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Reaction mechanism of dicofol removal by cellulase

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ABSTRACT

It remains unclear whether dicofol should be defined as a persistent organic pollutant. Its environmental persistence has gained attention. This study focused on its degradation by cellulase. Cellulase was separated using a gel chromatogram, and its degradation activity towards dicofol involved its endoglucanase activity. By analyzing the kinetic parameters of cellulase reacting with mixed substrates, it was shown that cellulase reacted on dicofol and carboxyl methyl cellulose through two different active centers. Thus, the degradation of dicofol was shown to be an oxidative process by cellulase. Next, by comparing the impacts of tert-butyl alcohol (a typical OH free-radical inhibitor) on the removal efficiencies of dicofol under both cellulase and Fenton reagent systems, it was shown that the removal of dicofol was initiated by OH free radicals produced by cellulase. Finally, 4,4′-dichloro-dibenzophenone and chloride were detected using gas chromatography mass spectrometry and ion chromatography analysis, which supported our hypothesis. The reaction mechanism was analyzed and involved an attack by OH free radicals at the orthocarbon of dicofol, resulting in the degradation product 4,4′-dichloro-dibenzophenone.

Introduction

Dicofol, with the chemical name 2,2,2-trichloro-1,1-bis (4-chlorophenyl) ethanol, is a low-toxicity pesticide used globally in many countries for farming and planting, such as apple and cotton (Zahm and Ward, 1998). Dicofol is synthesized by dichlorodiphenyltrichloroethane (DDT), a pesticide that has been restricted by the Stockholm Convention as a persistent organic pollutant (POP). Dicofol is structurally similar to DDT and is an endocrine disturbance that is difficult to degrade in the environment (Hoekstra et al., 2006). It is now believed to exert a negative influence on both animals (Jadaramkunti and Kaliwal, 2002; Kojima et al., 2004; Wiemeyer et al., 2001) and humans (Reynolds et al., 2005; Settimi et al., 2003). The toxicity and environmental persistence of dicofol have drawn attention, and it has been prohibited in most developed countries (Li et al., 2015).

Due to the lack of evidence regarding its environmental persistence, it remains unclear whether dicofol should be listed as a POP and regulated worldwide. Thus, the environmental persistence and degradation process of dicofol are of great interest to both academia and policy makers.

Soil receives various pesticides for agricultural purposes, and the microbial population may be either adapted to these pesticides or capable of degrading them (Barr and Aust, 1994; Bartha et al., 1967; Fogarty and Tuovinen, 1991). Thus, bioremediation is an effective pollution control technology, and many studies have focused on biodegradation of pesticides (Alexander, 1999; Johnston and Camper, 1991; Salama et al., 1999). Many biological approaches such as enzymatic

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remediation have been used to clean up DDT (Kantachote et al., 2004; Purnomo et al., 2008; Zhao et al., 2010). However, limited data are available on dicofol removal (Osman et al., 2008). Thus, we investigated the removal of dicofol from water using cellulose, and found that cellulase can catalyze the degradation of dicofol effectively without other substrates (Zhang et al., 2011).

This work is an extension of our former studies on dicofol removal by cellulase (Zhang et al., 2011), and depicted its degradation process and identified the reaction mechanism. We separated cellulase into various components and tested the features and reaction activities towards dicofol. Bisubstrates of dicofol and carboxyl methyl cellulase (CMC) were applied and reaction mechanisms were tested to identify the active center of dicofol. By contrast experiments of Fenton reagent and product detection using gas chromatography mass spectrometry (GC–MS) and ion chromatography, the reaction process was depicted.

1. Experimental methods

1.1. Materials and equipment

Cellulase (extracted from *Trichoderma longbrachiatum*) was purchased from Ningxia Heshibi Bio-tec. Co. Ltd., China. Dicofol (original drug) was purchased from Yangzhou Pesticide Factory, China. Potassium sodium tartrate, sodium sulfite, citric acid, N-hexane, sodium hydroxide, hydrochloric acid (all analytically pure), and 30% peroxide (excellent purity) were purchased from the Beijing Yili Fine Chemical Co. Ltd., China. Compound 3,5 dinitrosalicylic acid (DNS, chemically pure), ferrous sulfate (analytically pure), and methylene blue (indicator) were produced by Sinopharm Chemical Co. Ltd., China. Carboxyl methyl cellulase (CMC, chemically pure) and crystalline phenol (analytically pure) were produced by Beijing Xudong Chemical Co. Ltd., China. Sodium citrate (analytically pure) was produced by Beijing Chemical Regent Company, China. Pyridine (analytically pure) was purchased from Shantou Xilong Chemical Co. Ltd., China. Sephadex G-75 (with a grain size of 40–120 μm) was purchased from Beijing Ruida Henghui Co., Ltd., China. Tert-butyl alcohol (analytically pure) was purchased from Beijing Yili Fine Chemical Co., Ltd., China.

Instrumentation included a UV757CRT UV–vis spectrophotometer (Shanghai Lengguang Tech. Corp., China), a WMNK-404 temperature controller (Shanghai Huachen Medical Instrument Corp., China); a PHS-3A Digital pH meter (Hangzhou Huada Instruments Co., Ltd., China), and a chromatography column of 16 × 750 mm (Beijing Ruida Henghui Science & technology development Co., Ltd., China).

1.2. Extracting and determining dicofol in water

Dicofol in water was extracted and concentrated using *n*-hexane and determined using the colorimetric determination method; our former study provided the optimal working conditions (Yang et al., 2009). In this experiment, we conducted recovery tests before applying to the dicofol: the dicofol concentrations used were 35.6, 16.9 and 0 μg/L, with recovery rates in the range of 91.6% to 113.4%, 85.2% to 108.3%, and 87.9% to 108.9% and RSDs of 8.1%, 8.8% and 8.6%, respectively.

1.3. Separating cellulase and determining their features and activities

Cellulase is a complex enzyme containing three components. We separated cellulase components using gel chromatography and tested the active component that catalyzed the degradation of dicofol. The process was as follows: 10 g of Sephadex G-75 was added to 200 mL of deionized water, after which it was heated in a boiling water bath for 2 hr. Flocculent deposit was then added to the tube and kept in solution until reaching 40 cm. After stabilization for 15–20 min, buffer solution was added to neutralize the tube. Subsequently, the valve was closed after the solution was 1–2 mm higher than the gel. Next, 5 mL of enzyme and washing solution was added and washed at a rate of 12 mL/hr; 2 mL of elution was collected per tube. Finally, the absorbance of eluents in each tube was tested using an ultraviolet spectrophotometer with a 280-nm wavelength.

We tested the enzymatic activities of components in these three peak positions and reacted them with dicofol, respectively. We then compared their degradation efficiency and determined the active components during degradation.

1.4. Determining the active center of cellulase

1.4.1. Testing reaction kinetics of cellulase with CMC

Various concentrations of carboxyl methyl cellulase (CMC) were used to react with cellulase (at a concentration of 64.98 U/L) for 10 min at room temperature. The amount of glucose generated in the reaction system represented the hydrolytic reaction rate of cellulase. We then determined the Michaelis constant (K_m, mg/L) and maximum reaction rate (V_m, mg/(L·min)) based on the Lineweaver–Burk method.

1.4.2. Testing reaction kinetics of cellulase with dicofol (DCF)

Various concentrations of dicofol (DCF) were reacted with cellulase (at a concentration of 64.98 U/L) at room temperature in a 50-mL reaction system. Reaction solution was removed at various intervals, the concentrations of DCF were determined, and the DCF removal efficiency was calculated. We then applied the Lineweaver–Burk method to calculate K_m and V_m.

1.4.3. Testing reaction kinetics of cellulase with bisubstrates of DCF and CMC

The initial reaction rates of cellulase degrading bisubstrates of CMC and DCF were measured, and the concentrations of the various substrates were set as the equation below, where K can be expressed as (Keleti et al., 1987):

$$K_{m,CMC} \times C_{DCF} + K_{m,DCF} \times C_{CMC} = K \quad (1)$$

where, K is an arbitrary value close to K_m when concentrations of DCF and CMC are in an applicable range, $K_{m,CMC}$ (mg/L) is the Michaelis constant of enzyme hydrolyzing CMC, C_{DCF} (mg/L) is the concentration of DCF, $K_{m,DCF}$ (mg/L) is the Michaelis constant of enzyme degrading DCF, and C_{CMC} (mg/L) is the concentration of CMC. The concentrations of DCF and CMC are shown in Table 1.

Table 1 – Paired concentrations of dicofol (DCF) and carboxyl methyl cellulase (CMC) as mixed substrates.

Item	1	2	3	4	5	6
CMC (mg/L)	16.2	13.3	10.4	7.5	4.6	1.7
DCF (mg/L)	6	9	12	15	18	21

When one enzyme was acting with two different substrates, we applied the Keleti Theory and assumed that cellulase had one active center to catalyze two substrates, S_1 and S_2. During the reaction process, S_1 and S_2 competed for the same active center and the reaction mechanism could be expressed as:

$$E + S_1 \rightarrow ES_1 \rightarrow E + P_1,\ E + S_2 \rightarrow ES_2 \rightarrow E + P_2 \quad (2)$$

where, E is the enzyme, S_1 and S_2 are substrates 1 (CMC) and 2 (DCF), and P_1 and P_2 are products 1 and 2, respectively.

The initial rate V_0 (mg/(L·min)) follows Eq. (3) below (Keleti et al., 1987):

$$V_0 = \frac{V_1 K_2 C_{S_1} + V_2 K_1 C_{S_2}}{K_1 K_2 + K_1 C_{S_2} + K_2 C_{S_1}} \quad (3)$$

where, V_1 (mg/(L·min)) and V_2 (mg/(L·min)) are the maximum reaction rates of substrates S_1, and S_2; C_{S1} (mg/L) and C_{S2} (mg/L) are the concentrations of S_1, and S_2; K_1 and K_2 are the Michaelis constants of S_1 and S_2, respectively.

Eq. (1) is a prerequisite, which determines the linear relationship of V_0 to C_{S1} or C_{S2}. By applying the concentrations from Table 1 and theoretical $K_{m,CMC}$ and $K_{m,DCF}$ values into Eq. (3), the results under the assumption of one active center were obtained.

Assuming that cellulase has two active centers to catalyze substrates S_1 and S_2, the reaction mechanism of cellulase was described by Eq. (4):

$$\begin{aligned} &E + S_1 \rightarrow ES_1 \rightarrow E + P_1 \\ &E + S_2 \rightarrow ES_2 \rightarrow E + P_2 \\ &ES_1 + S_2 \rightarrow ES_1S_2 \rightarrow ES_1 + P_2 \\ &ES_2 + S_1 \rightarrow ES_1S_2 \rightarrow ES_2 + P_1. \end{aligned} \quad (4)$$

If two active centers acted separately, the starting rate could be calculated using Eq. (5):

$$V_0 = \frac{V_1 K_2 C_{S_1} + V_2 K_1 C_{S_2} + (V_1 + V_2) C_{S_1} C_{S_2}}{K_1 K_2 + K_1 C_{S_2} + K_2 C_{S_1} + C_{S_1} C_{S_2}}. \quad (5)$$

Applying the concentrations from Table 1 and K_{mCMC} and K_{mDCF} values into Eq. (5), we obtained results under the assumption of two different active centers.

We next prepared the mixed substrates of DCF and CMC based on the concentration ratio calculated according to factors discussed in Sections 1.4.1. We tested the initial reaction rate, obtained the kinetic curve of the bisubtrate reaction, and compared it with theoretical curves before identifying the active center of cellulase catalyzing DCF degradation.

1.5. Determining the reaction mechanism

1.5.1. Reactions in cellulase and Fenton systems

Fenton, a typical OH free radical reagent, was selected to react with dicofol and compared to cellulase. First, 100 mL of deionized water was mixed with hydrochloric acid to adjust the pH as 3. Then, $FeSO_4 \cdot 7H_2O$ and H_2O_2 (with an initial concentration of 10 mmol/L for $FeSO_4$ and 10 mmol/L for H_2O_2, respectively) were added to obtain Fenton reagent.

Previous studies have shown that in the absence of sensitizer, OH free radicals can react with Methylene Blue and produce stable and strong chemiluminescence signals (Cai et al., 2011), and the Methylene Blue method can be used to calculate OH free radical generated by the Fenton agent (Yang and Wang, 2005). In our study, we introduced this method to calculate the generation rates of OH free radical. The method was as follows: Methylene Blue solution was added to 100 mL of de-ionized water. The pH was adjusted to 8 and temperature was set as 25°C, these conditions were stable throughout the reaction process (Satoh et al., 2007). The absorbance value A_0 of de-ionized water at 508 nm was tested as a control. Subsequently, cellulase and Fenton reagent were added, respectively. Different absorbance values of A_i at various times were then tested, ΔA was calculated as A_0 minus A_i, and the apparent generation rate of OH (%) in the reaction system was calculated using Eq. (6):

$$\text{Apparent generation rate of OH} = (\Delta A / A_0) \times 100\%. \quad (6)$$

A typical OH inhibitor, tert-butyl alcohol at a concentration of 1 mmol/L, was then added into the cellulase and Fenton systems, respectively. Degradation rates of dicofol in cellulase and Fenton systems (along with an inhibitor) were tested. By comparing and analyzing these results, we determined the reaction mechanism of dicofol removal by cellulase.

1.5.2. Identifying reaction products

We extracted cellulase and Fenton solutions after reacting with *n*-hexane and detected the products in extracts using GC–MS (QP2010) after concentrating. The conditions were selected as: chromatographic column DB-5ms with a column length of 30 m × 0.2 mm, temperature of 50–250°C (increase by 20°C/min), split ratio of 1:10, injection port temperature of 250°C, ion source temperature of 200°C, electron energy of 70 eV, and scan scope of 20–650 m/z.

(2) We detected solutions in the cellulase system before and after degradation of dicofol using ion chromatography DIONEX ICS-900 (deionized water as blank). The conditions were selected as: chromatographic column AS14, electrical conductivity detector, Na_2CO_3 at 3.5 mmol/L and $NaHCO_3$ at 1.0 mmol/L for elution, and a sample size of 25 μL.

2. Results and discussion

2.1. Degradation activity of cellulase on dicofol

The separated cellulase was collected and the absorbance of each tube was tested at a wavelength of 280 nm. The results are shown in Fig. 1. Three protein peaks were observed after separation. The eluents of these three peaks were tested out of endo-1,4-β-D-glucanase (EG or CMC enzyme) activities (with CMC substrate), exo-1,4-β-D-glucanase (also called l,4-β-D-glucan cellobiohydrolase or CBH) activities (with cotton substrate), and β-D-glucosidase (BG) activities (with salicin

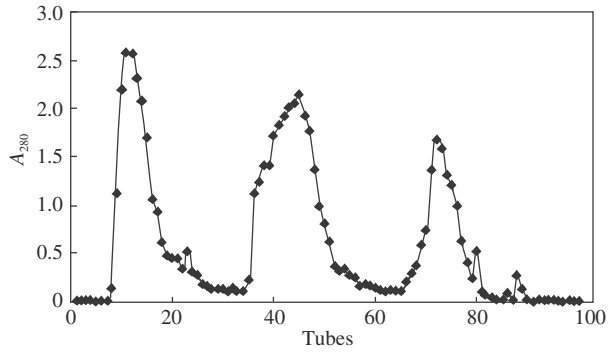

Fig. 1 – Gel filtration chromatography of cellulase on Sephadex G-75.

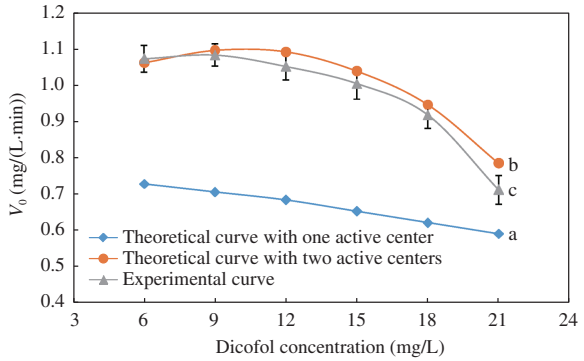

Fig. 3 – Theoretical and experimental curves of starting reaction velocity (V_0) to dicofol concentration (C).

substrate), respectively. Furthermore, these separated eluents were applied to degrade dicofol. Our results showed that the eluent in the second peak position could remove dicofol based on the noticeable endo-1,4-β-D-glucanase activity. Thus, we assumed that dicofol removal by cellulase required endoglucanase activity.

2.2. Active center of cellulase catalyzing degradation

The active component reacting with CMC and DCF was separated from cellulase, and cellulase was characterized with two functions (Mitsutomi et al., 1998). Cellulase reacted on the CMC based on hydrolytic enzyme activity, and the reaction mechanism involved the CMC enzyme hydrolyzing the β-1,4-glucoside bond in CMC, producing glucose. While dicofol is a small molecule, its reaction mechanism remains unclear. Whether cellulase reacted with bisubstrates CMC and DCF through the same active center or two active centers also remains unclear. We explored cellulase reacting with CMC, DCF, and mixed substrates, respectively, to identify the active center of cellulase on DCF.

2.2.1. Reaction kinetics of cellulase on CMC or DCF substrate

Fig. 2 shows the reaction kinetics of cellulase on CMC and DCF, respectively. By changing the concentration of substrates in the system and observing the reaction rate of cellulase, the value of the Michaelis constant K_m and the maximum reaction rate V_{max} were obtained based on the Lineweaver–Burk method. The results showed that for CMC removal, the K_m was 7.92 mg/L and V_{max} was 1.08 mg/(L·min). For the DCF reaction, the K_m was 8.18 mg/L and V_{max} was 0.79 mg/(L·min).

2.2.2. Reaction kinetics of cellulase on mixed substrates

By applying the concentration ratio in Table 1 with Eqs. (3) and (5), we determined the theoretical results as curve a (for one active center) and curve b (for two active centers), as shown in Fig. 3.

By reacting with mixed substrates, the initial rate of cellulase reacting with bisubstrates was detected and the results were plotted as curve c (for experimental results) in Fig. 3. The experimental result (curve c) showed high consistency with theoretical curve b, which assumed two separate active centers, after comparing with two theoretical curves. These results indicated that cellulase was acting on DCF and CMC *via* two different active centers, and the mechanism of cellulase catalyzing dicofol degradation was different from the mechanism of its degradation of CMC.

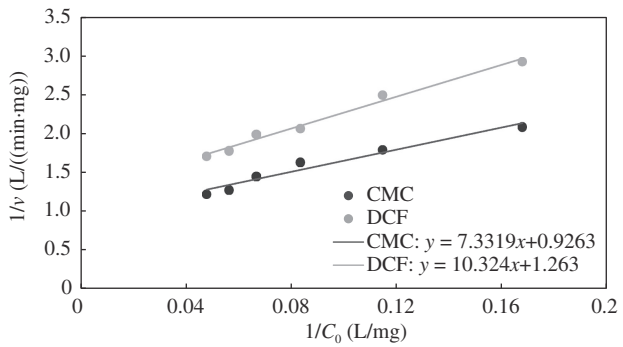

Fig. 2 – Lineweaver–Burk curves of CMC and dicofol removal by cellulase.

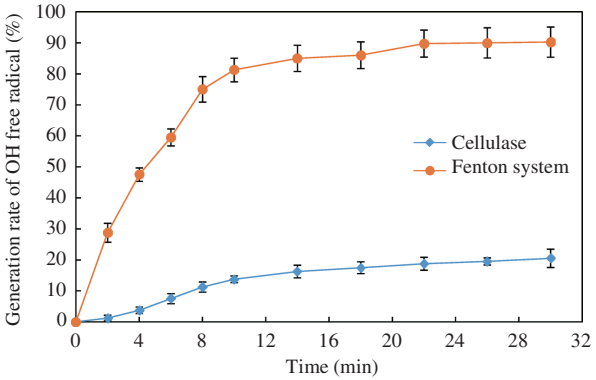

Fig. 4 – OH free-radical production efficiency for cellulase and Fenton systems.

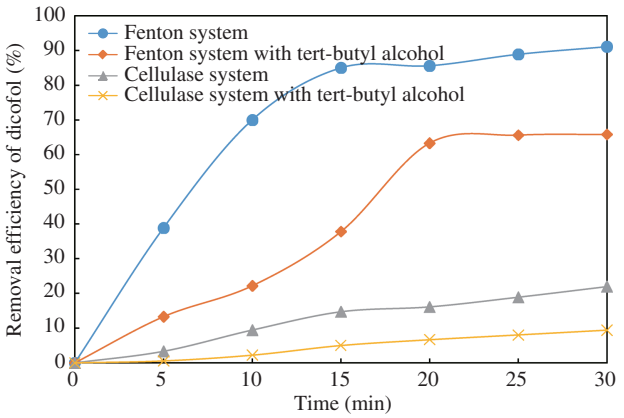

Fig. 5 – Effect of tert-butyl alcohol on dicofol removal in two systems.

2.3. Reaction mechanism of cellulase catalyzing the degradation of dicofol

The cellulase degradation process involved both oxidative degradation and hydrolytic degradation. According to our results, cellulase reacted with DCF and CMC based on two different active components, and hydrolytic activity did not function when degrading DCF. Thus, it can be assumed that dicofol was degraded by oxidative activity. In addition, it was speculated that the process was initiated by an attack from OH free radical. Wang *et al.* (Wang et al., 2002a,b) explored 11 strains of cellulolytic brown-rot fungi, which showed endoglucanase activity without exoglucanase activity. They found that OH free radicals were produced during cellulose degradation by these fungi. However, there is a lack of information on the OH oxidative mechanism, and we explored the role of OH free radical during the degradation process and increased our understanding of the oxidative mechanism.

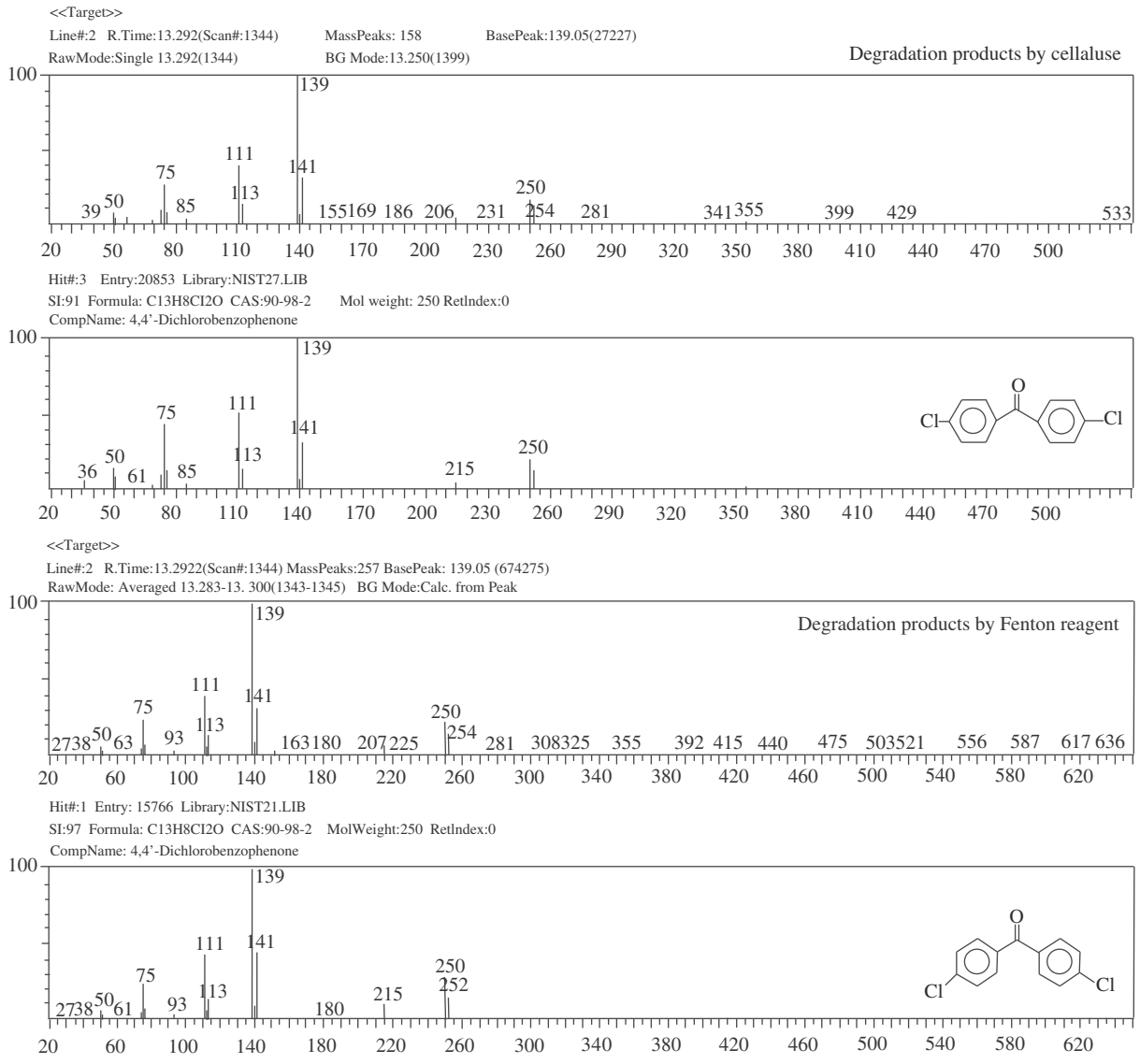

Fig. 6 – Mass spectrogram of degradation products by cellulase and Fenton reagent.

2.3.1. Production efficiencies of OH free radical by Fenton and cellulase

We tested OH free-radical production efficiency at various intervals in both cellulase and Fenton systems, respectively. Fig. 4 shows that these two systems could produce OH free radical, while the Fenton system had a faster rate and higher productivity.

2.3.2. Role of OH free radical in the degradation of dicofol

Fig. 5 shows that tert-butyl alcohol decreased the dicofol removal efficiency in these two systems. Tert-butyl alcohol was a typical OH inhibitor that rapidly reacted with OH to interrupt the free-radical reaction chain. OH free radical in the system decreased after adding inhibitors, which caused a decrease in dicofol removal efficiency. Thus, we speculated that it was the OH free radical that oxidized dicofol during its removal in both Fenton and cellulase systems, and the reaction mechanisms of these two systems were similar.

2.3.3. Reaction products

The mass spectrum of GC–MS is shown in Fig. 6, and the degradation product was determined to be 4,4′-dichloro-dibenzophenone. Results from ion chromatography are shown in Fig. 7. The ion chromatograms suggested that large amounts of Cl^- were produced by the degradation process. Detection results were consistent with our hypothesis that the degradation process was initiated by OH free radical. The mechanism was analyzed as shown in Fig. 8: the orthocarbon in dicofol was attacked by OH free radical and $-CCl_3$ was removed and further oxidized into Cl^- and CO_2, after which they were transformed into the stable product 4,4′-dichloro-dibenzophenone. Thus, dicofol was degraded and its toxicity was reduced.

3. Conclusions

Our work separated cellulase based on a gel chromatogram, tested characteristics of the eluents in three peak positions and their effects on dicofol, and showed that the degradation of dicofol by cellulase involved its endoglucanase activity. Furthermore, we analyzed the reaction kinetics of cellulase with DCF, CMC, and their mixture using gel column chromatography, compared experimental results with theoretical values, and found that cellulase reacted with CMC and DCF through two different active centers; thus, degradation may be an oxidative process.

We then measured the production efficiency of OH free radical during dicofol degradation in cellulase and Fenton systems, and evaluated the impacts of an OH inhibitor, tert-butyl alcohol, on dicofol removal efficiency in the two systems. Our results indicated that cellulase removal was initiated by OH free radical. Reaction products were detected using GC–MS and ion chromatography, and were identified as 4,4′-dichloro-dibenzophenone and Cl^-. The mechanism was shown to involve the production of OH free radical by an oxidative active center in cellulase, after which the free

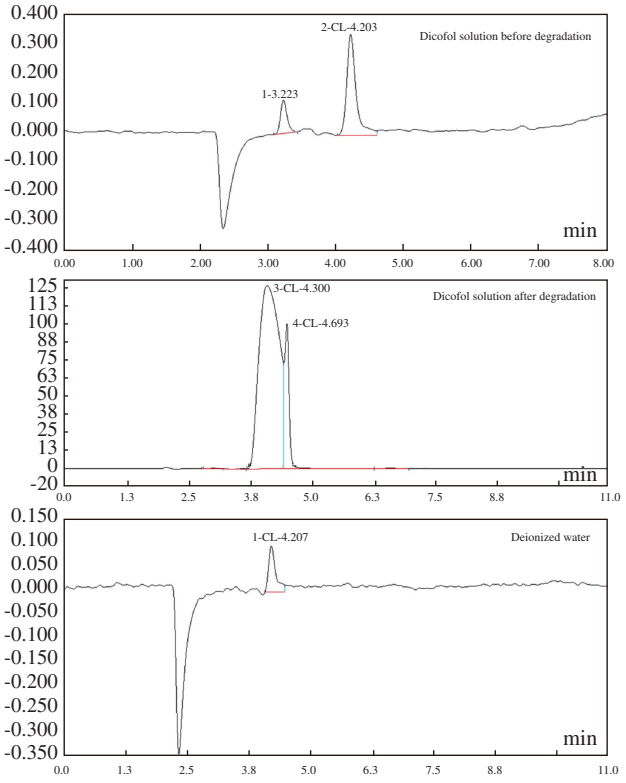

Fig. 7 – Ion chromatogram of dicofol before and after degradation and deionized water.

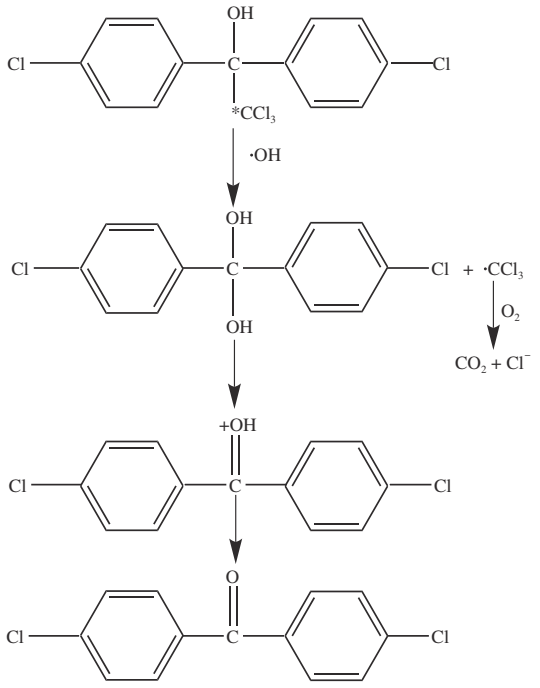

Fig. 8 – Pathway of dicofol degradation by ·OH produced from cellulase.

radical attacks the orthocarbon hydroxyl group in dicofol, finally producing 4,4′-dichloro-dibenzophenone.

Acknowledgments

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