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Isolated *Escherichia coli* Strain S591890 from a Wastewater Treatment Plant for Tetracycline Biodegradation

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Abstract

Tetracycline is a broad-spectrum antibiotic that is commonly used in both human and veterinary medicine. It is known to persist in the environment, including wastewater systems, where it can contribute to the development of antibiotic resistance in bacteria. This study aims to understand the mechanisms and potential of tetracycline removal through biodegradation by *Escherichia coli* strain S591890. The quantification of tetracycline was conducted using HPLC analysis. In addition, QTOF LC-MS was employed to investigate the biodegradation mechanisms of tetracycline. Analysis of tetracycline and its degradation products was conducted using liquid chromatography for separation and mass spectrometry for detection and identification. By monitoring the changes in the tetracycline peak and the appearance of degradation products over time, valuable insights into the degradation pathways and intermediates can be obtained. Furthermore, QTOF LC-MS enables the identification of specific metabolites formed during tetracycline biodegradation, providing valuable information about the transformation products and their potential toxicity.

Keywords Tetracycline, biodegradation, *Escerichia coli*, liquid chromatography, tetracyline by-product

1.0 INTRODUCTION

Tetracyclines (TCs) are a broad-spectrum bacteriostatic class of antibiotics that inhibit protein synthesis in bacteria by interfering with the binding of aminoacyl-tRNA with bacterial ribosomes to hinder bacterial protein production [1]. This involves the inhibition of polypeptide chain lengthening and therefore bacterial growth.

TCs degrading bacteria have been isolated from the environment since microbial biodegradation plays an important role in removing tetracyclines in both natural and artificial systems. Techniques used in wastewater treatment facilities to remove antibiotic residues include adsorption, precipitation, irradiation, membrane separation, hydrolysis and biodegradation [2]. The removal of antibiotics through biodegradation is receiving a lot of attention due to its potential benefits including ease of use and low cost.

Tetracyclines are stable and difficult to oxidise in the environment. However, they become unstable at high pH, which may indicate low volatility, which eventually denotes reduced degradability [3]. There are varieties of bacteria that are able to biodegrade a wide range of TCs waste releases by using biological techniques to achieve high efficiency in biodegradation with appropriate design and operational conditions. Additionally, TCs can be recycled from pharmaceutical manufacturing facilities,

as well as from homes, livestock and breeding industries, and wastewater. To solve the problem, the environmental fate of antibiotics has become a research area.

Furthermore, the long-term negative impacts of TCs will result in ecological imbalance because of the inadequate degradation of TCs by the current treatment techniques [3]. The enormous use of TCs in aquaculture, livestock and the treatment of human diseases has resulted in an increasing threat to both marine and terrestrial biodiversity worldwide. Excessive or inappropriate use of TCs can encourage the development of antibiotic-resistant bacteria (ARB). Bacteria can develop resistance through a variety of ways, including mutation or the acquisition of resistance genes from other bacteria. Tetracycline resistance decreases the ability of this class of medications to effectively treat bacterial infections. The current study aims to fill in these research gaps by gathering information on various degradation strategies, the mechanisms involved in biodegradable and non-biodegradable routes, the primary factors affecting degradation strategies and their potential impact on degradation.

2.0 EXPERIMENTAL

2.1 Materials

Tetracycline of analytical grade, containing 99% purity, was purchased from Sigma-Aldrich. The tetracycline powder was stored at 4°C. Tetracycline was prepared as a stock solution in methanol at a concentration of 5000 mg·L^{-1} and was stored at 4°C. For the purification and growth of axenic cultures, Luria-Bertani (LB) media with the following composition was routinely used: tryptone, 10.0 g; yeast extract, 5.0 g; and sodium chloride, 10.0 g. The pH of the LB media was adjusted to 7.0 and moisture sterilised at 121°C for 20 min. For the TC stock solution, it was prepared in sterilised water at a concentration of 10 mg/mL. The TC stock solution was filtered through a 0.22 µm syringe filter; it was stored at 4°C.

2.2 Bacterial growth measurement

The *Escherichia coli* strain S591890 was originally isolated from the Taman Tun Aminah treatment plant and characterised as a tetracycline-resistant bacteria. *E.coli* strain S591890 was then cultured from the glycerol stock on Luria-Bertani agar (LB). All the plates were incubated at 37 °C for 24 h. Bacterial suspensions were prepared via direct colony suspension method as described by the Clinical Laboratory Standards Institute [4]. A single colony was inoculated into the conical flask containing 100 mL of LB culture medium. The inoculum suspensions were then adjusted in broth to reach 0.5 MacFarland and further dilution was performed to reach a final inoculum of $5x10^{-5}$ CFU/mL in each tube which contained 100 mL of Luria Bertani (LB) medium with tetracycline (10 mg/mL) and 3 mL of bacterial inoculum suspension. A negative and positive control for each test were included. All the tubes were incubated in the shaking incubator at 37 °C, 120 rpm for 24 h, in which the change in bacterial population in the medium over time (growth curve) was recorded at 2-hour intervals for 24 h through the measurement of optical density (OD600) at time 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h [5].

2.3 Quantification of tetracycline biodegradation

In LB medium, both bacterial growth and tetracycline degradation were observed. A freshly prepared active culture of *E. coli* strain S591890 was inoculated into 100 mL of LB medium containing 10 mg L^{-1} tetracycline and the samples were prepared in triplicate. As a control, a conical flask with LB and tetracycline but no bacteria was prepared. A UV-Visible spectrophotometer was used to measure the bacterial growth at 600 nm. The remaining tetracyclines were extracted and put through a HPLC analysis from each of the treatments. With the aid of an extraction solution, tetracycline was twice removed from the samples using 4:1 water (containing 0.1% acetonitrile): acetic acid. The mobile phase consisted of acetonitrile and acetic acid (containing 0.1%); tetracycline was measured by using an Agilent 1260 HPLC system with a 4.6 x100 mm column (Poroshell 120 EC-C18, Agilent).

The mobile phase A was used to carry out gradient elution with 0.1% acetic acid in water with the mobile phase B (0.1% acetic acid in acetonitrile). The proportion of phase B varied with time (0–3 min, 10–20%; 3–8 min, 20%; 8–17 min, 20–100%; 17–18 min, 100–10%; 18–20 min, 10%), and a flow rate was determined at 0.3 mL/min. The column temperature was held constant at 35 °C and the detection PDA wavelength was set to 210–400 nm. The mass spectrometer received the complete LC flow. The MSe scan mode was used for positive ionisation mode scan with the capillary voltage set at 2.5 kV and MS detection was performed in the positive ESI mode in the MS range of m/z 50-1000. Temperatures at the source and during desolvation were 125 °C and 400 °C, respectively. Desolvation gas flow and cone gas flow were set at 800 L/Hr and 50 L/Hr [6] respectively.

2.4 Identification of biodegradation products

Three concurrent sets of tests were planned under the identical conditions to determine the TC's biodegradable products (30°C, 1.2 x g). To activate the strain S591890 to the logarithmic growth phase, sterile LB liquid culture was first used. Each conical bottle containing 1 mg mL^{-1} of TC the bacterial suspensions was then given a 10 % (v/v) inoculation dose. Lastly, daily solutions were collected to measure TC levels and bacterial growth (OD600). Additionally, solutions were sampled at 0, 4, 8, 12, 16, and 24 hours, respectively, to measure the biodegradable TC by-products.

Intermediate products were then identified using a quadrupole time-of-flight liquid chromatography mass spectrometer (QTOF LC-MS) (Agilent Technologies, Santa Clara, CA, USA). Separation was performed on an Acquity QTOF LC-MS C18 column (2.1 x 50 mm, 1.7 µm, Waters, USA) with a flow rate and injection volume of 0.3 mL min^{-1} and 3 µL. The mobile phase included acetonitrile and 0.1 percent acetic acid under gradient elution conditions. The Masslynx 4.1 programme was used to process the mass spectra (MS) (Thermo Scientific).The MS analysis was performed using a capillary cone 3000v and a heated electrospray ionisation (HESI) source in positive mode. The capillary temperature was 100 °C, and the cone gas flow rate was 50 L h^{-1}. Full scan mode was used for the MS acquisition and the data range was 50-1500 Da.

To identify the mechanism involved in the biodegradation of TC by-products, the Kyoto Encyclopedia of Genes and Genomes (KEGG) software was used. The function to find the possible by-product compound can be found using KEGG compound ID to retrieve the information. All the detailed information about the possible by-product compound including its chemical structure, formula, mass, and links to related pathways, metabolism and reactions were provided in the KEGG software.

3.0 RESULTS AND DISCUSSION

3.1 Growth curve measurement of E. coli strain S591890 under tetracycline stress

According to the growth profile (Figure 1), no growth was observed during the lag phase, between 0 and 6 hours. This lack of growth may be attributed to the action of tetracycline, which inhibits bacterial protein synthesis and effectively inhibit their growth. Tetracycline interacts with bacterial ribosomes, preventing aminoacyl-tRNA from binding to the ribosomal complex, thereby inhibiting protein production. The presence of tetracycline in the growth medium significantly extended the lag phase compared to bacteria cultures without antibiotic in the control experiment (Figure 1). *E. coli* strain S591890 cells needed longer time to adjust to the stress and develop defenses against the antibiotic's inhibitory effects. The amount of tetracycline present and the initial cell density can all affect the duration of the lag phase.

Next, tetracycline-exposed *E. coli* strain S591890 cells modified and activated stress response mechanisms during the lag phase to counteract the antibiotic's effects. The cells were ready for development under adverse conditions during this adaption phase. The microbial population actively divided and the growth rate reached its maximum from 7 to 12 hours during the log phase. An extended period of active growth resulted from the slower cell proliferation and slower growth rate. This is because *E. coli* strain S591890 cells took longer to recover from the inhibitory effects of tetracycline and adjust to the stress environment.

However, the bacterium reached the equilibrium phase when the microbial growth rate slowed down and the population reached equilibrium from 12 to 18 hours. Cell division and death were balanced in the stationary phase under tetracycline stress. In the presence of antibiotics, protein synthesis was inhibited hence it slowed down its cell division. Tetracycline exposure over a long period of time may simultaneously cause loss in cell viability. Despite the presence of the antibiotic, the metabolically active cells continue to carry out vital cellular tasks, repair cellular damage, and survive.

After 18 hours of incubation time, the microbial population began to decline due to nutrient depletion and accumulation of waste products during the death phase. The bacterial cells continued to lose viability if they were exposed to tetracycline during the stationary phase. More cells died than divided, leading to a decrease in the overall size of the population. The cells might slow down and lost their capacity to perform crucial metabolic functions. Waste materials built up in the growth medium as bacterial cells continued to metabolise and reproduce. Waste products including toxic chemicals and by-products of bacterial metabolism accumulated over time are harmful to bacterial cells.

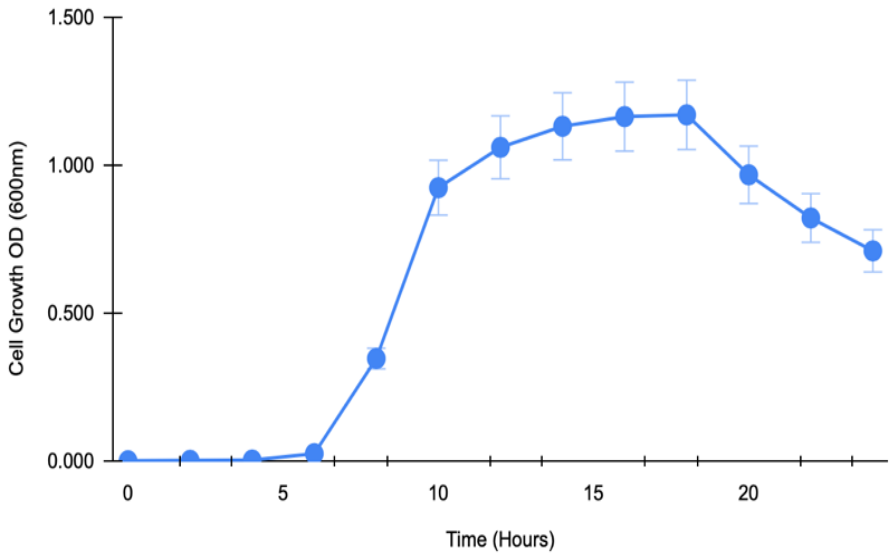

Figure 1 Bacterial growth profile rate of *E. coli* strain S591890 under TC stress for 24 hours incubation time.

3.1.2 Quantification of tetracycline concentration

Figure 2 shows that the concentration of TC is gradually decreased over incubation time for 24 hours. The initial TC concentration of 5.545 mg/ml was recorded, and began to decrease to 2.319 mg/ml after 24 hours of incubation time. The total of 5 samples of the tetracycline-containing *E. coli* strain S591890 bacteria were taken every 4 hours and it showed that the degradation rate of TC increased gradually from 0 hours to 24 hours with the degradation rate of 58.18%.

E. coli strain S591890 can produce enzymes that can break down tetracycline molecules. These enzymes include tetracycline-inactivating enzymes such as tetracycline-degrading enzymes which modify the chemical structure of tetracycline and render its inactivation. These enzymes can catalyse reactions such as oxidation, reduction, and hydrolysis leading to the degradation of tetracycline into simpler and less active compounds. Hence, TC can be actively transported out of bacteria's cells using efflux pumps. TC molecules are recognised by these pumps such as TetA and TetB efflux pumps which expel them from the bacterial cell ,lowering the intracellular concentration of TC and reducing its action.

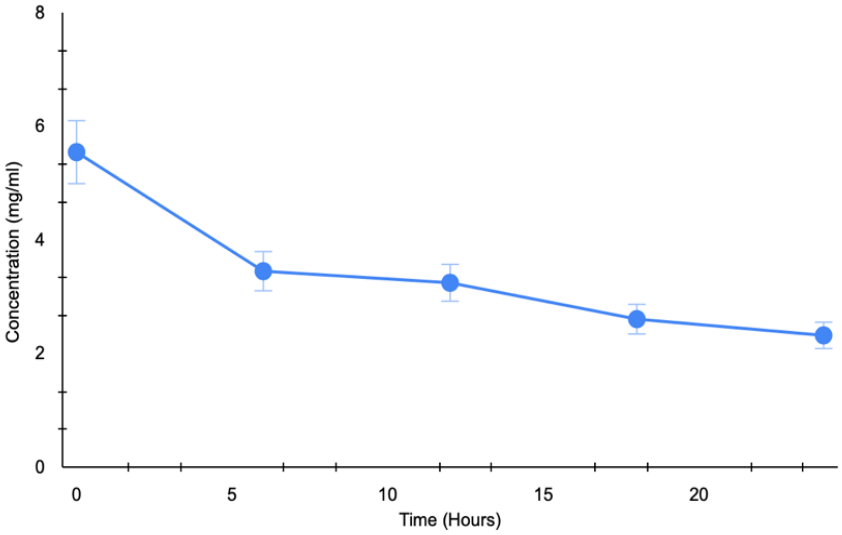

Figure 2 Degradation of tetracycline concentration by *E. coli* strain S591890 measured using high performance liquid chromatography (HPLC) over 24 hours

3.1.3 Determination of TC biodegradation by-products by using QTOF LC-MS

This qualitative method was performed to determine the presence of a by-product of biodegraded tetracycline by *E. coli* strain S591890 bacteria. The gradients of acetonitrile and acetic acid were used to chromatographically extract tetracycline to obtain good separation and peak shape in less than 18 minutes. Figure 3 displays a typical chromatogram produced from a sample of spiked feed. TC demonstrated strong UV absorption at 400 nm under specific circumstances with no matrix interference seen.

The retention time of the tetracycline peak in the sample chromatogram was compared with the retention times of the tetracycline standards shown in Figure 5 and 6. The results indicated that the retention time of TC compound was within the range of 7 to 8 min. The similarity in retention time suggests that the compound detected in the sample is likely tetracycline. Based on Figure 5, the product degraded by the bacteria was identified as macrozamin. This has not been reported before in literature. Macrozamin is toxic to human and animal [7], suggesting that the use of antibiotics may result in the production hazardous compounds.

The retention period is frequently used to distinguish one chemical from another by its feature. It is defined as the time taken by an analyte to elute from the detector after passing through the chromatographic column. It is possible to recognise and verify the presence of TC compounds in the sample by comparing the retention periods of tetracycline compound to the retention times of the reference standards because it acts as a fingerprint or a standard for identifying compounds.

Moreover, the separation of various chemicals in a mixture depends critically on the retention duration within 7 min to 8 min. However, the compounds can be changed to obtain the required separation by modifying the chromatographic parameters such as the mobile phase composition or column temperature. It is possible to successfully separate compounds with various retention durations for allowing the independent examination and quantification of each compound [8].

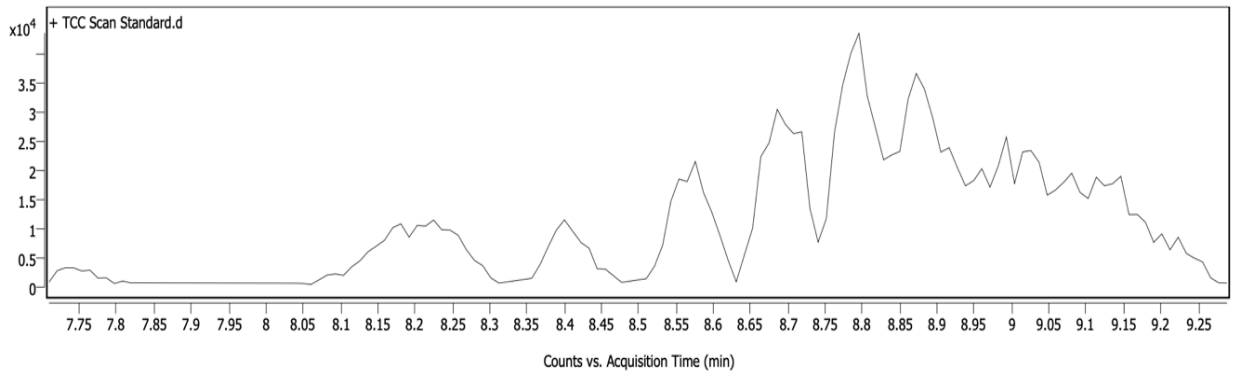

Figure 3 Chromatogram graph of TC standard by using QTOF LC-MS

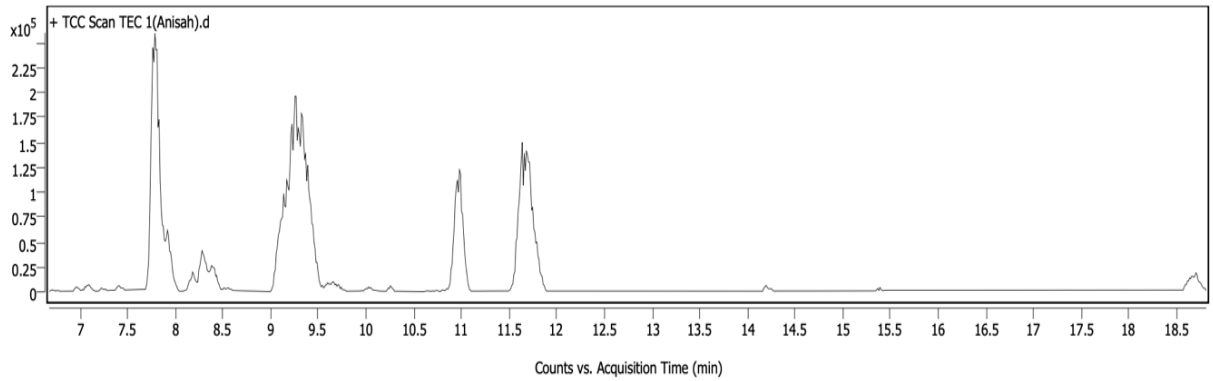

Figure 4 Chromatogram graph of TC by-product by using QTOF LCMS

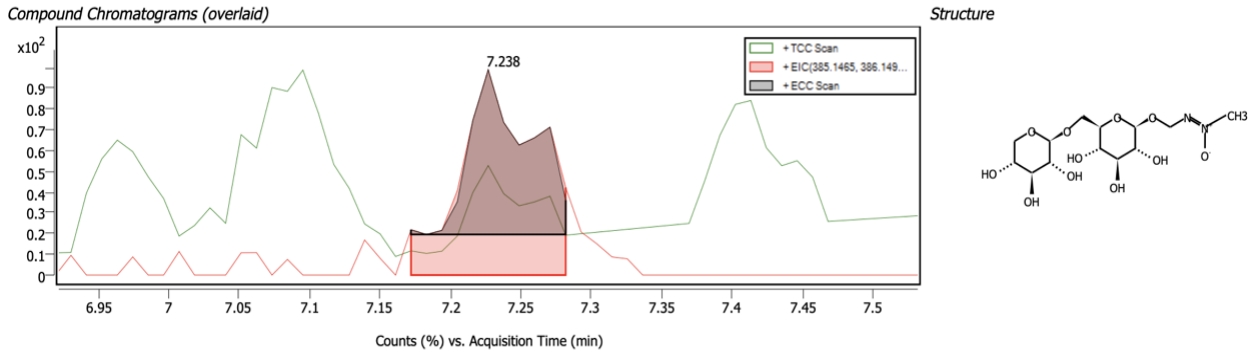

Figure 5 Compound chromatogram (overlaid) of macrozamin by using QTOF LC-MS

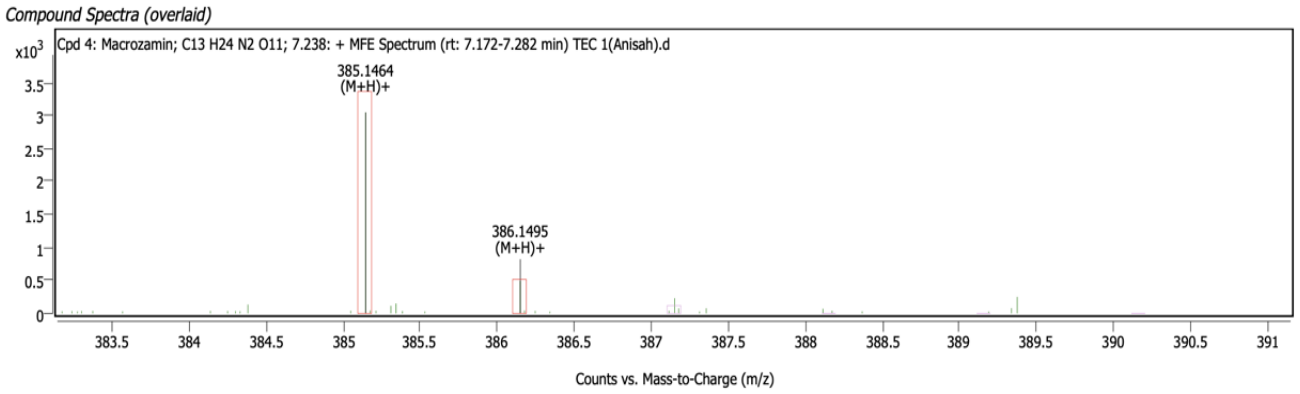

Figure 6 Compound spectra (overlaid) of macrozamin by using QTOF LC-MS

Appropriate software such as MassLynx or XCMS is needed in order to interpret and process the collected QTOF LC-MS data so that high-quality mass spectra, perform baseline correction, noise filtering, peak detection and alignment can be achieved. In order to recognise macrozamin compound, the expected mass-to-charge ratio (m/z) of macrozamin in the acquired data by looking for it using the molecule's chemical formula 385.1462 m/z. The chemical structure of macrozamin which is $C_{13}H_{24}N_2O_8$ can be used to determine its molecular composition (Figure 7). The retention durations range of macrozamin was recorded between 7.172 min to 7.82 min (Figure 7). The macrozamin fragmentation patterns in the compound of the overlaid spectra were superimposed (Figure 8).

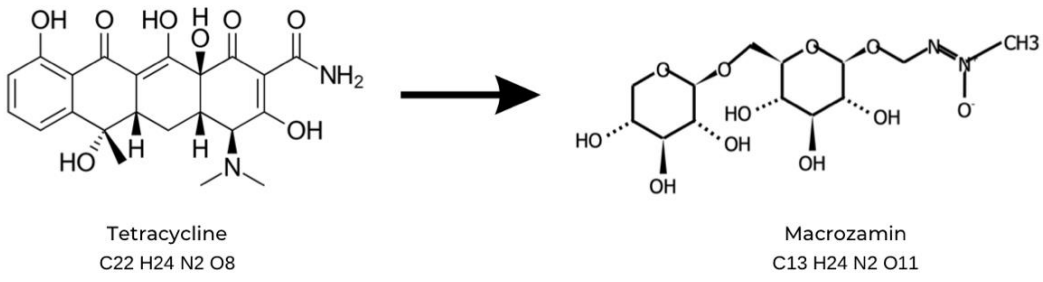

Figure 7 By-product of TC degradation by E. coli bacteria using QTOF LC-M

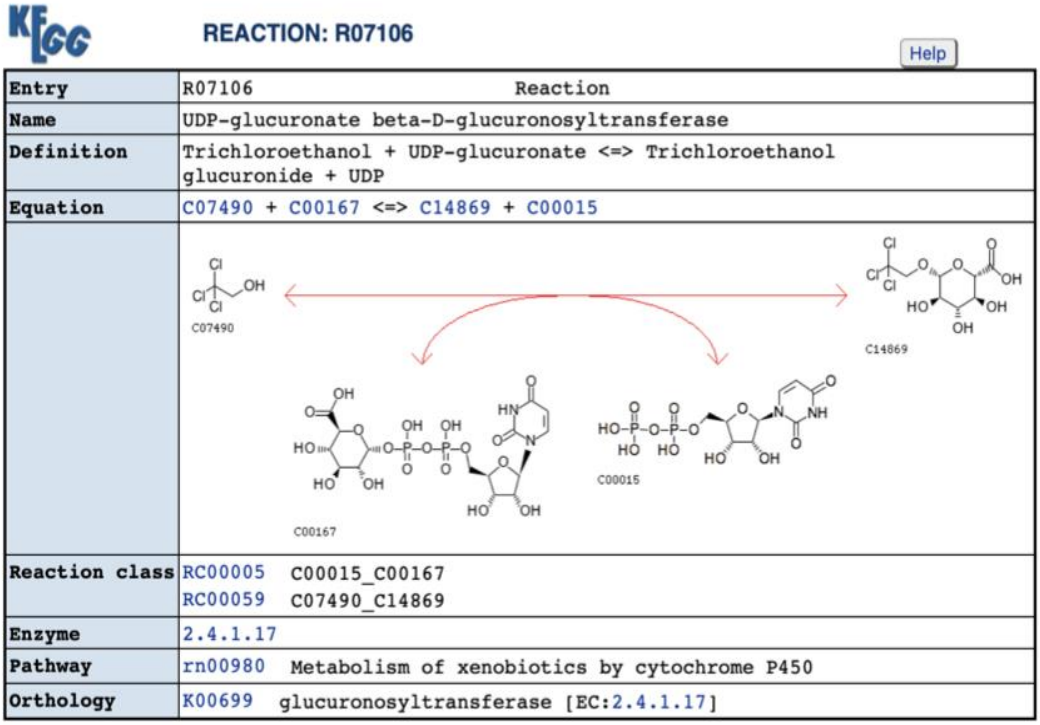

Figure 8 KEGG databased possible by-product

Based on the findings in KEGG software, macrozamin compound was identified as a potential by-product. One of the entry reactions is R07106 involved in the equation of C07490 + C00167 ↔ C14869 + C00015 (Figure 8). This reaction refers to Trichloroethanol + UDP-glucuronate ↔ Trichloroethanol glucuronide + UDP with the KEGG compound ID RC00059. The KEGG software provided all the detailed information about the macrozamin compound including its chemical structure, formula, mass, and links to related pathways and reactions.

Within the pathway information, the reaction networks that involve the macrozamin compound are able to identify the precursor compounds and potential by-products in the metabolic pathway. The metabolism that can be found for this reaction is metabolism of xenobiotics by cytochrome P450 where this metabolism is catalysed by glucuronosyltransferase enzymes (EC: 2. 4. 1. 17).

As shown in Figure 8, the analysis of differentially expressed genes by KEGG clearly shows the metabolic pathways that have been significantly altered by the biodegradation process of TC. Enrichment analysis of the KEGG metabolic pathway revealed various metabolisms involved during the degradation process of TC into possible by-product macrozamin. These included galactose metabolism, tyrosine metabolism, cyanoamino acid metabolism, starch and sucrose metabolism, biosynthesis of 12, 14-, and 16-membered macrolides including tyrosine biosynthesis and mycinamicin bi-synthesis, lipopolysaccharide biosynthesis, glycerolipid metabolism, ether lipid metabolism, sphingolipid metabolism and zeatin metabolism.

4.0 CONCLUSION

This study on TC degradation employing *E. coli* bacteria has yielded important insights into the microbial degradation of this common antibiotic. Enzymes and metabolic pathways involved in the breakdown of the antibiotic have been identified by analysing the capability of *E. coli* strains S591890 to biodegrade TC. *E. coli* bacteria can grow under TC stress for 24 hours. Within 24 hours of incubation time, *E. coli* bacteria has shown its ability to degrade TC at a considerable degradation rate. LCMS analysis revealed that the possible by-product of TC compound identified is macrozamin, a toxic compound that is associated with various metabolic pathways retrieved from KEGG software.

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