Biosynthesis of 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone by Yeasts

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4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF) is a character-impact compound of shoyu (Japanese soy sauce). This paper describes the biosyntheses of HEMF by yeasts. As precursors of the compound, intermediates of the pentose–phosphate cycle such as D-ribulose 5-phosphate barium salt, the combination of D-xylulose 5-phosphate sodium salt and D-ribose 5-phosphate, and D-sedoheptulose 7-phosphate barium salt are discussed. The pentose–phosphate cycle is necessary for yeasts to produce HEMF.

Shoyu is the Japanese name for soy sauce. It is made mostly by fermentative methods and mainly used as an all-purpose seasoning in Japanese cuisine. Five different types of shoyu are available in Japan: (1) Koikuchi, (2) Usukuchi, (3) Tamari, (4) Shiro, and (5) Saishikomi. Of all shoyu consumed in Japan, 85% is of the Koikuchi type. Koikuchi is made from a mixture of soybeans and wheat kernels of almost equal amounts and is characterized by deep reddish brown color and strong and pleasant aroma.

The first attempt to identify the flavor components of shoyu was made by Tahara (1887). Since then, many studies on shoyu flavor have been reported. To date, over 280 volatiles have been detected from shoyu, which include 168 new flavor components by us using gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC–MS) techniques (Nunomura et al., 1976a,b, 1977a,b, 1978, 1979, 1980, 1981, 1984; Nunomura and Sasaki, 1986; Sasaki and Nunomura, 1981; Yokotsuka 1986).

HEMF was isolated from shoyu for the first time from natural products (Nunomura et al., 1976b). HEMF represents the intense sweet flavor reminiscent of shoyu. It is presented in a high level in shoyu (ca. 50–100 ppm) and has the lowest flavor threshold in water (less than 0.04 ppb; Ohloff, 1978) of any compound found in shoyu. The odor value of HEMF is calculated to be more than 5 million. Moreover, its existence was found only in shoyu and not in other foods. Accordingly, HEMF significantly contributes to the shoyu flavor, and it is definitely a character-impact compound of shoyu.

The homologues 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 4-hydroxy-5-methyl-3(2*H*)-furanone (HMMF) have also been isolated from shoyu. The content of HDMF in shoyu is very low (ca. 10 ppm), but HMMF is present in a high level (100 ppm). HDMF was found in many kinds of foodstuff such as pineapples (Rodin et al., 1965), strawberries (Re et al., 1973), roasted almonds (Takei and Yamanishi, 1974), and beef broth (Tonsbeek et al., 1968). It was also found in various model systems such as the degradation of fructose (Shaw et al., 1968), the pyrolysis of D-glucose (Fagerson, 1969; Heyns et al., 1966; Johnson et al., 1969), and the roasting of alanine mixed with rhamnose (Shaw and Berry, 1977). HMMF was also found in many kinds of foodstuff such as beef broth (Tonsbeek et al., 1968), wild raspberries (Honkanen et al., 1980), and guava fruits (Idstein and Schreier, 1985) and in various model systems such as the degradation of L-dehydroascorbic acid (Velisek et al., 1976) and the roasting of a glycine and xylose mixture (Nursten et al., 1983). The formation of these compounds has been found to be caused by a nonenzymatic reaction. HDMF and HMMF in shoyu are probably formed from sugars and amino acids during a heating process such as the heat treatment of raw materials and the pasteurization of the liquid part of mash.

On the other hand, HEMF is presumed to be produced by shoyu yeasts (Sasaki et al., 1984). The present study reports the biosynthesis of HEMF by yeasts.

MATERIALS AND METHODS

Microorganisms. The yeast strains used in this investigation were from the laboratories of Kikkoman Corp.

Chemical Reagents. D-Glucose 6-phosphate monosodium salt, D-glucose 6-phosphate disodium salt, 6-phosphogluconic acid barium salt, D-ribulose 5-phosphate barium salt, D-xylulose 5-phosphate sodium salt, D-ribose 5-phosphate barium salt, D-sedoheptulose 7-phosphate barium salt, D-fructose 6-phosphate barium salt, D-erythrose 4-phosphate sodium salt, D-ribulose, and D-xylulose were purchased from Sigma Chemical Co. All other reagents were of reagent grade or the best grade available commercially.

Media. *Basal Medium.* One hundred grams of shoyu koji was dissolved in 900 mL of distilled water and incubated in a water bath at 58 °C for 6 h. The mixture was boiled for 15 min. The hot liquid was vacuum filtered through filter paper and sterilized under a pressure of 15 psi at 120 °C for 15 min. The pH of the filtrate was 6.5.

Medium A (Medium for Shoyu Yeasts). This medium was prepared by adding 17% NaCl (w/v) and 5% glucose (w/v) to the above basal medium, and then the pH was adjusted to 4.8 with diluted lactic acid. Each of the sugars or the phosphates was added to the basal medium at concentrations ranging from 0.5 to 6.0% (w/v). The medium was sterilized by passage through a 0.22-μm membrane filter.

Medium B (Medium for the Other Yeasts). This medium was prepared by adding 5% glucose to the basal medium and fillered through a membrane filter (pore size 0.22 μm). Then the medium was supplemented with 2% (w/v) of the sugar phosphates.

Cultivation. Five hundred microliters of the medium was dispensed into each 10-mL screw-cap vial. The vials were inoculated with a 10-μL cell suspension from the starter culture grown at 30 °C for 10 days without shaking. The vials were loosely capped and incubated stationarily at 30 °C for 12 days.

Determination of Volatiles. (*a*) *Extraction Procedures.* The method used was essentially the same as that described previously by the authors (Sasaki and Nunomura, 1980). Namely, 5.0 μL of 1-pentanol stock solution (10 mg in 10 mL of ethanol) was added to the 500-μL fermentation broth as the internal

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Table I. HEMF Production from Individual Sugars and Sugar Phosphates[a]

no.	reagent added to medium	EtOH,[b] %	HEMF,[c] ppm
1	DL-glyceraldehyde	1.85	5.25
2	DL-glyceraldehyde dimer	1.80	4.61
3	dihydroxyacetone dimer	3.23	2.88
4	D-erythrose	2.84	3.65
5	DL-glyceraldehyde + D-erythrose	2.07	4.92
6	DL-glyceraldehyde dimer + D-erythrose	1.59	2.92
7	glycerol + D-erythrose	2.73	3.07
8	dihydroxyacetone dimer + D-erythrose	2.66	2.36
9	L-arabinose	2.84	2.40
10	L-lyxose	3.12	2.61
11	D-ribose	2.62	2.33
12	D-ribulose	3.22	2.66
13	D-xylose	3.03	2.35
14	D-allose	2.87	2.73
15	D-altrose	3.11	2.36
16	D-fructose	3.36	2.54
17	D-galactose	2.90	2.82
18	D-glucose	3.69	2.30
19	D-gulose	3.55	2.64
20	D-idose	3.68	2.37
21	D-mannose	3.72	2.92
22	D-psicose	2.84	2.62
23	L-rhamnose	2.49	2.92
24	D-sorbose	2.94	2.61
25	D-tagatose	3.13	2.94
26	D-talose	2.73	2.63
27	D-sedoheptulose anhydride	2.90	2.42
28	D-sedoheptulose 7-phosphate barium salt	3.30	15.26
29	D-sedoheptulose 1,7-diphosphate sodium salt	3.22	3.03
30	D-cellobiose	2.81	4.12
31	D-gentiobiose	3.00	2.38
32	D-isomaltose 75% in water	3.20	2.39
33	D-lactose	1.37	3.05
34	D-maltose	3.42	2.72
35	D-melibiose	2.47	2.76
36	D-nigelose	3.03	2.39
37	D-sucrose	2.32	3.04
38	D-trehalose hydrate	2.53	3.07
39	isomaltose	2.48	2.78
40	maltotriose	2.63	2.54
41	panose	2.96	2.70
42	raffinose	2.44	2.28
43	stachyose	2.36	3.00

[a] The medium consisted of the following additions to the aqueous extract of shoyu koji (w/v): NaCl 17%, glucose 5%, sugar or sugar phosphates 2.0%. Organism and culture: *Z. rouxii* No. 210, ATCC 13356, was cultured in a 10-mL vial containing 0.5 mL of medium at 30 °C for 12 days in stationary culture. [b] Ethanol. [c] 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone.

standard. The sample was saturated with NaCl and blended with 500 μL of glass-distilled methyl acetate for 10 min in a Waring blender. The sample was then centrifuged at 3000 rpm for 10 min at 5 °C on a Hitachi centrifuge (Model 05 PR-22). One microliter of the organic layer was directly analyzed by GC.

(*b*) *Capillary Gas Chromatography.* Quantitative GC analysis was carried out on a Hewlett-Packard 5890A gas chromatography equipped with a flame ionization detector. A SIC Chromatocorder 12 integrator was used to determine the peak areas. A fused silica capillary column (Quadrex 0.25 mm i.d. × 50 m, film thickness 0.25 μm) coated with a FFAP phase was used. The oven temperature was held at 40 °C for the first minute and then programmed to 200 °C at 3 °C/min and held at 200 °C for 60 min. The column inlet pressure was 130 kPa of He. The flow rate of the carrier gas, helium, was 1.2 mL/min. The other operating conditions were as follows: make-up helium flow 30 mL/min; detector hydrogen flow 30 mL/min; injector and detector temperature 200 °C. The samples were injected in the splitless mode with purge time of 0.8 min.

Table II. HEMF Production from Sugar Phosphates[a]

no.	reagent added to medium	EtOH,[b] %	HEMF,[c] ppm
1	D-glucose 6-phosphate sodium salt	2.86	5.95
2	D-glucose 6-phosphate disodium salt	2.89	4.59
3	6-phosphogluconic acid barium salt	3.34	3.49
4	D-ribulose 5-phosphate barium salt	1.52	28.90
5	D-xylulose 5-phosphate sodium salt D-ribose 5-phosphate barium salt	1.83	44.34
6	D-fructose 6-phosphate barium salt D-erythrose 4-phosphate sodium salt	0.77	0.00
7	control	2.68	4.23

[a] The medium consisted of the following additions to the aqueous extract of shoyu koji (w/v): NaCl 17%, glucose 5%, sugar phosphate 2%. Organism and culture: *Z. rouxii* No. 210, ATCC 13356, was cultured in a 10-mL vial containing 0.5 mL of medium at 30 °C for 12 days in stationary culture. [b] Ethanol. [c] 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone.

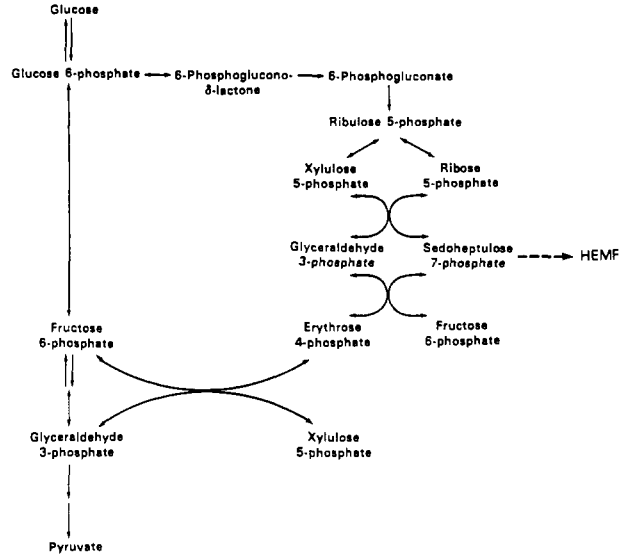

Figure 1. Pentose phosphate pathway and glycolysis.

(*c*) *Capillary Gas Chromatography–Mass Spectrometry.* A Hitachi Model M-80B combination mass spectrometer–gas chromagraph (Hewlett-Packard Model 5890A) equipped with Hitachi Model M-6010 and 1011A data system was used under the following conditions: ionization voltage 70 eV; emission current 80 mA; ion accelerate voltage 3100 V; ion source temperature 200 °C. The gas chromatographic conditions were as described above, with the capillary direct interface being heated to 280 °C.

(*d*) *Determination of Ethanol.* Quantitative determination of ethanol was accomplished by the GC method of Hamano et al. (1971). The sample was diluted 10-fold with 10% 1-propanol aqueous solution. The gas chromatograph was a Shimadzu 8A with a flame ionization detector and a Shimadzu Model AOC-8 autosampler. The integrator was a Shimadzu Model C-R3A. The column was 3.0 mm i.d. × 100 cm glass packed with 80–100 mesh Porapack Q. The other operating conditions were as follows: column oven 135 °C, injector and detector temperature 210 °C, nitrogen gas flow rate 40 mL/min, injection volume 1.0 mL.

RESULTS AND DISCUSSION

According to the author's previous research (Sasaki et al., 1984), the concentration of HEMF increased with the growth of yeasts in shoyu mash brewing. Moreover, HEMF was not formed from the filter-sterilized liquid part of mash before fermentation. From these results, HEMF was predicted to be biosynthesized by shoyu yeasts. From the standpoint of the chemical structure of HEMF, its

Table III. Influence of Intermediates of the Pentose–Phosphate Cycle on HEMF Production by *Z. rouxii* No. 210, ATCC 13356, in Stationary Culture[a]

no.	reagent added to medium	concn, %	EtOH,[b] %	HEMF,[c] ppm	HMMF,[d] ppm
1	D-glucose 6-phosphate monosodium salt	2.0	2.10	3.74	20.34
2		4.0	1.94	4.12	20.03
3		6.0	1.93	3.76	20.29
4	D-glucose 6-phosphate disodium salt	2.0	1.92	4.22	20.07
5		4.0	2.21	4.47	20.19
6		6.0	1.83	4.73	20.21
7	6-phosphogluconic acid barium salt	2.0	2.10	7.90	19.72
8		4.0	1.82	12.15	19.48
9		6.0	1.62	16.76	20.76
10	D-ribulose 5-phosphate barium salt	0.5	2.73	8.47	22.26
11		1.0	2.65	20.20	38.66
12		2.0	2.53	83.18	133.79
13		3.0	2.02	94.37	169.82
14		4.0	2.15	129.30	228.70
15		5.0	2.24	147.87	255.23
16	D-xylulose 5-phosphate sodium salt D-ribose 5-phosphate barium salt	0.5 0.5	2.47	24.76	58.38
17		1.0 1.0	2.38	66.17	120.50
18		2.0 2.0	2.29	82.74	152.28
19		3.0 3.0	2.29	111.92	221.20
20		4.0 4.0	2.18	82.26	231.04
21		5.0 5.0	2.46	92.54	388.50
22	D-sedoheptulose 7-phosphate barium salt	0.5	2.47	3.45	15.84
23		1.0	2.53	6.68	20.53
24		2.0	2.63	10.67	25.31
25		3.0	2.38	16.23	36.99
26		4.0	2.44	22.91	44.14
27		5.0	2.26	26.13	51.54
28	D-fructose 6-phosphate barium salt D-erythrose 4-phosphate sodium salt	2.0 2.0	2.96	2.68	tr
29		4.0 4.0	0.11	tr	tr
30		6.0 6.0	0.13	tr	tr
31	D-ribulose	5.0	2.68	8.78	24.22
32	D-xylulose D-ribose 5-phosphate barium salt	2.0 2.0	2.96	10.34	23.56
33		4.0 4.0	2.46	14.40	34.86
34		6.0 6.0	2.69	21.03	45.45
35	control		2.18	3.79	20.20
36	D-ribulose 5-phosphate barium salt	6.0	0.00	0.00	242.97
37	D-xylulose 5-phosphate sodium salt D-ribose 5-phosphate barium salt	6.0 6.0	0.00	0.00	299.90
38	D-sedoheptulose 7-phosphate barium salt	6.0	0.00	0.00	59.59

[a] The medium consisted of the following additions to the aqueous extract of shoyu koji (w/v): NaCl 17%, glucose 5%, sugar phosphates 0.5–6.0%. Organism and culture: *Z. rouxii* No. 210, ATCC 13356, was cultured in a 10-mL vial containing 0.5 mL of medium at 30 °C for 12 days in stationary culture. [b] Ethanol. [c] 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone. [d] 4-Hydroxy-5-methyl-3(2*H*)-furanone.

precursor may be mono- or oligosacchardies or the phosphates. To screen for the precursor of HEMF, the strain *Zygosaccharomyces rouxii* No. 210, ATCC 13356, representing the shoyu yeast was cultured in a 10-mL vial containing 500 μL of the preceding medium A supplemented with 2% each of the sugar or the phosphates shown in Table I. The content of HEMF in the fermented broth was determined by the GC method. Identification of HEMF and HMMF was established by comparison of mass spectra and retention times from the gas chromatography. To evaluate the extent of fermentation by yeast, ethanol in the fermented broth was also determined by the GC method. Table I shows that a fair amount of HEMF was produced only in the fermented broth in which D-sedoheptulose 7-phosphate barium salt was added. This result suggested that HEMF might be biosynthesized through the pentose–phosphate cycle.

To confirm the above presumption, the yeast was inoculated into the above-mentioned medium A containing 2% of the intermediates found in the pentose–phosphate cycle. The intermediates are shown in Table II, including D-ribulose 5-phosphate barium salt and the combination

Table IV. HEMF Contents in Culture Media Produced by Various Kinds of Yeasts[a]

		D-ribulose 5-P Ba salt		D-xylulose 5-P Na salt + D-ribose 5-P Ba salt		D-sedoheptulose 7-P Ba salt	
no.	yeast	EtOH,[b] %	HEMF,[c] ppm	EtOH, %	HEMF, ppm	EtOH, %	HEMF, ppm
1	*Z. rouxii* A	2.47	72.51	1.74	110.34	2.27	12.08
2	*Z. rouxii* 29B	2.70	51.72	2.24	56.76	1.86	7.78
3	*Z. rouxii* F	2.66	87.97	1.97	83.47	2.25	11.19
4	*Z. rouxii* no. 210, ATCC 13356	3.00	63.00	2.33	85.19	2.92	10.71
5	*Z. rouxii* no. 1-7	3.06	65.25	2.52	68.06	3.04	10.12
6	*Z. rouxii*	3.05	80.10	2.40	69.25	2.55	10.98
7	*C. halophila* A-1	2.00	17.23	2.06	27.10	2.03	7.47
8	*C. mannitofaciens* B-1	2.26	17.25	2.29	26.51	1.98	5.75
9	*C. mannitofaciens* C-1	2.52	25.28	2.11	59.28	2.16	9.80
10	*C. versatilis* D-1	2.46	11.32	1.93	26.89	2.45	6.54
11	*C. halophila* E-1	2.06	14.30	1.88	33.67	2.08	8.12
12	*C. halophila* F-1	2.05	14.38	1.90	23.28	1.37	6.83
13	*C. vanderwaltii* G-1	1.37	6.33	1.41	14.34	1.45	5.15
14	*C. etchellsii* IFO 100037	2.50	33.47	1.95	63.06	2.55	9.94
15	*C. halophila*	2.11	9.11	1.71	32.26	1.79	4.58
16	*C. halonitratophila*	2.46	6.68	1.07	31.22	1.96	6.94
17	*S. cerevisiae* AHU 3492 (sake yeast)	3.08	61.40	1.69	78.57	2.31	6.94
18	*S. cerevisiae* IFO 2164 (sake yeast)	3.49	28.15	3.61	72.56	2.28	6.69
19	*S. cerevisiae* IFO 2342 (sake yeast)	2.87	38.02	3.15	85.50	2.43	6.49
20	*S. cerevisiae* IFO 0216 (shyotyu yeast)	3.03	28.61	3.06	96.15	3.47	8.01
21	*S. cerevisiae* RIB 6600 (wine yeast)	2.85	48.89	2.83	86.38	2.06	7.93
22	*S. cerevisiae* OUT 7083 (wine yeast)	3.58	15.25	3.05	53.93	2.43	6.02
23	*S. cerevisiae* OUT 7892 (champagne yeast)	2.91	17.91	3.06	78.73	2.26	5.14
24	*S. cerevisiae* IFO 0262 (sherry yeast)	2.46	36.99	2.65	77.34	1.88	5.87
25	*S. cerevisiae* IFO 0233	3.15	14.32	3.57	48.17	2.74	4.59
26	*Y. lipolytica* ATCC 44601	2.37	4.65	2.68	17.09	1.81	3.77

[a] The medium consisted of the following additions to the aqueous extract of shoyu koji (w/v): samples 1–16, NaCl 17%, glucose 5%, sugar phosphates 2.0%; samples 17–26, glucose 5%, sugar phosphates 2.0%. Organism and culture: Yeasts was cultured in a 10-mL vial containing 0.5 mL of medium at 30 °C for 12 days in stationary culture. [b] Ethanol. [c] 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone.

of D-xylulose 5-phosphate sodium salt and D-ribose 5-phosphate barium salt. They were cultured stationarily at 30 °C for 12 days. As it is expected, the result in Table II indicates that HEMF was also formed from intermediates other than D-sedoheptulose 7-phosphate barium salt shown in Table I. As a next step, different concentrations of intermediates were examined. The conditions of cultivation for the yeast were the same as described above, except for the concentration of the added sugar phosphate to the medium. The result is presented in Table III, showing that HEMF production was found to increase in proportion to the concentration of the added sugar phosphate. That is to say, dose dependency was obviously recognized in all cases. When D-ribulose 5-phosphate barium salt was replaced with D-ribulose, HEMF production was reduced by ca. 94% (Table III, sample 31). When the combination of D-xylulose and D-ribose 5-phosphate barium salt (Table III, samples 32–34) was used instead of the combination of D-xylulose 5-phosphate sodium salt and D-ribose 5-phosphate barium salt (Table III, samples 16–21), the result was similar to that with D-ribulose. D-Ribulose 5-phosphate barium salt and D-xylulose 5-phosphate sodium salt are more suitable than D-ribulose and D-xylulose for the biosynthesis of HEMF. The yeast used in this experiment had no ability to use the combination of D-fructose 6-phosphate barium salt and D-erythrose 4-phosphate sodium salt for biosynthesis of HEMF (Table III, samples 28–30). When D-glucose 6-phosphate monosodium salt or D-glucose 6-phosphate disodium salt was added to medium A, the production of HEMF was small (Table III, samples 1–6). From this result, it is estimated that D-glucose 6-phosphate was located at a turning point between the glycolytic pathway and the pentose–phosphate cycle and was mainly metabolized through the glycolytic pathway. Moreover, no detectable amount of HEMF was produced in the samples that were not inoculated with the yeast (Table III, samples 36–38).

From the results described above, it could be expected that HEMF was formed by the biosynthesis of shoyu yeast via the pentose–phosphate cycle. However, the pathway for the metabolism of D-sedoheptulose 7-phosphate to HEMF has not been determined yet (Figure 1).

On the other hand, HMMF was found to be formed by the nonenzymatic reaction of several kinds of sugar and the phosphates used in this experiment other than D-ribose 5-phosphate, glycine, and xylose described in the literature (Peer and Van den Ouweland, 1968; Nursten and O'Reilly, 1983). Moreover, the results of tests 36–38 in Table III indicate that the formation of HMMF has no relation to yeast growth. Meanwhile, it was previously confirmed that HMMF is not present in the original medium.

Further, to check the ability for some strains of shoyu yeasts to produce HEMF from D-ribulose 5-phosphate barium salt the combination of D-xylulose 5-phosphate sodium salt and D-ribose 5-phosphate barium salt and D-sedoheptulose 7-phosphate barium salt, a total of 16 strains belonging to *Z. rouxii* and *Candida* (6 of *Zygosaccharomyces* and 10 of *Candida*) were selected from stock cultures at the Kikkoman Corp. Medium A described under Materials and Methods was employed to culture the yeasts.

The test result is shown in Table IV (No. 1–16). All strains tested produced a fair amount of HEMF compared with the control value, which is shown in Table III (sample 35). The control value of HEMF arises from the precursor in the basal medium. Then it was proved that most shoyu yeasts have the ability to biosynthesize HEMF. *Zygosaccharomyces* species have a tendency to produce more HEMF than *Candida*.

Besides shoyu yeasts, the production ability of HEMF

by 10 other strains of yeasts was also tested in the same way. The yeasts are used for alcoholic beverage fermentation and for single-cell protein. The yeasts were cultured in a 10-mL vial, containing 500 μL of medium B mentioned before, at 30 °C for 12 days, without shaking. The results are displayed in Table IV (no. 17–26). These yeasts tested also produced HEMF.

The results from the experiments above lead to the conclusion that HEMF in shoyu is biosynthesized through the pentose–phosphate cycle by yeasts. Not only shoyu yeasts but also other yeasts employed for alcoholic beverages and single-cell protein can change intermediates like D-ribulose 5-phosphate in the pentose–phosphate cycle to HEMF. Shoyu is the only food in which HEMF was formed, though other yeasts have the ability to produce HEMF. This is very interesting because HEMF is a character-impact compound in shoyu. Why does shoyu contain so much HEMF? Why has HEMF not been found in other fermented foods? Further research is required to answer these questions.

In this paper, it is clear that the pentose–phosphate cycle is essential for HEMF production by yeasts.

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