Effect of Two Humic Acids on Laccase-Driven 17β-Estradiol Conversion Kinetics and Polymerization Products at Varying pH Levels

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Abstract

Estrogens with tremendous ecological risks are proverbially found in water. Laccase can drive humification of estrogens to reduce their ecotoxicity and removability, but little investigation existed in exploring the effect of humic acids (HAs) on E2 conversion kinetics and polymerization products at different pH conditions. Here, *Trametes versicolor* laccase (*Tv* lac) was capable of efficiently converting a representative estrogen, 17β-estradiol (E2) with two different HAs, and the process followed a pseudo-first-order kinetics. The velocity constants were respectively 0.048, 0.022, and 0.020 min⁻¹ for HA-free, peat-derived HA, and commercial HA at pH 5.0. The changing pH not only impacted E2 coupling kinetics in *Tv* lac-evoked humification, but altered the aromaticity and humification degrees of HAs. A total of five intermediate species including estrone (E1), E2 dimer, trimer, and tetramer, as well as E1-E2 cross-linked products were tentatively detected, in which the dominating species were E2 self-oligomers resulting from radical-centered carbon-carbon/oxygen stepwise polymerization routes. Yields of dimeric, trimeric, and tetrameric species with increased molecular sizes were the highest at pH 5.0 in the given pH values, and they were readily handled by precipitation and filtration. Especially E2 was able to be covalently incorporated into humic constituents to generate new humified co-polymers, thereby accelerating E2 humification and detoxification. These findings demonstrate that pH exhibits a far-reaching influence on the conversion kinetics, humification degrees, and products distribution of E2 and HAs in *Tv* lac-reinforced polyreaction. Thus, there is need to reappraise the fate and transport of estrogens with HAs present in natural water at varying pH levels.

1. Introduction

Estrogens in essence are a category of steroid hormones, which have an extensive range of biological activities (Adeel et al., 2017; Yao et al., 2018). They not only act as a neuroprotective factor but as a signal, which regulate the function and organization of organisms (Dumas and Diorio, 2011; Brann et al., 2007). In general, the naturally-occurring estrogen such as 17β-estradiol (E2) is principally produced by animals and humans, it is continuously released into aquatic eco-environments owning to the incomplete elimination and conversion in sewage treatment plants (Bilal and Iqbal, 2019; Kolpin et al., 2002; Simpson, 2003). Currently, estrogens have drawn extensive concern because of their adverse effects on organisms, humans, and water ecosystems (da Silva et al., 2019). It is reported that the feminization of male fish was constantly observed in estrogen-polluted water even at an extremely low level (Harris et al., 2011; Vethaak et al., 2005). Therefore, it is particularly important to provide an approach with high efficiency and low cost for eliminating and converting estrogens in water (Bilal and Iqbal, 2019; Shrestha et al., 2012).

Humification refers to the process that the carbon of organic residues is converted into humus difficult to decompose by biochemical reactions (Chen et al., 2019; Lee et al., 2019). It is well-documented that natural enzyme can enhance humification and polymerization of organic compounds by reducing the reaction time and energy consumption (Lipczyńska-Kochany, 2018; Yang et al., 2018). Humic acids (HAs) are omnipresent in natural aquatic environments, and natural enzyme-evoked humification coupling not
only promotes oligomerization of estrogens but recombines humic constituents, which also contributes to the formation of new humified polymers between estrogens and HAs (Hur et al., 2013; Singh et al., 2015; Zhong et al., 2019). It follows that humification can serve as a vital natural decontamination and detoxification process of estrogens, and the core reaction is radical-centered oxidation and polymerization (Du et al., 2016; Feng et al., 2013). Thus, it is a novel strategy to utilize natural enzyme-centered humification of estrogens and HAs for generating humified polymers (Chen et al., 2019; Zhao et al., 2019). Nevertheless, how to select a high-efficient oxidation-reduction enzyme for participating in the humification coupling reaction is particularly important.

Laccase is a member of blue multicopper oxidases that can trigger a single-electron oxidation concomitantly with the four-electron reduction of \(O_2\) to \(2H_2O\) (Barrios-Estrada et al., 2018; Bilal et al., 2019). Laccase generally has four copper ions, including one type-1 Cu (T1-Cu), one type-2 Cu (T2-Cu), and two type-3 Cu (T3-Cu), in which the T2-Cu and T3-Cu sites are believed to close together and thus form a trinuclear cluster (Garavaglia et al., 2004). The substrates are oxidized by the T1-Cu site, and the extracted electron is transferred via a conserved His-Cys-His pathway to the T2/T3-Cu sites, where \(O_2\) as the second substrate binds to it. (Baldrian, 2006; Madhavi and Lele, 2009). Simultaneously, four unstable radicals from substrates are produced, and then they encounter a series of non-enzymatic coupling reactions to form a variety of dimers, trimers, tetramers, oligomers, and polymers (Chen et al., 2019; Kudanga et al., 2011). It is well-documented that laccase plays a far-reaching role in humification and polymerization processes, especially for the substrates that contain phenolic \(-\text{OH}\) (Bilal and Iqbal, 2019; Zhong et al., 2019). In addition, the electron acceptor of laccase is \(O_2\), which exists ubiquitously, it makes the application of laccase-driven humification coupling in bioremediation more green, economical, and eco-friendly (Chen et al., 2019; Lu et al., 2009).

Previous studies demonstrated that pH had a tremendous effect on laccase stability and activity (Baldrian, 2006; Schneider, et al., 1999; Xia et al., 2014). For instance, the enzyme activity at a greater pH is declined through the \(\text{OH}^-\) binding to the T2/T3-Cu sites, thus blocking the internal-electron transfer from T1-Cu to T2/T3-Cu cores (Munoz et al., 1997). It is thus necessary to investigate how pH impacts the conversion kinetics and products distribution of E2 with HAs during laccase-reinforced humification. In this study, the effect of two HAs on E2 removal kinetics was systematically explored at different pH levels by Trametes versicolor laccase (TvLac). TvLac-driven the aromaticity and humification degrees of HAs regardless of E2 presence were respectively investigated by the absorbance ratios of \(A_{280\text{ nm}}/A_{350\text{ nm}}\) and \(A_{250\text{ nm}}/A_{365\text{ nm}}\) using a UV-Vis spectrophotometry. Particularly, the probable intermediate species of E2 with HAs in TvLac-evoked humification were tentatively identified via Liquid Chromatography coupled with Triple Quadrupole Mass Spectrometry (LC-TQMS), and the impact of pH on the products distribution was assessed as well. Our results clearly revealed that HAs altered the conversion kinetics and products distribution of E2 at varying pH levels in TvLac-centered humification processes. Thus, it is extremely important to understand the geochemical fate and transport of estrogens with HAs present in water ecosystems at different pH conditions.
2. Materials And Methods

2.1. Chemicals and materials

E2 (≥ 98%), 2,6-dimethoxyphenol (2,6-DMP, 99%), and Tvlac (≥ 0.5 U·mg⁻¹) were purchased from Sigma-Aldrich. Previous studies reported that Tvlac exhibited a higher redox potential (RP) than other laccases, which was better for humification coupling of substrates (Kurniawati and Nicell, 2008). In this experiments, the peat-derived HA (P-HA) was extracted using the sodium pyrophosphate extraction method suggested by the International Humic Substance Society (IHSS). The commercial HA (C-HA) (technical grade) was obtained from Sigma-Aldrich. The stock solutions of HAs were filtered by 0.45 µm membrane before utilization. The spectral characteristics of both P-HA and C-HA are shown in Fig. S1 (Supplementary Information). UV-Vis spectra of two HAs were characterless, which revealed a decrease in absorbance with increasing wavelength (Fig. S1a). Notably, C-HA presenting higher absorbance values disclosed a better linearity than P-HA with smaller absorbance. As displayed in UV-Vis assays, FTIR spectra of P-HA and C-HA also showed some visible structural differences (Fig. S1b). High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were obtained from Fisher Scientific. All other reagents were analytical grade or higher and obtained from commercial suppliers.

2.2. UV-Vis spectrum analysis

Tvlac-triggered conversion of E2 was carried out in glass vials at room temperature (25°C) and 101.325 kPa. Each reactor was comprised of 5 mL citrate-phosphate buffer solution (C-PBS: 10 mmol·L⁻¹) mixture containing 5.0 µmol·L⁻¹ E2 at different pH values (i.e. 4.0, 5.0, 6.0, and 7.0). After adding 1.0 U·mL⁻¹ Tvlac, the solution was gently stirred to facilitate the mixture of Tvlac and E2 immediately. The control sample was prepared with no Tvlac added. To monitor changes in the absorbance spectrum in a range of 200–700 nm, 3.4 mL reaction mixture was taken from each treatment within predetermined time-intervals (i.e. 0 and 60 min), and subsequently, measured the absorption spectrum from 200 nm to 700 nm in a quartz cell via UV-Vis scanning spectrophotometer (UV-2550, Shimadzu, Japan) (Fonseca et al., 2011). Additionally, the characteristic absorption peak of E2 at 280 nm was detailedly recorded over time.

2.3. Effect of pH on Tvlac-evoked conversion kinetics of E2 and HAs

Reaction kinetics of E2 with HAs by Tvlac-evoked humification was conducted in a 5 mL C-PBS under different pH conditions (pH 4.0, 5.0, 6.0, and 7.0) containing 5.0 µmol·L⁻¹ E2 and 0 or 30 mg·L⁻¹ HA (i.e. P-HA or C-HA) at 25°C and 101.325 kPa (Sun et al., 2016; Xia et al., 2014). It is because the average level of HA was generally found in aquatic ecosystems at 30 mg·L⁻¹. 1.0 U·mL⁻¹ Tvlac was added into reaction solution, quickly mixed, and then incubated statically. The control sample was carried out with no Tvlac added. At preselected time intervals (i.e. 0, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, and 120 min), enzymatic reaction was promptly terminated with 5 mL methanol (Sun et al., 2016), and then filtered.
through 13 mm × 0.22 µm nylon syringe filters (Rasco, Hefei, China) for HPLC analysis. All experiments were treated in triplicate to minimize the experimental error.

In this research, the apparent pseudo-first-order velocity constant \( (k) \) and half-life \( (t_{1/2}) \) values of E2 regardless of HAs presence are calculated by the following equations:

\[
\ln \frac{C_0}{C_t} = k \cdot t \quad (1)
\]

\[
t_{1/2} = \frac{\ln 2}{k} \quad (2)
\]

where \( C_0 \) is the initial level of E2, and \( C_t \) represents the residual level of E2 at reaction time \( t \).

### 2.4. Activity test of \( T_{Vlac} \)

Activity of \( T_{Vlac} \) was monitored at 25°C and 101.325 kPa by oxidizing 2,6-DMP to form visible chromogenic species (Sun et al., 2020a). The reaction mixture contained 3.4 mL of 10 mmol·L\(^{-1}\) C-PBS (pH 3.8), 1.0 mmol·L\(^{-1}\) 2,6-DMP, and 20 µL of \( T_{Vlac} \). One unit of \( T_{Vlac} \) activity (U·mL\(^{-1}\)) was defined as the amount of enzyme that induced a unit change per minute in absorbance at 468 nm.

### 2.5. Quantification of E2 by HPLC

The level of E2 was analyzed and quantified by a Waters HPLC system equipped with a Waters 600 pump, a Waters 2707 autosampler, and a Waters 2998 photodiode array detector. An Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 150 mm, 5 µm particle size) was used for chromatographic separation at 40°C. The mobile phase consisting of acetonitrile and water (70: 30, v/v) was pumped via the column at a flow rate of 1.0 mL·min\(^{-1}\). The detection wavelength of E2 was set at 280 nm, which was the maximum absorption wavelength observed by UV-Vis spectrophotometer. 20 µL of injection volume was introduced onto the HPLC system every 10 min. The retention time of E2 was 3.529 min, and its level was quantified using a calibration curve. In this study, the quantification limit of E2 was below 10 nmol·L\(^{-1}\), and its recoveries in the presence of HA-free, P-HA, and C-HA were respectively 100.4%, 99.8%, and 101.3% (\( n = 5 \)).

### 2.6. Analysis of HAs spectral performances

As is well-known, the UV-Vis characteristic absorption peaks of HAs are connected with their aromatic structures and humification degrees (Liu et al., 2019; Rodríguez et al., 2016). Thus, we tested the spectral performances of P-HA and C-HA in \( T_{Vlac} \)-evoked systems at pH 4.0, 5.0, 6.0, and 7.0. The humification reaction system was a mixture of 5 mL C-PBS, 0 or 5.0 µmol·L\(^{-1}\) E2, 30 mg·L\(^{-1}\) HAs, and 1.0 U·mL\(^{-1}\) \( T_{Vlac} \). The control sample included parallel incubation but \( T_{Vlac} \)-free. The characteristic absorbance values of 250, 280, 350, and 365 nm were respectively detected at specific intervals (\( i.e. \) 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 min). The ratios of \( A_{280\,nm}/A_{350\,nm} \) and \( A_{250\,nm}/A_{365\,nm} \) were used to represent the aromatic structures and humification degrees of HAs during \( T_{Vlac} \)-triggered humification coupling reactions, respectively.
2.7. Identification of E2 humification products

The intermediate species of E2 conversion during Tvlac-evoked humification coupling were tentatively identified by LC-TQMS. After a 60 min incubation, reaction solution (5.0 µmol·L⁻¹ E2, 0 or 30 mg·L⁻¹ HA, and 1.0 U·mL⁻¹ Tvlac) was quenched by adjusting pH 1.5, and then concentrated by a Cleanert polar-enhanced polymer (PEP) column (500 mg / 6 mL, Bonna-Agela Technologies). The collected eluent was dried and re-dissolved in 1 mL methanol. An Agilent 1260 Infinity Liquid Chromatography coupled with an Agilent 6400 Triple Quadrupole Mass Spectrometry were established for the analysis of humification species. The separation was performed with a Poroshell 120 EC-C18 (3.0 mm × 100 mm, 2.7 µm particle size) column (Agilent Technologies). The mobile phase was set as gradient-elution by acetonitrile and water (0–6 min, 30: 70; 6–22 min, 60: 40; 22–22.5 min, 80: 20; 22.5–24.5 min, 90: 10; 24.5–25 min, 30: 70). The injection volume was 5.0 µL. Mass Spectrometry analysis was operated in the negative scan mode with the following parameters: drying gas temperature 300°C, sheath gas temperature 300°C, drying gas flow 6.0 L/min, sheath gas flow 11 L/min, nebulizer 45 psi, capillary entrance voltage 3.5 kV. The total ionization chromatography was collected in the m/z range of 50–1500. In this study, the polymerized species of E2 were not confirmed because of lack of analytical standards.

3. Results And Discussions

3.1. Impact of pH on E2 UV-Vis spectrum in Tvlac-evoked reactions

Conversion of E2 was qualitatively analyzed at pH 4.0, 5.0, 6.0, and 7.0 during Tvlac-triggered humification processes at 25°C and 101.325 kPa by UV-Vis absorption spectra. As shown in Fig. 1, the changes in the absorption spectra were a direct result of pH, and the maximum absorption peak of E2 was displayed at 280 nm (A₂₈₀ nm). Fonseca et al. (2011) also disclosed that E2 had a similar UV-Vis spectrum with a band centered at 281 nm. Moreover, the variations of A₂₈₀ nm were more significant for a 60 min reaction compared to the initial values (0 min). It has been reported earlier that bands around 275–290 nm were the the characteristic absorption peaks of phenolic compounds (Garcia-Morales et al., 2015). These results implied that pH played a far-reaching role in the removal and conversion of E2 by Tvlac-evoked oxidation. Thus, the absorbance change at 280 nm over time was further evaluated in Tvlac-triggered humification systems. Compared with pH 4.0, 6.0, and 7.0, the absorbance at 280 nm reached a maximum value for 65 min incubation at pH 5.0. However, the values of A₂₈₀ nm continued to increase at other pH conditions during the enzymatic reaction processes, revealing that the optimum pH for E2 removal and conversion was 5.0 under all the appointed pH conditions. It is probably because pH impacted the catalytic activity and stability of Tvlac, thus lowering the conversion efficiency of E2 (Baldrian, 2006; Margot et al., 2013).

3.2. Tvlac-evoked humification kinetics of E2 with HAs at varying pH levels
pH is a crucial factor governing the conversion kinetics of phenolic pollutants with HAs in fungal laccase-triggered humification systems (Gan et al., 2019; Spina et al., 2020; Wang et al., 2018). In this study, the effect of varying pH levels on the conversion kinetics of E2 was quantitatively analyzed in *Tv* lac-catalyzed humification processes by HPLC. As presented in Fig. 2, it is obvious that the level of residual E2 decreased with reaction time increasing. *Tv* lac-caused the conversion efficiency of E2 followed the order of pH 5.0 > pH 6.0 > pH 4.0 > pH 7.0. For example, *Tv* lac was capable of eliminating 99.3% E2 at pH 5.0 for a 120-min incubation, however, the removal efficiencies of E2 respectively were 94.9%, 98.0%, and 35.2% at pH 4.0, 6.0, and 7.0. Both P-HA and C-HA exhibited obviously inhibitory effects on the removal and conversion of E2 within 60 min. For instance, compared with HA-free, the conversion efficiencies of E2 reduced 24.9% and 27.4% in the presence of P-HA and C-HA for 60 min incubation, respectively. It can be due to the competition occurred between HA and E2 for the catalytic site of *Tv* lac (Dou et al., 2018; Sun et al., 2017). Moreover, the inhibitory effect of C-HA on E2 conversion was mildly higher than that of P-HA, implying that P-HA had a stronger binding ability with E2. Notably, overall the removal rate of E2 per unit time decreased with the increase of the reaction course. For example, the removal rates of E2 per minute without HA respectively reached 1.7%, 2.6%, 1.8%, and 0.5% for 30 min reaction at pH 4.0, 5.0, 6.0, and 7.0. However, the removal rates of E2 per minute reduced to 1.3%, 1.6%, 1.5%, and 0.4% after 60 min incubation, respectively. This is because *Tv* lac activity remained unchanged in humification reactions, while the levels of intermediate species continuously accumulated under the tested pH conditions and the apparent concentration of E2 gradually decreased until was completely soluble, thus resulting in the limited binding capacity for E2 at the reactive center of *Tv* lac (Beck et al., 2018; Wang et al., 2018; Xia et al., 2014).

Additionally, *Tv* lac-triggered removal and conversion of E2 satisfied the apparent pseudo-first-order kinetics within 120 min under different pH conditions (Fig. S2), which was consistent with laccase-caused reaction of other phenolic pollutants such as triclosan and tetrabromobisphenol A (Dou et al., 2018; Feng et al., 2013; Lu et al., 2017). It is because *Tv* lac remained relatively stable and its activity was hardly changed in this reaction process. As illustrated in Table 1, the *k* and *t*₁/₂ values at pH ranged from 4.0 to 7.0 were respectively 0.025, 0.048, 0.032, and 0.004 min⁻¹, and 27.73, 14.56, 21.58, and 173.29 min in *Tv* lac-evoked E2 reactions without HAs. The highest *k* value was obtained at pH 5.0, which was 12-fold higher than the reaction at pH 7.0. It follows that *Tv* lac had a higher reactivity to catalyze E2 conversion in acidic solution (pH 4.0, 5.0, and 6.0) than neutral or alkaline solution (pH ≥ 7.0), *Tv* lac-evoked E2 removal was thoroughly intercepted in pH ≥ 8.0 solution), which accorded with the results shown in Fig. 1. Compared with HA-free, the *k* values for P-HA and C-HA were much lower at the all measured pH conditions, demonstrating that the presence of HAs markedly restrained the conversion kinetics of E2 by *Tv* lac.
Table 1
The $k$ and $t_{1/2}$ values of E2 in $T_{\text{Viac}}$-catalyzed E2 humification kinetics

| pH | $T_{\text{Viac}}$+E2 | $T_{\text{Viac}}$+E2+P-HA | $T_{\text{Viac}}$+E2+C-HA |
|----|---------------------|-----------------------|---------------------|
|    | $k$ (min$^{-1}$)    | $t_{1/2}$ (min)       | $R^2$ | $k$ (min$^{-1}$)    | $t_{1/2}$ (min)       | $R^2$ | $k$ (min$^{-1}$)    | $t_{1/2}$ (min)       | $R^2$ |
| 4.0 | 0.025              | 27.73               | 0.9948 | 0.010            | 68.63               | 0.9952 | 0.008            | 85.57               | 0.9791 |
| 5.0 | 0.048              | 14.56               | 0.9681 | 0.022            | 31.22               | 0.9937 | 0.020            | 35.19               | 0.9881 |
| 6.0 | 0.032              | 21.58               | 0.9943 | 0.018            | 38.72               | 0.981  | 0.014            | 40.51               | 0.9762 |
| 7.0 | 0.004              | 173.29              | 0.9439 | 0.003            | 277.26              | 0.9731 | 0.002            | 462.10              | 0.8474 |

As reported earlier, the highest catalytic efficiency of fungal laccase for estrogens was displayed at pH range of 4.0–6.0 (the conversion efficiencies of estrogens were approximately 95%), and estrogens conversion decreased drastically outside these ranges (Kim and Nicell, 2006; Lloret et al., 2010). At present, the influence mechanism of pH on laccase-triggered the conversion kinetics of phenolic pollutants has been summarized (Baldrían, 2006; Sun et al., 2020b). It is suggested that the bell-shaped pH profile of phenolic compounds was produced by two opposite ways. On the one hand, the enzymatic reaction of phenolic contaminants is reduced at low pH (generally, pH was less than 5.0) due to the inhibition of the electron transfer between substrate and laccase T1-Cu site (Margot et al., 2013). Researchers pointed out that the RP of phenolic compound decreased with the increasing of pH, owning to the transfer of proton during the catalytic reaction (Wang et al., 2018). However, the RP of T1-Cu site in laccase had no significant effect by pH (Xu, 1997). The RP difference value between phenolic compound and T1-Cu site augmented with pH, thus improving the conversion kinetics of substrate (Mateljak et al., 2019). On the other hand, the enzymatic reaction of phenolic contaminants is also impeded at high pH (usually, pH was greater than 6.0) because of the limitation of the intramolecular electron transfer (Solomon et al., 2015). Some reports stated that OH$^-$ was beneficial to bind with T2/T3-Cu sites in laccase, which terminated the internal-electron transfer and thus suppressed the reactivity of laccase (Xu, 1997; Wang et al., 2018). Additionally, pH can also change the ionization and charge of substrate, and impact the electrostatic interactions of laccase and substrate (Su et al., 2019). This shows we need to make a concession to seek the optimal pH that allows the highest conversion efficiencies of estrogens and other contaminants.

### 3.3. Effect of pH on $T_{\text{Viac}}$-triggered HAs aromaticity and humification degrees

HAs are complex mixtures of large- to small-molecular-weight species held together through supramolecular interactions. Infrared spectra had testified that both P-HA and C-HA constituents presented a variety of functional groups such as –OH, –COOH, and C = O (Fig. S1b). A recent study suggested that aromaticity was an important structural characteristic of macromolecular HAs (Rodríguez et al., 2016; Sun et al., 2020b). Generally, the spectral change of a specific band can be used to describe
the aromaticity of HAs in enzyme-driven humification reactions (Deng, et al., 2019). In this study, we conducted batch tests to investigate how pH would impact the aromaticity of HAs without or with E2 in *Tv* lac-evoked reaction systems via evaluating the ratio of $A_{280\text{ nm}}/A_{350\text{ nm}}$. As shown in Fig. 3, the ratio of $A_{280\text{ nm}}/A_{350\text{ nm}}$ increased gradually as the reaction went on, indicating that the enzymatic reaction augmented the aromaticity of HAs by generating new stable macromolecular structures. An earlier report also stated that the ratio of $A_{280\text{ nm}}/A_{350\text{ nm}}$ displayed outstanding correlations for the aromaticity and molecular weight of HA (Rodríguez et al., 2016). Moreover, the change rate of $A_{280\text{ nm}}/A_{350\text{ nm}}$ ratio ($\Delta A_{280\text{ nm}}/A_{350\text{ nm}}$) at pH 5.0 was larger than that of pH 4.0, 6.0, and 7.0, implying that pH 5.0 was the optimal reaction condition for increasing the aromaticity of HAs by *Tv* lac in all the tested pH values. Notably, the treatment groups with E2 presented higher aromaticity than those of E2-free at varying pH levels. For instance, the values of $A_{280\text{ nm}}/A_{350\text{ nm}}$ ranged from 1.96 to 1.99 at pH 5.0 during *Tv* lac-evoked P-HA reactions. When adding E2, the absorbance ratio increased from 1.99 to 2.01. It is probable that E2 was covalently incorporated into humic constituents to generate new humified polymers by radical-centered cross-linking mechanism (Chen et al., 2019; Singh et al., 2015; Sun et al., 2016). Additionally, the aromaticity in treatments with P-HA was obviously greater than others with C-HA.

It is reported that the bonds of HAs could be destroyed and converted into radicals in the process of humification mediated by fungal laccase (Chen et al., 2019). These radicals are subsequently recombined to form new humified species through carbon-carbon, carbon-oxygen, and/or carbon-nitrogen coupling, resulting in an acceleration of the humification and electron conjugation degrees of HAs (Dou et al., 2018; Singh et al., 2015; Yoon et al., 2020). Consequently, the humification degrees of P-HA and C-HA in *Tv* lac-evoked reactions were also assessed by the ratio of $A_{250\text{ nm}}/A_{365\text{ nm}}$ (Liu et al., 2019; Rodríguez et al., 2016). As displayed in Fig. S3, the ratio of $A_{250\text{ nm}}/A_{365\text{ nm}}$ raised with the increase of reaction time, indicating that *Tv* lac caused the humification and electron conjugation of HAs to form new macromolecular humified species. Moreover, the value of $\Delta A_{250\text{ nm}}/A_{365\text{ nm}}$ at pH 5.0 was the largest compared to other experimental pH conditions, which was consistent with the result in Fig. 3. In addition, the treatment groups with E2 exhibited greater promotion in the humification degrees of HAs compared to the treatment groups without E2. For instance, the $A_{250\text{ nm}}/A_{365\text{ nm}}$ ratio varied from 2.91 to 2.96 and from 2.95 to 3.01 in *Tv* lac-caused humification of P-HA without and with E2 at pH 5.0, respectively. It is because HAs could cross-couple with E2 by combination of hydrogen bond and/or covalent bond (Chen et al., 2019; Du et al., 2016; Mao et al., 2010). These results manifested that *Tv* lac-triggered conversion and humification of HAs were similar to the coupling processes of phenolic compounds, which was markedly impacted by environmental pH.

### 3.4. Radical coupling mechanisms of E2 and/or HAs in *Tv* lac-triggered humification

*Tv* lac-evoked conversion species of E2 were tentatively identified by LC-TQMS. As displayed in Table 2, E2 dimer (at the peak of $m/z = 541.34$), trimer (at the peaks of $m/z = 811.48$), and tetramer (at the peaks of $m/z = 1081.69$) were identified as representative oligomeric species regardless of HAs presence during
TvIac-triggered humification coupling, in agreement with previous studies (Beck et al., 2018; Lloret et al., 2013; Sun et al., 2016; Xia et al., 2014). These results indicated that TvIac caused the oligomerization of E2 to yield a variety of self-linked products via radical-centered carbon-carbon/oxygen covalent binding. The molecular masses (MM) of E2 monomer as well as its self-polymerized products can be calculated by the following Eq. (3):

\[
MM = 272.18n - 2.02 \cdot (n - 1)
\]

where 272.18 is the MM of E2, 2.02 is the atomic mass of 2H, and \( n \) corresponds to the number of E2 units (\( n \) is integer, and \( n \geq 1 \)).

Additionally, estrone (E1) and E1-E2 cross-linked species were also detected in TvIac-evoked conversion of E2 according to the peaks of \( m/z = 269.15 \) and 539.31, respectively. Even so, the yields of these two products were very low.

| \( m/z \) value | Molecular formula | Intermediate products |
|-----------------|-------------------|----------------------|
| Theoretical     | Experimental      |                      |
| 271.17          | 271.17            | C\(_{18}\)H\(_{24}\)O\(_2\) | E2                   |
| 541.33          | 541.34            | C\(_{36}\)H\(_{46}\)O\(_4\) | Dimer                |
| 811.49          | 811.48            | C\(_{54}\)H\(_{68}\)O\(_6\) | Trimer               |
| 1081.66         | 1081.69           | C\(_{72}\)H\(_{90}\)O\(_8\) | Tetrramer            |
| 269.15          | 269.15            | C\(_{18}\)H\(_{22}\)O\(_2\) | Estrone (E1)         |
| 539.32          | 539.31            | C\(_{36}\)H\(_{44}\)O\(_4\) | E1-E2 cross-linked species |

As summarized in Fig. 4, we proposed the possible humification and oligomerization pathways for TvIac-evoked conversion of E2 and HAs. In the initial stage, TvIac triggered the one-electron oxidation of E2 and/or HAs to produce abundant unstable phenoxy radical intermediates (Bilal et al., 2019; Du et al., 2016; Sun et al., 2013). Subsequently, these unstable reactive intermediates coupled with each other outside the reactive site of TvIac T1-Cu to form E2 self-oligomers, new humified polymers, and E2-HA cross-linked products via radical-centered carbon-carbon, carbon-oxygen, and/or carbon-nitrogen covalent binding mechanisms (Kudanga et al., 2011; Xia et al., 2014; Yoon et al., 2020). In particular, if the steric effects did not eliminate the occupation of these polymeric species at the catalytic core of TvIac T1-Cu, the self/cross-polymerization would be continuous because these species still possessed the phenolic –OH (Beck et al., 2018; Qin et al., 2015; Sun et al., 2020a). As the reaction time increased, many more polymeric products with a greater degree of coupling were produced, and part of them would precipitate from reaction solution. Li et al. (2013) proposed a novel coupling mechanism between estrogen and HA.
by electro-enzymatic oxidative polyreaction. They pointed out the presence of HA could present chain reactions from estrogen self-coupling to co-polymerization. The formation of the polymerized precipitates dramatically reduced the ecotoxicity and removability of parent compound that had been confirmed by previous researchers (Feng et al., 2013; Lu et al., 2009), and these highly self/cross-linked precipitates were easily removed by precipitation and filtration. For example, the solubility of E2 oligomers in water was > 10^7 times lower than E2 monomer, and thus the bioavailability and transferability of these oligomers were much less than parent compound (Qin et al., 2015). Note that the functional substituents on HA might prominently impact the cross-linking with phenolic pollutants, as well as the type and stability of the polymerized species (Du et al., 2016). These results clearly demonstrated that Tvlac reinforced the humification and oligomerization of E2, and the presence of HAs changed the distribution of E2 self-linked products by generating E2-HA co-polymerization species in radical-centered cross-linking (Bialk et al., 2005; Chen et al., 2019; Li et al., 2013).

3.5. Influence of pH on the products distribution in Tvlac-evoked humification

pH plays a vital role in the formation of the polymeric species during laccase-triggered humification processes (Koschorreck et al., 2008). As is well-known, HAs can directly involve in laccase-induced humification coupling of phenolic pollutants, thereby exhibiting a significant influence on the distribution of E2 self-linked products (Chen et al., 2019; Lu et al., 2009; Mao et al., 2010). In this study, we calculated the relative abundance ratios (RARs) of E2 and its dimer, trimer, and tetramer at different pH conditions, due to lacking of analytical standard samples, these oligomer levels were not quantified. As shown in Fig. 5, the relative abundance of E2 dimer was higher relative to trimer and tetramer in all the treatment groups for a 60-min incubation, implying that E2 dimer was the most high-producing oligomeric species in Tvlac-evoked humification. Similar results were also obtained by Qin et al. (2015), they documented that the sum of dimer peak areas was much larger than that of trimer and tetramer, and the yield of dimer aggrandized fleetly and reached the maximum value for a 1-h incubation. Amazingly, the relative abundances of all oligomers in the presence of HAs were significantly lower than those of the HA-free. Such as the relative abundances of dimer, trimer, and tetramer were respectively 66.55%, 16.94%, and 2.30% in Tvlac-evoked E2 coupling without HA at pH 5.0, which were greater than that of the reaction with P-HA (or C-HA). These results revealed that HAs lowered the degree of E2 self-linking likely due to the proportional rise of E2-HA co-polymerization species. For instance, the RARs of E2 and its dimer, trimer, and tetramer at pH 5.0 changed from 1: 4.683: 1.192: 0.162 to 1: 0.501: 0.058: 0.012 and 1: 0.455: 0.052: 0.010 when P-HA and C-HA were respectively contained in Tvlac-caused humification. Previous reports indicated that HAs could compete with E2 for the catalytic space of laccase T1-Cu through generating new humified polymers and/or E2-HA cross-polymerized species (Li et al., 2013; Permana et al., 2019; Zhong et al., 2019). This process not only accelerated the humification of HAs but also promoted the cross-linking between E2 and HAs. In comparison, the relative abundance of E2 tetramer was extremely low, which might be explained by the rapid reduction of tetramer solubility/extractability in methanol with augmented chain length of oligomers (Beck et al., 2018; Qin et al., 2015). Additionally, the RARs of E2 oligomers at pH 5.0 were significantly greater than those of pH values at 4.0, 6.0, and 7.0 regardless of
HAs presence. These results revealed that pH presented a remarkable influence on the distribution of E2 and/or HAs self/co-polymerization species in water during \(Tvlac\)-evoked humification and oligomerization.

4. Conclusions

In this study, \(Tvlac\) was able to cause completely the single-electron transfer of four E2 molecules with the concomitant reduction of \(O_2\) to \(2H_2O\) regardless of HAs presence, thus accomplishing the decontamination and carbon sequestration of organic pollutants. Notably, HAs exhibited a strong effect on the conversion kinetics, humification degrees, and products distribution of E2 at different pH conditions during \(Tvlac\)-evoked reactions at 25°C and 101.325 kPa. The optimal pH for the catalytic systems was 5.0 in the specified pH values, with slow conversion kinetics of E2 and humification degrees of HAs for pH 7.0. Additionally, \(Tvlac\) accelerated the self-linking of E2 to form a variety of E2 oligomeric species such as dimer, trimer, and tetramer by radical-centered carbon-carbon/oxygen covalent binding. However, the presence of HAs altered the distribution of E2 self-oligomeric products by producing E2-HA co-polymerization species. The degree of oligomerization was highest at pH 5.0 during \(Tvlac\)-triggered humification coupling of E2 and HAs under all the experimental pH levels. These findings are beneficial to understand the pH dependent conversion kinetics and products distribution of E2 with HAs in laccase-driven humification processes.

Declarations

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Availability of data and materials

All data generated or analyzed during this work are included in this published article and its supplementary information files.

Author contributions

K.S. conceived the project and designed the experiments. K.S., R.Z., and H.W. performed the experiments. K.S. and S.L. performed statistical analyses. K.S., R.Z., H.W., M.G.W., S.L., and Y.S. wrote the paper. All authors edited and approved the final manuscript.

Ethical approval

All the authors deliberately approved the ethical rules adopted by the journal.

Consent to participate
All the authors expressed their solemn agreement to participate in the conception and elaboration of this work.

Consent to publish

All the authors deliberately consent to publish this work.

Declaration of interests

The authors declare no competing interests.

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