Degradation kinetics of chlortetracycline in wastewater using Ultrasonication assisted laccase

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Abstract: Chlortetracycline (CTC) is widely used as a veterinary antibiotic and is considered as a recalcitrant pollutant. In this study, spiked CTC (2 mg L^{-1}) in wastewater was degraded using laccase from the white rot fungi, *Trametes Versicolor* combined with ultrasonication (UlS). Over 60% of CTC was removed in 2h by UlS assisted laccase (UAL) treatment where laccase treatment alone took 2 days to degrade 87% of CTC under similar CTC concentration (2 mg L^{-1}), laccase dose (0.5 IU) and pH 6.0 conditions. UAL treatment showed 5.3 folds higher CTC degradation rate compared to laccase alone treatment at pH 6.0. Further, pH optimization of UAL treatment was done and pH 4.5 was found to be optimum wherein 80% of CTC degradation was obtained which is 2.6 folds higher compared to degradation at pH 6.0. The UAL treatment with optimized

pH was not only increased CTC degradation efficiency (~80%) but also reduced the degradation time to 2h. The obtained results highlighted the enhanced degradation rate, efficiency and unaltered stability of laccase during UAL treatment which can be used for oxidizing other tetracycline groups of antibiotics. Moreover, laccase and UAL treatments showed similar degradation products and no estrogenic activity.

Keywords: chlortetracycline; laccase; ultrasonication; degradation; toxicity

1. Introduction

The rapid increase in population, income, and urbanization has driven the global consumption of milk and meat in developing countries [1]. Current industrial farming with extensive use of antibiotics is likely helping to meet the growing demand for animal products. The current intake of antibiotics in animals has exceeded the human consumption [2]. This is due to their massive usage in growth promotion and disease prevention purposes apart from treating infections. As a result, animal farming became a key source for continuous release of veterinary antibiotics into the environment [3, 4]. Among antibiotics, tetracyclines (TCs) are traditional and extensively using broad-spectrum antibiotics in the market across the world. Chlortetracycline (CTC) is the first TC antibiotic that has been discovered and used for veterinary purposes. The low absorption (25-40%) of CTC in livestock led up to 70-90% release into the environment by animal excretions. This results in the detection of CTC in water sources across the world around 0.08−0.61 μg/L [5, 6]. The continuous detection of antibiotics and their exposure to microorganisms accelerate the development and spread of antibiotic resistance [7]. Apart from continuous release and detection, CTC is able to form stable metal complexes which have more toxicity and are persistent [8, 9].

Conventional biological treatment plants are not designed for effective removal of emerging and low concentration (ug/L-ng/L) contaminants including antibiotics. Studies have shown the negative effects of antibiotics on the biological treatment processes and other pollutant biotransformation processes [10, 11] even at low concentrations. Advanced physical and chemical methods, such as ultraviolet treatment, ozone treatment, adsorption and membrane filtration processes have been proven to some extent inefficient for the complete degradation/removal of antibiotics [12]. Studies so far have reported using higher amounts of chemical reagents to achieve enhanced degradation efficiencies incurring costs [13]. Hence, the research is continuously growing on these technologies to make them low cost, efficient, and safe applications. Specifically, the research is exploiting hybrid technologies which have synergistic effects on degradation of wide range of contaminants and are cost effective [14].

Enzyme based degradation of contaminants in wastewater is now emerging. Enzymatic treatment has benefits such as low energy requirement, easy process, no toxic effects and no adoption to conditions compare to conventional and/or chemical oxidation techniques[15, 16]. These advantages make enzymatic treatment as alternatives to conventional wastewater treatment [17]. It is well known that the extracellular ligninolytic enzymes (laccase, lignin peroxidase, and manganese peroxidase) of white rot fungi have great potential in degrading environmental pollutants [18]. Laccase belongs to the oxidases family of enzymes which oxidizes many organic contaminants having phenolic and non-phenolic aromatic structures. Literature reported the wide range of compounds that are susceptible to laccase. Main classes include antibiotics (tetracyclines, sulfonamides, quinolones, penicillins, fluoroquinolones), textile dyes, Micotoxins, endocrine disruptors, polycyclic aromatic hydrocarbons, petroleum hydrocarbons [19-22]. However, laccase oxidation alone showed lower degradation efficiency and slow degradation rate [23]. These

drawbacks can be overcome by combing with other degradation methods, specially simultaneous application of ultrasonication (UlS) with enzymes that showed higher degradation efficiency [24, 25].

Increase in enzymatic activity by UlS has been exploited for many enzyme families. In ultrasonic phenomenon, generation and growth of cavities and their subsequent collapse cause turbulence in the media generate heat and hydroxyl radicals [26]. This process enhances oxygen mass transfer owing to the enhanced interaction between enzyme and substrate molecules which is essential to speed up the degradation rate of contaminants. Apart from this, a portion of ultrasonic energy absorbed by the medium is converted into heat which increases media temperature intern increases the enzymatic activity in case of laccase. Studies have reported the enhancement and longevity of catalytic activity with the combined use of ultrasonication and enzyme [27].

Herein, crude laccase produced by the fungus, *Trametes versicolor* was used to degrade CTC which was selected as a typical veterinary antibiotic having wider application and less biodegradable nature. Several studies have reported the chemical oxidation methods to degrade CTC in wastewater. Chemical methods are non-selective, need excess reagents and harsh conditions (high temperature and pH) for efficient degradation[13]. Meanwhile, laccase treatment has high degree of specificity towards contaminants, needs mild conditions and low energy requirements, albeit lower rates of degradation [28]. The mentioned advantages makes the laccase treatment as a green technique over chemical methods. So far, very few studies were conducted on the application of ultrasonication assisted laccase (UAL) catalyzed the degradation of persistent pharmaceuticals [24, 29]. In this study, the authors combined the UlS with laccase treatment to overcome the slow degradation rate by enzyme treatment alone. Current study highlighted the efficient degradation of CTC without temperature control and also pH is optimized in the UAL

process to study the synergistic effect of OH radicals produced by UlS. In this context, the ultrasonic enhanced catalytic activity of laccase to degrade CTC was evaluated in wastewater. In addition, different operating parameters, such as pH, enzyme stability and kinetics during UlS were studied to determine optimum operating conditions for CTC degradation. Further, transformation products were identified by liquid chromatography-mass spectrometry (LC-MS/MS) and estrogenic activity of resulting treated streams was also determined in WW.

2. Materials and methods

2.1 Chemicals: Chlortetracycline hydrochloride was purchased from Toronto Research Chemicals (Toronto, Canada). Methanol (HPLC grade), Tween 80, disodium hydrogen phosphate, citric acid, sodium azide were purchased from Fisher Scientific (Ontario, Canada). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) N, N-Dimethyl-4-nitrosoaniline (RNO, 97%) were purchased from Sigma-Aldrich (Mississauga, Ontario, Canada). Millipore system (Milford, MA, USA) Milli-Q/Milli-RO was used for HPLC grade water preparation.

2.2 Wastewater sample

Wastewater samples were collected from wastewater treatment plant (WWTP) of Quebec Urban Community (CUQ) (Sainte-Foy, Quebec City, QC, Canada), with a sewage treatment capacity of 13,140 m^3/h. The plant treats the sewage using primary and physical-chemical treatments and further discharges the treated effluent into the Saint-Lawrence River. WW samples used in this study were collected before UV treatment and stored under dark conditions at 4 ± 1 ^oC to prevent CTC degradation. The basic characteristics of WW used in this study are presented in Table 1.

2.3 Solid state fermentation

2.3.1 Microorganism and culture conditions: White-rot fungus *T. Versicolor* (ATCC 20869) was selected for solid state fermentation (SSF) to produce laccase. Potato dextrose agar Petri plates were used to inoculate the fungus and incubated for 7 days at 30 ± 1 °C. The culture plates were stored at 4 ± 1 °C and sub-cultured for every 4 weeks.

2.3.2 Solid-state fermentation: Erlenmeyer flask (500 mL) containing 20 g of dried apple pomace containing Tween 80 (0.1%) and moisture content 75% (w/w) was sterilized at 121 °C for 15 min under 1 atm pressure. Subsequently, the culture medium was inoculated with the mycelia of *T. Versicolor* followed by incubation for 14 days at 30±1 °C [30]. Apple pomace was stored at -20 °C, until use for its conservation. After incubation for 14 days, 200 mL of 1M sodium phosphate buffer atpH 6.5 was added to the flask and mixed manually, then left to stirring at 250 rpm for 1 h to extract enzyme into the buffer. The supernatant was collected by centrifugation at 6500 × *g* for 20 min at 4 °C and stored at 4±1 °C.

2.4 Enzyme assay: Laccase activity was measured based on the oxidation of ABTS substrate (500 mM) at 420 nm (E_{max} = 3.6 × $10^4 M^{-1}$ cm^{-1}). The reaction medium having 0.1 mL laccase extract in 2.4 mL sodium phosphate-citrate buffer (pH 3.0) and 0.5 mL of ABTS (1.5 mM) were incubated at 45 °C for 10 min to measure the activity[31]. One unit laccase activity is equivalent to 1.0 µmol of product formation per min under the assay condition.

Enzyme stability: Laccase activity was tested during degradation studies at room temperature 21±2 ºC for 48 h. And also, kinetics of laccase activity during ultrasonication and temperature alone was tested with the respective pH degradation study of CTC. The specific amount of laccase was transferred immediately into pH 3.0 sodium phosphate-citrate buffer at each time and stored at 4±1 °C, until laccase activity measurement.

2.5 Experimental procedure: About 2 mg L^{-1} CTC spiked in WW was tested for UAL degradation. Laccase degradation kinetics of CTC was carried out for 48 h at room temperature 21±2 ^{o}C, 150 rpm, pH 6±0.5 using 0.5 IU laccase activity. Bath ultrasonication (Frequency:37 kHz, power:3200 W) was carried out for 2h without temperature control at pH 6±0.5 to find CTC degraded by UlS. Combined UlS and laccase degradation were performed at different pH including 3.0, 4.5, 6.0, 8.0 and 10.0 at constant 0.5 IU laccase activity and CTC concentration (2 mg L^{-1}) spiked in WW.

2.6 **Hydroxyl radicals (OH$^{\bullet}$) estimation:**

N, N-Dimethyl-4-nitrosoaniline (RNO) is an organic dye having a strong yellow color in aqueous solution. The selective bleaching of RNO by OH$^{\bullet}$ makes this compound specific for indirect estimation of OH$^{\bullet}$ [32]. The concentration of OH$^{\bullet}$ was determined by measuring the absorbance of RNO after the UlS for 2h at 440 nm by ultraviolet (UV)-vis absorption spectroscopy (UV 0811 M136, Varian, Australia). After UlS experiments, the resulting sample pH was adjusted to 7 with NaOH and centrifuged at 7000 × *g* to separate insoluble particles.

2.7 Analysis of CTC and its transformation products

Following laccase and ultrasonic assisted laccase degradation of CTC in wastewater, laccase activity was quenched by adding 0.1mM NaN_3 and further samples were diluted with methanol and stored at 4 ^{o}C in dark.

To analyze the CTC concentration in treated samples, LC-MS/MS (Thermo Fisher Scientific) coupled to a 4000 TSQ Quantum Access Mass Spectrometer equipped with an electrospray ionization source was used. Chromatographic separations were carried out in a reverse-phase Hypersil Gold C18 column (100 x 2.1mm, 3µm particle size). The mobile phase used for elution

of CTC consists of 0.1% acetic acid in MilliQ water (A) and 0.1% acetic acid in acetonitrile (B). Gradient elution technique used was as follows: 0 min, 90% A (10% B); 0–12 min, 30% A (70% B); and 12–18 min, 90% A (10% B), at a flow rate of 230 µL/min, 20 µL injection volume and column temperature of 30 °C. Electrospray ionization in positive ion mode was operated in the mass spectrometer and CTC acquisition was monitored by using multiple reaction transitions. Other parameters used were as follows: spray voltage, 4000 V; collision gas-Argon, 1.7 mTorr; capillary temperature, 340°C. The internal standard method was used to quantify the CTC. The five-point calibration curve (2 µg/L to 100 µg/L) was prepared from a stock solution of 500 µg/L CTC and sulfamethazine were used as internal standard.

The experiments were run in LC-MS/MS both positive and negative electrospray ionization modes with a mass scan range of m/z 50−1000 to identify the Transformation products (TPs) of CTC with the same column. These experiments were carried out in samples with (i) real WW without spiking CTC; (ii) WW with laccase (without spiking CTC); (iii) laccase degraded CTC; and UAL degraded CTC spiked in WW. A comparison was done between laccase and UAL TPs in order to identify the difference in the formed TPs.

2.8 Yeast Estrogen Screen (YES) assay

Estrogenicity test was performed to determine the toxicity of resulting treated WW which is important for understanding its effects on aquatic life as ultimately it will be discharged into the river, in this case. Moreover, there is a possibility that the transformation products of UAL degradation can be estrogenic leading to a secondary problem of endocrine disruption more than the resistance, which is CTC-derived. Hence, the estrogenic activity of untreated and treated CTC in WW samples (enzymatic and ultrasonication assisted enzymatic process) was tested using YES

assay described by Routledge and Sumpter (1996) [33]. In this YES assay, yeast strain is genetically modified to express the human estrogen receptor (hER) in an estrogen-dependent manner. Experiments were carried out in a 96-well plate (Costar Brand, NY, USA). All standards (17β-estradiol), stock solutions of CTC and samples were diluted with 70% alcohol prior to YES assay to prevent unwanted bacterial growth. Aliquots of 10 μL of each standard, stock, and samples diluted in alcohol were transferred to a 96-well plate under laminar flow chamber. The alcohol aliquots placed in 96 well was allowed to completely evaporate and further 200 μL aliquots of the yeast assay medium containing the recombinant yeast cells (hER-transfected) and chlorophenol red-β-d-galactopyranoside (CPRG) were placed after evaporation. Each plate contains one row of blanks (assay medium and alcohol evaporated) and a standard curve for standard 17β-estradiol. The plates were sealed with parafilm and shaken at 200 rpm for 5 min to mix assay contents yeast cells and further 3 days static incubation at 32±1 ^{o}C. Periodically the color development of the assay medium in plates was checked using a multi-reader microplate spectrophotometer (Epoch, BioTek, USA) at an absorbance of 540 nm.

2.9. Statistical analysis

All experiments were performed in triplicate. Statistical significance tests were performed using ANOVA (analysis of variance). *P*-values less than 0.05 were considered statistically significant. Zero, first and second order kinetics were calculated to find out the degradation rate (Table 2).

3. Results and discussion

3.1 Individual laccase and ultrasonication assisted CTC degradation

Considering the cost-effective aspect of using enzymes at industrial scale in degrading pollutants, current research has focused on production methods of enzymes to reduce the cost and

augmentation of their activity by combining with other technologies [34]. In this study, apple pomace industrial waste was used as a substrate for solid state fermentation to produce crude laccase enzyme to reduce the cost of enzyme production. Optimized incubation and extraction conditions from previous studies, such as 37 ± 1 ^{o}C for 14 days and 6.5 pH sodium phosphate buffer were used for laccase production, respectively [30].

Initial laccase degradation studies of CTC were carried out in 2 mgCTC L^{-1} spiked in WW using laccase of 0.5 U L^{-1} activity at room temperature 21±2 ^{o}C, pH 6 and agitation speed of 150 rpm for 48h. The physicochemical properties of WW have been given in table 1. The stability of laccase activity was studied during degradation of CTC and about 10% reduction in laccase activity was observed at 21±2 ^{o}C in 48h (Figure S1). Similarly, laccase degradation kinetics of CTC in WW is depicted in Figure 1. It has been observed that 87±2% of CTC was degraded in 48h without any mediators. Similar results were reported in previous studies of Suda et al., 2012 [35] where 48% CTC degradation with laccase alone was observed in 4h and 50% in 5h was recorded in this study.

Table 2 provides the rate constants and correlation coefficients of different kinetic models for laccase degradation of CTC. The second-order kinetic model described very well the degradation process of CTC by laccase oxidation. This kinetic behavior explains that the change in the concentration or dose of both laccase and CTC seems to be affecting the degradation rate of CTC. Similar results were obtained in the studies of Yang et al., 2017 [36], where increased enzyme loading increased the degradation and also different degradation efficiency among tested compounds due to varied transformation efficiencies with laccase. In this study, laccase, and CTC concentrations were kept constant to find the optimum conditions for the UAL degradation. Laccase was found to be stable ($p < 0.05$) after two days at room temperature 21±2 ^{o}C, at pH 6.0. UlS was performed to degrade CTC spiked in WW for 2h under similar conditions to enzymatic

degradation. UlS studies showed 18±5% of CTC degradation. Most of the literature on bath ultrasonication of CTC was focused on its extraction from complex media for 15-30 min [37]. In this study, the laccase and UlS lone techniques showed slow and lower degradation efficiencies which are not economically feasible to operate at industrial scale to degrade contaminants in WW. Authors thus combined these two techniques to get the synergistic effect on degradation efficiency. Studies used mediators, such as 1-hydroxybenzotriazole and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and immobilization techniques to speed up laccase oxidation. Increase in catalytic activity of laccase without the help of chemical mediator is preferable and advantageous because the formed mediator radicals affects its stability depending on the concentration and type of mediator. Furthermore, the mediators may be toxic, costly and might inactivate laccases [34, 38, 39]. This study pursues further ultrasonication process as a mediator to speed up the degradation process of CTC by laccase oxidation to avoid the disadvantages of the mediators.

3.2 Ultrasonication assisted laccase degradation

To investigate the combined effect of ultrasonication and laccase on CTC degradation, spiked WW with CTC was treated with 0.5 U L^{-1} laccase along with the bath sonication for 2h. As shown in figure 2, UAL showed degradation of over 60.2% of CTC compared to individual laccase (31±3%; 2h; figure 1) and UlS (18±5%; 2h; figure 2) under similar conditions of degradation, such as pH 6.0 and 2 mg L^{-1} CTC concentration. UAL degradation showed two and three folds increase in degradation efficiency compared to laccase and UlS degradation, respectively. Changes in temperature of WW during sonication are presented in supplementary file Figure S2. Increased temperature (25 to 52 ^{o}C) and bubble formation during UlS enhances the oxygen transfer rates which are responsible for the higher enzyme activity and the corresponding degradation efficiency

246 in the case of UAL. Effect of temperature without UlS on laccase activity at different pH are
247 reported in Figure S3. At pH 4.5 and 6.0, the enzyme activity remained stable. Meanwhile, at
248 acidic (3.0) and alkaline (>8.0) pH, the laccase was 60% and 50% inactivated, respectively.
249 Further, there was a constant heating of the laccase without any stirring which might have
250 decreased the dissolved oxygen in the solution[40]. Hence, the simple heating of laccase might not
251 have showed the synergistic effect in CTC degradation. Laccase catalyzed the oxidation of
252 aromatic compounds having electron-donating groups, such as amines (−NH2) and hydroxyl
253 (−OH) groups by using molecular oxygen as a terminal an electron acceptor in multi copper (Cu)-
254 electron redox reactions. Laccase has 4 copper atoms in three types, type I Cu takes the electron
255 from CTC and thus oxidizes it and transfers the electron to type II Cu and type III Cu (2 Cu atoms)
256 where reduction of oxygen to water takes place. CTC contains many electron donating groups,
257 such as OH, dimethyl amine (-N(CH3)2), amide (-NH2-C=O) groups in its structure (figure 4a)
258 which facilitates the oxidation of CTC. Further, the UlS cavitation enhances the interaction
259 between the laccase and CTC molecules and also produces hydroxyl radicals which play an
260 important role in degradation efficiency of CTC [41].

261 **3.2.1 Effect of pH on CTC degradation and laccase activity during the UAL**

262 pH is an important parameter to be studied which affects the enzyme activity/stability as well as
263 ionization state of CTC. No effort was made to maintain a constant temperature during the
264 experiments as laccase activity was increasing or maintained constant with temperature during UlS
265 (Figure S3). However, enzyme should be maintained at specific pH to retain its structural
266 integrityfor its specific activity and selectivity. Considering these facts, five different pH including
267 3, 4.5, 6, 8 and 10 have been studied to find out the optimum pH of UAL degradation. Effect of
268 pH on degradation kinetics of CTC during UAL is shown in Figure 2. pH 4.5 showed highest

degradation of CTC around 79%±1.5, where at pH 6.0 and 3.0, 60%±2.3 and 20%±3.7 CTC were degraded, respectively in 2h of UAL. No significant degradation of CTC was observed at alkaline pH of 8 .0 and 10.0. Laccase activity was simultaneously measured during UAL at different studied pH and results are presented in figure 3. Bath ultrasonication increased the laccase activity by 20% at pH 6.0 and the enzyme activity remained the same at pH 4.5 after 2h. At pH 3.0 and 8.0, laccase activity was reduced by 50% and 70%, respectively and the enzyme was deactivated at pH 10.

The increased temperature during UlS (figure 2S) enhanced the laccase activity but the activity is affected by the pH with time as shown in figure 3. Further, as shown in figure 2, the extent of CTC degradation was lower at acidic (pH 3.0, 20%) and alkaline pH (pH>8, no significant degradation) while at pH 4.5, higher degradation (80%) was obtained. To support these results of CTC degradation efficiency during UAL at different pH, OH radicals and corresponding CTC degradation at different pH during UlS were measured and the results are given in supplementary figure S4. As shown in figure S4, there is a continuous increase in OH radical production from pH 10 to 3 (10-50%) with corresponding increase in CTC degradation (7-25%). This indicated that the increase in laccase activity with temperature was asffected by generated hydroxyl radicals during UlS. At acidic pH 3.0, enzyme activity was reduced to 50% due to higher generation of OH radicals which deactivated the laccase[42], At pH >8, laccase was inactivated (figure 3) even though there was no generation of OH radicals. These results showed that at acidic pH, hydroxyl radicals and alkaline pH, the pH itself playing major role in UAL degradation efficiency. The enzyme activity is stable at pH 4.5 due to less production of OH radicals [43], compared to pH 3. The increased enzymatic activity due to temperature increase at pH 4.5 might be compromised by the OH radical effect. This type of enzyme activity at lower pH during UAL was further justified by comparing the results of heating on enzyme activity at different pH for 2h (figure S3). As shown

in figure S3, at pH 4.5, there was a slight increase in laccase activity which is a contrast to UAL degradation. Hence, at lower pH, generation of OH radicals during UlS decreasing the laccase activity[43]. The laccase activity was increased at pH 6.0 with increase in temperature, in both UAL (figure 3) and UlS (figure S3) showing no pH and OH radical effect at this pH. The optimum pH for the highest CTC degradation (80%) was found to be 4.5 even though the laccase activity was found to be highest at pH 6.0. This is due to the production of hydroxyl radicals at pH 4.5 which aids in the degradation of CTC whereas at pH 6.0 there was no significant hydroxyl radical production. Further, at pH 4.5, CTC exists in ionized form and at pH 6.0, CTC exists as zwitterionic form and the ionized form of CTC was more susceptible to degradation compare to zwitterionic form. The zwitterionic CTC would be able to form complexes with metals which decreases the availability of electron densities on the amide and OH groups [44], further reducing the probability of oxidation by laccase.

Considering the degradation kinetics of laccase and UAL treatments, all experiments at different pH followed the second order kinetics except at pH 4.5 (Table 2) where it followed first and second-order kinetics. This can be due to the fact that similar enzyme activity (0.5±0.04 IU) is maintained at pH 4.5 during UAL. On comparing the second order kinetics degradation rates of all studied experiments, pH 4.5 showed the highest degradation rate of 1.83 $M^{-1}s^{-1}$ while, degradation rates of 0.685 $M^{-1}s^{-1}$ and 0.163 $M^{-1}s^{-1}$ were found at pH 6.0 and 3.0, respectively which are 2.6 and 11.3 folds less compared to pH 4.5. However, as compared to laccase alone, UAL treatment showed degradation rate of 0.685 $M^{-1}s^{-1}$ which is 5.3 folds higher at similar conditions of degradation, such as pH 6.0. As explained earlier, pH and OH radical production play an important role in laccase activity during UAL. UAL treatments for other contaminants showed higher degradation rate similar to the current study [45].

This study concluded that in UAL degradation, UlS enhanced the laccase oxidation by increasing the media temperature and oxygen mass transfer (bubble formation) which is essential to speed up laccase activity. Further, UlS cavitation also aids in degradation by enhancing the interaction between laccase and CTC molecules and production of OH radicals.

Application of individual technology needs high energy as UlS need to be operated longer duration to degrade the contaminants. Similarly, laccase alone also needs longer duration. The combined ultrasonication with enzyme (UAL) system reduced the time from 48h to 2h which can lead to savings in the the energy and hence cost of the process. Further, low frequency bath sonication (38 kHz) and laccase production using agro-industrial residues can be scaled up. Future experiments should be carried out in pilot scale to compare the efficiency and cost effectiveness of the hybrid system. Hydraulic retention time, flow rate, enzyme activity during UlS should be monitored for developing large scale process.

3.3 Identification of CTC transformation products (TPs) and proposed degradation pathways

The possible part of CTC that was vulnerable to redox reactions and the summary of proposed degradation pathways are presented in figure 4. And also, the chromatographic peaks of CTC transformation products formed during laccase and UAL degradation are showed in supplementary data figure S5. This study is reporting for the first time the transformation products of CTC by UAL degradation. Laccase and UAL degradation showed similar oxidation sites, such as C2, C3, C4, and C6 (Figure 4A) for CTC. Laccase treatment showed 6 TPs having *m/z* 480.9, 430.8, 413.8, 41.9, 352.8 and 260.7 where UAL degradation showed 8 TPs of *m/z* 444.8, 437.8, 413.8, 403.9, 363.8, 260.7, 240.6, and 228.6. Similar TPs products, such as 413.8 and 260.7 were observed in

both treatment processes indicating the similar degradation processes. Additionally, the formation of smaller TPs, such as *m/z* 240.6, and 228.6 in UAL treatment indicated the potential of UAL degradation to form persistent end products. TPs are formed by hydrogenation (480.9), dehydroxylation (444.8) and demethylation (430.7), decarboxylation and de-ammoniation (451.9) reactions. These generated TPs involve redox reactions at C2, C3, C4, and C6 sites and this has been reported in previous studies of tetracycline degradation by laccase [36, 46]. To the best of authors' knowledge, these TPs have been detected for the first time during the UAL of CTC.

3.4 Estrogenic activity of laccase degraded CTC in WW samples

Figure 5 shows the response of the estrogenicity to the laccase, ultrasonication, ultrasonication assisted laccase-treated WW spiked and unspiked CTC. The blank (alcohol) samples absorbance range remained within 1 to 1.2 range after a 3-day incubation. Figure 5 showed that WW spiked CTC before and after treatment with different methods has no estrogenic activity compared to 17β-estradiol. The estrogenic samples are red colored with 17β-estradiol and other samples including blank (alcohol), WW spiked and unspiked CTC samples did not show any color change from yellow to red. Even though the TPs products were not completely similar, resulting laccase and UAL treatment samples were not estrogenic. These results are similar to the decreased estrogenic effect of other chlorinated endocrine disrupting compounds which are degraded by laccase [47]. This might be due to the similar degradation mechanisms, such as C-C and C-O bond oxidation which might be carried out during degradation of contaminants with electron-rich aromatic structures and chlorinated phenols[48]. Hence, CTC having aromatic rings, electron rich functional groups and chlorine atom can be efficiently degraded by laccase and showed no estrogenicity of resulting TPs. No estrogenic activity of treated WW indicates that the safe release of WW effluent into the surface water without causing any threat to the aquatic life.

4. Conclusion

Laccase treatment was combined with ultrasonication process to evaluate the efficiency of hybrid technologies in degrading CTC in wastewater. Laccase at 0.5 U L^{-1} showed 87% of CTC removal in 48h at 21±2 ^{o}C, whereas UAL treatment degraded over 60% of CTC in 2h at similar CTC concentration (2 mg L^{-1}) and pH 6.0. pH and OH radicals played an important role in judging the efficiency of UAL treatment. Optimum pH for UAL treatment was likely to be pH 4.5 wherein 80% of CTC was degraded. UAL and laccase treatments followed second order kinetics, which established that laccase degradation efficiency is dependent on the concentration of CTC and laccase dose. UAL showed 5.6 folds increase in degradation rate compared to laccase alone at pH 6.0. Further, optimization of pH in UAL treatment resulted in 2.6 folds increased degradation rate at pH 4.5 compared to pH 6.0. Even though the degradation rate and efficiency were different in laccase and UAL treatments, similar degradation products and pathways and no estrogenic activity was observed in both cases. This hybrid technology showed enhanced degradation and no toxic TPs, which highlighted the importance of combining two technologies in degrading other contaminants which are selectively degraded by laccase oxidation. This study signified the fact that UAL treatment is a promising technique which can enhance the degradation within a shorter time.

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506

507 **Table 1: Characterization of wastewater used in this study**

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Parameter	Value
pH	7.5±0.3
Total solids (g/L)	0.57±0.01
Suspended solids (g/L)	0.32±0.08
Volatile solids (g/L)	0.17±0.12
Volatile suspended solids (g/L)	0.11±0.05
Total organic carbon (g/L)	0.005±0.08
Alkalinity (g/L)	0.15±0.09
Ammoniacal nitrogen (g/L)	0.04±0.38
Chlortetracycline (µg/L)	8.54±0.20

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Table2: Correlation coefficients and rate constants of different degradation techniques used to degrade chlortetracycline in wastewater

Treatment method		Zero-order		First order		Second order	
		(R^2)	**K ($M\ s^{-1}$)**	**(R^2)**	**K (s^{-1})**	**(R^2)**	**K ($M^{-1}\ s^{-1}$)**
Enzymatic degradation		0.317	0.046	0.829	0.040	0.924	0.129
Ultrasonic assisted enzymatic treatment with different pH	3.0	0.806	0.648	0.946	0.132	0.960	0.163
	4.5	0.698	1.157	0.977	0.765	0.922	1.830
	6.0	0.699	0.695	0.917	0.390	0.963	0.685
	8.0	0.788	0.433	0.781	0.037	0.778	0.041
	10.0	0.340	0.343	0.314	0.317	0.317	0.015

K-rate constant

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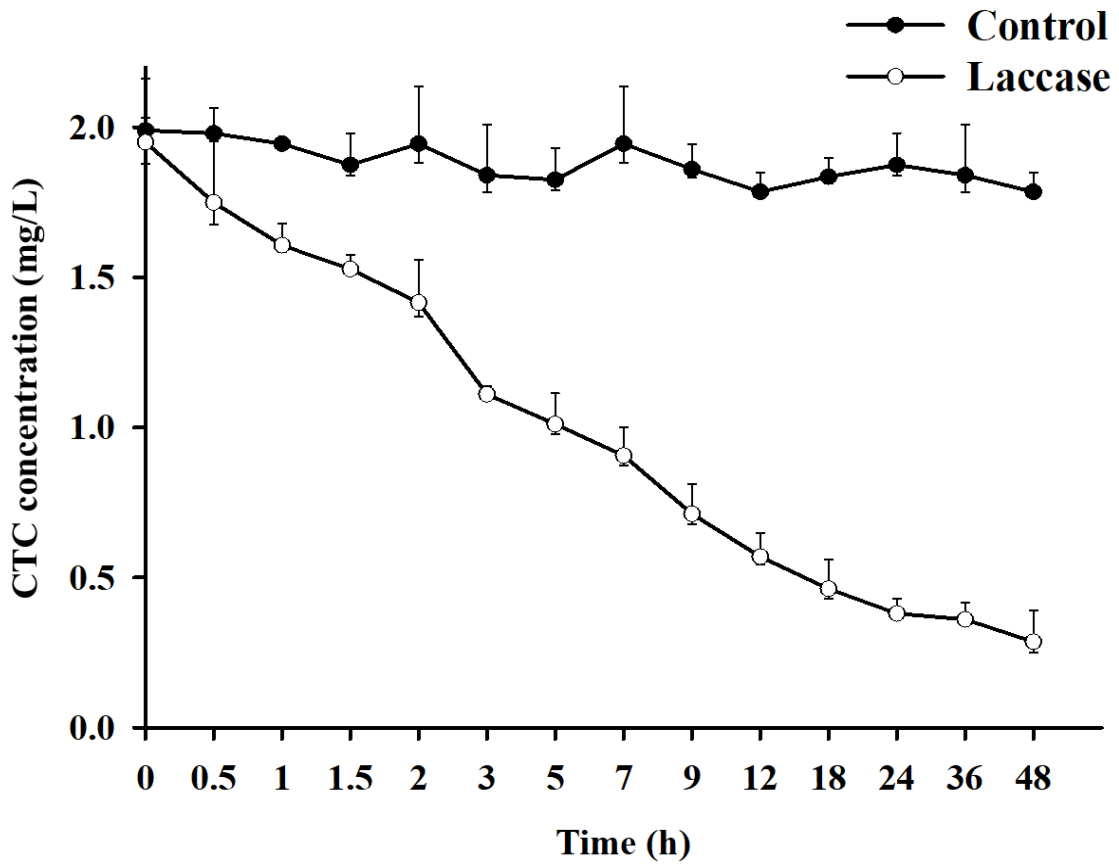

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528 **Figure1. Laccase degradation kinetics of chlortetracycline in wastewater at room**

529 **temperature** 21±2 ºC**.**

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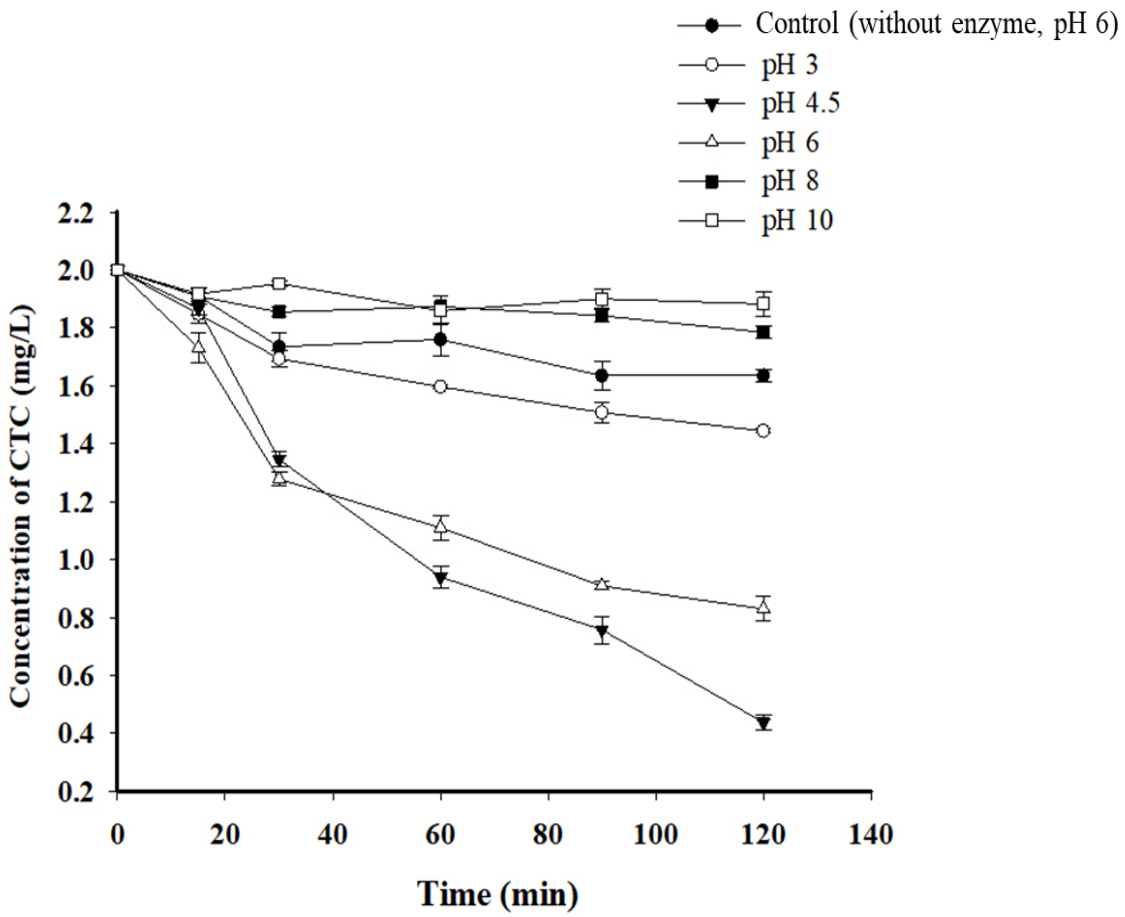

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534 **Figure 2. Effect of pH on ultrasonication assisted degradation kinetics of chlortetracycline**

535 **(2** mg L^{-1}**) in wastewater at 0.5 IU laccase activity**

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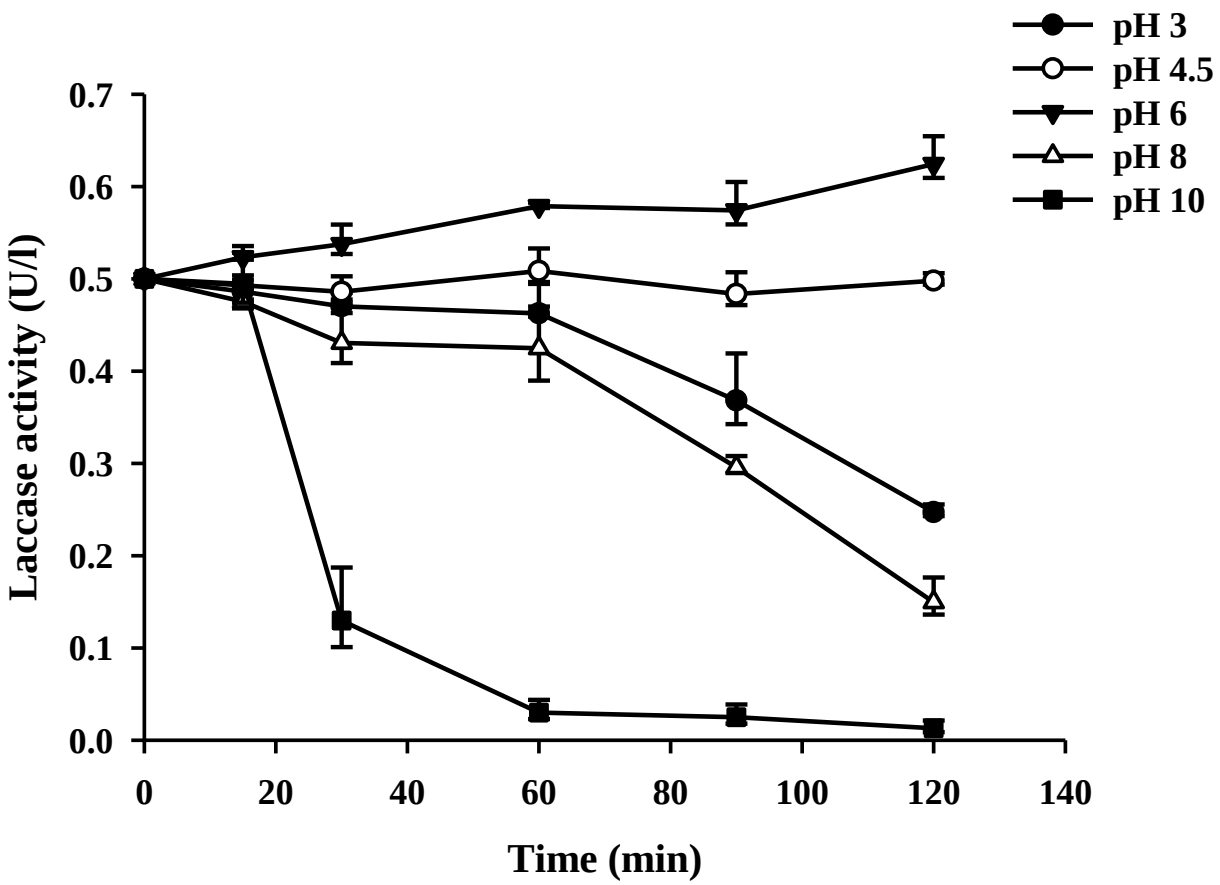

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544 **Figure 3. Effect of pH on stability kinetics of laccase (0.5 IU) during ultrasonication**

545 **assisted degradation of chlortetracycline (2** mg L^{-1}**) in wastewater**

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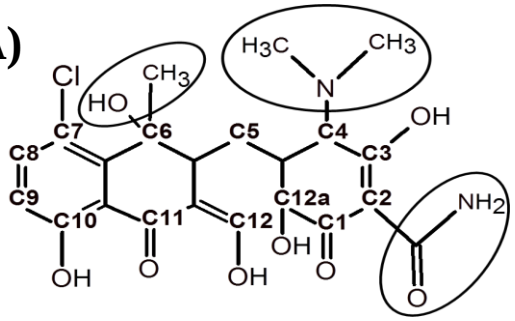
A)



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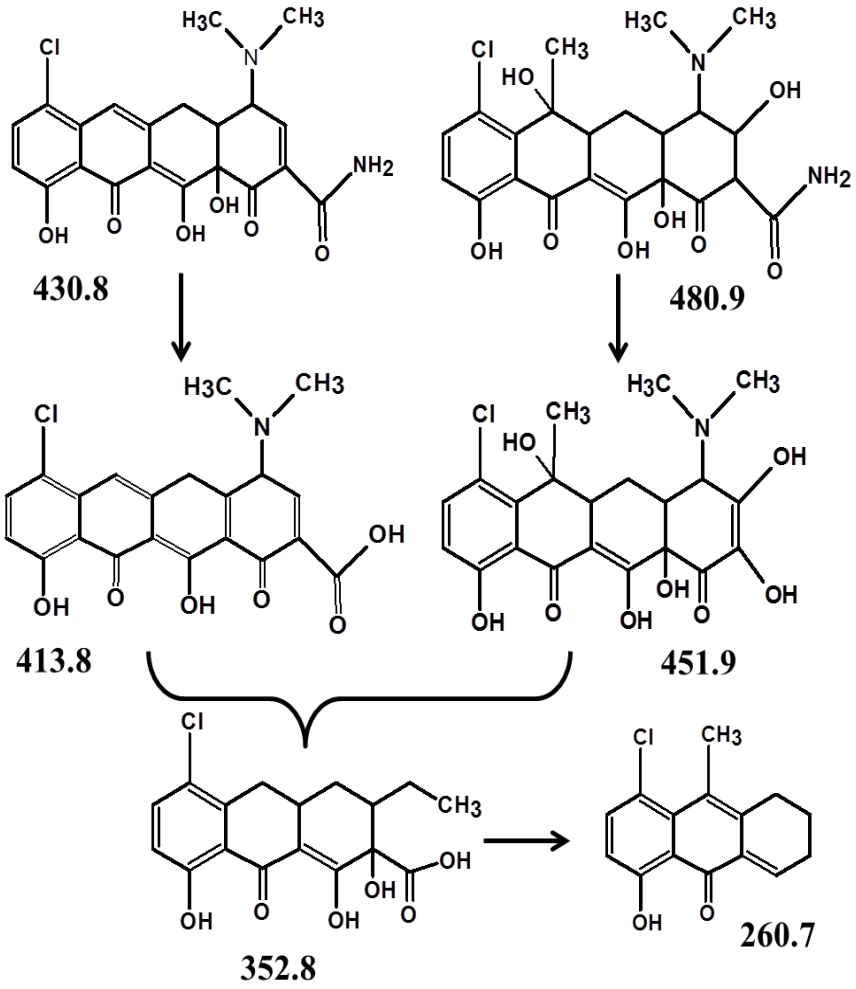
B)



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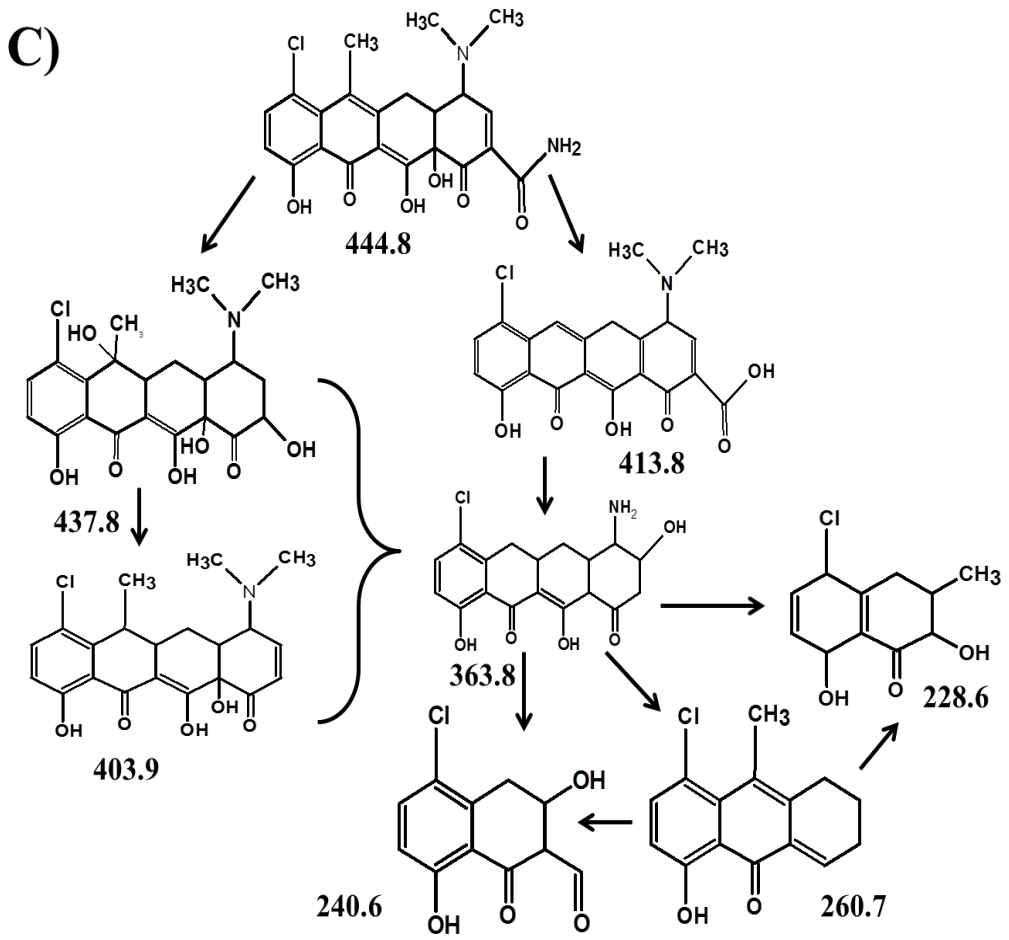

Figure 4: Degradation pathways and products (*m/z*) of CTC in wastewater; A) targeting sites of CTC; B) laccase degradation pathway of CTC(CTC-2 mg L^{-1}**; laccase 0.5 IU, time 48h; pH 6±0.5) and C) ultrasonication assisted laccase degradation pathway (CTC-2** mg L^{-1}**; laccase 0.5 IU, time 2h, pH 4.5±0.5).**

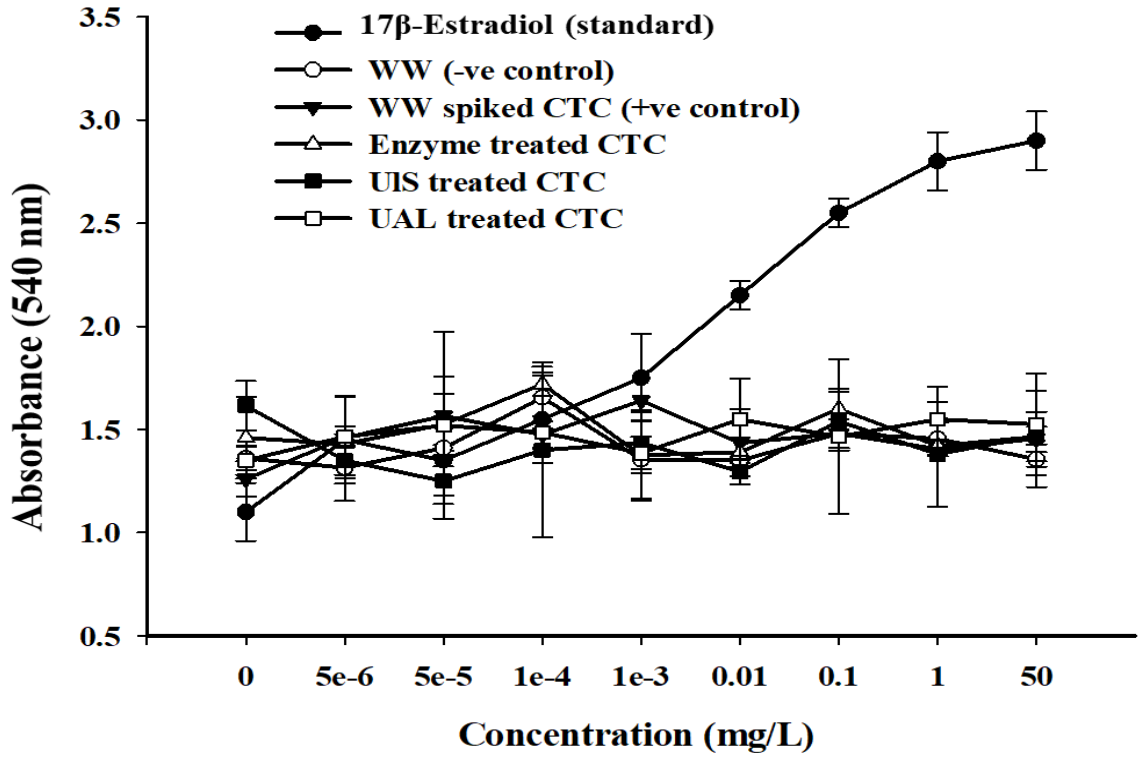

Figure 5: Estrogenic response of treated wastewater spiked with chlortetracycline (CTC,2 mg L^{-1}) using laccase (0.5 IU), ultrasonication (UlS, 2h) and, ultrasonication assisted laccase (UAL) treatment.