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Microbiological and biochemical survey on the transition of fermentative processes in Fukuyama pot vinegar brewing

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Traditional brewing of Fukuyama pot vinegar is a process that has been continued in Fukuyama, Kagoshima, Japan, for almost 200 years. The entire process proceeds from raw materials, including steamed rice, rice koji (steamed rice grown with a fungus, *Aspergillus oryzae*) and water, to produce vinegar in roughly capped large pots laid in the open air. No special fermentative manipulation is required, except for scattering dried rice koji (called furi-koji) on the surface of the mash to form a cap-like mat on the surface at the start of brewing. As the biochemical mechanism of the natural transition of the fermentative processes during brewing has not been fully explained, we conducted a microbiological and biochemical study on the transition. First, a distinct biochemical change was observed in the brewing of spring preparation; that is, a sharp decline in pH from 6.5 to 3.5 within the first 5 days of brewing was observed due to lactic acid fermentation. Alcoholic fermentation also proceeded with a sharp increase to 4.5% ethanol within the first 5 days under the acidic conditions, suggesting that saccharification and both fermentations proceed in parallel. Acidic conditions and ethanol accumulation restricted the growth of most microorganisms in the mash, and in turn provided a favorable growth condition for acetic acid bacteria which are acid resistant and “ethanol-philic.” Acetic acid was detected from day 16 and gradually increased in concentration, reaching a maximum of 7% at day 70 that was maintained thereafter. Empirically furi-koji naturally sinks into the mash after around day 40 by an unknown mechanism, allowing acetic acid bacteria to easily form pellicles on the mash surface and promoting efficient acetic acid fermentation. Dominant microbial species involved in the three fermentations were identified by denaturing gradient gel electrophoresis analysis using PCR-amplified defined-regions of small rDNA from microorganisms in the brewing mash or colony direct PCR applied to isolated microorganisms from the mash.

Key Words—acetic acid fermentation; alcoholic fermentation; denaturing gradient gel electrophoresis; Fukuyama pot vinegar; lactic acid fermentation

Introduction

Traditional brewing of Fukuyama pot vinegar is a process that has continued in Fukuyama, Kagoshima, Japan, since around 1820. The brewing occurs twice a year to produce spring and autumn preparations. In this process, raw materials including steamed rice,

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rice-koji (steamed rice grown with a fungus, *Aspergillus oryzae*) and water are combined in large earthenware pots. Then, dried rice-koji (called furi-koji) is laid on the surface of the mash, like a cap, to reduce the aerobic condition of the mash surface; this step is a characteristic technique. After being roughly covering with plastic sheets and ceramic lids, the pots were laid in the open air for several months.

Brewing comprises three fermentative processes: saccharification of rice starch to sugars, alcoholic fermentation from sugars to ethanol, and acetic acid fermentation from ethanol to acetic acid. The transition of the fermentative processes proceeds naturally.

Several biochemical or microbiological studies have already been reported on the enigmatic brewing of Fukuyama pot vinegar. One group reported the identification of yeasts, lactic acid bacteria and acetic acid bacteria isolated from mashes of Fukuyama pot vinegar, and the analytical data about flavor components with the changes of microbial flora of the mashes (Entani and Masai, 1985a, b). Another group studied the function of furi-koji (Koizumi et al., 1988, 1989). Further their group performed the identification of lactic acid bacteria, yeasts and acetic acid bacteria isolated from the mashes (Koizumi et al., 1996). Recently denaturing gradient gel electrophoresis (DGGE) has been used for culture-independent analysis of the microbial ecology in Fukuyama pot vinegar brewing (Haruta et al., 2006). However, the mechanism of the transition of fermentative processes is still unclear as no seed culture or sterilization is applied in the whole process. In this study we microbiologically and biochemically investigated with the reference to the mechanism of the transition of fermentative processes using spring preparations of Fukuyama pot vinegar.

Materials and Methods

Samples and reagents. Samples were isolated from Fukuyama pot vinegar brewed from April 3 to August 3, 2004 at the Da-te Breweries in Fukuyama, Kagoshima, Japan. Steamed rice (7.2 L), rice koji (3.6 L), and natural water (32.4 L) were combined in an earthenware pot (total volume, about 54 L), and then well-dried furi-koji (0.3 L) was placed on the surface of the culture. Each pot was covered with either a plastic sheet or ceramic lid. Approximate 50 ml of mash from each of defined three pots was periodically sampled at a depth of about 5 cm from the surface and sent to our

laboratory under refrigerated conditions. Before experimentation, samples were homogenized with a stomacher.

Unless otherwise stated, all reagents used in this study were reagent grade and obtained from Kanto Chemical Co., Inc. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma-Aldrich Corp. (St. Louis, MO, USA).

Biochemical analyses during brewing. Glucose, ethanol, acetic acid, and lactic acid concentrations were measured by enzyme kits (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Survey on microbial population of mash samples during brewing. Homogenized samples were serially diluted in phosphate buffered saline (PBS, 8 g/L sodium chloride, 2.9 g/L Na_2HPO_4 , 0.2 g/L potassium chloride, 0.2 g/L NaH_2PO_4) and spread onto the appropriate agar plates. The following culture media used in this study were obtained from Becton Dickinson and Co. (NJ, USA), except for potato dextrose agar (Eiken Chemical Co., Ltd., Tokyo, Japan): nutrient agar (pH 6.8) for viable cell counts, potato dextrose agar (pH 5.6) with chloramphenicol (100 mg/L) for mold counts, YPD agar (pH 6.5) with chloramphenicol (100 mg/L) and sodium propionate (2 g/L) for yeast counts, GYP agar (pH 6.5) with ethanol (10 ml/L) and CaCO_3 (5 g/L) for acetic acid bacteria counts (Koizumi et al., 1996), and MRS agar (pH 6.5) with sodium azide (10 mg/L) and cycloheximide (10 mg/L) for lactic acid bacteria counts (Okada et al., 1986). The composition of each code name medium is given in a handbook (Atlas, 1997). Cultivation was performed at 25°C for 3 to 5 days under aerobic conditions.

Extraction of DNA from brewing samples. Homogenized samples were neutralized to around pH 7.0 before DNA extraction; this step is indispensable for efficient extraction of microbial DNA because samples after day 5 of brewing are acidic (pH 3.5 or below). Bacterial DNA extraction was performed using the cetyltrimethylammonium bromide (CTAB) method (Meroth et al., 2003; Wilson, 2002) with modification. To harvest the bacterial cells, the neutralized samples were centrifuged at 4°C for 2 min at $10,000 \times g$, and the precipitated cells were resuspended in 0.6 ml of TESS (25 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, and 25% sucrose, pH 7.5) containing 5 mg/ml lysozyme. After incubation at 37°C for 30 min, 30 μl of 10% sodium dodecyl sulfate (SDS) solution and 5 μl of

proteinase K (20 mg/ml) were added to the cell suspension. After incubation at 37°C for 60 min, 100 µl of NaCl (5 M) and 80 µl of 10% CTAB (in 0.7 M NaCl) were added, and the mixture was further incubated for 10 min at 65°C. After incubation, an equal volume of phenol-chloroform-isoamyl alcohol (PCI) (25 : 24 : 1) was added, and the mixture was centrifuged for 10 min at $10,000 \times g$. Sodium acetate (final concentration 0.3 M) and $0.6 \times$ volume ice-cold isopropanol were added to the upper layer and mixed. After further centrifugation for 10 min at $10,000 \times g$, the pellet was washed with 70% ethanol, dried, and resuspended in 300 µl of Tris-HCl (10 mM; pH 8.0).

For the isolation of DNA from yeasts, the neutralized samples were centrifuged at 4°C for 2 min at $10,000 \times g$, and the precipitated cells were resuspended in 0.5 ml of 0.1 M EDTA solution (pH 7.5) containing sorbitol (1.0 M). Zymolyase 100T (50 µl; 1 mg/ml) and 50 µl of β -mercaptoethanol (0.28 M) were added to the suspension. After incubation at 37°C for 60 min, 50 µl of 10% SDS solution and 5 µl of proteinase K (20 mg/ml) were added to the treated suspension. After incubation, an equal volume of PCI was added, and then DNA was isolated in the same manner as that for the bacterial cells.

RNase A (5 µl; 10 mg/ml) was added to the resuspended DNA solutions. After incubation at 37°C for 30 min, PCI was added, and the mixture was centrifuged for 10 min at $10,000 \times g$. Sodium acetate (final concentration 0.3 M) and $2.5 \times$ volume ice-cold ethanol was added to the upper layer and mixed. After further centrifugation for 10 min at $10,000 \times g$, the pellet was washed with 70% ethanol, dried, and resuspended in 100 µl of Tris-HCl (10 mM; pH 8.0).

Denaturing gradient gel electrophoresis (DGGE). For DGGE (Fischer and Lerman, 1983), DNA extracted from brewing samples was PCR-amplified using a previously reported method (Muyzer et al., 1993) with some modification.

PCR amplification of the V3 region of 16S rRNA gene was performed using Ex Taq (TaKaRa Bio, Inc., Kyoto, Japan) according to the manufacturer's instructions. The primers used to amplify the V3 regions were 357F, 5'-CTACGGGAGGCAGCAG-3' (*Escherichia coli* positions 341 to 357), and 517R, 5'-ATTACCGCGGCTGCTGG-3' (517 to 534) (Muyzer et al., 1993). The primer 357F contained a GC clamp (Sheffield et al., 1989) sequence at the 5' end: CGCCGCGCGCGCGGCGGCGGGGCGGGGGGACGGGGGGG. Amplification of

the 18S rRNA gene of fungi was performed using the primers: 1427F, 5'-TCTGTGATGCCCTTAGATGTTCTGGG-3' (*Saccharomyces cerevisiae* positions 1427 to 1452), and 1616R, 5'-GCGGTGTGTACAAAGGGCAGGG-3' (1637 to 1616). The primer 1427F contained the same GC clamp sequence as the 357F primer. The products were examined by agarose gel electrophoresis before DGGE analysis.

DGGE was performed with the DCode system (Bio-Rad Laboratories, Hercules, CA, USA). To survey the microbial community, 10% of the polyacrylamide gel with 40% to 50% denaturant gradient was used. Gels were stained with 10 mg/ml ethidium bromide for 15 min. The gel image was recorded with a Typhoon 9400 (GE Healthcare UK, Ltd., England). Distinct gel bands were excised with a clean razor blade, and PCR re-amplification was performed with the same primers as described above. The rRNA gene sequences obtained from the DGGE bands were compared with those from the DDBJ nucleotide sequence database using the BLAST program.

Identification of isolated bacteria. The 16S rRNA gene sequences of isolated bacteria were determined by direct colony PCR (Plourde-Owobi et al., 2005) using universal bacterial primers: 8F (forward), 5'-AGAGT TGTATCCTGGCTC-3' (*E. coli* positions 8 to 25), and 1492R (reverse), 5'-GGTTACCTGTTACGACTT-3' (1510 to 1492). The amplified DNA samples were purified by ethanol precipitation and then sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Chiba, Japan) with an ABI 3100 genetic analyzer (Applied Biosystems). The 16S rRNA gene sequences obtained from the isolated bacteria were compared with those from the DDBJ nucleotide sequence database using BLAST.

Results

Biochemical analyses during brewing

To determine the biochemical changes during brewing of the Fukuyama pot vinegar preparation sampled in spring of 2004, pH and concentration of lactic acid, ethanol and acetic acid were monitored periodically. Samples taken from three defined pots were not mixed but analyzed individually. The data obtained were quite similar to each other among the three pots. The analytical data were expressed as the mean of those obtained from the three pots. The results are summarized

Fig. 1. Fermentation profile of Fukuyama pot vinegar brewing.

Symbols used are: open circle for pH, filled triangle for ethanol, filled box for lactic acid and filled diamond for acetic acid.

in Fig. 1. One of the most distinct changes was the sharp decline in pH from 6.5 to 3.5 within the first 5 days. This decline solely depended on the rapid increase in the D- and L-lactic acid concentration (up to 0.4%). Alcoholic fermentation proceeded coincidentally with lactic acid fermentation as the concentration of ethanol also increased up to 4.5% within the first 5 days. Acetic acid became detectable at day 16 of brewing and its concentration gradually increased to 7% around day 70, where it remained thereafter. The concentration of lactic acid in the later stage of brewing remained stable at about 0.25% and pH was 2.8 at day 90.

Microbial population of mash samples during brewing

To determine the approximate number of culturable microorganisms during brewing, we adopted plate count methods using approximately diluted samples and appropriate agar plates mentioned in MATERIALS AND METHODS. Fungal colonies formed on the nutrient agar plates were also counted. The data of viable counts among three pots were also practically identical. The results of mean values of three pots are summarized in Fig. 2. Lactic acid bacteria and yeasts grew rapidly within the first 5 days to comprise large populations reaching maximums of about 10^8 cells/ml. Between days 10 and 40, however, the populations of lactic acid bacteria and yeasts were present at a much lower range (around 10^5 cells/ml). Coinciding with these results, total viable cell counts also reached a

Fig. 2. Profile of microbiological flora during Fukuyama pot vinegar brewing.

Symbols used are: filled circle for total viable count on nutrient agar, shaded diamond for lactic acid bacteria on MRS medium, shaded box for yeasts on YPD medium, open triangle for acetic acid bacteria on GYP medium, asterisk for fungi on PDA medium and shaded circle for fungi colony numbers on nutrient agar (NA).

maximum of about 10^8 cells/ml at day 5 and thereafter gradually decreased to below 10^5 cells/ml by day 40. In contrast, the number of acetic acid bacteria was very low until day 10 but markedly increased at day 16, to a level between 10^4 and 10^5 cells/ml, which was maintained until day 40. By day 50, the cell number markedly decreased to below 10^2 cells/ml. This decrease will be discussed later.

DGGE analysis for microorganisms present during brewing

Using the PCR-amplified defined regions of small bacterial and yeast rDNA found in the brewing mash, DGGE analysis was performed to determine the dominant microorganisms in the mash samples of pot 1 irrespective of culturability. As microbial profiles were anticipated to change greatly especially in the early period of brewing, we mainly analyzed the samples obtained between day 0 and 20. Using a prokaryote-specific primer set for amplification of these defined regions, four distinct bands were observed (Fig. 3, left panel). As two of these bands (open arrow heads in Fig. 3) were distinct in the starting mash (designated as day 0) and then later disappeared, they were determined to be rDNA from dominant bacterial species in the raw materials. On the other hand, the other two bands (bands A and B in Fig. 3) were not present or distinct on day 0 and were only notable after day 0, suggesting they represent rDNA of dominant species during fermentation. The A and B rDNA bands were thus extracted from the gel, PCR-amplified and sequenced for species determination. Band A was determined to be small rDNA of *Lactococcus lactis* (100% identity: 147/147 bp); band B rDNA could not be identified.

Using a eukaryote-specific primer set for amplification of small rDNA, four distinct bands were observed

on day 0 (Fig. 3, right panel). Three of these bands (open arrow heads in Fig. 3) disappeared after day 0. The remaining band (band C in Fig. 3) was distinct throughout brewing. This band was extracted from the gel, PCR-amplified and sequenced for species determination. Band C was found to be derived from *Saccharomyces cerevisiae* (98% identity: 147/150 bp).

Colony direct PCR for isolated acetic acid bacteria

We failed to identify the dominant species of acetic acid bacteria of pot 1 by DGGE analysis. Therefore, we conducted colony direct PCR to identify culturable, dominant species of acetic acid bacteria. We picked up 8 and 9 colonies of acetic acid bacteria isolated from 40- and 50-day samples of pot 1, respectively. All sequence data from the 17 colonies invariably identified *Acetobacter pasteurianus* (99% identity: 214/215 bp) as the isolated species.

Discussion

Fukuyama pot vinegar brewing is mainly composed of three fermentative processes: 1) saccharification of rice starch to sugars by carbohydrases supplied from rice koji, 2) alcoholic fermentation from sugars to ethanol, and 3) acetic acid fermentation from ethanol to acetic acid. These processes were found to partially proceed in parallel. Therefore, it has been proposed to call the vinegar brewing (parallel) triple fermentation (Yanagida, 1990).

Our results suggested that another factor, lactic acid fermentation, plays a key role in this process. Within the first 5 days of brewing, lactic acid fermentation and alcoholic fermentation proceeded coincidentally, as clearly indicated in the fermentation profile shown in Fig. 1. The microorganisms involved in lactic acid and alcoholic fermentation might be derived from the raw material, koji, as indicated by the DGGE analysis which clearly shows that amplified rDNA of a dominant lactic acid bacterium and a yeast were visible from the start of brewing (Fig. 3, bands A and C).

The acidic condition elaborated by lactic acid bacteria may result in an unfavorable condition for proliferation of diverse contaminating microorganisms (see Fig. 2). Even under this acidic condition, acid-tolerant *Saccharomyces* yeasts proliferated, leading to alcoholic fermentation (Figs. 1 and 3). The importance of lactic acid fermentation in ecological selection has been previously reported for traditional yeast seed (ki-

Fig. 3. PCR-DGGE profile during Fukuyama pot vinegar brewing.

Open arrow heads represent the position of PCR-amplified rDNA bands which appeared only in 0 day lanes.

moto or yamahai-moto) making in the brewing of Japanese rice wine (Akiyama, 1978). The main difference between yamahai-moto (or ki-moto) seed making and the early stage of Fukuyama pot vinegar brewing is the much faster accumulation of lactic acid in Fukuyama pot vinegar brewing, probably due to the higher temperature conditions. The average temperature in Kagoshima City near Fukuyama was 17.7°C in April 2004 (data from the Japan Meteorological Agency), while that in the early stage of yamahai-moto seed making is around 8–10°C (Ishikawa, 2002). The peak cell number of lactic acid bacteria was reported to occur earlier in yamahai-moto seed making, while that of sake yeasts occur later (Akiyama, 1978). In contrast, lactic acid and alcoholic fermentation proceeded in almost parallel fashion in the early stage of Fukuyama pot vinegar spring brewing (Figs. 1 and 2).

The occurrence of lactic acid fermentation in Fukuyama pot vinegar brewing has already been pointed out (Entani and Masai, 1985a; Koizumi et al., 1996). The meaning of lactic acid fermentation, however, has not been clearly discussed in regards to its contribution to ecological selection. Another report (Haruta et al., 2006) discussed the contribution of lactic acid fermentation to the ecological selection but not to the transition of fermentative processes.

In addition to the acidic environment, the relatively high concentration of ethanol (above 4.5%) between days 5 and 20 may additionally or cooperatively restrict the growth of existing microorganisms, although the killing activity was not significant (see the total bacterial count in Fig. 2). The resulting environment thus provides a favorable condition for acetic acid bacterial growth as these bacteria are well known to be acid resistant and “ethanol-philic.” In fact acetic acid was detectable on day 16 and a typical precursor-product (ethanol-acetic acid) relationship was observed in the brewing profile (Fig. 1). Therefore, the entire process of Fukuyama pot vinegar brewing appears to roughly compose two phases: the early parallel fermentative phase (especially marked in the first 5 days) and the later acetic acid fermentation phase. The responsible acetic acid bacteria are supposedly derived from the environment, especially from pots which have been rinsed but not sterilized before every brewing. From the fermentation profile (Fig. 1), band B in Fig. 3 is the most probable candidate for rDNA from an acetic acid bacterium. However, we failed to identify its origin. Colony direct PCR for 17 isolated strains of acetic acid

bacteria from 40- and 50-day samples was also conducted to determine the origin of 16S rDNA. All the sequence data coincided with that of *A. pasteurianus*, suggesting that it was the dominant species in the acetic acid fermentation phase.

According to the empirical data of the brewery, mash surface-floating koji (furi-koji) slowly sinks into the mash from one side after about 40 days of brewing in the spring preparation of Fukuyama pot vinegar. The disappearance of the surface cap could provide a favorable condition for proliferation of pellicles (biofilm) of acetic acid bacteria. The early proliferation of acetic acid bacteria before day 40 may therefore occur at the periphery of furi-koji. Studies on the functional effect of furi-koji on acetic acid fermentation (Koizumi et al., 1988, 1989) showed that no addition of furi-koji results in a higher ethanol concentration due to excessive yeast growth, which thus causes slower and less acetic acid production. On the other hand, floating styrene-foam plates on the surface instead of furi-koji inhibited acetic acid fermentation (Koizumi et al., 1988, 1989). These results clearly suggest that the sinking of furi-koji into the mash is an important step for more efficient acetic acid fermentation and that furi-koji is a natural regulator of Fukuyama pot vinegar brewing. In fact, acetic acid fermentation seems to proceed in a biphasic manner (Fig. 1), that is, the first increase in acetic acid concentration from day 16 to day 33, the lag between day 33 and day 40, and the second increase in acetic acid after day 40. The second increase might reflect the increased acetic acid fermentation by pellicles of acetic acid bacteria. However, the marked decrease in cell number of acetic acid bacteria after day 40 (Fig. 2) is controversial. This marked decrease may reflect a change in life style of acetic acid bacteria from nomadic (free living) to sedentary (static or biofilm forming) (Kolter and Greenberg, 2006). Unfortunately, our method of monitoring bacterial populations by sampling of mashes from the depth of about 5 cm from the mash surface was not suitable for estimating the cell number of acetic acid bacterial pellicles.

It was suggested that the appearance of the acetic acid bacterial pellicle at the periphery of furi-koji could lead to the sinking of furi-koji (Yanagida, 1990). The mechanism of sinking of furi-koji, however, has not been discovered. It remains to be elucidated.

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