hassanshahian, Isolation and characterization of phenol degrading yeasts from wastewater in the coking plant of Zarand, Kerman, Braz. J. Microbiol., 47 (2016) 18–24.
[4] J. Nesvera, L. Rucka, M. Patek, Catabolism of phenol and its derivatives in bacteria: genes, their regulation, and use in the biodegradation of toxic pollutants, Adv. Appl. Microbiol., 93 (2015) 107–150.
[5] Y. Zhang, J. Tay, Toxic and inhibitory effects of trichloroethylene aerobic co-metabolism on phenol-grown aerobic granules, J. Hazard. Mater., 286 (2015) 204–210.
[6] S. Bera, A.S. Roy, K. Mohanty, Biodegradation of phenol by a native mixed bacterial culture isolated from crude oil contaminated site, Int. Biodeter. Biodegr., 121 (2017) 107–113.
[7] Y. Cui, X.Y. Liu, T.S. Chung, M. Weber, C. Staudt, C. Maletzko, Removal of organic micro-pollutants (phenol, aniline and nitrobenzene) via forward osmosis (FO) process: Evaluation of FO as an alternative method to reverse osmosis (RO), Water Res., 91 (2016) 104–114.
[8] J. Wang, Z. Yao, Y. Wang, Q. Xia, H. Chu, Z. Jiang, Preparation of immobilized coating Fenton-like catalyst for high efficient degradation of phenol, Environ. Pollut., 224 (2017) 552–558.
[9] Y. Jiang, K. Yang, H. Wang, Y. Shang, X. Yang, Characteristics of phenol degradation in saline conditions of a halophilic strain JS3 isolated from industrial activated sludge, Mar. Pollut Bull., 99 (2015) 230–234.
[10] W.S. Shin, Adsorption characteristics of phenol and heavy metals on biochar from *Hizikia fusiformis,* Environ. Earth Sci., 76 (2017) 782.
[11] P.M. Fernández, M.M. Martorell, M.G. Blaser, L.A. Ruberto, L.I.Figueroa, W.P.M. Cormack, Phenol degradation and heavy metal tolerance of Antarctic yeasts, Extremophiles, 21 (2017) 445–457.
[12] T. Aman, A.A. Kazi, M.U. Sabri, Q. Bano, Potato peels as solid waste for the removal of heavy metal copper (II) from waste water/industrial effluent, Colloids Surf B, 63 (2008) 116–121.
[13] H. Aydm, Y. Bulut, C. Yerlikaya, Removal of copper (II) from aqueous solution by adsorption onto low-cost adsorbents, J. Environ. Manag., 87 (2008) 37–45.
[14] L. Martínkov, M. Kotik, E. Markov, L. Homolka, Biodegradation of phenolic compounds by *Basidiomycota* and its phenol oxidases: A review, Chemosphere, 149 (2016) 373–382.
[15] J.Q. Sun, L. Xu, Y.Q. Tang, F.M. Chen, X.L. Wu, Simultaneous degradation of phenol and n-hexadecane by Acinetobacter strains, Bioresour. Technol., 123 (2012) 664–668.
[16] L. Wang, C. Xue, L. Wang, Q. Zhao, W. Wei, Y. Sun, Strain improvement of Chlorella sp. for phenol biodegradation by adaptive laboratory evolution, Biores. Technol., 205 (2016) 264–268.
[17] H.P. Bacosa, K. Suto, C. Inoue, Bacterial community dynamics during the preferential degradation of aromatic hydro carbons by a microbial consortium, Int. Biodeter. Biodegr., 74 (2012) 109–115.
[18] S.K.F. Marashi, H.R. Kariminia, I.S. Savizi, Bimodal electricity generation and aromatic compounds removal from purified terephthalic acid plant wastewater in a microbial fuel cell, Biotechnol. Lett., 35 (2013) 197–203.
[19] S. Shi, Y. Qu, Q. Ma, X.W. Zhang, J. Zhou, F. Ma, Performance and microbial community dynamics in bioaugmented aerated filter reactor treating with coking wastewater, Bioresour. Technol., 190 (2015) 159–166.
[20] M.G. Waigi, F. Kang, C. Goikavi, W. Ling, Y. Gao, Phenanthrene biodegradation by sphingomonads and its application in the contaminated soils and sediments: A review, Int, Biodeter, Biodegr., 104 (2015) 333–349.
[21] M.T. García, A. Ventosa, E. Mellado, Catabolic versatility of aromatic compound-degrading halophilic bacteria, FEMS Microbiol Ecol., 54 (2006) 97–109.
[22] Y.Q. Xu, X. Fang, M. Chen, W. Zhang, J.M. Li, M. Lin. The detection of phenol degrading strain in environment with specific primer of phenol hydroxyl asegene, Acta Microbiol Sinica., 41 (2001) 298–303.
[23] APHA, Standard methods for the examination of water andwaste water, 20th ed. American Public Health Association, Washington DC, WA .(2005).