Isolation, Identification, and Bioaugmentation of an Oxytetracycline-Degrading Bacterium *Staphylococcus* sp. TJ-1 in Composting of Swine Manure

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***Abstract*—To enable hazard-free treatment of swine manure, a bacterium capable of using oxytetracycline as the sole source of carbon (*Staphylococcus* sp. TJ-1) was isolated. The organism was able to degrade 80.7% of oxytetracycline in 5.5 d when cultured at pH 7.0, 30°C and 100 mg/L of oxytetracycline. For its application in engineering, the key conditions for composting were optimized using the custom design. When setting the conditions as 70% of the initial percentage of moisture, an initial C/N of 30, inoculation with 1% TJ-1 and the stack-turning every 1.5 d, the degradation of oxytetracycline reached 98.2%, indicating that the organism could be used for agriculture application to reduce the oxytetracycline pollution.**

Keywords-oxytetracycline degradation; compost of swine manure; bioaugmentation; Staphylococcus sp. TJ-1

I. INTRODUCTION

Veterinary antibiotics (Vas) are widely used in many countries to treat disease, protect the health, and promote the growth of animals. In China, approximately 6000 tons of Vas is used per year [1]. According to Yang et al., 50–90% of the feeding-antibiotics could not be absorbed by the gut of the animal, and the output of antibiotics in manure by livestock and poultry is reportedly 2170 tons per year [2]. After their output, they enter into soil, ground water, river sediment and so on. If these antibiotics could not be efficiently degraded, they possible increased in antibiotic resistant genes in the environment, which will lead to serious environmental problems including ecological risk and human health damage [3, 4]. Thus, it could be paid more attention to the Vas degradation in environment.

There are many technologies that reduce or degrade Vas in manure, including aerobic composting and anaerobic composting. Composting is an effective method of reducing Vas in animal manure that has the advantages of low cost, reduced secondary pollution and the ability to treat large amounts of Vas [5].

Oxytetracycline is one of the most commonly used Vas in agriculture. Zhang et al. reported that residual oxytetracycline could be more than 100 mg/kg in manure, which bring significant effect on environment [6]. Moreover, the stability of oxytetracycline was significantly higher than that of other Vas. Its half-life in composting could be up to 30 d, and it could still be detected after five months [7, 8]. Thus, in order to reducing the hazardous effect of oxytetracycline on environment, some strategies should be adopted to accelerate oxytretracycline degradation in composting.

Bioaugmentation using specialized bacteria has proven effective in improving the efficiency of bioremediation by rapidly reducing recalcitrant compounds [6]. Therefore, the first aim of present work was to isolate, identify and characterize a bacterium with a high capacity for degradation of oxytetracycline. The conditions for bioaugmentation with the oxytetracycline-degrading bacterium for swine manure treatment was then determined as a further aim. Overall, the results presented here are beneficial for the hazard-free treatment of swine manure, which is the cause of many serious ecological problems.

II. MATERIALS AND METHODS

A. Media

Inorganic salt medium of oxytetracycline (ISM) (g/l) was composed of $(NH_4)_2SO_4$ (2.0), K_2HPO_4 (0.5) and NaH_2PO_4 (0.5) adjusted to pH 7.0. The medium was autoclaved for 30 min at 121°C and allowed to cool to 55°C, at which time filtrate-sterilized oxytetracycline was added. LB culture medium (g/l) was composed of beef extract (5.0) and peptone (10.0), and NaCl (10.0) adjusted to pH 7.0. Solid medium contained 20g agar per liter.

B. Isolation of oxytetracycline-degrading bacterium

A sample of swine manure was obtained from Changxing Huzhou Zhejiang Province. Enrichment of the oxytetracycline-degrading bacteria from the swine manure was conducted by transferring 1 g of swine manure to a 300-ml flask containing 100 ml ISM with 100 mg/l oxytetracycline and incubating the mixture on a reciprocal shaker at 30°C and 140 r/min for 7 d. Next, 1 ml of the swine manure culture was transferred to fresh oxytetracycline-ISM. After 1 month of enrichment, the culture was serially diluted and spread onto oxytetracycline-ISM agar plates containing 100 mg/l oxytetracycline. Following incubation at 30°C for 48 h,

individual colonies were selected and streaked onto new agar plates repeatedly until a bacterium capable of using oxytetracycline as the sole source of carbon was isolated.

C. Identification of strain TJ-1

The genomic DNA was extracted using a Bacterium Genomic DNA Extract Kit (Bioteke, China), after which the 16S rDNA was amplified by polymerase chain reaction (PCR) using the method described by song et al. [9].

The obtained 16S rDNA sequence was aligned in GenBank using the BLAST program, then subjected to multiple sequence analysis using Clustal X (Version 1.8). Phylogenetic and distance analysis of the aligned sequences was then performed using Molecular Evolutionary Genetics Analysis (MEGA, Version 5.05). Finally, the maximum parsimony method was used to develop a phylogenetic tree, which was evaluated by bootstrap analysis based on 1,000 resamplings.

D. Bacterial growth and oxytetracycline degradation by strain TJ-1

The dynamic curve of the isolate was developed as follows: 3% pre-cultured bacteria (OD_{600}=1.251) was inoculated into 500 ml flasks with 150 ml of ISM containing 100 mg/l oxytetracycline at a pH of 7.0 and then cultured on a reciprocal shaker at 30°C and 130 rpm. The culture was then sampled to measure the cell growth and oxytetracycline degradation.

To determine the optimum pH for TJ-1 cell growth and its degradation of oxytetracycline, the initial pH of the ISM was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and.10.0, after which 3% pre-cultured bacteria (OD_{600}=1.387) was inoculated into ISM containing 100 mg/l oxytetracycline. To evaluate the dependence of cell growth and oxytetracycline degradation on culture temperature, 3% pre-cultured bacteria (OD_{600}=0.807) was inoculated into ISM containing 100 mg/l oxytetracycline and then cultured at 4°C, 25°C, 30°C, 35°C, 40°C, 45°C and 55°C. In general, the bacterial strain cannot grow at 4°C. However in order to make sure of it, we set this temperature so we could store the samples. Every treatment has three replicates. Except the optimization of temperature, all cultures were incubated on a reciprocal shaker at 30°C and 130 rpm. Finally, all cultures were sampled at 96 h for cell growth and oxytetracycline degradation.

E. Bioaugmentation with strain TJ-1 in composting of swine manure

Strain TJ-1 was inoculated in the composting system with aim to accelerate the oxytetracycline degradation in swine manure. The swine manure for this study was collected from Huajiachi, Hangzhou, Zhejiang, China. The fresh swine manure was tested without oxytetracycline, which was tested by high performance liquid chromatography (HPLC) as mentioned in Section 2.6. In addition, sawdust used to adjust C/N ratio, was purchased from a timber mill in the suburbs of Hangzhou. The basic properties of both materials are shown in Table I.

TABLE I. BASIC PROPERTIES OF RAW MATERIALS.

Material	Moisture%	C% (in dry weight)	N% (in dry weight)	C/N
Swine Manure	69.8	39.4	1.8	21.9
Sawdust	8.5	49.4	0.74	66.8

TABLE II. LEVELS AND CODES OF VARIABLES SELECTED FOR THE CUSTOM DESIGN.

Variables	Symbols[a]		Coded Levels		
	Uncoded	Coded	–1	0	1
Moisture (%)	X_1	x_1	50	60	70
C/N	X_2	x_2	20	25	30
Microbial inoculation (%)	X_3	x_3	0	0.5	1
Stack-turning (times/d)	X_4	x_4	1	2	3

[a] $x_i=(X_i-X_0)/\delta X$, where X_0 is value of the X_i at the center point and δX presents the step change.

Based on our previous investigations, the % moisture, C/N ratio and stack-turning frequency have the important effects on common compost of swine manure. Thus, in order to good-performance of bioaugmentation of strain TJ-1 into the swine manure compost, the three aforementioned factors and the rate of inoculation were optimized using a custom design. The levels of the variables are given in Table II.

According to the experimental design, the % moisture and initial C/N ratio were adjusted using the sawdust. Prior to composting, 150 mg/kg of oxytetracycline was added to the 5 kg swine manure, which was subsequently placed in darkness for 24 h to equilibrate. Afterward, the mixture was inoculated with strain TJ-1 with an OD_{600} value of 1.376 according to the designed proportions. The mixture was then composted at 30 ± 5°C in a warm house. All composts were stack-turned for aeration according to the experimental design. All composts were sampled at 0 and 12 d to monitor oxytetracycline degradation. Every treatment has three replicates. Meanwhile, the compost with initial water rate of 70%, initial concentration of oxytetracycline 150 mg/L, and initial C/N ratio of 30 was taken as black.

F. Analytical methods

Bacterial growth was measured based on the absorbance at 600 nm using a Unico UV-2000 spectrophotometer. Oxytetracycline concentration was determined using a Waters high performance liquid chromatography (HPLC) with a Waters e2695 spectrophotometer and a Waters 2489 UV/Visible detector at a detection wavelength of 365 nm and a temperature of less than 30°C. The mobile phase, which contained 25% acetonitrile and 75% 0.01 M oxalic acid, was applied to an XBridge™ C18 column (5.0 μm and 4.6

mm×250 mm) at a flow rate 1.000 ml/min. The sample injection volume was 20 ul.

G. Statistical analysis

All data are expressed as the mean ± standard error (SE). Statistical analyses were performed using one-way ANOVA conducted with SPSS (version 16.0) and surface regression (JMP 7.0.1, SAS Institute Inc., Cary, North Carolina, USA) based on fitting to a second-order polynomial model. Differences were considered significant at $p < 0.05$.

III. Results and Discussions

A. Isolation and identification of oxytetracycline-degrading bacterium

A bacterium using oxytetracycline as the sole source of carbon was isolated from swine compost samples and denoted TJ-1. On the plates containing oxytetracycline, strain TJ-1 formed yellow round colonies with smooth surfaces. Microscopic observation revealed that strain TJ-1 was a Gram-positive, round and non-flagellum bacterium.

The 16S rDNA sequence was amplified from the total DNA of TJ-1 by PCR, after which its sequence was determined and deposited in the GenBank database under accession number JN794602. BLAST searches of the NCBI website were subsequently used for analysis of the 16S rDNA gene and MEGA 5.05 was used to construct a phylogenetic tree. As shown in Figure 1, the 16S rDNA sequence of strain TJ-1 had 97% similarity to that of Staphylococcus sp. R-25657; accordingly, strain TJ-1 was classified as Staphylococcus sp.

B. Bacterial growth and oxytetracycline degradation by strain TJ-1

In preliminary study, 100 mg/l oxytetracycline ISM without inoculation was cultured in 30°C and 130 rpm to estimate its natural lose. After 72 h, there was 97.7–99.5 mg/l oxytetracycline in ISM detected. Thus, its natural lose was limited. All in following study we think the loss of oxytetracycline was mainly degraded by bacteria.

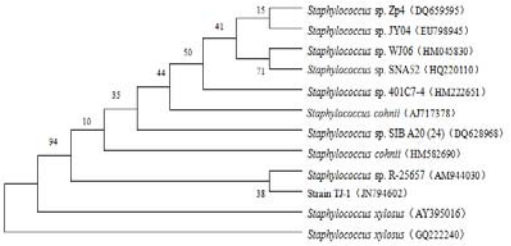

Figure 1. Phylogenetic tree of *Staphylococcus* sp. TJ-1 based on its 16S rDNA sequence.

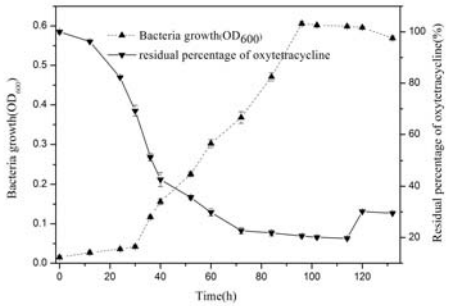

Figure 2. Cell growth and oxytetracycline degradation curves.

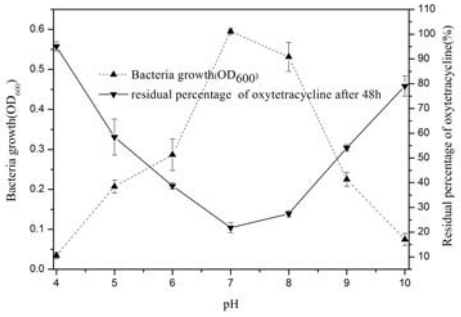

Figure 3. Effect of initial pH on cell growth and oxytetracycline degradation.

Dynamic curves of growth and oxytetracycline degradation by strain TJ-1 are shown in Figure 2. There was a 30 h lag phase in growth and oxytetracycline degradation by strain TJ-1, after which the bacterium entered the logarithmic phase. The maximum growth and oxytetracycline degradation occurred after culture for 96 h, as indicated by an OD_{600} of 0.606 and a decrease in concentration from 100 mg/l to 22.3 mg/l. The bacterium then entered a stationary phase. Regression analysis of the increase in OD_{600} and the decrease in oxytetracycline concentration showed a solid linear relationship between growth and oxytetracycline degradation, with an R^2 value of 0.97 ($p < 0.01$), indicating that oxytetracycline degradation was primarily dependent on growth of the isolate. Up to now, few oxytetracycline-degrading bacteria were isolated [10]. According to Li et al., compost composed of a mixture of soil and swine manure required 30 to 41 d for 50% oxytetracycline degradation, while 100 to 137 d were required for 90% degradation [8]. Thus, the new isolated bacterium has a great potential on swine manure composting to accelerate oxytetracycline degradation.

The character of bacterial growth and oxytetracycline of strain TJ-1 has been shown in Figure 3 and 4. As shown in Figure 3, strain TJ-1 could grow at pH 5–9, with an optimal pH of 7.0. Under the optimal pH, the strain could also rapidly degrade oxytetracycline, as indicated by a decrease from 100 mg/l to 21.8mg/l. This optimum pH range (pH 5-9) is broader than that of Actinomucor elegans

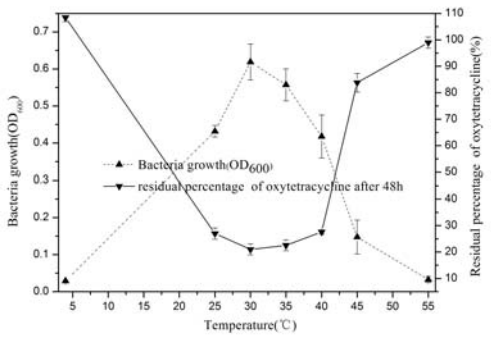

Figure 4. Effect of temperature on cell growth and oxytetracycline degradation.

TABLE III. THE RESULTS OF THE CUSTOM DESIGN.

Operation	Moisture (%)	C/N	Microbial Inoculation (%)	Stack-turning (d/times)	Degradation of Oxytetracycline (%)
1	50	20	0	3	59.18
2	50	30	1	2	79.86
3	70	30	0.5	3	89.08
4	50	25	0.5	1	81.81
5	70	25	0	2	83.08
6	60	20	0.5	2	72.25
7	60	30	0	1	86.96
8	70	20	1	1	87.76
9	60	25	1	3	80.76
Optimal Predict	70	30	1	1.5	98.2

BFL-5, which has an optimal pH of 4.5 to 5.0 [11]. As shown in Figure 4, temperature had a great effect on the growth of strain TJ-1. Specifically, this organism could grow at 25–45°C, with the optimal growth occurring at 30°C; however, it could not grow at greater than 45°C. Generally, Staphylococcus have the potential to cause disease; however, since the temperature can reach 55°C or higher after long periods of time during composting, the organism could not survive the process, resulting in decreased risk to the environment.

C. Bioaugmentation of strain TJ-1 to composting of swine manure

Definitely, oxytetracycline could be absorbed by swine manure. Thus, 150 mg/kg oxytetracycline was added into sterilized swine manure and sampled to monitor oxytetracycline concentration for 7 d in preliminary study. The result showed that the absorption of oxytetracycline mainly occurred at the first 12 h and the ratio of absorption maintained in the range of 7.67% to 12.53%. The degradation rate of

TABLE IV. ANALYSIS OF VARIANCE (ANOVA) OF THE FITTED LEAST SQUARE MODEL OF OXYTETRACYCLINE DEGRADATION.

Source	DF	Sum of Squares	Mean Square	*F* Value	*p* Value>*F*
X_1	1	0.0254	0.0254	32.1969	0.0048**
X_2	1	0.0225	0.0225	28.4247	0.0060**
X_3	1	0.0061	0.0061	7.7432	0.0497*
X_4	1	0.0126	0.0126	15.9628	0.0162*
Regression	4	0.0666	0.0167	21.0819	0.0060**
Error	4	0.0032	0.0008		
Total	8	0.0698			
R^2=0.9547					

**Extremely significant at $p<0.01$.
*Significant at $p<0.05$.

Oxytetracycline in different composts after 12 d is shown in Table III. As the environmental factors changed, the degradation rate of oxytetracycline changed from 59.18% to 89.08%. Compared to the absorption detected in preliminary study, the aerobic compost of swine manure was efficient to oxytetracycline degradation.

Multiple regression analysis revealed that the following second-order polynomial equation described oxytetracycline degradation.

$$y=0.1640+0.6512x1+0.0122x2+6.3867x3-0.0459\ x4 \quad (1)$$

where y was the predicted response of the degradation rate of oxytetracycline, x1 was the % moisture, x2 was the C/N ratio, x3 was the inoculation amount and x4 was the stack turning frequency.

ANOVA was used to evaluate the goodness of fit of the model. The correlation coefficient (R^2) was equal to 0.9547 (Table IV), which implies that more than 95% of the sample variation can be attributed to the variables. In addition, the model was significant in statistics ($p<0.01$) (Table IV). Taken together, these findings demonstrate that the model-predicted values agreed well with the experimental values. As shown in Table IV, all four variables had a significant effect on the degradation of oxytetracycline by strain TJ-1, which suggests that they should be carefully controlled when designing compost systems to improve the degradation of oxytetracycline. Finally, based on the regression equation, at an initial moisture content of 70%, C/N ratio of 30, inoculation amount of 1% and stack-turning of 1.5 d, the degradation of oxytetracycline would be 98.2%. Overall, these findings indicate that inoculating Staphylococcus sp. TJ-1 into manure compost is an effective method of reducing the release of oxytetracycline into the environment.

IV. CONCLUSIONS

An Oxytetracycline-degrading bacterium (denoted TJ-1) capable of using oxytetracycline as the sole source of carbon was isolated and investigated. The 16S rDNA sequence showed that it belonged was a Staphylococcus sp. with an oxytetracycline degradation rate of 77.7% at 96 h when cultured at pH 7.0, 30°C and 100 mg/l oxytetracycline. According to regression equation, the

optimal conditions for bioaugmentation with strain TJ-1 in composting to degrade oxytetracycline were as follows: 70% initial moisture, initial C/N of 30, microbial inoculation value of 1% and stack-turning every 1.5 d. Under the optimal conditions, the degradation of oxytetracycline was 98.2%, indicating that the organism could be used for agriculture application to reduce the oxytetracycline pollution.

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