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ORIGINAL PAPER

Decolorization and treatment of *Kokuto-shochu* distillery wastewater by the combination treatment involving biodecolorization and biotreatment by *Penicillium oxalicum* d, physical decolorization by ozonation and treatment by activated sludge

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Abstract *Kokuto-shochu* is a traditional Japanese distilled liquor made from brown sugar. *Kokuto-shochu* distillery wastewater (KDW) contains high concentrations of organic compounds and brown pigments (called molasses pigments) which are hardly decolorized by general biological wastewater treatment. A fungus, *Penicillium oxalicum* d, which we isolated in a previous study, decolorizes 47% of the color from KDW without the addition of any nutrients. *P. oxalicum* d decolorizes KDW by absorbing the pigments into its mycelia. Here we describe a KDW treatment system that combines biodecolorization and biotreatment by *P. oxalicum* d with treatment by activated sludge and physical decolorization by ozonation. Adding HClO to suppress bacterial growth and replacing fresh seed sludge at regular intervals helped to maintain the dominance and decolorization ability of *P. oxalicum* d. In a laboratory-scale demonstration, 48 cycles (12 days) achieved a decolorization ratio of 90% and removed more than 97% of dissolved organic carbon (DOC), dissolved total nitrogen (DTN) and dissolved total phosphorus (DTP). A major feature of our system is that it uses only 6% of the water used in an activated sludge-ozonation system.

Keywords *Kokuto-shochu* distillery wastewater · Molasses pigments · Decolorization · *Penicillium oxalicum* · Continuous experiment · Ozonation

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Introduction

Current biological wastewater treatment system are not effective at removing molasses pigments, which are found in various sugar products, such as sugar cane molasses and sugar beet molasses (Pant and Adholeya 2007a). *Kokuto* (brown-sugar), which is made from sugar cane juice, also contains molasses pigments. *Kokuto shochu* distilled wastewater (KDW), a distilled liquor waste, has high concentrations of organics and molasses pigments. Many of the organics are organic acids, which make KDW acidic (pH 4.1–4.3). The Amami islands, which lie between

Kyushu and Okinawa islands, are the site of major sugar cane plantations and *Kokuto shochu* distilleries. In this region, annual discharge of KDW is about 24,000 tons (Watanabe et al. 2009b). KDW is disposed of by disseminating onto farmland (73.9% of total discharge volume), composting (18.6%), treating by methane fermentation (7.3%), and other means. However, the volume of discharged of KDW has been gradually increasing. Thus, new treatment systems are needed, especially for small *Kokuto shochu* manufacturers who cannot afford to treat KDW with expensive methods.

Conventional activated sludge treatment does little to decolorize molasses pigments and cannot treat high-concentration wastewater. As a result, treating KDW with the activated sludge method requires a high dilution (by a factor of 100), a large treatment space and a large amount of energy for aeration. Other treatment methods, such as physico-chemical methods, adsorption, precipitation, advanced chemical oxidation and membrane treatment have high operational costs (Pant and Adholeya 2007a). Adsorption by activated carbon is impractical because of the large amount of activated carbon that would be needed. Ozone treatment, while effective at decolorizing molasses pigments (Peña et al. 2003), requires a large amount of electric power. Therefore, before KDW can be treated with ozone, it must be decolorized and treated to remove organic compounds. There have been some attempts to decolorize molasses pigments by using bacteria such as *Bacillus* (Nakajima-Kambe et al. 1999) and acetogenic bacteria (Sirianuntapiboon et al. 2004), but maintaining the dominance of a single bacteria strain in wastewater is too difficult. On the other hand, it is relatively easy to control the dominances of yeasts in wastewater treatment systems by keeping the low pH and by adding HClO or SO_2 (Yoshizawa et al. 1980).

Microbial decolorization of molasses pigments in molasses distilled wastewater (MDW) has been well studied. Molasses pigments can be decolorized by several species of fungus (Table 1), which produce lignin-degrading enzymes such as lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) and laccase. However, a problem with decolorization of MDW by the above species is that it requires pre-treatment such as activated sludge treatment and anaerobic treatment, to remove nutrients and then the addition of nutrients (mainly carbon sources) and/or long treatment periods, making them impractical for industrial wastewater treatment. Physical decolorization treatment and conventional activated sludge treatment are also required following fungal decolorization before the wastewater can be released into the environment. This problem also occurs with KDW treatment.

Table 1 Fungal strains capable of molasses pigments decolorization

Species	Reference
Trametes versicolor[a]	Aoshima et al. (1985)
Thanatephorus cucumeris Dec1[b]	Kim and Shoda (1999)
Mycelia sterilia D90	Sirianuntapiboon et al. (1988)
Aspergillus niger	Miranda et al. (1996)
	Pant and Adholeya (2007b)
Penicillium pinophilum	Pant and Adholeya (2007c)
Alternaria gaisen	Pant and Adholeya (2007b)

[a] Formerly: *Coriolus versicolor*

[b] Obsolete name: *Geotrichum candidium*

We recently isolated *Aspergillus tubingensis* DCT6 that decolorized 44% of the molasses pigments in MDW without needing any added nutrients (Watanabe et al. 2009a). We then developed a combination treatment system involving biodecolorization and biotreatment by *A. tubingensis* DCT6, together with physical decolorization by ozonation after treatment by activated sludge (Fig. 1). This system is well suited for decolorizing and treating MDW because it decreases the amount of electrical energy needed for decolorization by ozone and decreases the space needed for activated sludge treatment. Although, adding HClO inhibited the growth of *A. tubingensis* DCT6, the decolorization ability was kept by replacing the contaminated sludge with the fresh seed sludge at regular intervals. *A. tubingensis* DCT6 was found to be ineffective at decolorizing KDW (data not shown).

One the other hand, we recently isolated and identified *Penicillium oxalicum* d as a decolorization candidate and showed that it decolorized 47% of the molasses pigments in KDW without the needing any added nutrients (Watanabe et al. 2009b). *P. oxalicum* d grows rapidly on the organic compounds in KDW. The decolorization mechanism of *P. oxalicum* d is

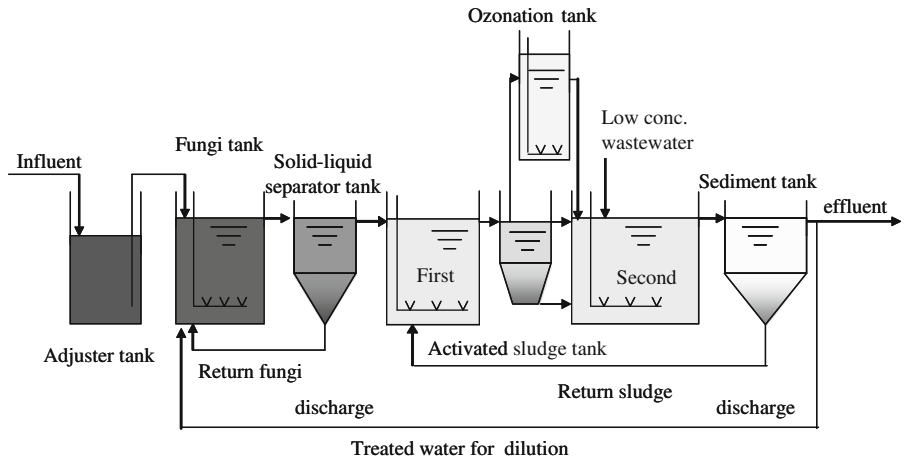

Fig. 1 Flow diagram of BSW treatment process with three sub-processes

mainly biosorption (Watanabe et al. 2009b). We also demonstrated the effectiveness of ozonic decolorization. The combined *P. oxalicum* d-activated sludge treatment shortened the ozonation time, probably because it decolorized much of the pigments and removed much of the organic nutrients. However, these results were obtained from batch experiments using sterilized KDW. Additionally, each fungus is usually inhibited by different type acids and chemicals for suppressing bacterial growth. Therefore, further studies such as the methods keeping the dominance of *P. oxalicum* d, estimation of the ability of whole treatment systems were needed to make it more practical.

In the present study, we demonstrate the inhibition of growth of bacteria by addition of HClO and acids (HCl, H_2SO_4, HNO_3, H_3PO_4) to stabilize the abilities of color removal and dominance of *P. oxalicum* d in a semi-batch continuous process. We also examined the efficiency of the combination KDW treatment system with a laboratory bench-scale experiment.

Materials and methods

Strain and activated sludge

A laboratory stock of *P. oxalicum* d, (Watanabe et al. 2009b), was used in all experiments. Fungi were maintained on potato dextrose agar (PDA) plates at 30°C. Fungal spores were suspended in sterilized distilled water. The initial concentration of spores in the culture medium was 10^6 spores ml^{-1} in all cases. Activated sludge was obtained from a sewage treatment plant in Higashihiroshima city.

Wastewater

Kokuto-shochu distillery wastewater (KDW) used in this study was sampled from a *shochu* factory in Japan. The KDW medium was diluted with ion-exchanged water. The physico-chemical characteristics of this KDW are shown in Table 2. Fungal treated wastewater which was discharged at a semi-batch continuous experiment described below was stored in a polyethylene tank at 4°C. Synthetic low concentration wastewater (1.25×10^{-2}% glucose, 6.3×10^{-3}% peptone, 4.4×10^{-3}% CH_3COONa, 6.3×10^{-3}% $(NH_4)_2SO_4$, 6.3×10^{-3}% NaCl, 9.4×10^{-3}% $NaHCO_3$, 2.2×10^{-3}% KH_2PO_4, 3.1×10^{-3}% $CaCl_2{\cdot}2H_2O$, 4.6×10^{-3}% $MgSO_4$, 2.3×10^{-4}% $FeSO_4{\cdot}7H_2O$) was used for the combination KDW treatment experiment.

Analytical methods

The decolorization ratio was defined as the decrease in absorbance at 475 nm (A_{475}) against the initial absorbance at the same wavelength, after adjusting the pH to 5.0 with 0.1 M sodium acetate buffer and centrifuging at 3500 rpm for 10 min (Ohmomo et al. 1988). pH was measured by a glass electrode with a pH meter (F-52, HORIBA). Dissolved organic carbon (DOC) was measured using total organic carbon

Table 2 Physico-chemical characteristics of *Kokuto-shochu* wastewater

Parameter	Value
A_{475}	2.98 ± 0.02
pH	4.21 ± 0.01
MLSS (mg l^{-1})	1187 ± 34
Citric acid (mg l^{-1})	3608 ± 75
Pyruvic acid (mg l^{-1})	583 ± 50
Malic acid (mg l^{-1})	1926 ± 54
Succinic acid (mg l^{-1})	652 ± 14
Lactic acid (mg l^{-1})	885 ± 56
Fumaric acid (mg l^{-1})	178 ± 8
Acetic acid (mg l^{-1})	523 ± 30
DOC (mg l^{-1})	17475 ± 452
DTN (mg l^{-1})	918 ± 22
DTP (mg l^{-1})	76.2 ± 0.1

analyzer (TOC-5000A, Shimadzu). Mixed liquor suspended solid (MLSS), dissolved total nitrogen (DTN), and dissolved total phosphorus (DTP) were measured using standard methods (APHA, AWWA, WEF 1998).

Batch treatment

KDW culture broth (50 ml) was placed into 200 ml baffle flasks and sterilized. Each flask was inoculated with a spore suspension to an initial spore density of 10^6 spore ml^{-1}, then incubated on a rotary shaker (130 rpm). Generally, the dilution ratio, cultivation time and incubation temperature were 33%, 72 h and 30°C.

Semi-batch continuous treatment

For the semi-batch continuous experiment, a water tank (W320 × D200 × H250 mm) was used. The total volume of the water tank is 16 l with a working volume of 6 l. Air was pumped through an air-stone sitting on the bottom of the water tank. Wastewater was agitated by a stirrer turning at 200 rpm. The water tank was set in an incubator box at 30°C. A semi-batch continuous experiment was conducted as described below.

(1) Fresh seed sludge of *P. oxalicum* d was pre-cultivated into 1 l of sterilized KDW medium (dilution ratio was 33%) using 2 l flasks (each flask contained 500 ml KDW medium), shaking at 130 rpm at 30°C for 72 h.
(2) 5 l of non-sterilised KDW medium and 1 l of fresh seed sludge were mixed in the water tank.
(3) Every 6 h, new KDW medium was replaced with the same volume of water that had been treated with fungal sludge using a siphon. Replaced ratios were 1 l and 2 l for phase 1 (1–40 cycles) and phase 2 (41–60 cycles), respectively. At each cycle, 2.5 ml of antifoam (Wako) and 2.5 ml of 5% HClO solution were added to the water tank. HClO was added to keep yeast as the dominant microorganism in the tank (Yoshizawa et al. 1980).

KDW ozonation experiment

500 ml of wastewater was ozonated in a 1-l glass vessel as an ozone reactor at room temperature. Wastewater was mixed with a magnetic stirrer. Ozone was generated from dry air by electrical discharges using an ozone generator (ED-OG-R4, EcoDesign Corporation). The inflow gas rate (1 l min^{-1}) was fed through an air stone nozzle in order to break the foam and dissolve ozone efficiently. The ozone concentration in the inflow (41 O_3 mg l^{-1}) and outflow (22 O_3 mg l^{-1}) gas was determined using an Ozone Detector Tube (IM0018LJ1, GASTEC Corporation) and PUMP SET (GV-100S, GASTEC Corporation).

Activated sludge treatment

Two water tanks (the first and second activated sludge tanks), each with a working volume of 6 l, were used for the activated sludge treatment. Air was supplied as described in the semi-batch continuous experiment. Each tank was maintained at 20°C in an incubator box.

In the first tank, 1 l of the first tank influent [mixture of fungal-treated wastewater (500 ml) and the second tank effluent (500 ml)] was replaced with 1 l of the first tank at first tank using a siphon every 6 h. In the second tank, 2 l of the second tank influent [mixture of the first tank effluent (500 ml), ozonated wastewater (500 ml) and the synthetic low concentration wastewater (1 l)] were replaced with 2 l of the second tank effluent every 6 h. The combination

KDW treatment experiment was operated for 48 cycles (12 days).

Results and discussion

Change of spectrum by fugal treatment and ozonation

Under the optimal cultivation conditions (Watanabe et al. 2009b), *P. oxalicum* d grew rapidly and decolorized 47% of molasses pigments without any nutrient addition. Treatment with *P. oxalicum* d resulted in shortening of the ozonation time by half, as found in our previous study (Watanabe et al. 2009b). The absorbance values of KDW in the range 300–600 nm dropped after treatment by *P. oxalicum* d (Fig. 2).

The main KDW decolorization mechanism of *P. oxalicum* d is biosorption (Watanabe et al. 2009b). Zhang et al. (2003) reported that *P. oxalicum* biosorbed reactive dyes by the mycelium pellets. We also confirmed that *P. oxalicum* d efficiently biosorbed reactive black 5, reactive blue 19, and reactive green 19 (data not shown). Therefore, *P. oxalicum* d appears to biosorb a variety of pigments.

The absorbance spectra values of KDW also dropped after ozonation following fungal treatment (treated by *P. oxalicum* d). The DOC values of KDW were reduced by 51% by fungal treatment and by 61% by fungal-ozonation treatment (data not shown). These results indicated that the ozonic decolorization would be enhanced by removal molasses pigments and organic compounds from KDW by fungal treatment.

Stabilization of color removal and dominance of *P. oxalicum* d with chemicals

Bacterial contamination in the wastewater treatment tank using yeasts can be inhibited by controlling low pH and adding HClO or SO_2 Yoshizawa et al. (1980). Therefore, we conducted a repeated-batch experiment to investigate the effect of the dominance of *P. oxalicum* d by the addition HClO and acids (HCl, H_2SO_4, HNO_3, H_3PO_4).

Preliminary experiments (data not shown) proved that reuse of mycelia of *P. oxalicum* for KDW treatment shortened the time needed to treat KDW. The best results were obtained by replacing 25% of the wastewater by the same volume of influent every

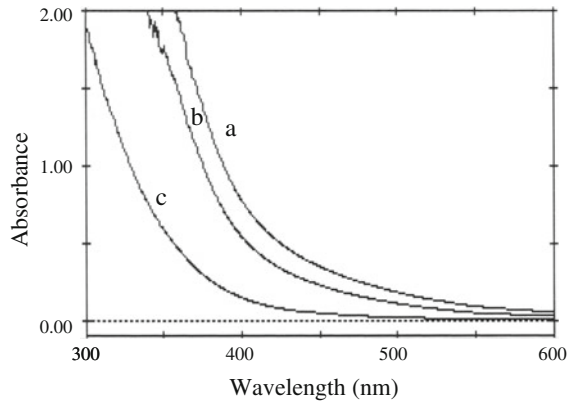

Fig. 2 Spectrum patterns of BSW (control) (*a*), treated wastewater with fungi (*b*) and treated wastewater with fungi-ozone (*c*)

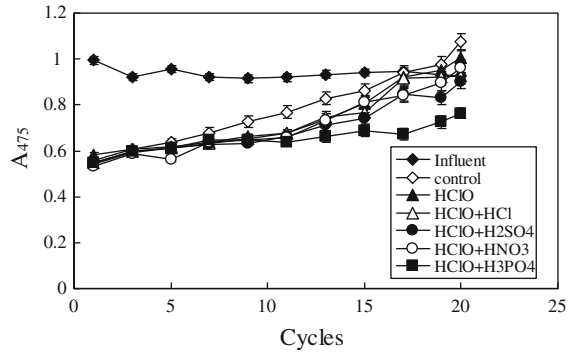

Fig. 3 Effect of additional chemical compounds on the dominance and decolorization ability of *P. oxalicum* d. Time course of change of A_{475} of influent (*filled diamond*), control (no addition, *open diamond*), HClO addition (*filled triangle*), HClO + HCl addition (*open triangle*), HClO + H_2SO_4 addition (*filled circle*), HClO + HNO_3 (*open circle*) and HClO + H_3PO_4 addition (*filled square*). The results were shown as average of three different experiments. The *error bars* shows standard deviations

6 h. Under these conditions, the time needed to treat the water was shortened by 60%. Adding 30 ppm HClO delayed the decrease of color removal by bacterial contamination (Fig. 3). Color removal inhibition was most strongly decreased when 30 ppm H_3PO_4 was added.

Although H_3PO_4 is more expensive than HCl and H_2SO_4, an additional physico-chemical removal process such as removing H_2S (desulfurization) would not be needed. KDW has a little phosphorus, which is essential for cell growth. Therefore, it seemed that H_3PO_4 addition would be useful not only

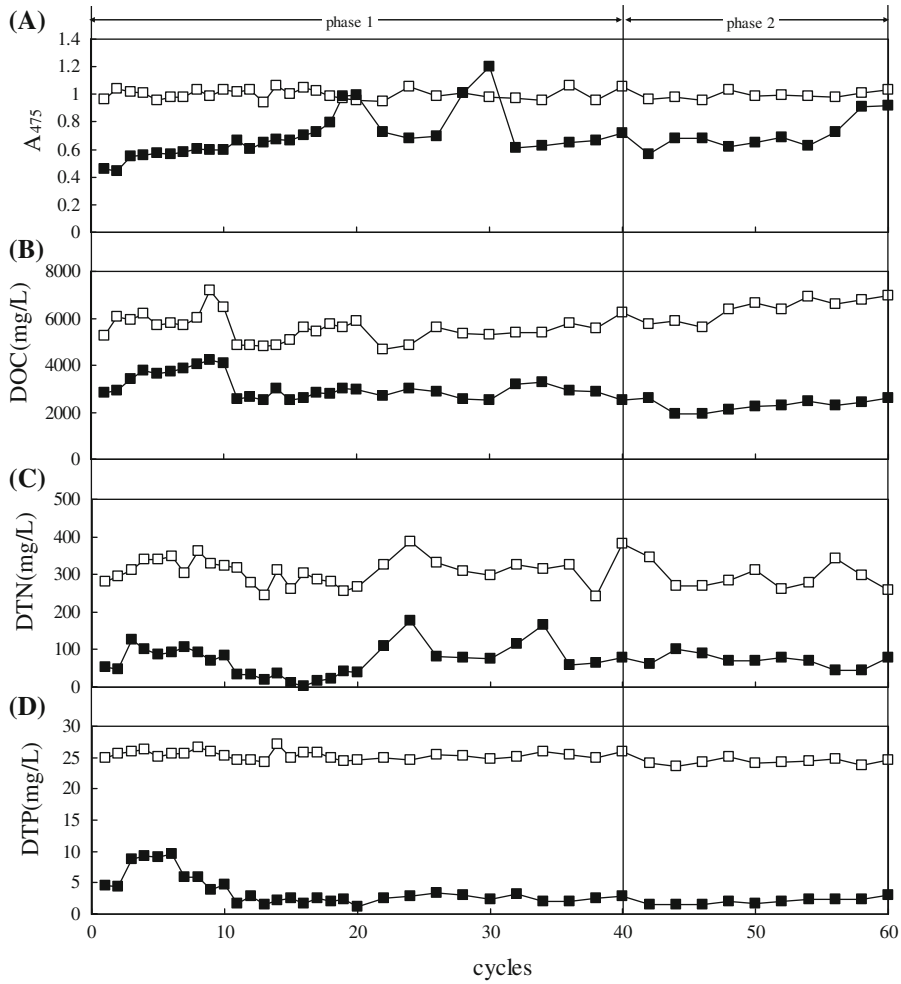

Fig. 4 Time course of changes of A_{475} (**a**), DOC (**b**), DTN (**c**), and DTP (**d**) at continuous treatment of BSW using *P. oxalicum* d with semi-batch operation. *Symbols*: influent (*open square*) and effluent (*filled square*)

to maintain the dominance of *P. oxalicum* d, but also to accelerate removal of organic compounds following cell growth.

Yeasts have been successfully used to treat food and beverage industry wastewater (Yoshizawa 1978) and olive mill wastewater (Lanciotti et al. 2005). However, using filamentous fungi to treat wastewater has so far not been practical because of the spore formation and difficulty in maintaining the dominance of a fungus in the reactor tank. Our results to suppress bacterial growth by adding HClO and H_3PO_4 appear to make fungal treatment more practical.

Semi-batch continuous operation by *P. oxalicum* d

The average decolorization ratio for the entire operation was about 30% (Fig. 4). This value was lower than that of the batch experiment (47%), probably because the decolorization was decreased by bacterial contamination. The average removal ratios for DOC, DTN, and DTP for the entire operation were 51, 77, and 87%, respectively (Table 3). The pH rose from 4.2 to 6.6, probably because *P. oxalicum* d removed organic acids (data not shown). Additionally, it was found that a little contamination by bacteria enhanced the DOC removal ability.

At the start of phase 1, the decolorization ability was gradually decreased. After 18 cycles, the number of flocks of *P. oxalicum* d dropped dramatically, the turbidity increased and decolorization ratio dropped, probably because of bacterial contamination. Wastewater treatment studies that used other microorganisms also reported losses of decolorization effectiveness with time (Watanabe et al. 2009a; Miranda et al. 1996;

Table 3 Summary performance of semi-batch continuous operation by *P. oxalicum* d

Characteristic	Type	Phase 1 Cycle 1–40 RR[a] = 1	Phase 2 Cycle 41–60 RR = 2
A_{475}	Influent	1.00 ± 0.02	0.99 ± 0.01
	Effluent	0.69 ± 0.08	0.71 ± 0.06
DOC	Influent (mg l^{-1})	5579 ± 283	6401 ± 244
	Effluent (mg l^{-1})	3084 ± 263	2287 ± 127
	Removal (mg l^{-1})	2494 ± 273	4114 ± 186
	Day removal (mg l^{-1} day^{-1})	1663 ± 182	5485 ± 124
DTN	Influent (mg l^{-1})	309.5 ± 18.3	291.8 ± 15.9
	Effluent (mg l^{-1})	70.8 ± 21.5	71.4 ± 9.0
	Removal (mg l^{-1})	238.7 ± 19.9	220.3 ± 12.4
	Day removal (mg l^{-1} day^{-1})	159.1 ± 13.3	293.8 ± 8.3
DTP	Influent (mg l^{-1})	25.36 ± 0.01	24.28 ± 0.01
	Effluent (mg l^{-1})	3.78 ± 0.01	2.04 ± 0.01
	Removal (mg l^{-1})	21.58 ± 0.01	22.24 ± 0.01
	Day removal (mg l^{-1} day^{-1})	14.38 ± 0.01	29.65 ± 0.01

[a] Replacement ratio (l/6 h)

Ohmomo et al. 1985). The decolorization ability of *P. oxalicum* d recovered when the fresh seed sludge was replaced with 1/3 volume of treated water.

Fujita et al. (1994) analysed pellet formation of *A. niger* based on shear stress and studied fungal wastewater treatment. They placed a pellet-cultivation tank upstream of the fungal treatment tank. Because *P. oxalicum* d grew rapidly in KDW, KDW seemed like a good choice for the medium for a small scale pellet-cultivation tank. Because fungal treatment is a developing method, further studies, such as continuous flow treatment experiments and pilot-plant operations, are needed to make it more practical.

The amounts of removal of each component on a daily basis were higher in phase 2 than in phase 1. Additionally, the daily removal of DOC in phase 2 (5500 mg l^{-1}) was comparable to that of the upflow anaerobic sludge blanket (UASB) system (3000–6000 mg l^{-1}) which is considered as high-efficiency anaerobic wastewater treatment method (Yamada et al. 2006). We expect that the treatment efficiency could be improved by improving the air flow ratio and the replacement ratio.

Combination treatment of KDW

A_{475} of the second tank effluent remained stable at around 0.12 through the experiment (Fig. 5). The decolorization ratio through the combination KDW treatment experiment was around 90%. The effluents of DOC, DTN, and DTP from the second tank were 134 mg l^{-1} (removal ratio: 98%), 8.9 mg l^{-1} (removal ratio: 97%), and 0.41 mg l^{-1} (removal ratio: 98%), respectively (Table 4).

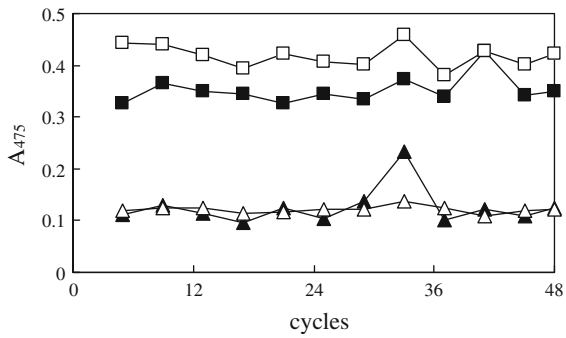

Fig. 5 Changes of A_{475} over time with the combination treatment. *Symbols*: first activated sludge tank influent (*filled square*), first activated sludge tank effluent (*open square*), second activated sludge tank influent (*filled triangle*) and second activated sludge tank effluent (*open triangle*)

The activated sludge treatment was not effective at treating high-concentration wastewater. Thus, the activated sludge-ozone treatment requires a high degree of dilution (100 times) of KDW and a large treatment space. On the other hand, the volume of wastewater treated with our presented system requires only a six times dilution (three times dilution with *P. oxalicum* d treatment and two times dilution with

Table 4 Summary of performance of continuous combination treatment of KDW

Type of wastewater	A_{475}	pH	DOC (mg l^{-1})	DTN (mg l^{-1})	DTP (mg l^{-1})
Influent of fungal treatment tank	1.00 ± 0.02	4.21 ± 0.01	5795 ± 243	309.5 ± 19.1	25.40 ± 0.02
Effluent of fungal treatment tank	0.61 ± 0.07	5.49 ± 0.22	3276 ± 229	71.2 ± 21.2	3.77 ± 0.01
Influent of first activated sludge tank	0.35 ± 0.03	6.05 ± 0.04	1663 ± 29	51.9 ± 10.4	3.68 ± 0.01
Effluent of first activated sludge tank	0.42 ± 0.02	8.55 ± 0.19	370 ± 20	22.7 ± 6.9	0.43 ± 0.01
Influent of second activated sludge tank	0.12 ± 0.04	5.95 ± 0.25	209 ± 16	31.4 ± 7.4	1.51 ± 0.01
Effluent of second activated sludge tank	0.12 ± 0.01	7.09 ± 0.20	134 ± 15	8.9 ± 4.8	0.41 ± 0.01

following treatment). Because our results and those of other decolorization studies using fungi were based on lab-scale experiments, estimating the costs of a full-scale system is difficult. However, the present system appears to drastically decrease the pond area and operating costs (e.g., the cost for adding nutrients, aeration, and ozonation).

These results suggest that the combination treatment involving biodecolorization and biotreatment by fungal treatment, together with physical decolorization by ozonation after treatment by activated sludge would be effective for decolorizing and removing organic compounds from KDW.

Conclusions

In the present study, we demonstrated a KDW treatment system with combination treatment involving biodecolorization and biotreatment by *P. oxalicum* d, together with physical decolorization by ozonation after treatment by activated sludge (Fig. 1). This is the first report about a lab-scale entire combination treatment experiment to decolorize and treat KDW.

This system doesn't require the addition of any nutrients to enhance biodecolorization or pre-treatment for fungal treatment. It also requires less treatment space because it needs only a low amount of dilution. Because the mycelia of *P. oxalicum* d grow rapidly on the organic compounds of KDW, their biosorb ability would not become saturated through the continuous treatment.

Spectral analyses show that fungal treatment enhance the ozonic decolorization by removing molasses pigments and organic compounds from KDW without any new pigments.

The decolorization ratio of the combination treatment was around 90%. The second tank effluent contained 134 mg l^{-1} DOC (removal ratio: 98%), 8.9 mg l^{-1} DTN (removal ratio: 97%), and 0.41 mg l^{-1} DTP (removal ratio: 98%).

Maintaining the dominance and ability of the fungal strain in the fungal-treatment tank is essential. The decolorization ability and dominance of *P. oxalicum* d could be maintained by adding HClO, keeping the pH low with H_3PO_4 and replacing the contaminated sludge with fresh seed sludge at regular intervals.

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