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Decolorization of malachite green, decolorization kinetics and stoichiometry of ozone-malachite green and removal of antibacterial activity with ozonation processes

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ABSTRACT

This study aimed to identify degradation intermediates and to investigate the stoichiometry of decolorization and degradation, decolorization kinetics, and removal of antibacterial activity of *malachite green* (*MG*) using ozonization processes. The decolorization of *MG* was optimal at an acidic pH value of 3 based on molecular ozone attack on *MG* molecules. The *stoichiometric ratio of decolorization* between ozone and *MG* was calculated to be 7.0 with a regression coefficient of 0.995, whereas the ratio for degradation was calculated as 13.1 with a regression coefficient of 0.998. With *MG* concentrations in the range of 0.30–1.82 mM, the concentration of decolorized *MG* increased with higher initial concentrations of *MG*, whereas the ozonolytic decolorization rates of *MG*, decreased with increasing initial concentration. The *pseudo*-first-order degradation rate constants (k') decreased with the initial concentration and ranged from 0.769 to 0.223 min^{-1}. Twelve different intermediates were produced during the ozonation of *MG* with ozonation times between 5 min and 30 min and were identified by GC–MS. Although 86% of *MG* in the reaction mixture was removed by ozonation after 10 min, the decrease of antibacterial activity was very low (10%) for *Bacillus subtilis* and *Staphylococcus epidermidis* because the degradation intermediates, phenol and benzoic acid, also have antibacterial activity. The antibacterial activity of both *MG* and its intermediates were removed successfully with ozonation times above 26 min.

1. Introduction

The world produced 29.4 million tons of dye during the last year. A growth rate of 5 percent is estimated by 2011, with production expected up to 35 billion tons [1]. This increasing production rate will be reflected directly or indirectly in environmental pollution. Thus, effective methods are needed to deal with the waste containing toxic dyes. Many researchers have used various processes, such as photocatalysis, electro-Fenton, wet-air [2,3], Fenton [4,5] and ozonation applications [6–10] to deal with this waste. Especially, ozonation has been one of the most effective oxidation processes. Some researchers [6] have reported that ozone increased the rate of decolorization for two azo dyes when compared with H_2O_2/UV process in their study. Alaton and Alaton [7] exposed that detoxification and biodegradability of effluents from textile preparation were improved by ozonation.

Dyes are readily water soluble due to the SO_3^- and $=N^+R_2$ groups present. Even at low concentrations, reactive dyes which have azo, anthraquinone and phthalocyanin as defined chromophoric systems, give color to water. Solar irradiation can be absorbed by this chromophoric system. In deep water solar irradiation may not reach the bottom or the intensity will be very low. For photosynthetic activity solar irradiation intensity is vitally important. Some studies have shown that azo dyes are very slow to biodegrade under aerobic conditions [11].

Ozonation is one method that gives the best results in the degradation of these dyes and eliminates any problem to a great extent [12]. Oxidation with ozone is known to be a powerful method for decolorizing reactive dyes by destroying the chromophoric system. The reaction mechanisms of ozonalytic decomposition follow two possible degradation pathways. Both molecular ozone attack (i.e. direct reaction) and the free radical mechanism (i.e. indirect reaction) have been found to simultaneously exist during the reaction processes [13]. The oxidation potential of ozone is 2.07 V and its high oxidation potential allows it to degrade most organic compounds [14]. At basic pH, ozone rapidly decomposed to yield

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hydroxyl radicals (2.8 V) was reported by Lopez-Lopez et al. [10] during ozonation of sulphonated some azo dyes.

Ozonolysis, the reaction of ozone with a carbon–carbon double bond (C=C), is well known. The C=C bonds are very attractive centers for addition reactions by ozone to yield unstable intermediates. Therefore, stoichiometric relationship may be considered between ozone and the double bonds. Kusvuran et al. [15] recently reported that relation between ozone and double bonds, C=C, C=N and N=N, in the decolorization study of some dyes. Decolorization stoichiometry must be different from degradation stoichiometry since the double bonds may be still present in dye molecule after decolorization. The necessary ozone amount may be predicted according to dye chemical structure to produce non-hazardous waste, relatively, as compared with other intermediates during ozonation.

The ozone and dye reaction is shown by Eq. (1):

$$\text{Dye} + aO_3 \rightarrow \text{OP} \quad (1)$$

where OP are the oxidized products.

The ozone concentration in water is limited by its solubility and is inversely proportional to ionic strength intermediates or final products that may be formed during the ozonation of dye molecules. Because the dissolved ozone in water reacts with Dye, the ozone concentration is constant and is related to the partial pressure and Henry's constant. The concentration of dissolved ozone can be written as:

$$P = k_H[O_3] \quad (2)$$

where P is the partial pressure of ozone and k_H is Henry's constant of ozone. If Eq. (2) is written as a second-order kinetic equation, Eq. (3) is obtained:

$$-\frac{d[\text{Dye}]}{dt} = k[\text{Dye}]\left[\frac{P}{k_H}\right] \quad (3)$$

where P and k_H are constants in the experimental conditions. The final kinetic equation can be arranged to be:

$$-\frac{d[\text{Dye}]}{dt} = k_{app}[\text{Dye}] \quad (4)$$

$$k_{app} = k\frac{P}{k_H}$$

where k_{app} is the apparent *pseudo*-first-order rate constant, which can be calculated from the slope of the plot of $-\ln([\text{Dye}]/[\text{Dye}]_0)$ versus time [13,15–17].

Malachite green (*MG*) is a synthetic dye used to color silk, wool and leather. In medicine, dilute solutions of *MG* are used as local antiseptics and are also effective against fungal and bacterial infections [18]. However, *MG* is extremely toxic to fish as it is a respiratory poison and accumulates in the tissues [19].

This study aimed to (1) investigate the degradation of *MG* using the ozonation method; (2) determine the degradation kinetics and stoichiometry of ozone-*MG* and (3) study the antibacterial effect of *MG* on *Bacillus subtilis* (NRRL B-354) and *Staphylococcus epidermidis* (NRRL B-4268) and its relationship to ozonation processes.

The stoichiometric ratios of ozone-*MG* for decolorization and degradation were determined; in addition, we characterized the kinetics of decolorization of *MG* degradation in water by ozonolytic processes. The removal of the antibacterial effect of *MG* was studied, and the degradation products of *MG* during ozonation process were identified.

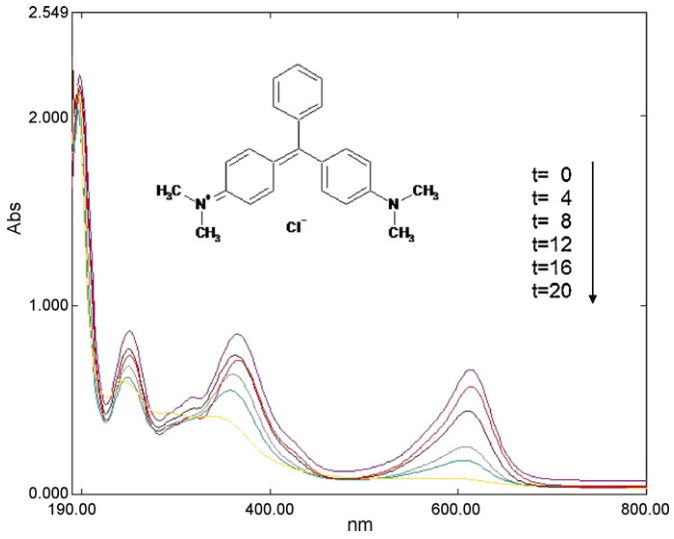


Fig. 1. Chemical structure of *MG* and UV spectra of *MG* at various ozonolysis times ($[MG]_0$ = 1.21 mM, pH = 3, T = 19 °C).

2. Experimental

2.1. Materials

MG dye, Color Index No.: 5 g mol^{-1} of C.I. Basic Green 4 (42000) with a molecular weight of 364 was purchased from Riedel-de Haen AG (96%, Germany) (Fig. 1) and used without further purification. Ozone gas was produced from pure oxygen (99.99%) by an Ozo-1VTT model ozone generator (Ozomax, Canada). The gas flow rate was monitored by a flow meter on the oxygen gas tube. Other chemicals were purchased from Merck (Germany). The experimental set-up for the decolorization reaction is shown in Fig. 2. A cylindrical glass reactor with a 1.000 L volume was used.

2.2. Ozonation experiments

2.2.1. Effect of pH on decolorization of MG

The dye solutions used were buffered to obtain a stable pH. Necessary pH adjustments were made by titrating 0.10 M orthophosphoric acid with a 1.00 M sodium hydroxide solution while the pH was monitored by a pH meter. *MG* solution (1.82 mM) was prepared in different phosphate buffers, pH 3, 7 and 10.

The dye solution (0.50 L) was placed into a 1.00-L reaction tube (diameter and height of 6 and 35 cm, respectively) with a water-cooled jacket (Pyrex) and the ozone gas was diffused into the reaction mixture through a glass sparger at the bottom of the reactor. The ozone flow rates and concentration of ozone gas used during all experiment were 720 mL min^{-1} and 0.3505 mmol min^{-1}. One-milliliter samples were taken every 2 min for 30 min.

2.2.2. Ozonolytic decolorization kinetics of MG

Kinetic experiments were carried out at six concentrations ranging from 0.30 to 1.82 mM in the phosphate buffer at pH 3. A constant temperature of 19 °C was maintained *via* cooling water because the room temperature was 19 °C. For a constant O_2 gas inlet velocity, 1 mL samples were taken every minute for 30 min. The dye solution absorbance was analyzed using a Shimadzu UV-1800 PC scanning spectrophotometer at 618 nm.

2.2.3. Stoichiometry of MG-ozone

2.2.3.1. Ozonolytic decolorization of MG. In order to determine the decolorization stoichiometry, additionally to the procedure described in Section 2.2.2, ozone traps containing 0.50 L of a potas-

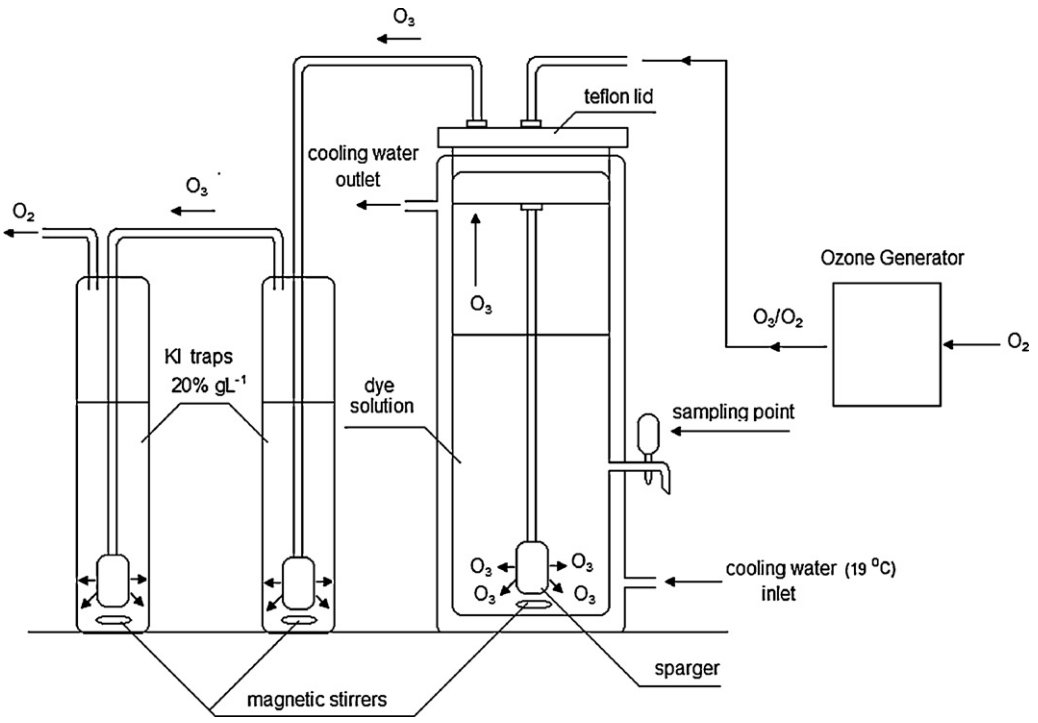


Fig. 2. Experimental set up for decolorization reaction.

sium iodide solution (KI) (20.00 g L^{-1}) were used with the outlet of the reaction mixture tube to measure the excess ozone concentration produced. The content of the traps was titrated with standardized sodium thiosulphate ($Na_2S_2O_3$) (0.01 M), and the ozone concentrations were determined *via* titrant volume [20]. To determine the total ozone concentration diffused in the reaction mixture at a constant O_2 gas inlet velocity, 0.50 L of a KI solution instead of the dye was added to the reaction tube. The KI in the reaction tube and trap were titrated again with $Na_2S_2O_3$ at the end of the experiment. The amount of consumed ozone (in mmol) by the dyes was calculated for each initial dye concentration (Eq. (3)) [15]:

$$O_3^{Consumed} = O_3^{Total} - O_3^{Trap} \quad (5)$$

where $O_3^{Consumed}$ is the amount of ozone (mmol) that reacted with dye molecules, O_3^{Total} is the total amount of ozone (mmol) generated by the ozone generator in an experiment and O_3^{Trap} is the excess ozone captured by the KI traps. The trap contents titrated by 0.010 mol L^{-1} $Na_2S_2O_3$ were normalized to a reaction volume of 0.50 L.

2.2.3.2. Ozonolytic degradation of MG. In degradation reaction, the stoichiometric ratio between ozone and *MG* was determined in the phosphate buffer solution (pH 3) saturated with ozone. First, 0.50 L of the buffer solution was measured in a 1.00-L reaction tube, and the ozone gas was diffused in the tube for 10 min. Next, 0.20 L of the saturated buffer solution was poured in a 1.00-L Erlenmeyer, and 1 mL of the *MG* solution (0.304 mmol L^{-1}) was added and mixed. Although the color of *MG* was disappeared in about 3–5 s, the mixture was left to stir for 5 min to allow the degradation reactions to finish. Then, 0.20 L of the KI solution was added to the reaction mixture and titrated with the 0.0100 M $Na_2S_2O_3$ solution to determine the amount of ozone remaining. The same procedure was applied to other *MG* solution volumes (2, 3, 4 and 5 mL). The ozone concentration in the ozone-saturated buffer solution during the degradation reaction time (5 min) was checked by a blank experiment. For this purpose, 0.20 L of a saturated buffer solution placed in a 1.00-L Erlenmeyer and stirred under air for 5 min before 0.20 L of the KI solution was added to the solution and then titrated with an $Na_2S_2O_3$ solution. The results from the blank experiments revealed that the concentration of ozone remained stable during the degradation reaction time.

To determine the ozone solubility in the buffer (pH 3, with an ionic strength of 0.12 M), 0.50 L of the buffer solution without dye was placed in the reaction tube and ozone gas was diffused for 10 min. Next, 0.20 L of the saturated ozone solution in buffer was placed in a 1.00 L Erlenmeyer, and 0.20 L of KI (20.00 g L^{-1}) was added. The mixture was shaken vigorously in the dark and then titrated with $Na_2S_2O_3$. Under these conditions, the solubility of ozone was determined to be 0.136 mmol L^{-1} (6.54 mg L^{-1}). According to the Debye–Hückel theory [21], the activity coefficient is 0.75 for an ionic strength of 0.12 mM. The solubility of ozone in water was theoretically determined to be 0.135 mmol L^{-1} (6.46 mg L^{-1}) *via* the activity coefficient multiplied by the ozone solubility in water (0.178 mmol L^{-1}, 8.57 mg L^{-1}) at 20 °C, 0.12 M ionic strength, and pH 4.75 [22].

2.2.4. Mineralization of MG

Mineralization of the *MG* solution, 1.82 mM, during ozonation treatment was followed by measuring the total organic carbon contents (TOC) using a Tekmar Dohrmann Apollo 9000 instrument. In a typical application, an aliquot of 10 mL was withdrawn from the reaction medium at certain intervals. The samples were ignited at 700 °C on a platinum-based catalyst, and the carbon dioxide formed was swept by pure oxygen as the carrier gas through a nondispersive infrared (NDIR) detector.

2.3. Gas chromatograph–mass spectrometry analysis (GC–MS) for degradation products of MG

The intermediates produced during ozonolysis of *MG* were determined by GC–MS (Schimadzu, 2010) with a TRB-WAX (100% polyethylene glycol) column (0.25 μm, 30 m × 0.30 mm) and elec-

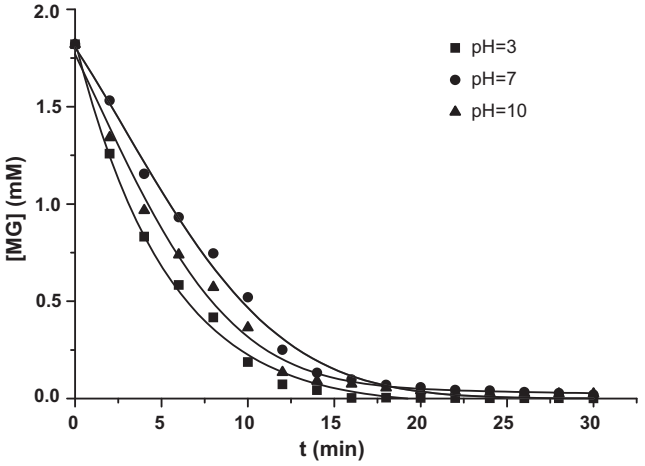


Fig. 3. Ozonolytic degradation of *MG* at various pH values ($[MG]_0$ = 1.21 mM, T = 19 °C).

tron impact (EI) detector (70 eV). The run temperature was 50 °C (1 min)–9 °C min^{-1}–260 °C (15 min), and the temperatures of the inlet, interface and ion sources were 260, 260 and 230 °C, respectively. A 0.500-L sample of *MG* with a 1.82 mM initial concentration at pH 3 was placed into the reactor before ozone gas was diffused into solution. The ozone flow rates and concentration of ozone gas used during all experiment were 720 mL min^{-1} and 0.3505 mmol min^{-1}. The ozonation reaction was aborted after 5 min, and 250 mL of the reaction mixture was measured in a separation funnel. To increase the extraction efficiency, 2 mL of a saturated NaCl solution was added to the separation funnel. The extraction was carried out using diethyl ether (75 mL × 3 times) and was concentrated to 5 mL after being dried by Na_2SO_4. Next, 50 μL of the silylation reagent (Supelco, n-trimethylsilylimidazole) was pipetted on this extract and then heated at 80 °C for 20 min in a 10-mL glass vial with a screw cap. Finally, the concentrated solution (1 μL) was injected to GC–MS. The same procedure was applied for samples with ozonation times of 30 and 100 min.

2.4. Bacterial strains and antimicrobial susceptibility testing

The disc diffusion technique was used for antimicrobial susceptibility testing [23]. Antibiotic paper discs (Oxoid) were used for testing. *B. subtilis* (NRRL B-354) and *S. epidermidis* (NRRL B-4268) were used as standard organisms. Bacterial strains were cultured on LB-agar plates, and the plates were incubated for 24 h at 37 °C. After incubation, the bacterial concentration was adjusted to a McFarland standard (0.5) and swabbed onto Müller–Hinton agar plates. The antibiotic paper discs were then put onto the agar plates. The pH of the sample was adjusted to 7 with an NaOH solution because acidic pH values prevent bacterial activity. In addition, 10 μL of the dye and/or oxidation products of the dye were inoculated onto the paper disk, and all plates were incubated for 24 h at 37 °C. The antimicrobial activity was evaluated by measuring the zone of inhibition. Tetracycline and cefuroxime antibiotics were used as positive controls.

3. Results and discussion

3.1. The effect of pH on ozonolytic degradation of MG

The experiments were carried out with 1.82 mM dye at pH values of 3, 7, and 10 for 30 min (Fig. 3). Decomposition of *MG* was obtained under the 5% level at each pH for the first 15 min of the ozonation time. A maximum decomposition (99.1%) of *MG* was observed at pH 3 and the efficiency of the reaction followed the order of pH 3 > pH 10 > pH 7. Ozone reacts with organic compounds in two different ways depending on the pH. Direct attack occurs at acidic pH whereas free radical takes place at basic pH [24]. Although there were no major differences in the degradation of *MG* at each pH, slightly more degradation occurred at acidic pH than at neutral and basic pH. In basic solution, the decomposition of ozone is initiated by means of one or more of the three reactions [25,26]:

$$O_3 + OH^- \rightarrow O_3^{\bullet -} + OH^{\bullet} \quad (6)$$

$$O_3 + OH^- \rightarrow HO_2^{\bullet} + O_2 \quad (7)$$

$$O_3 + OH^- \rightarrow O_2^{\bullet -} + HO_2^{\bullet} \quad (8)$$

Reactions (6) and (7) are thermodynamically more favorable than reaction (8). In reaction (8), when the alkali pH becomes 10, $HO_2^{\bullet}$ will be in rapid equilibrium with $O_2^{\bullet -}$:

$$HO_2^{\bullet} \rightleftarrows H^+ + O_2^{\bullet -} \quad pK = 4.8 \quad (9)$$

In a series of continuing reactions, peroxide anion (HO_2) and superoxide anion ($O_2^{\bullet -}$) react further with ozone to form ozonide ion ($O_3^{\bullet -}$):

$$HO_2^{\bullet} + O_3 \rightarrow O_3^{\bullet -} + HO_2^{\bullet} \quad (10)$$

$$HO_2^{\bullet} + OH^- \rightarrow O_2^{\bullet -} + H_2O \quad (11)$$

$$O_2^{\bullet -} + O_3 \rightarrow O_3^{\bullet -} + O_2 \qquad K_1 = 1.6 \times 10^9\ M^{-1}\ s^{-1} \quad (12)$$

Furthermore, the ozonide ion ($O_3^{\bullet -}$) ultimately decomposes to form $OH^{\bullet}$ radicals by means of the reactions:

$$O_3^{\bullet -} + H^+ \rightarrow HO_3^{\bullet} \qquad K_2 = 5.0 \times 10^{10}\ M^{-1}\ s^{-1} \quad (13)$$

$$HO_3^{\bullet} \rightarrow OH^{\bullet} + O_2 \qquad K_3 = 1.4 \times 10^5\ s^{-1} \quad (14)$$

$$O_3^{\bullet -} \rightarrow O^{\bullet -} + O_2 \quad (15)$$

$$O^{\bullet -} + H_2O \rightarrow OH^{\bullet} + OH^- \quad (16)$$

The $OH^{\bullet}$ radical can further react with more ozone to form more superoxide anion (O_2^-) directly. On the other hand, the mechanism of ozone decomposition was proposed by Staehelin and Hoigne [27] as shown below:

$$O_3 + OH^- \rightarrow HOO^{\bullet} + O_2 \quad (17)$$

Recently, Lopez-Lopez et al. [10] reported that self-decomposition of ozone to form hydroxyl radical was proportional relationship with increase of pH. They also observed that the decay efficiency of water soluble four azo dyes due to the SO_3^- groups present increased with increases in pH. Similar results were reported by Kusvuran et al. [15] for sulphonated azo dyes, the charge of main structure of these dyes is negative in water solutions because of anionic dye. However, they observed that decay efficiency was higher at acidic pH 3 than basic pH 10 for cationic dye such as Basic Yellow 28. Finally, the decay reaction between molecular ozone and *MG* was slightly more favorable than that of radical formation by decomposition of ozone in alkali pH.

3.2. Ozonolytic degradation of MG

3.2.1. Stoichiometric ratio between ozone and MG

In this section, two types of experiments were designed for measuring the stoichiometry of ozone-*MG*. A decolorization reaction was carried out with a mixture of excessive dye and limited ozone, which is soluble in water. A degradation reaction was performed with excess ozone and limited dye.

For the decolorization reactions, the stoichiometric ratio of ozone-*MG* was determined for each initial *MG* concentration and is reported in Table 1 and Fig. 4. Although the times of decolorization were linear in proportion to the initial *MG* concentrations, all the experiments were conducted until the decolorization time with an

Table 1
Ozone consumption by *MG* with various initial *MG* concentrations and ozonation time for decolorization reactions.

Initial *MG* (mmol)[a]	Ozonation time (min)	0.0100 M $Na_2S_2O_3$ (mL)	Excess O_3 in trap (mmol)	O_3 consumed by *MG* (mmol)
0.911	20	95.71	0.67	6.33
0.759		245.58	1.72	5.28
0.607		330.00	2.31	4.69
0.455		575.71	4.03	2.97
0.304		652.86	4.57	2.43
0.152		850.00	5.95	1.05

[a] Decolorization reaction was carried out at pH 3 phosphate buffer and in 500 mL reaction volume.

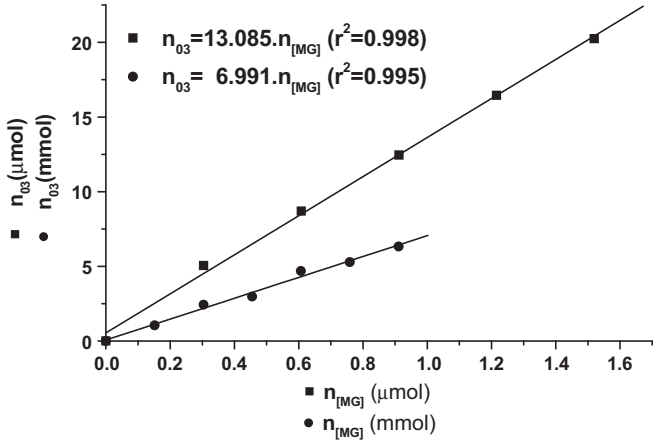


Fig. 4. Stoichiometric relationship between ozone and *MG* (■: μmol *MG* concentration versus μmol ozone in a saturated ozone solution, ♦: mmol *MG* concentration versus mmol ozone in the reaction mixture, pH 3, T = 19 °C).

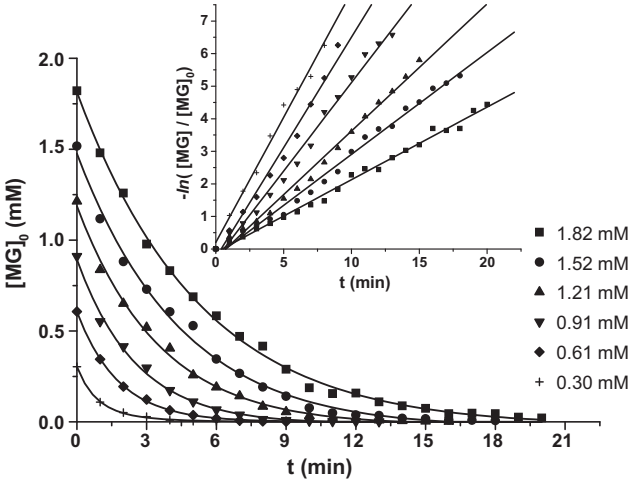


Fig. 5. Decolorization of *MG* in solutions with various initial concentrations for ozonization applications (pH 3, T = 19 °C) and their linear transforms $-\ln([MG]/[MG]_0)$ versus time (zoomed out).

MG concentration of 1.82 mM for 20 min to calculating stoichiometry of ozone-*MG*. During this time, 7.01 mmol ozone was produced by the ozone generator with a constant O_2 gas velocity. As shown in Table 1, over the range of 1.05–6.33 mmol, the amount of the ozone consumed by *MG* is linear in proportion to the increasing initial *MG* concentrations varying from 0.911 to 0.152 mmol. The regression coefficient and slope were calculated as 0.995 and approximately 7.0, respectively (Fig. 4). The slope corresponds to a stoichiometric ratio for the decolorization reaction between ozone-*MG*.

The stoichiometric ratio of the degradation reactions of *MG* with ozone was similarly calculated to be approximately 13.1. The ozone concentration of the solution saturated by ozone was determined as 136 μmol *via* iodometric titration. Different concentrations of *MG* (0.304, 0.608, 0.912, 1.216, and 1.520 μmol) were separately reacted with a solution saturated with ozone. The ozone consumed by *MG* was calculated by subtracting the concentration of the remaining ozone after reaction with *MG* from the total ozone concentration in solution. For the different concentrations of *MG* (0.304, 0.608, 0.912, 1.216, and 1.520 μmol), the obtained results were 5.05, 8.70, 12.45, 16.45, and 20.25 μmol, respectively (Table 2).

Comparison of both reaction mixtures revealed that the stoichiometric ratio for the degradation reaction is approximately twice that of the decolorization reaction. The ozone diffuses into the reactor and dissolves into the reaction mixture with different rates, depending upon the solubility equilibrium, before reacting with the substrate. The solubility of ozone is inversely proportional to the ionic strength of the solutions [22]. The dye molecules that react with ozone are degraded to yield small organic molecules, such as oxalic acid or acetic acid [7,28,29]. These products cause a salting-out effect [30] with an increase in ionic strength of the solutions, thereby decreasing the solubility of ozone. Therefore, a significant proportion of ozone is removed from the reaction mixture as it becomes insoluble. In other words, the rate of soluble ozone reacting with the substrate is less than the rate of the gas-phase ozone leaving the solution. Because competitive reactions occur between intermediates of *MG* and unreacted *MG* molecules, further reactions may not be carried out to produce organic acids with one, two and three carbons. As a result, the stoichiometric ratio between *MG* and ozone in the decolorization reaction was observed to be lower than in the degradation reaction because the change in ionic strength in Erlenmayer was negligible.

3.2.2. Ozonolytic decolorization kinetics of MG

Fig. 5 shows the decolorization of *MG* at different initial dye concentrations with varying ozonation times. The more diluted

Table 2
Ozone consumption by *MG* with various initial amount of *MG* for degradation reactions.

O_3 concentration in Erlenmeyer (μmol)[a]	Amounts of *MG* (μmol)	0.0100 M $Na_2S_2O_3$ (mL)	Excess O_3 in Erlenmeyer (μmol)	O_3 consumed by *MG* (μmol)
27.20	0.000	5.44	27.20	0.00
	0.304	4.43	22.15	5.05
	0.608	3.70	18.50	8.70
	0.912	2.95	14.75	12.45
	1.216	2.15	10.75	16.45
	1.520	1.39	6.95	20.25

[a] μmol in 200 mL volume.

Table 3
Pseudo-first-order rate constants and initial *MG* concentrations during ozonation experiments.

Initial *MG* (Mm)	First order, k' (min^{-1})
1.821	0.223
1.518	0.313
1.214	0.390
0.911	0.543
0.607	0.677
0.304	0.769

the initial solution, the faster that the degradation occurred. For instance, at an initial concentration of 0.30 mM, all of the dye molecules in the dye solution were degraded after a reaction time of 6 min. With an initial concentration of 1.82 mM, the concentration of dye molecules was 0.58 mM.

The kinetics of *MG* were analyzed using the kinetic equation $-d[MG]/dt = k'[MG]$. *Pseudo*-first-order rate constants were calculated from the slope of the plot of $-\mathrm{Ln}([MG]/[MG]_0)$ versus time for each compound.

An inverse relationship was observed between the initial dye concentration and the pseudo-first-order kinetic rate constants (Fig. 5 and Table 3). For initial *MG* concentrations of 1.82–0.30 mM, *pseudo*-first order rate constants were in the range of 0.223–0.769 min^{-1}, respectively. In addition, it was noted that k_{app} (or overall reaction coefficient) was also inversely proportional to the ozone consumed. When k_{app} was plotted versus ozone consumed, linearity whose regression coefficient was calculated as 0.992 was observed and reported in Fig. 6. The decrease rate of k_{app} dependent on ozone consumed was obtained as 0.054 min^{-1} mM^{-1}. Naturally, the amount of ozone consumed by *MG* increased with the increase of initial *MG* concentrations. The amount of ozone that reacted with *MG* is limited by dissolving ozone in reaction mixture. If the reaction between dissolved ozone and *MG* carried out in an instant of time zone is assumed a cycle, the number of cycle will increase with increase in *MG* concentration. In a cycle, dissolving ozone in reaction mixture and reaching equilibrium will need time [8], even very short. Moreover, number of cycles will also have effect on over all reaction time or vice versa until decolorization of *MG* is completed. In conclusion, as the higher initial *MG* concentration was increased, the smaller k_{app} was observed. Furthermore, the intermediates of MG occurred such as acetophenone, phenol, benzoic acid (Tables 4 and 5) may compete with MG to react with ozone. As a result, while the amount of consumed ozone and reaction time increased, k_{app} decreased.

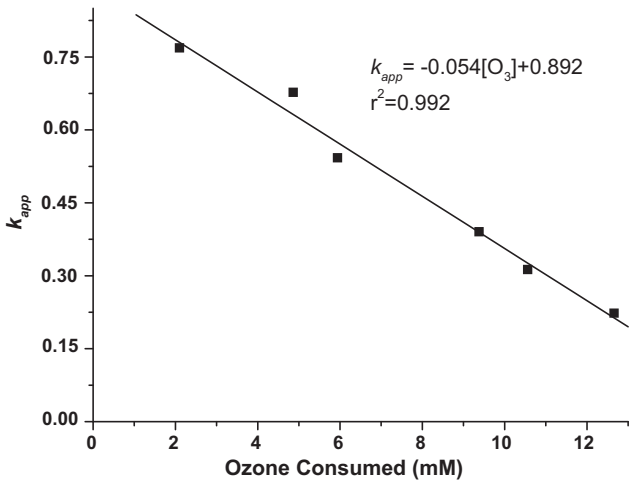


Fig. 6. Overall rate constants of dye ozonation at various consumed ozone.

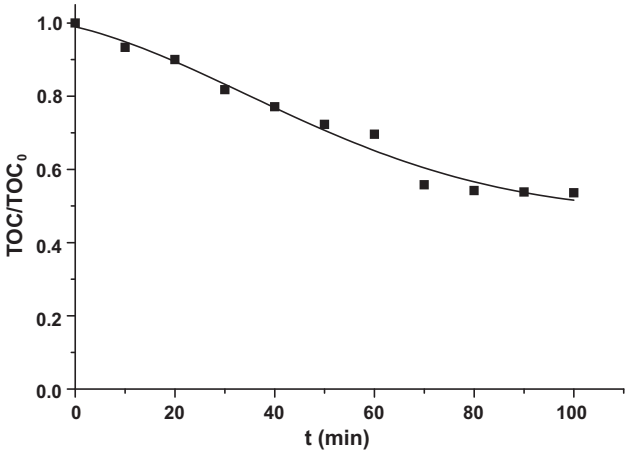


Fig. 7. Decreasing TOC of *MG* aqueous solutions during ozonation application ($[MG]_0$ = 1.82 mM, pH 3, T = 19 °C).

Fig. 7 shows that the TOC decreased by 47.4% during the ozonation process of 100 min and with an initial *MG* concentration of 1.82 mM. Although the decolorization of *MG* was completed in 20 min, the removal of the TOC was determined to be 10% TOC. These findings imply that mineralization using ozone is not successful for all types of dye molecules and their intermediates, although colorless solutions can be obtained. Decolorization reactions carried out on dye solutions can produce many intermediates, such as aldehydes and phenols, which are reactive with ozone by degradation mechanisms [7,28,29]. Moreover, intermediate products of dyes can be toxic to aquatic organisms [31–33].

3.2.3. Degradation products of MG during ozonation processes

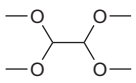
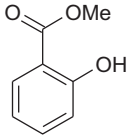
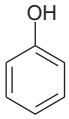
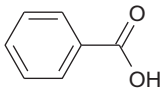
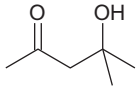
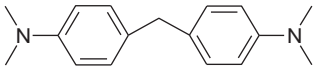
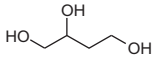
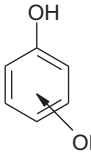
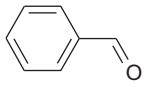
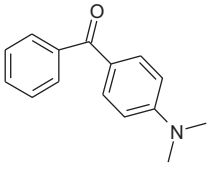
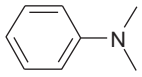
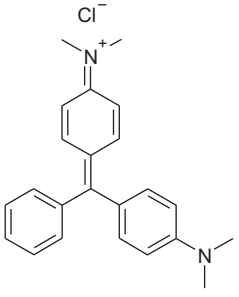
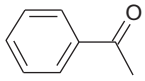
In this section of the study, 12 different intermediates formed during the ozonation of *MG* were identified *via* GC–MS after a trimethylsilylating reagent was used to derivatize non-volatile compounds, such as alcohols, phenols, or carboxylic acids, by substituting a trimethylsilyl group (TMS) for a hydrogen in the hydroxyl groups of the compounds (Table 4). Fig. 7a–e contains the total ion chromatogram of *MG* (initial concentration of 1.82 mM) treated with ozone for 5 min. In this chromatogram, 13 peaks were identified although one peak was not an intermediate (peak number (PN) 13, referring to *MG*) (Fig. 8a–e).

The reaction between molecular ozone and carbon–carbon double is obvious. The mechanism of ozone addition to alkenes begins with the formation of unstable compounds called initial ozonides. The process occurs vigorously and leads to the spontaneous rearrangement to form compounds known as ozonides. Ozonides are very unstable compounds that are degraded to functional groups composed of a carbon atom double-bonded to an oxygen atom (carbonyl groups) [34].

Tetramethoxy ethene (peak number1 (*PN1*)), 4-hydroxy-4-methyl-2-pentanone (*PN2*) and butane-1,2,4–triol (*PN3*) were identified among the intermediates in the first 5 min of the ozonation time. Unlike *PN2*, the area of *PN1* and *PN3* increased in the total ion chromatograms (TICs) (Table 5) with an ozonation time of 100 min.

Although *PN2* did not involve any carbon–carbon double bonds, which are attractive groups for reacting with ozone, decreased area was observed for ozonation times of 30 and 100 min. PN2 is assumed to have been consumed by other intermediates as a reagent. Except for PN 7, the other intermediates containing an aromatic ring, PN4-13, were reduced by ozonation at the relevant times. The area of PN7 decreased with ozonation for the first 30 min and later increased at the end of the process (100 min).

Table 4
Organic intermediates identified by GC–MS.

Peak number	Name	Open structure	Peak number	Name	Open structure
1	Tetramethoxy ethene		8	2-Hydroxy-methylbenzoate	
2	Phenol		9	Benzoic acid	
3	4-Hydroxy-4-methyl-2-pentanone		10	4-Dimethylamino-diphenylmethane	
4	Butane-1,2,4-triol		11	Dihydroxy benzene	
5	Benzaldehyde		12	p-Benzoyl-N,N-dimethylaniline	
6	N,N-Dimethyl-benzenamine		13	C.I. Basic green 4	
7	Acetophenone				

PN5, 8, 10, 11, and 12 disappeared on the TICs after ozonation for 30 min, while PN 13 disappeared after 100 min of ozonation. The proposed degradation mechanism of *MG* is shown in Fig. 9. The structure of *MG* (PN13) is composed of a central carbon atom (C^C) bonded to three aromatic rings, two anilinic (C^N) and a phenylic (C^P) group and all carbon atoms in the structure form sp^2 hybridization. Therefore, a conjugation composes throughout dye molecule and central carbon atom in each case has double bond regard-

Table 5
The determined intermediates *via* TICs (GC/MS) in the reaction media during ozonation process at decolorization. The beginning time, decolorization completing time, and mineralization time were 5, 30, and 100 min, respectively.

Peak number	Intermediates	5 min	30 min	100 min
1	Tetramethoxy ethane	√	Unchanged	Increased
2	4-Hydroxy-4-methyl-2-pentanone	√	Decreased	Decreased
3	Butane-1,2,4-triol	√	Unchanged	Increased
4	Benzaldehyde	√	Decreased slightly	Decreased
5	N,N-Dimethyl-benzenamine	√	Decreased slightly	None exist
6	Acetophenone	√	Decreased	Decreased
7	2-Hydroxy-methylbenzoate	√	Decreased	Increased
8	Phenol	√	Decreased	None exist
9	Benzoic acid	√	Decreased	Decreased
10	4-Dimethylamino-diphenylmethane	√	Decreased	None exist
11	Dihydroxybenzene	√	Decreased	None exist
12	p-Benzoyl-N,N-dimethylaniline	√	Decreased	No exist
13	C.I. Basic Green 4	√	None exist	None exist

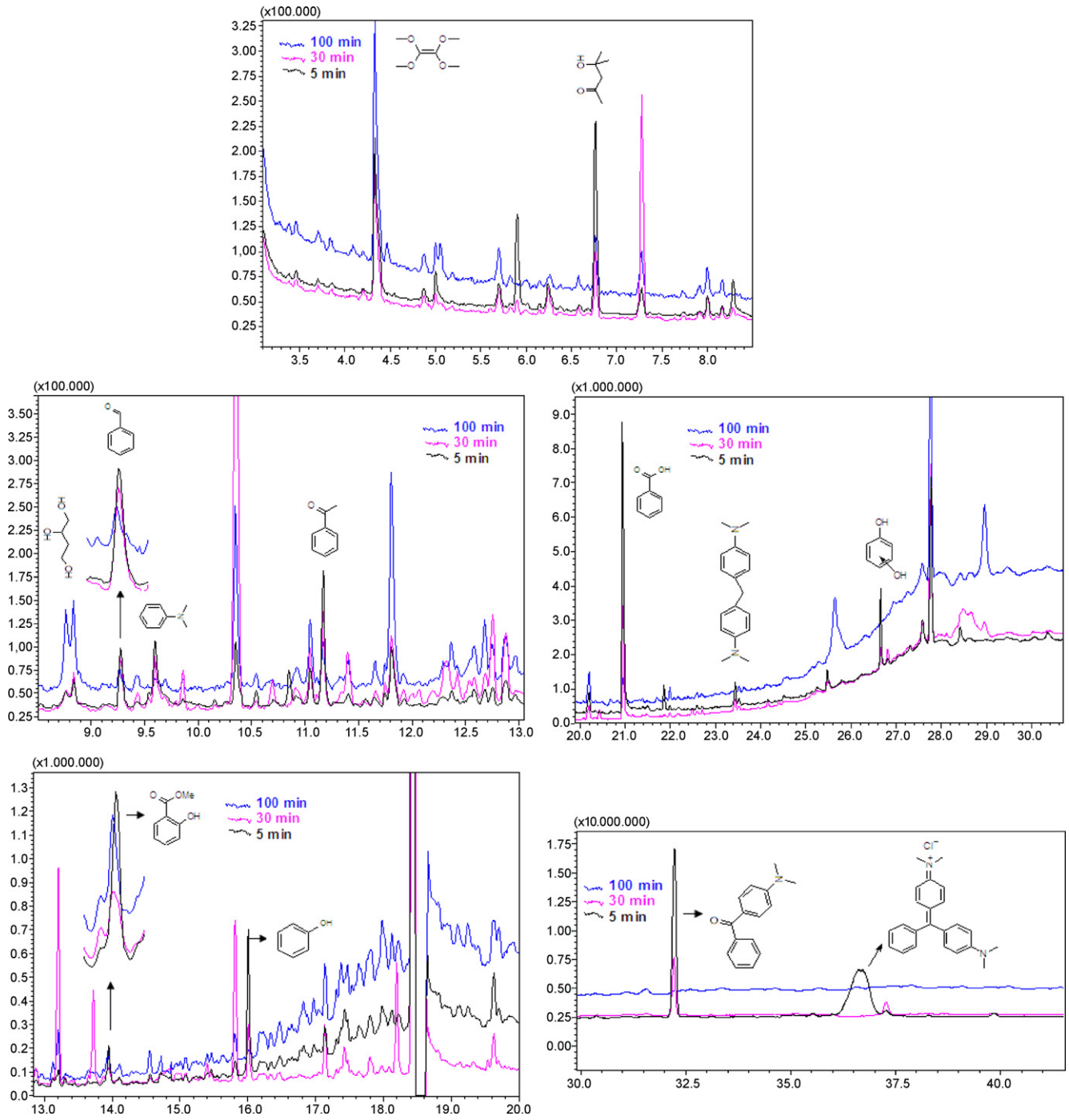


Fig. 8. (a) First 8.5 min of total ion chromatogram. (b) Between 8.5 and 13 min of total ion chromatogram. (c) Between 12.8 and 20 min of total ion chromatogram. (d) Between 18 and 31 min of total ion chromatogram. (e) Between 30 and 42 min of total ion chromatogram.

less of possible resonance structures. This situation makes the central carbon atom π electron-rich and also creates an attractive center on this carbon atom for electrophilic ozone attacks. As shown in Fig. 7a–e, the PN12 was the highest peak among the stabile intermediates. This result shows that ozone preferred the central carbon atom for electrophilic attacks and the degradation started *via* pathway 1 or pathway 2 (Fig. 9). TIC analysis showed that the ratio of relative abundance of PN 12 to PN 10 was about to 15. As a result, ozone preferred C^C–C^N bond for electrophilic attacks which has more π electron than C^C–C^P bond due to electron donor nitrogen atom. After electrophilic attack of ozone to PN12, secondary stabile intermediates formed the reaction of ozone with PN12. It was reported that these intermediates were assumed to be alcohols, aldehydes or ketones and carboxylic acid [35,36].

3.2.4. Antibacterial activity of degradation products of MG using ozonation processes

The ozonation and antimicrobial activity of *MG* were determined at an *MG* concentration of 1.82 mM. The antimicrobial activity of *MG* and the intermediate products after ozone treatment were evaluated against *S. epidermidis* (NRRL B-4268) and *Bacillus*

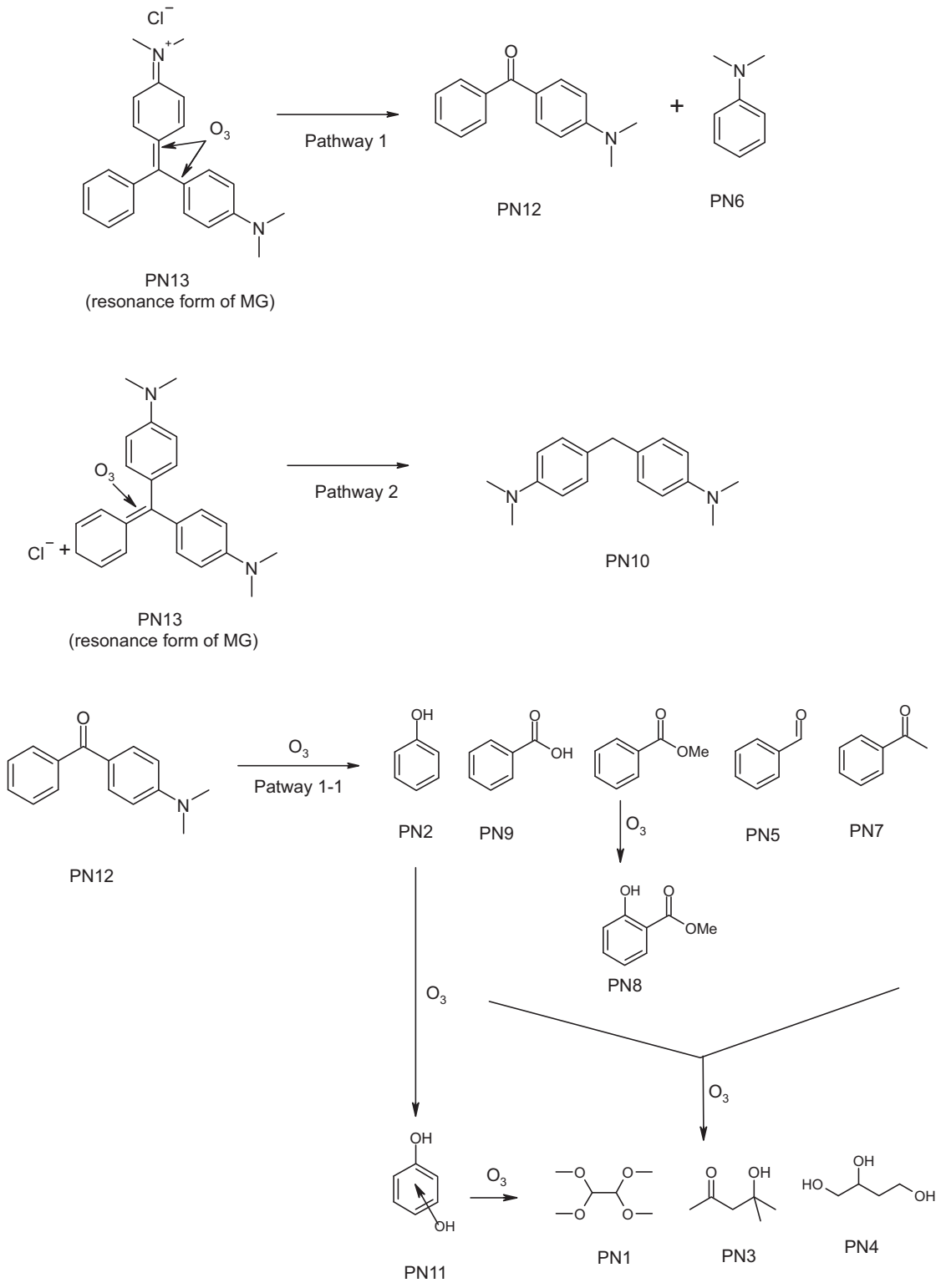


Fig. 9. Proposed degradation mechanism of *MG*.

cereus (NRRL B-354). *MG* was used as an antimicrobial agent to treat microbial infections. Tetracycline (30 mg L^{-1}) and a second-generation cephalosporin (cefuroxime, 30 mg L^{-1}) were used as positive controls.

The antimicrobial activity test indicated that the degradation products of *MG* were active against all of the test organisms (Table 6 and Fig. 10). The zones of inhibition of B-354 and B-4268 were 21 and 20 mm, respectively, for tetracycline and 23 mm for cefuroxime.

The zones of inhibition of B-354 and B-4268 were both 25 mm for *MG* (Fig. 11). The zones of inhibition of *MG* were 19.04% greater than those for tetracycline and 8.69% greater than those for cefuroxime for both B-354 and B-4268. *MG* showed greater antimicrobial activity than tetracycline and cefuroxime.

Table 6
Antibacterial activity of *MG* and intermediates during the ozonolytic experiment.

Sampling number	Sampling time (min)	Bacteria zone size (mm)	
		B-354	B-4268
0	0	25	25
1	2	23	24
2	4	23	24
3	6	23	24
4	8	22	23
5	10	23	22
6	12	22	22
7	14	19	21
8	16	19	20
9	18	18	18
10	20	17	17
11	22	15	15
12	24	10	11
13	26	0	0

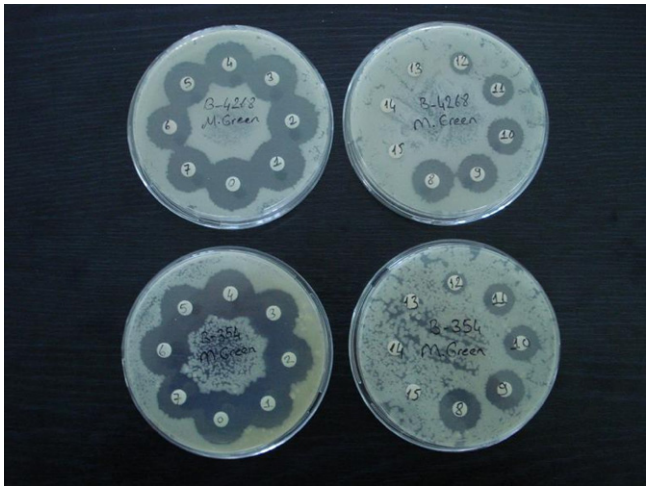


Fig. 10. Antibacterial activity of *MG* and intermediates during ozonization applications for *B. subtilis* (B-354) and *S. epidermidis* (B-4268).

This *in vitro* study showed that *MG* could exhibit antibacterial activity at low concentrations. However, the antimicrobial activity of *MG* degradation products, after an ozonation time of 10 min, was decreased, depending on the contact time of the dye-ozone interaction. Although 86% of *MG* in the reaction mixture was removed by ozonation after 10 min (Fig. 4), the decreased antibacterial activity could be very low (10%) for each bacterium (Fig. 11). We assumed that some intermediates formed during the decolorization process

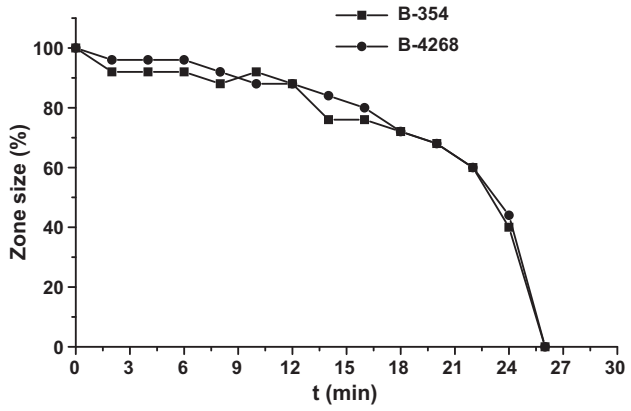


Fig. 11. Inhibition zone size of *MG* and intermediates during ozonization applications versus time for both B-354 and B-4268 ($[MG]_0$ = 1.82 mM).

exhibited antibacterial effects on B-354 and B-4268. Phenol and benzoic acid are especially well-known antibacterial agents [37]. The antibacterial activity remained by both intermediates, which formed during the first 5 min of ozonation time even though the concentration of *MG* was as low as 0.25 mM.

4. Conclusions

In this experimental work, the degradation of *MG* dye was investigated by ozonation processes. Decolorization of *MG* by ozonation processes was observed to be optimal at an acidic pH of 3. In this process, *pseudo*-first-order degradation rate constants were determined to increase from 0.223 to 0.769 min^{-1}. Stoichiometric ratios between ozone and *MG* were calculated to be 7.0 and 13.1 for the decolorization and degradation reactions, respectively. The degradation reactions of *MG* did not go to completion even after the decolorization reactions were completed. Although decolorization of *MG* was completed in 20 min, removal of TOC was determined to be 10% TOC. Thus, mineralization using ozone is not effective for all types of dye molecules and their intermediates, although colorless solutions could be obtained.

Twelve different intermediates were identified by GC–MS during the ozonation of *MG*. The area of *PN1* and *PN3* increased on TIC during ozonation time of 100 min although PN4-13 was decreased by ozonation at relevant times. The results suggest that the intermediates and *MG* had competitive effect on reaction with ozone during ozonation process.

Although 86% of MG in the decolorization reaction was removed by ozonation after 10 min, the antibacterial activity remained due to intermediates in each bacterium. However, the antibacterial activity of *MG* and its intermediate products, such as phenol and benzoic acid, was completely removed from the solution after 26 min of ozone treatment.

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