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# Isolation of a Microorganism to Oxidize 5-Hydroxymethylfurfural

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**A microbe of *Pseudomonas syringae* pathovar *cannabina* SF 4-17 converting 5-hydroxymethylfurfural (HMF) to 5-hydroxymethyl-2-furancarboxylic acid (HMFA) was isolated from the soil. When HMF was incubated with this microbe for 1 day, the absorbance at 283 nm, the absorption maximum of HMF, decreased by 99%. This microbe also decomposed furfural by about 97%, but not 2-furancarboxylic acid or acetylfuran. After food samples with added 0.01% HMF were incubated with *P. syringae* SF 4-17, the amount of HMF in each sample was estimated from the absorbance at 283 nm before and after cultivation. The amounts of HMF of heated orange juice and caramel were estimated to be about 2 mg% and 900 mg%, respectively.**

Keywords: 5-hydroxymethylfurfural (HMF), microbial oxidation, screening, *Pseudomonas syringae* pathovar *cannabina*

Brown pigments in foods are formed by various interactions of food components. The increase of brown pigments and intermediates in browning reactions during food processing or preservation have a definite effect on the quality of the food. However, these brown pigments are heterogeneous polymers and difficult to chemically analyze. Our group has screened for microbes decolorizing the brown pigments to develop a new biological method to analyze the pigments (Murata *et al*., 1992; Terasawa *et al*., 1994; Terasawa *et al*., 1996).

5-Hydroxymethylfurfural (HMF) is one of the major intermediates of the Maillard reaction or caramelization, and is formed during the heating or preservation of foods. HMF is considered one indicator of the browning reaction and quality parameters of foods, especially for the flavor deterioration of such alcoholic drinks as wine, whisky, rum, and sake, and of such foods as fruit juice and caramel. The amount of HMF in foods is often measured by colorimetric method or HPLC method. However, there is no report on the analytical method of HMF using microbes.

During the screening of microbes degrading melanoidins or intermediates of the Maillard reaction, we isolated a soil strain of bacteria degrading HMF. The strain decomposes HMF and furfural, but not other furan derivatives or melanoidins. In this paper, we report the microbial conversion of HMF into 5-hydroxymethyl-2-furancarboxylic acid (HMFA, Fig. 1) and the determination of HMF in some foods using the microbial oxidation of HMF.

## Materials and Methods

*Microbes* Soil microbes were isolated using a modified Pridham medium consisting of 0.2% HMF (Wako Pure Chemical Industries, Osaka), 0.2% $(NH_4)_2SO_4$, 0.2% $CaCO_3$, 0.1% $K_2HPO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, and 1.2% agar (pH 7.0). Soil samples (0.1 g) were suspended in 10 ml of sterile water and sonicated for 30 s. Each suspension was allowed to stand for about 30 min to 1 h, and the supernatant was spread on the modified Pridham medium, which was incubated at 27°C for 2–7 days.

*Screening of microbes degrading HMF* Isolated microbes were cultured in Davis medium consisting of 0.7% $K_2HPO_4$, 0.2% $KH_2PO_4$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, and 0.2% glucose at 27°C for 1–2 days. This seed culture (0.2 ml) was transferred into 3 ml of modified Davis medium, in which glucose had been replaced by 0.1% HMF, and cultured at 27°C for 3 days. The culture was done in a test tube ($\phi$18×180 mm) on a reciprocating shaker (120 oscillations/min, 5 cm stroke). After the culture was centrifuged at 2000×*g* for 10 min, the absorbance at 280 nm ($OD_{280}$) of the broth supernatant was measured. The decrease ratio of $OD_{280}$ was calculated against the control culture in which no microbes had been inoculated. The $OD_{280}$ of the broth supernatant with no addition of HMF was almost zero. Cultured broths showing a large decrease in $OD_{280}$ were applied to TLC (silica gel $60F_{254}$, Merck), which was developed with *n*-butanol/acetic acid/ water (3:1:2, v/v/v). Spots were detected by spraying $H_2SO_4$/ethanol/water (8:1:1, v/v/v).

*Identification of a soil microbe* Strain SF 4-17 grown in the Davis medium was morphologically observed under a microscope (Olympus VANOX, model AHB-LB, Tokyo). The general morphological and physiological characteristics were investigated according to "Bergey's Manual of Determinative Bacteriology" (Holt *et al*., 1994) and "Bergey's Manual of Systematic Bacteriology" (Krieg & Holt, 1984). Utilization of the carbon source by the microbe was evaluated by the turbidity ($OD_{600}$) of a liquid medium consisting of 0.1% $NH_4NO_3$, 0.1% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% KCl, and the carbon source, the concentration of which was 1% for sugars and sugar derivatives, and 0.2% for aromatic compounds. Each medium was cultured at 27°C for 1–3 days on a reciprocating shaker.

*Time course for HMF degradation by P. syringae SF 4-17* A loopful of the bacteria from a slant culture of *P. syringae* SF 4-17 was transferred into a seed medium (the Davis medium without glucose) and incubated at 27°C for 1 day to give a seed

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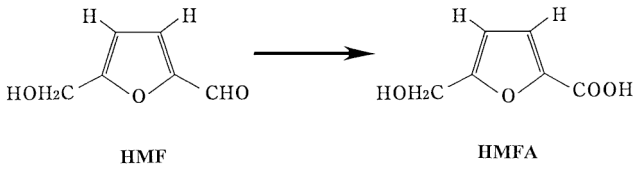


**Fig. 1.** Conversion of 5-hydroxymethylfurfural (HMF) into 5-hydroxymethyl-2-furancarboxylic acid (HMFA) by *P. syringae* SF 4-17.

culture. This seed culture was transferred into 100 ml of the Davis medium in which glucose had been replaced by 0.1% HMF. The culture was carried out at 27°C in a 500-ml Sakaguchi flask on a reciprocating shaker. The broth was measured for $OD_{600}$ to represent the growth of the microbe. The broth supernatant obtained by centrifugation (2000×*g* for 10 min) was then measured for $OD_{280}$ and $OD_{250}$, and its pH value every 6 h.

*Isolation of the converted product of HMF* The seed culture of *P. syringae* SF 4-17 (3 ml) was transferred into 100 ml of the Davis medium in which glucose had been replaced by 0.1% HMF. Culture was carried out at 27°C for 2 days in a Sakaguchi flask (500 ml) on a reciprocating shaker. The supernatant of the cultured broth was concentrated *in vacuo* and applied to an ion-exchange column (ϕ13×150 mm, Amberlite IR-120 [$H^+$], Organo, Tokyo), which was developed by distilled water. The fractions containing the converted compound, which was monitored by $OD_{250}$, were concentrated *in vacuo* and then applied to a Sephadex G-10 column (ϕ8×120 mm), which was developed by distilled water. The fractions containing the converted compound were collected and concentrated *in vacuo*, the yield of the converted product being 40 mg.

*Instrumental analyses and properties of the converted product of HMF* Spectroscopic measurements to identify the converted product of HMF were taken with the following instruments: Hitachi 200-20 (UV), Jeol JNM-GX 270 (NMR), and Hitachi M80 (EI-MS).

*Preparation of HMFA* HMFA was prepared from HMF by oxidation with $Ag_2O$ according to the procedure for the oxidation of aldehydes to acids (Thomason & Kubler, 1968). HMF (7.9 mmole) was added to a suspension of $Ag_2O$ (7.9 mmole; Sigma, St. Louis, MO) in 100 ml of 4% NaOH. The mixture was stirred at 60°C for 30 min. After filtration, the solution was washed three times with ether, the resulting residue was adjusted to pH 2 and extracted with ether. The extract was evaporated to yield HMFA. Data of the instrumental analyses were identical with those for the converted product of HMF.

*Oxidation of furan derivatives* Glucose in the Davis medium was replaced by 0.1% of HMF, furfural, furfuryl alcohol, 2-furancarboxylic acid, 2-acetylfuran, or 2-acetyl-5-methylfuran. Each medium (3 ml) was inoculated with 0.2 ml of the seed culture of *P. syringae* SF 4-17 and then incubated at 27°C for 3 days on a reciprocating shaker. As a control, the medium not inoculated was incubated under the same conditions. The absorbance of the supernatant of cultured broth was measured at the wavelength of absorption maximum of each furan derivative. The decrease ratio was calculated against the control culture in which no microbe was inoculated. Cultured broths were also checked by the TLC as already described.

*Determination of HMF in food samples* HMF in food samples was determined by the microbial method in which HMF is oxidized by *P. syringae* SF 4-17 and then residual HMF was determined by $OD_{283}$, the absorption maximum of HMF.

The Davis medium (2.8 ml) free of glucose and containing 0.01% HMF (standard addition) and a food sample was inoculated with a seed culture (0.2 ml) of *P. syringae* SF 4-17. No seed culture was inoculated in the control culture. Each culture in a test tube was incubated at 27°C for 1–2 days on a reciprocating shaker. The supernatant of the cultured broth was filtered by a Molcut II ultra-filter (Millipore, Cut off Mw 5000) and measured for $OD_{283}$. The HMF amount in a food sample was calculated as follows;

$$\text{Add+Food+Back=Tbe},$$
$$(1\text{–D})\times(\text{Add+Food})+\text{Back=Taf}.$$

where Add=HMF amount of standard addition, Food=HMF amount in a food sample, Back=amount of a background having the absorption at 283 nm, Tbe=apparent total amount of HMF before microbial cultivation, D=decreasing ratio of HMF by the microbial cultivation (0.990), Taf=apparent total amount of residual HMF after microbial cultivation. In this microbial cultivation, "D" is assumed to be 0.990, which was determined from the experiment without food samples. "Back" is assumed to be unchanged by the microbial conversion. "Tbe" and "Taf" are assumed to contain a material overlapping with HMF at 283 nm. "Tbe" and "Taf" were determined from the $OD_{283}$ before and after cultivation, respectively. Then "Food" and "Back" were calculated from the formulae above described.

All samples were cultured in duplicate and the cultivation was repeated three times.

## Results and Discussion

*Screening and identification of strain SF 4-17* Eleven strains of bacteria were isolated from soil, and one strain (SF 4-17) showed a high HMF-decreasing activity of more than 90%. When the supernatant of the cultured broth was analyzed by a silica gel TLC, a new spot with a lower $R_f$ value (0.56) than that of HMF (0.68) was detected.

The taxonomic properties of strain SF 4-17 are as follows: gram-negative aerobic motile, rods, O-F test was oxidative, the color on the Pridham medium was white-pale yellow, and fluorescence was observed by irradiating with UV light. The other characteristics are summarized in Table 1. Based on the taxonomic properties, strain SF 4-17 is believed to belong to the *Pseudomonas* genus (Hasegawa, 1995). From the utilization of carbon sources by this strain (Table 2), it was identified as *Pseudomonas syringae* pathovar *cannabina* according to "Bergey's Manual of Determinative Bacteriology" (Holt *et al.*,

**Table 1.** Characteristics of strain SF 4-17.

| Property | Response[a)] |
|---|---|
| Fluorescent, diffusible pigment | + |
| Diffusible, non-fluorescent pigment | – |
| Non-diffusible, non-fluorescent pigment | – |
| Growth at 41°C | – |
| Growth at pH 4 | – |
| Oxidase reaction | – |
| Denitrification | – |
| Hydrolysis of gelatin | – |
| Hydrolysis of starch | – |

[a)]+ active, – inactive.

**Table 2.** Utilization of carbon sources by strain SF 4-17.

| Carbon source | Utilization[a)] |
|---|---|
| D-Glucose | + |
| D-Trehalose | – |
| Geraniol | – |
| β-Alanine | ± |
| L-Arginine | + |
| D(–)-Tartrate | – |
| meso-Erythritol | – |
| DL-Homoserine | – |
| D-Mannitol | – |
| Betaine | + |
| myo-Inositol | – |
| D-Sorbitol | – |
| D-Quinate | – |

[a)]+ utilized, – not utilized.

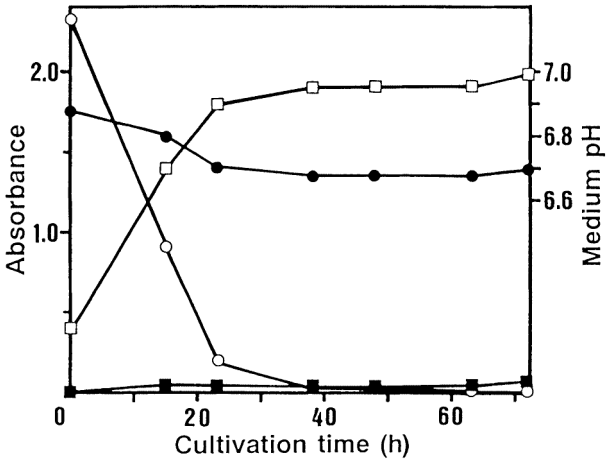


**Fig. 2.** Time-course for the conversion of HMF into HMFA by *P. syringae* SF 4-17.
○: 280nm, □: 250nm, ■: 600nm, ●: pH.

1994) and "Bergey's Manual of Systematic Bacteriology" (Krieg & Holt, 1984). Although *Pseudomonas syringae* pathovar *cannabina* did not utilize β-alanine according to these reports, strain SF 4-17 did, this being the only difference. Another species, *P. viridiflava* was similar to strain SF 4-17. However, there are several differences in the carbon source utilization between these species, *P. viridiflava* utilizing erythritol, D-tartrate and quinate, and SF 4-17 not utilizing them.

*Identification of the converted product of HMF* After HMF was incubated with *P. syringae* SF 4-17, the converted compound was purified by chromatographies of Amberlite IR-120 and Sephadex G-10. The converted compound was obtained as white powder. This compound was water-soluble and had an UV absorption maximum at 250 nm. The instrumental data were as follows; UV λ max ($H_2O$) nm (ε): 250 (133,000); $^1$H-NMR δ ($D_2O$): 4.45 (2H, s), 7.10 (1H, d, *J*=3.7), 6.38 (1H, d, *J*=3.7); $^{13}$C-NMR $\delta_C$ ($D_2O$): 162.1, 158.3, 143.9, 120.0, 110.3, and 55.8; EI-MS(*m/z*): 142($M^+$), 97($M^+$-COOH ).

In the $^1$H-NMR analysis, this compound did not show a proton signal of an aldehyde, while HMF showed a signal at δ 9.28 ppm; other signals of this compound were similar to those of HMF. The $^{13}$C-NMR spectrum of this compound was also similar to that of HMF. However, the signal of a carboxyl group was detected at $\delta_C$ 162.1 ppm and the signal of an aldehyde was not detected in this compound. These results suggest that the aldehyde group of HMF is oxidized to a carboxyl group. This was ascertained from the MS (*m/z* 142 ($M^+$), 97 ($M^+$-COOH)). We therefore prepared HMFA by the chemical oxidation of HMF. The spectral data of HMFA coincided with those of the converted compound, and the converted compound was thus identified as HFMA (Fig. 1).

*Time-course for the conversion of HMF into HMFA by P. syringae SF 4-17* A typical time-course for the conversion of HMF (0.1%) by strain SF 4-17 is shown in Fig. 2. The $OD_{280}$ and $OD_{250}$ were used as indicators of HMF and HMFA, respectively. The $OD_{280}$ decreased by about 99.0% after 24 h, showing that the conversion of HMF into HMFA occurred within 24 h. The increase in $OD_{600}$, which is an indicator of growth of the bacteria, was minimal, and the medium pH value remained almost constant during the cultivation. The decreasing ratios of HMF after 1 day cultivation were about 99% in the presence of 0.1% or lower HMF concentration and about 13% in 0.2% of HMF concentration with cultivation for 1 day. Therefore, the HMF concentration in food only requires an adjustment of less than 0.1% to determine HMF by the following microbial method.

*Substrate specificity of oxidation of furan by P. syringae SF 4-17* The oxidations of several furan derivatives by *P. syringae* SF 4-17 were compared. The decrease of each compound was estimated by the decrease in absorbance at the absorption maximum of each compound (Table 3). Furfural and furfuryl alcohol decreased by 97% and 44%, while decreases of 2-furancarboxylic acid, 2-acetylfuran, and 2-acetyl-5-methlfuran were only slight. Straight chain aldehydes such as acetaldehyde were not decreased by this microbe, as was also true of some melanoidins (data not shown).

*Determination of HMF by the microbial method* As there is no report on HMF determination using a microbial method, we examined the possibility of applying *P. syringae* SF 4-17 for this purpose in foods.

The $OD_{283}$ of the medium with added 0.01% HMF decreased by 99.0±0.2% when cultivated with *P. syringae* SF 4-17. We estimated the amount of HMF in heated orange juice and caramel

**Table 3.** Decrease in the maximal absorbance value of furan derivatives by *P. syringae* SF 4-17.

| Furan derivatives | Maximal absorbance (nm) | Decrease (%) |
|---|---|---|
| HMF | 283 | 99.0±0.2 |
| Furfural | 277 | 96.8±0.1 |
| Furfuryl alcohol | 216 | 43.6±1.0 |
| 2-Furancarboxylic acid | 245 | –2.8±1.4 |
| 2-Acetylfuran | 275 | 2.4±0.9 |
| 2-Acetyl-5-methylfuran | 290 | 1.1±1.5 |

**Table 4.** HMF contents (mg%) in foods determined from the decrease in $OD_{283}$ of the cultured supernatant and by the HPLC method.

| Food samples | Decrease in $OD_{283}$ | HPLC method |
|---|---|---|
| Heated orange juice A[a)] | 1.7±0.22 | 4.6±0.01 |
| Heated orange juice B[a)] | 2.6±0.06 | 4.3±0.15 |
| Caramel A | 850 ±4.9 | 900 ±1.1 |
| Caramel B | 940 ±1.8 | 1000 ±0.44 |

Values are shown as mean±SD. [a)]heated at 120°C for 15 min.

from the decrease in $OD_{283}$ after this cultivation, and found them to be about 2 mg% and 900 mg%, respectively. These data were compared with the HMF contents determined by the conventional direct HPLC injection method. The values of caramel samples with a high amount of HMF were similar by both methods, the difference being less than 10%. However, the absorbance was affected by slight turbidity even after ultra-filtration, and the HMF contents in heated orange juice samples determined by microbial method were much lower than those determined by the conventional HPLC method (Table 4). According to these results, the microbial method was effective in determining HMF in food such as caramel in which the amount is high and there is no turbidity. Using this method, the final concentration of HMF in culture medium must be adjusted to between 0.01–0.1%. On the other hand, to determine the HMF in a food or beverage such as fruit juice which has a low amount of HMF and is turbid, a column may have to be used to remove the turbidity. This microbial method is expected to improve the accuracy and sensitivity because of the use of an enzyme of this microbe.

There has been a report on the conversion of furfural into 2-furancarboxylic acid by various bacteria (Yamatoya *et al*., 1969), but our study is the first to report the microbial production of HMFA from HMF in high yield and utilization of a microbial treatment to determine HMF in foods. There are some problems with this method, however, the decreasing ratio of HMF is not 100%, and the technique is not a time-saving one. Yet this report does suggest that an enzyme method for HMF analysis is possible. It is expected that a purified enzyme of this strain can be utilized for the convenient determination of HMF in food samples. Further, the strain may prevent browning of food by removing HMF and furfural.

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