Decolorization of Brown Pigments in Foods by Immobilized Mycelia of *Coriolus versicolor* IFO 30340 and *Paecilomyces canadensis* NC-1

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ABSTRACT: Three microorganisms were cultured to investigate their decolorizing activity on mixtures of 2 types of model brown pigment and 2 types of browned food. The mixture of model brown pigments of the Maillard reaction type and phenol type was not decolorized by cultivation with *Coriolus versicolor* IFO 30340, but it was decolorized by *Paecilomyces canadensis* NC-1 and by *Streptomyces werraensis* TT 14. The immobilized mycelia of *C. versicolor* IFO 30340 and *P. canadensis* NC-1 were incubated simultaneously or successively with each mixture of 2 types of browned food. It was found that the mixed culture of these 2 mycelia showed a higher decolorization rate than their successive culture.

Key Words: Maillard reaction, browning, brown pigment, microbial decolorization, immobilized mycelia

Introduction

Sugar and fermentation industries use cane molasses, creating colored wastewater that poses serious problems. The brown-colored substances in the wastewater from these industries are mainly brown pigments formed by the Maillard reaction. The main substances, called melanoidins, are difficult to remove or degrade by normal treatments such as the activated sludge process. Although these colorants can be chemically or physically removed by adsorption to activated carbon, precipitation by lime, and ultrafiltration, the cost of these treatments is high (Galeno and Agosin 1990). The microbial decolorization of browned wastewater has been investigated, and *Coriolus versicolor* Ps4a (Aoshima and others 1985), *Aspergillus fumigatus* G-2-6 (Ohmomo and others 1987), *Aspergillus oryzae* Y-2-32 (Ohmomo and others 1988), and *Mycelia sterilia* D90 (Sirianuntapiboon and others 1988a, 1988b) decolorized wastewater from molasses, while *Phanerochaete chrysosporium* (Galeno and Agosin 1990; Sayadi and Ellouz 1992) and *Trametes versicolor* (Bajpai and others 1993) decolorized pulp waste, olive mill wastewater, and Kraft effluents. More than 74% of the pigments were decolorized by these microbial treatments. However, there have been no reports on the decolorization of mixtures of various types of brown pigments, such as wastewater from the kitchen.

We have also screened microorganisms to decolorize model melanoidin and coffee and isolated a soil actinomycetes, *Streptomyces werraensis* TT 14 (Murata and others 1992), and a fungus, *Paecilomyces ca-*

nadensis NC-1 (Terasawa and others 1994). We reported (Terasawa and others 1996) that the brown pigments in foods could be categorized by comparing the microbial decolorization pattern for each food using 3 microorganisms: *S. werraensis* TT 14, *P. canadensis* NC-1, and *C. versicolor* IFO 30340. The objective of this study is to determine the ability of fresh and immobilized mycelia of these microorganisms to decolorize mixtures of synthetic brown pigments and brown pigments in foods under various mixing ratios of these pigments. We also investigated the effect of pH of the medium, pre-incubation, and polyethylenimine and glutaraldehyde treatments of the immobilized mycelia on the decolorizing activity. We tested the hypothesis that immobilized mycelia of these microbes may be usable to decolorize wastewater including various types of browned foods.

Results and Discussion

Decolorization of the mixtures of synthetic brown pigments or browned foods

We previously found that *C. versicolor* IFO 30340 decolorized model melanoidins and Maillard reaction-type pigments such as cane molasses, soy sauce, and miso, *P. canadensis* NC-1 mainly decolorized phenol-type model brown pigments such as coffee and black tea, and *S. werraensis* TT 14 decolorized the model melanoidins of xylose-glycine and glucose-lysine, and caramels (Terasawa and others 1996). In the present work, mixtures of model brown pigments were not decolorized by

C. versicolor IFO 30340 when the proportion of the phenol-type pigment was more than 25% of the mixture (Fig. 1ab). The phenol-type model brown pigments turned darker brown by culturing with this strain in our previous study (Terasawa and others 1996). Some basidiomycetes, *C. versicolor* IFO 4937, *C. hirsutus* IFO 4917, and so on, have polyphenol oxidase (PPO), and cane molasses turned darker brown by the PPO in the early stage of cultivation (Tamaki and others 1986). It seems that the phenol-type pigments in the media tested might have been oxidized and polymerized by PPO of *C. versicolor* IFO 30340, resulting instead in a darkening effect. The mixtures of model brown pigments were decolorized more readily by *P. canadensis* NC-1 and *S. werraensis* TT 14 than by *C. versicolor* IFO 30340 (Fig. 1). *P. canadensis* NC-1 decolorized these mixtures of brown pigments because this strain could decolorize not only phenol-type pigments but also the model melanoidins. The decolorization rates for the glucose-glycine (G-G) and glucose-tryptophan (G-T) model melanoidins were about 37% and 95%, respectively. In contrast, the decolorization rate for polymerized chlorogenic acid by this strain was about 32% (Terasawa and others 1996). For the polymerized chlorogenic acid, the decolorization rate by this strain was almost same as for G-G. Although *S. werraensis* TT 14 decolorized the xylose-glycine (X-G) model melanoidin and turned the brown color of polymerized chlorogenic acid darker in our previous study (Terasawa and others 1996), this strain mainly decolorized mixtures of

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these Maillard reaction-type and phenol-type model brown pigments in the present study (Fig. 1c).

The mixture of instant coffee and cane molasses (MCM) was decolorized by *P. canadensis* NC-1 and *C. versicolor* IFO 30340.
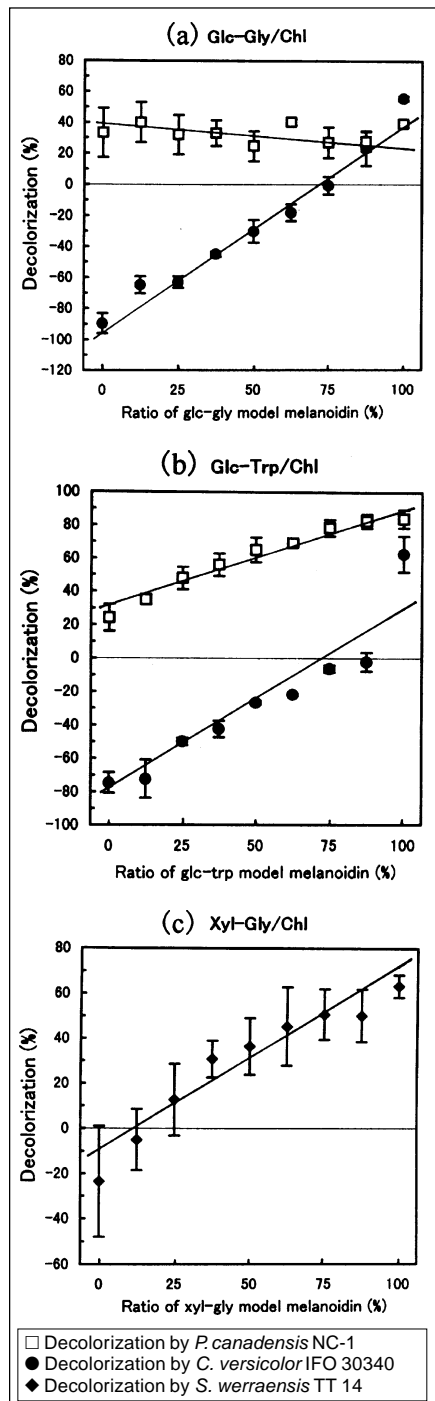

Fig. 1—Decolorization rate for mixtures of model brown pigments. (a) Mixture of glucose-glycine model melanoidin and polymerized chlorogenic acid. (b) Mixture of glucose-tryptophan model melanoidin and polymerized chlorogenic acid. (c) Mixture of xylose-glycine model melanoidin and polymerized chlorogenic acid. The OD$_{500}$ of each mixture was adjusted to about 1.

The decolorization rate for MCM was about 50% by these strains when the mixing ratio was 1:1 (Fig. 2a). It seems that the decolorization rate for cane molasses by *C. versicolor* IFO 30340 was higher than that for coffee. The decolorization rates for cane molasses and coffee by this strain were about 73% and -13% (darkening), respectively (Terasawa and others 1996).

The decolorization rate for the mixture of barley tea and black tea (MBB) by *C. versicolor* IFO 30340 (Fig. 2c) increased with increasing proportion of barley tea. This decolorization pattern is almost the same as that for MCM (Fig. 2a), but the mixtures with added model phenol-type pigments turned darker brown by *C. versicolor* IFO 30340 for every mixing ratio (Fig. 1ab). These results do not agree, possibly because the decolorization of foods by these microbes was influenced by some of the food constituents. On the other hand, the mixture of soy sauce and miso (MSM; Fig. 2b) was decolorized by about 70% with

C. versicolor IFO 30340 and turned darker brown by *P. canadensis* NC-1 for every mixing ratio. In our previous study, *P. canadensis* NC-1 decolorized the model melanoidins and turned the color of miso and cane molasses darker brown (Terasawa and others 1996). *S. werraensis* TT 14 turned the color of the mixture of caramel and Worcestershire sauce darker brown when the concentration of Worcestershire sauce was higher than that of caramel.

It is apparent that the results from our previous study (Terasawa and others 1996) on microbial decolorization almost agree with those for the decolorization of mixtures of brown pigments in this study, however, the decolorization of mixtures of model brown pigments and mixtures of browned foods do not agree between the 2 studies. The decolorization rate for mixtures of Maillard reaction-type and phenol-type browned foods was higher than that for the model brown pigments by *C. versicolor* IFO 30340. Furthermore, the de-
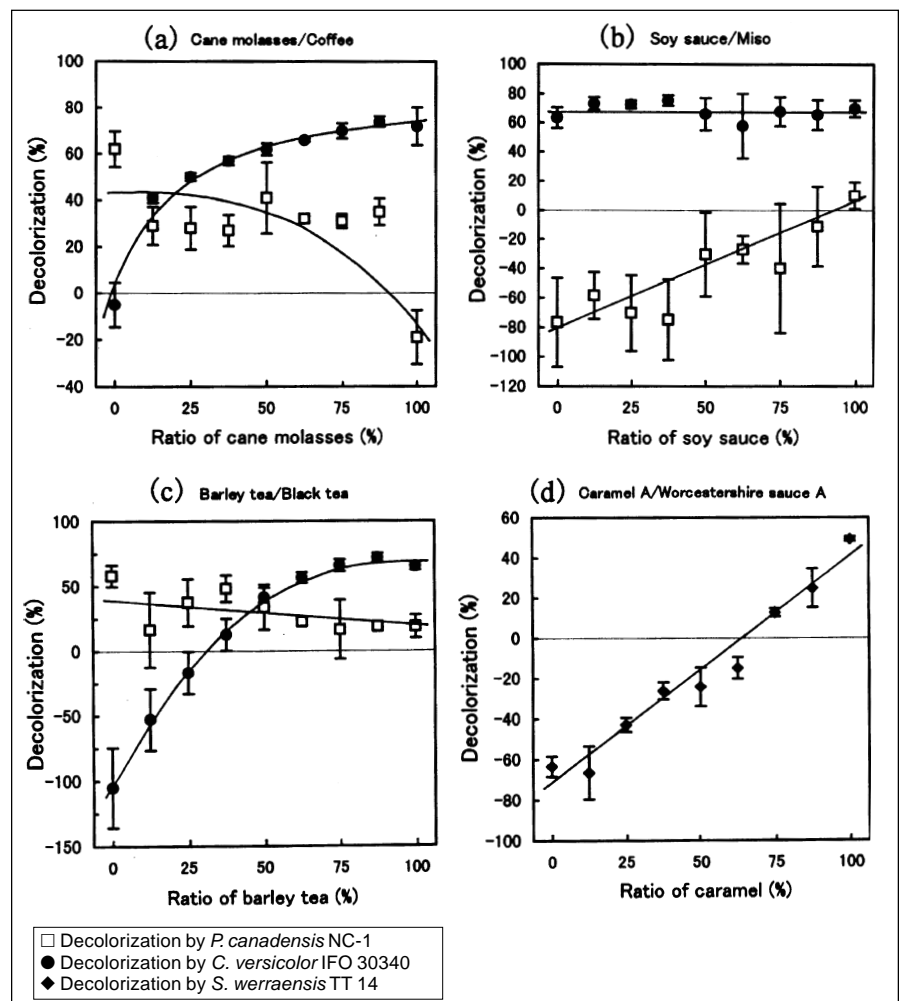

Fig. 2—Decolorization rate for mixtures of browned foods. (a)Mixture of instant coffee and cane molasses. (b) Mixture of soy sauce and miso. (c) Mixture of barley tea and black tea. (d) Mixture of caramel and Worcestershire sauce. The OD$_{500}$ of the mixture of instant coffee and cane molasses was adjusted to about 4, and that of the other mixtures was adjusted to about 1.

colorization rates for MCM and MBB by *C. versicolor* IFO 30340 were not linearly related to the mixing ratio of these foods, and these results do not agree with those for the mixtures of model brown pigments. These differences in decolorization rate between the model pigments and foods may have been caused by the effect of some components such as sugar, salt, or elements, and so on, in the foods on the decolorizing activity of the microbe.

Fig. 3—Effect of pH on the decolorizing activity of *P. canadensis* NC-1 and *C. versicolor* IFO 30340

Fig. 4—Effect of pre-incubation on the decolorizing activity of *P. canadensis* NC-1 and *C. versicolor* IFO 30340

Fig. 5—Successive decolorization by repeated culture of 3 types of immobilized mycelia

Effect of pH and pre-incubation on the decolorizing activity of fresh and immobilized mycelia

Since immobilized mycelia are easy to separate from a medium after the decolorization of brown pigments, immobilized mycelia of the 2 microbes were prepared and used for decolorizing wastewater.

The optimum pH value for immobilized mycelia of both *P. canadensis* NC-1 and *C. versicolor* IFO 30340 for decolorization was 5.5, however, the optimum pH values for fresh mycelia of these microbes were 3 and 3.5, respectively (Fig. 3). The results show that the media did not need to be acidified as much when immobilized mycelia were used for decolorizing the wastewater.

Since wastewater from the kitchen is sometimes high in temperature, the heat resistance of immobilized mycelia of these microorganisms was also investigated for their potential use in decolorizing browned wastewater from the kitchen. While the decolorization rates by fresh mycelia of *P. canadensis* NC-1 that had been pre-incubated at 30 to 55 °C for 10 min were almost same among the different media, the decolorization rate by immobilized mycelia of this microbe that had been pre-incubated at 55 °C for 10 min was higher. The fresh and immobilized mycelia of *C. versicolor* IFO 30340 that had been pre-incubated at 40 °C for 10 min had the strongest decolorizing effect (Fig. 4). The results show that these immobilized mycelia would be heat resistant to hot wastewater for a short time to exert their decolorizing activity.

Effect of treating immobilized mycelia with PEI and GA on the decolorizing activity

The effect of treating with PEI and GA on the decolorizing activity of immobilized mycelia of the 2 microbes is shown in Fig. 5. Since the decolorization by *P. canadensis* NC-1 was due to the adsorption of pigment to the surface of the mycelia (Terasawa and others 1994), the decolorizing activity almost disappeared by repeating the culture 3 times. On the other hand, the PG-treated immobilized mycelia of *P. canadensis* NC-1 decolorized the medium more than the untreated immobilized mycelia did; the half-life for the decolorizing activity of the untreated immobilized mycelia of this strain was in the 2nd-repeat culture, while that of the PG-treated immobilized mycelia was estimated to be in
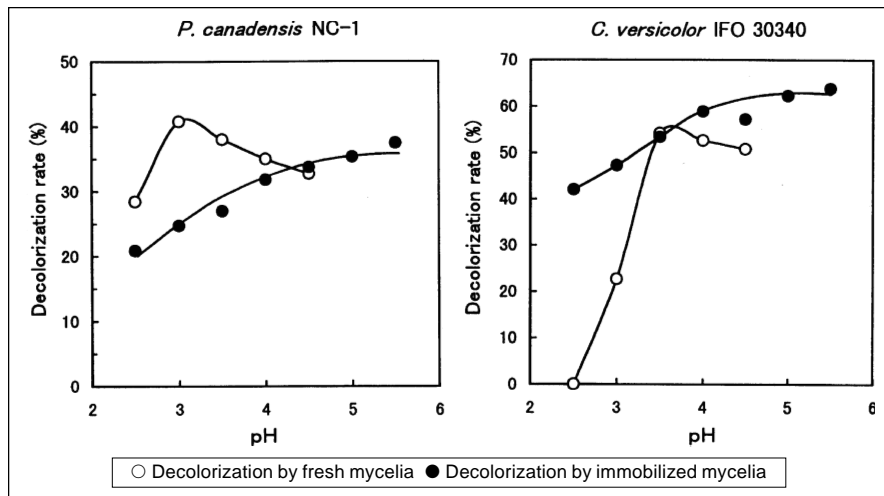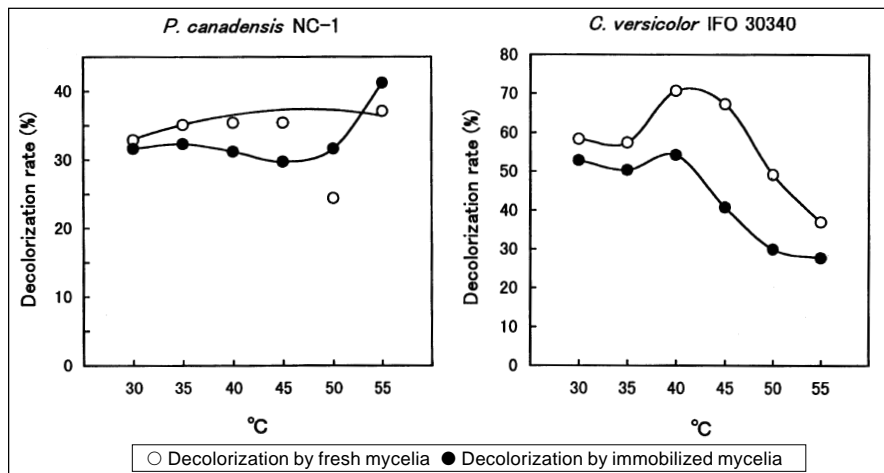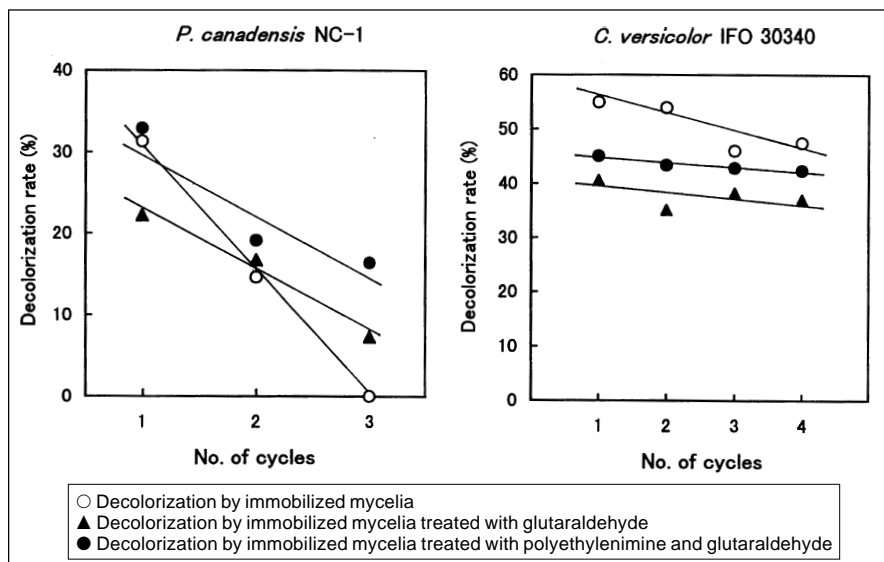
the 3rd-repeat culture.

The decolorizing activity of the untreated immobilized mycelia of *C. versicolor* IFO 30340 was higher than that of the PG-immobilized mycelia of this strain in the early stage, but they showed almost the same activity at the 3rd-repeat culture stage (Fig. 5). The half-life for the decolorizing activity of the immobilized mycelia of this strain was estimated to be at the 9th-repeat culture. Ohmomo and others (1985) have reported that the decolorizing activity of immobilized mycelia of *C. versicolor* Ps4a for molasses had decreased to 2/3 at the 6th-repeat culture. The half-life for the decolorizing activity of the PG-immobilized mycelia of *C. versicolor* IFO

30340 was estimated to be at the 24th-repeat culture (data not shown). It is apparent that the immobilized mycelia were stabilized by treating with PEI and GA, and the half-life for the activity was extended. Nakajima (1983) has reported that gelation occurred with the combination of GA and amino groups in the structure of PEI, resulting in a more dense cross-linkage in the gel of calcium alginate.

Decolorization of the mixtures of browned foods by immobilized mycelia

The immobilized mycelial gel without the PEI and GA treatment was destroyed by cultivation in some media, and the decolorization rate for these media did not increase. It seems that the immobilized mycelia needed the treatment with PEI and GA to stabilize the gel for decolorizing brown pigments.

The decolorization rates for the mixtures of browned foods by the PG-immobilized mycelia are shown in Fig. 6. With incubation method C, which involved a mixed culture of the PG-immobilized mycelia of *C. versicolor* IFO 30340 and *P. canadensis* NC-1, the decolorization rates for MCM and MBB were higher than those by the other incubation methods. These media were a mixture of Maillard reaction-type and phenol-type pigments, and they seemed to show the same tendency for decolorization. The decolorization rates for MCM and MBB by incubation method A, which successively involved the PG-immobilized mycelia of *C. versicolor* IFO 30340 and *P. canadensis* NC-1, were lower than those by the other incubation methods.

To decolorize a mixture of browned foods of the Maillard reaction type and phenol type, it would be necessary to first incubate the PG-immobilized mycelia of *P. canadensis* NC-1 to decrease the phenol-type pigments and then to incubate the PG-immobilized mycelia of *C. versicolor* IFO 30340 (incubation method B) because the phenol-type pigment in the media

turned darker brown by PPO of *C. versicolor* IFO 30340. However, a mixed culture of these mycelia (incubation method C) decolorized these media more than the other 2 incubation methods did.

The decolorization rate for MCM (the mixing ratio was 1:1) was about 60% for *C. versicolor* IFO 30340 and about 40% for *P. canadensis* NC-1 (Fig. 2a), and that for MBB (the mixing ratio was 1:1) was about 30% to 40% for both microbes (Fig. 2c). The decolorization rates for the mixtures of foods by mixed culture of the PG-immobilized mycelia of these microbes (Fig. 6) did not reflect those findings. The decolorization rate for MCM by mixed culture of the PG-immobilized mycelia of these microbes was lower than that by individual cultivation of these microbes, and the rate for MBB was higher than that by individual cultivation of these microbes.

Incubation method A decolorized more MSM than the other 2 methods did. This medium was decolorized by *C. versicolor* IFO 30340 and turned darker brown by *P. canadensis* NC-1 (Fig. 2b) because *P. canadensis* NC-1 turned the brown color of miso darker. Incubation method A for cultivating PG-immobilized mycelia of *C. versicolor* IFO 30340 at first decrease the miso pigment, and the total decolorization rate for this mixture seems to have been increased by successive incubation of the PG-immobilized mycelia of *P. canadensis* NC-1.

Conclusions

THE IMMOBILIZED MYCELIA OF *C. VERSIcolor* IFO 30340 and *P. canadensis* NC-1 showed the highest decolorizing activity at pH 5.5, and they also showed heat resistance to pre-incubation at 40 or 55 °C for 10 min. These results indicate that the immobilized mycelia would be very easy to use and would be useful to decolorize mixtures of brown pigments in foods. They would also be useful to prevent eutrophication of rivers because these microorganisms consumed the sugars and amino acids in wastewater.
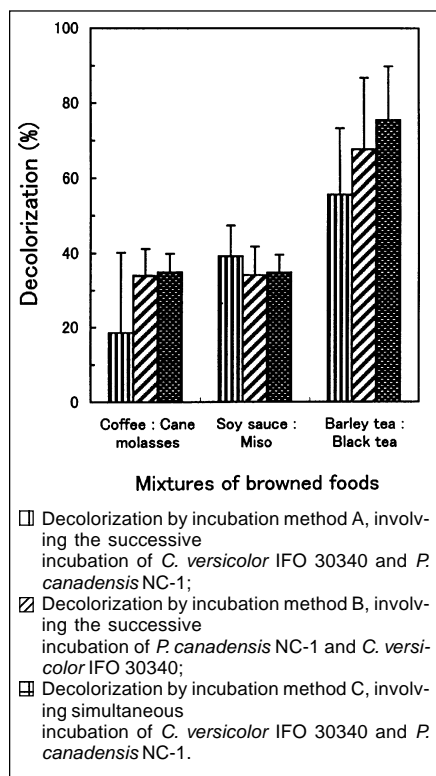
□ Decolorization by incubation method A, involving the successive incubation of *C. versicolor* IFO 30340 and *P. canadensis* NC-1;

▨ Decolorization by incubation method B, involving the successive incubation of *P. canadensis* NC-1 and *C. versicolor* IFO 30340;

▦ Decolorization by incubation method C, involving simultaneous incubation of *C. versicolor* IFO 30340 and *P. canadensis* NC-1.

Fig. 6—Decolorization rate for mixtures of browned foods by immobilized mycelia treated with polyethylenimine and glutaraldehyde

Materials and Methods

Preparation of the model melanoidins

The preparation procedure for non-dialyzable model melanoidins was reported previously (Terasawa and others 1996). Two types of reaction mixture were prepared: a mixture of 2 M D-glucose or D-xylose, 2 M glycine, and 2 M NaHCO$_3$, and a mixture of 1 M D-glucose, 1 M tryptophan, and 1 M NaHCO$_3$. Each mixture was refluxed by heating

for 2 h. The resulting brown-black reaction mixture was dialyzed by membrane filtration (Amicon PM 10) or by using seamless cellulose tubing (Viskase Sales Corp., Chicago, Ill., U.S.A.) against double-distilled water and then lyophilized.

Preparation of the phenol-type model brown pigment

The preparation procedure for the phenol-type model brown pigment was reported previously (Terasawa and oth-

ers 1996). Briefly, chlorogenic acid was oxidized with KIO$_3$ in double-distilled water for 3 d, and the resulting filtrate was dialyzed against double-distilled water and then lyophilized.

Browned food samples

Food samples of cane molasses, soy sauce, miso (fermented bean paste), caramel, barley tea, instant coffee, black tea, and Worcestershire sauce were purchased from local markets in Tokyo.

Test microorganisms and composition of the assay media

Streptomyces werraensis TT 14 (Murata and others 1992) was cultured in a starch-yeast medium consisting of 2% starch, 1% yeast, 0.3% NaCl, and 0.3% CaCO$_3$ at a pH of about 5.5. *Paecilomyces canadensis* NC-1 (Terasawa and others 1994) and *Coriolus versicolor* IFO 30340 were cultured in a glucose-peptone medium consisting of 5% glucose, 0.5% peptone, 0.1% KH$_2$PO$_4$, and 0.05% MgSO$_4 \cdot$ 7H$_2$O at pH 5.0 to 5.5.

To each medium was added a model melanoidin, oxidized chlorogenic acid, or a browned food (soy sauce, miso, barley tea, black tea, caramel, or Worcestershire sauce) to give an OD$_{500}$ for the medium of about 1. The media were also adjusted to an OD$_{500}$ of about 4 with instant coffee or cane molasses. The mixing ratio between each kind of colored material and food was changed from 1:7 to 7:1 based on the color intensity at 500 nm. Mixtures of brown-colored media were prepared in the following combinations: model melanoidin and oxidized chlorogenic acid, soy sauce and miso, barley tea and black tea, caramel and Worcestershire sauce, and instant coffee and cane molasses. The extraction method for barley tea and black tea was reported in a previous paper (Terasawa and others 1996).

Cultivation of microorganisms to decolor the mixture of synthetic brown pigments or browned foods

A seed culture (0.2 mL) of each microorganism was transferred into 4 mL of the assay medium and incubated at 37 °C for 5 d on a reciprocating shaker (120 oscillations/min, 5-cm stroke) for *Streptomyces werraensis* TT 14 and at 27 °C for 10 d on a reciprocating shaker (110 oscillations/min, 5-cm stroke) for *Paecilomyces canadensis* NC-1 and *Coriolus versicolor* IFO 30340. As a control, sterilized water was added to the assay medium instead of the seed culture. The composition of the seed media and cultivation methods were reported previously (Terasawa and others 1996).

All samples were cultured in duplicate, and the entire procedure was repeated 3 times. The culture broth was centrifuged (2000 × g for 10 min), and the supernatant was measured for OD$_{500}$, the decolorization rate being expressed as the percentage decrease in OD$_{500}$ compared with the control, and each value being shown as the mean ± SD of 3 cultivations.

Preparation of immobilized mycelia of *Paecilomyces canadensis* NC-1 and *Coriolus versicolor* IFO 30340

Four mL of the seed culture of *P. canadensis* NC-1 were transferred into 100 mL of the glucose-peptone medium and incubated at 27 °C for 5 d in a Sakaguchi flask (500 mL) on a reciprocating shaker (110 oscillations/min, 5-cm stroke). Ten mL of the seed culture of *C. versicolor* IFO 30340 were transferred into 100 mL of the glucose-peptone medium for cultivating for 4 d under the same conditions as those for *P. canadensis* NC-1. Each culture broth was homogenized in Polytron apparatus (Kinematica, Lucerne, Switzerland), and the mycelia were collected by filtration on No. 2 filter and washed with saline. The collected mycelia were immobilized within calcium alginate gel as described by Tamaki and others (1989). Briefly, the wet mycelia were mixed with sodium alginate (500 cps, Nakarai tesque Co., Kyoto, Japan), and the mixture was added drop-wise to a calcium chloride solution by a pipette (2.4 mm i.d.).

Cultivation of immobilized or fresh mycelia and measurement of the decolorization rate

To the glucose-peptone medium without KH$_2$PO$_4$ was added 0.5% instant coffee for cultivating the immobilized mycelia of *P. canadensis* NC-1, and 1.5% cane molasses was added to the same medium for cultivating the immobilized mycelia of *C. versicolor* IFO 30340.

Fresh mycelia were cultivated by using the same glucose-peptone medium with KH$_2$PO$_4$ added. The immobilized or fresh mycelia were prepared from the culture broth of 2 Sakaguchi flasks. Incubation of the immobilized or fresh mycelia of *P. canadensis* NC-1 was carried out at 27 °C for 6 d while occasionally stirring, and cultivation of the immobilized or fresh mycelia of *C. versicolor* IFO 30340 was carried out at 27 °C for 6 d on a reciprocating shaker (110 oscillations/min, 5-cm stroke). Each culture broth was centrifuged (2000 × g for 10 min). The resulting supernatant was measured for OD$_{500}$, and the decolorization rate expressed as the percentage decrease in absorbance compared with the initial value.

Effect of pH on decolorizing activity of fresh and immobilized mycelia

The above growth media were adjusted to pH 2.5 to 5.5 with HCl or NaOH. The same weight of fresh or immobilized mycelia was transferred into 28 mL of the medium for subsequent cultivation.

Effect of pre-incubation on the decolorizing activity of fresh and immobilized mycelia

The above growth media were to pH 3.5 or 5.5 for the fresh mycelia or immobilized mycelia, respectively. The same weight of fresh or immobilized mycelia was transferred into 4 mL of the medium. The mycelia were pre-incubated at 30 to 55 °C for 10 min and then immediately cooled to less than 20 °C for subsequent cultivation.

Effect of PEI and GA treatments of the immobilized mycelia on the decolorizing activity

The immobilized mycelia described above were further treated with the following reagents as described by Nakajima (1983): (1) treated with glutaraldehyde (GA) (G-immobilized mycelia), and (2) treated with both polyethylenimine (PEI) and glutaraldehyde (GA) (PG-immobilized mycelia). These mycelia were incubated in 50 mL of the medium at pH 5.5 in a 500-mL Sakaguchi flask, the settings of the reciprocating shaker for cultivating *C. versicolor* IFO 30340 being 80 oscillations/min with a 3-cm stroke. These immobilized mycelia were separated from each assay medium (the glucose-peptone medium without KH$_2$PO$_4$ with added instant coffee or cane molasses as already described) after 6 d of cultivation and then transferred into a fresh medium for cultivation under the same conditions.

Decolorization of the mixtures of browned foods by the immobilized mycelia

The OD$_{500}$ of the glucose-peptone medium without KH$_2$PO$_4$ was adjusted to about 1 by adding instant coffee, cane molasses, soy sauce, miso, barley tea, or black tea, and the pH of each of these media was adjusted to 5.5. By mixing equivolumes of 2 kinds of these brown-colored media, 3 types of decolorization assay media were prepared: instant coffee and cane molasses, soy sauce and miso, and barley tea and black tea.

The same weight of PG-immobilized mycelia was transferred into 40 mL of each assay medium. Incubation of these mycelia was carried out by the following 3 incubation methods: With

method A, the PG-immobilized mycelia of *C. versicolor* IFO 30340 was first incubated for 6 d in a 100-mL Erlenmeyer flask, and then the mycelia were removed by filtration. Freshly prepared PG-immobilized mycelia of *P. canadensis* NC-1 were added to the filtrate and incubated for 6 days. With method B, the order of cultivation for method A with these microbes was reversed, that is, 1st with the PG-immobilized mycelia of *P. canadensis* NC-1 and 2nd with that of *C. versicolor* IFO 30340 under the same cultivation conditions. With method C, the PG-immobilized mycelia of both microbes were added simultaneously in each assay medium and incubated for 6 d on a reciprocating shaker. Each cultivation was repeated 3 times, and each value shown is the mean ± SD.

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