The advanced treatment of wastewater containing tetracycline resistance genes by Fenton oxidation process

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Abstract

Fenton reaction as an advance oxidation process was applied to treat SBR effluent containing tetracycline resistance genes (tet genes) since it has been a threat to human health. The optimal parameters of pH=3, 0.2mol/L H2O2 with 30mmol/L FeSO4.7H2O reacting for 30minutes have been determined by a series of single factor experiments. After treated in the optimal condition, bacterial community diversity and equitability all have an apparent decrease. While Quantity Polymerase Chain Reaction(QPCR) experiments described Fenton regent lead to a remarkable efficiency on tetracycline resistant genes removal and have stronger damage on that genes encoding efflux pump proteins and genes that can modified enzyme, but have relative week damage effect on that genes that encode ribosomal protection proteins. And in the treated sample, tet genes/16SrRNA ratio have obvious drop, except tet(M). It also described that comparing with other treatments, Fenton regents have more fierce scavenging efficiency.

Keywords

Fenton regent, tetracycline resistance genes, DGGE, QPCR

INTRODUCTION

Recently, the abuse of various antibiotics for human, veterinary and agricultural purposes has resulted in the development of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs), which is a sever threat to human health(Rahman et al., 2008; Munir et al., 2011a). Till now, many kinds of antibiotic resistance bacteria and genes have been detected in various environments over the worldwide scale (Rodriguez-Mozaz et al., 2015). Among the various resistance genes, the diversity and occurrence frequency of tetracycline resistance genes are the focus of most study

because its wide spread in environment (Aarestrup et al., 2000; Aminov et al., 2001; Chee-Sanford et al., 2001).

Four habitats have been universally recognized as the pool where antibiotic resistance bacteria and genes evolved: human and animal microbiota, hospitals, wastewater facilities, surface and ground water. Among those habitats, urban wastewater treatment plants represent the major hotspots of antibiotic resistance bacteria and genes. On the one hand, antibiotic resistant bacteria and resistance genes are heavily discharged into the municipal sewage system (Zhang et al., 2011). On the other hand, wastewater treatment is designed to decrease the Carbon Organic Demand (COD) and conventional indexes, not aimed at ARB or ARGs. So it leads to the final effluents containing ARB or ARG, sometimes even higher than the concentration of influent (Munir et al., 2011a; Rizzo et al., 2013; Al-Jassim et al., 2015).

As a consequence, further and effective treatment focusing on ARB or ARG is necessary. Conventional chlorine, ozone and UV disinfection are commonly used as a method to remove the organism in the effluent to decrease the health risk (Macauley et al., 2006; Malato et al., 2009; Rizzo et al., 2014; García-Fernández et al., 2015). The effectiveness of chlorine, ozone and UV disinfection on ARG had not come to a comparative conclusion. ozone can damage DNA by the shifting supercoiled plasmid DNA to open circular plasmid DNA(Wolff et al., 1986). UV radiation can also damage DNA and result in inhibition of cell replication, but bacteria may recover replication activity just under visible light through a process (for example photo reactivation) catalysed by the DNA repair enzyme photolyase (Oguma et al., 2001).

Fenton reaction as an advance oxidation process has been successfully investigated for the removal of a wide range of contaminants (Zapata et al., 2010; Rizzo, 2011). In the Fenton oxidation process, hydroxyl radicals catalytically produced by hydrogen Peroxide can oxidize a large number of cellular constituents, even destructing cell membranes and cytoplasmic components (Ananthaswamy and Eisenstark, 1977; Wolff et al., 1986). However, the research about the effectiveness of Fenton oxidation process in decreasing tetracycline resistance genes quantities is yet insufficient by now.

Considering the absence information about Fenton oxidation process on tetracycline resistance genes, this research was launched on the performance of Fenton oxidation process for the destruction of 5 tetracycline resistance genes(tet(A)、tet(C)、tet(M)、tet(G)、tet(X). Through a series experiments, the optimal parameters as pH, time, the dosage of $FeSO_4 \cdot 7H_2O$ and H_2O_2 have been investigated in terms of Total organic Carbon （TOC ）which was the indexe for organic removal. Quantity Polymerase Chain Reaction (Q-PCR) has been investigated for the detection of removal efficiency of tetracycline resistance genes by Fenton oxidation process. And denaturing gradient gel electrophoresis (DGGE) has been applied to compare the Shannon-Wiener diversity index (SDI) and the equitability index (EI) of raw water with treated sample to reflect its effect.

METHODS and MATERIALS

Samples

Water samples containing tetracycline resistance genes were collected from the effluent of a lab-scale sequencing bath reactor (SBR) feed with synthesized tetracycline and glucose wastewater. The COD concentration of synthesized wastewater was 1000 mg/L including 63.4 mg/L tetracycline. The samples were stored in 4 ^{o}C refrigerator before experiment. The characteristics of water samples were listed in Table.1.

Table 1. Initial conditions of SBR effluent

Parameters	value
Concentration of tetracycline（mg/L）	ND
TOC(mg/L)	14.216
16SrRNA(log(copies/mL)	7.68
tet(A)(log(copies/mL)	5.76
tet(C) (log(copies/mL)	6.29
tet(M) (log(copies/mL)	1.56
tet(G) (log(copies/mL)	5.85
tet(X) (log(copies/mL)	6.72

ND: non-detected

Fenton process

A series of Fenton oxidation experiments were carried out at room temperature (25±2℃) and atmospheric pressure in magnetically stirred batch reactors With 400 ml effluent of SBR. After the pH of the wastewater was adjusted to a target value with 98% H_2SO_4 or 0.1 M NaOH solutions, the ferrous sulfate and hydrogen peroxide (30%, w/w) were added to initiate the series of Fenton reactions. To halt the Fenton reactions, a 10.0 M NaOH solution was added in the system to increase the wastewater pH value to 11. After settling down, the supernatant was filtered through a 0.20 μm filter paper (Millipore, Billerica, MA). The filter liquor was then analyzed for TOC，tetracycline resistance genes (tet(A), tet(C), tet(X) and tet(G)) removal. All experiments were conducted in triplicate.

Analytical methods

TOC of water samples were determined by TOC-VCPN analyzer (Shimadzu, Japan).

DNA extraction and QPCR experiments

Before analysis, water samples were filtered through a 0.20 μm filter (Millipore, Billerica, MA) which were cut into small pieces by sterilized scissors and then added directly to the extraction tubes (Lysing Matrix E, MP Biomedicals) for DNA extraction. Before DNA extraction, a lysis step was carried out with the samples using SLX Buffer, glass beads and XP2 Buffer, then put in the heating block at 70 °C for 10 min. The obtained lysed mixture was used for DNA extraction. DNA extraction was performed with E.Z.N.A®. Water DNA Kit（omega bio-tek，USA）as described by the manufacturer. The final extracts were dissolved in Elution buffer. All extracted DNA samples were stored at -20 °C until used. The quality and concentration of the DNA was determined by agarose gel electrophoresis and NanoDrop(NanoDrop2000, NanoDrop, USA).

Five tetracycline-resistance gens (tet A, tet C, tet M, tet G, tet X) and 16SrRNA in wastewater samples were quantitatively detected using Light Cycler 7000 (applied biosystems, USA). Five target tetracycline resistance genes and 16SrRNA were determined using specific primers listed in Table 2, Table 3. The genes were cloned to plasmids to generate q-PCR standard curves (Table 2), which were used to determine the abundance of genes in the filtered samples. The q-PCR were conducted in 96 plates tubes with a system of 20 ul mixture containing 10 ul SYBR Green QPCR Master Mix(SG Fast QPCR Master Mix（2x）, BBI), 0.4 ul for each primer(10μM), 7.2 ul ddH_20, 2 ul DNA template. PCR amplification was performed as follows: initial denaturation at 95 **°C** for 3 min, followed by 40 cycles including denaturation at 95 **°C** for 15 s, annealing at melting temperature (Table 2) for 20 s, and extension at 72 **°C** for 30 s, and a final extension step at 72 **°C** for 7 min. The data were analyzed by Light Cycler 480 Software Setup (Roche). Each reaction was run in triplicate for every sample. The specificity of the q-PCR products was checked by melt curves and agarose –gel electrophoresis.

Table 2 Information of target genes and 16SrRNA

Target Genes	Primer Sequence（5'-3'）	Melting Temperature（°C）	Amplicon Size（bp）
16SrRNA（F357/R518）	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	53	169
tet（A）	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	55	210
tet（C）	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	55	418
tet（G）	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC	55	468
tet（M）	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	55	406
tet（X）	CAATAATTGGTGGTGGACCC TTCTTACCTTGGACATCCCG	55	468

Table 3 Information of standard curve and the final concentration of target genes

Target genes	Equation	R^2	Amplification efficiency	Error	Log(copies/ml)(RSD)
16SrRNA	Y=-3.235*X+35.6	0.990	103.8%	0.163	5.30(0.3119)
tet(A)	Y=-3.375*X+38.8	0.994	97.8%	0.186	2.53(0.3229)
tet(C)	Y=-3.326*X+39.3	0.995	99.8%	0.159	1.70(0.5681)
tet(M)	Y=-3.232*X+37.2	0.997	103.9%	0.077	1.16(0.4692)
tet(G)	Y=-3.228*X+36.8	0.996	104.1%	0.094	1.87(0.2790)
tet(X)	Y=-3.54*X+39.2	0.997	91.7%	0.191	2.18(0.5248)

DGGE-PCR Amplification

PCR primers P2 and P3 (containing 40 bp of GC clamp, the sequence of P2 is 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CA-3′ and the P3 is 5′-ATT ACC GCG GCT GCT GG -3′) were used to amplify the variable V3 (Muyzer et al., 1993) region of bacterial 16S

rRNA gene (corresponding to positions 357–518 in the Escherichia coli sequence). Amplification was performed with a Mastercycler gradient (Eppendorf AG, Hamburg, Germany) using a 50 µl (total volume) mixture containing 1.25 U of Taq polymerase (Promega), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 µM deoxynucleoide triphosphate (dNTP), 10 pmol of each primer, and 1 µl of DNA solution (20 ng μl^{-1}). Touchdown PCR was employed with a Mastercycler Gradient thermal cycler (Eppendorf A. G., Hamburg, Germany), which involves 10 min of activating the polymerase at 94 ºC, 1 min at 65 ºC, and 2 min at 72 ºC. The annealing temperature was subsequently decreased by 1°C for every second cycle until it reached 55 ºC, at which point 20 additional cycles were carried out, finally, a 10 min extension step at 72 ºC was performed. The amplicons obtained were confirmed by electrophoresis through 2% agarose gel in 1µ TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM Na_2-EDTA, pH 8.0) stained with ethidium bromide.

Denaturing Gradient Gel-electrophoresis (DGGE)

For DGGE, the DNA amplified fragments were separated on polyacrylamide gels (8%, 38:1 acrylamide-bisacrylamide) with a 40 to 60% linear gradient of denaturant (100% denaturant=40% [vol/vol] formamide plus 42%[wt/vol] urea) depending on their different molecular weight. Gels were run for 15 min at 120 V and 16 hours at 60 V in 1xTAE buffer solution at 60°C. Then the gels were stained in the mixture of 1xTAE buffer with SYBR Gold nucleic acid stain (Molecular Probes) for 1 hour and visualized with UV radiation using a Fluor-S Multi Imager and the Multi Analyst imaging software (Bio-Rad). Quantity One software (Bio-Rad) was used for band pattern analysis. Different DNA samples were distinguished by Lanes and bands. Their Shannon-Wiener diversity index (SDI) and equitability index (EI) were analyzed. As described by Eichner et al.(Eichner et al., 1999).

RESULTS and DISCUSSION

Optimization of organic maters by Fenton oxidation

Effect of reaction time

TOC of the filtrate liquor were determined after reacting for 1min, 5 min, 10 min, 30 min and 60 min of Fenton reactor system. It described as following Figure 1 (a). As illustrated in Figure 1 (a), Fenton regent have apparent effectiveness on TOC removal over 50%. Even in 30 min and 60 min, the removal rates were up to 62.5% and 65.6%. So 30 min was selected as an economic and feasible time for subsequence experiments.

Effect of pH value

pH=2, 3, 5, 8 have been set in pH single factor experiments to investigate the effect of pH on whole Fenton system. TOC values also has been detected, and the development tendency was illustrated as Figure 1(b).

pH value have a fluctuant effect in TOC removal rate. It is known that acidic conditions

can widespread prompt Fenton system to release more ·OH and remove more TOC. From Figure 1(b), pH=3 resulted in highest TOC removal efficiency and when the pH exceeded 5, TOC removal efficiency fell down fiercely. The decrease may be due to the increasing rate of auto decomposition of H_2O_2 and deactivation of iron ions into iron oxyhydroxides. At low pH (pH=2), the TOC removal efficiency was low primarily due to the increased scavenging effect of carbonate and bicarbonate on ·OH by H^+, and/or the decreased oxidation potential of ·OH by higher concentrations of H^+(Szpyrkowicz et al., 2001). Considering TOC transformation, pH=3 is the optimal initial pH.

Optimal H_2O_2 dosage parameter determination

In order to determine the effect of H_2O_2 dosage, 5 experiments about the concentration ranging from 0.05mol/L to 0.3mol/L were conducted. The relationship of H_2O_2 dosage with TOC was shown as Figure 1(c).

Appropriate H_2O_2 dosage is necessary to make sure that H_2O_2 have been completely consumed and/or decomposed and TOC have been ultimately removed. As Figure 1.(c), more than 50% TOC removal rate have been achieved with any H_2O_2 dosage, and 74% removal rate were achieved with adding 0.2 mol/L H_2O_2. It described that a large proportion of TOC fragments have been oxidized into simple chemical compounds with this dosage H_2O_2 oxidation. Further increase of H_2O_2 dosage (0.25 mol/L and 0.3 mol/L) did not show significant removal improvement, rather an obvious decrease. This result might be attributed to the scavenging effect of peroxide on the hydroxyl radicals, which presumably became stronger as H_2O_2 dosage increased. Another explanation can be the formation of hydroperoxyl radicals ($\cdot HO_2$), which are less reactive in attacking the recalcitrant compounds in the liquid phase (Kurniawan and Lo, 2009). To ensure the maximum amount of ·OH radicals is available for the oxidation of organic compounds, 0.2 mol/L H_2O_2 was selected for further experiments.

Optimal $FeSO_4.7H_2O$ dosage parameter determination

Fe^{2+} produced by the hydrolyzation of $FeSO_4{\cdot}7H_2O$ can promoted active oxidant ·OH radicals formation by hydrogen peroxide as a catalyst. Appropriate Fe^{2+} will enhance the amount of ·OH radicals, but excessive Fe^{2+} will lead H_2O_2 invalid decomposed. Appropriate $FeSO_4{\cdot}7H_2O$ dosage was investigated by single factor experiments varying from 20 mmol/L to 40 mmol/L. The efficiency on TOC and DOM removal are illustrated as Figure1 (d).

As can be seen in Figure1 (d), the effect of $FeSO_4{\cdot}7H_2O$ dosage on the mineralization of organic represented by TOC removal is obvious. With the increase of $FeSO_4{\cdot}7H_2O$ dosage, the removal rates of TOC have been enhanced significantly, except a slight deceasing in 35mmol/L. However, with the increase of $FeSO_4{\cdot}7H_2O$ dosage, the chemical residue also increased which will lead to a new contaminant. The effect on DOM removal is different with TOC removal. Considering all information, 30 mmol/L $FeSO_4{\cdot}7H_2O$ is the favor dosage.

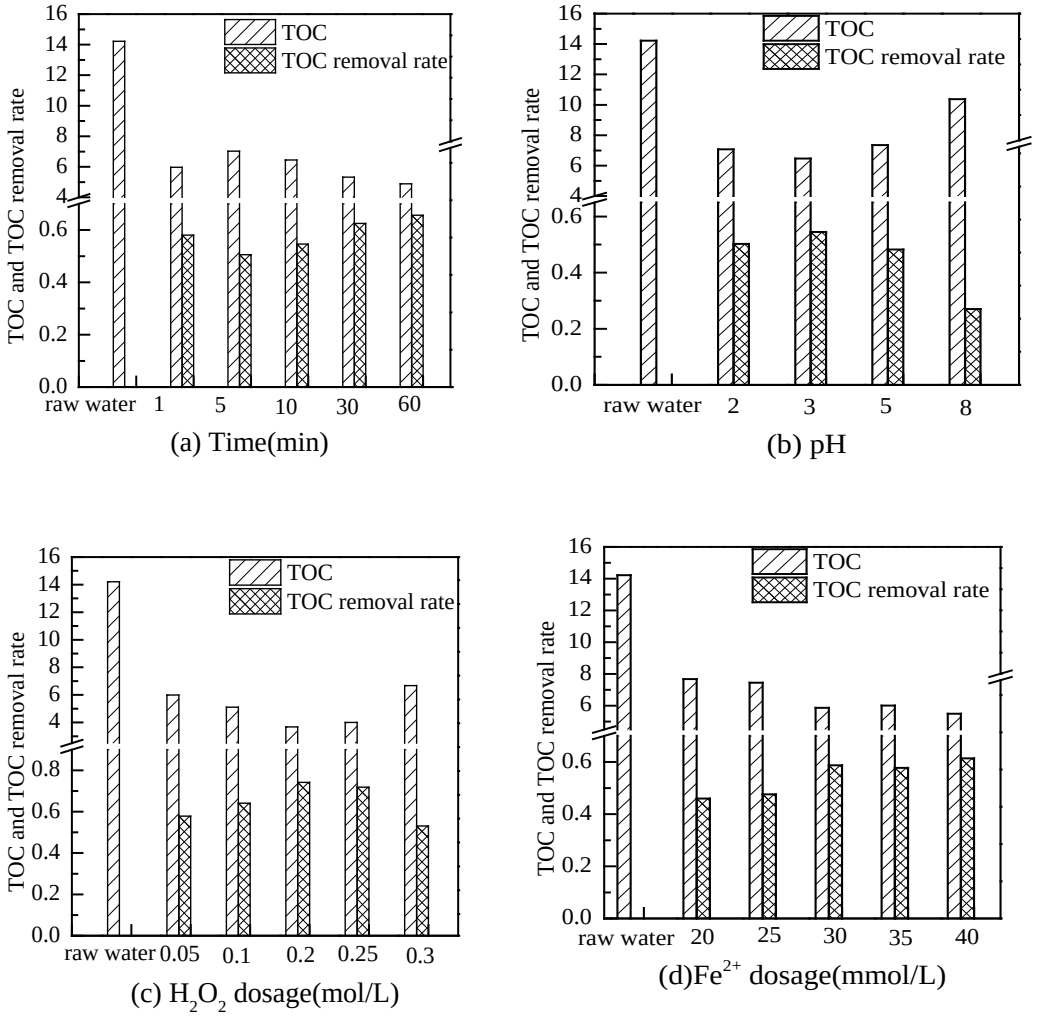

Figure 1 Single factor experiments of Fenton reaction on TOC for (a) time, (b) pH, (c) H_2O_2 dosage and (d) Fe^{2+}dosage

QPCR detection of tetracycline resistance genes

Three more experiments have been carried out in the optimal parameters group as pH=3, 0.2 mol/L H_2O_2 with 30 mmol/L $FeSO_4{\cdot}7H_2O$ reacting for 30min to investigate the efficiency of Fenton regent on tetracycline resistance genes. Comparing the target genes copies of treated sample with standard curve acquired by plasmid DNA, the final concentration of target genes have been shown in Table.3. The correlation coefficients are all above 0.99, and the amplification efficiencies are all among 90%-105%. It further confirms the equations are available and the results are correct.

Fenton regent resulted in a remarkable disinfection of target genes, as Figure 2 illustrated. The removal rate of target genes absolute concentration almost excessed 99%, except tet(M) of 48%. In log(copies/ml), tet(A), tet(C), tet(X) and tet(G) all have been eliminated 2.4-4.5logs. According to literatures(Zhang et al., 2009; Munir et al., 2011b; Yang et al., 2014; Huang et al., 2015), tetracycline resistance genes exist in wastewater as two ways, one for located in the organism intracellular as genetic material, another one located extracellular as naked DNA or independent DNA which

can be integrated by some bacterial such as Escherichia Coli. As shown in Figure 2,

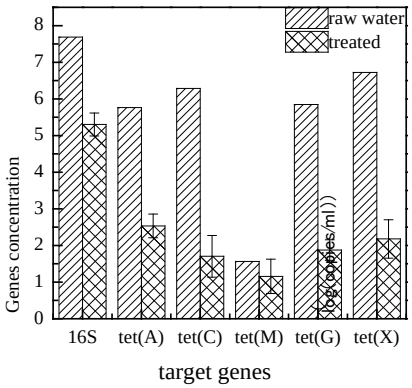

Figure 2. The effect of Fenton system reaction on target genes and 16SrRNA

Fenton regent not only can disinfect antibiotic resistance bacteria inactive, also can oxidize genes conformation.

PCR-DGGE analysis of bacterial community structure

The DGGE profiles of the bacterial communities for raw water and treated sample are shown as Figure 3. Many DGGE bands have been detected. There are slight different in raw water sample with treated sample. 11bands are their mutual bands, 5bands special for raw water and 2 bands special for treated sample. The relationship with raw water and treated sample are also displayed in Shannon-Wiener Diversity Index (SDI) and Equitability Index (EI). After Fenton regent treated, SDI has slight decrease from 1.14 for raw water to 1 for treated sample. It declared that bacterial community abundance decreased. And EI is also decreased from 0.945 for raw water to 0.877 for treated

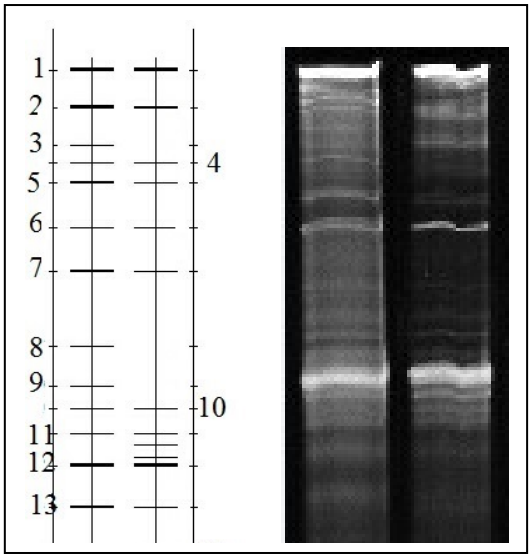

Figure 3. DGGE files of raw water and treated

sample. It indicated that the evenness of species distribution after treated is lower than raw water. And the bands similarity of treated with raw water is 65.2%. It expressed the bacterial community in treated sample have 65.2% similarity shown in bands location

and intensity. So Fenton regent have an apparent effect in destroying the bacterial community.

Conclusions

The optimal parameters of Fenton regents was listed as follows: pH=3, 0.2 mol/L H_2O_2 with 30 mmol/L $FeSO_4 \cdot 7H_2O$ reacting for 30 min. DGGE files have illustrated bacterial community diversity and equitability had decreased after Fenton oxidation. And the similarity is only 65.2% for treated to raw water. While the results of QPCR experiments described Fenton regent lead to a remarkable efficiency on tetracycline resistant genes removal. Comparing different mechanism, Fenton regent have stronger damage on that genes encode efflux pump proteins and modified enzyme and have relative week damage effect on that genes that encode ribosomal protection proteins. Moreover, along with the deceased of 16SrRNA, tetracycline resistant genes also have been oxidized and removed. And in the treated sample, tet genes/16SrRNA ratio have obvious drop, except tet(M). It also described that comparing with other treatments, Fenton regents have more fierce scavenging efficiency.

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