Biodegradation (2008) 19:489–494
DOI 10.1007/s10532-007-9153-3

ORIGINAL PAPER

Degradation of reactive dyes by ozonation and oxalic acid-assimilating bacteria isolated from soil

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Received: 20 June 2007 / Accepted: 17 September 2007 / Published online: 10 October 2007

Abstract Ozonation and treatment of wastewaters with oxalic acid-assimilating bacterium was attempted for the complete degradation of reactive dyes. Oxalic acid-assimilating bacterium, *Pandoraea* sp. strain EBR-01, was newly isolated from soil under bamboo grove and was identified to be a member of the genus *Pandoraea* by physicochemical and biochemical tests including 16S rDNA sequence analysis. The bacterium was grown optimally at pH 7 and temperature of 30°C under the laboratory conditions. Reactive Red 120 (RR120), Reactive Green 19 (RG19), Reactive Black 5 (RB5) and Remazol Brilliant Blue R (RBBR) were used in degradation experiments. At the initial reactive dye concentrations of 500 mg/l and the ozonation time of 80 min, it was confirmed that 75–90 mg/l oxalic acid was generated from reactive dyes by ozonation. Microbial treatment using EBR-01 greatly decreased the amount of oxalic acid in the mixture after 48 h, but it was not removed completely. TOC/TOC_0 of reactive dye solutions was also decreased to 80–90% and 20–40% by ozonation and microbial treatment using EBR-01, respectively. The study confirmed that consecutive treatments by ozone and microorganisms are efficient methods to mineralize reactive dyes.

Keywords Ozonation · Reactive dye · Decolorization · Oxalic acid-assimilating bacteria

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Introduction

Ozone is one of the chemical reagents capable of oxidizing a variety of organic compounds in aqueous solutions. Ozonation requires no post-treatment because ozone dissociates into oxygen (Nakamura et al. 2004). There are several research reports on ozonation in the field of degradation of persistent aromatic compounds such as reactive dyes and agrichemicals whereby ozone reacts with organic matter and cleavages aromatic rings and C=C bonds (Gutowska et al. 2007; Lackey et al. 2006; Lopez-Lopez et al. 2007; Shu and Chang 2005).

Synthetic dyes are widely used in textile and paper industries (Vinodgopal et al. 1998). The textile industry is the largest user of synthetic dyes consuming about 56% of the total annual world production (7×10^5 tons) (Hutzinger 1980; Vaidya and Datye 1982). Among the available dyes, about 50% of the industrial dyes produced in the world are azo dyes. Reactive group of azo dyes are mostly used in textile dyeing due to their superior fastness to the

applied fabric, high photolytic stability, and resistance to microbial degradation. However, reactive dyes exhibit low levels of fixation with the fiber and about 10–20% of total dye used in dyeing process remain left in the spent dye bath with accessory chemicals (Gouvea et al. 2000; Murugesan et al. 2006). Colored effluents from textile wastewater which are discharged from textile industries pose a significant environmental pollution problem. Even at low concentrations, textile wastewater are intensely colored. It has been reported that colored effluents can be effectively degraded by ozonation (Hitchcock et al. 1998; Wu and Wang 2001), and oxalic acid is among the products of ozonation of aromatic compounds such as reactive dyes (Andrew Hong and Zeng 2002; Koch et al. 2002; Nakamura et al. 2004; Zhang et al. 2004).

Oxalic acid reacts with metals dissolved in water to produce metal oxalates that result to scale problems (Fukuzawa et al. 2003; Kowata 2003; Suzuki 2003). Scales are thick metal oxide films such as calcium oxalate, ferric oxide and calcium carbonate which can stick to the inside walls of iron pipes of industrial fittings such as heat exchangers. These scales affect flow quantity and thermal conductivity of the pipes. Furthermore, scale formation results to more complicated phenomena including simultaneous nucleation, growth and/or adhesion of crystals (Kowata 2003). Therefore, it is necessary to develop technical means of removing oxalic acid from industrial wastewater.

In this work, the oxalic acid-assimilating bacterium isolated from soil was identified by base sequence analysis of the 16S rDNA and its properties were clarified. In addition, the biological removal of the oxalic acid generated by ozonation of reactive dyes was investigated.

Materials and methods

Materials

Reactive Red 120 (RR120), Reactive Green 19 (RG19), Reactive Black 5 (RB5) and Remazol Brilliant Blue R (RBBR) were used as reactive dyes and their concentrations were adjusted to 500 mg/l in the degradation experiments. RR120 (λ_{max} = 535 nm), RG19 (λ_{max} = 630 nm) and RB5 (λ_{max} = 596 nm) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RBBR (λ_{max} = 595 nm) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Microorganism and growth condition

An oxalic acid-assimilating bacteria, strain EBR-01, was isolated from soil under bamboo grove in the neighborhoods of Kanazawa University, Japan. Characteristics of strain EBR-01 were investigated using Gram strain, oxidase test and catalase test. Gram strain was examined using a victria blue stain and fuchsine blue stain after an immobilization of cells. Motility was investigated by the hanging drop method (Quinn et al. 1994). Oxygen requirement of bacterium was investigated using semi-fluid agar basal medium including 2 g/l oxalic acid. It was identified based on the nucleotide sequence of its 16S rDNA. Nucleotide sequencing was carried out by the dideoxy chain termination method (Sanger et al. 1977) with an automated 377A DNA sequencer (Applied Biosystems), after which the nucleotide sequence was analyzed by using the GENETYX gene analysis software (software Development, Tokyo Japan). Tested strain was aerobically cultivated in a basal medium (3.0 g/l KH_2PO_4, 7.0 g/l K_2HPO_4, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l $MgSO_4 \cdot 7H_2O$) containing oxalic acid in the range from 0 to 10 g/l at pH 7.0. Incubation experiments were carried out in 300 ml Erlenmeyer flasks containing 100 ml of medium on shaken state at 100 rpm. The effect of different temperature range from 10 to 50°C was estimated in a basal medium containing 2 g/l oxalic acid at pH 7. The ability of aerobic growth of tested strain in basal medium with oxalic acid under various acidifications was tested in the range from pH 4.0 to pH 9.0. Furthermore, the effect of different sodium chloride range from 0 to 10% was estimated in a basal medium containing 2 g/l oxalic acid at pH 7.0. After every 3 h, 3.0 ml culture samples were withdrawn from the flasks for the estimation of bacterial growth by measuring the cell density, as optical density at 660 nm (OD_{660}) in a spectrophotometer. The specific growth rate was estimated using the standard curve between OD_{660} and dry cell weight. One hundred ml of ozone-pretreated reactive dyes wastewaters were inoculated with 18 h old culture 1 ml of EBR-01 and

were aerobically cultivated at 30°C and pH 7.0 for 48 h on shaken state at 100 rpm.

Ozonation and analytical method

In the ozonation experiments on reactive dyes, the mixture of ozone and air was introduced into the reactor at a concentration flow rate of 0.4 m^3/h. The ozone concentration was 15 g/m^3. The volume of reactor was 2 L (working volume 1 L). The ozonolysis was carried out at pH 6.87 and 30°C. Decolorization efficiency of ozonation was determined by measurements of absorbance at wavelengths ranging from 190 to 900 nm by spectrophotometer (UV-240, *Shimadzu*, Japan). The maximum visible λ_{max}, (535, 630, 596 and 595 nm), respectively for RR120, RG19, RB5 and RBBR were employed as a base for characterization of color reduction percentage. The oxalic acid was measured by HPLC (*Shimadzu* LC-10AD, *Shimadzu* Co. Ltd.,Kyoto, Japan) with UV detector (SPD-10AV, *Shimadzu*, Japan) at 220 nm using KC-811 column (*Shodex*., Japan). Total organic carbon (TOC) was measured by TOC analyzer (TOC-VCSH, *Shimadzu*, Japan). All reported data were the mean values and standard deviations ($P < 0.05$) corresponding to triplicate sample measurements.

Result and discussion

Characteristics of oxalic acid-assimilating bacteria (EBR-01)

Table 1 shows characteristics of oxalic acid-assimilating bacteria EBR-01 from soil. EBR-01 was shown to be a Gram-negative and aerobic rod-shaped bacterium. EBR-01 was positive in motility, catalase and oxidase tests. This strain was negative for growth under anaerobic conditions and positive for growth in the presence of 1, 2, 5, 7% NaCl. The incubation pH and temperature range were 5.0–8.0 and 20–40°C, respectively; the optimal pH and temperature for growth being pH 7.0 and 30°C, respectively. The phylogenetic tree based the 16S rDNA sequences as shown in Fig. 1 indicates that the EBR-01, although very close to the genus *Pandoraea* sp., may be a member of a new species.

Table 1 Characteristics of oxalic acid-assimilating bacteria from soil

Proprieties	Bacteria
Shape	Rod
Motility	+
Gram staining	–
Oxidase activity	+
Catalase activity	+
Growth at 10°C	–
at 20°C	+
at 30°C	+
at 35°C	+
at 40°C	+
at 45°C	–
Growth at pH 4	–
at pH 5	+
at pH 6	+
at pH 7	+
at pH 8	+
at pH 9	–

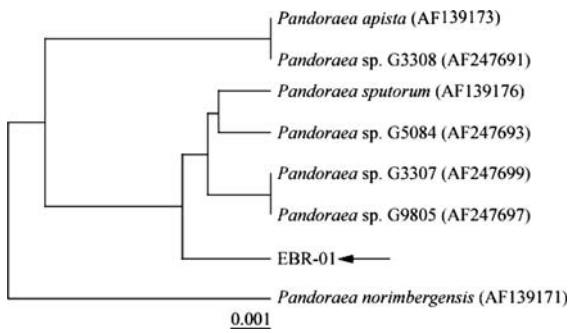

Fig. 1 Phylogenetic tree for EBR-01 strain and its relative constructed by using 16S rDNA gene sequences. GenBank accession numbers are described in the parentheses. The isolate (EBR-01 strain) in indicated by an arrow. The scale bar indicates a distance percentage of sequence of divergence

Effect of substrate concentration

Figure 2 shows the effect of initial oxalic acid concentration on specific growth rate of EBR-01. It was found that EBR-01 was capable of using oxalic acid as a carbon source. Eight different initial oxalic acid concentrations were used. It was shown that as the initial concentration of oxalic acid increased, the specific growth rate increased to a value of 0.34 h^{-1}, and then it started to decrease with further increase of

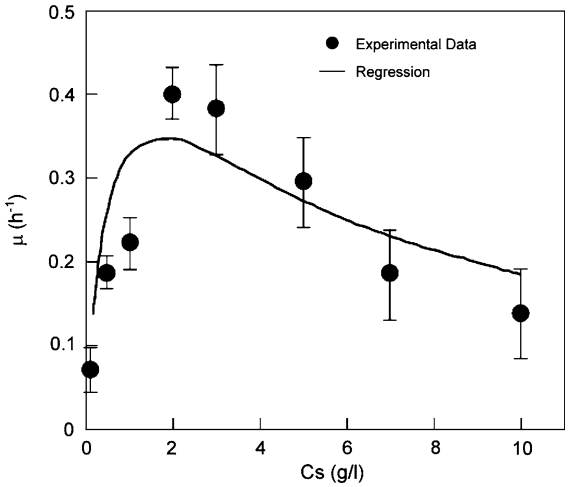

Fig. 2 Effect of initial oxalic acid concentration on specific growth rate of EBR-01

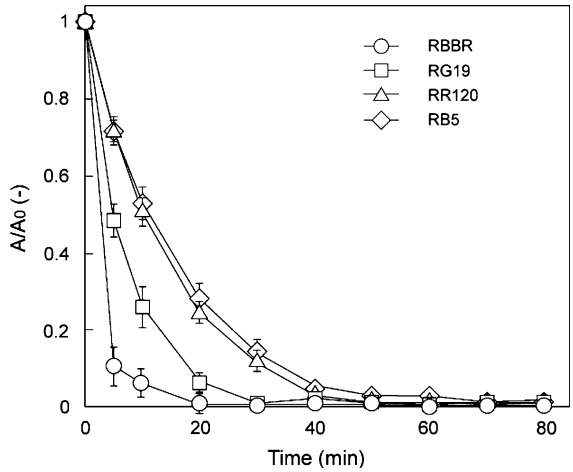

Fig. 3 Time course of UV absorption on ozonation of reactive dyes at pH 6.87 and 25°C

oxalic acid. This trend is attributed to the fact that the microbial cells were inhibited with further increase in the oxalic acid concentration. Several kinetic models were attempted in order to describe the trend. The Haldane model was applied and could best describe the inhibitory effect as follows (Beydilli and Pavlostathis 2005; Khleifat 2006; Kus and Weismann 1995):

$$\mu = (\mu_{\max} C_S)/(K_I + C_S + C_S^2/K_P)$$

where $\mu_{\max}$ is the maximum specific growth rate that could be attained, K_I (g/l) is the half saturation concentration constant, which represents the oxalic acid concentration when μ is equal to half $\mu_{\max}$ and K_P (g/l) is the inhibition constant of substances. The constants of the equation were determinate from the Lineweaver–Burk plot as $\mu_{\max} = 0.63\ h^{-1}$, $K_P = 4.35$ g/l and $K_I = 0.69$ g/l. From the above equation, the optimal oxalic acid concentration was 2.0 g/l, and it was confirmed that this bacteria could assimilate oxalic acid of as high concentration as 10 g/l. Therefore, it is thought that this bacterium can be used for treatment of ozone-pretreated wastes containing a high concentration of aromatic compound.

Treatment of ozonation waste by oxalic acid assimilating bacteria (EBR-01)

Figure 3 shows time course of UV absorption on ozonation of reactive dyes at pH 6.87 and 25°C. The UV absorption of reactive dyes decreased monotonously with the increase of ozonation time. The ease of degradation of reactive dyes by ozonation were in the following order: RBBR, RG19, RR120 and RB5. While RBBR and RG19 were completely degraded within 20 and 30 min, respectively, RR120 and RB5 were completely degraded in 50 min. The variance in degradation rates could be attributed to the difference of their chemical structures, i.e. various numbers and positions of N=N bond and molecular weights. Since the reactive dyes were readily degraded by ozonation, it was confirmed that ozonation was an effective method for the degradation of reactive dyes.

Figure 4 shows the change of oxalic acid concentration in reactive dyes solution by ozonation and microbial treatment using EBR-01. The initial reactive dye concentrations were 500 mg/l, and the ozonation time was 80 min. It was observed that 75–90 mg/l oxalic acid was generated from reactive dyes by ozonation. The difference in the amounts of oxalic acid produced was due to differences in their chemical structures. As the dyes were completely degraded, the oxalic acid concentrations reached their maximum levels. Ozonation could not degrade oxalic acid, as its levels remain unchanged. From the results, it has been confirmed that ozonation facilitates ring cleavage of aromatic molecules, but it does not degrade the aliphatic compound such as oxalic acid. When *Pandoraea* sp. strain EBR-01 was used, the oxalic acid decreased significantly after 48 h of

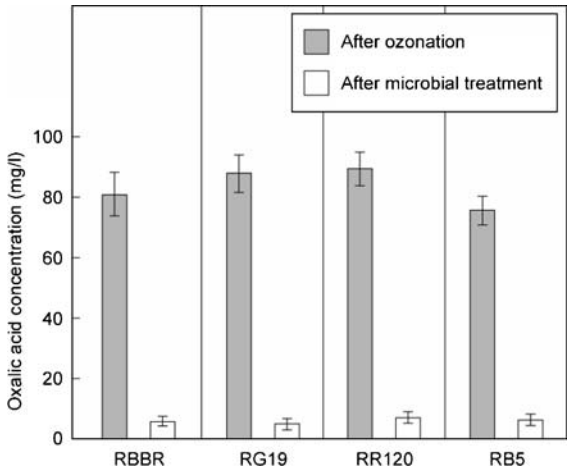

Fig. 4 Change of oxalic acid concentration in reactive dye solution by ozonation and microbial treatment using EBR-01

incubation. However, the complete removal of oxalic acid was not observed even when the EBR-01 culture incubation time was longer than 48 h, probably due to the presence of non-degradable residual compounds which are analogous to oxalic acid.

Figure 5 shows the change of TOC/TOC_0 of reactive dye solution by ozonation and microbial treatment using EBR-01. Ozonation reduced 10–20% of TOC/TOC_0 of reactive dye solutions mainly due to the generation and volatilization of volatile compounds such as formaldehyde. The difference in the decrease rate was due to differences in their chemical structures. TOC/TOC_0 of reactive dye solutions decreased further to 60–80% by when the reaction mixture was subjected to *Pandoraea* sp. strain EBR-01 biotreatment. The amount of oxalic acid from RR120 solution by ozonation was highest as shown Fig. 3, but the decrease rate of TOC/TOC_0 of RR120 solution after microbial treatment using EBR-01 was lowest though oxalic acid was almost metabolized by EBR-01. It is thought that this is because low molecular substrates other than oxalic acid generated by ozonation were not metabolized by EBR-01. The amount of oxalic acid and decrease rate of TOC/TOC_0 of RB5 solution after ozonation were lowest. But the decrease rate of TOC/TOC_0 after microbial treatment was lower than that of RR120. It is thought that the chloroorganic compounds were generated by ozonation because RR120 includes chlorine more than the other dyes i.e. RG17. The difference in the decrease rate of TOC/TOC_0 after microbial treatment was due to the structures of low molecular substrates other than oxalic acid generated by ozonation. The study confirmed that strain EBR-01 was capable of significantly assimilating oxalic acid. Our work is consistent with another study which demonstrated that soil microorganisms are capable of fermenting oxalic acid and other low molecular weight substances (Sahin 2003). With regard to nitrogen metabolism, the diamine groups in the dyes were mineralized by EBR-01 (data not shown), leaving behind some residual nitrogen that does not contribute to generation of a harmful nitroso compounds (Ishizaki et al. 1983).

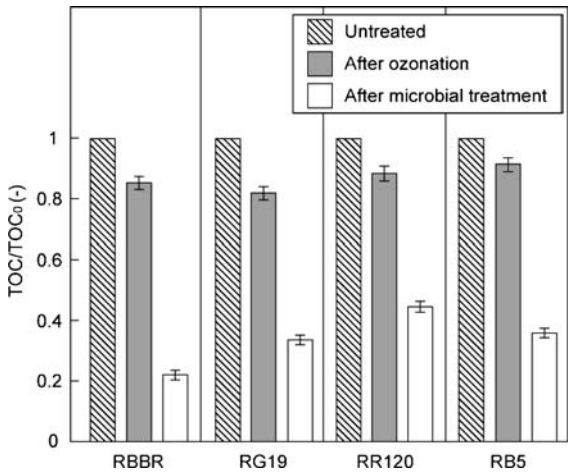

Fig. 5 Change of TOC/TOC_0 of reactive dye solution by ozonation and microbial treatment using EBR-01

Conclusion

Reactive dyes such as RR120, RG19, RB5 and RBBR were treated using ozonation and oxalic acid-assimilating bacteria isolated from the soil. The following findings were obtained:

(1) The isolated bacterium from soil was identified as *Pandoraea* sp. strain EBR-01 and the optimal pH and temperature for growth were pH 7.0 and 30°C, respectively.
(2) Reactive dyes were degraded by ozonation to oxalic acid, which was completely degraded due to metabolic activity of introduced bacterial strain EBR-01.

(3) Ozonation and microbial treatment using EBR-01 could decrease to 80–90% and 20–40% of the TOC/TOC_0, respectively.
(4) The study confirmed that consecutive treatments by ozone and microorganisms are efficient methods to mineralize reactive dyes.

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