Bacteriological Properties of and Amine-Production Conditions for Tyramine- and Histamine-Producing Bacterial Strains Isolated from Soybean Paste (Miso) Starting Materials

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The conditions for production of amines by tyramine- and histamine-producing bacterial strains (T- and H-strains) isolated from soybean paste (miso) starting materials were examined. These strains grew and produced tyramine and histamine under various conditions which may occur during miso fermentation. Namely, tyramine and histamine were produced at pH above 5.1, at salt concentrations of less than 6% and 4%, under either aerobic or anaerobic conditions and at 15–37°C in MRS medium. Both strains consumed possible precursor amino acids of the respective amines.

It is assumed that tyramine and histamine were produced by T- and H-strains during the early stages of miso aging and within a short period, when the salt concentration and pH conditions were optimal.

Based on their bacteriological properties, the T-strain was identified as *Enterococcus faecium* and the H-strain was identified as *Lactobacillus* sp..

Keywords — tyramine ; histamine ; tyramine-producing strain ; histamine-producing strain ; miso fermentation ; *Enterococcus faecium* ; *Lactobacillus* sp.

Introduction

We previously reported that miso contained high levels of tyramine (Tym) and histamine (Him).^{1,2)} In particular, the toxicity of Him is enhanced by coexistence with other amines, making it necessary to know the origins of these amines in order to remove them from foods. We examined the formation of amines in miso, isolated a gram-positive coccus (T-strain) producing Tym and a gram-positive rod (H-strain) producing Him from miso starting materials, and suggest that these strains are related to the presence of amines in miso. Of the microorganisms related to the fermentation of miso, *Enter-*

ococcus faecalis is the only bacterium that contains amino acid decarboxylase and that produces Tym.^{3,4)} In this study, to clarify the mechanism of amine production during miso fermentation, we examined the various conditions under which amines are produced by T- and H-strains and also identified the properties of these two strains.

Materials and Methods

1. Miso Medium — One gram of peptone, 0.6 g of yeast extract, 0.2 g of glucose and 8 g of miso (kome miso : rice-koji miso) were added to 200 ml distilled water (pH 6.5) and autoclaved.

2. BBL Gas Pak Pouch — Becton Dickinson Microbiology Systems Co.

3. Strain Identification Kit — api STREP 20, manufactured by API System S.A., France.

4. Determination of Tym and Him in Culture Broth — Water (1.9 ml) was added to 100 μ l of culture medium, which was then dansylated and analyzed by HPLC as we previously reported²⁾ (detection limit: 5 μ g/ml).

5. Determination of Amino Acid — This was performed as previously described.²⁾

6. Test of Media at Various pH Values — 1.6 ml of one-tenth M citrate buffer (pH 3, 4, 5, 6, 7, and 8) was added to 0.4 ml of miso medium. One platinum loopful each of the T- and H-strains cultured in medium was inoculated into each of the miso culture media, which were allowed to stand at 25°C for one week, after which the levels of Tym and Him were measured.

7. Test to Determine Dependence on Salt Concentration — The T- and H-strains were each inoculated into de Man, Rogosa, Sharpe broth (MRS) media (salt ratio: 0, 2, 4, 6, 8 and 10%) as described above, followed by incubation at 25°C for 3 d. The levels of Tym and Him were measured, and bacterial growth was assessed by examining the turbidity and sedimentation of each medium.

8. Incubation Test under Aerobic and Anaerobic Conditions — The T- and H-strains were inoculated into the MRS medium and incubated under both aerobic (open-air) and anaerobic conditions (BBL Gaspak Pouch) at 25°C and 37°C for 5 d. Tym and Him levels were then measured.

9. Temperature Dependent Incubation Test — The T- and H-strains were inoculated into 10 ml of the MRS medium and incubated at 15, 25 and 37°C. The culture broth was collected aseptically at various times and the levels of Tym and Him were measured. Bacterial growth was assessed based upon the turbidity of the medium.

Results and Discussion

1. Effects of pH

Table I shows Tym production by the T-strain and Him production by the H-strain

at various pH values. Miso medium free of buffer was used as a control.

The T-strain produced Tym at a level of 85 to 172 μ g/ml when the pH was above 5.1. At pH 4.2, this strain produced negligible amounts of Tym and barely proliferated, and at pH 3.3 growth ceased. Both the production of Tym and the growth of the strain considerably increased as the pH approached neutral. The H-strain thrived and produced Him within a pH range of 4.2 to 6.9. At pH 6.0, this bacterium produced a particularly large amount of Him at a level of 14.5 μ g/ml.

The final pH value of T-strain media starting at pH 6 or above changed to alkaline, while the pH of other T-strain media did not change. No change in pH value was observed during growth of the H-strain. In the control medium with no buffer, however, the original pH dropped to an acidic 4.0 (T-Strain) and 6.1 (H-strain), suggesting that both of these strains liberated acids. Based upon the result of the control study, Tym did not affect the pH value. Thus, the fact that the final pH of the Tym culture medium was alkaline might

TABLE I. Effects of Medium pH on Formation of Tyramine and Histamine by T-and H-Strains

(μg/ml)			
T-strain pH	Tym	Him	pH After culture
3.3	0	—	3.3
4.2	3	—	4.2
5.1	85	—	4.9
6.0	86	—	8.6
6.7	100	—	9.0
6.9	172	—	9.2
Control (6.5)	136	—	4.0
H-strain pH	Him	Tym	pH After culture
3.3	0	—	3.4
4.2	5.0	—	4.2
5.1	2.1	—	5.1
6.0	14.5	—	6.1
6.7	3.9	—	6.7
6.9	4.2	—	6.9
Control (6.5)	14.0	—	6.1

Culture conditions: 25°C, 1week.

have been due to the unbalanced composition of the buffer solution resulting from growth of the bacteria. Hanke and Koessler⁵⁾ reported that a bacterium produces amines in order to neutralize its acidic environment. However, the production of large amounts of amines in the acidic region is not constant, nor do large amounts of amines necessarily make the environment alkaline. Thus the amines probably did not contribute to the increased pH value.

During the aging period, the pH value of the miso changed from around 6.2 at mashing to 5.7 after 30 d, 5.5 after 90 d, 5.2 after 200 d and 5.1 after 450 d.²⁾ A pH value of around 6.0 at the mashing was suitable for the growth of both the T- and H-strains and for the production of amines by these strains. In particular, the H-strain produced Him within a narrow pH range of around 6.0. Thus, the pH value did not affect the production of Tym by the T-strain, but affected the production of Him by the H-strain. From the viewpoint of pH alone, the T-strain grew continuously and produced Tym throughout the aging period. On the other hand, Him was produced in large amounts during the early stage of H-strain growth when the pH value was high, but its productivity tended to decrease as the pH value became acidic.

2. Effects of Salt Concentration

Table II shows amine production at various salt concentrations.

The T- and H-strains thrived at salt

TABLE II. Effects of Salt Concentration of Formation of Amines and Growth of T- and H-strains

Salt (%)	(μg/ml)			
	T-strain		H-strain	
	Tym	growth	Him	growth
0	144	(+)	91	(+)
2	188	(+)	74	(+)
4	184	(+)	88	(+)
6	172	(+)	—	(±)
8	—	(—)	—	(—)
10	—	(—)	—	(—)

concentrations of under 6 and 4%, respectively, and produced Tym and Him, respectively; the production was virtually independent of the salt concentration. That is, salt did not affect the production of amines but affected only the growth of the bacteria. Based on their field studies, Takagi and Okamoto⁶⁾ reported that the amine content of farmers' homemade miso increased with a decrease in the salt concentration. Further, Chin and Koehler⁷⁾ reported that amine production was promoted during the aging process in miso as the salt concentration was lowered. However, our studies using the producing bacteria alone indicated no relationship between salt concentration and amine production. This might be because the aging of miso is affected by various factors, including other bacteria. In particular, the salt concentration (about 12%) at the time the miso is mashed exceeds the growth limits of the T- and H-strains. In the early stage of the aging process following the mashing, however, it seems that the salt concentration is not uniform and therefore these bacteria can grow in some parts of the mash where there is a low salt concentration, thus producing Tym and Him. This assumption agrees with the reports of Takagi and Chin that the growth of amine-producing bacteria in miso appears to rise with a reduction in salt concentration.

3. Effects of Aerobic and Anaerobic Conditions

Since a weight is placed on the miso during the aging period, the conditions are almost anaerobic. Thus we measured the amine production under aerobic and anaerobic conditions (Table III). Both the T- and H-strains grew well under both conditions, producing Tym and Him, respectively.

Tym production under both conditions ranged from 127 to 162 μg/ml, while the Him production range was from 33 to 51 μg/ml. Thus no difference in production was observed due to the different conditions.

TABLE III. Formation of Amines in Aerobic and Anaerobic Cultures

Condition	($\mu\text{g/ml}$)	
	T-strain Tym	H-strain Him
<u>Aerobic</u>		
25°C	132, 127	44, 42
37°C	148, 153	39, 40
<u>Anaerobic</u>		
25°C	144, 162	42, 51
37°C	138, 143	33, 41

4. Effects of Temperature

Although our studies were carried out at 25 and 37°C, we conducted another investigation at 15°C since, in practice, miso would be exposed to a lower temperature during the aging period. Amine production over time at this temperature was measured (Fig. 1).

At 15°C, 25°C, and 37°C, the T- and H-strains grew and produced Tym and Him, respectively. When incubated at 15°C for 23 hours, the T-strain did not produce Tym and no growth of the strain was observed. After incubation at this temperature for 39 hours, Tym was detected, but there was no apparent growth. After incubation for 47 h, the strain started to grow and the amount of Tym rose

to 65.2 $\mu\text{g/ml}$. The production of Tym then slowly increased and became almost constant after 4 d.

When incubated at 25°C for 23 h, the T-strain did not grow, but produced 97.4 $\mu\text{g/ml}$ of Tym. After incubation at this temperature for 39 h, growth started and Tym production became almost constant (197 $\mu\text{g/ml}$).

When incubated at 37°C, the strain grew quickly to maximum levels within 14 h. The production of Tym reached an almost constant level of 195 $\mu\text{g/ml}$.

When incubated at 15°C for 23 h, the H-strain did not grow, but started to do so after 39 h. After incubation for 47 h, however, no Him was detected. After incubation for 64 h, Him was detected for the first time (44.3 $\mu\text{g/ml}$) and its production reached an almost constant level after 5 d.

When incubated at 25°C for 39 h, the H-strain grew well and produced 50.2 $\mu\text{g/ml}$ of Him. After 64 h at this temperature production became almost constant.

When incubated at 37°C for 14 h, the H-strain grew but no Him was detected. Him was first detected after incubation for 23 h, becoming almost constant after 64 h.

Although the growth rates of both of the T- and H-strains were elevated with an increase in temperature, the final amine production levels were constant (Tym, about 200 $\mu\text{g/ml}$; Him, about 60 $\mu\text{g/ml}$) and not temperature-dependent.

However, these strains differed greatly in the following characteristic. The T-strain began to produce Tym in the inductive phase period (*i.e.*, before the initiation of growth), whereas the H-strain produced Him in the log phase, while the strain was growing. That is to say, the number of T-strain cells required to produce Tym is less than of the H-strain to produce Him. This finding suggests that even a small number of T-strain cells can produce Tym in miso, which contains various competing microorganisms, and that there is a higher probability of detecting Tym than Him in

Fig. 1. Curves of Amine Formation by T- and H-strains at Various Temperatures

—▲— 37°C, —◆— 25°C, —●— 15°C.

miso products.

5. Effects of Amino Acids

The amino acid content of the culture broth of each strain was examined with an amino acid analyzer after incubation. Table IV shows the content of 6 of 17 amino acids: tyrosine (Tyr), histidine (His), serine (Ser), glutamic acid (Glu), arginine (Arg) and proline (Pro), which changed remarkably.

In the culture broth of the T-strain, amino acids including Tyr, which is the precursor of Tym, Ser and Arg decreased, whereas Glu and Pro increased with increasing Tym production. His was not affected.

In the culture broth of the H-strain, the amino acid His, which is the precursor of Him, decreased with increasing Him production. However, the extreme decrease in Ser and Arg observed in T-strain broth was not seen. Glu increased, while Pro and Tyr were scarcely changed.

Thus both of these strains consumed the precursor amino acids corresponding to the respective amines. These findings suggest that the amine production depended on the Tyr and His content. His and Tyr were therefore added to the MRS medium at a final concentration of 1000 ppm of each and the strains were incubated. Table V shows the results of the amine determination.

Tym production by the T-strain was about 5 times greater than that without added Tyr, whereas Him production by the H-strain was about 12 times greater than that when His was not added. These results indicate that the production of Tym and Him would be raised depending on the content of the substrates (*i.e.*, Tyr and His). In the practical production of miso, Tyr and His are supplied in sufficient amounts; that is based on a miso product during the early stage of aging, Tyr was present at a level of several hundred ppm to several thousand ppm, while the level of Him was tens to several hundred ppm. However, neither Tym nor Him was detected in the product. Thus it is considered that a

TABLE IV. Relationship Between Formation of Amines and Consumption of Amino Acids in Liquid Media

(μg/ml)						
T-stran						
Levels of Tym	0	3	85	86	100	172
Amino acids						
Tyr	65	53	—	—	—	—
Ser	209	144	134	5.7	7.4	5.6
Arg	246	248	7.2	7.8	—	—
Glu	204	233	385	489	532	499
Pro	57	70	105	175	224	201
His	70	57	57	84	88	76
H-strain						
Levels of Him	0	5.0	2.1	14.5	3.9	4.2
Amino acids						
His	57	37	43	11	43	33
Ser	144	146	139	148	144	140
Arg	246	229	196	221	220	203
Glu	206	258	283	295	287	282
Pro	62	63	61	60	64	58
Tyr	53	55	54	58	57	44

TABLE V. Formation of Amines in Medium Supplemented with Tyrosine and Histidine (μg/ml)

Strain	Addition	Tym	Him
T	Tyr	660, 672	—
	—	130	—
H	His	—	508, 522
	—	—	43

variation in the Tym and Him levels of up to 1000 ppm might depend on whether or not the strains grow during the aging period.

6. Identification of Strains

We attempted to identify the T- and H-strains by conventional methods. Table VI summarizes their properties and characteristics and compared them with those of standard species with similar properties.

The T-strain, which was a gram-positive coccus, was a catalase-negative facultative anaerobe which was asporogenic and changed the color of bromocresol purple (BCP) agar plate to yellow. It was identified as *Streptococcus*. On the other hand, the H-strain, which was a gram-positive rod and had some prop-

TABLE VI. Bacteriological Properties of T- and H-strains

Characteristics	T-strain	<i>E. faecium</i>	H-strain	<i>L. sanfrancisco</i>
Gram stain	+	+	+	+
Spore	—	—	—	—
Cell morphology	cocci	cocci	rods	rods
Gas from glucose	—	—	+	+
Growth at 45°C	+	+	+	—
Growth at 15°C	+	+	+	+
Catalase	—	—	—	—
Oxydase	—	—	—	—
Growth in Litmus milk	+	+	—	—
Arginine hydrolysis	+	+	—	—
Growth on BCP plate	+	+	+	—
OF-test (Hugh & Leifson)	F	F	F	F
Aerobic growth	+	+	+	+
Anaerobic growth	+	+	+	+
Production of EtOH	—	—	+	+
Tyrosine decarboxylated	+	+	—	—
Histidine decarboxylated	—	—	+	—
Acid from				
Arabinose	—	+	—	—
Fructose	+	+	—	—
Galactose	+	+	+	—
Glucose	+	+	+	+
Gluconate	+	+	—	+
Lactose	+	+	—	—
Maltose	+	+	+	+
Mannose	+	+	—	—
Raffinose	+	—	—	—
Ribose	+	+	—	—
Salicin	—	—	—	—
Mannitol	+	+	—	—
Sorbitol	+	—	—	—
Sucrose	+	+	—	—
Trehalose	+	+	—	—
Xylose	—	—	—	—

+ : positive; — : negative; F : fermentation type

erties in common with the T-strain, was identified as *Lactobacillus* sp. In order to further identify the strains, their ability to ferment sugars was examined.

The T-strain was identified as *Enterococcus faecium*. According to Bergey's Manual,⁴⁾ however, *E. faecium* never produces Tym from Tyr, i.e., the Tym production in the T-strain differed from that of *E. faecium*. *E. faecium* was obtained from the Japan Collection of Microorganisms at the Institute of Physical and Chemical Research and its prop-

erties were compared with those of the T-strain. We found that these strains were in fairly good agreement. Furthermore, *E. faecium* produced Tym in a manner similar to the T-strain. The T-strain was also identified using the identification kit, api STREP 20.

Thus it is considered that *E. faecium*, which is a lactic acid bacterium frequently found together with *E. faecalis* in other fermented foods such as pickles,^{3,8,9)} might be closely related to the presence of Tym in foods other than miso. *E. faecalis*, which can

produce Tym, is related to the aging process in fermented foods, although this strain was not detected in this study. It is therefore assumed that *E. faecalis* is also related to the presence of Tym in miso. *Pediococcus halophilus*, a lactic acid bacterium, is also related to the aging of miso;¹⁰⁾ however, we examined this species, we found that it did not produce Tym or Him.

The properties of the H-strain observed in this study were compared with those in Bergey's Manual and with the methods for identifying *Lactobacillus* specified in Standard Methods of Analysis in Food Safety Regulation.¹¹⁾ The H-strain properties and characteristics most closely agreed with those of *L. sanfrancisco*. However, this strain could not be identified further. We are therefore examining the DNA homology of this strain with others that have similar properties.

Conclusion

It is assumed from this study that the behavior of the strain relating to the production of Tym or Him in miso fermentation is as follows:

The T- and H-strains may predominate during the early stage of miso aging. These

strains then proliferate, producing Tym and Him within a short period under optimum salt and pH conditions.

The H-strain has a long inductive phase with a slow growth rate, requiring severe growth conditions, namely, an increased salt concentration and lowered pH value compared with the T-strain or other species, and thus had less opportunity for producing Him during miso fermentation. This assertion is consistent with the results of our study of marketed miso products in which Him was detected at lower concentrations than Tym.

Furthermore, based on their properties, we identified the T-strain as *Enterococcus faecium*, and the H-strain as *Lactobacillus* sp.. These strains, which are frequently observed in various fermented foods other than miso, might be closely related to the presence of amines in these foods.

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