STUDIES ON BIOTRANSFORMATION OF TETRACYCLINE IN THE PRESENCE OF STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI.

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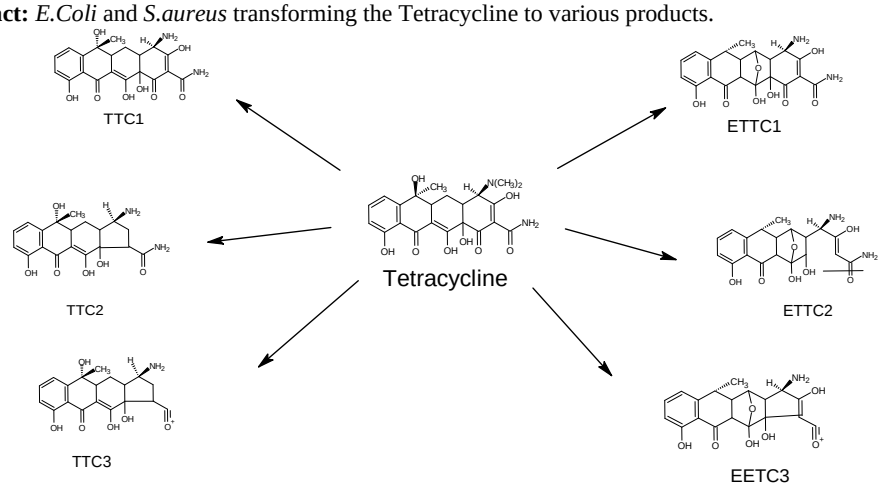
Article History: **Received:** 28.07.2022 **Revised:** 28.08.2022 **Accepted:** 27.09.2022

Graphical abstract: *E.Coli* and *S.aureus* transforming the Tetracycline to various products.



Highlights:

- Staohylococcus aureus and Escherichia Coli strains are capable to transform the Commercially available Tetracycline.
- Biotransformed Tetracycline products were found to be identified by using Spectral analysis.
- Maximum Biotransformation occurred at Temp 30ºc, P^H 9
- Microbial Biotransformation involves the removal of N-Methyl groups, Carbonyl group, and Amino group.

Abstract: Background: Even though numerous antibiotic approaches were suggested that, they can transform antibiotics, little is known about whether or not and how microbiological techniques may degrade antibiotics inside the surroundings. **Methods:** This work involves the Transformation of Tetracyclines using bacteria such as, Staphylococcus aureus and Escherichia. Coli. And characterized the biotransformation of tetracycline via that microorganism below various environmental situations. The biotransformation rate was the very best while the initial pH become 9 and the reaction temperature was at 30ºC which may be described by the usage of the Michaelis-Menten model underneath distinct preliminary tetracycline concentrations. Whilst the extra substrate turned into a gift, the substrate that triggered expanded biomass led to a decreased biotransformation rate of tetracycline. **Results:** Six feasible biotransformation products have been recognized, and a capacity biotransformation pathway turned into proposed that included sequential removal of N-methyl, carbonyl, and amine useful businesses. **Conclusion:** Results from this examination can result in a better estimation of the destiny and shipping of antibiotics to modified/transformed which will be having more advantages than that of existed forms.

Keywords: Tetracycline; biotransformation; hydrolysis; Staphylococcus aureus, E. Coli, transformation products.

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DOI: 10.31838/ecb/2022.11.10.007

INTRODUCTION

"Microbial Biotransformation (MBT) can be defined as stereoselective and stereospecific chemical transformations that are catalyzed by biological systems comprising of various Bacterial, fungal, and plant cells through their effective enzymatic nature and structures" [7]. **MBT has been progressively utilized as a means to produce noble therapeutic compounds at mild laboratory conditions as and when required, it is a targeted synthesis for the desired products with a high degree of purity and quality** [8]**.** It is a technique to synthesize precursor molecules or synthons, intermediates, and sometimes with the desired stereoselectivity or stereospecificity that are pharmacologically more effective than their racemates [8]. **Biotransformation has wide scope for synthesizing stereo-specific compounds such as pure enantiomeric/diastereomeric compounds of our interest instead of being prepared racemic mixtures that lead to serious side effects along with desired pharmacological action** [8]**.** The best example is Thalidomide Crisis that occurred in the 1960s [9]. at that time, Thalidomide was given as a racemic mixture, but only (S)- enantiomer is responsible for morning sickness treatment in Pregnant women, so due to (R)- form, severe side effects were reported such as pregnant ladies giving birth to limbless babies [9]**.**

Tetracyclines are broad-spectrum antibiotics that are used for human and animal health [1]. Tetracyclines are produced in large amounts (6800 tons worldwide) every year and used for human beings as well as animals [2]. So that these tetracyclines are excreted through Urine or feces to the environment [3]. Because of high amounts, these tetracyclines cause resistance to several organisms by changing their structure and function [4]. This is possible with tetracyclines and transformed products of Tetracyclines. But as per the previous literature, It was found that the Marine-derived fungi Paecilomyces Species is very effective to transform the Tetracyclines into Hemicyclines and Secocycline derivatives [5]. Among all these derivatives some of them show potential antifungal activity just like the initial tetracyclines [6]. But the fungal enzymes cannot degrade these derivatives to another form which gives lead that is the derivatives are very useful anti-fungal agents by which we can overcome the fungal resistance [5].

In this work, we use Microorganisms such as Gram-Positive Bacteria *Staphylococcus aureus*, and Gram-negative Bacteria Escherichia.coli [10]. For the transformation of Tetracyclines. It was found that Tetracycline is getting converted to 6 metabolites mostly. Among these two Bacteria, Gram-positive Bacteria Staphylococcus aureus was found to be strong in transforming Tetracycline to other derivatives. Through this work, we tried to explain that, what are the suitable microbes from a taken group of organisms, favorable environmental conditions such as temperature and P^H [11], suitable substrate, recovery of the transformed derivatives of tetracyclines [12], structural analysis of them [13], and the influence of concentration of the Tetracyclines [14].

MATERIALS AND METHODS

Experimental Methods:

Procurement and Maintenance of selected Organisms:

Stenotrophomonas maltophilia DT1 and Paecilomyces Species have been reported for Tetracycline Biotransformation [5,10]. In this work we studied the transformation of commercially available Tetracycline (Resteclin 250mg Capsules from Abott) by using Gram-positive bacteria such as Staphylococcus aureus (MTCC 3160), Gram-negative bacteria such as Escherichia Coli (MTCC 293), obtained from Institute of Microbial Technology, Shanthi Path, 39A, Sector 39, Chandigarh-160036.

These organisms have been maintained in our Laboratory by doing Subculturing time to time which can provide the required nutrients to the organisms [15]. LB Media for Bacteria The following are the required nutrient Media for Bacteria.

All the chemicals were procured from Qualigens fine Chem. Mumbai and Himedia Lab, Mumbai.; Luria Berthani broth prepared by using 10gm/L of Sodium Chloride, 10gm/L of Peptone, 5gm/L of Yeast extract, Distilled water upto 1000 ml. LB Medium has prepared by using LB broth 5gm/L, 1.5% of Bact agar, Deionised Distilled water 150ml/L, make up the volume upto 200 ml.

Subculturing done by using Streaking. Required medium prepared and sterilized for Bacteria then slants prepared aseptically. On these slants, carefully streaking done then incubated. Like that time-to-time sub culturing done and in between motility and growth measurement, viability of microorganisms was observed by using Hanging drop method and Direct inoculation method, serial dilution technique respectively [16].

Biotransformation of Tetracyclines: Microbial Biotransformation of tetracyclines were observed in submerged fermentation. Strains Staphylococcus aureus and Escherichia Coli were grown in LB medium containing 20 mg L -1 tetracycline at 30°C on a shaker set at 150 rpm. After reaching the mid-exponential phase, cells were harvested and washed twice with sterilized physiological saline (0.9 % NaCl). Cells were adjusted to OD600nm = 1.00 prior to being added to 50 mL sterilized Tetracycline (1%, v/v) in a 250 mL flask [17]. Two series of experiments were designed based on results from preliminary screening. First, two bacteria Staphylococcus aureus, Escherichia Coli, were tested. Second Immobilized cells of Staphylococcus aureus, Escherichia Coli were tested.

For two series, the following conditions were maintained.

(1) The initial tetracycline concentration was less than 20 mg L-1, P^H 9.0, Temperature 30^0C. unless stated otherwise.

(2) All tests were performed in triplicates.

(3) A blank control without bacteria and immobilized cells were included in all tests.

(4) All flasks were covered in aluminium foil to prevent photo degradation.

(5) Liquid samples were collected daily for 7 days and the concentration of the parent compound was measured using HPLC.

Rate of Biotransformation of Tetracyclines: Submerged fermentation was the procedure we conducted for biotransformation of Tetracyclines with different concentrations. The decrease in Tetracycline concentration was attributed to both Hydrolysis and Biotransformation in the presence of organisms. Such as two Bacteria. Without organisms decrease in Tetracycline concentration was attributed only because of Hydrolysis. In this work we are not added much about the first order kinetics. But in previous literature it was mentioned that various organisms were transforming the Tetracyclines into number of derivatives and explained about the first order kinetics. While we are doing the

two series of experiments, we have observed that as increasing the tetracycline concentration, biomass also increased. But transformation rate decreased. i.e., transformed products formed in less concentrations. Compared to whole cells, immobilized cells showed decreased transformation even we maintained same P^H and temperature. Among two organisms, Staphylococcus aureus given 3 derivatives, Escherichia Coli given 3 derivatives. Immobilized cells also given the same number of derivatives. But they only differ in concentration of transformed products.

HPLC oftransformed Tetracyclines: Using HPLC To measure tetracycline, 1 mL solution from each batch reactor was centrifuged at 8,000 rpm at 4°C for 10 min. Mcilvaine-Na2EDTA buffer was added to the sample supernatant in an equal volume fashion to chelate metal ions [18], and the mixture was filtered through 0.22 µm filters prior to being preserved at -20°C. Stored samples were loaded onto a Shimadzu LC-06A system. A C18 reversed-phase column (4.6×250 mm, 5 µm, Agilent Technologies) was operated at 40°C, with a 1 mL min-1 mobile phase consisting of 67% (v/v) 0.1 M ammonium oxalate in high purity water, 22% (v/v) acetonitrile and 11% (v/v) methanol. The injection volume was 20 µL, and the column isocratic elution was monitored by a UV detector at 355 nm.

Identification of the Transformation Products: Two sets of reaction conditions were evaluated: in first Set reactions the three conditions we followed are 1) BM-T solution, containing Whole Cell Microorganisms such as Bacteria and (hydrolysis plus biotransformation), 2) BA-T solution and containing no bacteria and (hydrolysis), and 3) BM (no tetracycline); in second Set of reactions the above three steps followed but the only difference is instead of BM-T solution, we used IBM-T solution which means Immobilized Cells taken instead of Whole cells. Solutions from each reaction condition were collected on Day 0 and Day 3, and the transformation products from the three conditions were identified using LC tandem mass spectrometry Q Extractive hybrid Quadrupole-Orbitrap mass spectrometer (Thermo scientific, Bremen, Germany). Aqueous samples were extracted using solid-phase extraction cartridges (Oasis HLB, 6cc/150mg, Waters) as described in previous studies [19]. Separation was performed on a C18 column (4.6×250 mm, 5 µm, Agilent Technologies) with an injection volume of 5 µL. Flow rate was set at 0.3 mL min-1 at the isocratic mode for 30min. Mobile phase consisted of 0.1% formic acid, acetonitrile, and methanol at 67:22:11. Mass spectra were processed using the X calibur 11 2.1 software (Thermo Scientific). The mass accuracy accepted for the experiments was estimated to be below 5 ppm. The MS analysis was performed with a heated electrospray ionization (HESI) source in positive mode with a spray voltage of 3.5 kV, S-lens RF level of 50%, and a capillary temperature of 300°C. The MS acquisition was performed in full scan mode 50-1000 Da with a mass resolution of 70000 [20-21]. The molecular structure for tetracycline and their transformation products were tentatively proposed by the detection of predicted mass, changing in full scan ion intensity, and ring double bound equivalent number (RDB) from the reacted sample (Table 1).

RESULTS

Staphylococcus aureus, Escherichia coli were selected for further analysis due to their ability to bio transform tetracycline. these are Gram-Positive, Gram-negative Bacteria. They were grown on LB media [15]. They could grow on CM-T agar plate which contained 20 mg L-1 tetracycline. Growth was observed over a temperature range of 20-40°C, a pH range of 6.0–10.0, and a salinity range of 0-5 % NaCl. They showed positive results in the methyl red test, in the catalase/oxidase/urease test, in indole production and nitrate reduction, and showed negative results in gelatine hydrolysis and Voges–Proskauer test.

Insights of Tetracycline Biotransformation: Because the biotransformation experiments were conducted in submerged fermentation, hydrolysis was an intrinsic part of the experiment. Hence, biotransformation cannot be one and only method to alternate the Tetracycline Concentrations in Medium. Hence, to estimate the biotransformation, we conducted different experiments that contained strains individually are *Staphylococcus aureus*, *Escherichia Coli* whole cells and Immobilized cells and a control experiment that contained no bacterial cells. The differences in the tetracycline profiles between the experiments were attributed to biotransformation (Scheme. 1,2,3,4). Both the overall transformation and the hydrolysis followed first order reaction kinetics. The fastest overall transformation appeared at 30°C, where 20 mg L -1 tetracycline was transformed in 3 days. The changes of tetracycline concentrations due to hydrolysis and due to combined hydrolysis and biotransformation plotted in Scheme. 1,2,3,4. The initial biotransformation rate was the highest at 30°C with a value of 10.13 mg L-1 d -7 (Scheme. 2). it is difficult to estimate bacterial biomass. Because the bacterial strains are very possible to form the biofilm on the inner wall of the fermentation vessels. The hydrolysis and the biotransformation of tetracycline were also affected by solution PH. The maximum hydrolysis occurred when the initial pH was 10. The hydrolysis rate will be increases with the increasing initial pH. The Maximum initial biotransformation rate was at pH 9. At the end of the transformation experiment, the final pH of all two sets was close to 9. If the initial p^H is <7, it leads to lagging of biotransformation fo Tetracyclines.

Tetracycliine Transformed Products: From the fermentation vessels, where both hydrolysis and biotransformation occurred, the initial concentraion of the tetracycline reduced from 20.00 mg L-1 to approximately 4.23 mg L-1 by Day 7 (1,2,3,4). In comparison, in the control reactors where only hydrolysis occurred, residual tetracycline dropped from 20.00 mg L-1 to 9.93 mg L-1 by Day 7. Both organisms are involved in the transformation of Tetracycline, the residual concentrations are nearer to the above values. T A peak corresponding to either an epimer or isomer of tetracycline was identified (Fig:1 and Table 1) in Day 3 of the experiment. Tetracycline eluted at 7.40 min with an m/z value of 445.1601 for its protonated form, while the peak for either 6-epi-tetracycline (ETC) or is tetracycline (ISO-TC) 5, had the same m/z value but an earlier retention time (i.e., 6.49 min). Previous work in aqueous systems at near neutral pH suggests that formation of ISO-TC is unusual [22]. Six possible biotransformation products were also identified (Fig. 1 to 6 and Table 1) and the mass spectra of the parent compound and proposed transformation products are shown in Fig. 1 to 7. A putative biotransformation pathway of tetracycline by S. aureus and E. coli was proposed in Fig 1 to 7. Formation of compound STTC1 could occur when the N-(CH3)2 group on epi tetracycline C-4 is de-methylated. Formation of STTC2 may occur via dehydrogenation. After losing a carbonyl group, STTC2 may be converted to compound

an intermediate. Finally, compound STTC3 may form when an amine group was further removed from compound STTC2 (Fig. 2,3,4). Biotransformation may also start with de-methylation at C-4 of ISO-TC and form compound ETTC1. After losing a carbonyl group, compound ETTC1 was converted to compound ETTC2. It is noted that the intensity of ETTC1 and ETTC2 was lower than STTC1 and STTC2. More over from ETTC2, ETTC3 will be formed due to loss of amino group from ETTC2. (Table 1).

Table 1. Characteristicsoftheparentcompoundandthetransformedproductsfromhydrolysisand/or biotransformation:

Retention time	Compound	Ion	Predicted mass m/z	Measured mass m/z	Elemental composition	Double bond equivalents (RDB)	Intensity
6.49	ETC/ISO-TC	[M+H]+	445.1601	445.1580	C22H25O8N2	11.5	2.75E+07
7.40	TC*	[M+H]+	445.1601	445.14801	C22H25O8N2	11.5	4.79+05
6.90	STTC1	[M+H-CH2]+	431.1445	131.1327	C21H23O8N2	11.5	4.24E+05
9.84	STTC2	[M+H-CH2-CH2-2H]+]+	415.1131	415.1116	C20H19O8N2	11.5	5.57E+06
11.43	ETTC1	[M+H-CH2-CH2-	415.1131	415.1120	C20H19O8N2	12.5	1.08E+04
21.09	INTERMEDIATE	[M+H-CO-CH2-CH2-2H]+]+	387.1181	287.1170	C19H19O7N2	12.5	5.69E+05
22.91	STTC3	[M+H-CO-NH3-CH2-CH2-2H]+]+	370.0990	370.0980	C19H16O7N	11.5	1.37E+06
24.67	ETTC2	[M+H-CO-CH2-CH2-2H]+	387.1180	387.1080	C19H19O7N2	11.5	6.54E+05
25.23	ETTC3	[M+H-CO-NH3-CH2-CH2-2H]+]+	370.0990	370.0970	C19H16O7N	12.5	1.12E+06

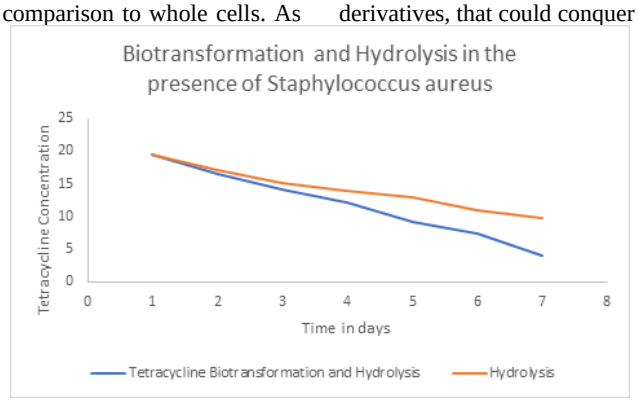
RESULTS AND DISCUSSION

Different from sulphonamide compounds, whose degradation by pure bacterial cultures [23-25]. and microbially mediated abiotic processes have been reported [26], little is known about how microbial processes may transform tetracycline compounds. Several Stenotrophomonas strains have demonstrated the ability to degrade organic pollutants, such as pesticide, insecticide [27], BTEX [28], PAH [29], and steroid hormones. These strains can transform toxic pollutants by opening their loop structure or by cutting functional groups [30]. For example, Stenotrophomonas Sp. THZ-XP can bio transform the pesticide acetamiprid by cutting it cyano substituent [2]. We noticed that although strains of Staphylococcus and Escherichia could form colonies on LB-T agar plates, they could not grow in LB-T liquid medium [31]. This suggests that strains may not be able to use tetracycline as sole carbon and energy source. The growth of strains on LB-T agar plates might be due to the utilization of the trace number of organic matters in agar [32]. The biotransformation of tetracycline by Bacterial strains might be attributed to the detoxification mechanism [23], which relies on the flavin-dependent monooxygenases [33]. Temperature and pH can influence the hydrolysis of tetracycline [11]. Similar to the findings from a previous study, this study demonstrated that high temperatures accelerated tetracycline hydrolysis. According to the collision theory, higher temperatures lead to higher reaction rates by causing more collisions between particles. High pH also accelerated the hydrolysis of tetracycline. Tetracycline is an amphoteric molecule with three dissociation constants (pKa=3.3, 7.7, 9.7)[34]. As pH increased from 6 to 10, different reactive species with various degrees of ionization might have dominated the solution and led to different hydrolysis rates. For the temperature and pH tested (except pH 6), biotransformation increased in the first four days before levelling off [35]. Despite the buffer system in the solution, strains changed the final pH of the solution to ~9 at the end of the experiment regardless the initial pH values. Bacterial cells can break down peptone in the reaction medium and release alkaline compounds such as ammonia and amines, which can cause increase in pH. Previous studies showed that the tetracycline transformation products from photolysis had higher toxicity than the parent compound [35]. In contrast, the tetracycline transformation products from fungal laccase had lower toxicity than the parent compound [36]. Hence, microbial transformation of tetracycline may be more desirable from an environmental perspective than physicochemical processes as it reduces the biological activities of the antibiotic [23]. During hydrolysis, under the action of hydroxide ion from water the hydroxyl group on C6 may attack the carbonyl group on C12 and form iso-tetracycline (ISO-TC) irreversibly in alkaline pH range. Also, tetracycline can undergo reversible epimerization on C6 under certain acidic conditions and form the corresponding 6-epi-tetracyline (ETC) with the participation of hydrogen ions from water at acidic pH conditions. Moreover, tetracycline can turn into ISO-TC or ETC at neutral or weak alkaline conditions (pH =6.5–9), causing decrease in tetracycline concentrations during hydrolysis [fig. 1]. The intensity of subsequent transformation products ETTC1 and ETTC2 were nearly an order of magnitude lower than STTC2 (Table 1), suggesting the pathway through ISO-TC was not the dominant pathway.

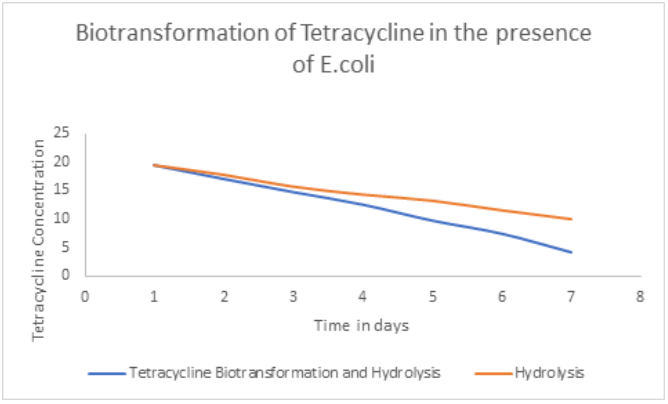
CONCLUSION

Biotransformation is the premise of lifestyles. Microbes had been widely applied for steroid biotransformation to prepare precise derivatives, the manufacturing of that's difficult via conventional artificial methods. Biotransformation is also proper to deal with the environmental problems like degradation of xenobiotics and petroleum hydrocarbons as they may be actual world trouble. Consequently, primarily based on the existing evaluation, it is able to be concluded that microbial biotransformation is a boon for the present day world with its huge range of applications. In this examine, Gram wonderful

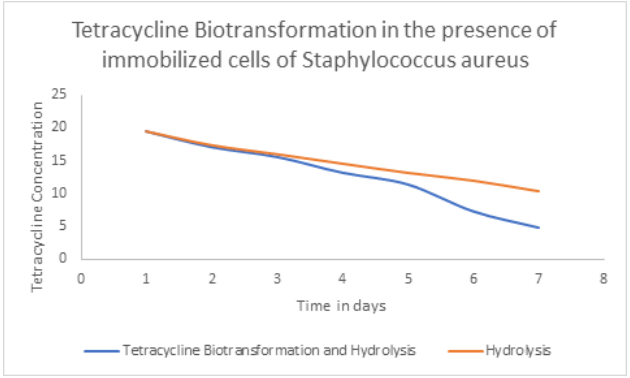
micro-organism Staphylococcus aureus and Gram-bad bacteria Escherichia Coli each are able to transforming tetracycline. Each micro-organism had been displaying highest transformation of Tetracycline underneath pH 9 and Temperature 30^0 C. Immobilized bacterial Cells additionally showed the transformation of Tetracyclines. But the rate of transformation turned into reduced in the presence of Immobilized bacterial Cells in comparison to whole cells. As growing the attention of Tetracyclines, the Biomass expanded however the price of changes reduced. Six possible biotransformation merchandise had been recognized and a potential transformation pathway became supplied. Via focussing on stereospecific and stereoselective reactions and recovery of Biotransformation products, in close to destiny there is a large scope to expand the brand-new Tetracycline derivatives, that could conquer the bacterial resistance.



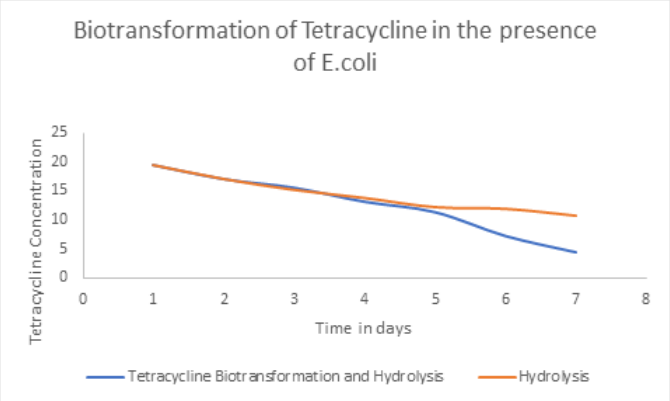
Scheme:1 Residual Concentration of Tetracycline in the presence and absence of Gram-Positive Bacteria Staphylococcus aureus at PH 9 and Temperature 300C.



Scheme:2 Residual Concentration of Tetracycline in the presence and absence of Gram-negative Bacteria Escherichia Coli at PH 9 and Temperature 300C.



Scheme:3 Residual Concentration of Tetracycline in the presence and absence of Gram-negative Bacteria Immobilized Cells of Staphylococcus aureus at PH 9 and Temperature 300C.



Scheme:4 Residual Concentration of Tetracycline in the presence and absence of Gram-negative Bacteria Immobilized Cells of Escherichia Coli at PH 9 and Temperature 300C.

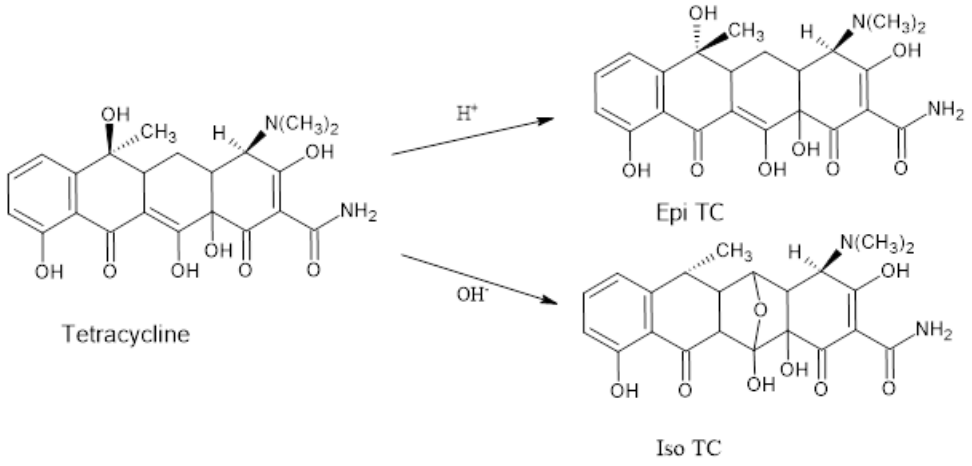


Figure 1: Hydrolysis of Tetracycline occurs in the absence of Bacteria and Immobilized cells.

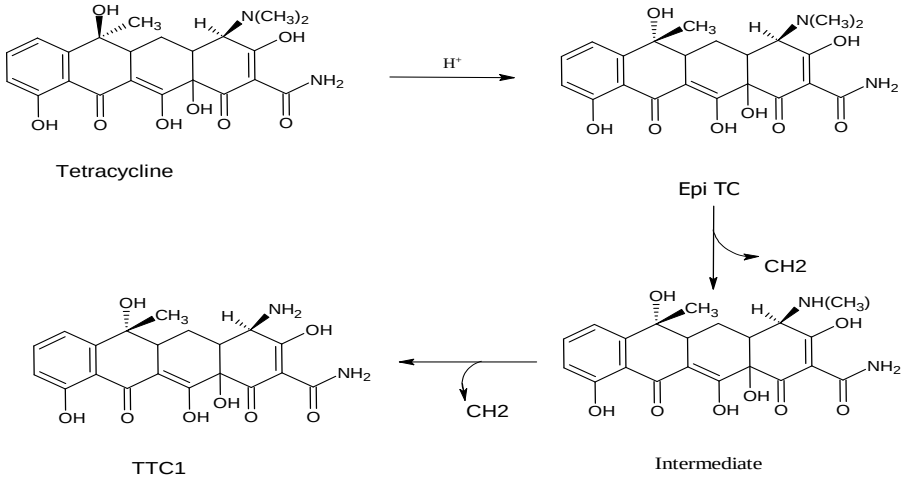


Figure 2: Biotransformation of Tetracycline to TTC1 (Transformed Tetracycline 1). In the presence of Staphylococcus aureus.

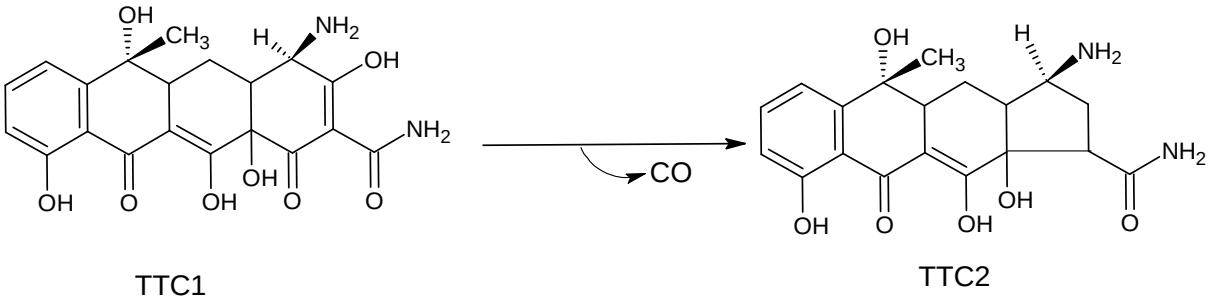


Figure 3: Biotransformation of TTC1C to TTC2, in the presence of Staphylococcus aureus.

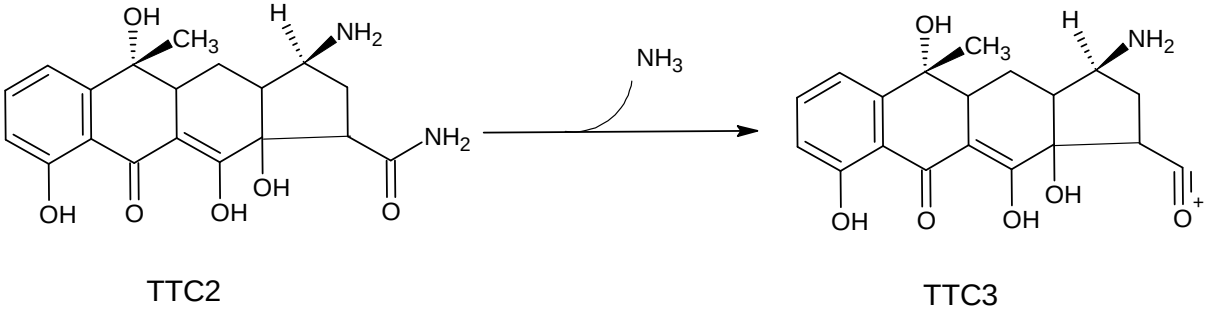


Figure 4: Biotransformation of TTC2 to TTC3, in the presence of Staphylococcus aureus.

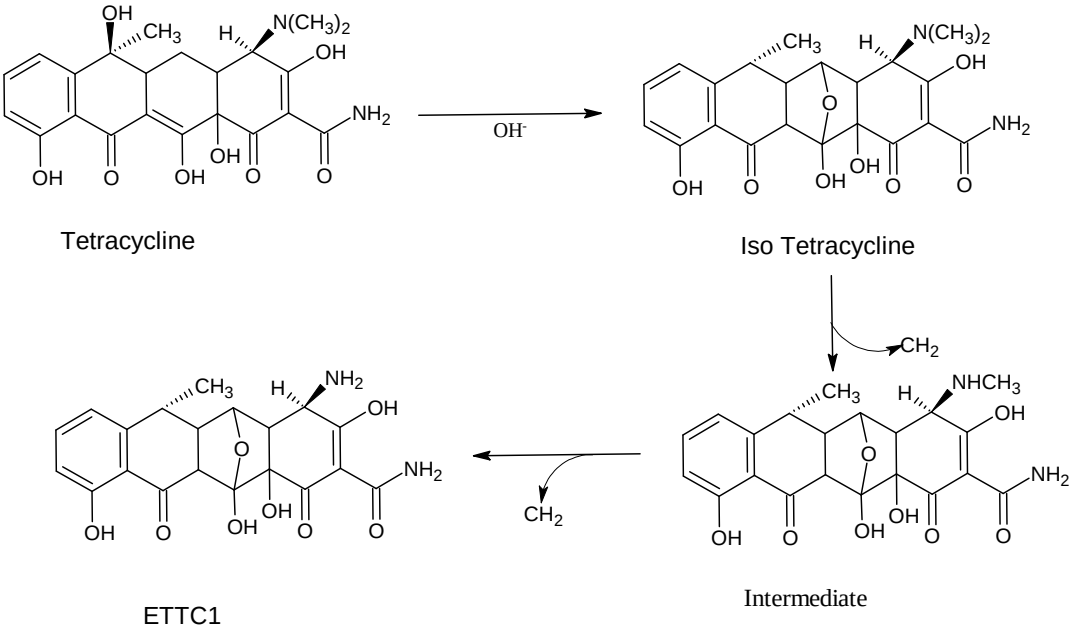


Figure: 5 Transformation of Tetracycline to ETTC1 by Escherichia Coli.

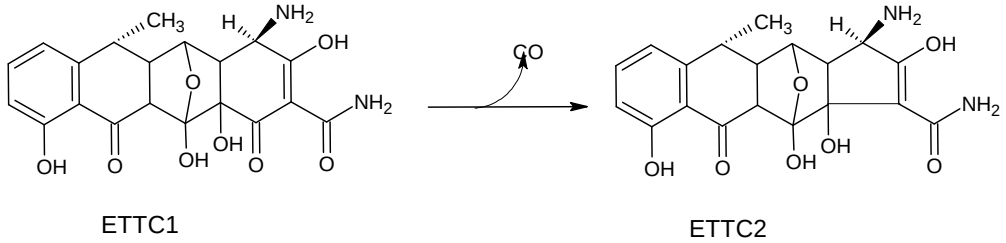
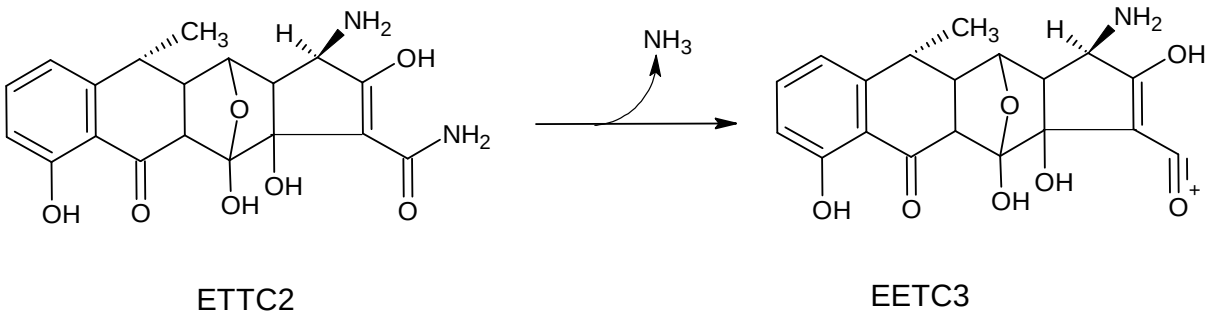


Figure :6 Transformation of ETTC1 to ETTC2 in the presence of Escherichia Coli.



ETTC2 EETC3

Figure: 7 Transformation of ETTC2 to ETTC3 in the presence of Escherichia Coli.

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