DEVELOPMENT OF PURE CULTURE STARTER FOR KECAP, AN INDONESIAN SOY SAUCE

[Pengembangan Ragi Kecap Khas Indonesia Dengan Biakan Murni]

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ABSTRACT

In order to prevent aflatoxin contamination during the production of *kecap,* an Indonesian soy sauce, a white-spored mutant K-IA strain induced from an aflatoxin-negative koji mold was applied to koji making process *of kecap.* While *kecap* koji making process took 9 days by conventional method without inoculum, the use of K-IA strain as a starter shortened the process to 3 days at room temperature. The koji prepared with this starter contained 2.3 x 10^7 cfu/g of *Aspergillus* sp. K-IA as the dominant species and some contaminants from the environment such as other species *of Aspergillus* (2.0 x 10" cfu/g) and Mucorales (1.4 x 10^5 cfu/g). The mash prepared with *kecap* koji inoculated with this starter contained higher formol nitrogen (FN) and water-soluble nitrogen (WN) than those prepared with koji inoculated without any starter. These results demonstrated that the use of this starter did not only contribute to the prevention of aflatoxin contamination but also improved the conventional *kecap* fermentation process.

Key words: *Aspergillus, ragi kecap,* soybean fermentation, non-aflatoxin.

Untuk mencegah teijadinya kontaminasi aflatoksin selama pengolahan kecap di Indonesia, biakan mutan BAf berspora putih yang diinduksi dari kapang negatif aflatoksin, digunakan untuk membuat ragi kecap. Dibanding cara konvensional, penggunaan biakan K"1A dapat mempersingkat proses fermentasi koji dari 9 hari menjadi 3 hari pada suhu ruang. Hasil menunjukkan bahwa koji yang difermentasi menggunakan ragi mutan mengandung 2,3 x 10^7 cfu/g *Aspergillus* sp., dimana biakan K-IA ditemukan sebagai spesies yang dominan serta beberapa kontaminan berasal dari spesies lain *Aspergillus* (2,0 x 10" cfu/g) dan Mucorales (1,4 x 10^5 cfu/g). Sampel sari kecap yang difermentasi dengan koji hasil inokulasi ragi mutan, mengandung formol nitrogen (FN) dan nitrogen terlarut (WN) yang lebih tinggi dibanding sampel yang difermentasi menggunakan koji dari ragi konvensional. Hasil tersebut menunjukkan bahwa penggunaan ragi mutan tidak hanya bermanfaat dalam mencegah kontaminasi aflatoksin tetapi juga meningkatkan proses fermentation kecap konvensional.

Kata kunci: *Aspergillus,* ragi kecap, fermentasi kedelai, bebas-aflatoksin.

INTRODUCTION

Kecap is a fermented soybean food known as Indonesian soy sauce. Whole soybeans, specially the varieties of black soybeans, are used as raw material for processing *kecap.* Kecap is prepared by spreading cooked soybeans on bamboo trays and leaving them for a period of time until molded soybeans *(koji)* are formed. Kecap manufacturers do not usually use any inoculum *(tane-koji, ragi kecap)* for *koji* preparation. During processing, molds grow on the cooked soybeans as the result of contamination from the environment such as the air and the previously used trays. Judoamidjojo (1986) reported that molds isolated from kecap *koji* were mostly from the genus of *Aspergillus.* It is known that agricultural food commodities such as peanut and maize are widely contaminated with aflatoxins, natural carcinogens, produced by fungi belong to *Aspergillus* section *Flavi,* in Indonesia (Dharmaputra, 1991). It was also reported that 15 out of 32 samples of Indonesian kecap contained aflatoxin Bj at concentrations of more than 5 µg/kg (Sadjonoefa/., 1992). Since the possibility of aflatoxin contamination cannot be ruled out in the traditional way of *koji* processing, thus, it is necessary to use a pure culture starter, *ragi* kecap from the standpoint of preventing aflatoxin contamination.

In our previous study (Nikkuni *et al.,* 2002), we induced white-spored mutants from *koji* molds by UV-irradiation and used them in kecap fermentation. We evaluated the possibility of distinguishing the mutant from aflatoxin-producing molds and revealed that white-spored mutant can be used as a starter for preparation

of kecap *koji* from the standpoint of preventing aflatoxin contamination.

In this study, we prepared *ragi* kecap using a white-spored mutant, K> 1A and applied it for kecap production at Zebra Kecap factory in Bogor, Indonesia. We will analyze and evaluate the quality of *kecap* moromi during fermentation.

MATERIALSANDMETHODS

Preparation of ragi kecap

Soaked rice (4 kg as raw material) was autoclaved at 121°C for 30 min, allowed to cool down until 40°C, inoculated with the *ragi* kecap *(Aspergillus oryzae* K-1A Strain) that had been prepared at JIRCAS in Tsukuba, Japan in 2001, left it overnight at room temperature, spread on a aluminum tray, and covered with a bamboo tray. After 4-day incubation at room temperature, it was dried at 42°C for 3 days.

Kecap production

Kecap were prepared by the conventional and traditional methods without inoculum and newly developed methods using the *ragi* kecap prepared at JIRCAS in 2001 at "Zebra Kecap" Factory in Bogor, Indonesia (Fig. 1). Microbial analysis and isolation of fungi Sample (lOg) was mixed with 90 ml of autoclaved 0.05% Tween 80. For counting filamentous fungi, serial dilutions of the samples were prepared in the same solution and the diluted suspensions (0.1ml) were spread on the plates of potato dextrose agar (PDA, Difco, USA) containing 100 ppmchloramphenicol. The plates were incubated at room temperature for 1 to 3 days at room temperature.

To count bacteria, 1 ml of diluted suspension was mixed with molten plate-count agar consisting of 0.5% peptone (Difco, USA), 0.25% yeast extract (Difco, USA), 0.1% glucose and 1.5% agar. One to 6 colonies of *Aspergillus* were isolated from a PDA plate and further purified.

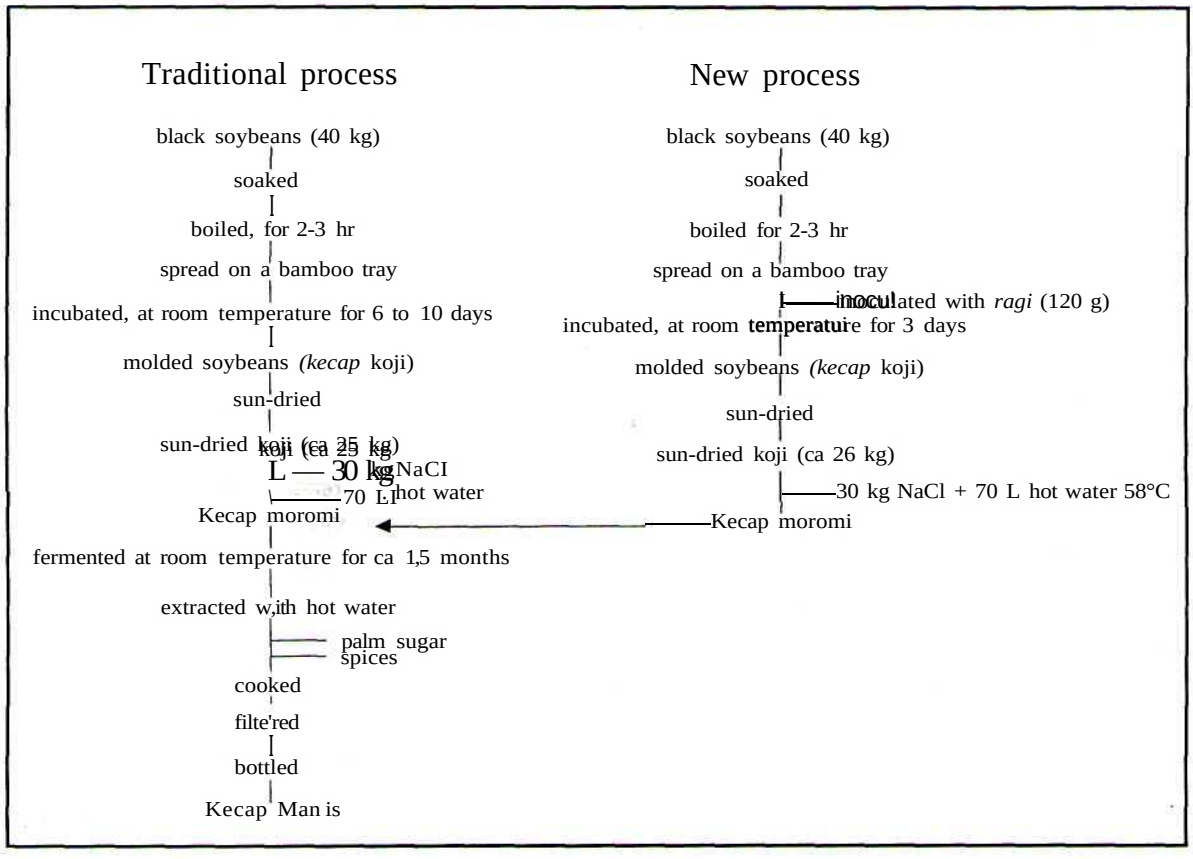

Figure 1. Kecap production process at Zebra Kecap Factory in Bogor, Indonesia.

Analytical methods

Moisture and Nitrogen

Moisture was determined by the aluminum foil cup method. A piece of aluminum foil (15 - 20 cm square) was formed into a cup shape (ca 5 cm in diameter, ca 5 cm deep) using a bottom of a 100 ml beaker. Five grams of sample were weighed into an aluminum foil cup and heated at 105°C overnight in an oven and cooled in a desiccator for 30 min.

After homogenizing kecap mash *(moromi)* preparations, total nitrogen (TN) was determined by Kjeldahl method. To determine water-soluble nitrogen (WN), lOg of the homogenized *moromi* was diluted to 250 ml with distilled water. The resulting diluted suspension was centrifuged at 18,000 rpm for 10 min and the supernatant was filtered. Nitrogen present in 20 ml of the filtrate was determined by Kjeldahl method. Formol nitrogen (FN) was determined according to Official Method of Miso Analysis (1986). After neutralizing the supernatant (50 ml) with 0.1 N NaOH to pH 8.5, formaldehyde (20 ml, pH 8.5) was added and the mixture was titrated with 0.1 N NaOH to pH 8.5.

Sodium chloride

Sodium chloride was determined according to the method described in Shokuhin Bunseki-Ho (1985). The supernatant (5 ml) as prepared above was taken in a porcelain evaporating dish and titrated with N/20 silver nitrate in presence of 1 ml 2% potassium chromate as an indicator.

RESULTS

Preparation of **ragi kecap**

To study the capacity of *ragi* kecap on the production of kecap comparing to the traditional way of kecap production at Zebra Kecap factory, it was required the *ragi* kecap, containing less than 10% moisure, more than 10^8 spores of *Aspergillus* and less than 10^4 bacteria for the pilot scale experiment purposes on kecap production at the factory. The *ragi kecap* was prepared from rice using the white-spored mutant K-1A strain (Photo 1).

Fig. 2 shows the moisture contents in *ragi* kecap during drying at 42°C after 4 day-fermentation at room temperature. Moisture of *ragi* decreased with drying time and reached the level of less than 10% after 3 days.

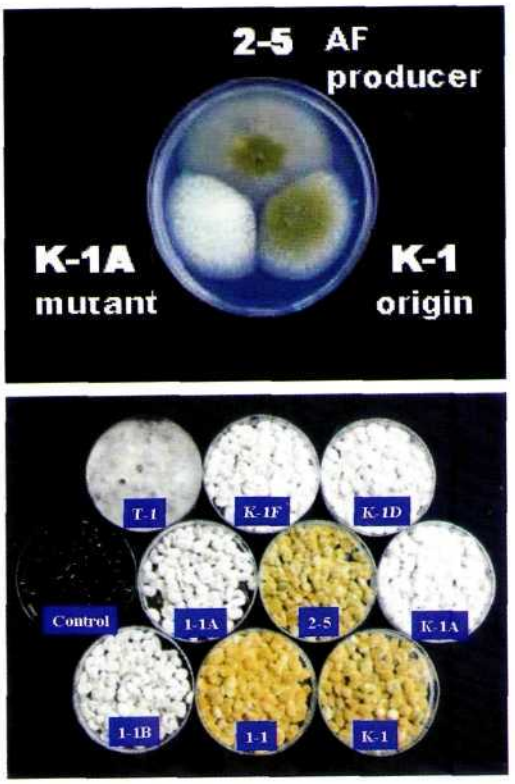

Photo 1. Kecap *koji* preparation using white-spored mutant *Aspergillus oryzae* K-l A *(above)* on rice-substrate for *ragi* kecap of various strains *(below).*

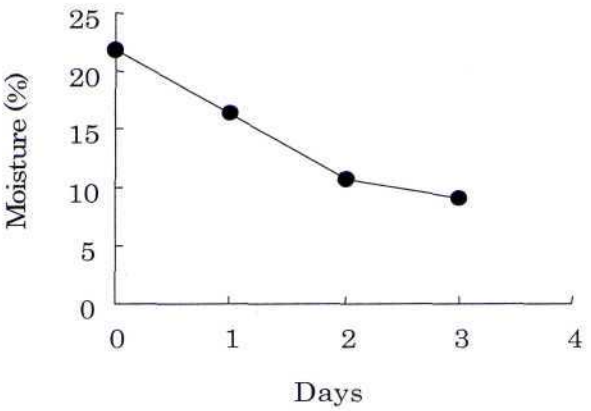

Figure 2. Moisture contents of *ragi* kecap during drying at 42°C.

Table 1 shows the yields, moisture contents, mold counts and total viable counts in prepared *ragi.* Four kg rice was converted into 3.3 to 3.7 kg *ragi.* Ragi contained 7.7% to 10.4% moisture, more than 10^8 spores of *Aspergillus* and less than 10^3 bacteria.

Preparation of kecap production at Zebra Kecap Factory

Kecap *kojis* were prepared by the conventional methods and the newly developed methods using the *ragi* kecap. Moisture contents and microbial counts in *koji* preparations are shown in Table 2. Both samples contained approximately 10^8 cfu/g of bacteria. The traditional *koji* sample contained 2.3 x 10^7 cfu/g of *Aspergillus* sp. as dominant mold and 5.0 x 10^6 cfu/g of Mucorales. After sun-drying, these values decrease to one-tenth. The *koji* prepared using the *ragi* kecap contained 2.3 x 10^7 cfu/g of *Aspergillus* sp. K-1A as the dominant mold, even though it contained contaminants: 2.0 x 10^6 cfu/g of the other *Aspergillus* spp and 5.0 x 10^6 cfu/g of Mucorales from the environment and the moisture contents decreased to 7 to 8 %.

Kecap mash *(moromi)* fermentation

Use of the *ragi* kecap for a large scale preparation of kecap koji is becoming important procedure by overgrowing soaked, cooked, cooled soybeans coated with ground roasted wheat with K-1A strain belonging to the *Aspergillus oryzae* species. The kecap koji contains proteases, amylases, and lipases that hydrolyse their respective substrates in the subsequent submerged fermentation in approximately 20% weight per volume salt brine. During the submerged fermentation, some halophilic microorganisms such as *Pediococcus cerevisiae, Lactobacillus delbruekii,* and salt-tolerant *Saccharomyces rouxii* naturally developed, since in a fact that kecap fermentation depends on proteolytic enzymes derived from those halophilic microbial strains to hydrolyse the proteins in the substrate to the constituent amino acids and peptides (Photo 2).

Table 1. Preparation of *ragi* kecap using rice as substrate

Ragi No.	**Yield* (kg)**	**Moisture** (%)	*Aspergillus* **sp (cfu/g)**	**TVC** (cfu/g)**
1	**3.7**	7.7	2.3 x 10^8	<3000
2	**3.4**	10.4	3.5x10*	<3000
3	3.3	9.0	3.9 x 10^8	<3000
4	3.3	8.9	5.8x10*	<3000

*) Four kg of rice was used as substrate ; **) Total viable bacterial counts.

Table 2. Moisture and microbial counts in kecap *koji*

Cooked and Fermented Soybean	**Moisture** (%)	**Mold count (cfu/g)**			**TVC (cfu/g)**
		Aspergillus sp.		*Rhizopus* sp. / *Mucor* sp.	
		K-lA	**Others**		
Cooked soybean	68.5	–	–	–	–
Traditional kecap *koji*					
Fermented (9 d) *koji*	36.5	-	**2.3** x 10^7	5.0 xlO^6	4.1 xlO^9
Sundried *koji*	7.3	-	**2.8** xlO^6	1.5 xlO^5	2.6 x 10^9
K-lA kecap *koji*					
Fermented (3 d) *koji*	54.9	2.3 xlO^7	**2.0** xlO^6	1.4 xlO^5	8.6x10*
Sundried *koji*	8.1	8.6 xlO^6	**1.0** xlO^6	1.3 xlO^5	1.8 xlO^9

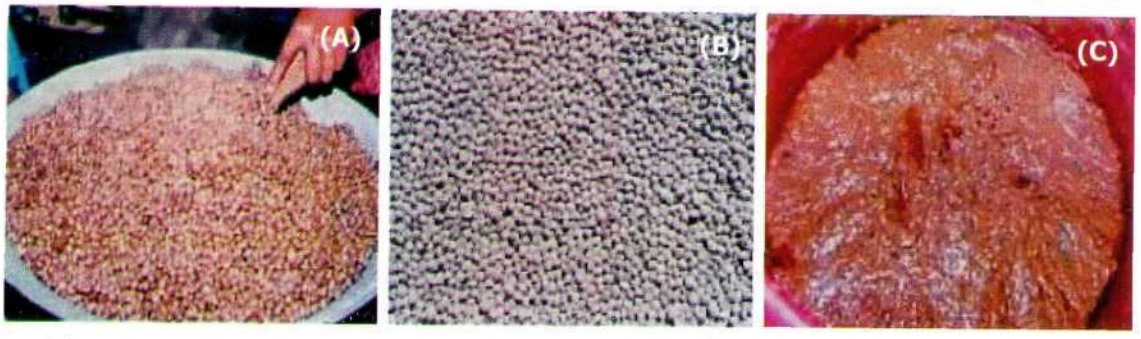

Photo 2. Preparation of kecap initiating with cooking soybean (A), inoculating with *ragi* kecap (koji) (B) and kecap mash fermentation *(moromi)* in salt-brine (C).

Table 3. Formol nitrogen (FN), water-soluble nitrogen (WN), total nitrogen (TN), NaCl contents, and pH values of kecap *moromi.*

Fermentation Period (days)	pH	NaCl (%)	FN (%)	WN (%)	TN (%)	FN/TN (%)	WN/TN (%)	FN/WN (%)
Control*(42)	5.53	20.8	0.17	0.68	1.65	10.3	41.21	25.00
Control (60)	5.54	21.0	0.26	0.83	1.98	13.1	41.92	31.33
*K-IA*** (2)	5.73	21.0	0.15	0.40	1.12	13.4	35.7	37.5
K-IA (14)	5.64	20.9	0.24	0.65	1.35	17.8	48.2	36.9
K-IA (42)	5.51	20.3	0.41	0.77	1.57	22.3	49.0	45.5
K-IA (60)	5.45	20.6	0.42	0.91	1.61	26.1	56.5	46.2

*) Traditionally processed *moromi.*
**) Newly processed *moromi.*with *ragi* K-IA.

Kecap *moromi* were prepared with the kecap *koji* and allowed to ferment in order to have more favourable kecap product enriched with amino acid constituents. Free amino acids are known to contribute the taste of kecap and glutamic acid is especially important for flavor. As FN value comprises ammonia and amino nitrogen, FN and TN of each kecap *moromi* were determined. Total nitrogen (TN) contents, water-soluble nitrogen (WN), formol nitrogen (FN), sodium chloride and pH during fermentation are shown in Table 3.

After 2 months submerged fermentation, the ratio of FN to TN (FN/TN) and the WN to TN (WN/TN) of kecap *moromi* prepared without *ragi* kecap according to Zebra Kecap procedure were 13.1% and 41.9%, respectively. However, the values of FN/TN and the WN/TN of kecap *moromi* inoculated with *ragi* kecap K-IA were 26.1% and 56.5%, respectively. *Aspergillus* strain K-1A apparently contributed well to hydrolysis of the soybean proteins during the fermentation rather than that of kecap from Zebra factory.

DISCUSSION

Kecap mashes *{moromi)* were prepared with each *ragi* kecap and allowed to ferment. It influenced the results of total nitrogen contents, water-soluble nitrogen, formol nitrogen, sodium chloride and pH values during fermentation. The pH value of the both mashes indicated 5.5 after 2 months of fermentation and they contained about 21% salt. The contents of formol nitrogen and water-soluble nitrogen increased with the fermentation time. After 2 months of fermentation, the *moromi* prepared with *ragi* kecap inoculated with the K-1A strain showed higher contents of formol nitrogen and water-soluble nitrogen than that prepared without the starter. These results demonstrate that the use of the white-spored mutant as the starter not only contributes to the prevention of

aflatoxin contamination but also improves the conventional kecap fermentation process.

The water-soluble nitrogen of the total nitrogen reached 56.5% after 2 months of fermentation when the starter culture was used. However, this value was lower than that of the result described in our previous paper[5'] using a 500 mL Erlenmeyer flask as a fermentation vessel at the laboratory. The temperature of the hot water used for the *moromi* preparation was 58°C in this study, while the water at room temperature was used in our previous work[5']. The hot water might have inactivated proteases of the *ragi* kecap when the *moromi* was prepared at Zebra Bogor Co. There was much room to improve kecap production process.

The *ragi* kecap that was prepared using K-lA was the white-spore mutant of strain K-l isolated from the Japanese *tane-koji* for soy sauce production. However, the WN content of the *moromi* samples prepared using both of the original strain (K-l) and the mutant one (K>1A) was not so much difference (Nikkuni *et al.*, 2002). Since the mutants develop white conidia during *koji* fermentation and exhibited enough activity for digesting soybean proteins, this white-spored *koji* of K- 1A strain was expectedly useful as a *ragi* kecap for manufacturing kecap *koji* from the standpoint of preventing aflatoxin contamination, since it could be distinguised from those prepared with the original *koji* strain and and the aflatoxin producer by their appearances (Nikkuni *et al.*, 2002).

CONCLUSION

While kecap *koji* making process took 9 days by conventional method (without inoculum), the use of K-lA strain as a starter, *ragi* kecap, shortened the process to 3 days at room temperature.

CFU of wild *Aspergillus* in kecap *koji* caused by contamination from environment decreased to 1/10 by using *ragi* kecap of K"lA strain.

The kecap mash prepared with kecap *koji* inoculated with *ragi* kecap contained higher formol nitrogen (FN) and water-soluble nitrogen (WN) than those prepared with *koji* inoculated without any starter.

These results demonstrate that the use of white-spored mutant as a starter, *ragi* kecap, does not only contribute to the prevention of aflatoxin contamination but also improves the conventional kecap fermentation process.

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