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Yeast-immobilized SPV device for koji quality control in sake brewing process

T. Chiyo [a,*], K. Matsui [a], Y. Murakami [b], K. Yokoyama [b], E. Tamiya [b]

[a] *Fukumitsuya Sake Breweries, 2-8-3 Ishibiki, Kanazawa, Ishikawa, 920-8638 Japan*

[b] *Japan Advanced Institute of Science and Technology, Hokuriku, 1-1, Asahidai, Tatsunokuchi, Ishikawa 923-1292, Japan*

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Abstract

The malted rice, koji, is an indispensable material for the brewing of sake. It saccharifies rice starch and supplies vitamins for the yeast in sake brewing. Since the quality of sake depends strongly on the quality of koji, quality control of koji is very important in the brewing. There are some methods to measure the activity of enzymes and the quantity of vitamins with the quality of koji. None of these methods, however, directly relate to the yeast metabolism. We constructed a sensor system to monitor the yeast metabolism in sake brewing by use of immobilized *Saccharomyces cerevisiae* and a Surface PhotoVoltage device (SPV). In this system, *S. cerevisiae* K701 and K9, designed for use in sake brewing by the Brewing Society of Japan, were employed as immobilized microbe. The pH change due to the production of organic acids in sake brewing is measured using the SPV. A linear relationship was observed between decrease in the photocurrent (the metabolism response) and the concentration to less than 60 mM of glucose ($r = 0.990$). Then we measured the koji extract and observed the difference of response between K701 and K9 which corresponded to the productivity of acidic substances by batch test. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Sake; Koji; *Saccharomyces cerevisiae*

1. Introduction

The biosensor consists of biomaterial to sense analytes selectively and a transducer to change the chemical/physical information produced by the biomaterial into electric information. The technology is finding wide application today in various fields of analytical science, such as the measurement of environmental, medical and the quality control of foods. Various biosensors that measure glucose, galactose, lipids and other chemicals are applied to the control of food quality such as taste, smell, and freshness (Scheller et al., 1997). To control the quality of the sake, those sensors are applied to analyze the components of sake and the mash of sake.

In the traditional sake brewing, koji, the malted rice, is one of the most important materials for the quality control of the sake. *Aspergillus oryzae* in the koji produces proteolipids, vitamins and enzymes. These components are necessary for the fermentation of the mash, and affect the production of acidic substances that give taste and smell to the sake. These acidic substances, such as carbonate ions and organic acids, are produced from the metabolic pathway of sake yeast in the sake brewing. Therefore, measurements of the components of the koji have been used for the quality control of the mash (Seisyukoubo kenkyukai, 1980).

The Surface PhotoVoltage (SPV) device is the transducer for the biosensor that measure the metabolism of microorganisms and cells. The SPV device transduces the surface potential of the device, especially pH of the solution near the surface (Hafeman et al., 1988; Adami et al., 1992; Owicki and Parce, 1992; Sartore et al., 1992). The device consisted of only a silicon chip. One side of the chip has an insulator layer such as silicon dioxide and/or sili-

* Corresponding author. Tel.: +81-76-223-1161; fax: +81-76-222-3769.

E-mail address: chiyo@fukumitsuya.co.jp (T. Chiyo).

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con nitride as sensor side. The other side (back side) has a partially deposited metal layer for ohmic contact and the other part has no typical layer. Illumination of the backside of the SPV device induces photovoltage. When only sensor side of the SPV device is immersed in the analyte solution, and the potential of the solution is biased against the bulk silicon of the device using the ohmic contact, the silicon device acts as MIS (metal insulator semiconductor) structure.

Since the depletion layer at the surface of the device has certain capacitance depending on the surface potential, the modulated photovoltage induces a photocurrent that depends on the sum of the bias potential and the surface potential of the device. Thus, modulated illumination induces modulated photovoltage and AC photocurrent. The SPV device has advantages against other pH sensors. For example, (1) the fabrication process is simple; (2) by multiplexing different light sources in different locations, the device can be multi-sensor without additional process complexity; and (3) encapsulation is easier and less critical. The SPV devices have been used in some research such as the chemical sensor to measure pH and ion concentrations, detection of bacterial growth, quantification of enzyme-linked immunoassays (Libby and Wada, 1989; Briggs and Panfili, 1991), and measurement of metabolism of animal cell (Parce et al., 1989; Owicki et al., 1990; McConnell et al., 1992; Wada et al., 1993; Chan et al., 1995). Thus, when the sake yeast is immobilized on the sensor side of the SPV device, the system is expected to sense the biological information of the koji extract solution for the quality control of the sake.

In this research, we constructed the biosensor system that consists of the SPV device and yeast layer. The yeast for sake brewing was employed for this purpose. First, we confirmed that the sensor indicated the metabolism of the yeast by the change of the AC photocurrent. Second, the response of the sensor in the measurement of the koji extract was compared with the response of the glucose solution. This sensor system will not only give us the important information in the sake brewing processes, but can be applied for the quality control of other fermentation processes.

2. Experimental

2.1. Materials

Saccharomyces cerevisiae Kyokai No. 701 (K701) and Kyokai No. 9 (K9) were purchased from Brewing Society of Japan. The koji was sampled from the brewery in Fukumitsuya Sake Breweries. All other chemicals were laboratory grade and used without purification.

2.2. Cultivation and immobilization of the cells

S. cerevisiae K701 and K9 were cultured in YEPD medium containing 20 g/l glucose, 10 g/l yeast extract and 10 g/l polypepton at 30 °C for 2 days. The cells were collected by centrifugation at 1000 rpm for 5 min (himac CF7D2, Hitachi Koki) and washed two times with 0.5 mM phosphate buffer with 0.15 M NaCl (pH 5.0) using centrifugation. The washed cells were suspended in the phosphate buffer with NaCl and its optical density of the suspension was adjusted to 2.0 at 600 nm with 10 mm cell (DR2000, HACH). A double-sided adhesive tape with a diameter of 6 mm having a 4 mm hole was put on a polytetrafluoroethylene (PTFE) membrane filter (H202A025A, pore size 0.2 μm, Advantec) as shown in Fig. 1. The 100 μl of the yeast suspension was filtered through the membrane, and the filtered cell was covered with the other PTFE membrane. This yeast-immobilized membrane was stocked in the phosphate buffer at 4 °C.

2.3. Preparation of the sample solution

The glucose solution was prepared as following. 0.50–5.0 ml of 1.0 M glucose solution, 5.0 ml of 100 mM phosphate buffer and 10 ml of 1.5 M NaCl solution were mixed, and its pH and volume were adjusted to 5.0 and 50 ml. The 20 g (dry weight) of koji, that was sampled from the brewery, was immersed in 100 ml of distilled water at 25 °C for 2 h. Then, the koji extract was filtered from the mixture with a filter paper. The sample solution contained 12.5 ml of the extract, 5.0 ml of 100 mM phosphate buffer and 10 ml of 0.15 M NaCl solution and its pH and volume were adjusted to 5.0 and 100 ml, and was sterilized with a sterilized membrane filter (DISMIC-25cs, pore size 0.2 μm, Advantec).

The measurement of glucose concentration was taken from the sample solution by glucose meter (GLU-11, TOA Electronics).

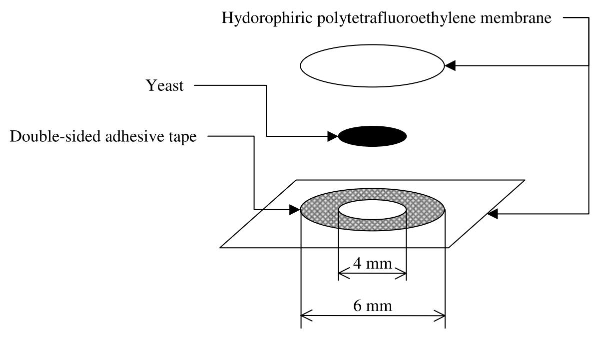

Fig. 1. Schematic diagram of yeast-immobilized biosensor system using surface photovoltage device.

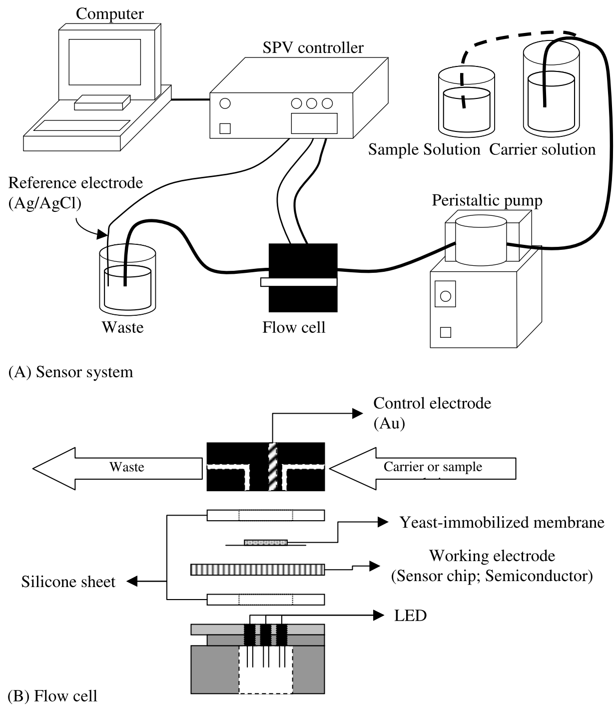

Fig. 2. Schematic diagram of microbial membrane for yeast-immobilized biosensor system using surface photovoltage device.

2.4. Sensor system

The sensor system was constructed as shown in Fig. 2. As reported earlier (Murakami et al., 1998), the microbial membrane was placed on the SPV device (Shindengen) and held between the device and a silicone sheet to give a flow cell. A peristaltic pump (M315, Gilson) was employed to pump carrier or sample solution from their reservoir to the flow cell. The flow cell was connected to a SPV controller (SE1030, Technologic) and a computer (PC9801BA, NEC). A carrier solution (0.5 mM phosphate buffer with 0.15 M NaCl) was pumped at a flow rate of 200 μl/min until a photocurrent became stable. The photocurrent was first measured at increasing bias potentials to obtain a capacitance versus voltage ($C–V$) curve. The bias potential between the bulk silicon of the device and the solution in the flow cell was adjusted to be the middle photocurrent of its $C–V$ curve (2.0–2.1 V). As the pH in the fermentation of sake brewing was from 3.0 to 5.0, all of artificial sample solution's pH was adjusted to 5.0 after adequate dilution with the same solution as the carrier solution. After 5 min data acquisition, the carrier reservoir was replaced with a sample reservoir for 5 min and then replaced with the carrier reservoir again. The photocurrent was monitored until it returned to the initial photocurrent and became stable. The difference between the average value of the photocurrent from 5 min before the replacement to the time of the replacement and the minimum photocurrent as the sample response.

2.5. Glucose response

The measurement of the photocurrent, which is a response to glucose solution, with the sensor system was as follows. First, the microbial membrane, in which the autoclaved *S. cerevisiae* K701 was immobilized, was attached in the flow cell. The photocurrent data were acquired for 5 min to calculate the initial photocurrent, and then the carrier reservoir was replaced with the sample reservoir. Next, the microbial membrane was replaced with the other, in which the living *S. cerevisiae* K701 was immobilized, and then the measurement was done as above.

2.6. Fermentation test

The koji medium for the examination was prepared as following. About 250 g of the koji was immersed in 1000 ml of distilled water at 55 °C for 4 h for saccharification. After the saccharification, the liquid for the medium was filtrated from the mixture with paper filter. Its specific gravity was adjusted to 5.0 Bh (Bh = 144.3 × (1 − 1/d), d: density [g/cm^3]) with distilled water and then its pH was adjusted to 5.0 with lactic acid. Characteristics of the yeast were examined as following. For this experiment, *S. cerevisiae* K701 or K9 were cultured in 50 ml of the koji medium at 30 °C for 2 days. The cells were collected by centrifugation at 1000 rpm for 5 min and washed two times with distilled water using centrifugation. The cells were suspended in distilled water and the its optical density at 600 nm with 10 mm cell was adjusted to 0.5. The Koji (0.8 g), 20 ml of the koji medium and 2.0 ml of the yeast suspension were mixed for the preparation of the test mash. This mash was fermented at 15 °C for 3 weeks, and then its sake meter, acidity, amino acidity, pH and content of alcohol and glucose were measured for characterization of *S. cerevisiae* K701 and K9. The acidity indicates a volume (ml) of 0.1 N NaOH for neutralization of 10.0 ml of a sample solution to 7.0–7.3 of its pH. The amino acidity indicates a volume (ml) of 0.1 N NaOH for neutralization of the sample, which was neutralized in the measurement of the acidity, to 8.2 of its pH. The sake meter is an index of density and the value was calculated by a formula ((Sake meter) = (1/d − 1) × 1443, d: density [g/cm^3])

3. Results and discussion

3.1. Glucose response

As a result with autoclaved-yeast membrane, the photocurrent was retained at the initial value (Fig. 3).

On the other hand, the photocurrent of the living microbial membrane decreased. The decrease in the photocurrent indicated a decrease in the pH on the surface of the SPV device. The decrease in the pH is consistent with the production of acidic substances that were caused by the microbial metabolism. As stated above, this sensor system could sense the metabolism of *S. cerevisiae*. However, the longer the measurement of the photocurrent takes, the more the photocurrent become unstable. It would be caused by the difference between the initial metabolism of the yeast and the later metabolism. Stabilization of the photocurrent is necessary to estimate the quality of the koji. Therefore, we decided to measure only initial metabolism, because photocurrent after long time incubation was unstable. In the following experiment, the sample solution was pumped into the flow cell for only 5 min in order to measure only initial metabolism.

3.2. Stability of yeast immobilized membrane

The Fig. 4 shows the typical photocurrent response to the glucose solution. As shown in this figure, the photocurrent decreased at first and then increased to the initial value. The difference between the average value of the photocurrent from 0 to 5 min and the minimum photocurrent was looked on as the sample response the replacement. To estimate the stability and the reappearance of the yeast-immobilized SPV sensor, 100 mM and 50 mM glucose solution were applied to this experiment. At first, response to 100 mM glucose solution was measured and then response to 50 mM glucose solution was measured. A set of these two experiments was repeated until the response curve to be unstable.

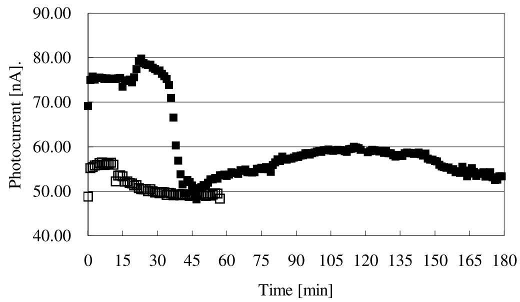

Fig. 3. Comparison of sensor response between autoclaved and living *Saccharomyces cerevisiae* Kyokai Number 701. The immobilized-yeast (*Saccharomyces cerevisiae* Kyokai Number 701) in microbial membrane corresponded with 100 µl of its suspension whose optical density at 600 nm with 10 mm cell was 2.0. The measurement was done with living (filled square) or autoclaved (closed square) yeast. After the stabilization of the response with the flow of 0.5 mM phosphate buffer (pH 5.0) containing 0.15 M NaCl at 200 µl/min, carrier solution was changed to sample solution containing 10 g/l glucose in the buffer. The time when the solution changed is 15 in horizontal axis.

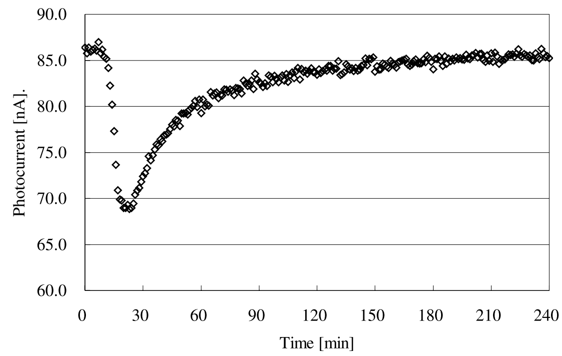

Fig. 4. Typical photocurrent response to glucose solution with SPV sensor system. The sample solution was pumped into the flow cell for 5 min in order to measure only initial metabolism, and then it was replaced with the carrier solution until the photocurrent returned to the initial one and became stable.

As shown in Fig. 5, the first curve of the measurement of 100 mM glucose solution was different form other curve. Especially, the response did not return to the initial value and though the other responses returned. After the first measurement of the 100 mM glucose solution, there was no difference in each response and the response became stable. At the first measurement, the yeast in the membrane would be in a resting, because the yeast-immobilized membrane had been stocked in a refrigerator with no carbon and nitrogen source. And the yeast would be in active stage and stable after the first measurement because of the same reason. The responses was stable from the first measurement of 50 mM glucose solution to the fifth

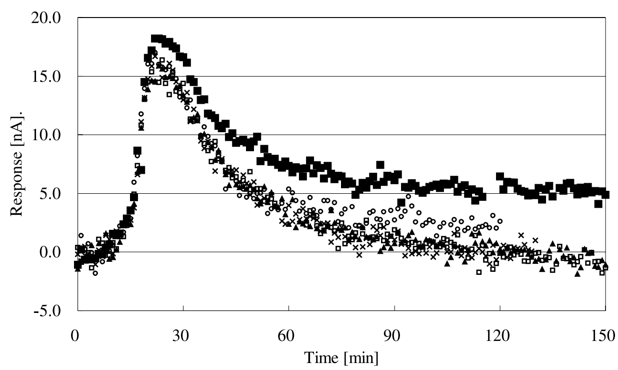

Fig. 5. Reappearance of response to 100 mM glucose solution. The immobilized-yeast (*Saccharomyces cerevisiae* Kyokai number 701) in microbial membrane corresponded with 100 µl of its suspension whose optical density at 600 nm with 10 mm cell was 2.0. After the stabilization of the response with the flow of 0.5 mM phosphate buffer (pH 5.0) containing 0.15 M NaCl at 200 µl/min, carrier solution was changed to sample solution containing 100 or 50 mM glucose in the buffer. Then sample solution was changed to carrier solution. The period of time when the solution changed to sample solution was from 5 to 10 in horizontal axis. As a cycle, 100 mM glucose solution was measured at first and, then, 50 mM glucose solution was measured. About five cycles of measurement were done (■; 1st, □; 2nd, ▲; 3rd, ×; 4th, ○; 5th).

Table 1
Stability of maximum response

Test number	Response (nA)	
	100 mM glucose	50 mM glucose
1	18.2	9.8
2	15.1	9.3
3	15.5	9.6
4	16.1	9.5
5	16.4	9.6
Average	15.8	9.6
S.D.[a]	0.6	0.2

Responses to 100 mM glucose solution were averaged except 18.2, because the microbial membrane was not steady state at its measurement.
[a] S.D., standard deviation.

Table 2
Comparison between koji extract and glucose solution

Immobilized yeast	Sample	Response (nA)	Glucose (mM)
Kyokai Number 701	Glucose[a]	7.0	30
	Koji[b]	9.1	41
Kyokai Number 9	Glucose[a]	6.2	30
	Koji[b]	9.2	51

[a] The response and the glucose concentration were determined from the calibration curve which was obtained by the measurement of 21, 45, 68 mM glucose solution.
[b] The response was obtained by the measurement of the koji extract solution which has 30 mM glucose and the glucose concentration was determined from the calibration curve of glucose.

response of 100 mM glucose solution as shown in Table 1. It indicates that the sensor system could measure eight samples per membrane at least and had enough strength to estimate a koji extract solution against the standard solution of glucose.

3.3. Calibration with glucose solution

Various concentrations of the glucose solutions were applied to obtain a calibration curve as shown in Fig. 6. *S. cerevisiae* K701 was immobilized in the microbial membrane. The response increased with increasing glucose concentrations, and the relation is in a linear relationship between the decrease of photocurrent (the metabolic response) and the glucose concentration from 10 to 60 mM. It indicated that the measurement of the

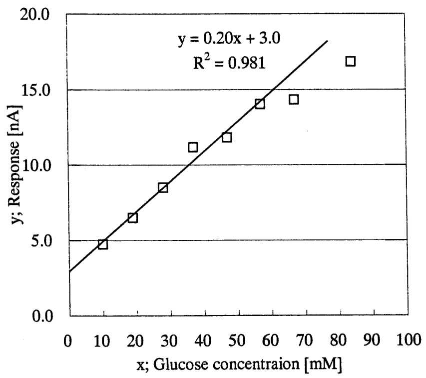

Fig. 6. Calibration curve of sensor with *Saccharomyces cerevisiae* Kyokai Number 701. The immobilized-yeast (*Saccharomyces cerevisiae* Kyokai Number 701) in microbial membrane corresponded with 100 μl of its suspension whose optical density at 600 nm with 10 mm cell was 2.0. After the stabilization of the response with the flow of 0.5 mM phosphate buffer (pH 5.0) containing 0.15 M NaCl at 200 μl/min, carrier solution was changed to sample solution containing 10–100 mM glucose in the buffer. Then sample solution was changed to carrier solution. The period of time when the solution changed to sample solution was 5 min.

photocurrent and the estimation of the quality of a koji should be done less than 60 mM of the glucose concentration. Furthermore, the calibration should be done prior to use, because the slope of the calibration curve was changed on each occasion of replacing the microbial membrane.

3.4. Estimation of koji extract solution

The calibration curve of glucose and the response to the koji extract solution using the microbial membrane, in which *S. cerevisiae* K701 or K9 was immobilized, were shown in Table 2. The glucose concentration of the koji extract solution was 30 mM, but the response to the koji solution with each microbial membrane was larger than the response, which was calculated from the calibration curve, to 30 mM glucose solution. We supposed that substances in the extract solution except for the glucose caused this difference, because the koji solution includes many substances as vitamins and organic acids. Therefore, this difference suggested that the sensor system would be able to estimate the quantity of substances except for glucose in koji.

The glucose concentration of the koji solution with each microbial membrane was calculated from the calibration curve of glucose and the response to the koji solution, and the calculated concentration of glucose with the K701-immobilized membrane was less than the K9-immobilized membrane. It indicated that the pH more decreased in the estimation with K9-immobilized membrane than K701-immobilized membrane, and it suggested that acidic substances in the estimation with K9-immobilized membrane were more produced than K701-immobillized membrane. Furthermore, this suggestion was consistent with the following result of the fermentation test. As Table 3 shows, there was no difference in the pH of fermentation test, nevertheless the acidity of the fermentation test with K9 was larger than K701. The difference in the acidity indicated that

Table 3
Comparison of characteristics between *Saccharomyces cerevisiae* Kyokai Number 701 and Kyokai Number 9

	Yeast	
	Kyokai number 701	Kyokai number 9
Sake meter[a]	+8.2	+9.0
pH	4.46	4.44
Acidity	3.92	4.11
Amino acidity	3.72	3.79
Alcohol % (v/v)	19.9	19.2
Glucose % (w/v)	0.82	0.55
Brix %	12.6	12.0

[a] The index of density which can be calculated by content of alcohol and Brix scale ([sake meter] = 3.609 × [alcohol % (v/v)] − 5.524 × [Brix %] + 6.022).

acidic substances, such as organic acids, were more produced in the metabolism of K9 than K701. However, buffer effect of organic acids caused no difference in the pH.

These results suggested that our sensor system indicated a difference between productivity of acidic substances by K9 and K701. Therefore, the sensor system could estimate the differences in the characteristics of sake yeast. Furthermore, it would be able to estimate the koji quality. As the quantity of substances in koji would be estimated by the system and it was one of the index of the koji quality. It is true that the measurement of the koji quality every sample takes about 3 h, but it is shorter than the measurement, which takes a few weeks, by test mash or industrial mash.

4. Conclusion

In this research, we employed the SPV method for the measurement of acidic substance, and combined it with a microbial membrane to construct a sensor system that estimate the quality of koji. This system responds to the production of acidic substances due to the metabolism of the sake yeast. However, the longer the measurement of the photocurrent takes, the more the response become unstable. Therefore, the sample solution was pumped into the flow cell for 5 min in order to get stable response. As a result, the response became stable, and the system indicated the linear relationship between the response and the glucose concentration from 10 to 60 mM.

Then we measured the response of the koji, which was sampled from sake breweries, extract solution with the sensor. The system indicated the metabolism of the yeast against not only glucose but other substances. The response of *S. cerevisiae* K701 against substances except for glucose was more than the one of K9 relatively. It is consistent with their capacity for the producing acidic substances. This sensor will be able to measure the koji quality easily and fast. The construction of the database from the quality data and the knowledge of the sake master make it possible to forecast the condition of the sake mash at the end of fermentation which takes a few weeks. Thus, the system was expected to estimate not only the quality of the koji but the process for fermenting with the microorganisms.

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