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# Production of Mirin by Mutants of *Aspergillus* sp.

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Abstract

**To improve the quality of mirin, various molds were screened for mutants with high acid carboxypeptidase (ACPase) by the method of ultraviolet radiation. Mutants, *Aspergillus oryzae* 9-12 and *Aspergillus shiroussamii* 6082-60 showed activities of ACPase about 2~6 times higher than their parent strains. *Aspergillus oryzae* 9-12 and *Aspergillus shirosamii* 6082-60 were the most suitable strains for preparing *koji* in mirin by the conventional or improved methods. The results showed that total sugar, reducing sugar and total nitrogen were almost the same values in mirin prepared by both methods. The yield of mirin was higher in the improved method than in the conventional method. The clouding formation of mirin appeared in the conventional method ; however, mirin prepared with the mutant *koji* by the improved method did not show clouding formation.**

**Key words** : *Aspergillus oryzae, Aspergillus shirousamii,* mutation, mirin

## INTRODUCTION

Mirin, a conventional light yellow liquor, has been consumed as a seasoning in cooking rather than as an alcoholic beverage. It tastes very sweet owing to the high content of glucose and has a unique flavor mostly produced by the *koji* employed as one of the raw materials[1]. Conventionally, mirin is manufactured by fermenting the mixture of steamed rice, *koji* and brewing alcohol. Quality and productivity of mirin depend mainly on the enzymatic digestibility of steamed rice[2]. Since low quality and productivity of mirin-manufacture were considered to be the result of the insufficient enzymatic digestion of the raw materials, research focused not only on the properties of various enzymes in *koji,* but also on the conditions for soaking and steaming the rice to improve digestibility[3,4]. *Koji* plays a very important role in the mirin quality and productivity as sources of enzymes or flavor in the mirin-preparation[5]. In this regard, the mold selected for *koji* preparation directly affects the digestion of raw materials of mirin[6,7].

In mirin-preparation, *koji* has been traditionally prepared by *Aspergillus* sp. *Koji* prepared with *Aspergillus usamii mut. shirousamii* decreased the glucose concentration in mirin by transglucosidation[6]. Mirin prepared with *koji* of *Aspergillus kawachii* contained a large amount of unfermentable sugar and citric acid[8]. Various *koji* were prepared with *A. oryzae, A. awamori, A, usamii mut. shirousamii* and *Rhizopus oligosporus*[6,9]. Among them, *A. usamii mut. shirousamii,* was the most appropriate strain for preparing *koji* in mirin-preparation without reducing productivity[6].

To improve the productivity and availability of mirin, mirin was made with *koji* of a new mutant of *A. usamii mut. shirousamii*[10]. *A. usamii mut. shirousamii* has been treated by UV radiation, resulting in the mutant which produces twice as much citric acid as the original strain[7].

*Koji* prepared with a mutant of *A. usamii mut. shirousamii* had higher transglucosidase activity as compared with the original strain, and its mirin had a pleasantly sour taste[10]. Mutant *Aspergillus oryzae* derived from *Aspergillus oryzae* IFO 4079 had low acid carboxypeptidase activity in the *koji* prepar-

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ation process[11].

In the present study, mutants having higher enzyme activity were selected among various molds, and then we evaluated the quality of mirin prepared by *koji* of mutant, *A. oryzae and A. shirousamii.*

## MATERIALS AND METHODS

### Materials

#### Steamed glutinous rice

Polished glutinous rice was washed and soaked in tap water for 12~20hr at room temperature. After straining off the excess water through a stainless steel net, the soaked rice was steamed in an autoclave at 100° C for 20min. The steamed rice was then cooled to about 45° C.

#### Microorganisms

*Aspergillus awamorii* IFO 4122, *A. awamorii* IFO 4125, *A. niger* IFO 4034, *A. niger* IFO 6661, *A. niger* IFO 3390, *A. sojae* IFO 4200, *A. sojae* IFO 8875, *A. sojae* IFO 30112, *A. sojae* IFO 4359, *A. oryzae* IFO 3079, *A. oryzae* IFO 5238, *A. oryzae* 10, *A. usamii* IFO 4359, *A. usamii* IFO 4397, *A. usamii* mut. *shirousamii* IFO 6082, *A. usamii* IFO 8875, and *A. oryzae-9* (obtained from Institute of Fermentation, Japan) were used in this experiment.

#### Media

Malt agar medium (CM medium), pH 6.0 used for this experiment contained 20g glucose, 1.0g peptone, 15g agar per 1L distilled water. Czapek's agar medium contained 20g $NaNO_3$, 1.0g $K_2HPO_4$, 0.5g KCl, 0.5g $MgSO_4 \cdot 2H_2O$, 0.01g $FeSO_4 \cdot 7H_2O$, 3.0g sucrose and 15g agar per 1L distilled water.

#### *Koji*-preparation

Polished regular rice was washed and soaked in the tap water for 12~20 hr at room temperature and followed by steaming at 100° C for 20min. The steamed rice (200g) was inoculated with 0.2g of the spores of mold obtained from a slant culture. Static cultivation was carried out at 30° C for 48hr or 72hr under 90~100% relative humidity.

#### Preparation of crude enzyme from *koji*

*Koji* (10g) was mixed with 25ml of 10mM acetate buffer (pH 5.0) containing 0.5% NaCl. The mixture was filtered with filter paper No. 2 (Toyo Co.) and 5ml of the filterate was dialyzed against 10mM acetate buffer (pH 5.0) at 5° C for 24hr. Then dialyzate was brought to 10ml with 10mM acetate buffer (pH 5.0). This solution was used as the crude enzyme.

#### Enzyme activity

The activities of $\alpha$-amylase and glucoamylase were assayed using alkali-gelatinized potato starch, and the activity of protease was assayed using alkali-dispersed milk casein dissolved in 0.1M lactic acid or alkali[12].

The activity of acid carboxypeptidase (ACPase) was assayed using carbobenzoxy-glutamyl-tyrosine. One unit of ACPase was defined as the amount of enzyme needed to form 1$\mu g$ of tyrosine from carbobenzoxy-glutamyl-tyrosine at pH 5.0 and 30° C for 60min[11].

The activity of transglucosidase (TGase) was assayed by the official method of the National Research Institute of Brewing (Tax Administration Agency, Japan). One unit of enzyme activity is defined as the amount of enzyme needed to form 1mM of glucose from $\alpha$-methyl-D-glucoside at pH 5.0 at 40° C per 60min[13].

#### Mutation

Mutants were isolated from *A. oryzae* 9 after UV irradiation with a 15W lamp at the distance of 30cm according to the method of Hara and Sugama[14].

A suspension of spores was irradiated for 10min and inoculated in Czapek's medium. The mycelia were removed with a glass filter. The filterate was cultured in Czapek's, medium containing 1.5% agar at 30° C for 24h. The colonies on the plates with good growth were marked, then 10ml of Czapek's medium containing 1.5% agar and either arginine or proline at the concentration of 50$\mu g$/ml was

overlayed on the plate, and cultivation was continued at 30° C for 48h. The mutants were selected from colonies on the plate.

### Preparation of mirin

Mirin was prepared according to Uchida *et al.*[15]. A conventional mash composed of 93g *koji*, 62g of steamed glutinous rice, and 279g of 35% ethyl alcohol was incubated at 30° C for 30 days. Improved mash was composed of 93g *koji* and 62g steamed glutinous rice, 201g of 12.5% ethyl alcohol and commercial enzyme (α-amylase 40mg (4,000DU.g$^{-1}$), and protease 48.3mg (6,000DU. g$^{-1}$) obtained from Taepeungyang Co.) Improved mash was incubated at 30° C for 25 days (Table 1).

### Analytical methods

Total sugar, reducing sugar, and nitrogen were analyzed by the methods of AOAC[16]. Acidity was represented by the amount of 0.1N-NaOH required to neutralize 10*ul* of a sample to pH 7.0. Alcohol content was determined by measuring the specific gravity of the distillate[17]. Specific gravity was measured by the ostwald viscosimeter.

### Detection of clouding materials

Mirin was filtered with filter paper No. 131 (Toyo Roshi).

Alcohol clouding (detection of clouding · materials with alcohol) : 3.5ml of the appropriate concentration of alcohol was added to 5ml of filtered mirin to bring it to 44.5% (v/v) alcohol concentration. Its turbidity was measured at 660nm. Water clouding(detection of clouding materials with water) : 5ml of distilled water was added to 5ml of mirin. After, the mixture was stirred well and left for 2~3h.

Heat clouding (detection of clouding materials with heat) : 100ml of mirin was heated until only half remained. The results were evaluated by visual inspection. Turbidity was recorded as positive(+) or negative (-)[18].

**Table 1. Compositions of feeding materials for fermentation of Mirin**

| Raw materials | Preparation of mash | | | |
|---|---|---|---|---|
| | Conventional method | | Improved method | |
| Steamed glutinous rice (g) | 628 | 628 | 628 | 628 |
| *Koji* (g) | 93 | 93 | 93 | 93 |
| 35% Ethyl alcohol (g) | 279 | 279 | 0 | 0 |
| 12.5% Ethyl alcohol (g) | 0 | 0 | 201 | 201 |
| 93.2% Ethyl alcohol(g) | 0 | 0 | 78 | 78 |
| α-Amylase (mg) | 0 | 0 | 40 | 40 |
| Protease (mg) | 0 | 0 | 48.3 | 48.3 |

α-Amylase (4,000DU.g$^{-1}$) and protease (6,000DU.g$^{-1}$) obtained from Taepuengyang Co.

## RESULTS AND DISCUSSION

### Screening of molds

Enzymes such as α-amylase and protease released from *koji* are important to increase the productivity of the mirin. ACPase is also an important enzyme in its contribution to the quality of the mirin[11]. To screen the mold for high activity of ACPase, various molds used in food or alcoholic beverages were evaluated for the activity of ACPase. Molds were cultured on the steamed rice at 30° C for 48h, and then to evaluate their suitability for use in the mirin preparation, the ACPases in different preparations of *koji* were assayed with or without 13.5% alcohol (Table 2). Among them, *koji* made from *A. oryzae* 9 and *A. shirousamii* IFO 60 82 had the highest level of ACPase activities. ACPase activities of *Koji* made from *A. oryzae* 9 were 6,900units/g without alcohol and 2,560units /g with alcohol. *A. shirousamii* IFO 6802 was 6,820units/g without alcohol and 2,480 units/g with alcohol.

*A. oryzae* 9 and *A. shirousamii* IFO 6082 have almost the same high levels of ACPase activities. Therefore, *A. oryzae* 9 and *A. shirousamii* IFO 6082 were selected for further study of mutation by using UV irradiation.

### Isolation of mutants

To increase the ACPase activity, *A. oryzae* 9 and *A. shirousamii* IFO 6082 were treated with UV irradiation. Generally, mutants of brewing molds grew well on the Czapek's medium containing certain amino acids[11]. In this experiment, mutation

was carried out by UV irradiation. Mutants were cultured in the Czapek's agar medium and about 200 colonies of mutants were obtained. When each colony was incubated at 30°C for 48h, most mutants grew slowly on the Czapek's medium. However, some colonies grew well in the Czapek's medium containing arginine or aspartic acid. Mutants of the highest ACPase activities were incubated at 30 for 48h. Harvested spores of the mutants were inoculated on the steamed rice and cultivated at 30°C for 48h.

**Table 2. Activity of ACPase in *koji* prepared with different strains**

| *Koji* strains | | Activity of ACPase unit/g of *koji* | |
|---|---|---|---|
| | | Alcohol concentration 0% | 13.5% |
| *A. awamorii* | IFO 4122 | 634 | 308 |
| *A. awamorii* | IFO 4125 | 102 | - |
| *A. niger* | IFO 4034 | 1160 | 305 |
| *A. niger* | IFO 6661 | 1140 | 200 |
| *A. niger* | IFO 3390 | 1640 | 140 |
| *A. oryzae* | IFO 3079 | 5700 | 2480 |
| *A. oryzae* | IFO 5238 | 6630 | 1050 |
| *A. oryzae* | 9 | 6900 | 2560 |
| *A. oryzae* | 10 | 4700 | 1640 |
| *A. sojae* | IFO 4200 | 6280 | 2340 |
| *A. sojae* | IFO 8875 | 6340 | 2450 |
| *A. sojae* | IFO 30112 | 6400 | 2400 |
| *A. sojae* | IFO 4359 | 3200 | 1640 |
| *A. usamii* | IFO 4359 | 3820 | 2100 |
| *A. usaii* | IFO 4397 | 1030 | 362 |
| *A. shirousamii* | IFO 6082 | 6820 | 2480 |
| *A. usami* | IFO 8875 | 1370 | 980 |

*A. oryzae* 9 and *A. oryzae* 10 were obtained from Institute of Fermentation, Japan

**Table 3. Effects of amino acids on the growth of *A. oryzae* 9 and *A. shirousamii* IFO 6082**

| Amino acids | Growth (O.D.660nm) | |
|---|---|---|
| | *A. oryzae* 9 | *A. shirousamii* IFO 6082 |
| Lysine | 0.06 | 0.17 |
| Histidine | 0.12 | 0.14 |
| Arginine | 0.50 | 0.49 |
| Aspartic acid | 0.38 | 0.36 |
| Threonine | 0.02 | 0.06 |
| Serine | 0.20 | 0.24 |
| Glutamic acid | 0.23 | 0.28 |
| Proline | 0.30 | 0.32 |
| Glycine | 0.26 | 0.28 |
| Alanine | 0.17 | 0.09 |
| Valine | 0.21 | 0.20 |
| Methionine | 0.23 | 0.25 |
| Tryptophan | 0.04 | 0.05 |
| Tyrosine | 0.08 | 0.04 |

Spores($10^5$)of *A. oryzae* 9 and *A. Shirousamii* IFO 6082 were inoculated into 10ml of Czapek's medium containing the above amino acids to give final concentration of 20μg/ml for each amino acid. The mixture was incubated in the shaker at 30°C for 18h. The culture was homogenized with a glass homogenizer and the degree of growth was measured by the absorbance at 660nm

Mutants growing well on the steamed rice were selected for *koji* preparation. Mutants from *A. oryzae* 9 and *A. shirousamii* IFO 6082, were chosen based on requirement of arginine or aspartic acid for growth, and then designated *A. oryzae* 9-21 and *A. shirousamii* 6082-60.

The morphological characteristics of the mutants on the Czapek's sugar and malt agar medium were almost the same in shape, size, and content as the parent strains.

Activities of mutants, *A. oryzae* 9-12 and *A. shirousamii* 6082-60 were 6,980units/g, 6,900 units/g without alcohol, respectively and 2,700 unit/g, 2,567units/g with 13.5% alcohol, respectively.

*Aspergillus* sp. was treated by UV irradiation, resulting in a mutant which produced twice as much enzymes of several types as the parent strains[10,14]. *Koji* prepared with the mutant of *A. oryzae* had a high level of transglucosidase activity[15]. The mutant induced by UV irradiation from *A. oryzae* IFO 4079 had three times as much acid carboxy pepticase (ACPase) activity as did that of parent strains[11]. These mutants were used for further study.

### ACPase activity of mutant *koji*

With *koji* prepared with mutant, *A. oryzae* 9-12 and *A. shirousamii*, 6082-60 ACPase activities were compared, and their parent strains were inoculated separately on steam rice and cultivated at 30°C for 80h. ACPase activities of *Asp. oryzae* 9-12 was 19,200units/g of *koji* which was about 2.7 times higher than that of the parant strain after 48h of cultivation. In case of *A. shirousamii* 6082-60, ACPase activity was 18,200unit/g of *koji* which was about 2.6 times higher than that of the parent strain after 48hr of cultivation. Tanaka *et al.* reported that the mutant was obtained by UV irradiation

from *A. oryzae* IFO 4079, and had three times as much ACPase activity than that of the parent strain[11].

In the connection, the activities of α-amylase, glucoamylase and protease combined to improve the productivity of the mirin preparation. Table 4 shows the activities of α-amylase, glucoamylase, protease, ACPase and transglucosidase of *koji* prepared with the mutant. *Koji* prepared with the mutant had high activities of α-amylase, and glucoamylase-as active as those of parent strains, and the protease at pH 6.0 and pH 3.0 were more active than the parent strains.

Oyashiki *et al.*[11] suggested that α-amylase, glucoamylase and protease increased the productivity of mirin. In addition, increases in the concentration of oligosaccharide, amino acids and peptides would probably improve the taste in mirin[6]. Therefore, the *koji* prepared with *A. oryzae* 9-12 and *A. shirousamii* 6082-60 were suitable to improve the taste of mirin.

**Mirin preparation**

Mirin was prepared with *A. oryzae* 9-12, *A. shirousamii* 6082-60 and their parent strains by the conventional or improved methods. As shown in Table 5 and 6, the amounts of total sugar and reducing sugar were almost the same when fermented by the conventional or improved methods. The total nitrogen was at a higher level in the conventional mirin produced by the *A. oryzae* 9-12 than in the parent strain in the improved mirin prepared with *A. oryzae* 9-12. Nitrogen was higher than that of parent strain. A significantly higher level of amino-nitrogen was found in mirin prepared by the improved method ; however, in mirin prepared by the conventional method, amino-nitrogen was low.

The yield of mirin prepared with *A. oryzae* 9-12 and *A. shirousamii* 6082-60 was the highest through the improved method. These results suggested that the yield of mirin prepared by the improved method was high because of the addition of α-amylase and protease[15]. The limiting factor of the

**Table 4. Enzyme activities in *koji* prepared from *A. oryzae*-9, *A. shirousamii* IFO 6082 and their mutants**

| Strains | α-Amylase (DU/g) | Glucoamylase (GU/g) | Protease (PU/g) | | ACPase | TGase |
|---|---|---|---|---|---|---|
| | | | pH 3.0 | | pH 6.0 | (unit/g) |
| *A. oryzae* 9 (parent) | 210 | 32 | 114 | 68 | 6,820 | 56 |
| *A. oryzae* 9-12 (mutant) | 214 | 36 | 223 | 73 | 19,200 | 58 |
| *A. oryzae* 9-29 (mutant) | 209 | 34 | 210 | 70 | 19,000 | 53 |
| *A. shirousamii* IFO 6082 (parent) | 209 | 33 | 160 | 68 | 6,700 | 53 |
| *A. shirousamii* 6082-28 (mutant) | 214 | 31 | 213 | 74 | 11,200 | 50 |
| *A. shirousamii* 6082-60 (mutant) | 219 | 38 | 214 | 68 | 18,200 | 63 |

DU : Dextrinogenic unit, GU : Glucose Unit, PU : Proteolytic unit

**Table 5. Yield and chemical components of mirin made from different *koji* of *A. oryzae*, *A. shirousamii* 6082 and their mutants, *A. oryzae* 9-12, *A. shirousamii* 6082-60 by the conventional method**

| Components | Conventional method | | | |
|---|---|---|---|---|
| | Parents | | Mutants | |
| | *A. oryzae* 9. | *A. shirousamii* 6082 | *A. oryzae* 9-12 | *A. shirousamii* 6082-60 |
| Total sugar as glucose (%) | 42.1 | 42.5 | 40.0 | 40.2 |
| Reducing sugar as glucose (%) | 34.3 | 34.8 | 36.9 | 36.8 |
| Nitrogen (%) | 0.63 | 0.58 | 0.76 | 0.75 |
| Amino-nitrogen (%) | 0.016 | 0.012 | 0.043 | 0.048 |
| Ethanol (%, v/v) | 10.3 | 0.3 | 10.3 | 10.3 |
| Acidity (ml, 0.1N-NaOH) | 0.46 | 0.46 | 0.46 | 0.47 |
| Specific gravity at 15°C | 1.160 | 1.161 | 1.161 | 1.161 |
| pH | 5.7 | 5.7 | 5.6 | 5.6 |
| Yield of mirin (g.mirin/g.mash) | 0.60 | 0.64 | 0.69 | 0.69 |

**Table 6. Yield and chemical components of mirin made from different *koji* of *A. oryzae-9*, *A. shirousamii* 6082 and their mutants, *A. oryzae* 9-12, *A. shirousamii* 6082-60 by the improved method**

| Components | Improved method | | | |
|---|---|---|---|---|
| | Parents | | Mutants | |
| | *A. oryzae* 9. | *A. shirousamii* 6082 | *A. oryzae* 9-12 | *A. shirousamii* 6082-60 |
| Total sugar as glucose (%) | 43.0 | 43.8 | 41.0 | 41.0 |
| Reducing sugar as glucose (%) | 35.8 | 36.0 | 40.0 | 40.0 |
| Nitrogen (%) | 0.40 | 0.40 | 0.60 | 0.063 |
| Amino-nitrogen (%) | 0.028 | 0.023 | 0.063 | 0.07 |
| Ethanol (%, v/v) | 10.3 | 10.3 | 10.3 | 10.3 |
| Acidity (ml, 0.1N-NaOH) | 0.47 | 0.48 | 0.48 | 0.48 |
| Specific gravity at 15°C | 1.166 | 1.167 | 1.163 | 1.163 |
| pH | 5.6 | 5.6 | 5.6 | 5.6 |
| Yield (g.mirin/g.mash) | 0.74 | 0.70 | 0.85 | 0.85 |

**Table 7. Cloudings in mirin made from *A. oryzae 9*, *A. shirousamii* 6082 and their mutants, *A. oryzae* 9-12 and *A. shirousamii* 6082-60 by the improved method**

| Components | Clouding of mirin | | | |
|---|---|---|---|---|
| | Parents | | Mutants | |
| | *A. oryzae* 9. | *A. shirousamii* 6082 | *A. oryzae* 9-12 | *A. shirousamii* 6082-60 |
| Alcohol clouding | 0.650 | 0.840 | 0.475 | 0.402 |
| Water clouding | 0.830 | 0.720 | 0.481 | 0.360 |
| Heat clouding | (++) | (++) | (-) | (-) |

(++) : positive(high clouding), (-) : negative (no clouding)

ACPase and protease depended on the high alcohol concentration in the conventional mirin. In the improved mirin with 12.5% alcohol and the addition of enzymes, the limiting factor of the action of ACPase seemed to be solid material in the mash compared to the solution containing substrate for the ACPase assay *koji* and steamed glutinous rice[10], and brewing alcohol was used as the raw materials in mirin preparation.

### The degree of clouding in mirin

Mirin prepared by the conventional or improved method often clouded during storage or heating[19]. This phenomenon is undesirable for commercial mirin. As shown in Table 7, a clouding formation was observed in mirin prepared by the conventional method. The clouding in the mirin was much less in the *koji* prepared with the mutants by the improved method.

Yamashita and Doi[18] reported that clouding in mirin was caused by solubilized protein which had a molecular weight more than 4,000Kd, and mechanisms of the clouding formation caused the protein of low molecular weight. The clouding formation decreased the quality of mirin. Mirin prepared with *koji* of *A. sojae* 9-12 and *A. shirousamii* 6082-60 did not show clouding in the improved method. Therefore, the *koji* prepared with the mutants was suitable for mirin when prepared by the improved method, and the taste of mirin was improved by use of *koji* prepared with the mutants.

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# *Aspergillus* sp.의 변이주에 의한 미린의 생산

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## 요 약

본 연구는 품질이 우수한 미린을 생산하기 위하여 미린의 생산에 필요한 *koji*제조 균주를 검색하여 이들 중 ACPase활성이 높은 균주는 *Aspergillus oryzae* 9와 *Aspergillus shirousamii* IFO 6082를 얻었다. 이 두 균주는 UV조사에 의하여 변이주인 *Aspergillus oryzae* 9-12와 *Aspergillus shirousamii* IFO 6082-60을 얻었는데 acid carboxypeptidase의 활성은 친주에 비하여 2.6배 높았다. 이 균주로 재래식과 개량식 방법으로 미린을 제조한 결과 총당, 환원당 및 총질소의 양은 비슷하였으나 아미노태질소는 재래식보다 개량식이 높았고 수율도 높았다. 미린의 품질에 지대한 영향을 미치는 혼탁의 형성은 개량식에서 변이주로 만든 미린에서는 나타나지 않았다.