types of microorganisms present in the two animals, rather than an animal difference, caused the difference in pH.

Acknowledgement

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Literature Cited


Miso

III. Pure Culture Fermentation with Saccharomyces rouxii

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Abstract

Hesseltine, C. W. (Northern Regional Research Laboratory, Peoria, Ill.), and K. Shibasaki. Miso, III. Pure culture fermentation with Saccharomyces rouxii. Appl. Microbiol. 9:515-518. 1961.—Excellent mizo has been prepared with soybean grits inoculated with a pure culture of Saccharomyces rouxii strain NRRL Y-2547. Pure culture inoculum of this osmophilic yeast was prepared by growing the culture in aerated flasks on a yeast extract medium with a salt concentration equal to that used in the manufacture of mizo. It has also been found possible to make mizo from whole beans with the above culture. The advantages of pure culture fermentation in producing mizo are discussed.

As previously described (Shibasaki and Hesseltine, 1961c), the manufacture of mizo is a process involving two separate and distinct fermentations. The first involves the aerobic pure culture fermentation of rice with selected strains of Aspergillus oryzae (Ahlburg) Cohn to prepare koji (mold rice) as a source of enzymes and nutrients for the second fermentation—the fermentation of mold rice, salt, and soybeans. In the second fermentation the inoculum used is a suitable sample of good mizo from an earlier fermentation. Such a mixed inoculum has two obvious disadvantages:

1) Contaminating microorganisms are carried from one fermentation to the next.

2) Since most types of mizo require months to ferment, followed by an aging period, it is apparent that the proper microorganisms for the fermentation become reduced in number and vigor; hence, a longer period is required for them to grow actively and multiply.

Since most modern fermentations are started with pure cultures, an obvious improvement in the manu-
facture of miso would be the use of a pure culture starter. Several different organisms might be mixed together at the time of inoculation if a mixed culture fermentation is required.

Previous reports suggested that a variety of bacteria and yeasts are present in miso (Shibasaki and Hesseltine, 1961a), and the implications are that several species of yeast and bacteria are needed to make a suitable product.

**Materials and Methods**

Microscopic examination of miso purchased in Japan and used in our earlier fermentations (Shibasaki and Hesseltine, 1960; 1961a, c) showed fragments of mycelium (from the koji) yeast cells but very few bacterial cells. Miso from Japan, as well as miso prepared in our laboratory, was plated out on a variety of media used for the isolation of fungi, yeasts, and bacteria, including lactic-acid bacteria and, in some instances, with as high an NaCl content as that used in miso. Although various bacteria grew, yet in each case the dominant type of organism always proved to be yeasts. Among the yeasts present only one species occurred in large numbers, whereas among the bacteria there was obviously a mixture of a number of kinds, with no single type predominating. In any case the bacterial totals did not exceed the yeast population. Therefore, we selected a single yeast culture of the predominant yeast present in miso for our first experiment. This was a strain of *Saccharomyces rouxii* Boutroux strain NRRL Y-2547, selected by L. J. Wickerham and reported on elsewhere (Wickerham and Burton, 1960).

Koji used throughout the experiments reported in this paper was prepared from American rice inoculated with a mixture of strains of *Aspergillus oryzae* and prepared in the manner described elsewhere (Shibasaki and Hesseltine, 1961c).

**Preparation of pure yeast culture.** *S. rouxii* strain NRRL Y-2547 was grown on slants of yeast extract-malt extract agar for several days. The slants were used to inoculate Erlenmeyer flasks containing the following ingredients: 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 10.0% NaCl, and distilled water. The pH was not adjusted. Each flask contained 150 ml of medium. The inoculated flasks were placed on a Gump shaker in a 28 C incubator and harvested after 10 days for inoculum.

**Preparation, cooking, and fermentation of soybeans.** Soybean grits were prepared as previously described (Shibasaki and Hesseltine, 1961c). The grits were made from the soybean variety Jackson harvested in 1959. Two 1-kg lots of dry grits were placed in large beakers and 1.5 liters of tap water were added. The grits were allowed to soak for 2.5 hr at room temperature. Occasional stirring was necessary for the grits to uniformly absorb water. At the end of this time, the grits were soft and nearly all the water had been absorbed. The grits were then wrapped in cheesecloth bags and placed together in the soybean cooker described elsewhere (Shibasaki and Hesseltine, 1961b).

Steam was allowed to flow from the top of the cooker through the bottom for 10 min with the bottom drain open. Then the bottom valve was closed and pressure was allowed to rise to 5 psi. Cooking then continued for 1 hr. At the end of this time, the steam was shut off and the beans were allowed to stand in the cooker for 25 min. Next, the cooked grits were removed, placed in glass beakers and allowed to cool. The two lots of grits now weighed 2,096 and 2,100 g. Then, 400 g of salt and 800 g of moist koji were added to each lot of cooked grits and thoroughly mixed.

Fermentation 1 was inoculated by weighing out 50 g of miso from a previously good fermentation and suspending it in 215 ml of sterile tap water; this was used for inoculum. After inoculation, the paste was tightly packed into a single glass fermentor, described elsewhere (Shibasaki and Hesseltine, 1961c), and covered with parafilm. The lid was tightly closed on the fermentor before placing it in a 28 C incubator. Fermentation 2 was inoculated with about 145 ml of a pure culture of *S. rouxii* prepared as described above. This inoculum was mixed thoroughly with the above materials and placed in a fermentor. Conceivably during this operation a few air contaminants might have entered the fermentor. This fermentor was tightly closed and placed at 28 C.

**Results**

After 48 hr, the contents of both fermentors appeared to be alike. However, at 96 hr, fermentor 2 had a strong yeast and ester odor, and the fermentation was obviously going on at a rapid rate. Fermentor 1 had no odor other than that present at the time of inoculation. At 1 week, both fermentors were placed in a 35 C incubator. On the eighth day fermentor 1 had a typical odor of esters and alcohol and on the tenth day a white mycelium of the yeast appeared on the surface of the miso; this is characteristic of traditional miso fermentations as carried out on a small scale in our laboratory. However, in fermentor 2 no mycelium appeared, even though its contents had started to ferment earlier. At the end of 60 days, both fermentors were removed from the 35 C incubator and allowed to age for 2 weeks at room temperature. At this time the material in both fermentors was harvested. The results are summarized in Table 1.

At the time of harvest about 100 g were tightly packed into glass containers and sent for evaluation to K. Shibasaki at Tohoku University, Sendai, Japan. He and his associates judged both products (fermentation 1 and 2) to be high quality miso, as compared to Japanese miso. The analytical data on these samples are summarized in Table 2.
This analytical comparison points out the close agreement between traditional type inoculum and that conducted with pure culture inoculum. Taste, appearance, and odor tests by other qualified persons in Japan confirmed the fact that the pure culture miso product compared favorably with the traditional miso.

With the pure culture NRRL Y-2547 and soybean variety Hawkeye, the above experiment was repeated in identical fashion with similar excellent results.

**Pure culture fermentation with whole soybeans.** Since we had shown that pure culture fermentation of soybean grits gave excellent miso, it seemed desirable to determine what would happen if whole soybeans were fermented with the above strain of *S. rouxii* NRRL Y-2547. The pure culture inoculum, the koji, and the amounts of each ingredient in the fermentation were kept the same. Whole soybeans (Hawkeye) in 1-kg amounts were placed in large containers with 1.5 liters of tap water and left for approximately 24 hr at 25 C. The whole, soaked beans were placed in the soybean cooker; steam was allowed to flow through the mass for 15 min; and the beans were then cooked at 5 psi for 1 hr and 15 min. After the soybeans had cooled, salt and koji were added, and the whole mixture was lightly mashed by passing it through a food grinder. One lot was inoculated with a pure culture of *S. rouxii* prepared as before and the second lot was prepared with 50 g of miso and water to make about 150 ml of inoculum. After thorough mixing, each lot of medium was packed into glass fermentors and placed in a 25 C incubator. On the fourth day the fermentors were examined. Fermentor 12, inoculated with the pure culture, had a distinct odor of alcohol and esters indicating an active fermentation. Fermentor 13, on the other hand, gave no evidence of fermentation even on the sixth day. On the seventh day the fermentors were removed to a 35 C incubator, where they were allowed to ferment for 2 months. At the end of this time the fermentors were removed to room temperature for 2 weeks and then harvested. Fermentor 12 showed the substrate to be dark at the surface with very little white oxidative yeast growth; the odor seemed slightly off, like that of burnt sugar. The miso also seemed to have dried slightly and pulled away from the glass wall of the fermentor at the top. When this surface material was removed, the miso below had a bright, light yellow-brown color and the flavor and odor were excellent. In Fermentor 13 there was the usual heavy growth of the oxidative yeast, but below the surface the color was darker than in fermentor 12. The color and flavor were good. When the jar was opened, the odor was superior to that of fermentor 12, but below the surface the odor was similar to fermentor 12. The two lots of miso were both acceptable.

**DISCUSSION**

Ordinarily, in the manufacture of miso, inoculum, consisting of some miso from a previous fermentation, is used in the second fermentation. This inoculum is added to the ingredients used to make miso, to start a new fermentation. Its disadvantages have already been pointed out in the introduction. Now it was found that with an appropriate culture of an osmophilic yeast, *S. rouxii*, such as NRRL Y-2547, alone can carry out the fermentation unaided, with a resulting product equal to that produced by the old method in all known respects. This strain was picked at random from yeast colonies from a sample of good miso. With proper selection an even better strain could probably have been isolated.

A pure culture fermentation eliminates all the contaminating organisms which are introduced by using as a starter the old miso that has been handed down through the centuries. For example, we noticed that all our previous fermentations resulted in a filamentous dirty white growth of an oxidative yeast on the surface of the miso. When pure culture fermentations were used, this was almost completely eliminated, even though the mat growth is actually a phase of *S. rouxii* which is carried from one fermentation to the next. As the data indicate above, when a vigorous, large inoculation of yeast cells grown in the presence of the same salt

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**TABLE 1. Comparison of pure culture versus mixed culture fermentation of miso using soybean grits**

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Saccharomyces rouxii strain NRRL Y-2547</th>
<th>Miso used as inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surface:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td>Little or no visible yeast growth</td>
<td>Heavy grayish white mycelium of oxidative yeast</td>
</tr>
<tr>
<td>Color</td>
<td>Darker and the color extended further into the miso</td>
<td>Lighter</td>
</tr>
<tr>
<td>Odor</td>
<td>Less pronounced</td>
<td>More pronounced</td>
</tr>
<tr>
<td><strong>Below surface:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavor</td>
<td>Excellent</td>
<td>Excellent</td>
</tr>
<tr>
<td>Color</td>
<td>Reddish yellow</td>
<td>Reddish yellow</td>
</tr>
<tr>
<td>Odor</td>
<td>More pronounced and good</td>
<td>Less pronounced</td>
</tr>
</tbody>
</table>

**TABLE 2. Comparison of miso fermented with a pure culture of Saccharomyces rouxii strain NRRL Y-2547 and with miso from a previous fermentation**

<table>
<thead>
<tr>
<th></th>
<th>Uninoculated medium</th>
<th>S. rouxii strain NRRL Y-2547</th>
<th>Miso used as inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture, %</td>
<td>52.0</td>
<td>56.2</td>
<td>58.0</td>
</tr>
<tr>
<td>Total nitrogen, %</td>
<td>1.89</td>
<td>2.03</td>
<td>2.05</td>
</tr>
<tr>
<td>Soluble nitrogen, %</td>
<td>0.67</td>
<td>1.49</td>
<td>1.28</td>
</tr>
<tr>
<td>Amino nitrogen, %</td>
<td>0.13</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>Total acid calculated as lactic acid, %</td>
<td>0.57</td>
<td>1.05</td>
<td>1.12</td>
</tr>
<tr>
<td>Reducing sugars calculated as glucose, %</td>
<td>11.05</td>
<td>9.17</td>
<td>10.01</td>
</tr>
<tr>
<td>pH</td>
<td>5.7</td>
<td>5.12</td>
<td>5.0</td>
</tr>
</tbody>
</table>
concentration as miso is used as inoculum, the fermentation starts much faster and undoubtedly is completed sooner. To reduce the variables above, we purposely allowed the pure culture fermentation to continue for the same period of time as with the older method. Further studies are needed to determine how much the processing time can be shortened.

Another advantage we noted is that the fermentation as judged by odor always began much sooner in the pure culture fermentation than in the older method. The speed of initiation of fermentation could be improved by determining the number of yeast cells which would be most economical and practical.

The pure culture fermentation appears to work with either grits as developed at our laboratory (Smith, Hesseltine, and Shibasaki, 1961) or with whole soybeans. However, it appears the most economical method would be to use soybean grits since they shorten soaking, cooking, and fermenting times. Another possible savings in cost would be to find a cheap substrate for growing the pure inoculum, such as soybean meal or flour. This medium might help condition the yeast for its fermentation of miso. It might be feasible, especially for small factories, to have a company with the proper technical personnel grow large amounts of the yeast in pure culture and prepare fresh yeast cake as inoculum, which would give reliable and reproducible results. Then, even in small poorly equipped factories, a reproducible product could be manufactured economically.

Even greater than these improvements might be the tremendous possibilities of improving strains of \textit{S. rouxii} by genetic studies through breeding. Since it has been proven that pure culture fermentations can be accomplished and since the discovery that \textit{S. rouxii} is heterothallic, the background is complete for almost unlimited production of useful crosses. Thus, a search should be made of various lots of superior miso for strains of \textit{S. rouxii} which have one or more desirable genes. In this way, a strain with the ability to grow and ferment faster could be mated with one producing more flavor or odor, resulting in a haploid cross that would grow and ferment faster and, at the same time, produce better taste. Strains with a greater salt or heat tolerance could be brought into the breeding program, if these features were desirable. It is possible that wild strains with genes for higher vitamin content could be introduced. Methods for producing ascis and ascospores, and then isolating individual ascospores, are available. Once a desirable haploid cross was found, it could be maintained indefinitely. Of all the microorganisms used in industrial fermentations now known, probably none offers any better possibilities for real culture improvement through hybridization than does \textit{S. rouxii}.

\textbf{LITERATURE CITED}


