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Tetracycline Biodegradation by *Shigella flexneri* strain TA_E_3 Isolated from Wastewater Treatment Plant

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Abstract

Although the transformation of tetracycline (TEC) by abiotic mechanisms has been extensively reported in literature, knowledge on the potential degradation of TEC by microbial activities in aquatic environments is still scarce. Some limitations are present for tetracycline biodegradation including the typical metabolite products that have been produced by the *Shigella flexneri* TA_E_3. Therefore, the purpose of this study is to investigate the by-products produced during biodegradation of tetracyclines by using *Shigella flexneri* TA_E_3 as model, and to determine the possible TEC degradation by-products and metabolic pathway. The changes in the concentration of the TEC were measured within 6 hours of degradation. TEC degradation by-products produced from biodegradation by strain TA_E_3 have been studied. The result shows that danazol is the main biodegradation product identified using LC-MS Q TOF analysis. Potential degradation pathways were then proposed including the removal of amide, carbonyl, and amine groups, alongside the isomerisation of the parent compound. The findings of this work can serve as a theoretical foundation for more accurate predictions of antibiotic destiny, transit, and degradation in aquatic environments.

Keywords Tetracycline, *Shigella flexneri* TA_E_3, biodegradation, tetracycline by-product, metabolic pathway

1.0 INTRODUCTION

Tetracyclines are known as the common antibiotic drugs in treating many different kinds of bacterial infections affecting the skin, intestines, respiratory tract, and other parts of the body. It is also widely used in animal husbandry and aquaculture to control diseases that affect livestock and as additives to enhance animal growth rate and disease resistance. It was reported that the tetracycline usage in Malaysia, United Kingdom, Korea, and Japan are approximately 43.97% [1], 90%, 50%, and 43%, respectively [2]. Even though tetracyclines have been shown to benefit human and animal health, its overconsumption may lead to allergic reactions in humans, bacterial resistance, and serious fluctuations in environmental microflora which is detrimental to environmental health [3].

Globally, tetracyclines (TECs) are among the most widely used antibiotics. Along with treating a range of bacterial diseases, this broad-spectrum class of antibiotics has been shown to prevent bacteria from synthesising proteins. About 20 different chemicals known as antibiotics called TECs have been introduced into the market and are generated from different species of *Streptomyces*. Tetracyclic rings with diverse hydroxyl, methyl, keto, as well as di-methylamino functional groups make up the fundamental structural component. Based on their composition, dose, and rate of elimination, TECs are subdivided into three classes: oxytetracycline (OTC), chlortetracycline (CTC), and tetracycline (TEC) [3].

Due to limited ability to consume or metabolise TECs; between 30% and 90% of TECs will end up in animals' faeces and urine and expelled into the environment. Several studies revealed significant levels of TECs residues in manure (78.6 mg/kg TEC, 764.4 mg/kg CTC, and 78.5 mg/kg doxycycline (DO). The degradation efficiency of TECs using conventional treatments, including compost along with activated sludge, is still relatively low [2]. They also reported that only 27% of the CTC were removed from hog manure following 42 days of composting. Wastewater treatment plants eliminate only around 12% of TEC, 35% of demeclocycline (DC), as well as 28% of OTC.

Biodegradation is important in the treatment of antibiotic contamination in the environment. Tan et al. [2], also found that removing veterinary medications from swine wastewater by aerobic and anaerobic biodegradation was effective. Existing research demonstrated that crude lignin and manganese peroxidases from *Phanerochaete chrysosporium* may effectively remove TEC and OTC, respectively [2]. Nevertheless, the enzymatic reaction system needs a variety of chemicals, such Mn^{2+} and H_2O_2, which raises the input cost for large-scale use. Aside from the elimination of TECs, there is additional concern on the toxicity of the biodegradation products. Therefore, it appears that microbial breakdown of TECs is a safer process than enzymatic conversion. In addition, a species of microorganism such as *Trichosporon mycotoxinivorans* strain XPY-10 [4] is one example of a bacterium where the toxicity of TEC breakdown products reduces. However, as most research focuses on specific bacterial species, little is known about the mechanism by which the *Shigella flexneri* strain degrades TEC.

The results of this study may greatly contribute to existing techniques in managing tetracycline pollution in the environment. Tetracycline removal from wastewater, soil, and other contaminated environments might benefit from the development of novel bioremediation methods using *S. flexneri* strain TA_E_3. Additionally, the information gleaned from this work may help in the design and optimisation of related biotechnological applications, such the creation of microbial consortiums or genetically modified strains for improved tetracycline breakdown. This study will provide a thorough study on tetracycline biodegradation by *S. flexneri* strain TA_E_3 isolated from the wastewater treatment plant.

2.0 EXPERIMENTAL

2.1 Materials

The strain used in this study is *Shigella flexneri* TA_E_3. This strain is previously isolated and characterized from the Indahwater wastewater treatment plant, effluent 3, at Taman Tun Aminah, Johor, Malaysia. The broth media was prepared based on Bertani [5], by mixing 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride, NaCl, into 1 L of distilled water in a 1 L Schott bottle. The solution was then autoclaved at 121 °C, 15 lb/sq.in. (psi) for 20 minutes. LB media was left at room temperature to cool and stored in a chiller (4°C) for future use. As for nutrient agar (NA), the nutrient agar was prepared (Nutrient Agar 500 g, Oxoid) by weighing 7 g of Nutrient Agar powder (composition: agar, 15 g/L, meat extract, 1 g/L, peptone, 5 g/L, sodium chloride, 5 g/L, and yeast extract, 2 g/L) using an electronic balance and mixed with the distilled water at volume 250 mL in 250 mL Schott bottle. The mixture is stirred until the agar is completely dissolved and autoclaved at 121°C, 101.3 kPa for 20 minutes. The autoclaved medium was left at room temperature to cool prior to pouring into the sterile petri dishes. The agar is allowed to solidify. The solidified agar plates are stored in the refrigerator at temperature 4°C for future use. Tetracyclines were purchased from (VNK Supply & Services, Taman Kota Masai, Johor, Malaysia).

2.2 Bacteria growth profiling

The UV-Vis spectral analysis was performed using the spectrophotometer (Thermo Scientific) for the determination of bacterial growth profile based on the measurement of bacteria absorbance at OD_{600}·· according to method by Shao et al., [6]. *S. flexneri* TA_E_3 were cultured in 100 mL of LB broth containing 6 mg/mL of TEC and incubated at 37°C at 150 rpm. The growth profile data were recorded for every 2 hours within 24 hours of incubation and later tabulated.

2.3 Tetracylines biodegradation

All samples were characterized using Fourier Transform Infrared (FTIR) Spectroscopy, X-Ray Diffraction (XRD), and Energy Dispersive X-Ray (EDX). Thermo Scientific Nicolet iS10 with Smart iTR Diamond crystal FT-IR (Fourier Transform Infrared) Spectrometer was used for the structural analysis. About 1-2mg of sample was placed on the sample area. The FTIR spectrum was then recorded using OMNIC software in the range of 4000 to 650 cm^{-1} using Attenuated Total Reflectance (ATR) technique. The modified kaolinite powders were characterized by the XRD (X-ray Diffraction) instrument (Bruker, D8 advance). The XRD patterns were recorded with a CuKα radiation at λ = 1.5406 Å at 40 kV and 20 mA in the range of 2θ = 5° to 35° with a scanning speed of 0.05° per second. The morphology of kaolinite and modified kaolinite was characterized by an image taken by CARL ZEISS 35 VP Supra FESEM.

2.4 Tetracycline and by-product analysis

In order to prepare standard samples, a serial dilution was carried out for HPLC analysis use. The standards were prepared with the following concentration such as 2 ppm, 1.6 ppm, 1.2 ppm, 0.8 ppm, 0.6 ppm and 0.4 ppm of TEC solution and stored in 2 mL vials.

An HPLC system consisting of an HP 1100 Series (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump with four solvent channels, degasser system, automatic injector, column thermostat, and diode array detector was used for the analysis. The chromatographic conditions were chosen in terms of peak shape, column efficiency, retention time, resolution, and sensitivity. Separations were performed on a reverse-phase C18 column (2.1 mm x 150 mm, 5 µm silica) from Eclipsed, maintained at 35°C. The flow rate was 0.3 mL/min and the injection volume 3.0 µL. The composition of mobile phases A and B was set as 0.1% acetic acid (A), and acetonitrile (B). The intermediate products were identified using LC-MS Q-TOF (Agilent Technologies, Santa Clara, CA, USA). Separation was performed on a C18 column (2.1 x 50 mm, 1.7 µm, Eclipse) with a flow rate and injection volume of 0.3 mL min^{-1} and 3 µL. The mobile phase consisted of 0.1% acetic acid, and acetonitrile at the gradient elution conditions [6].

KEGG Software was also employed to search for the compound ID resulting from the TEC degradation. The related metabolic pathway of the selected possible degradation compound was then determined.

3.0 RESULTS AND DISCUSSION

3.1 Bacterial growth under TEC stress

The cell growth and the changes in TEC concentration during biodegradation under aerobic conditions were presented in Figure 1 and Figure 2. From hours 0-5, the bacterial growth was still in a lag phase until after 6 hours of cultivation under TEC stress. Then, bacterial growth appeared to be in a logarithmic phase as evidenced by the exponential increase in OD_{600} value from 0.015 to 1.117. Meanwhile, as shown in Figure 2, the strain TA_E_3 has effectively degraded TEC in solution over the course of 6 hours, with a rapid fall in concentration from 6 g/L to 0.01 g/L with almost 100% reduction in Tet concentration.

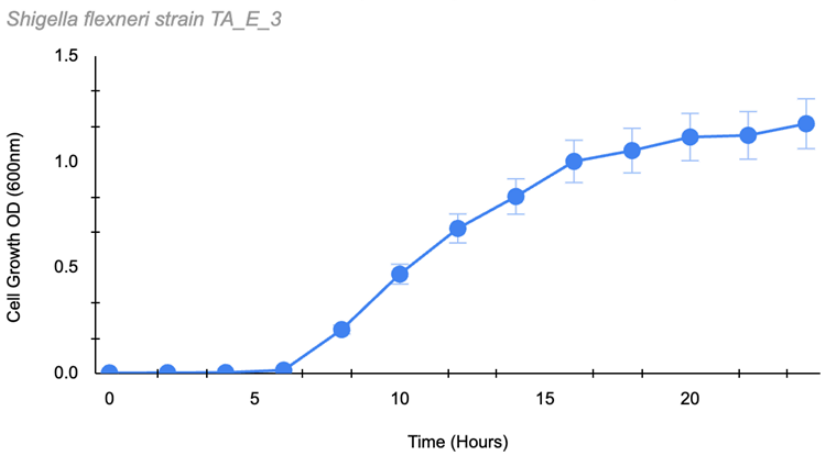


Figure 1 Growth profile of *S. flexneri* strain TA_E_3 with absorbance at wavelength 600 nm over time (hours).

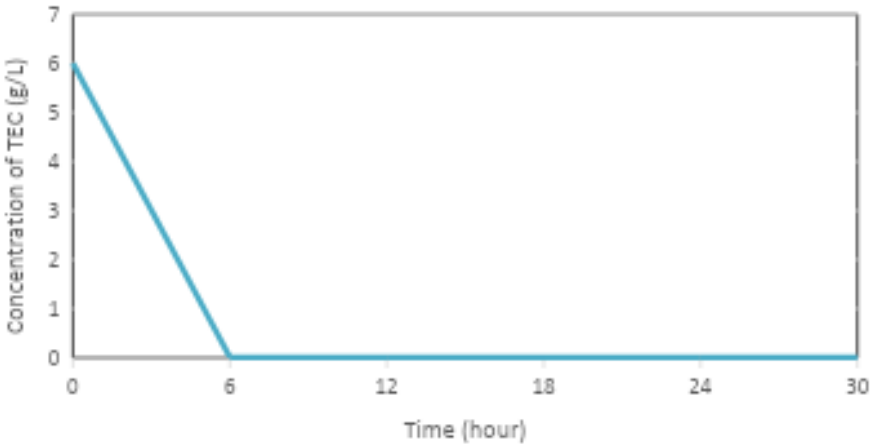


Figure 2 Tetracycline concentration over time (hours)

3.2 By-product of TEC biodegradation

A possible biodegradation product was identified with retention time of 4.85 min, as shown in Figure 3. The biodegradation products of TEC in terms of mass, parent component, and composition of elements from the reacted sample are also tentatively proposed. Figure 4 suggested potential biodegradation routes for TEC by strain TA_E_3.

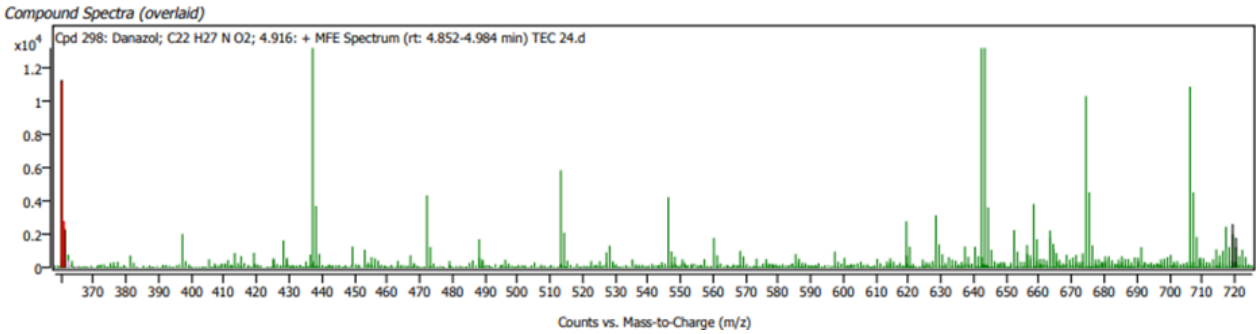
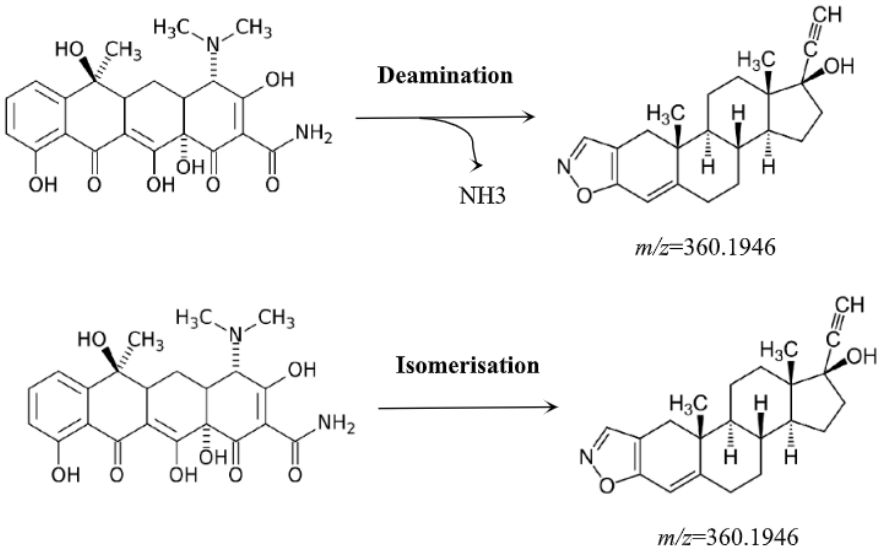


Figure 3 Retention time for danazol compound from LC-MS Q TOF analysis.



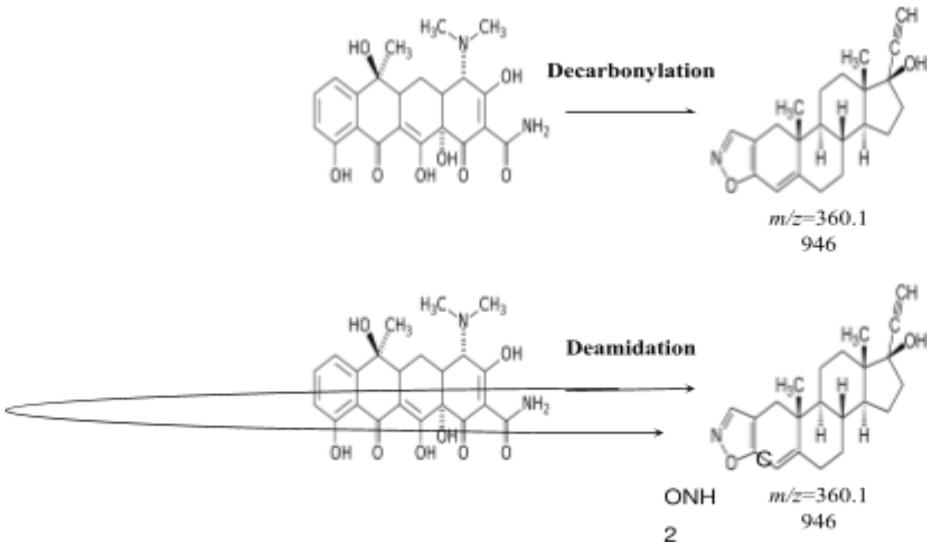


Figure 4 Proposed biodegradation pathways of TEC by strain TA_E_3

The proposed biodegradation pathways are shown in Figure 4, where the parent compound TEC was transformed into danazol (*m/z* = 360.1946) through deamination, isomerisation, decarbonylation, and deamidation alternatively. Deamination involves the removal of the amine group at position C4. This is followed by the isomerisation of the parent compound through hydrolysis by isomerase. Figure 5 revealed the map of the biosynthesis pathway of danazol and the position of the isomerase enzyme with E.C. number 5.3.3.1 in the KEGG metabolic pathway is shown as the red line, indicating it transpose the C-C and C=C bonds in TEC molecular structure. According to Zhong et al. [8], the epimerization of tetracyclines, which results in the creation of the matching 4-epimers through epimerization at position C4, is a significant early transformation event. Previous studies revealed that hydroxyl groups at the C6 of tetracyclines may attack the carbonyl group at the C11 to isomerize the corresponding iso-tetracyclines [8]. Decarbonylation on the other hand, involves the quick dissociation of CH_3CO once the C-C bond in acetylacetone was broken, releasing CO [8] (Zhong et al., 2022).

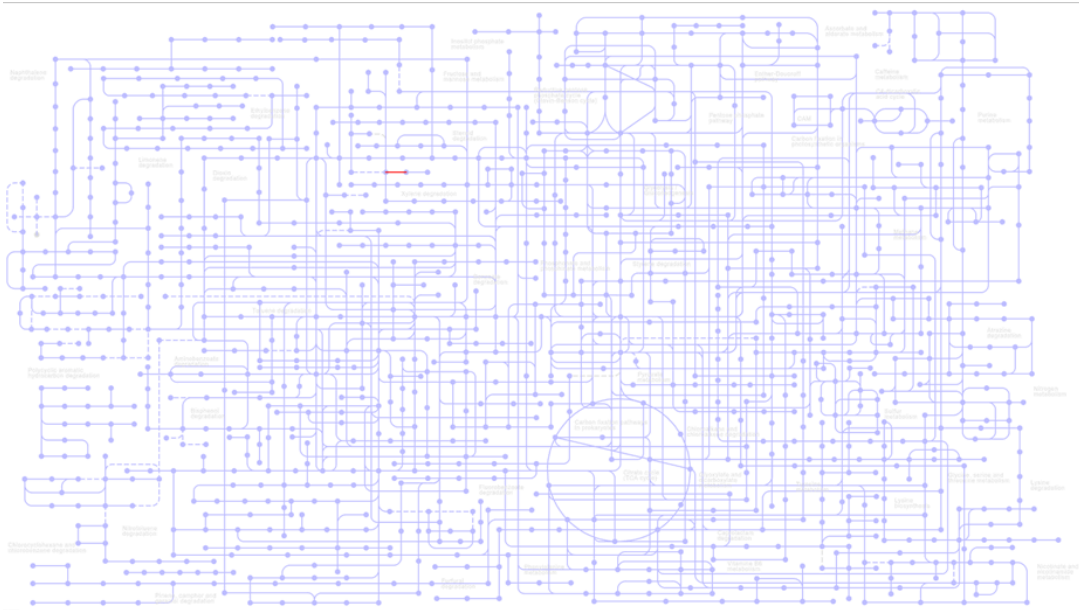


Figure 5 Biosynthesis pathway of danazol. Retrieved from https://www.genome.jp/pathway/rn01120+RC00762

Finally, the possible pathway would be deamidation where an amide bond (-$CONH_2$) was cleaved from C2 of TEC. Zhong et al., [8], has reported that TEC deamidation products were produced via an $e^{-1}aq$ reaction with an amide group in the location of ring A. They proposed that the anion radical may be created by the addition of $e^{-1}aq$ to the keto-tautomer, followed by the elimination of the formamide radical and the enol product. Since deamidation may only occur in acidic or alkaline environments, these hydrolysis products have only been occasionally documented before. Additionally, the susceptibility of the identification of low-abundance deamidation compounds is enhanced by high-resolution mass spectrometry. However, the hypothesised degradation route might not be sufficiently thorough given that certain products could not be detected and

quantitative analysis may pose certain challenges. Further studies on other intermediates of the TEC biodegradation by strain TA_E_3 are required in order to obtain a full degradation pathway of TEC, and can be reused this work, to study on the other effects of factors on TEC degradation by *Shigella flexneri*, such as temperature, pH, and inoculation dosage.

4.0 CONCLUSION

This study has successfully developed a single microbial culture, *Shigella flexneri* TA_E_3 to biodegrade tetracycline (TEC). The bacteria showed the ability to biodegrade about 100 % of TEC within 6 hours of cultivation, at the temperature of 37 °C. TEC was almost completely transformed by strain TA_E_3 after 6 hours of incubation, and its concentration rapidly dropped from 6 g/L to 0.01 g/L. A possible biodegradation product also which is danazol was identified, and few potential degradation pathways were presented and discussed accordingly.

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