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Vinegar production from post-distillation slurry deriving from rice *shochu* production with the addition of caproic acid-producing bacteria consortium and lactic acid bacterium

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To establish a zero emission process, the post-distillation slurry of a new type of rice *shochu* (NTRS) was used for the production of health promoting vinegar. Since the NTRS post-distillation slurry contained caproic acid and lactic acid, the effect of these two organic acids on acetic acid fermentation was first evaluated. Based on these results, *Acetobacter aceti* CICC 21684 was selected as a suitable strain for subsequent production of vinegar. At the laboratory scale, acetic acid fermentation of the NTRS post-distillation slurry in batch mode resulted in an acetic acid concentration of 41.9 g/L, with an initial ethanol concentration of 40 g/L, and the acetic acid concentration was improved to 44.5 g/L in fed-batch mode. Compared to the NTRS post-distillation slurry, the vinegar product had higher concentrations of free amino acids and inhibition of angiotensin I converting enzyme activity. By controlling the volumetric oxygen transfer coefficient to be similar to that of the laboratory scale production, 45 g/L of acetic acid was obtained at the pilot scale, using a 75-L fermentor with a working volume of 40 L, indicating that vinegar production can be successfully scaled up.

[**Key words:** Rice *shochu*; Post-distillation slurry; Vinegar production; Acetic acid fermentation; Zero emission]

Since 2004, *honkaku shochu* output has decreased dramatically, especially rice *shochu* output, which has decreased by 30% between 2004 and 2012 (1). To increase the popularity of rice *shochu*, a process was developed to produce a new type rice *shochu* (NTRS) that is rich in both ethyl caproate and isoamyl acetate (2). In this process, a caproic acid-producing bacteria (CAPB) consortium enriched from the Chinese liquor pit mud and lactic acid bacterium (LAB) isolated from a lactic acid beverage were added on the first day of the second stage fermentation. Caproic acid was produced by the CAPB consortium, and ethyl caproate was formed by *Saccharomyces cerevisiae* in the presence of ethanol (3). This new type rice *shochu* product was favourably commented by the panellists from Japanese *shochu* distilleries (2).

Shochu post-distillation slurry is the main by-product of the *shochu* industry, and approximately 1.3 tonnes of post-distillation slurry is generated per 1 tonne of *shochu* produced (4). Generally, the *shochu* post-distillation slurry is disposed of by feeding animals, methane fermentation, and incineration. In Japan, vinegar beverage has a similar price as *shochu*; therefore, it is worthwhile to produce vinegar product using the *shochu* post-distillation slurry. The *shochu* post-distillation slurry is rich in physiological activators derived from the raw material, *koji*, and yeast (4). In our previous study, a process was developed to produce vinegar from traditional rice *shochu* (TRS) post-distillation slurry by acetic acid fermentation. The vinegar product had radical scavenging activity (4,5), the ability to inhibit angiotensin I converting enzyme (ACE) and advanced glycation end products (AGE) (4), induce apoptosis (6), and antitumour properties (7,8), both *in vitro* and *in vivo*. However, *shochu* post-distillation slurry contains organic acids, which are potential physiological stressors of microorganisms. In our previous study of acetic acid fermentation from TRS post-distillation slurry (containing 3000–5000 mg/L of citric acid), using 4 strains of *Acetobacter aceti*, each strain produced a significantly different amount of acetic acid (9). Thus, screening of acid-tolerant strain is important for efficient vinegar production. In the NTRS post-distillation slurry, there are organic acids like caproic acid and lactic acid, and their effect on acetic acid fermentation is still unknown.

This study aimed to establish a zero emission process for production of NTRS, by producing vinegar, using its post-distillation slurry. To this end, the effects of caproic acid and lactic acid on acetic acid fermentation were investigated, and vinegar was produced from the NTRS post-distillation slurry, both at the laboratory scale and at the pilot scale. The beneficial properties of the vinegar were also tested by measuring its ACE inhibition activity *in vitro*.

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MATERIALS AND METHODS

Preparation of NTRS post-distillation slurry *Shochu* production was carried out with the addition of CAPB consortium, LAB, and yellow *koji* prepared from *Aspergillus oryzae*, as described previously (2). The fermented mash was distilled at 45°C and 65 mmHg, using a rotary evaporator (NE-1001; Eyela, Tokyo, Japan) with a pressure controller (NVC-2200; Eyela, Tokyo, Japan). Distillation was terminated when 40% (v/v) of the fermented mash was distilled. The post-distillation slurry was used for acetic acid fermentation without filtration or centrifugation. At the laboratory scale, three kinds of NTRS post-distillation slurry were prepared from *shochu* prepared with the addition of 4% (v/v) CAPB consortium, 4% (v/v) CAPB consortium and 0.8% (v/v) LAB, and 4% (v/v) CAPB consortium and 2% (v/v) LAB, respectively. At the pilot scale, NTRS was produced with the addition of 4% (v/v) CAPB consortium and 0.8% (v/v) LAB, and its post-distillation slurry was used for vinegar production.

Microorganisms Acetic acid bacteria purchased from China Centre of Industrial Culture Collection were used for vinegar production. They were *Acetobacter pasteurianus* CICC 20056, *A. pasteurianus* CICC 20064, *A. pasteurianus* CICC 20672, *A. pasteurianus* CICC 23563, and *Acetobacter aceti* CICC 21684, respectively. *A. pasteurianus* NBRC 3283, which was used in our previous studies, was used as control. Hereafter, these strains are referred to as 20056, 20064, 20672, 23563, 21684, and 3283, respectively.

Strains 20056, 20064, 20672, and 23563 were stored on slant CM0001 media (yeast extract, 10 g/L; glucose, 100 g/L; $CaCO_3$, 10 g/L; agar, 15 g/L), and strain 21684 was stored on slant CM0831 media (yeast extract, 5 g/L; peptone, 10 g/L; glucose, 5 g/L; beef extract, 3 g/L; NaCl, 5 g/L; agar, 15 g/L), as recommended by the collection centre. Stain 3283 was stored on slant yeast extract-peptone-dextrose (YPD) media (yeast extract, 5 g/L; peptone, 5 g/L; glucose, 5 g/L; agar 15 g/L).

Preparation of acetic acid bacterial inoculum The acetic acid bacteria strains stored on slant media were separately inoculated into 50 mL of sterilized media (yeast extract, 10 g/L; glucose, 10 g/L; peptone, 20 g/L; ethanol, 40 g/L) in 300-mL conical flasks. After incubating at 30°C for 48 h with shaking at 200 rpm on a rotary shaker (TB-9R-2F; Takasaki Kagaku, Saitama, Japan), these were served as the acetic acid bacterial inocula.

Acetic acid fermentation using artificial synthetic media Effect of caproic acid and lactic acid on acetic acid fermentation was investigated using artificial synthetic media. The composition of artificial synthetic media used is shown in Table 1. Tests were carried out in 500-mL conical flasks. Ninety-five millilitres of the artificial synthetic media without the addition of ethanol were sterilized at 121°C for 15 min. After the media had cooled, 4 g (5 mL) of ethanol and 5 mL of acetic acid bacterium inoculum were added to initiate acetic acid fermentation. Acetic acid fermentation occurred at 30°C with shaking at 200 rpm for 72 h in a rotary shaker (TB-9R-2F; Takasaki Kagaku, Saitama, Japan). Samples were periodically taken to measure the concentrations of ethanol and acetic acid. Preliminarily, the effects of covers for the 500 mL conical flask on acetic acid fermentation were evaluated, including cotton wool, silicon plug (T-38; Shin-Etsu Polymer Co. Ltd., Tokyo, Japan), and sterile and breathable sealing film (12×12 cm^2, $\Phi = 0.22$ μm).

Screening of acetic acid bacterial strains for vinegar production from NTRS post-distillation slurry Screening of acetic acid bacterial strains was carried out using the NTRS post-distillation slurry. A 95-mL sample of NTRS post-distillation slurry, produced with 4% (v/v) CAPB consortium and 2% (v/v) LAB, was mixed with 1 g of $CaCO_3$ in a 500-mL conical flask and sterilized at 121°C for 15 min. After the mixture had cooled, ethanol was added to a final concentration of 40 g/L. Acetic acid fermentation was induced by the addition of 5 mL of acetic acid bacterial inoculum. Acetic acid fermentation was carried out under the same conditions as described above.

Vinegar production at the laboratory scale Effect of sterilization on acetic acid fermentation was firstly evaluated. NTRS post-distillation slurry (produced with 4% (v/v) CAPB consortium and 2% (v/v) LAB) was combined with ethanol to a final concentration of 40 g/L, as well as 1 g of $CaCO_3$. Five millilitres of acetic acid bacterial inoculum was added to initiate acetic acid fermentation in non-sterilized NTRS post-distillation slurry, as well as in NTRS post-distillation slurry sterilized at 121°C for 15 min (as described above) as the control.

Batch acetic acid fermentation was carried out in a 3-L jar fermentor (BMS03PI; ABLE Corporation, Tokyo, Japan) containing 1.38 L of unsterilized NTRS post-distillation slurry with 40 g/L ethanol. Acetic acid fermentation was initiated by the addition of 75 mL of *A. aceti* CICC 21684 inoculum, and carried out at 30°C, with shaking at 600 rpm and an aeration rate of 0.5 vvm (=0.75 L/L/min) for 20 h. The pH was automatically controlled at >4.0 by the addition of 3 M NaOH solution using a pH controller (HBM 100A; Toa DKK, Tokyo, Japan). Dissolved oxygen (DO) concentration was monitored using a DO sensor (SBO; ABLE).

Fed-batch acetic acid fermentation was also carried out in the 3-L jar fermentor containing 1.38 L of unsterilized NTRS post-distillation slurry, but with an initial ethanol concentration of 20 g/L. Then, 75 mL of *A. aceti* CICC 21684 inoculum was added to initiate vinegar fermentation. During the fermentation process, ethanol was fed equally three times to give a final ethanol concentration of 40 g/L. Acetic acid fermentation was carried out at 30°C with shaking at 600 rpm for 20 h, with aeration rate of 0.5 vvm. The pH was automatically controlled at >4.0 by the addition of 3 M NaOH solution, using a pH controller. During the fermentation process, DO concentration was monitored using a DO sensor as described above.

Vinegar production at the pilot scale A 37 L sample of unsterilized NTRS post-distillation slurry produced with 4% (v/v) CAPB consortium and 0.8% (v/v) LAB was added to a 75-L fermentor, and combined with ethanol to a concentration of 40 g/L. Acetic acid fermentation was initiated by the addition of 2 L of *A. aceti* CICC 21684 inoculum. Acetic acid fermentation was carried out at 30°C for 20 h with an aeration rate of 0.5 vvm. During the fermentation process, the k_La was dynamically determined. When the DO concentration decreased to 3–4 mg/L, the exact value was recorded and used to determine the k_La. The shaking revolution rate was then increased, and the DO concentration also increased. When the DO concentration decreased to 3–4 mg/L again, the k_La was determined. This process was repeated until a k_La was similar to that obtained at the laboratory scale. The pH was automatically controlled at >4.0 by the addition of 3 M NaOH solution, using a pH controller. DO concentration was monitored using a DO sensor.

Determination of volumetric oxygen transfer coefficient During acetic acid fermentation with DO concentration of 3–4 mg/L, the volumetric oxygen transfer coefficient (k_La) was determined using Eq. 1 (10),

$$kLa = \frac{V(dc/dt) + R(C_{in} - C_{out})}{V(C^* - C)} \quad (1)$$

where V is the working volume (1.5 L); R is the aeration rate (0.75 L/L/min); C_{in} is the oxygen content of air supplied, which was 20.9%; C_{out} is the oxygen content of the gas exhausted, which was determined using an oxygen indicator (XP-3180; New Cosmos Electric, Osaka, Japan); dc/dt is the slope of DO concentration curve at time t; C^* is the saturated DO concentration at 30°C (7.56 mg/L) and C is the measured concentration of DO.

Calculation of acetic acid recovery efficiency Based on the chemical reaction equation of $2CH_3CH_2OH + O_2 \rightarrow 2CH_3COOH + 2H_2O$, the acetic acid recovery efficiency (E) after fermentation was calculated using Eq. 2,

$$E = \frac{CA}{CE \times 60/46} \times 100\% \quad (2)$$

where C_A is the acetic acid concentration measured in the fermented broth (g/L); C_E is the total ethanol concentration used for acetic acid fermentation, which was 40 g/L; 60 is the molecular weight of acetic acid; 46 is the molecular weight of ethanol.

Analytical methods All samples were centrifuged at 10,000 ×g at 4°C for 10 min using a MX-301 centrifuge (Tomy Tech, USA). The supernatant was filtered using a 0.45-μm membrane filter (Jinteng, Tianjin, China), and the filtrate was used for subsequent analysis.

The ethanol concentration was measured by gas chromatography (GC) (GC353B; GL Sciences, Tokyo, Japan) with a flame ionization detector and a TC-1 capillary column (0.25 mm i.d. 60 m L; d.f.: 0.25 mm) using an internal standard method (with isopropanol), as previously described (10). The GC was run with a 50°C oven temperature and 180°C injection and detector temperatures using He as the carrier gas and H_2 as the flaming gas.

The lactic acid concentration was measured by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a post-label method. Samples were separated by Shimpack SCR-101H column (Shimadzu) using a 0.003 M $HClO_4$ solution as mobile phase at 0.5 mL/min and 60°C. After separation, lactic acid reacted with a bromothymol blue solution, and the absorbance was measured at 450 nm by an ultraviolet–visible detector (SPD-10AV) (11).

The concentrations of *shochu* flavour compounds were measured by internal standard (with *n*-butyl acetate) using a GC system (GC353B; GL Science, Tokyo, Japan) equipped with a flame ionization detector and an InertCap Pure Wax capillary column (30 m × 0.25 mm i.d., 0.25 μm d.f.; GL Science), as previously described (2). The GC was operated using the following conditions: injector temperature, 230°C;

TABLE 1. Composition of artificial synthetic media used for screening of acetic acid bacterial strains in new-type rice *shochu* (NTRS) post-distillation slurry.

Composition	Content			
	Blank[a]	CA[a]	LA[a]	CA + LA[a]
Yeast extract (g/L)	10	10	10	10
Peptone (g/L)	10	10	10	10
Glucose (g/L)	10	10	10	10
Ethanol (g/L)	40	40	40	40
Caproic acid (mg/L)	0	50	0	50
Lactic acid (mg/L)	0	0	2000	2000
$CaCO_3$ (g/L)	10	10	10	10
Initial pH	6.8–7.0	6.8–7.0	6.8–7.0	6.8–7.0

[a] Blank, medium without addition of caproic acid or lactic acid; CA, medium with addition of caproic acid; LA, medium with addition of lactic acid; CA + LA, medium with addition of caproic acid and lactic acid.