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Diploid Hybridization in a Heterothallic Haploid Yeast, *Saccharomyces rouxii*

HARUHIKO MORI AND HIROSHI ONISHI
Noda Institute for Scientific Research, Noda-shi, Chiba-ken, Japan

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By crossing of a heterothallic haploid yeast, *Saccharomyces rouxii*, we have succeeded in obtaining diploid hybrids. This paper shows one possible method of breeding heterothallic haploid yeasts for industrial application. *S. rouxii* is highly salt-tolerant and plays an important role in shoyu and miso fermentation. Therefore, genetic improvements of the properties are of commercial importance. Since newly isolated *S. rouxii* could neither conjugate nor sporulate on sporulation media commonly used, a suitable medium for conjugation and sporulation of *S. rouxii* was firstly investigated. A 5% NaCl Shoyu-koji extract agar was found to be most efficient. Next, we tried to get diploid strains by mass culture of two mating types on the conjugation medium, but several phenomena made this difficult: (i) zygotes quickly sporulated before budding; (ii) several zygotes showed terminal budding, but the buds could not grow into diploid cells, suggesting they would be heterocaryon; and (iii) a few zygotes lost their viability. After trying to isolate and cultivate a large number of zygotes in various combinations of crossing by micromanipulation, we fortunately recognized that large cells arose from some combinations. The analysis of ploidy suggested that the large cells would be diploid. Also, they showed sporulation of typical *Saccharomyces*, i.e., two to four spores in an unconjugated ascus. The diploid strains thus obtained were highly salt-tolerant and stable in liquid medium. Therefore, the procedure presented here would be effective for breeding salt-tolerant *S. rouxii*.

Yeast hybridization has been investigated by many researchers (12, 22), and their results have suggested that one of the most effective methods for breeding excellent yeast strains for application to fermentation and brewing industries would be to get hybrids of higher ploidy by crossing parent strains of excellent properties. Improvements of bakers' or brewers' yeasts along this line have been attempted, and results useful to industry have been achieved (6). Recently, it has been suggested by Gunge (7-9) that bakers' yeast can be improved in some important properties for industrial application. Johnston (10, 11) showed that haploid ascospores of brewers' yeast have low viability, but their hybrids sometimes have higher attenuating ability than the parental strains.

Genetic improvement of *Saccharomyces rouxii* (17), a commercially important yeast for shoyu and miso fermentation, has not yet been tried. This is largely attributable to the fact that *S. rouxii* spends its vegetative state in the haploid phase and, after isolation from its habitat, apparently becomes nonascosporogenous. Based upon their important finding of heterothallism in *S. rouxii*, Wickerham and Burton (21) suggested the possibilities of obtaining diploid *S. rouxii* by crossing opposite mating types. They suggested that yeast strains could be made more useful for practical application by use of this breeding procedure.

The present report describes the establishment of suitable media and cultural conditions for conjugation and sporulation of *S. rouxii*, breeding procedures, and means of obtaining diploid *S. rouxii*. We have succeeded in obtaining diploid *S. rouxii* and have shown that this hybridization method can be applied to salt-tolerant *S. rouxii*.

MATERIALS AND METHODS

Isolation and identification of S. rouxii. Salt-tolerant yeasts were isolated from shoyu mashes and miso pastes by use of 10% NaCl Sake-koji agar medium. The taxonomic study of pure cultures, obtained by repeating the dilution plate method on 18% NaCl Sake-koji agar medium, was carried out according to the method of Lodder and Kreger-van Rij (13); *S. rouxii* cells thus identified were used in the following experiments. *S. rouxii* NRRL-2547 and 2548, which were opposite mating types, were kindly supplied by L. J. Wickerham. Incubation temperature, except as specifically noted, was 25 C.

Media. NaCl (18%) Sake-koji agar medium was used for stock culture of salt-tolerant yeasts. Four modifications of Shoyu-koji media were used for preculture, conjugation, sporulation, and isolation of zygotes: for preculture, 2% NaCl Shoyu-koji agar (2.0 % reducing sugar and 0.6% total nitrogen); for sporulation, 5% NaCl-diluted Shoyu-kohi agar (0.25% reducing sugar and 0.07% total nitrogen); for conjugation, the sporulation medium supplemented with 5% glucose; for isolation of zygotes, the conjugation medium without agar (liquid medium). For harvesting yeast cells for ploidy determination, 18% NaCl synthetic medium of the following composition (13) was used: glucose, 5.0%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; $CaCl_2$, 0.01%; Vitamin Free Casamino Acids, 0.4%; yeast extract, 0.1%; NaCl, 18.0%. The *p*H of these media was adjusted to 5.0 to 5.2.

Preparation of Shoyu-koji extract. For this process, 55 g of defatted soy beans and 45 g of cracked and parched wheats were mixed thoroughly with 50 ml of tap water. After about 0.5 hr, the mixture was autoclaved at 15 psi for 30 min, and the cooked materials were inoculated with *Aspergillus sojae* and incubated at 30 C for 3 days. Thus, we get "Shoyu-koji." Shoyu-koji was extracted with water of four times its weight at 45 C for 5 hr. After clarification with egg white, clear shoyu-koji extract, which contained about 2.0% reducing sugar and 0.6% total nitrogen, was obtained.

Preparation of Sake-koji extract. After steeping rice in tap water over night, swelled rice was cooked for 1 hr, and the cooked materials were inoculated with *A. oryzae* and incubated at 30 C for 2 days. During the incubation, they were mixed once. The product is called as "Sake-koji." Sake-koji was then extracted with water of four times its weight at 60 C for 6 hr, and clear Sake-koji extract was obtained after the clarification by the same method used for Shoyu-koji extract. This extract, which contained about 15% reducing sugar and 0.1% total nitrogen, was diluted to 10% reducing sugar and adjusted to *p*H 5.0 to 5.2 after the addition of 18 or 10% sodium chloride. In Japan, Sake-koji medium is commonly used as an excellent medium for yeasts.

Determination of mating types. Isolated strains of *S. rouxii* were transferred to the preculture medium several times every 2 or 3 days. Each of random pairs of these well-nourished cells was mixed on the sporulation medium, and after 4 days of incubation their conjugation and sporulation were observed by phase-contrast microscopy. Opposite mating types were distinguishable on the basis of the formation of copulation canals, ascospores, and bar-bell-like cells in their mixtures. In mixed culture of the same mating types, only the formation of durable cells as shown in Fig. 2 was observed.

Determination of ploidy. Yeast cells were cultivated in 18% NaCl synthetic medium (13) with shaking at 30 C for 3 days, and heavy cell suspensions for the analysis of ploidies were prepared by washing the harvested cells three times with distilled water. Ploidies were judged from cell shapes, cell sizes, cell dry weights, 2-deoxyribose contents, and mating responses for their parent strains. 2-Deoxyribose was estimated according to the indole method (2, 3) with deoxyribonucleic acid (DNA) fraction which was prepared by the method of Schmidt, Thanhauser, and Schneider (20). Cell counts were measured by Thoma's hematometer. Cell sizes and shapes were observed by use of cells cultivated in the conjugation medium for 2 days.

RESULTS

Isolation of S. rouxii. About 1,200 salt-tolerant yeasts were isolated from 127 samples of shoyu mashes and miso pastes collected from 49 places throughout Japan; of these yeasts, 423 strains were identified as *S. rouxii* according to the taxonomic studies. *S. rouxii* var. *halomembranis* (17), a film-forming type in the saline media, was omitted from this study. Tested strains were of the glistening type; cells were round to oval, 3 to 7 μ in diameter.

Establishment of suitable media and cultural conditions for conjugation and sporulation of S. rouxii. Isolated *S. rouxii* cells have been maintained on the 18% NaCl Sake-koji agar medium. None of the strains tested exhibited appreciable conjugation and sporulation on common sporulation media (5, 16, 18, 19), even after careful observation over a long period of time. It was suggested that their abilities to sporulate were lost, or the sporulation media were unsuitable. To investigate the restoration of the ability to conjugate and sporulate, each strain was transferred several times on 2% NaCl Shoyu-koji agar; then two well-nourished cells of each strain were mixed at random on this medium and incubated for 7 to 10 days. Conjugating cells, spores, and haploid cells with copulation canal were found on the culture at a rather high frequency. Therefore, 2% NaCl Shoyu-koji agar seemed to be suitable as a preculture medium or as a conjugation and sporulation medium. Spore formation was tested by mass culture of well-nourished cells from the preculture medium of 2% NaCl Shoyu-koji agar on various commonly used sporulation media, such as Gorodokowa agar (18), yeast autolysate-agar (16), diluted shoyu agar (19), and acetate agar (5). Sporulation media containing riboflavine and sodium glutarate (15) were also tested. However, conjugation and sporulation were observed rarely or not at all on all media tested. Thus, it became apparent that a more effective medium for conjugation and sporulation of *S. rouxii* should be investigated. It was considered possible that *S. rouxii* might sporulate under specific circumstances, such as in shoyu-mashes. We used Shoyu-koji medium as the basal medium and observed its effects on various factors of sporulation (14). In the following experiment on mass cultures of opposite mating types, a cross of 537 $\times$ 1028 was used.

Effect of sugar concentration on sporulation. For

the preparation of the basal medium, Shoyu-koji extracts were diluted about 10 times (0.2% reducing sugar and 0.07% total nitrogen). The 5% NaCl-diluted Shoyu-koji agar media with various sugar concentrations (10.0, 5.0, 2.0, 1.5, 1.0, 0.75, 0.5, and 0.25%) were examined. After 4 days of incubation, a good result was obtained on the medium containing 0.25 to 1.5% reducing sugar. In this case, the more reducing sugar the medium contained, the slower the cells conjugated and sporulated. Therefore, a reducing sugar concentration of 0.25% was found to be suitable for sporulation.

Effect of total nitrogen concentration on sporulation. The diluted Shoyu-koji agar containing 0.25% reducing sugar and 0.07% total nitrogen was used as the basal medium. Total nitrogen concentration was sometimes raised by the addition of polypeptone. Sporulation was inhibited in proportion to the increase in total nitrogen concentration. Thus, 5% NaCl diluted Shoyu-koji agar containing 0.07% total nitrogen was most effective for sporulation. When Shoyu-koji medium contained high levels of nitrogen and sugar, sporulation was delayed.

Effect of sodium chloride concentration on sporulation (21). On the Shoyu-koji agar (0.25% reducing sugar and 0.07% total nitrogen) without salt, sporulation did not occur, and only a few conjugating cells of bar-bell shape were seen. However, many asci with spore or copulation canal, and some conjugating cells, were formed on the media containing 2 to 13% sodium chloride. The best spore formation was observed on 5% NaCl medium. On 15% NaCl medium, many cells with copulation canals and a few cells with unhealthy spores were recognized; on 18% NaCl medium, some cells with copulation canals were observed, but little sporulation occurred. Thus, 5% sodium chloride was most suitable for sporulation.

Effect of pH on sporulation. On the diluted Shoyu-koji agar of *p*H 7.0 and 8.0, formation of copulation canals was good but spore formation was poor. The spores seemed to be unhealthy. Many cells with spore or copulation canals appeared at *p*H 6.0. At *p*H 5.0, the best sporulation and the production of healthy spores was recognized.

Effect of temperature on sporulation. Conjugation and sporulation were not found when the mixtures were incubated at 30 C or higher, but most cells with healthy spores appeared at 25 C or lower, even as low as 7 C. At 25 C, cells gradually began to conjugate and sporulate after 48 hr of incubation.

In conclusion, the best sporulation was observed when different mating types of haploid *S. rouxii* were mass-cultured on diluted Shoyu-koji agar containing 5% sodium chloride, 0.25% reducing sugar, and 0.07% total nitrogen at *p*H 5.0 and 25 C for 4 days. As the conjugation medium, the sugar-rich medium with the further addition of about 5% of glucose to the sporulation medium seemed to be most satisfactory. This medium was used as conjugation medium in the following experiments.

TABLE 1. *Mating type of isolated Saccharomyces rouxii*

Type	No.	Type culture
I	19	484, 537, NRRL-2548
II	49	630, 1,028, NRRL-2547
III (nonmating type)	8	458
IV (mixture of two mating types)	29	—
Total tested strains	105	

Mating type and conjugation of isolated S. rouxii, and breeding of diploid S. rouxii. According to the methods described in Materials and Methods, determination of mating types of 105 isolated strains was carried out. Some mass cultures in which two of the tested stains were mixed, at random, on the sporulation medium and then incubated gave many conjugating cells and many asci with spores. In this case, some of the single cultures of each strain did not show any conjugation and sporulation. Thus, 68 strains were classified into two mating types (type I and II, as shown in Table 1). Type I could mate with type II, and their sporulation ratios were different with each pair. Eight strains of type III could not conjugate

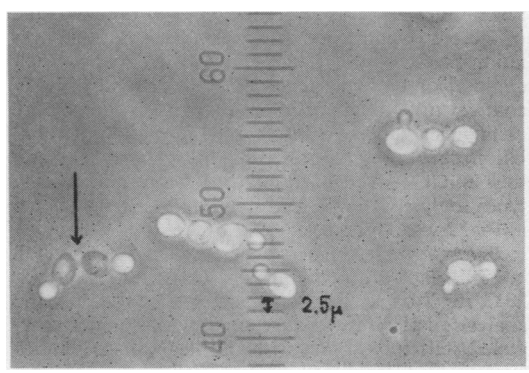

FIG. 1. *Heterocaryon-like zygote formed on filmy agar of the conjugation medium. Terminal budding is shown. (Cross: 537 × 1,028.)*

with type I or II, and seemed to be nonmating. The remainder (29 strains) showed conjugation and sporulation even in single culture, and were assumed to be mixtures of two mating types. From this result, it appears possible that a wild strain of *S. rouxii* exists generally in haplophase and still has abilities to conjugate and sporulate.

It is generally know that the diploid strain grows much faster than the parent haploid (4) and that diploid colonies, if sufficiently isolated and aged, show some difference in size, degree to which they glisten, or color. Therefore, it was expected that diploid *S. rouxii* could be easily obtained by cultivation of the conjugating cells on plates of the nutrient-rich agar and selection of large colonies. According to this principle, we tried to get diploid strains from the possible combinations of one mating type of (484, 537) and another mating type of (630, 1,028). There were many colonies of different sizes on the plate. During 4 to 6 weeks of incubation, we examined microscopically the center part of the large colonies every week, and recognized only many conjugated asci with spores. By finding the unconjugated asci with spores, diploid strains would be obtained by cultivating the cell taken from the edge of the colony (L. J. Wickerham, *personal communication*). The result was contrary to our expectation. Next we observed the reproduction process of the conjugating cells and found that most of the conjugating cells began to sporulate very quickly, before budding, suggesting that these would be "committed to" sporulation as pointed out by Miller and Hoffmann-Ostenhof (14). Several zygotes showed terminal budding (Fig. 1). However, the reproduction of these buds gave only haploid cells, suggesting that the zygotes were still heterocaryons in which nuclear fusion had not yet occurred. We could not see the buds from the center part of the zygotes, and this kind of bud would grow into diploid cells. A few zygotes could neither sporulate nor grow, showing loss of viability. These phenomena indicate that many practical difficulties exist in obtaining diploid cells. We continued our attempts to obtain diploid cells by use of many combinations of isolated *S. rouxii* cells by micromanipulation; a large number of each zygote were picked and transferred to 5% NaCl-diluted Shoyu-koji liquid medium. Most of the cultures gave no growth or yielded only haploid cells, showing the same phenomena as stated above. Fortunately, however, large cells (Fig. 6) were found in the combination of 510 × 1,028 (Fig. 3–5) with an isolation efficiency of about 20%. These large cells could grow in 18% NaCl medium and multiplied well in liquid medium. Several large cells derived from the other combinations were successively obtained in the same way.

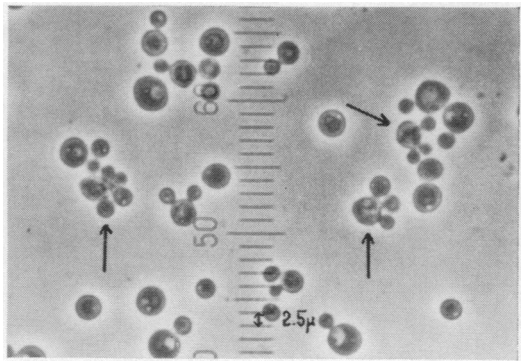

FIG. 2. *Durable cells formed on the sporulation medium. (Haploid strain of one mating type, 510.)*

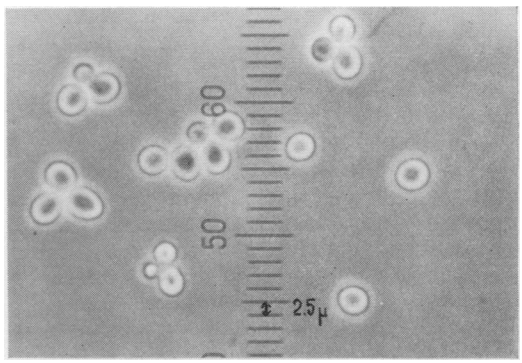

FIG. 3. *Vegetative cells of haploid Saccharomyces rouxii in the conjugation medium. (One mating type, 510.)*

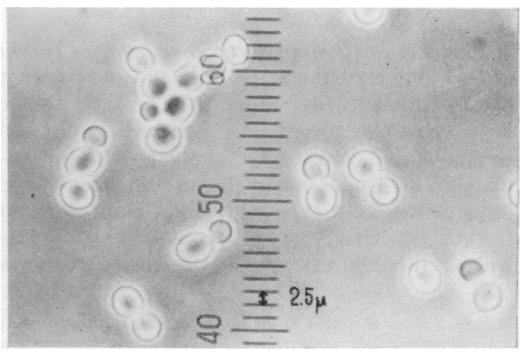

FIG. 4. *Vegetative cells of haploid Saccharomyces rouxii in the conjugation medium. (The other mating type, 1,028.)*

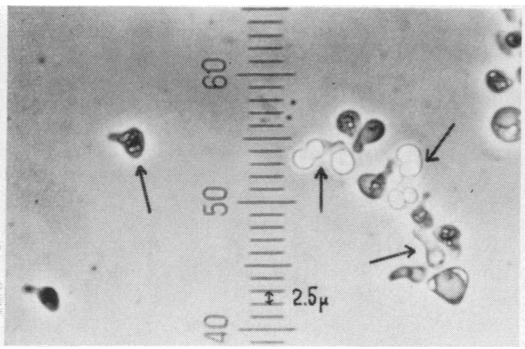

FIG. 5. *Bar-bell-like asci (three or four ascospores) formed by two mating types on the sporulation medium; haploid cells with copulation canal failed to conjugate. (Cross: 510 × 1,028.)*

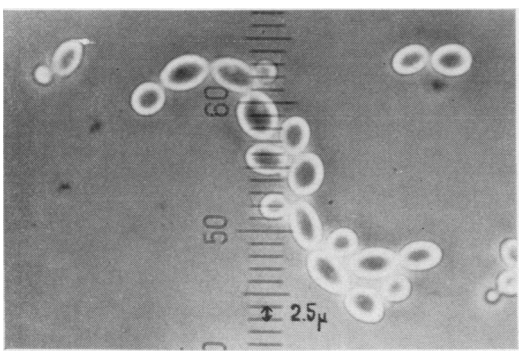

FIG. 6. *Vegetative cells of diploid Saccharomyces rouxii in the conjugation medium. (MD-6 derived from 510 × 1,028.)*

Ploidy estimations of the large cell and the parent cells. Ploidies of the large cells and their parent cells were estimated (Table 2). The ratio of the large cell to each haploid parent cell in dry cell weight and 2-deoxyribose content was approximately 1.67 to 1.72. The shapes and sizes of the large cells were distinctly different from those of the parent. The large cells could not conjugate with each parent cell and they formed ascospores

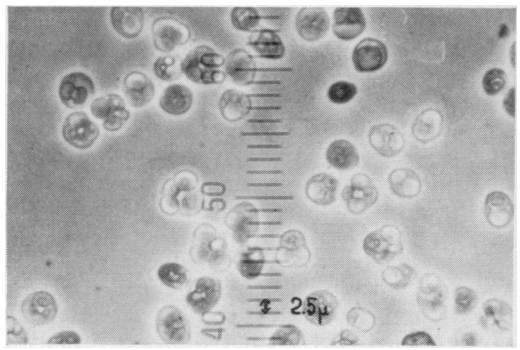

FIG. 7. *Unconjugated asci of diploid Saccharomyces rouxii (MD-6).*

only in the unconjugated asci. Their sporulation showed diploid *Saccharomyces* type, as shown in Fig. 7. Thus, the large cells proved to be heterozygous diploid cells by estimations of ploidy and of mating responses.

DISCUSSION

Salt-tolerant *S. rouxii* (17), which have been described as *Zygosaccharomyces major* or *Z. soya*, is the commercially important yeast for shoyu and miso fermentation. Genetic studies by tetrad analysis were difficult because of the strong tendency of *S. rouxii* to live in haplophase in media with high concentrations of salts or sugar and to lose easily the ability to form ascospores (13). Wickerham and Burton (21) showed the heterothallism in *S. rouxii* and suggested the possibility of deriving their diploid hybrids. Thus, we attempted to obtain diploid hybrids by crossing opposite mating types of haploid *S. rouxii*, newly isolated from shoyu mashes and miso pastes. We were trying to devise a procedure for breeding yeasts with more useful properties for application to shoyu and miso industries. At the beginning of our study, we found that conjugation and sporulation could scarcely be observed by mass culture on sporulation media commonly used. It was supposed that some special media for the conjugation and sporulation would be required

TABLE 2. *Ploidy estimations of the large cell and the parent cells*

Strain no.	Cell shape	Cell size (major axis, μ)	Dry cell wt (mg/cell)	2-Deoxyribose content (μg/10^{10} cells)	Mating response	Ploidy
NRRL-2548	Round-ovoid	3–6	1.41×10^{-8}	159.24	+	Haploid
510	Round-ovoid	3–6	1.35×10^{-8}	151.80	+	Haploid
1,028	Round-ovoid	3–7	1.39×10^{-8}	150.98	+	Haploid
MD-20[a]	Ovoid-ellipsoid	8–12	2.32×10^{-8}	253.16	−	Diploid

[a] MD-20 strain was derived from the combination of 510 × 1,028.

in the case of salt-tolerant *S. rouxii*, and that our first step should be to establish an effective medium. Nickerson and Thimann (15) reported that an extract from *Aspergillus* had a stimulatory effect on conjugation and sporulation of *Zygosaccharomyces*, and that riboflavine and sodium glutarate in the extract were the stimulators. However, in our experiment, neither substance showed any stimulatory effect toward shoyu and miso *S. rouxii*. As the result of our examination, 5% NaCl-diluted Shoyu-koji agar was found to be suitable for the purpose. The effect of our medium would probably depend upon a suitable concentration of some other nutrient in Shoyu-koji extract and sodium chloride. It was also apparent that heterothallism was prevalent in our shoyu and miso *S. rouxii*.

In our investigation of the reproduction process of conjugating cells, we observed that the diplophase of haploid yeasts, in which vegetative growth occurs essentially in the haploid condition, is limited, in the majority of cases, to the zygote formed by the mating of two haploid cells. The diplophase is immediately followed by sporulation. Such is the case with shoyu and miso *S. rouxii*. In our experiments, the following phenomena were usually observed when conjugating cells were transferred to fresh nutrient-rich medium to get diploid hybrids: (i) most of zygotes were "committed to" sporulation (14) and quickly sporulated before budding; (ii) several zygotes showed terminal budding, but the buds could not grow into diploid hybrids, as if the zygotes were still heterocaryon; (iii) a few zygotes had lost their viability. The first phenomenon would be quite natural, showing that diplophase of normal *S. rouxii* is very short. The second is seemed to be a phenomenon similar to that of *Hansenula*, a primitive heterothallic yeast, as suggested by A. Herman (M.S. Thesis, Western Reserve Univ., Cleveland, Ohio, 1959). The last one would be caused by abnormal conjugation. These phenomena suggest that it would be difficult to obtain diploid *S. rouxii* by plating culture of the conjugating cells, since they had the strong tendency to sporulate on agar media. However, when the conjugating cells were transferred to liquid media, such as 5% NaCl-diluted Shoyu-koji liquid medium, except for cells that became "committed to" sporulation, they did not sporulate but grew into diploid cells. The stability of the diploid *S rouxii* thus obtained is another matter of concern. It is desirable that they continue diploid growth but do not sporulate in such an environment as shoyu mashes and miso pastes, as well as in the stock culture. Our diploid *S. rouxii* cells sporulated on ordinary agar media, but not at all or very little in liquid medium or on saline media such as 18% NaCl medium. Therefore, they are considered very promising for practical utilization in the shoyu and miso industries. It goes without saying that the nonsporulating diploid derived from haploid *S. rouxii*, as suggested by Wickerham and Burton (21), would be more beneficial. Our present study, which showed a detailed procedure for successfully obtaining diploid *S. rouxii*, is an encouraging example of the possible genetic improvements of more useful shoyu and miso yeasts.

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