Consumption of Trimethylamine and Trimethylamine N-Oxide by *Aspergillus oryzae*

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An attempt was made in order to examine the consumption of trimethylamine (TMA) and trimethylamine N-oxide (TMAO), the main components of fishy odor and its precursor, by *Aspergillus oryzae*.

The organism is one of the most useful molds utilized in making "koji" and is widely used in the fermentative industry in Japan.

First, a pressed cake of heated sardine was molded with the organism, and TMA, TMAO, and ammonia content in the molded cake were determined. The results indicated that TMA and TMAO were consumed markedly after a certain cultivation period.

Second, the consumption of TMA and TMAO in both the modified malt extract and Czapek's medium were examined. From the results obtained, it was concluded that TMA and TMAO were utilized as a nitrogen source for the growth of *A. oryzae*.

Fishy odor is one of the most serious barriers to the improved utilization of fish itself as well as fish products. Fishy odor is derived from a variety of components contained in fish. Trimethylamine (TMA) may be especially predominant in fishy odor.¹⁾ Although TMA has been found even in many fresh fishes, its concentration is extremely low.²⁾ In most cases, TMA is produced from trimethylamine N-oxide (TMAO) by microorganisms during the storage of the fish.³⁾

In order to remove TMA contained in fish materials and products, some investigations have been reported.⁴⁻⁸⁾*² Fujii *et al.*^{4,5)} reported that an amount of TMA did not increase when molds grew abundantly on the surface of salted fish muscle homogenate, and that *Penicillium* isolated from the homogenates utilized TMA rapidly. They furthermore found that, of 23 authentic strains of molds tested, 8 of 11 strains of *Penicillium*, 1 of 8 strains of *Aspergillus*, and none of *Rhizopus*, *Mucor*, *Citromyces*, or *Alternaria* have the ability to utilize TMA. Therefore, they suggested the possible application of TMA-utilizing molds to the food industry wherever it is desirable to remove TMA from food materials. But they reported that *A. oryzae*

has no ability to utilize TMA. Fujii *et al.* also found that, of 6 strains of molds isolated from "kusaya" brine, all strains except the strain of *Aspergillus* had a TMA-utilizing ability and 4 strains of *Penicillium* were more powerful.⁷⁾

On the other hand, Horie *et al.*⁸⁾ reported that TMA in the mixture of fish soluble and "shironuka", which is a kind of rice bran rich with albumen, was completely consumed during molding with *Aspergillus sojae*. A similar finding was reported in the mixture of the dark muscle of skipjack *Katsuwonus pelamis* and "soy sauce kodi."⁹⁾ Although the strain name was not described in the report, generally, *A. oryzae* or *A. sojae* are used for making "koji". However, little attention has been paid to the uniformity of micro-organisms used in these studies, because it has been reported that the commercial product of mold spores, "tanekoji" in Japanese, is contaminated by many bacteria.⁸⁻¹⁰⁾ Furthermore, some bacteria and a yeast take part in making koji.¹⁰⁻¹²⁾

In our course of studies on the improvement of fish products by molding, it was ascertained in a preliminary organoleptic test that "sardine koji" molded by *A. oryzae* displayed no TMA odor. Therefore, it is necessary to clarify the

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consumption of TMA and TMAO by *Aspergillus oryzae*, since the organism is one of the most useful molds utilized in making "koji" and is widely used in the fermentative industry in Japan.

Materials and Methods

Organism

Aspergillus oryzae IFO 4204 (*A. oryzae*) was used as the test organism.

Chemicals

TMA hydrochloride, dimethylamine (DMA) hydrochloride, methylamine hydrochloride, and TMAO were purchased from Tokyokasei Ind. Co., Ltd. Malt extract was obtained from Difco Lab. Co., Ltd. (Detroit, USA). Other chemicals were of special grade.

Sardine Meal as Natural Medium

The whole body of the sardine *Sardinops melanosticta* was heated at 90°C for 30 min. After heating, the sardine was pressed to dehydrate about 55% of its water content. Added to the pressed cake was 10 or 20% soluble starch which was mixed in by hand. The mixture was passed through a 5 mm hole size screen twice, using a chopper to granulate.

Media as Undefined and Defined Media

Malt extract medium was used to prepare the washed mycelia of *A. oryzae*. Approximately 0.1 mg N/ml medium of TMA or TMAO were added to malt extract media to ascertain the utilization of these in the undefined media. Czapek's medium, in which NaNO_3 was replaced by approximately 0.1 mg N/ml medium of TMA or TMAO, was used to ascertain the utilization of these in the defined media.

Cultivation

For the preparation of "sardine koji", 90 g of the granulated sardine meal were put into a 1 l flask, and then autoclaved at 121°C for 20 min and a loopful spore of the organism was inoculated. The inoculum was shaken vigorously and cultivated at 30°C. Mean while, malt extract medium was autoclaved at 121°C for 16 min and the modified malt extract and the Czapek's media were sterilized by filtration with a Milipore filter of 0.22 μm . After sterilization, both malt extracts and modified malt extract media were inoculated with a loopful spore of the organism

and then cultivated with reciprocal shaking at 110 rpm and at 25°C. The modified Czapek's medium was used for the following preparation of the washed mycelia suspension. After cultivation for 48 h in malt extract medium, the mycelia were harvested by filtration through double sheets of gauze and washed twice with sterilized water. After washing, 6 g of wet mycelia were put into 50 ml of the modified Czapek's medium containing TMA or TMAO and cultivated under reciprocal shaking at 25°C.

Determination and Identification of TMA, TMAO, and Ammonia

A test sample was prepared as follows. Of "koji", 10 g of "sardine koji" were macerated with 15 ml of 20% trichloroacetic acid in mortar. After 30 min, the mixture was filtrated with Toyo filter paper No. 5 A. Of the cultured fluid, a sufficient volume of cultured fluid was filtrated with a Milipore filter 0.45 μm to remove the mycelia, and then mixed with the same volume of 10% trichloroacetic acid, and furthermore, the mixture was filtered with Toyo filter paper as above. Both the filtrates from "koji" and cultured fluid were applied to determine TMA, TMAO, and ammonia (NH_3) by the micro-diffusion method.¹³⁾ Identification of TMA and other amines was carried out by thin-layer chromatography according to the method by Amano *et al.*¹⁴⁾

Determination of Dextrose, pH, Amino acids, and Mycelia

Dextrose and pH of the culture fluids were determined by phenol-sulfuric acid method and pH meter, respectively. Amino acids were determined by the hydrindantin method and presented as leucine. Mycelia were determined by the weighing method after drying for 24 h at 105°C.

Results and Discussion

Consumption TMA and TMAO by *A. oryzae* in Natural Medium

The sardine meal, which supplemented starch as a carbon source, was molded with *A. oryzae* at 30°C. The changes in the amount of TMA, TMAO, and NH_3 in the molded sardine meal, namely "sardine koji", are shown in Fig. 1. The amounts of TMA and NH_3 decreased with time except for large amounts of NH_3 at 60 h irrespective of the amount of added starch. On the other hand, the amount of TMAO remained

Fig. 1. Changes in the amount of TMA, TMAO, ammonia, and pH in the "sardine koji" molded on the sardine meal containing 10 and 20% starch.

TMA, TMAO, and NH_3 were determined by the micro-diffusion method.

Fig. 2. Time courses of various chemical factors during cultivation of *A. oryzae* in the malt extract medium with added TMA.

TMA and ammonia were determined by the micro-diffusion method, while dextrose, amino acid, and mycelia were determined by the phenol-sulfuric acid, hydrindantin, and weighing methods, respectively.

unchanged for 36 h, but decreased rapidly after that time. The pH values of the "sardine koji" did not change significantly in the case of 20% soluble starch. However, it increased rapidly after 48 h molding in the case of 10% soluble starch.

The amounts of TMA at 36 and 48 h were almost the same irrespective of the amount of added starch. However, TMA at 48 hours might be derived from TMAO, because at the same time the levels of TMAO decreased rapidly, and TMAO was reduced to TMA, as shown in Figs. 4 and 5. As for the large amount of NH_3 , it was considered that the added starch was completely consumed between 48 to 60 h of molding, and amino acids may have been utilized as the carbon

Fig. 3. Thin-layer chromatogram of volatile amines in the malt extract medium with added TMA during the cultivation of *A. oryzae*.

Fig. 4. Time courses of various chemical factors in the malt extract medium with added TMA during the cultivation of *A. oryzae*.

source. A similar phenomenon has been found in a *Penicillium* sp. isolated from the fish muscle homogenate. Fujii *et al.* reported that, in a medium containing TMA and nitrate, TMA was utilized after the nitrate had disappeared from the medium.⁴⁾ These findings suggested that competition was involved in the utilization of nitrogenous compound as the nitrogen source. With respect to the accumulation of NH_3 , Ishigami *et al.*¹⁰⁾ reported that the ratio of carbon source to nitrogen source for growth has the effect of producing NH_3 in *A. oryzae*. If an excess of carbon source is present in the medium, organic acids were produced and the pH value decreased. Besides, the presence of an excessive nitrogen source brings out the contrary effect by producing NH_3 from amino acids. The phenomenon observed coincided with the results as described in the report above.

The results above demonstrate indirectly the consumption of TMA, TMAO, and NH_3 by *A. oryzae* to the same extent.

Consumption of TMA and TMAO by *A. oryzae* in Undefined Medium

"Sardine koji" was made from natural medium and a solid culture lacking uniformity. Thus, the consumption of TMA and TMAO was tested to obtain further confirmation. As shown in Fig. 2, when *A. oryzae* was cultivated in the malt extract medium containing TMA, the amount of TMA decreased during cultivation and utilized 95% of added TMA during an 80-h period. The amount of amino acids decreased very rapidly, and they were consumed within 20 h. On the other hand, the amount of NH_3 was almost the

Fig. 5. Thin-layer chromatogram of volatile amines in the malt extract medium with added TMAO during the cultivation of *A. oryzae*.

same during the cultivation. The amount of dextrose also decreased with time but remained at 43% of the added amount at 80 h. Furthermore, the amount of mycelia increased concomitantly with the decrease of TMA, amino acids, and dextrose. The results indicate that amino acid was more favored as a nitrogen source for growth than TMA. Although TMA may be utilized slowly in coexistence with amino acids, it was utilized rapidly after the consumption of amino acids. Besides, the accumulation of NH_3 did not occur in this medium, which contained dextrose in a higher concentration than amino acids. It seemed that the excess dextrose may suppress the production of NH_3 , similar to Ishigami's findings¹⁰⁾ as described above.

As shown in Fig. 3, only TMA was detected by thin layer chromatography, while DMA and monomethylamine were not. In addition to

Fig. 6. Changes in the amount of TMA and ammonia in the modified Czapek's medium with added TMA during the cultivation of mycelia of *A. oryzae*.

Cultivation was performed in Czapek's medium consisting of 2% dextrose, 0.1% K_2HPO_4 , 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, trace $FeSO_4$, and 0.1% TMA by using precultivated mycelia. The conditions of analysis were the same as shown in Fig. 2.

Fig. 7. Changes in the amount of TMA, TMAO, and ammonia in the modified Czapek's medium with added TMAO during the cultivation of mycelium of *A. oryzae*.

Conditions of cultivation and analysis were the same as shown in Fig. 6.

the above, when the cultivated fluid was reduced with titanous chloride, the amount of volatile amines did not change before or after the reduction. This result indicated that the formation of TMAO from TMA did not occur.

The utilization of TMAO in malt extract medium by *A. oryzae* was also examined as with TMA. As shown in Fig. 4, the changes in the amounts of TMAO, dextrose, amino acids, NH_3 in addition to mycelia during the cultivation were almost the same as that of TMA. However, TMA and NH_3 were accumulated in the latter part of the cultivation. As shown in Fig. 5, TMA was the only volatile amine detected by thin-layer chromatography. These results indicate that the route of degradation of TMAO may be the reduction of TMAO to TMA and the demethylation of TMA to NH_3 . Furthermore, NH_3 derived from TMAO may be utilized as a nitrogen source, since the amount of the mycelia was increased for 70 h irrespective of the exhaustion of amino acids in the medium after 20 h of cultivation.

No chemical decomposition of TMA and TMAO contained in the medium was found during the cultivation. Namely, the amount of TMA and TMAO in the case of the medium without inoculation was not changed.

Consumption of TMA and TMAO by *A. oryzae* in Defined Medium

The suspension of washed mycelia was prepared with 6 g of wet mycelia and 50 ml of modified Czapek's medium, which was made from synthetic materials, as described previously.

As shown in Fig. 6, the amount of TMA decreased immediately and was completely consumed within 20 h.

As shown in Fig. 7, the decrease of the amount of TMAO was very slow during the early period of cultivation and almost complete consumption of the amount of TMAO was necessary for 48 h. During the cultivation in these media, the amount of mycelia increased approximately 2.0 times in the medium containing TMA and 1.5 times in the medium containing TMAO. These results also show that TMA and TMAO were utilized for growth of the mycelia as a sole nitrogen source.

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