Decolorization and semi-batch continuous treatment of molasses distillery wastewater by *Aspergillus tubingensis* DCT6

T. Watanabe, M. Tanaka, K. Masaki, T. Fujii and H. Iefuji

ABSTRACT

Large quantities of deeply pigmented molasses distillery wastewater (MDW), are discharged during the production of bio-ethanol from molasses. Conventional biological wastewater treatment is not effective in removing the molasses pigments. In the present study, a MDW treatment system was developed with combination treatment involving biodecolorization and biotreatment by *Aspergillus tubingensis* DCT6, together with physical decolorization by ozonation after treatment by activated sludge. *A. tubingensis* DCT6, which was isolated from soil, decolorized 44% of the pigments in MDW without adding any nutrients. The combination treatment with *A. tubingensis* DCT6 and activated sludge method (fungi-activated sludge treatment) removed about 90% of organic compounds from MDW and appears to reduce the amount of space and water required for treatment. The fungi-activated sludge treatment reduced the time needed for decolorization by ozone by 83%. Replacing fresh seed sludge at regular intervals was useful to maintain the dominance and decolorization ability of *A. tubingensis* DCT6. The entire treatment obtained a decolorization ratio of 89–94% and removed more than 90% of each of DOC, DTN, and DTP.

Key words | *Aspergillus tubingensis*, continuous experiment, decolorization, molasses, ozonation

T. Watanabe
M. Tanaka
T. Fujii
H. Iefuji (corresponding author)
Graduate School of Biosphere Science Hiroshima University,
Kagamiyama 1-4-4 Higashihiroshima,
Hiroshima 739-8527,
Japan

T. Watanabe
M. Tanaka
K. Masaki
T. Fujii
H. Iefuji (corresponding author)
National Research Institute of Brewing,
Kagamiyama 3-7-1 Higashihiroshima,
Hiroshima 739-0046,
Japan
E-mail: *iefuji@nrib.go.jp*

INTRODUCTION

CO_2 released from the burning of petroleum and coal is considered a major contributor to global warning. There is thus a need for alternative energy sources that are carbon neutral. One such energy source is bio-ethanol, which is typically produced from corn-starch and sugar cane juice. Okinawa prefecture in Japan has promoted the production of bio-ethanol using sugar cane molasses. However, every litre of bio-ethanol that is produced generates 15 litres of molasses distillery wastewater (MDW) that must be discharged. Thus, the cost to treat MDW is an important factor in production of bio-ethanol from molasses. MDW has a high chemical oxygen demand (COD) and a high concentration of brown pigments (such as melanoidin and polyphenol), called molasses pigments, which are hardly decolorized by general biological wastewater treatment (Wolfrom *et al.* 1953). Ozone treatment, while effective at decolorizing MDW (Peña *et al.* 2003), requires a large amount of electronic power.

MDW can also be decolorized by several species of fungus, such as *Trametes versicolor* (formerly *Coriolus versicolor*) (Aoshima *et al.* 1985), *Aspergillus niger* (Miranda *et al.* 1996), *Thanatephorus cucumeris* (formerly *Geotrichum candidium*) Dec1 (Kim & Shoda 1999), and *Mycelia sterilia* D90 (Sirianutapiboon *et al.* 1988). However, a problem with microbial decolorization of MDW by the above species is that it requires pre-treatment such as activated sludge treatment, to remove nutrients from MDW and then the addition of nutrients (mainly

doi: 10.2166/wst.2009.240

carbon source) and/or long treatment periods, making them impractical for industrial wastewater treatment. Physical decolorizing treatment and conventional activated sludge treatment are also required following the fungal decolorization prior to releasing the wastewater into the environment.

We recently isolated and identified *Aspergillus tubingensis* DCT6 as a decolorization candidate and showed that it decolorized 44% of molasses pigments without the need to add any nutrients (Watanabe *et al.* submitted). We also demonstrated the effectiveness of ozonic decolorization. The fungal (treated by *A. tubingensis* DCT6)-activated sludge treatment shortened the ozonation time, probably because it decolorized much of the pigments and removed much of the organic nutrients. *A. tubingensis* DCT6 doesn't produce Ochratoxin A (OTA), a toxic agent (van der Merwe *et al.* 1965). Therefore, *A. tubingensis* DCT6 could be safely used in practical wastewater treatment systems. However, in order to apply to pilot plant scale experiments, our proposed treatment system needs to confirm the stability of color removal by *A. tubingensis* DCT6.

In the present study, an MDW treatment system was developed with combination treatment involving biodecolorization and biotreatment by *A. tubingensis* DCT6, together with physical decolorization by ozonation after treatment by activated sludge (Figure 1). We examined the stability of color removal and dominance of *A. tubingensis* DCT6 in a semi-batch continuous process. We also examined the efficiency of the combination MDW treatment system with the bench scale experiment.

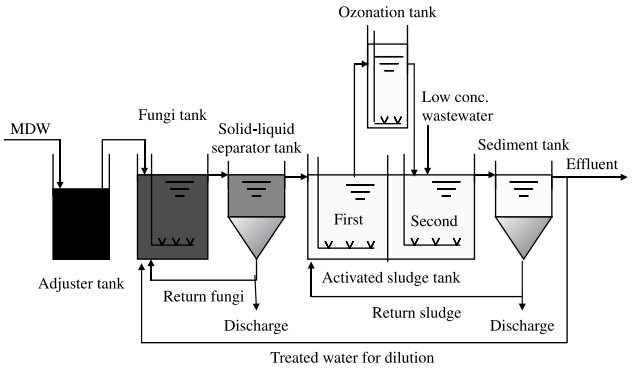

Figure 1 | Flow diagram of MDW treatment process with three sub-processes.

MATERIALS AND METHODS

Strain and activated sludge

A. tubingensis DCT6, stocked in laboratory (Watanabe *et al.* submitted), 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 l^{-1} in all cases. Activated sludge was obtained from a sewage treatment plant in Higashihiroshima city.

Wastewater

Molasses distillery wastewater (MDW) used in this study was sampled from a pilot factory in Japan. The MDW medium was diluted with ion-exchanged water. The physico-chemical characteristics of this MDW are shown in Table 1. Fungal-treated wastewater which was discharged at a semi-batch continuous experiment described below was stored in 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 MDW treatment experiment.

Semi-batch continuous experiment

For the semi-batch continuous experiment, a water tank (W320 × D200 × H250 mm) was used. The total volume of the water tank is 16 litres with a working volume of 6 litres.

Table 1 | Physico-chemical characteristics of MDW

Parameters	Value	Unit
A_{475}	12.5	–
pH	4.45	–
MLSS	1,700	$mg\,l^{-1}$
DOC	40,000	$mg\,l^{-1}$
DTN	2,700	$mg\,l^{-1}$
DTP	180	$mg\,l^{-1}$
SO_4^{2-}	1,000	$mg\,l^{-1}$

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. Fresh seed sludge of *A. tubingensis* DCT6 was pre-cultivated into 1 litre of sterilized MDW medium (dilution ratio was 20%) using 2 litres flasks (each flask contained 500 ml MDW medium), shaking at 130 rpm at 30°C for 72 h. Five litres of non-sterilised MDW medium and 1 litre of fresh seed sludge were mixed in the water tank. Every 6 h, 1–2 litres of new MDW medium was replaced with 1–2 litres of treated water with fungal sludge using a siphon. At each cycle, 2.5 ml of antifoam (Wako) was added to the water tank. After 20–50 cycles, 2 litres of fresh seed sludge was replaced with 2 litres of treated water with fungal sludge.

Activated sludge treatment

Two water tanks (the first and second activated sludge tanks), each with a working volume of 6 litres, 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 litre of the first tank influent (mixture of fungal-treated wastewater and the second tank effluent) replaced 1 litre of the first tank using siphon every 6 h. In the second tank, 2 or 4 litres of the second tank influent (mixture of the first tank effluent, ozonated wastewater (described below) and the synthetic low concentration wastewater) replaced 2 or 4 litres of the second tank effluent for every 6 h. Wasted sludge was discharged from the second tank every 6 h. The combination MDW treatment experiment was operated in 3 phases, in which phase 1 consisted of cycles 1–23, phase 2 consisted of cycles 24–130 and phase 3 consisted of cycles 131–140 (Table 2).

Ozonation experiment

Semi-batch experiments consisting of an ozone generator and an ozone reactor were operated. Ozone was generated from dry air by electrical discharges using an ozone generator (ED-OG-R4, EcoDesign Corporation). Wastewater (800 ml) was ozonated in a 2 litres glass vessel as an ozone reactor at room temperature. The ozone concentrations in the inflow (41 O_3 mg l^{-1}) and outflow (22 O_3 mg l^{-1}) were determined with an Ozone Detector Tube (IM0018LJI, GASTEC Corporation) and PUMP SET (GV-100S, GASTEC Corporation). The inflow gas (1 litre min^{-1}) was fed through an airstone nozzle in order to break the foam and dissolve the ozone efficiency. Wastewater was mixed with a magnetic stirrer to provide homogeneous conditions.

Analytical methods

The decolorization ratio was determined 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 3,500 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 was measured using total organic carbon analyzer (TOC-5000A, Shimadzu). Mixed liquor suspended solid (MLSS), dissolved total nitrogen (DTN), and dissolved

Table 2 | Conditions characteristic of influents in each tank under the combination MDW treatment experiment

Tank	Parameter		Phase 1 Cycles 1–16	Phase 2 Cycles 17–32	Phase 3 Cycles 33–64
1	Mixture ratio	Fungal-treated wastewater	25%	25%	25%
		The second tank effluent	75%	75%	75%
	Replacing volume (litre cycle^{-1})		1	1	1
2	Mixture ratio	The first tank effluent	12.5%	20%	20%
		The ozone tank effluent	37.5%	30%	30%
		Synthetic low wastewater	50%	50%	50%
	Replacing volume (litre cycle^{-1})		2	2	4

total phosphorus (DTP) were measured using standard methods (APHA *et al.* 1998).

RESULTS AND DISCUSSION

Decolorization and wastewater treatment by *A. tubingensis* DCT6 and activated sludge

A. tubingensis DCT6 grew rapidly and decolorized 44% of molasses pigments without any nutrient addition, under the optimal cultivation condition. Additionally, *A. tubingensis* DCT6 removed 65% of DOC, 51% of DTN, and 95% of DTP from MDW (diluted 20%). The mycelia were stained brown, indicating that they had adsorbed molasses pigments (data not shown). After fungal-treatment, the wastewater was treated with activated sludge (fungi-activated treatment) to remove residual nutrients of fungal-treated wastewater (data not shown). However, the activated sludge treatment increased rather than decreased the color (data not shown). Therefore, decolorization treatment after fungi-activated sludge treatment was needed.

Improvement of decolorization by ozone treatment

Figure 2 shows the change in A_{475} with reaction time with ozone. In the control (Initial) solution, after 1 h of reaction the A_{475} increased, then decreased gradually, reaching a value of 0.02 after 18 h. Treatment with *A. tubingensis* DCT6 resulted in shorting of the ozonation time to 9 h. Post-treatment by activated sludge following fungal treatment also resulted in shortening of the ozonation time to 3 h. These results indicated that pretreatment by fungi-activated sludge can decrease the amount of electrical energy needed to decolorize MDW, probably due to biodecolorization and the removal of organic compounds. Thus, a combination of fungal treatment, activated sludge treatment and ozone treatment appears to be effective for decolorizing and removing organic compounds from MDW.

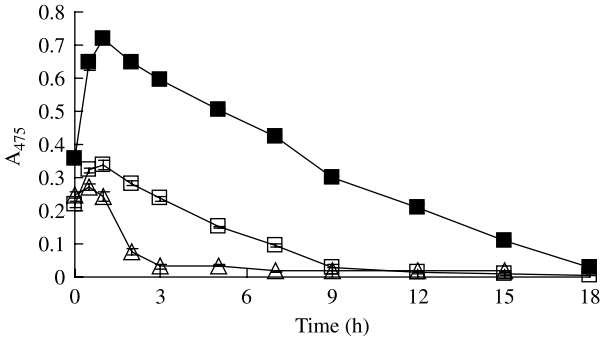

Figure 2 | Change of A_{475} over reaction time with ozone. Symbols: ■, initial (control) solution; □, solution treated by *A. tubingensis* DCT6; Δ, solution treated by activated sludge following treatment by *A. tubingensis* DCT6.

Semi-batch continuous operation by *A. tubingensis* DCT6

The average decolorization ratio for the entire operation was about 20% (Figure 3A). Although the decolorization ratio was low during continuous experiments, the ozonation time to decolorize MDW was shortened by half by treatment with *A. tubingensis* DCT6 (Figure 3B). The average removal ratios for DOC, DTN, and DTP for the entire operation were 40%, 50%, and 85%, respectively (Table 3). Additionally, it was found that a little contamination by bacteria enhanced the DOC and DTN removal ability.

During phase 1, we confirmed the stability of the decolorization ability of *A. tubingensis* DCT6 under the optical condition which was obtained from the batch experiment (data not shown). During the first half of phase 1, the mycelia of *A. tubingensis* DCT6 grew poorly, and the decolorization ratio was very low. During the

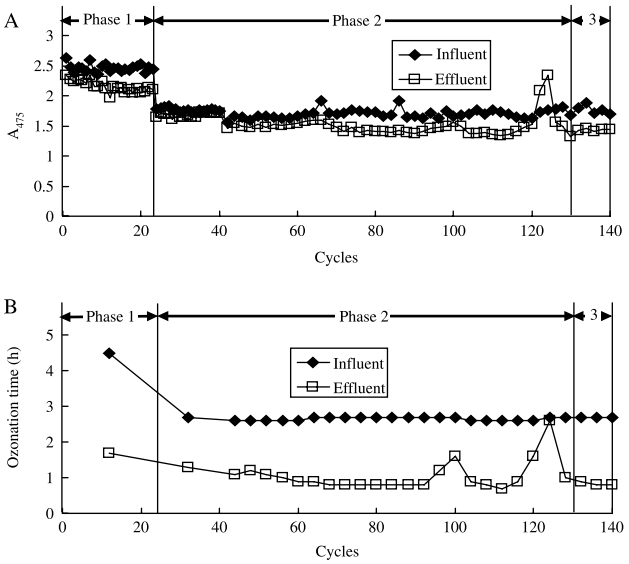

Figure 3 | Continuous treatment of MDW using *A. tubingensis* DCT6 with semi-batch operation. A: change of A_{475} over time, B: time course of the change of ozonation time.

Table 3 | Summary performance of semi-batch continuous operation by *A. tubingensis* DCT6. Ozonation time: each solution was diluted by 8 times with ion-free water. Then, 400 ml of each solution was treated with ozone until A_{475} of the solution decreased to 0.1

Characteristic	Type	Phase 1 Cycles 1–23 DR* = 20 RR† = 1	Phase 2 Cycles 24–130 DR = 14 RR = 1	Phase 3 Cycles 131–140 DR = 14 RR = 2
A_{475}	Influent	2.46	1.71	1.77
	Effluent	2.16	1.55	1.44
Ozonation time	Influent (h)	4.5	2.7	2.7
	Effluent (h)	1.7	1.1	0.8
DOC	Influent ($mg\,l^{-1}$)	7210.9	4983.5	4907.2
	Effluent ($mg\,l^{-1}$)	5296.2	3200.0	2086.2
	Removal ($mg\,l^{-1}$)	1914.7	1783.5	2821.0
	Day removal ($mg\,l^{-1}\,d^{-1}$)	1276.5	1189.0	3761.3
DTN	Influent ($mg\,l^{-1}$)	385.1	284.2	254.7
	Effluent ($mg\,l^{-1}$)	266.0	140.6	80.0
	Removal ($mg\,l^{-1}$)	119.1	143.6	174.7
	Day removal ($mg\,l^{-1}\,d^{-1}$)	79.4	95.7	233.0
DTP	Influent ($mg\,l^{-1}$)	35.4	23.7	22.8
	Effluent ($mg\,l^{-1}$)	5.8	3.9	3.1
	Removal ($mg\,l^{-1}$)	29.6	19.8	19.7
	Day removal ($mg\,l^{-1}\,d^{-1}$)	19.7	13.2	26.3

*Dilution ratio (%).
†Replacement ratio (litre per 6 h).

second half of phase 1, *A. tubingensis* DCT6 grew better and the decolorization ratio was around 25% (Figure 3A).

During phase 2, we changed the dilution ratio of MDW to 14%. At the start of phase 2 (40 cycles), bacterial contamination was observed and the decolorization ability was gradually decreased. Similar results were reported by several studies (e.g. Ohmomo *et al.* 1985; Miranda *et al.* 1996) using other microorganisms. HClO was added to keep yeasts as the dominant microorganism in the tank (Yoshizawa *et al.* 1980). Therefore, we tried using HClO to maintain the dominance of *A. tubingensis* DCT6. However, HClO inhibited the growth of *A. tubingensis* DCT6 so we stopped to add HClO halfway through phase 2. The decolorization ability of *A. tubingensis* DCT6 recovered when the fresh seed sludge was replaced with 1/3 volume of treated water. These result indicated that the decolorization ability and dominance of *A. tubingensis* DCT6 could be maintained if the contaminated sludge was replaced with fresh seed sludge at regular intervals. Because *A. tubingensis* DCT6 could grow at acidic pH (data not shown), using an acidic pH might be a way to maintain the dominance of *A. tubingensis* DCT6.

During phase 3, we changed the replacement volume to 2 litres in each cycle to enhance the treatment performance. The decolorization ratio and nutrient removal ratios were not decreased. Therefore, the amount of nutrients removed per day in phase 3 was twice as much as the amount removed in phase 2.

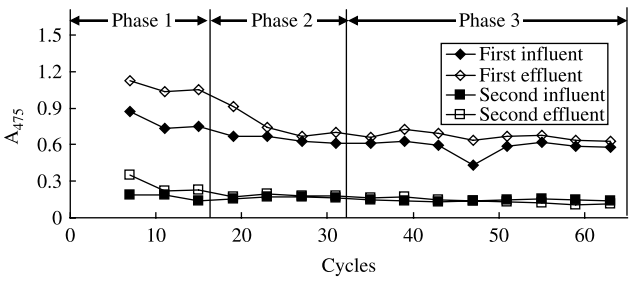

Figure 4 | Change of A_{475} over time with the continuous combination treatment.

Combination treatment of MDW

A_{475} of the second tank effluent remained stable at around 0.2 through the experiment (Figure 4). The decolorization ratio of the combination treatment was 89–94%. The second tank effluent of DOC, DTN, and DTP during phase 1–3 were 134–160 mg l^{-1} (removal ratio: 97%), 21–56 mg l^{-1} (removal ratio: 90%), and 0.8–2.0 mg l^{-1} (removal ratio: 97%) respectively (Table 4). Each removal ratio was calculated from the decrease number between MDW and the second tank effluent. These results suggest that the combination treatment would be effective in decolorizing and removing of organic compound from MDW.

The volume of wastewater treated with *A. tubingensis* DCT6 was doubled by adding synthetic low concentration wastewater to the second activated sludge tank. Therefore, the final discharge volume was 10 times greater than the initial volume of MDW. On the other hand, the activated

Table 4 | Summary of performance of continuous combination treatment of MDW

Characteristic	Type	Phase 1 Cycles 1–16	Phase 2 Cycles 17–32	Phase 3 Cycles 33–64
A_{475} (−)	Initial MDW	2.43	2.43	2.43
	Fungal-treated wastewater	1.99	1.99	1.99
	The first tank influent	0.78	0.65	0.58
	The first tank effluent	1.07	0.76	0.66
	The second tank influent	0.17	0.16	0.14
	The second tank effluent	0.27	0.18	0.14
pH (−)	Initial MDW	4.45	4.45	4.45
	Fungal-treated wastewater	3.60	3.60	3.60
	The first tank influent	3.73	3.38	3.58
	The first tank effluent	7.35	6.72	7.09
	The second tank influent	2.62	3.01	2.73
	The second tank effluent	4.86	5.18	4.32
DOC (mg l^{-1})	Initial MDW	7993.0	7993.0	7993.0
	Fungal-treated wastewater	3935.0	3935.0	3935.0
	The first tank influent	843.0	792.6	1120.1
	The first tank effluent	381.4	361.6	429.2
	The second tank influent	163.6	187.4	202.2
	The second tank effluent	134.0	146.8	158.7
DTN (mg l^{-1})	Initial MDW	527.1	527.1	527.1
	Fungal-treated wastewater	329.7	329.7	329.7
	The first tank influent	156.3	127.7	109.4
	The first tank effluent	74.2	86.1	64.8
	The second tank influent	115.5	111.3	78.8
	The second tank effluent	49.2	55.5	21.0
DTP (mg l^{-1})	Initial MDW	29.9	29.9	29.9
	Fungal-treated wastewater	10.8	10.8	10.8
	The first tank influent	7.3	4.5	7.9
	The first tank effluent	3.5	1.8	1.6
	The second tank influent	4.4	2.6	3.2
	The second tank effluent	2.0	0.9	0.8

sludge treatment was not effective at treating high-concentration wastewater. Thus, the activated sludge-ozone treatment requires a high degree of dilution with MDW and a large treatment space. Compared with previous studies (Ohmomo *et al.* 1987; Miranda *et al.* 1996), the present treatment does not need activated sludge as pre-treatment for fungal decolorization or additional nutrients, both of which are expensive.

The second tank influent pH was acidic, probably because MDW contains high concentration of SO_4^{2-} and organic acid produced by the ozone treatment. Organic acid can be removed by activated sludge treatment. However SO_4^{2-} could not be removed by activated sludge treatment. Thus, desulfurization treatment would be required before MDW is discharged into the environment. Desulfurization treatment consists of evolution of H_2S from wastewater to the air under anaerobic conditions and scrubbing of H_2S by the desulfurizer. Thus, anaerobic treatment appears to be more effective than the first activated sludge tank.

CONCLUSION

In the present study, we developed an MDW treatment system with combination treatment involving biodecolorization and biotreatment by *A. tubingensis* DCT6, together with physical decolorization by ozonation after treatment by activated sludge (Figure 1). 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. The decolorization ratio of the combination treatment was 89–94%. The second tank effluent contained 134–160 mg/l DOC (removal ratio: 97%), 21–56 mg/l DTN (removal ratio: 90%), and 0.8–2.0 mg/l DTP (removal ratio: 97%).

Maintaining the dominance and ability of the fungal strain in the fungal-treatment tank is essential. The decolorization ability and dominance of *A. tubingensis* DCT6 could be maintained if the contaminated sludge was replaced with fresh seed sludge at regular intervals. Because *A. tubingensis* DCT6 can grow at acidic pH, keeping a low pH might be a way of maintaining its dominance in the fungal treatment tank.

We confirmed that *A. tubingensis* DCT6 does not produce OTA. Therefore, using *A. tubingensis* DCT6 for the wastewater treatment system should be safe.

ACKNOWLEDGEMENTS

We thank Ryuseki Co. Ltd. for kindly providing MDW.

REFERENCES

Aoshima, I., Tozawa, Y., Ohmomo, S. & Ueda, K. 1985 Production of decolorizing activity for molasses pigment by *Coriolus versicolor* Ps4a. *Agric. Biol. Chem.* **49**, 2041–2045.

APHA, AWWA, WEF 1998 *Standard Methods for the Examination of Water and Wastewater*. APHA, AWWA, WEF, Washington, DC.

Kim, S. J. & Shoda, M. 1999 Decolorization of molasses and a dye by a newly isolated strain of the fungus *Geotrichum candidium* Dec1. *Biotechnol. Bioeng.* **62**, 114–119.

Miranda, P. M., Benito, G. G., Cristobal, N. S. & Nieto, C. H. 1996 Color elimination from molasses wastewater by *Aspergillus niger*. *Bioresour. Technol.* **57**, 229–235.

Ohmomo, S., Noboru, I., Watanabe, Y., Kaneko, Y., Tozawa, Y. & Ueda, K. 1985 Continuous decolorization of molasses waste water with mycelia of *Coriolus versicolor* Ps4a. *Agirc. Biol. Chem.* **49**, 2551–2555.

Ohmomo, S., Kaneko, Y., Sirianuntapiboon, S., Somachai, P., Atthasampunna, P. & Nakamura, I. 1987 Decolorization of molasses waste water by a thermophilic strain, *Aspergillus fumigatus* G-2-6. *Agric. Biol. Chem.* **51**, 3339–3346.

Ohmomo, S., Kainuma, M., Kamimura, K., Sirianuntapiboon, S., Aoshima, I. & Atthasampunna, P. 1988 Adsorption of melanoidin to the mycelia of *Aspergillus oryzae* Y-2-32. *Agric. Biol. Chem.* **52**, 381–386.

Peña, M., Coca, M., González, G., Rioja, R. & García, M. T. 2003 Chemical oxidation of wastewater from molasses fermentation with ozone. *Chemosphere* **51**, 893–900.

Sirianuntapiboon, S., Somachai, P., Sihanonth, P., Atthasampunna, P. & Ohmomo, S. 1988 Microbial decolorization of molasses waste water by *Mycelia sterilia* D90. *Agric. Biol. Chem.* **52**, 393–398.

van der Merwe, K. J., Steyn, P. S., Fourie, L., Scott, D. B. & Theron, J. J. 1965 Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. *Nature* **23**, 1112–1113.

Watanabe, T., Tanaka, M., Masaki, K., Fujii, T. & Iefuji, H. submitted. Decolorization and wastewater treatment of molasses distillery wastewater by *Aspergillus tubingensis* DCT6. *Biosci. Biotechnol. Biochem.*

Wolfrom, M. L., Kolb, D. N. & Langer, A. W., Jr. 1953 Chemical interactions of amino compounds and sugars VII, pH dependency. *J. Am. Chem. Soc.* **75**, 3471–3473.

Yoshizawa, K., Momose, H., Hasuno, T., Suzuki, O. & Takano, K. 1980 Disinfection of microorganism in a yeast tank using sterilizers during wastewater treatment. *Hakkokogaku (in Japanese)* **58**, 139–144.