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**Note**

# Identification of Turbid Compounds Generated in Sugarcane Vinegar

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**Abstract: Sugarcane vinegar is produced in various countries of southern Asia. It is also a niche product of the Kagoshima and Okinawa Prefectures in Japan. Turbid compounds are sometimes found in sugarcane vinegar, thereby lowering the market value. In this study, the turbid compounds were precipitated with a 1:2 (v/v) volume of ethanol, and they were identified as α-1,6-glucan using enzymatic digestion tests and $^{13}C$ nuclear magnetic resonance analysis. Moreover, *Lactobacillus nagelii* was isolated from sugarcane juice, and it produced α-1,6-glucan when grown with sugar. The turbid compounds found in sugarcane vinegar were assumed to be α-1,6-glucan produced from sugar by lactic acid bacteria that exist in sugarcane juice.**

**Key words: sugarcane vinegar, glucan, dextran, *Lactobacillus nagelii***

Sugarcane vinegar is traditionally produced in the sugarcane-growing districts of southern Asian countries such as the Philippines, Thailand, and India. It is also produced in the Amami and Okinawa Islands located in southwestern Japan. The vinegar is not only used as a condiment but also consumed as a beverage. Sugarcane vinegar is a natural vinegar produced by two fermentation processes, ethanol fermentation from sugar followed by acetic acid fermentation.[1)] Various beneficial properties such as antioxidative, antidiabetic, antimicrobial, antitumor, antiobesity, antihypertensive, and cholesterol-lowering effects have been reported in natural vinegar products.[2)] The radical scavenging activity and repression of human leukemia cells have also been reported in sugarcane vinegar.[3)] The manufactured sugarcane vinegar is ideally expected to be clear; however, the product occasionally has a cloudy appearance due to the generation of white turbid compounds. We have previously attempted to prevent the formation of these compounds to preserve the quality of sugarcane vinegar.[4)] Here, we report on the isolation and identification of the turbid compounds in detail and discuss the possible reasons for turbidity in sugarcane vinegar.

In the current study, 14 white cloudiness formed sugarcane vinegar samples shown in Table 1 were used. The turbid compounds in all the samples were almost completely precipitated by adding a 1:2 (v/v) volume of ethanol. Each precipitate was collected by centrifuging at 34,000 × G. They were then dried at 60 °C and redissolved in water. The amounts of protein, polyphenol, and total sugar were measured by the bicinchoninic acid assay Protein Assay Reagent Kit (Pierce Distribution Services Company, Inc., Rockford, USA)[5)] using bovine serum albumin as the standard, the Folin–Ciocalteu method,[6)] and the Phenol-Sulfate method,[7)] respectively. As shown in Table 1, all the precipitates were mostly composed of carbohydrates [approximately 90 % (w/w)], whereas proteins and polyphe-

**Table 1.** Amounts of protein, polyphenol, and total sugar per 1 g dried ethanol precipitates[a)] from sugarcane vinegar samples.

| Sugarcane vinegar sample number[b)] | Protein (mg/g) | Polyphenol (mg/g) | Total sugar (mg/g) | Others (mg/g) |
|---|---|---|---|---|
| 1 | 8.4 | 1.4 | 952.9 | 47.1 |
| 2 | 9.0 | 0.9 | 898.3 | 101.7 |
| 3 | 10.0 | 1.2 | 919.3 | 80.7 |
| 4 | 10.7 | 1.3 | 888.9 | 111.1 |
| 5 | 16.0 | 0.9 | 908.0 | 92.0 |
| 6 | 18.7 | 1.0 | 893.0 | 107.0 |
| 7 | 22.1 | 1.2 | 878.7 | 121.3 |
| 8 | 18.7 | 1.0 | 907.9 | 92.1 |
| 9 | 24.6 | 0.8 | 911.3 | 88.7 |
| 10 | 17.1 | 0.9 | 917.2 | 82.8 |
| 11 | 10.0 | 1.0 | 905.7 | 94.3 |
| 12 | 24.0 | 1.4 | 934.4 | 65.6 |
| 13 | 19.7 | 1.1 | 935.7 | 64.3 |
| 14 | 23.7 | 1.2 | 916.8 | 83.2 |

[a)] Dried ethanol precipitates were prepared by ethanol precipitation of sugarcane vinegar samples followed by heating and drying, as described in the text. [b)] Sugarcane vinegar number 1−7 were collected from the vinegar pots at Shigehara Manufacturing Store on January 20, 2009. Number 8−12 were collected from the experimental tanks at Kagoshima Prefectural Institute of Industrial Technology on January 10, 2009. Numbers 13 and 14 were the commercial products obtained at a retail shop in Kirishima City, Kagoshima on February 15, 2009.

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Abbreviations: CI, cycloisomaltooligosaccharide; CITase, CI glucanotransferase; HBDase, highly branched dextran hydrolase; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

nols comprised minor proportions. The major constituents of the turbid compounds were, therefore, assumed to be carbohydrate polymers.

Three ethanol precipitate samples, namely Shigehara 1, Shigehara 7, and Kougi 7 (sample numbers 1, 7, and 12 in Table 1, respectively) were further digested by enzymes, and the degraded products were analyzed by thin-layer chromatography (TLC). As shown in Fig. 1, none of the three samples were digested by α-amylase from porcine pancreas and by glucoamylase, indicating that the turbid compounds were not α-1,4-glucan. In contrast, they were digested by dextranase to produce isomaltooligosaccahrides. In addition, they were digested by a highly branched dextran hydrolase (HBDase),[8] an exoglucanase which digests α-1,6-; α-1,4-; α-1,3-; and α-1,2-glycosidic linkages at the non-reducing end, to produce glucose. Cycloisomaltooligosaccharide glucanotransferase (CITase, EC 2.4.1.248) catalyzes intramolecular transglucosylation of α-1,6-glucan to produce cycloisomaltooligosaccharides (CIs) exclusively.[8] Furthermore, *Escherichia coli* recombinant CITase from *Bacillus circulans* T-3040 was prepared, and the production of CIs from glucan samples was measured using high-performance liquid chromatography (HPLC), as previously described.[9] CIs were produced from the three ethanol precipitate samples. All enzymatic degradation patterns of the turbid compounds were similar to that of a control α-1,6-glucan, dextran 40 (GE Healthcare, Little Chalfont, UK; Fig. 1).

Dextran is produced from sucrose by certain lactic acid

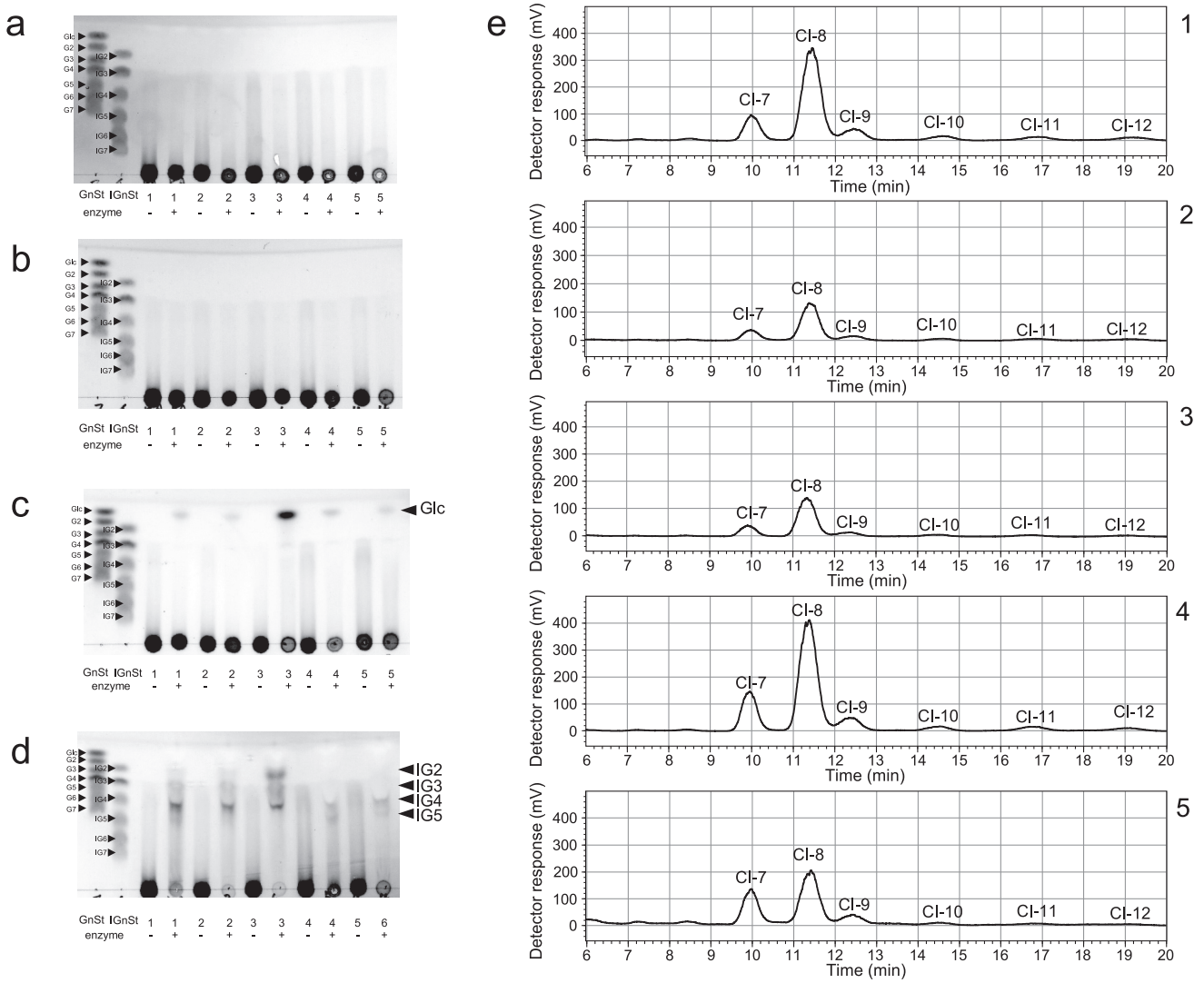


**Fig. 1.** TLC (a–d) and HPLC analysis (e) of enzymatic degradation products from dextrans and turbid component samples taken from sugarcane vinegar.

Appropriate amounts of α-amylase from porcine pancreas (Sigma-Aldrich Corporation, St. Louis, USA) (a), glucoamylase (Oriental Yeast Co., Ltd., Tokyo, Japan) (b), HBDase (kindly donated by Dr. Oguma and Mr. Kawamoto) (c), dextranase (Amano Enzyme Inc., Nagoya, Japan) (d), or *E. coli* recombinant CITase from *B. circulans* T-3040 prepared as described before[9] (e) were added respectively to 20 mg/mL dextran samples in 50 mM sodium acetate buffer (pH 5.5), and they were incubated at 40 °C for 30 min. Glucose and oligosaccharide production was detected by TLC with a silica gel 60 $F_{254}$ (Merck Millipore, Billerica, USA). Each plate was developed with a solvent system of acetonitrile:water (7 : 3) two times, dried, and soaked in 5 % sulfuric acid in methanol and heat at 200 °C. CI-production was analyzed by HPLC with evaporative light scattering detection system (LC Workstation Class-VP, Shimadzu Corporation, Kyoto, Japan) with a TSK gel Amide-80 column (4.6 × 250 mm; Tosoh Corporation, Tokyo, Japan) as described before.[9]

Glc, glucose; G*n*, IG*n*, and CI-*n*, maltooligosaccharide, isomaltooligosaccharide, and cycloisomaltooligosaccharide, respectively, where *n* indicates the number of glucose molecules. Dextran 40 (1); dextran produced by *L. nagelii* (2); turbid component sample from sugarcane vinegar Shigehara 1 (3), Shigehara 7 (4), and Kougi 7 (5); incubated with (+) and without ( − ) enzyme.

bacteria.[10] We tested the possibility that a lactic acid bacterium was responsible for the production of dextran in sugarcane juice, and then attempted to isolate the lactic acid bacterium from sugarcane juice which was stored after boiling.[4] The boiled sugarcane juice was incubated with 0.001 % cycloheximide containing MRS agar (Oxioid Ltd., Cheshire, England) under an anaerobic condition using AnaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan) at 30 °C for 3 days. Three lactic acid bacteria were isolated, and based on their partial 16S ribosomal DNA sequences, two of them were determined as *Lactobacillus nagelii* (accession numbers LC064107 and LC064108). The third species was identified as *Lactobacillus* sp. (LC064109). A strain belonging to *L. nagelii* was reported to produce dextran from sucrose.[11] One of the isolated *L. nagelii* strains (LC064107) was cultured with sucrose, and the produced dextran was purified from the culture supernatant by ethanol precipitation, as previously described.[12] The enzymatic

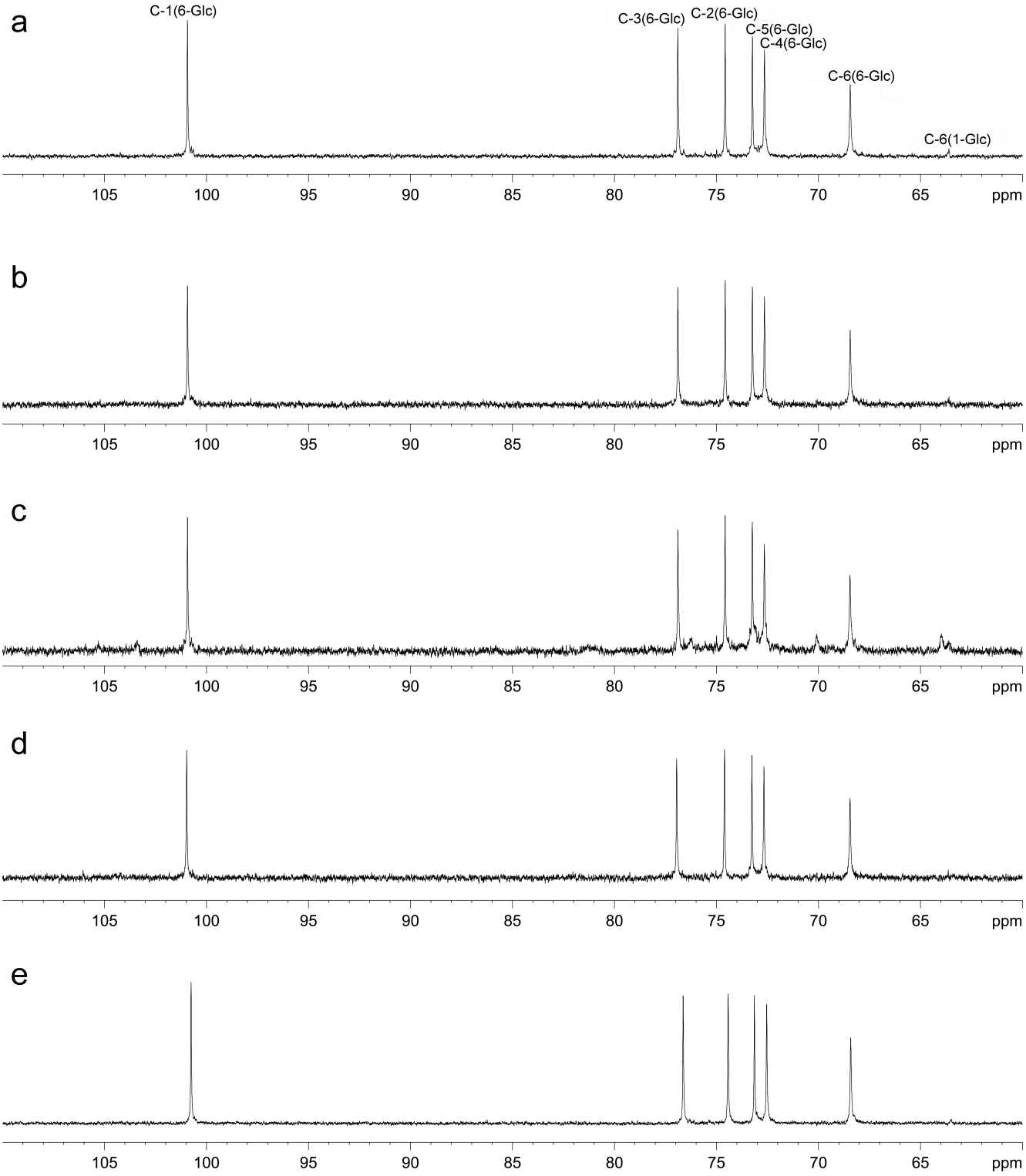


**Fig. 2.** $^{13}$C NMR spectra of dextrans and turbid component samples taken from sugarcane vinegar.

Dextran 40 (a), dextran produced by *Lactobacillus nagelii* (b), and turbid component samples from sugarcane vinegar, Shigehara 1 (c), Shigehara 7 (d), and Kougi 7 (e) were prepared, reduced, and lyophilized, and $^{13}$C NMR spectra of each sample was measured, as described in the text.

degradation pattern of *L. nagelii* dextran was similar to that of dextran 40 and the ethanol precipitates from sugarcane vinegars (Fig. 1).

Dextran 40, *L. nagelii* dextran, and Shigehara 1, Shigehara 7, and Kougi 7 samples were reduced by $NaBH_4$, and $^{13}C$ nuclear magnetic resonance (NMR) analysis was conducted using a Bruker DRX600, as previously described.[12] Dextran produced by *L. nagelii* and all the ethanol precipitate samples from sugarcane vinegars showed spectra indicative of containing only an α-1,6-linked glucopyranoside, such as dextran 40 (Fig. 2).

We concluded that the turbid compounds in sugarcane vinegar were α-1,6-glucan produced by lactic acid bacteria in sugarcane juice. Although the sugarcane juice was sterilized by boiling in advance, lactic acid bacteria were isolated in the current study. One possible reason could be bacterial contamination from outside during storage. Another reason could be incomplete boiling sterilization because of which the bacterial strains in the sugarcane juice were still alive. Pasteurization temperatures used for lactic acid bacteria are typically not very high. For *L. nagelii* isolated from sugarcane juice, living colonies were not detected after boiling the full-growth culture for 5 min.[4] However, viable but non-culturable lactic acid bacteria in sulfited wine was reported.[13] There is a possibility that the lactic acid bacteria obtained from sugarcane juice were the restored cells which were in a viable but non-culturable state during storage. Further studies are required to clarify the entire mechanism of contamination of sugarcane vinegar; nevertheless, it is important to use sugarcane juice immediately after sterilization to reduce the risk of contamination by lactic acid bacterial-derived turbid compounds.

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