# 低温塩蔵魚肉中の糸状菌によるトリメチルアミンの消費


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# Consumption of Trimethylamine by Molds in Salted Fish during Storage at Low Temperature*1

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The TMA-utilizing ability of molds grown on fish muscle homogenates stored with or without salt under low temperature was examined. Two strains of *Penicillium* (F2 and F4) which grew on the salted fish muscle homogenates as the dominant strains utilized TMA, but 2 strains of *Mucor* and Fungi-imperfecti (F1 and F3) which were isolated from the homogenates without salt did not. Strain F2 grew well in CZAPECK's medium which was prepared to contain 5 mg TMA-N/100 m*l* as the sole source of nitrogen in place of $NaNO_3$. Intact mycelia of strain F2 showed the TMA-utilizing activity after a lag period.

Among 23 authentic strains of molds, 8 of the 11 strains of *Penicillium*, 1 of the 8 strains of *Aspergillus*, and none of the *Rhizopus, Mucor, Citromyces* and *Alternaria* strains utilized TMA.

Based on the data obtained, the reasons why the amount of TMA produced in the salted fish muscle homogenates stored at low temperature did not exceed the threshold value of spoilage (3.0–3.8 mg-N/100 g) were discussed.

In the previous paper[1] we have studied the changes of microflora during storage in order to elucidate the reason why the amount of trimethylamine (TMA) produced during storage of salted fish muscle homogenates at a low temperature did not exceed the so-called threshold value of spoilage (3.0–3.8 mg-N/100 g). During these studies we found that TMA produced through the trimethylamine N-oxide (TMO) reduction by bacteria was at least partially consumed by molds grown on the salted fish homogenates at the low temperature. A preliminary note on this phenomenon has already been published[2]. This paper deals with the further study on the mechanism involved in the TMA consumption by molds.

## Materials and Methods

### *Organisms*

To examine the ability to utilize TMA, 4 strains of molds; *Mucor* sp. (F1), *Penicillium* sp. (F2), Fungi-imperfecti (F3) and *Penicillium* sp. (F4) were employed. These strains were isolated from the fish muscle homogenates stored under the conditions of 0% NaCl–0°C, 5% NaCl–0°C, 0% NaCl–10°C and 5% NaCl–10°C, respectively. Besides these strains, 23 authentic strains of molds belonging to *Penicillium camenberti, P. citrinum, P. casei, P. roqueforti, P. spinulosum, P. expansum, P. digitatum, P. puberulum, Aspergillus flavus, A. tamarii, A. sulfureus, A. wentii, Eurotium repens, A. niger, A. oryzae, Rhizopus chinensis, R. tokinensis, P. glaucum, P. notatum, P. caseicolum, Citromyces casiae, Mucor* sp. and *Alternaria* sp. were used. Of these the first 13 strains were provided by the Institute for Fermentation (Osaka), and the others were maintained in Laboratory of Microbiology, Department of Fisheries, Faculty of Agriculture, Kyoto University.

### *Media*

NB medium of which composition was previously described[3] was used in order to determine the ability of molds to utilize TMA and to determine the effect of pH and NaCl concentration on the growth of molds. For determining the ability to utilize various nitrogen sources, CZAPECK's medium[4] in which $NO_3$ was replaced by desired nitrogen sources was used.

### *Determination of the Ability of Molds to Utilize TMA*

Each strain of molds was inoculated to the NB medium which was prepared to contain NaCl at the designated concentration and TMA at the concentration of 10 mg-N/100 m*l*, and incubated

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under shaking condition at 5°C or 20°C for a desired period. After the incubation the amount of TMA which remained in the medium and the dry weight of mycelia grown in the medium were determined. The mycelia were separated by filtering the culture fluid through a Whatman GF/C glass fiber filter.

*Utilization of Various Nitrogen Sources by Molds*

In place of $NaNO_3$ as the nitrogen source, the same amount of TMO, TMA, dimethylamine, methylamine, $NH_4Cl$, choline, betaine, dimethylglycine, sarcosine or glycine was added to the CZAPECK's medium which contained per 1,000 m*l* of distilled water: $K_2HPO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $NaNO_3$, 0.6 g; sucrose, 30 g; and NaCl, 25 g. The strain to be tested was inoculated into 300 m*l* Ehrenmeyer flask containing 100 m*l* of the medium, and incubated for 10 days at 20°C using a rotary shaker (220 r.p.m., 4 cm amplitude). The growth of mold was measured as dry weight of mycelia.

*Conditions used for Determining the Effect of pH and NaCl Concentration on the Mold Growth*

To examine the effect of pH on growth of the mold, the NB medium containing 2.5% NaCl of which pH value was adjusted to 3.0–9.0 was employed. To examine the effect of NaCl concentration the NB medium which was prepared to contain NaCl in the concentration of 0, 2.5, 5.0, 7.5, 10.0, 12.5 or 15.0% (pH 6.0) was used. After being cultivated at 20°C for 3 days on a rotary shaker, the culture was filtrated and the dry weight of mycelia was measured.

*Preparation of Cell Suspension and Starved Cells*

The cells grown in 500 m*l* of NaCl-free NB medium at 20°C for 2 days on a shaker were harvested and washed twice with phosphate buffer (pH 6.0). Ten g of the washed cells were resuspended in 100 m*l* of the same buffer and used as the cell suspension. The cell suspension was incubated on a reciprocal shaker at 20°C for 20–24 hr, and then used as the starved cells.

*Preparation of Sonicate and Homogenate*

The starved cell suspension was sonicated by using an ultrasonic disintegrator (20 kc). The sonication was made intermittently in an ice-water bath to prevent warming of the suspension; total time of the ultrasonic treatment was 4 min. The product was used as sonicate. The starved cell suspension was homogenized with glass beads on ice by using Potter-Elvehjem Homogenizer. To this homogenate, when necessary, both 10 mM $MgCl_2$ and 2 mM $CaCl_2$, 5 mM EDTA, or 0.5 m*l*/*l* of mercaptoethanol were added.

*Measurement of TMA-utilizing Activity*

The reaction mixture was prepared to contain 10 m*l* phosphate buffer (pH 6.0) containing 0.5–5.0 mg/100 m*l* TMA-N, and 10 m*l* of either the cell suspension, the sonicates, or the homogenates in 20 m*l*. To test the optimal condition for the TMA utilization by starved cell suspension, phosphate buffer solutions of various pH values which contained NaCl in different concentrations were used. The effect of temperature was examined in Temperature Gradient Incubator Model TN-3 (Toyo Kagaku Sangyo Co. Ltd.). The reaction was stopped by adding 5 m*l* of 25% TCA after 6 hr incubation. Trimethylamine in the reaction mixture was measured by DYER's picrate method[5] modified by HASHIMOTO and OKAICHI[6] after filtration through glass filter. The activity was expressed as relative value (%) of the TMA utilization.

## Results and Discussion

Utilization of TMA by the mold strains isolated from the salted fish muscle homogenates stored under the low temperature is shown in Table 1. Among the strains employed F2 (*Penicillium* sp.) and F4 (*Penicillium* sp.) showed high TMA-utilizing activity. These two strains were isolated from the homogenates stored under the conditions of 5% NaCl–0°C and 5% NaCl–10°C, respectively, in which the amount of TMA produced did not exceed the threshold value of spoilage. Strains F1 and F3 which were isolated from the homogenates stored under conditions of 0% NaCl–0°C and 0% NaCl–10°C did not utilize TMA. These results coincide with the observation that the amount of TMA during storage increased in the fish muscle homogenates stored without salt but did not in the homogenates with salt.

The distribution among molds of the ability to utilize TMA was observed with 23 authentic strains. As shown in Fig. 1, 8 of the 11 strains of genus *Penicillium*, and 1 of the 8 strains of genus *Aspergillus* showed the activity to utilize TMA, but any strain of genera *Rhizopus, Mucor, Citromyces* and *Alternaria* did not. The strains which were found to have the ability to utilize TMA

**Table 1.** Utilization of TMA by molds isolated from the fish muscle homogenates stored under various conditions

| Strain (Genus) | Cultivation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5°C, 60 days | | | | | | 20°C, 10 days | | | | | |
| | 0% NaCl[a] | | 2.5% NaCl | | 5% NaCl | | 0% NaCl | | 2.5% NaCl | | 5% NaCl | |
| | Growth[b] | TMA[c] | Growth | TMA | Growth | TMA | Growth | TMA | Growth | TMA | Growth | TMA |
| Control | | 10.0 | | 10.0 | | 10.0 | | 10.0 | | 10.0 | | 10.0 |
| F1 (*Mucor*) | 363 | 9.3 | 104 | 9.5 | NG | — | — | 9.9 | 68 | 9.9 | — | — |
| F2 (*Penicillium*) | 231 | 2.0 | 338 | 1.3 | 346 | 6.3 | 209 | 1.7 | 260 | 1.7 | 259 | 3.3 |
| F3 (Fungi-imperfecti) | 35 | 10.9 | NG[e] | — | NG | — | — | — | NG | — | — | — |
| F4 (*Penicillium*) | —[d] | — | 173 | 0.2 | — | — | — | — | 181 | 0.5 | — | — |

[a] Concentration of NaCl in the medium.
[b] Dry weight of mycelia: mg/100 m*l* medium.
[c] TMA remained in the medium: mg-N/100 m*l*.
[d] —: not tested.
[e] NG: no growth.

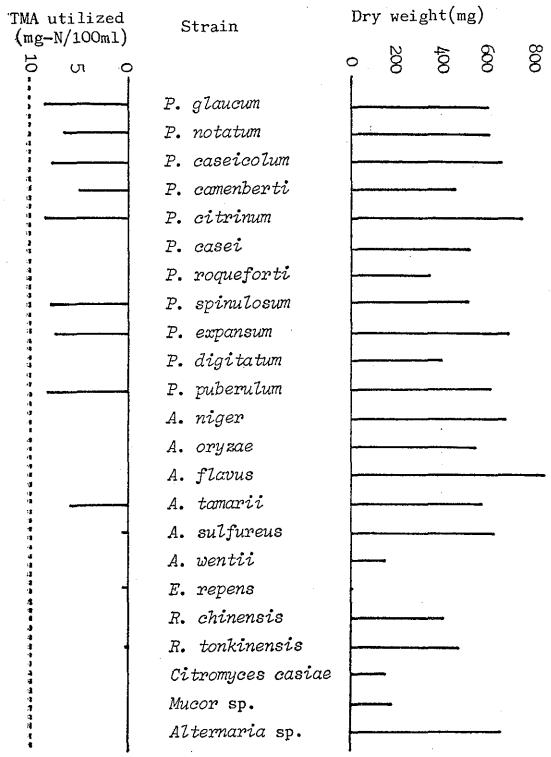


**Fig. 1.** Growth and TMA-utilizing activity of authentic strains of molds.

**Table 2.** Compounds utilized by strain F2 (*Penicillium*) as the sole nitrogen source

| Nitrogen source (10mg-N/100m*l*) | Dry weight (mg/100 m*l*) | | |
|---|---|---|---|
| | Not-shaking | Shaking | |
| | | Expt. 1 | Expt. 2 |
| None | NG* | | NG |
| $NO_3$ | 182 | 404 | 278 |
| TMO | NG | NG | NG |
| TMA | NG | 68 | 53 |
| Dimethylamine | NG | | 102 |
| Methylamine | | 123 | 154 |
| $NH_4Cl$ | | 195 | 224 |
| Choline | 44 | | 174 |
| Betaine | | 207 | |
| Dimethylglycine | | 159 | 144 |
| Sarcosine | | 304 | 164 |
| Glycine | 182 | 313 | |
| $NO_3$+TMO | 191 | | |
| $NO_3$+TMA | 197 | 390 | 301 |
| $NO_3$+Dimethylamine | 164 | | 286 |
| $NO_3$+Methylamine | | 453 | 282 |
| $NO_3$+$NH_4Cl$ | | 330 | 360 |
| $NO_3$+Choline | 241 | | 360 |
| $NO_3$+Betaine | | 394 | |
| $NO_3$+Dimethylglycine | | 411 | 294 |
| $NO_3$+Sarcosine | | | 140 |
| $NO_3$+Glycine | 134 | 528 | |

* NG: no growth.

were *P. glaucum, P. notatum, P. caseicolum, P. camenberti, P. citrinum, P. spinulosum, P. expansum, P. puberulum* and *A. tamarii.*

In the field of food science, it has generally been believed that TMA is not utilized by microorganisms. Recently a few papers[7-11] have been published on the presence of TMA-utilizing microorganisms in soil, water etc. The ability to consume TMA of microorganisms is noteworthy in considering the preservation of food, especially sea food.

As described in the previous paper[2], strain F2 (*Penicillium*) grew well in CZAPECK's medium which was prepared to contain 5 mg/100 m*l* TMA-N as a sole source of nitrogen in place of $NaNO_3$. In the presence of nitrate TMA in the culture was utilized after nitrate had completely been utilized. This strain, as shown in Table 2,

utilized, as a sole source of nitrogen, dimethylamine, methylamine, ammonia, choline, betaine, dimethylglycine, sarcosine or glycine too. However, TMO was not utilized by this organism during the first 10 days' cultivation. These results suggest that TMO is not an intermediate during TMA utilization by this organism.

About the metabolism of TMA, COLBY and ZATMAN[12] suggested that there are two different pathways for oxidation of TMA, and that the obligate methylotrophs use a TMA dehydrogenase-dependent route and facultative methylotrophs use a TMA monooxygenase-dependent route. However, the oxidation of TMA by F2 strain which is not an obligate methylotroph is probably done through TMA dehydrogenase, which catalyses the oxidation of TMA to dimethylamine and folmaldehyde. BOULTON *et al.*[13] also reported the existence of TMA dehydrogenase in a culture of *Hyphomicrobium vulgare* NQ which is not an obligate methylotroph. YAMADA *et al.*[11] recently studied the metabolism of amines by a facultative methylotroph belonging to genus *Candida* and reported that degradation of TMA is dependent on the route of oxidative demethylation directly yielding dimethylamine and formaldehyde.

Strain F2 grew well in the media of which pH value were between 4.0 and 8.0 and NaCl concentrations were between 0 and 5.0%. The best growth was obtained under the conditions of pH 5.0–7.0 and 0–2.5% NaCl.

Cell suspension of strain F2, as shown in Fig. 2, did not show TMA-utilizing activity immediately after the start of incubation. But after a lag period of about 10–20 hours, regardless of the presence of TMA (100 mg-N/100 m*l*) in the growing medium, TMA was utilized by the cell suspension. The lag period does not seem to be related to the induction of TMA-utilizing enzymes. It was not clear why the lag period is necessary for the TMA-utilizing activity of strain F2. From the data shown in Fig. 2, the rate of TMA utilization per g of wet weight of mycelia was calculated to be about 0.13 mg TMA-N/hr.

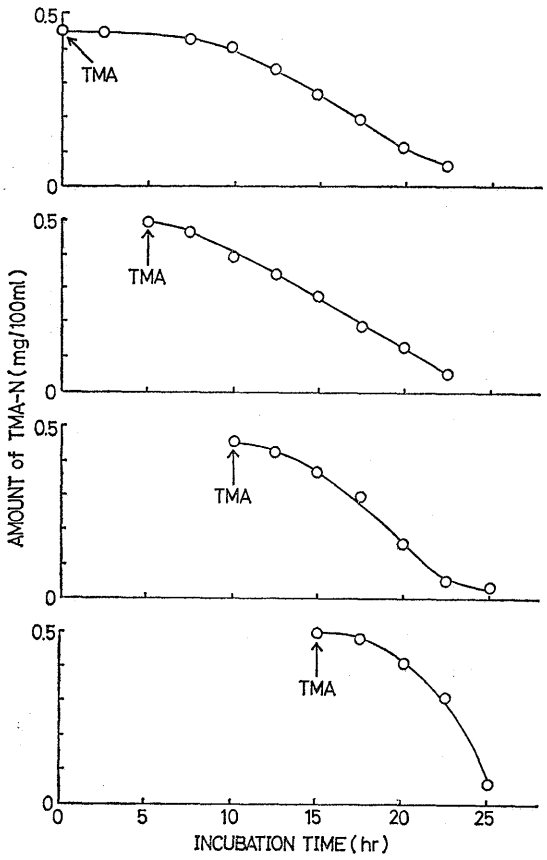


**Fig. 2.** Utilization of TMA by cell suspension of strain F2. TMA (0.5 mg-N/100 m*l*) was added to the suspension at the time indicated by the arrow.

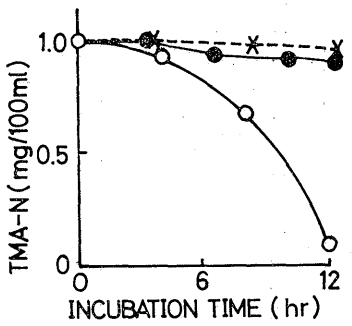


**Fig. 3.** Utilization of TMA by starved cells of strain F2, and their sonicates and glass-homogenates. ○—○: starved cells, ●—●: sonicates, ×---×: glass-homogenates.

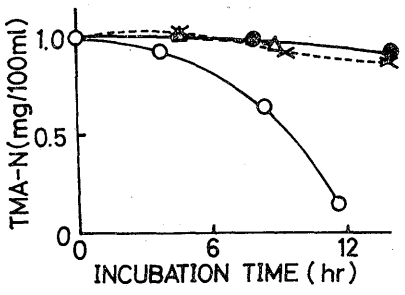


**Fig. 4.** Effect of additives on the TMA-utilizing activity of homogenates. ○—○: starved cells, ●—●: $MgCl_2+CaCl_2$, ×---×: EDTA, △—△: mercaptoethanol.

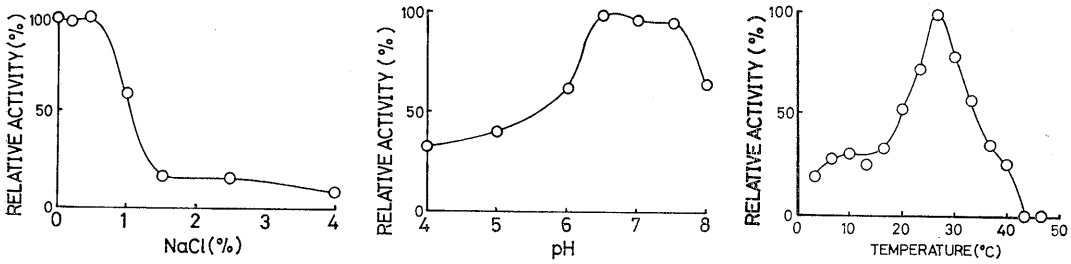


Fig. 5. Effect of NaCl concentration, pH and temperature on the TMA-utilizing activity of cell suspension.

To obtain further information about the mechanism of TMA utilization by strain F2, the TMA-utilizing activity was observed with the sonicated and homogenated mycelia. It was found by this experiment that the sonicates and homogenates did not show the activity (Fig. 3). Addition of 10 mM $MgCl_2$ and 2 mM $CaCl_2$, 5 mM EDTA, or 0.5 ml/l of mercaptoethanol to the homogenates did not affect the activity (Fig. 4). The optimal conditions for the TMA-utilizing activity of the starved cell suspension were examined. The optimal pH, NaCl concentration and temperature for TMA utilizing activity were 6.5–7.5, 0–0.5% NaCl and around 25°C, respectively, as shown in Fig. 5.

When crude extracts of the mycelia were employed the TMA-utilizing activity could not be detected. This might be due to the instability of the enzyme. JARMAN and LARGE[10] reported with *Pseudomonas aminovorans* that the enzyme catalyzing the oxygenation of TMA had a half-life in crude extracts of 11 min at 35°C and its purification resulted in major losses in activity. YAMADA *et al.*[11] also reported that their attempt to solubilize the enzymes responsible for the oxidative demethylation of TMA in the yeast has not been successful and that the enzymes may be located on an organella in the cells and be inactivated during the disruption of the cells.

Based on all the data obtained we[1,2] have discussed about the reasons why the amount of TMA in the salted fish muscle homogenates did not exceed the threshold value of spoilage, whereas in the absence of NaCl it rapidly increased. In the homogenates stored without NaCl, a bacterial population (A group in the previous paper[1]) which produced TMA became dominant. Some molds (F1 or F3 strain) grew on these homogenates, but they did not utilize TMA. These data may explain why TMA accumulated in the homogenates without salt. In the homogenates added with 5% NaCl, some yeasts became dominant but they did not produce TMA. In this case TMA-producing activity of bacteria was depressed by the combined effect of NaCl and low temperature of which details will be described in the following paper. A small amount of TMA produced by bacteria in the salted homogenates was probably utilized by molds (F2 or F4 strain) which grew abundantly on the surfaces of the homogenates. In the homogenates stored with 15% NaCl, yeasts which did not produce TMA became dominant during storage and the bacterial activity to produce TMA was almost completely inhibited.

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