A New Process for Soy Sauce Fermentation by Immobilized Yeasts

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Using bioreactors with yeasts, *Zygosaccharomyces rouxii* that undergoes ethanol fermentation and produces 2-phenyl ethanol, and *Candida versatilis* that produces 4-ethyl guaiacol, adsorbed-immobilized on a ceramic carrier, the total time required for production of soy sauce was shortened to 8 days without affecting the product's quality.

Recently, in addition to conventional techniques, the use of bioreactors with immobilized enzymes and/or immobilized microorganisms in food industry has been popular. A new process using bioreactors with immobilized cells which was different from the conventional soy sauce fermentation was investigated. Osaki *et al.*¹⁾ reported that the use of bioreactors packed with calcium alginate-entrapped yeasts shortened the aging of soy sauce fermentation.

Although there would not be any safety problems when calcium alginate gel is used in the food industry, calcium alginate gel is unstable to heat, and therefore can not be sterilized completely by steam. Furthermore, owing to its gel conformation, calcium alginate gel is weak and can be easily damaged by high salt concentrations. Hence, continuous fermentation for a long period of operation would not be feasible. Besides, it is impossible to recycle

because the calcium alginate gel is an organic material and therefore would impose a disposal problem. On the basis of these facts, we decided to develop a carrier for immobilization to replace calcium alginate. A new ceramic carrier was developed to get rid of the defects described above. With the new bioreactor, the aim is also to shorten the fermentation time and at the same time producing a good quality of soy sauce.

Materials and Methods

Microorganisms. The soy sauce yeasts used in this experiment were *Zygosaccharomyces rouxii* IFO 1877 as a main producer of ethanol and aromatic components like 2-phenyl ethanol (2-PE), and *Candida versatilis* G001 in charge with the production of 4-ethyl guaiacol (4-EG).

Culture solution and feed solution for fermentation. Growth medium for soy sauce yeasts consists of 20 g of glucose, 3 g of malt extract, 5 g of Polypeptone, 5 g of yeast extract,

Fig. 1. Schematic Flow Diagram of Reactors for Soy Sauce Production by Immobilized Yeasts with Ceramic Carrier.

(1) feed tank; (2) pump; (3) reactor I; (4) ceramic carrier; (5) ceramic filter; (6) reactor II; (7) reservoir.

and 60 g of NaCl, dissolved in 50 ml of raw soy sauce and diluted to 1 liter at pH 5.0 before sterilization. The feed solution used for the bioreactor was prepared by the method of Takamatsu *et al.*²⁾; 10% saline solution was added to the soy sauce koji, made from steam-boiled soybeans, roasted wheat, and koji microbes, and digested below 55°C. The digest was fermented with lactic acid bacterium, *Pediococcus halophilus* for lactic acid fermentation. The cell-free filtrate that was passed through a membrane filter was used as a feed solution for fermentation. The feed solution used in this experiment was kindly supplied by Kikkoman Corporation.

Cultivation of yeasts. Soy sauce yeasts (*Zygo. rouxii* and *C. versatilis*) were inoculated separately into 100 ml of growth medium in a 500-ml flask and cultivated aerobically at 30°C for 60 hr (6-cm stroke, and 133 reciprocations/min). This comprised the seed culture. The main culture was placed in a 2-l jar fermentor containing 1.2 l of growth medium and the yeast harvested from the seed culture by centrifugation. Cultivation was done at 500 rpm with stirring aeration at a rate of 5 l/min for 24 hr at 30°C.

Reactors and method of immobilization. The reactors used for fermentation are shown in Fig. 1. These column reactors are made of stainless steel (height, 30 cm; diameter, 10 cm), and a working volume of 900 ml. The ceramics carrier used for cell immobilization consists of 95% alumina and 5% silica, filamentous in structure with an average pore size of 60 μ m and a porosity of 90%. The carrier forms a column with an outer diameter of 7.5 cm, inner diameter of 5.5 cm and height of 25 cm. It was a product of Basella Co., Ltd., Japan. Regeneration of used ceramics was done by heating at 900°C for 2 hr. Bioreactors with the ceramic carrier were sterilized in an autoclave at 120°C for 30 min. *Zygo. rouxii* was adsorbed-immobilized in reactor I and *C. versatilis* in reactor II. A ceramic membrane filter was used to avoid the transfer of free cells from reactor I to reactor II. Both yeasts were harvested by centrifugation, resuspended in 1.3 l of feed solution (cell counts, 5×10^7 cells/ml) and placed into separate reactors. Microtube pumps were used to circulate the cell suspension at a flow rate of 20 ml/min for 12 hr. By this treatment, the yeast cells were immobilized on the ceramic carrier.

Analyses. Measurements of sugar, total nitrogen, formol nitrogen total acidity, and NaCl were done by Hirose's method.³⁾ The concentration of ethanol was measured by Shimadzu gas chromatography GC-4C using a flame ionization detector with chromosorb 101 (60–80 mesh)-packed column (2000 $\times$ 3 ϕ mm). Aroma components such as ethyl acetate, *n*-propyl alcohols, *iso*-butyl alcohol, and *iso*-amyl acetate were analyzed by a Shimadzu head space auto analyzer (GC-9A; HSS-2A). The analyzer column (2000 $\times$ 3 ϕ mm) was packed with PEG-20M on chro-

mosorb W (AW-DMCS, 60–80 mesh). *n*-Amyl alcohol and ethyl acetate were used as internal standard solutions. Two characteristic aroma components of soy sauce, 2-PE and 4-EG, were extracted with methyl acetate, and then analyzed by the Shimadzu head space auto analyzer (GC-9A; HSS-2A), where 2,3,5-trimethyl phenol was added as a standard solution.

Preparation of sample for scanning electron micrograph. Soy sauce yeast adsorbed on ceramics (3 $\times$ 5 $\times$ 1 mm) was fixed with glutaraldehyde at 4°C for 1 hr, and then immersed in 60, 80, and 100% acetone for 15 min, each. After substitution with amyl alcohol for 10 min, samples were dried in a Hitachi critical point drying unit HCP-2. Dry samples were then fastened to the sample plate by silver paste and coated with Pt-Pd by vacuum evaporation in a Hitachi E102 ion sputter. Observations were done under a Hitachi H-800 electron microscope at an accelerating voltage of 75 keV.

Results and Discussion

Soy sauce fermentation by free yeasts

Before immobilized yeasts were used for soy sauce fermentation, we mixed up 2 strains of yeasts, *Zygo. rouxii* and *C. versatilis*, under normal conditions as a model experiment simulating the conventional fermentation. Feed solution of 1.3 l, which contained 2.5% direct-reducing sugar and 1% lactic acid, was placed in 3-l Erlenmeyer flasks and resuspended with *Zygo. rouxii* and *C. versatilis* with a total cell count of 5×10^7 cells/ml in both reactors. Fermentation was done at 30°C in stationary culture. The course of this fermentation is presented in Fig. 2. According to this figure, mixed cultivation of the two yeasts readily produced 13 mg/l of 2-PE, 1.5% ethanol, and 20 mg/l of *iso*-amyl alcohol on the fourth day of fermentation.

Nevertheless, 4-EG, a unique aroma component in soy sauce could not be detected even after 15 days of cultivation. The reason for this may be due to the unfavorable effect of *Zygo. rouxii* and *C. versatilis* in mixed culture. From this result, it is necessary to separate these two yeasts and use a ceramic membrane filter between the two bioreactors.

Scanning electron micrographs of immobilized yeasts

The scanning electron micrographs of soy

Fig. 2. Course of Residual Sugar, Ethanol, and Aroma Component Production by Mixed Fermentation Using Whole Cells of *Zygo. rouxii* and *C. versatilis*.

●, residual sugar; ○, ethanol; ▲, 2-PE; □, iso-amyl alcohol; ■, ethyl lactate; ○, furfuralcohol.

Fig. 3. Scanning Electron Micrographs of Yeasts Adsorbed on Ceramic Carrier. (a) *Z. rouxii* (reactor I); (b) *C. versatilis* (reactor II).

Fig. 4. Course of Ethanol and Aroma Component Production by Reactor I in Continuous Fermentation Using a Ceramic Carrier.

The flow rate is 10 ml/hr; retention time, 4 days; reactor temperature, 30°C. ○, ethanol; □, ethyl acetate; ●, *n*-propyl alcohol; △, iso-butyl alcohol; ▲, iso-amyl alcohol; ▼, 2-PE.

sauce yeasts, *Zygo. rouxii* and *C. versatilis*, which were immobilized in the ceramic carrier, are shown in Fig. 3. Obviously, the ceramics used in this experiment is filamentous in a structure 3–5 μm in width. Yeasts were adsorbed and spread throughout the ceramic carrier.

Continuous fermentation by bioreactors

For cell immobilization, yeast cells (6.5×10^{10}) were immobilized in the ceramic carrier (2.2×10^8 cells/cm³). After 5 days of fermentation, immobilized cells on ceramic carrier was 2.2×10^8 cells/cm³ and free cells in reaction medium of bioreactors was 5×10^6 cells/cm³. So, this bioreactor system is thought to be a prolific immobilized system.

Reactor I

Zygo. rouxii was immobilized in reactor I. The course of concentrations of ethanol, 2-PE, and major aroma components of soy sauce in

reactor I are presented in Fig. 4.

Reactor I contained about 2.5% ethanol and 20 mg/l 2-PE on the first day. This was very fast compared to 4 days using free cells. The other aroma components reached about 15 mg/l of iso-amyl alcohol, 10 mg/l of iso-butyl alcohol, 5 mg/l of *n*-propyl alcohol, and 2 mg/l of ethyl acetate.

Reactor II

The course of concentrations of aroma components in reactor II, containing immobilized *C. versatilis*, is presented in Fig. 5. As can be seen in Fig. 5, about 2.5% ethanol was produced in reactor I while that in reactor II remain unchanged. In addition, 4-EG was produced at a rate of 2 mg/l. More than 15 mg/l of iso-amyl alcohol, 10 mg/l of iso-butyl alcohol, and 5 mg/l of *n*-propyl alcohol were produced steadily. So also with ethyl acetate at 2 mg/l.

Fig. 5. Course of Ethanol and Aroma Component Production by Reactor II in Continuous Fermentation Using a Ceramic Carrier.

The flow rate, retention time, and reaction temperature were the same as in reactor I. ○, ethanol; □, ethyl acetate; ●, *n*-propyl alcohol; △, *iso*-butyl alcohol; ▲, *iso*-amyl alcohol; ▼, 2-PE; ▽, 4-EG.

Table I. GENERAL CHARACTERISTICS OF SOY SAUCE PRODUCED BY CONVENTIONAL AND IMMOBILIZED METHODS

Characteristics	pH	Total acidity	Total N (%)	Formol N (%)	NaCl (%)	Reducing sugar (%)
Conventional method (A)*	4.84	2.80	1.58	1.18	15.54	0.91
(B)*	4.92	2.49	1.61	1.24	14.28	0.77
(C)*	4.83	2.40	1.54	1.14	16.25	0.84
Immobilized method (A)**	4.89	2.52	1.86	1.29	13.84	1.12

* Commercial products.

** Our data.

Comparison of aroma components

Products from continuous fermentation in reactors I and II were compared with commercial soy sauce with regard to the major constituents and aroma components. The results are shown in Tables I and II. As can be seen in Table I, there is not much difference in the major constituents between our product and the commercial products. But Table II indicates that concentration of 4-EG, the unique aroma component of soy sauce, is

lower in Osaki's data than any other product, while concentrations of *iso*-amyl alcohol and *iso*-butyl alcohol of his data are too high compared with other products. However, in contrast to Osaki's data, ours show better result with regard to aroma components compared with commercial soy sauce. Sensory evaluations conducted gave a high commendation of our product.

From the above-mentioned facts, our process with soy sauce yeasts, *Zygo. rouxii* and *C.*

Table II. COMPARISON OF AROMA COMPONENTS OF SOY SAUCE PRODUCED BY CONVENTIONAL AND IMMOBILIZED METHODS

Aroma components ^a	I	II	III	IV	V	VI	VII	VIII	IX	X
Conventional method (A)*	2.29	5.3	1.4	17.7	25.0	5.0	13.3	4.9	3.5	6.5
(B)*	2.32	4.5	1.4	16.0	14.4	8.2	2.1	7.3	3.0	14.7
(C)*	2.49	4.6	1.6	18.4	12.6	4.0	10.1	9.4	3.4	4.5
Immobilized method (A)**	2.21	16.0	2.4	14.7	10.5	4.9	1.2	1.5	4.3	3.2
(B)***	2.40	21.6	0.5	63.2	35.3	—	—	8.3	5.2	4.6

^a I, ethanol (%); II, 2-PE (mg/l); III, 4-EG; IV, iso-amyl alcohol; V, iso-butyl alcohol; VI, *n*-propyl alcohol; VII, ethyl acetate; VIII, ethyl lactate; IX, furfuralcohol; X, *n*-butyl alcohol.

* Commercial products.

** Our data.

*** Data from Osaka *et al.*

versatilis immobilized on ceramic carrier, the first time in Japan, works effectively.

The total time required for fermentation by immobilized-cell reactors was shortened to only 8 days, which is much shorter than 6 months in the conventional process, while at the same time producing good quality of soy sauce. With this as a foundation, we would like to do the scale-up experiment in the future. Results of this investigation have already been patented,⁴⁾ and were presented during the annual meetings of Nippon Nogeikagaku Kai.^{5,6)}

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