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Chemical Characteristics of Dark-Brown Humic-like Substances Formed from the Abiotic Condensation of Maillard Precursors with Different Glycine Concentrations

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Abstract: The Maillard reaction is a type of nonenzymic browning process, and it is also an abiotic humification process of sugars, amino acids and phenols catalyzed by δ-MnO_2. It is considered to be one of the possible pathways for the formation of humic-like substances (HLS). The change in the ratio of the Maillard precursors inevitably affects the chemical characteristics of HLS, among which the effect of amino acids concentration on the humification pathway and HLS formation has not yet been reported. In view of this, the glucose, glycine and catechol were chosen as tested objects for the present study, and the method of liquid shake-flask culture was adopted. Both catechol and glucose with fixed concentrations were added into a phosphate buffer solution (pH 8.0) containing δ-MnO_2, and only the glycine concentration was adjusted in the sterile culture system. The supernatant solution and dark-brown residue were collected dynamically within 360 h through the centrifugation method. The E_4/E_6 ratio and total organic C (TOC) of the supernatant solution, the C content of the humic-like acid (C_{HLA}), C_{HLA}/C_{FLA} (C content of fulvic-like acid) ratio and FTIR spectra of the dark-brown residue and the E_4/E_6 ratio and atomic ratio of humic-like acid (HLA) extracted from the dark-brown residue were systematically analyzed to reveal the effect of different glycine concentrations on the abiotic humification pathways and the characteristics of related products from the Maillard reaction under abiotic processes. The results showed that (1) Under the influence of the addition of different glycine concentrations, the structure of organic molecules in the supernatant after culture tended to be simplified, and the addition of three lower concentrations (0, 0.03 and 0.06 mol/L) of glycine made the E_4/E_6 ratios increase by 100.4%, 57.7% and 33.0%, respectively, and obtained a simpler structure of organic molecules in the supernatant than that of 0.12 and 0.24 mol/L glycine, which made the E_4/E_6 ratios increase by 5.6% and 18.0%, merely. After culture, the TOC content in the supernatant solution of each treatment decreased to varying degrees, and the addition of Maillard precursors effectively inhibited the loss of TOC in the supernatant solution, especially the addition of glycine at a concentration of 0.06 mol/L, which only reduced the TOC content by 0.1%. (2) The greater the concentration of glycine added, the higher proportion of aromatic C structure existed in the dark-brown residue. O-containing functional groups from the dark-brown residue and δ-MnO_2 were bound to each other through hydrogen bonding, and (3) During the culture process, the C_{HLA} treated with the addition of Maillard precursors was significantly higher than that of the CK control. Compared with the result at 0 h, the addition of higher concentrations of glycine (0.12 and 0.24 mol/L) were more conducive to the formation of HLA, making the C_{HLA} increase by 666.2% and 422.7%, which were much more than these results for 256.6%, 282.2% and 360.0% from three concentrations of glycine (0, 0.03 and 0.06 mol/L) at the end of culture. After culture, the structure of HLA molecules treated by the addition of Maillard precursors became more complex, and the overall performance showed that the higher the concentration of added glycine, the more complex the HLA molecules became. Under the coexistence of glucose and catechol, the addition of glycine could promote abiotic condensation and improve the condensation degree of HLA molecules, among which the addition of 0.12 mol/L glycine had the most significant effect. Compared with the

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CK control, the addition of Maillard precursors could achieve a higher increase in the C_{HLA}/C_{FLA} ratio, which was more beneficial to the improvement of humus quality.

Keywords: Maillard precursors; glycine; abiotic condensation; humic-like substances; chemical characteristics

1. Introduction

Humic substance (HS) endows the soil with the ability to produce crops, is the material basis for soil fertility, the foundation for ensuring agricultural production and is also a hot topic in agronomy. There are many classical theories about the pathways of HS formation, including biotic and abiotic processes. Usually, with products obtained in abiotic processes, the molecular structure is not condensed to the complexity of natural soil, therefore, it is called humic-like substance (HLS). The phenol-protein and Maillard theories can be used to explain the abiotic pathways of (HLS) formation [1]. The Maillard reaction is a type of nonenzymic browning process that involves the reaction of carbonyl compounds, especially reducing sugars, with compounds that possess free amino groups, such as amino acids, amines and proteins [2]. The browning process involves the polycondensation of sugar and amino acids and is considered an important abiotic pathway for the formation of HLS [3]. During the formation of HLS, biomacromolecules such as proteins, polysaccharides, lignin and polyphenols are hydrolyzed into low-molecular-weight compounds such as amino acids, sugars and quinones, which are called humic precursors [4]. These precursors can be polymerized through oxidative and nucleophilic reactions to form dark polymers, whose structures are similar to that of HLS [5].

Many scholars have reported on the relationship between the Maillard reaction and the formation of HLS. Pal et al. [6] pointed out that in the Maillard reaction, MnO_2 could act as an electron acceptor, convert phenolic compounds and indirectly catalyze the formation of HLS. Wu et al. [1] also pointed out that MnO_2 could be used as a chemical catalyst in the composting process of corn stover, and its addition could rapidly reduce the concentration of reducing sugars and significantly increase the content of HLS within 5 days. Zhang et al. [7] pointed out that the reduction of MnO_2 played a dominant role in the formation of fulvic-like acid (FLA), and the subsequent reduction products, MnO(OH) and Mn(II), acted as catalysts in the formation of humic-like acid (HLA). Jokic et al. [8] showed that δ-MnO_2 could promote the reaction between glucose and glycine to form fructosylglycine (Maillard reaction) and accelerate the formation of HLS. Jokic et al. [9] also studied the abiotic humification process of glucose, glycine and catechol catalyzed by δ-MnO_2 at room temperature and normal pH and pointed out that the above three substances were the precursors of the Maillard reaction, and the catalysis was closely related to the adsorption and condensation of the mineral surface. Additionally, exogenous precursors were significant in promoting the humification process, among which protein-like precursors (amino acids) were the key linkers [10]. Zhang et al. [11] added different concentrations of catechol to investigate the mechanism of abiotic humification by tracking the fate of the precursors in systems containing glucose, glycine and various catechol concentrations, and the results showed that the N-containing organic molecules could significantly contribute to controlling the darkening effect. Increasing the catechol concentration could promote the formation of FLA and HLA and contribute to the increase in the degree of unsaturation in HLA. Hardie et al. [12] demonstrated the important role of sugars in affecting abiotic humification pathways and related products in natural environments. In his report, he also pointed out that increasing the molar ratio of polyphenols to Maillard reagents substantially enhanced humification reactions and promoted the formation of HS with a stronger aromatic character. Hardie et al. [12] reported that increasing the molar ratio of glucose to catechol and glycine in the integrated catechol-Maillard system could enhance the formation of low-molecular-weight, and strongly aliphatic carboxylic Maillard reaction products in the supernatant, which were similar to natural humic acids. The humification process could be promoted by increasing the number of precursors, and the maturity of composting and the generation of HS during composting could be enhanced [13].

Compared with biotic processes, abiotic processes have attracted extensive attention although it is not comparable to the formation of HS in the natural environment [14,15]. Even humic-like polymers can be formed in the absence of microbial life [16]. It was originally thought that amino acids control the formation of HS through the sugar-amine condensation theory in biotic processes (reducing sugars and amino acids produced by microbial metabolism undergo nonenzymatic polymerization to form brown nitrogen-containing polymers). However, under the abiotic condensation reaction (i.e., the Maillard reaction), changes in glycine (the simplest type of amino acid) concentration would promote the humification process and enhance the production of HLS. This topic has always puzzled and fascinated us. In view of this, the method of liquid shake-flask culture was adopted. Both catechol and glucose solutions with fixed concentrations were inoculated into a phosphate buffer containing δ-MnO_2, and only the concentration of glycine was controlled in the sterile culture system. The supernatant solution and dark-brown residue were separated and collected dynamically by centrifugation, and the E_4/E_6 ratio and total organic C (TOC) of the supernatant solution, the C content of humic-like acid (C_{HLA}), C_{HLA}/C_{FLA} (C content of fulvic-like acid) ratio and FTIR spectra of the dark-brown residue and the E_4/E_6 ratio and atomic ratio of humic-like acid (HLA) extracted from the dark-brown residue were analyzed. The aims of this study are (1) o evaluate the effects of different glycine concentrations on the abiotic humification pathway of the Maillard reaction, and (2) to reveal the chemical characteristics of related products under abiotic stress. The results may help to preliminarily understand the effect of glycine on the abiotic pathway of HLS formation and provide a useful strategy for promoting agriculture waste composting.

2. Materials and Methods

2.1. Materials

δ-MnO_2 was synthesized with reference to the method of Parida et al. [17]: 180 g of $MnSO_4 \cdot H_2O$ was dissolved into 1500 mL of H_2SO_4 solution with a concentration of 29 mmol·L^{-1}. Another 120 g of $KMnO_4$ was dissolved into 1500 mL of distilled water, and the solution of $KMnO_4$ was slowly added into the $MnSO_4$ solution while stirring. The formed precipitate was collected by centrifugation, dried at 55 °C, and ground and passed through a 0.01 mm sieve for use.

Analytical grade catechol ($C_6H_6O_2$), glucose ($C_6H_{12}O_6$) and glycine ($C_2H_5NO_2$) were obtained from Sinopharm Chemical Reagent Co., Ltd. in China.

Preparation of 0.2 mol/L (pH 8.0) phosphate buffer: 5.3 mL of 0.2 mol/L NaH_2PO_4 and 94.7 mL of 0.2 mol/L Na_2HPO_4 were mixed thoroughly, and 0.02% thimerosal ($C_9H_9HgNaO_2S$) was added.

2.2. Methods

Sterile conditions were maintained throughout the experiments to ensure that abiotic transformation dominated. The laboratory glassware or something like that were autoclaved prior to use. A certain number of 500 mL conical flasks were prepared. A total of 250 mL of 0.2 mol/L phosphate buffer was introduced into each flask, and δ-MnO_2 was added to each system at 2 g per flask. Catechol and glucose were both added at a concentration of 0.06 mol/L and incubated with increasing glycine concentrations (0, 0.03, 0.06, 0.12 and 0.24 mol/L) under the constant temperature of 28 °C with the initial pH was 8.0. The above treatments of different glycine concentrations are represented by Gly0, Gly0.03, Gly0.06, Gly0.12 and Gly0.24. The CK control was set as follows: 2 g of δ-MnO_2 was added to the phosphate buffer only. All reactions were conducted in duplicate.

The liquid shake-flask culture was initiated under sterile operating conditions, with the rotation speed kept at 150 rpm. During the culture process, a 2 mL aliquot of the supernatant solution was drawn from each system at 0, 3, 6, 18, 28, 48, 76, 124, 172, 240 and 360 h and centrifuged at a high speed of RCF 23,470× g for 5 min. A 1 mL aliquot of the supernatant solution was withdrawn from it and diluted to 25 mL, and the absorbances at 465 and 665 nm (E_4 and E_6) were measured. Then, the E_4/E_6 ratio was further calculated. Its TOC content was determined with the total organic C analyzer vario TOC cube. Another 10 mL aliquot of the supernatant solution was dynamically drawn from the conical flasks after culture for 0, 18, 48, 76, 124, 172, 240 and 360 h and then centrifuged at high speed (RCF 23,470× *g*) for 10 min. The obtained dark-brown residue was acidified to pH 1.0 with concentrated HCl (1.0 mol/L) and equilibrated for 24 h in order to extract the humic-like acid (HLA) that was insoluble in acidic conditions. After the appearance of a flocculation-like precipitate, high-speed centrifugation (RCF 23,470× *g*, 10 min) was performed again. The obtained supernatant was fulvic-like acid (FLA), the pH of which was adjusted to neutral and the solution was diluted to 25 mL. The remaining precipitate was HLA, which was dissolved with a 0.1 mol/L NaOH solution at 60 °C, adjusted to neutral and diluted to 25 mL (HLA liquid sample). The organic C contents of HLA and FLA (C_{HLA} and C_{FLA}) liquid samples were both measured by the TOC analyzer, and the C_{HLA}/C_{FLA} ratio could be further calculated. After culture for 360 h, the remaining reaction medium in the conical flask was centrifuged and adjusted to pH 1.0 to separate HLA and FLA. The solid phase was HLA, which was dissolved in 0.1 mol/L NaOH solution, rinsed with a 150 mL mixture of HCl (0.72 mol/L) and HF (3.42 mol/L) to remove its inorganic impurities, magnetically stirred for 24 h and centrifuged at 16,000 rpm for 10 min. After removing impurities, the HLA was freeze-dried with a freeze dryer after electrodialysis to prepare a solid sample of HLA, which was ground and passed through a 0.01 mm sieve for use.

2.3. Analysis

The absorbances of diluted aliquots of the supernatant solution and HLA-diluted liquid sample at 465 and 665 nm (E_4 and E_6) were measured by a UV-visible spectrophotometer (TU-1900, Beijing Purkinje General Instrument Co., Ltd., Bejing, China), and then the C_{HLA}/C_{FLA} ratio was calculated. The percentages of C, H, O and N in the solid HLA samples were measured by an elemental analyzer (PerkinElmer PE 2400II CHNS/O). The above samples were tested three times and an average of the three results was quoted. Spectroscopic characterization of dark-brown residue was measured by FT-IR spectrophotometry (Model: FTIR-850, Tianjin Gangdong Sci & Tech Development Co., Ltd., Tianjin, China). FTIR spectra were taken in the wavelength region from 400 to 4000 cm^{-1}, analyzed with FTIR 850 software and presented as graphs with Origin 8.0 software.

2.4. Statistical Analysis of the Data

Data analysis and spectrum processing were performed using Excel 2003 and Origin 8.0. The statistical analysis of the data was conducted with SPSS 18.0 (ANOVA) with a least significant difference (LSD) test.

3. Results

3.1. E_4/E_6 Ratio and Total Organic Carbon (TOC) of the Supernatant Solution

The E_4/E_6 ratio was related to the condensation degree of the aromatic C network [18]. As shown in Figure 1, with the culture, the change in the E_4/E_6 ratio of the supernatant solution was different under the addition of glycine at different concentrations. The E_4/E_6 ratio of the Gly0 treatment increased drastically from 1.65 to 1.92 drastically from 0~3 h of culture and then began to stably fluctuate, after which it decreased significantly at 28 h of culture. Nevertheless, it was still higher than the result at 0 h and then gradually increased until the end of the culture. The E_4/E_6 ratio of the Gly0.03 treatment was relatively stable from 0~28 h of culture and enhanced significantly after 48 h until the end. The E_4/E_6 ratio of the Gly0.06 treatment fluctuated between 2.72 and 2.90 from 0~124 h of culture and increased significantly after 240 h of culture until the end. A fluctuating decrease in the E_4/E_6 ratio of the Gly0.12 treatment occurred from 0~18 h, which then experienced a gradual increase. There was no regular change in the E_4/E_6 ratio of the Gly0.24 treatment during this period; it only changed in the range from 3.61 to 3.89 from 0~124 h. After 240 h of culture, the E_4/E_6 ratio increased significantly until the end of the culture. The E_4/E_6 ratio of the CK control had a decreasing trend in the fluctuation. The E_4/E_6 values of all the treatments were higher than that of the CK control throughout the culture. Compared to the results at 0 h, at the end of the culture, the E_4/E_6 ratios of the Gly0, Gly0.03, Gly0.06, Gly0.12 and Gly0.24 treatments increased by 100.4%, 57.7%, 33.0%, 5.6% and 18.0%, respectively, among which the E_4/E_6 ratios of the Gly0, Gly0.03 and Gly0.06 treatments had higher ratios than those of the Gly0.12 and Gly0.24 treatments, whereas the E_4/E_6 ratio of the CK control decreased by 46.7%.

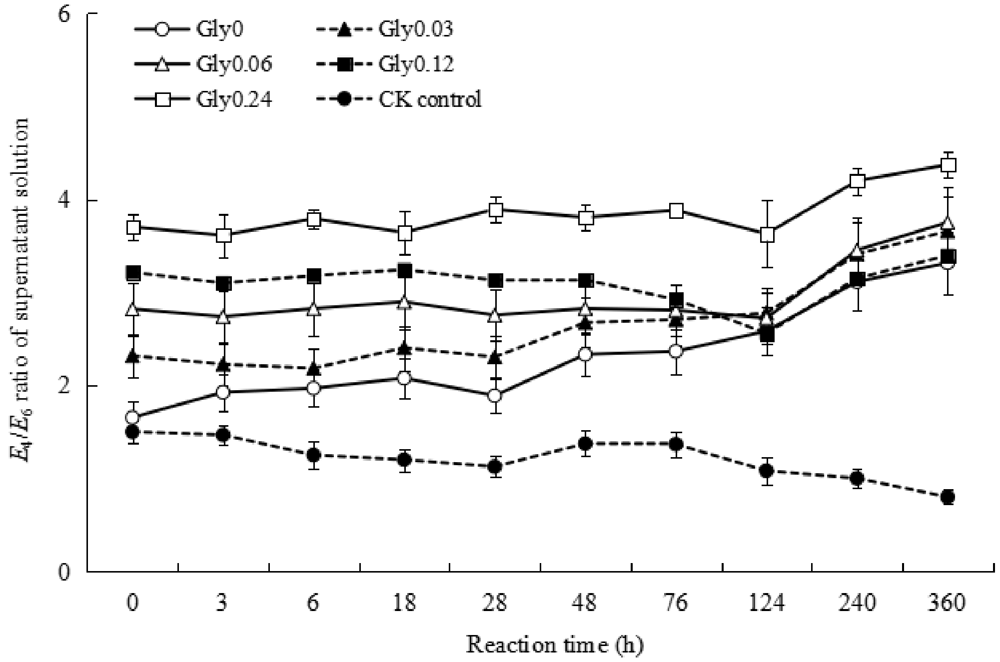

Figure 1. Effects of the glycine with different concentrations in the Maillard precursors on the E_4/E_6 ratio of the supernatant solution. Note: The treatments added different glycine concentrations (0, 0.03, 0.06, 0.12 and 0.24 mol/L) are represented by Gly0, Gly0.03, Gly0.06, Gly0.12 and Gly0.24, respectively. The error bars in the scatter plots indicate the standard deviation for each data point. The same below.

As shown in Figure 2, with the culture, the change in TOC content in the supernatant solution was different under the addition of different glycine concentrations. The TOC content in the supernatant solution from the Gly0 treatment dropped drastically from 8.6 to 4.8 g/L from 0~6 h of culture and then gradually increased until the end. The TOC content in the supernatant solution of the Gly0.03 treatment decreased from 8.5 to 6.8 g/L within 18 h and then increased to 8.3 g/L at the end. The TOC content in the supernatant solution from the Gly0.06 treatment increased from 8.9 to 9.9 g/kg within 3 h, decreased from 9.1 to 7.9 g/L from 6~48 h of culture and then increased to 8.8 g/L at the end. The TOC content in the supernatant solution from the Gly0.12 and Gly0.24 treatments had a similar trend, peaked after 3 h of culture and then decreased in the fluctuation until the end. The TOC content in the supernatant solution from the CK control remained at a low level (0.4~0.9 g/L) in the fluctuation. Compared to 0 h, at the end of the culture (360 h), the TOC contents in the supernatant solution of the Gly0, Gly0.03, Gly0.06, Gly0.12 and Gly0.24 treatments and the CK control decreased by 13.0%, 2.8%, 0.1%, 6.3%, 12.9% and 34.3%, respectively. Compared with the CK control, the addition of Maillard precursors effectively reduced the loss of TOC in the supernatant solution, and Gly0.06 treatment was more helpful for the stabilization of TOC content in the supernatant solution.

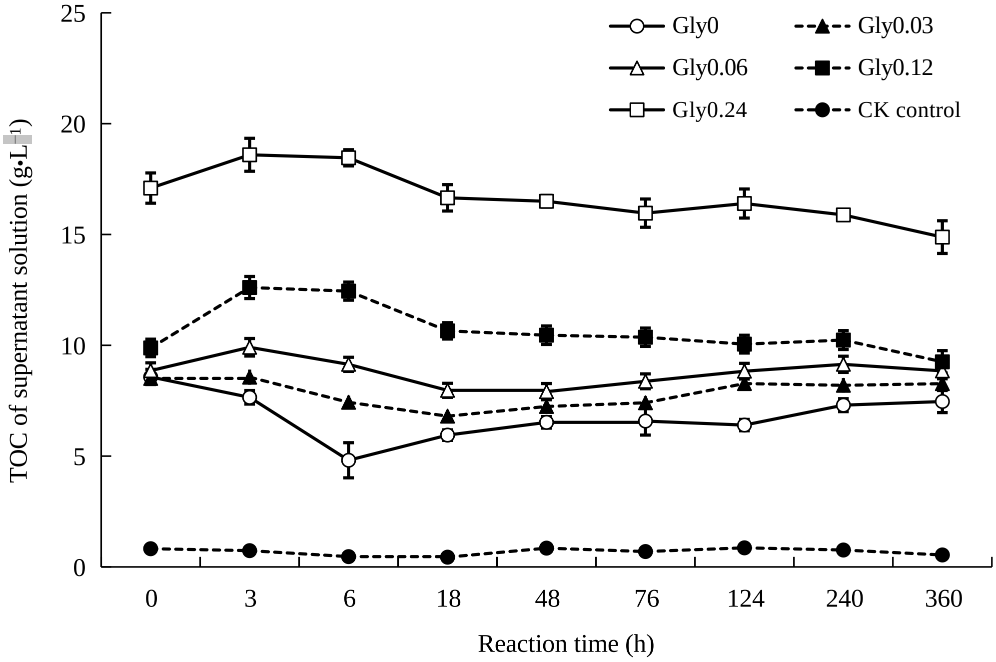

Figure 2. Effects of the glycine with different concentrations in the Maillard precursors on the TOC of the supernatant solution.

3.2. C Content (C_{HLA}), C_{HLA}/C_{FLA} Ratio and FTIR Spectra of the Dark-Brown Residue

As shown in Figure 3, the C_{HLA} treated with the addition of Maillard precursors was significantly higher than the CK control during culture. As the culture progressed, the C_{HLA} of the CK control changed steadily, and the fluctuation range was between 0.05 and 0.08 g/L. All C_{HLA} values from treatments with different concentrations of glycine showed significant upward trends. Compared to 0 h, at the end of culture (360 h), the C_{HLA} values of Gly0, Gly0.03, Gly0.06, Gly0.12 and Gly0.24 treatments and CK control increased by 256.6%, 282.2%, 360.0%, 666.2%, 422.7% and 72.8%, respectively, of which Gly0.12 treatment increased the most, followed by Gly0.24 treatment.

Different change rules in the C_{HLA}/C_{FLA} ratio during the liquid shake-flask culture were observed, as shown in Figure 4, under the addition of glycine at different concentrations. The C_{HLA}/C_{FLA} ratios of the Gly0, Gly0.06 and Gly0.12 treatments increased first and then decreased and peaked at 240 h of culture. The C_{HLA}/C_{FLA} ratios of the Gly0.03 and Gly0.24 treatments and the CK control showed gradually increasing trends. Compared with the results at 0 h, at the end of culture (360 h), the C_{HLA}/C_{FLA} ratios of the Gly0, Gly0.03, Gly0.06, Gly0.12 and Gly0.24 treatments and the CK control had increased by 102.1%, 395.1%, 720.9%, 1072.0%, 701.7% and 84.6%, respectively, among which the C_{HLA}/C_{FLA} ratio of the Gly0.12 treatment had increased the most. The increase in the C_{HLA}/C_{FLA} ratio from the treatments with addition of Maillard precursors was higher than that of the CK control, which was more conducive to improving humus quality.

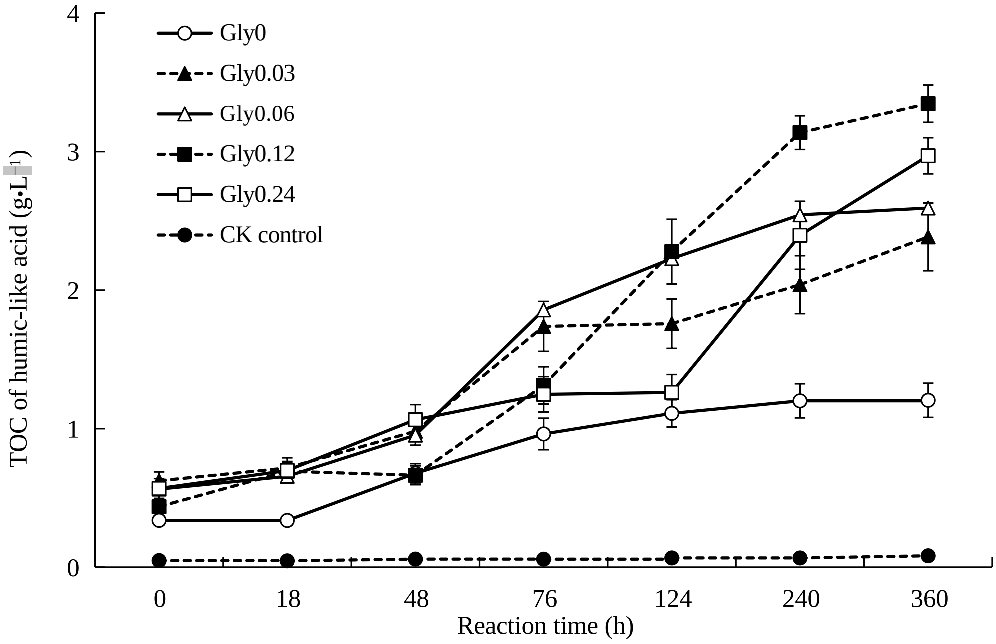

Figure 3. Effects of the glycine with different concentrations in the Maillard precursors on C_{HLA} extracted from the dark-brown residue.

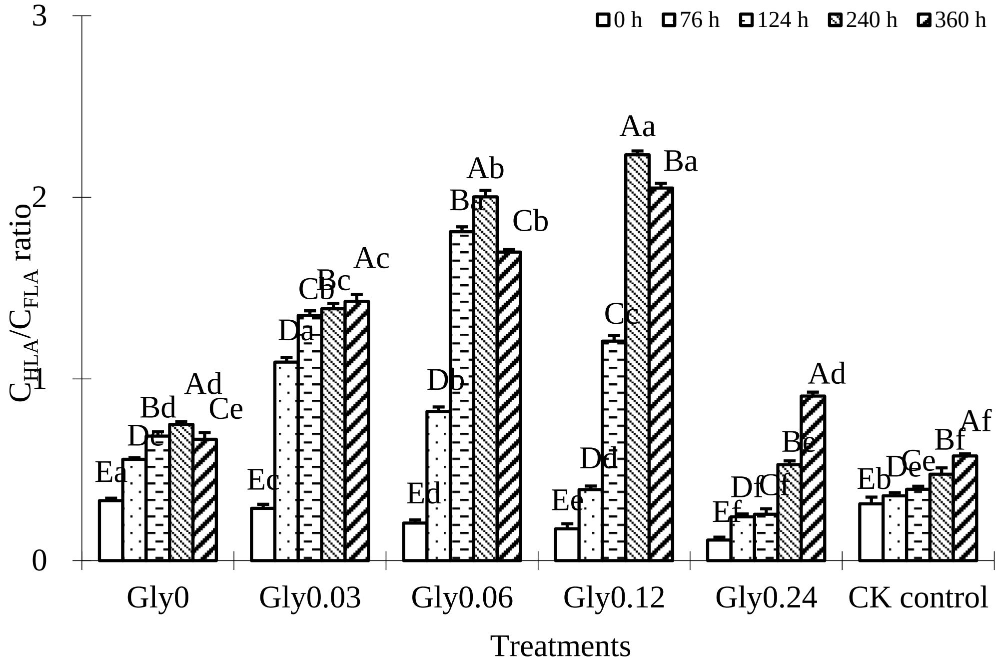

Figure 4. Effects of the glycine with different concentrations in the Maillard precursors on the C_{HLA}/C_{FLA} ratio of the dark-brown residue. Note: Different capital letters indicated significant differences among the different reaction times within the same treatment ($p < 0.05$); Different lowercase letters indicated significant differences among the different treatments under the same reaction time ($p < 0.05$).

The FTIR spectra of the dark-brown residue treated with glycine at different concentrations are shown in Figure 5. The broad peak at 3419~3435 cm^{-1} could not only represent the -OH stretching vibration of δ-MnO_2 or its interlayer water molecule but also the H–bonded O–H stretching vibration of hydroxyl groups from alcohols, phenols and organic acids and H-bonded N–H groups [19]. The bands of 2922 cm^{-1} and 2850 cm^{-1} were ascribed to the aliphatic C–H stretching of CH_3/CH_2 groups [20], which were only found in the CK control. The band at 1606~1633 cm^{-1} was assigned to the bending vibration of surface-adsorbed water of δ-MnO_2 or aromatic C=C skeletal vibrations and the C=O stretching of quinines [21]. The band at 1369~1387 cm^{-1} was attributed to the stretching vibration of COO^- groups [22]; The peak at 1101 cm^{-1} was assigned to the stretching vibration of O–H [23] and was only found in the CK control. The sharp absorption peak located at 619~669 cm^{-1} was attributed to the lattice vibration of the layered Mn–O bond.

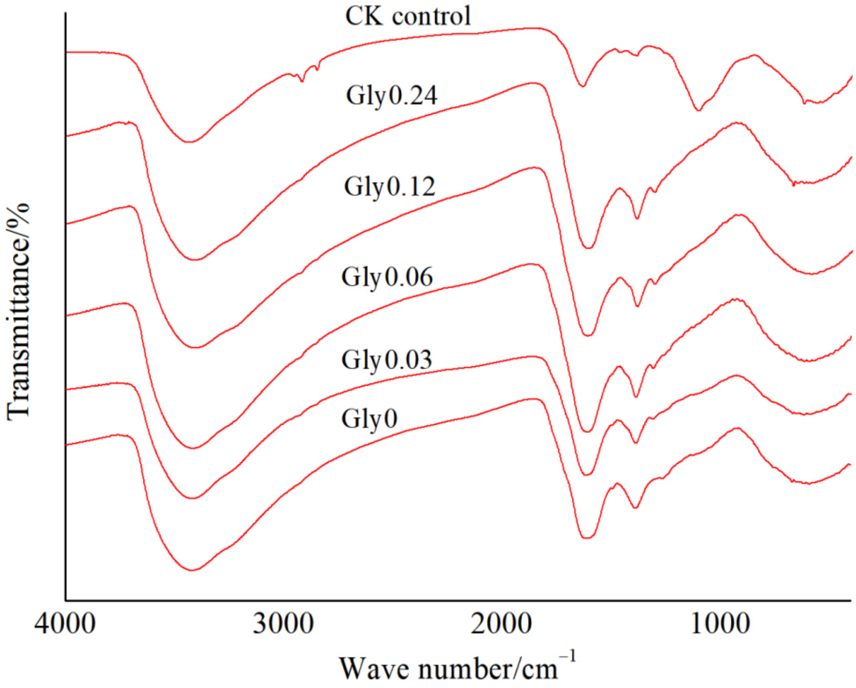

Figure 5. Effects of the glycine with different concentrations in the Maillard precursors on the FTIR spectra of the dark-brown residue.

The common features in the FTIR spectra are summarized in Table 1. Compared with the CK control, the intensity of the broad band at approximately 3419~3435 cm^{-1} from the treatments with the addition of Maillard precursors increased to varying degrees. The stretching vibrations from -OH of δ-MnO_2 or -OH of its interlayer water molecules and the stretching vibrations from alcohol or phenolic hydroxyl -OH could jointly explain the enhancement of the absorption peak here. The two bands at 2922 cm^{-1} and 2850 cm^{-1} attributed to the aliphatic C structure only appeared in the CK control, which was related to the addition of 0.02% thimerosal ($C_9H_9HgNaO_2S$). The relative absorption intensity of 1606~1633 cm^{-1} was enhanced, whereas the intensity of the peak at 1369~1387 cm^{-1} was weakened.

Table 1. FTIR relative intensities (% of total area) for the dark-brown residue under different glycine concentrations in the Maillard precursors.

Wavenumbers (cm^{-1}) Treatments	3419~3435	2922	2850	1606~1633	1369~1387	1101	619~669
Gly0	74.2	0.0	0.0	12.3	4.9	0.0	8.6
Gly0.03	73.7	0.0	0.0	13.5	4.0	0.0	8.9
Gly0.06	74.1	0.0	0.0	14.1	1.6	0.0	9.0
Gly0.12	74.7	0.0	0.0	14.7	1.5	0.0	9.5
Gly0.24	74.0	0.0	0.0	14.4	1.4	0.0	9.8
CK control	62.2	0.8	0.2	10.6	0.5	22.5	0.4

3.3. E_4/E_6 Ratio and Atomic Ratios of the Humic-like Acid

The E_4/E_6 ratio was negatively related to the humification degree (degree of aromaticity) and molecular weight of HLA [18]. As shown in Figure 6, with culture, the E_4/E_6 ratio of the diluted liquid HLA sample in all treatments showed a trend of an initial decrease and then an increase, indicating that the molecular structure of HLA first became complicated and was then simplified. The E_4/E_6 ratio of HLA from Gly0 treatment decreased from 4.31 to 1.59 from 0~48 h and then gradually increased to 3.38 by the end of culture (360 h). The E_4/E_6 ratio of HLA from Gly0.03 treatment decreased from 3.09 to 1.17 from 0~240 h and had reached 1.81 at the end of culture. The E_4/E_6 ratio of HLA from Gly0.06 treatment decreased first from 0~124 h and then gradually increased until the end of culture. The E_4/E_6 ratio of HLA from Gly0.12 treatment decreased from 2.76 to 1.18 from 0~240 h and had increased to 1.38 at the end of culture. The E_4/E_6 ratio of HLA from Gly0.24 treatment decreased from 4.19 to 1.26 from 0~240 h and had increased to 1.32 at the end of culture. The total E_4/E_6 ratio of HLA from the CK control decreased from 2.13 to 0.99 from 0~76 h of culture and then had increased from 0.99 to 3.33 at the end of culture. Compared with 0 h, at the end of the culture (360 h), the E_4/E_6 ratios of HLA from the Gly0, Gly0.03, Gly0.06, Gly0.12 and Gly0.24 treatments decreased by 21.8%, 41.5%, 44.4%, 50.0% and 68.4%, respectively, of which the Gly0.24 treatment had the largest decrease. The overall result was that the greater the concentration of glycine added, the greater the decrease in the E_4/E_6 ratio of HLA, indicating that the complexity of the HLA molecule was higher; however, the E_4/E_6 ratio of HLA from the CK control increased by 56.3%, indicating that the HLA molecular structure tended to be simpler.

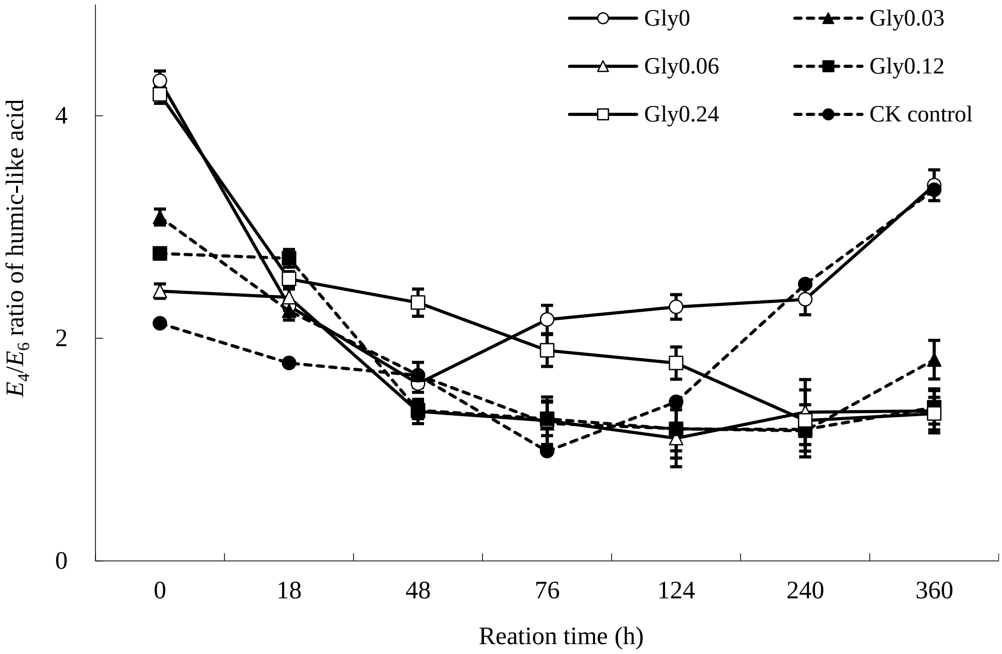

Figure 6. Effects of the glycine with different concentrations in the Maillard precursors on the E_4/E_6 ratio of diluted liquid sample of humic-like acid extracted from the dark-brown residue.

The CK control contained only δ-MnO_2 in the phosphate buffer of 0.2 mol/L because no Maillard precursor had been added, so HLA could not be extracted, and corresponding elemental analysis data could not be obtained. Here, only Gly0 could be treated as the control. Table 2 shows that under the addition of different concentrations of glycine, compared with Gly0 treatment, the H/C ratio of HLA extracted from the dark-brown residue decreased to different degrees, indicating that more aromatic structures formed, among which the H/C ratio of Gly0.12 treatment was the smallest. With increasing glycine concentration, the C/N ratio of the HLA molecule gradually decreased, whereas the O/C ratio gradually increased, which generally indicated a higher degree of humification [18].

Table 2. Atomic ratios of the humic-like acid extracted from the dark-brown residue under different glycine concentrations in the Maillard precursors.

Treatments	H/C Ratio	C/N Ratio	O/C Ratio
Gly0	1.06 ± 0.02 a	14.8 ± 0.5 a	0.61 ± 0.01 e
Gly0.03	1.02 ± 0.01 b	13.2 ± 0.3 b	0.76 ± 0.02 d
Gly0.06	0.99 ± 0.02 c	10.6 ± 0.2 c	0.89 ± 0.01 c
Gly0.12	0.93 ± 0.01 e	8.5 ± 0.1 d	0.92 ± 0.02 b
Gly0.24	0.94 ± 0.01 d	8.2 ± 0.1 e	0.98 ± 0.03 a

Note: Different lower-case letters indicated significant differences among the different treatments with different glycine concentration ($p < 0.05$).

4. Discussion

4.1. E_4/E_6 Ratio and TOC of the Supernatant Solution

Abiotic humification reactions could provide a promising approach to synthesize an HLS. Whether through the polyphenol pathway (polycondensation of catechol and glycine) or the Maillard pathway (polycondensation of glycine and glucose), both pathways emphasized the essential importance of glycine in the formation of HLS [24,25]. Glycine-catechol and glycine-catechol-glucose systems were used most commonly to synthesize HLS [18]. This was enough to see the contribution of glycine in abiotic humification. Glycine, as the simplest type of amino acid and an important precursor, could participate in HLS formation by the Maillard reaction and be considered the key linker to promote the humification process [25]. The increase in the E_4/E_6 ratio may be due to the appearance of simpler hydroxyl, carbonyl and carboxyl groups in the supernatant solution [26]. Owing to the addition of glycine at different concentrations, the complicated molecules in the reaction system might transferred from the supernatant solution to the dark-brown residue, leaving the simpler molecules in the supernatant solution after the culture. Compared with the addition of 0.12 or 0.24 mol/L glycine, the addition of 0, 0.03 or 0.06 mol/L glycine was more likely to promote the simplification of organic molecules in the supernatant solution. In contrast, the CK control was more conducive to the complication of organic molecules in the supernatant solution. After the culture, the TOC content in the supernatant solution from all the treatments decreased to varying degrees, similar to a previous study by Okabe et al. [27]. The TOC content in the reaction mixture of catechol and glycine in the presence of an allophanic soil after the incubation was significantly lower than that before the incubation. Compared with the CK control, the addition of precursors effectively reduced the loss of TOC in the supernatant solution. The participation of δ-MnO_2 could cause the organic matter content to decrease during the co-composting of chicken manure and rice straw [28] and simultaneously accelerate the condensation of low-molecular-weight substances through chemical catalysis. Evidently, the catalytic power of δ-MnO_2 in affecting C turnover and HS formation occurred via abiotic processes. Among all the treatments, the Gly0.06 treatment was more beneficial for maintaining the TOC content in the supernatant solution.

4.2. C_{HLA}, C_{HLA}/C_{FLA} Ratio and FTIR Spectra of the Dark-Brown Residue

Since the polycondensation between phenols and amino acids could yield a dark-brown residue, to clarify its relationship with HLS, we extracted HLA from it and performed C content analysis and structural characterization. During the whole culture process, the C_{HLA} treated by the addition of precursors was significantly higher than that of the CK control. Compared with 0 h, the C_{HLA} from each treatment increased to varying degrees at the end of culture (360 h), in which the increase in C_{HLA} under the Gly0.12 treatment was the largest, reaching 666.2%, followed by 422.7% from the Gly0.24 treatment. The addition of a higher concentration of glycine was more favorable for the increase in C_{HLA}.

Catechol, a typical phenolic compound, can be effectively transformed into HLS [14]. Some phenols were oxidized and cleaved to form aliphatic fragments that then also participate in condensation with other phenols or amino acids, resulting in the formation of HLS. The coupled catechol and glycine products, such as aminophenol and imine derivatives, were produced as a result of nucleophilic addition. Subsequent polymerization of these compounds via oxidative coupling likely results in the production of HLA [29].

The C_{HLA}/C_{FLA} ratios from all the treatments increased to different degrees after culture, among which the C_{HLA}/C_{FLA} ratio in the sample treated with Gly0.12 had the largest increase. The increase in the C_{HLA}/C_{FLA} ratio in the sample treated with Maillard precursors was higher than that of the CK control, which was more conducive to the improvement of humus quality. The addition of amino acids could greatly improve the transformation efficiency during composting and significantly promote the formation of more mature humic components [25]. Zhang et al. [30] reported that amino acids and polyphenols were both reactive precursors in the humification process, so oxidative polymerization between glycine and catechol would preferentially take place. The quinones derived from catechol oxidation and the amino group of glycine underwent nucleophilic addition to form the corresponding Schiff base (imines) [31]. Polyphenols with two hydroxyl groups could induce the Strecker degradation of amino acids. In summary, in the presence of glucose and catechol, glycine greatly influenced and improved the evolution of dark polymers, and the darkening effect was crucial for humification reactions in nature through intermolecular favoring (or cross-linking) polymerization. The number of visible light-absorbing chromophores facilitated the browning process of humus [32].

The addition of glycine was positively correlated with the browning feature of the cysteine-xylose reaction, resulting in the formation of dark-brown residue through a nonenzymatic browning reaction [33]. Under the present experimental conditions, the dark-brown residue obtained from the liquid shake-flask culture was analyzed by FTIR. There was a contradiction between the weakening of the absorption peak representing the carboxyl group and the addition of increasing concentrations of glycine. The reason for this was that δ-MnO_2 could enhance the dealkylation of the alkyl C and especially the decarboxylation of the carboxyl C of glycine [34]. Additionally, the absence of significant amounts of free COOH, probably ionized as carboxylate groups and partially complexed with Mn(II) originating from the reduction of Mn(IV), could explain the lack of strong adsorption bands in the 1740~1710 cm^{-1} region [35]. Thus, the rule was expressed as the higher the concentration of glycine that was added, the stronger the intensity of the peak at 1606~1633 cm^{-1}, and the weaker intensity from the peak at 1369~1387 cm^{-1}, indicated that the greater the concentration of glycine that was added, the higher the proportion of aromatic C structure in the dark-brown residue, and at the same time, the lower proportion of carboxyl groups. The dark-brown residue formed by abiotic condensation involving the participation of Maillard precursors could wrap up the surface of δ-MnO_2 to mask its O–H stretching vibration of the peak at 1101 cm^{-1}, which was only found in the CK control. The intensity of the peak at 619~669 cm^{-1}, which is characteristic of the Mn–O bond lattice vibration of the δ-MnO_2 layered structure, became stronger owing to the addition of Maillard precursors for abiotic condensation and was enhanced with increasing glycine concentration. Combined with the enhanced intensity of peaks at 3419~3435 cm^{-1}, it could be judged that the O-containing functional groups in the dark-brown residue and δ-MnO_2 were bound to each other through hydrogen bonding, thereby increasing the lattice vibration of the Mn–O bond. This result was consistent with the conclusion that hydrogen bonding might be involved in the formation of humic acid because the peak at 3450 cm^{-1} appeared in the reaction system reported by Zou et al. [36]. The greater the concentration of glycine added, the higher proportion of aromatic C structure existed in the dark-brown residue, and the O-containing functional groups in it were hydrogen-bonded with δ-MnO_2. The various constituents ("building blocks" or "structural units") in the macromolecular structure can be held together by covalent and hydrogen bonding [37]. The polycondensates and fragments, including aliphatic components, transformed from the phenolic compounds through oxidative polymerization and were able to form an organic coating on the δ-MnO_2 granules [38].

4.3. E_4/E_6 Ratio and Atomic Ratios of the Humic-like Acid

During the culture, the molecular structure of HLA from each treatment was complicated at first and then gradually became simpler, and finally, the molecular structure of HLA treated with Maillard precursors became more complicated. δ-MnO_2 was believed to first promote the ring cleavage of polyphenols, facilitate the deamination of amino acids, and then accelerate the polycondensation of these compounds to form HLS [36]. The polyphenols acted as the backbone and underwent coupling reactions to initiate the oxidative binding of the phenols or amino acids [9]. The overall performance was as follows: the higher the concentration of glycine added, the higher the complexity of the HLA molecular structure. Wu et al. [39] reported that compared with HLA derived from biotic processes, it was still much more complex in abiotic processes. Under the coexistence of glucose and catechol, the addition of glycine could promote the abiotic condensation reaction and improve the degree of condensation of HLA molecules, among which the addition of 0.12 mol/L glycine had the most significant effect. The oxidation of the amino N of glycine to amide N occurred during the humification reaction. The amide N was the dominant species in the synthetic HLA, and synergistic effects occurred between N-containing groups and groups containing C–C/C–O double bonds [30]. Additionally, δ-MnO_2 could promote the interaction of glycine and catechol and the resulting simultaneous decarboxylation and dealkylation of glycine, the ring cleavage of catechol and polycondensation. The carboxyl C and especially the alkyl C of glycine were incorporated into the humic polycondensates [34]. In contrast, the HLA molecular structure from CK control tended to be simpler.

The elemental analysis of HLA showed that under the coexistence of glucose and catechol, the abiotic condensation reaction with the addition of glycine catalyzed by δ-MnO_2 could improve the degree of condensation of HLA molecules, in which the addition of 0.12 mol/L glycine had the most significant effect in improving the condensation degree of HLA molecules. With increasing glycine concentration, the contents of O-containing functional groups and N-containing compounds in HLA molecules increased, resulting in a decrease in the C/N ratio and an increase in the O/C ratio. N-containing compounds could take part in the synthesis of dark-colored HLSs by the abiotic pathway [40]. Organic N (e.g., glycine) could serve as a source of N in the HLS [41] and play an important role in the darkening process [11,25]. Catechol played a critical role in promoting N involvement in HLA, which significantly contributed to promoting humification and controlling the emission of organic N [42]. That is, the N mineralization of amino acids could be inhibited by polyphenols [34]. The catalytic action of δ-MnO_2 on mixtures of polyphenols and amino acids leading to the formation of N-containing polycondensates has been demonstrated. Therefore, in the presence of catechol, increasing the concentration of glycine was more conducive to increasing the N content in HLA molecules through oxidative polymerization [11].

5. Conclusions

(1) The addition of glycine with an increasing concentration simplified the molecular structure of the supernatant solution after the culture, in which the addition of three lower concentrations of glycine (0, 0.03 and 0.06 mol/L) showed more obvious performance. After the culture, the TOC content in the supernatant solution from each treatment decreased to varying degrees, and the addition of Maillard precursors could effectively inhibit the loss of TOC.

(2) The greater the concentration of glycine added, the higher the proportion of aromatic structures in the dark-brown residue. The O-containing functional groups from the dark-brown residue and δ-MnO_2 were bound to each other through hydrogen bonding.

(3) During the culture, the C_{HLA} treated with the addition of Maillard precursors was significantly higher than that in the CK control. Compared with 0 h, the addition of a higher concentration of glycine was more conducive to the formation of HLA at the end of the culture. After culture, the molecular structure of HLA treated by the addition of Maillard precursors was more complex, and the overall performance showed that the higher the concentration of glycine added, the higher the complexity of the HLA molecule. Under the coexistence of glucose and catechol, the addition of glycine could promote the abiotic condensation reaction and improve the condensation degree of the HLA molecule, in which the addition of 0.12 mol/L glycine had the most significant effect. Compared with the CK control, the addition of Maillard precursors could achieve an increase in the C_{HLA}/C_{FLA} ratio and was more beneficial to the improvement of humus quality.

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