KOJI - THE KEY PRODUCT IN JAPANESE ALCOHOLIC BEVERAGES AND FERMENTED FOODS

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ABSTRACT

Koji is a culture of koji mold, *Aspergillus* grown on cereal substrates. Koji making process is one of the most typical solid state cultivation. In Japan, both alcoholic beverages and fermented foods are made with koji, although different strains and substrates are used depending on the final product. It is the most important role of koji to supply various enzymes. These enzymes generally decompose complex compounds, including carbohydrates, proteins and fats, into smaller molecules. In addition, koji is an important contributor of flavor, aroma and color materials to the final products. In submerged culture, none of such substances are produced in them. Accordingly, attempts to use a submerged culture broth as a substitutes for koji were unsuccessful. It is worth noting that no aflatoxin-producing strains are found among industrial strains of koji mold.

Conventionally, koji is prepared by manpower using small wooden trays. This method, however, is very trouble some and needs much labor. Therefore, in order to save labor, automatic methods have been devised successfully for commercial application. In koji preparation, enzyme production of koji mold is much influenced by temperature. Being cultured at a relatively high temperature, e.g. 45°C, it produce amylase in greater amount. On the contrary, at a relatively low temperature, e.g. 35°C, it produces protease more abundantly. Improvement of strains by mutation was also carried out. To prevent excess color development of snake, a mutant strain of *Aspergillus oryzae* lacking deferriferrichrysin productivity was selected by UV irradiation of conidia. As for *A. oryzae* used in soy sauce production, a strain having higher productivity of protease than ordinary strains was made by UV irradiation followed by protoplast fusion.

INTRODUCTION

Koji is a culture of *koji* mold, *Aspergillus* grown on cereal substrates, such as rice, barley or soybean. *Koji* making process is one of the typical solid state cultivation.

In many Asian countries, *Aspergillus* are used to produce seasoning agents, such as soy sauce. To produce alcoholic beverages, however, molds other than *Aspergillus* generally used (Table 1). In Japan, both alcoholic beverages and fermented foods are produced using *koji,* although different strains and substrates are used depending on the final products (Table 2).

Aspergillus oryzae

Though there are several species being industrially used in the genus *Aspergillus,* the most important one is *A. oryzae.* This mold produces many kinds of enzymes, among which amylase and protease are the most important.

Aspergillus oryzae includes many strains in which there are slight graded variations in morphological and physiological properties from strain to strain. One strain may produce more amylase and less protease and *vice versa.* Strains producing much amylase are used for *sake* brewing and others producing much protease are used for making fermented foods, such as *shoyu* and *miso.*

Table 1. Names given in various countries to a starter used to manufacture certain food product.

Name	Country	Microorganism*
Bubod	Philippines	*Rhizopus*
Ragi	Indonesia	*Absidia*
Ragi	Malaysia	*Mucor*
Luk-pang	Thailand	*Amylomyces*
Chuzu	China	*Endomycopsis*
Nurook	Korea	*Sacchromyces*
Murcha	Nepal	*Candida*

* Microorganisms listed here were isolated from these starters by many workers.

Table 2. Koji used in Japanese indigenous beverages and fermented foods.

Name	Micro-organism	Substrate	Note
Sake	*A. oryzae*	white rice	
Shochu	*A. kawachii*	rice	spirits
Awamori	*A. awamori*	broken rice	spirits made in Okinawa
Miso	*A. oryzae*	rice, barley, soy bean	soy paste
Shoyu	*A. oryzae* *A. soyae*	soy bean + toasted and ground wheat	soy sauce

Non-productivity of Aflatoxin by Aspergillus oryzae

It is worth noting that no aflatoxin producing strains are found among industrial strains of *koji* mold.

The *koji* molds have long been considered to belong to *Aspergillus oryzae* and/or *Aspergillus flavus* by many investigators. Because almost all of the Japanese indigenous alcoholic beverages and fermented foods are made using *koji* molds as enzyme sources, outbreak of aflatoxin being produced by *A. flavus* had become a serious problem for Japanese. Therefore, the Japaneses have extensively looked at the productivity of aflatoxins by *A. oryzae,* and none of *koji* mold has been found to produce them.

Murakami *et al.* (1967, 1968) investigated 214 industrial strains of *Aspergillus* (100 for *sake*, 32 for *miso*, 44 for *shoyu*, 16 for spirits and 22 wild strains) for the production of aflatoxin after cultivation in agar slants and in a liquid medium by shaking. No aflatoxin producers was found, though there were some strains producing compounds that were very similar to those of aflatoxins in fluorescence spectra and R_f values in thin layer chromatograms. (Table 3).

Yokotsuka *et al.* (1967) likewise studied the aflatoxin production in 73 industrial strains of *Aspergillus* and found all of them to be negative.

Later, the *koji* mold were proved to belong to the *A. oryzae* group and definitely discriminated from the *A. flavus* group producing aflatoxin, by a multivariate statistical analysis. (Murakami, 1971).

Table 3. Number of industrial strains of *Aspergillus* examined for their aflatoxin productivity by Murakami and Yokotsuka.

Investigator	Murakami	Yokotsuka
Strains		
For *sake*	100	28
For *miso*	32	16
For *shoyu*	44	27
For spirits	16	—
Total	214	73

TANE-KOJI (SEED KOJI)

To make *koji, tane-koji* is used as inoculum. None of *koji* is made with spontaneous fermentation.

Tane-koji is a *koji* which has a great deal of spore and yellow-greenish color, and made by special makers in Japan. For the past millenium, *tane-koji* makers have been isolating a strain of *A. oryzae* which has superior quality. Once they got such a strain as they had expected, they preserved it in a form of *tane-koji* for a long time. So, in Japan, neither alcoholic beverages nor fermented foods are made with wild strains of *A. oryzae.* It is reasonable to say that *A. oryzae* is one of the oldest horticultural plant in the world.

Tane-koji is made of brown rice. The steamed brown rice is mixed with wood ashes and inoculated with spores of some specific molds which have been prepared in advance, and cultivated at about 30°C. The cultivation is finished after 5—6 days when the materials became abundunt with spores; the *tane-koji* may contain about 4×10^9 spores per g. These are dried to 10% moisture content in the sun or by fire, then packed. At present, *tane-koji* is produced and distributed to saké brewers or food factories by some ten or so makers. An objective of using ashes is to give molds some nutrients and to prevent the media from bacterial contamination by giving a high pH to the media. *Tane-koji* contains at least two or three different strains of *Aspergillus oryzae* which have been mixed by each maker secretly as his particular product.

The Role of Koji

Undoubtedly, it is the most important role of *koji* to supply various enzymes. These enzymes generally decompose complex compound, including carbohydrates, proteins and fats, into smaller molecules. In addition, *koji* is an important contributor of flavor, aroma and color materials to the final products. In submerged culture broth, none of such substances are produced. Accordingly, attempts to use a submerged culture broth as a substitutes for *koji* were unsuccessful yet.

Conventional Koji Making Process

Conventionally, *koji* is made with small wooden trays by manpower.

After the steamed rice has been cooled to about 35°C, it is transferred into the cultivation room, in which temperature (28°C) and humidity are controlled at suitable level for the growth of mold. *Tane-koji* is sprayed on to rice at a rate of 0.1%. Then the rice is mixed thoroughly and heaped on a wooden bed. After 10—12 hours, the rice is mixed again and heaped. At 20 hours, a slight mycerial growth is apparent; at this stage the developing *koji* is distributed in small shallow wooden trays to about 5 cm in depth, and the trays are stacked on a shelf.

Thereafter, at 6—8 hour intervals, the *koji* mass in the trays is mixed by hand. As mold growth continuesly, the temperature of the *koji* mass could rise to 40°C or higher. Therefore, the *koji* mass should be stirred periodically to maintain uniform temperature, moisture and airation. Forty four hours after the inoculation, the *koji* making process usually is finished. At this stage, rice grain is completely covered with white mycelium of the inoculated *A. oryzae strains*. *Koji* has a pleasant smell, and is quite sweet in taste.

Cultural conditions influence the formation of enzymes. In general, the higher the cultivation temperatures, the greater the activities of amylase (Suzuki *et al.* 1956) (Table 4), and lower temperatures favor the development of protease activities (Suzuki *et al.* 1957) (Table 5).

Table 4. Effect of cultivation temperature on protease activity of rice *koji*.

Temp.	Acid protease	Alkaline protease
32°C	165*	239*
42°C	100*	100*

* Numerical value shows relative activity of enzymes.

Table 5. Effect of cultivation temperature on amylase activity of rice *koji*.

Temp.	α-Amylase	Glucoamylase
32°C	100*	100*
43°C	140*	132*

* Numerical value shows relative activity of enzyme.

RECENT PROGRESS OF KOJI MAKING TECHNOLOGY

Recent progress of *koji* making technology goes toward two directions. One is the improvement of used strains, and the other is the development of *koji* making machine.

Deferriferrichrysin Non-Producing Mutants of *Aspergillus oryzae*

For the past 100 years, it has been well known that iron containing water is not suitable for *saké* brewing, because of excess color development of produced *saké*, but its mechanism was not elucidated before Tadenuma *et al.* (1967) found ferrichrysin, red colorant containing iron, in *saké*. *A. oryzae* used in rice-*koji* making produces deferriferrichrysin, which chelates ferric ion to form ferrichrysin.

Based on the above described reason, it was concluded that the brewing water for *saké* should not contain more than 0.02 ppm of iron, but it is difficult to decrease iron in water to this level. To prevent *saké* from excess color development, another trial was attempted to get a mutant strain of *A. oryzae* lacking deferriferrichrysin productivity.

Table 6. Deferriferrichrysin productivity of *Aspergillus oryzae*.

Strain	Conc. of DFC* (mg/kg of koji)
RIB 203 (parent)	410
RIB 203—27 (mutant)	6
RIB 155 (parent)	324
RIB 155—11 (mutant)	41
RIB 647 (parent)	316
RIB 647—88 (mutant)	26
RIB 326 (parent)	370
RIB 326—77 (mutant)	35

* DFC: Deferriferrichrysin.

By UV irradiation of conidia on the surface of Czapek's medium, Hara *et al.* (1974) selected 6 mutant strains having lower deferriferrichrysin productivity than their parent strains. Rice-*koji* made with one of these 6 strains (RIB 203—27) contains no detectable amount of deferriferrichrysin and shows equal amylase activity to that of parent strain.

Table 6 shows deferriferrichrysin productivity of *Aspergillus oryzae*, both parent and its mutant strains.

The mutant strain RIB 203—27 now put into commercial use.

Breeding of *Aspergillus oryzae* by Protoplast Fusion For Soy Sauce Production

A. oryzae var. No. 13 for soy sauce production exhibits fast growth. Mutant strain No. 8536, which was derived from strain No. 13 using UV irradiation before, produces 8-times more protease than strain No. 13, but exhibits slow growth.

Furuya *et al.* (1983) carried out by protoplast fusion to breed a new strain which produced more protease than strain No. 13 and exhibited faster growth than strain No. 8536. At first, they derived new mutant strains having nutritional marker using UV irradiation (Table 7). Strain ac7 derived from strain No. 13 was cysteinless and had yellow conidia. Strain ac14 derived from strain No. 8536 was nicotinic acidless and had white conidia.

Table 7. Properties of used strains.

Strain No.	Phenotype		Growth* (mm)	Protease (x10^2 unit/g)
	Color	Requirement		
No. 13	green	—	78.5	4.30
ac7	yellow	cys^-	78.0	4.14
No. 8536	green	—	49.5	33.38
ac14	white	$n.a.^-$	50.2	32.76

* Growth was indicated by measuring the diameter of colonies of 5 days culture on koji extract agar.

Table 8. Properties of green strains.

Strain No.	Growth (mm)	Protease (x10^2 unit/g)
g 201	76.1	9.68
g 344	76.9	9.85
No. 8536	49.5	33.38
No. 13	78.5	4.30

Protoplasts of two mutant strains were obtained using snail gut juice. When the protoplasts of the two strains were treated with a solution containing polyethylene glycol, they fused effectively and formed heterocaryons. The fusion frequency was 1%. Two of the green strains derived from the heterocaryon showed high stability, fast growth, producing 2.3—times more protease than strain No. 13 (Table 8).

Development of Koji Making Machine

In Japan, many fermentation factories are so large that they usually produce five or more k*l* of *saké* or *shoyu* a day, and make one or more ton of *koji* a day.

Since the conventional *koji* making process is very troublesome and needs much labor, automatic *koji* making machine have been devised successfully for commercial application.

Fig. 1 shows the semi automatic *koji* making machine having a fixed bed. The bottom of the bed is perforated and a steady stream of forced and filtered air blow through *koji* mass. Probes buried in the *koji* monitor the temperature and humidity of the air blast to maintain conditions in the *koji* optimal for mold growth and enzyme production. In this machine, however, charge of the steamed rice and discharge of *koji* are carried out by manpower.

Fig. 2 shows the fully automatic *koji* making machine, which has two cultivation chambers, upper and lower, and each chamber has the rotary bed.

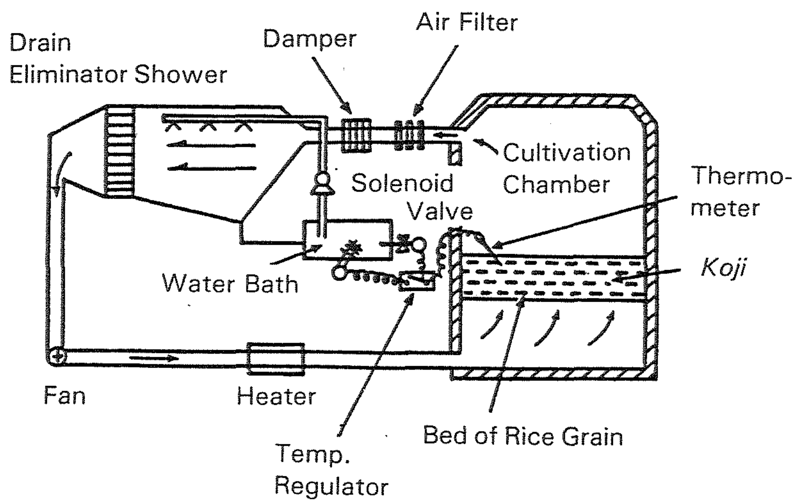
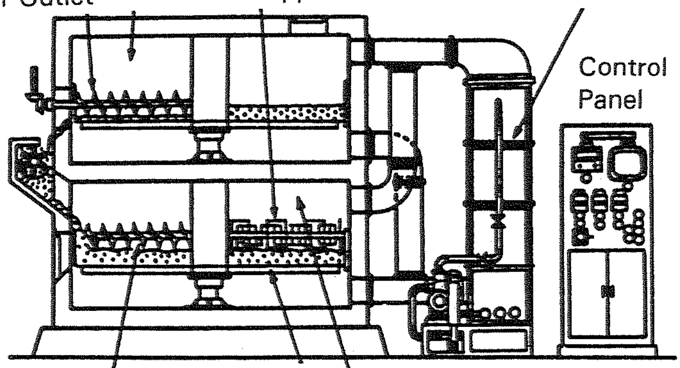


Fig. 1. Semi automatic *koji* making machine.



(By courtesy of NAGATA BREWING MACHINE Co., SAGAMIHARA, JAPAN)

Fig. 2. Fully automatic *koji* making machine.

In this fully automated machine, charge of the steamed rice, inoculation, stirring and discharge of *koji* are carried out mechanically. The steamed rice is charged into the upper chamber. After 20 hours cultivation, *koji* mass are transferred into the lower chamber, and cultivated for 24 hours with stirring at intervals. The diameter of the largest machine is ten meter and has the capacity of producing 10 ton of *koji* a day.

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