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Taro *koji* of *Amorphophallus konjac* enabling hydrolysis of konjac polysaccharides to various biotechnological interest

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ABSTRACT

Due to the indigestibility, utilization of konjac taro, *Amorphophallus konjac* has been limited only to the Japanese traditional konjac food. *Koji* preparation with konjac taro was examined to utilize konjac taro as a source of utilizable carbohydrates. *Aspergillus luchuensis* AKU 3302 was selected as a favorable strain for *koji* preparation, while *Aspergillus oryzae* used extensively in *sake* brewing industry was not so effective. *Asp. luchuensis* grew well over steamed konjac taro by extending hyphae with least conidia formation. *Koji* preparation was completed after 3-day incubation at 30°C. D-Mannose and D-glucose were the major monosaccharides found in a hydrolyzate giving the total sugar yield of 50 g from 100 g of dried konjac taro. An apparent extent of konjac taro hydrolysis at 55°C for 24 h seemed to be completed. Since konjac taro is hydrolyzed into monosaccharides, utilization of konjac taro carbohydrates may become possible to various products of biotechnological interest.

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Konjac taro has been exclusively used for many years for the Japanese traditional konjac food, devil's tongue starch paste, due to the indigestibility. Annual domestic gross product of konjac taro was recorded to be 65,000 t (as of 2017) and most of them was used for konjac food production. If the konjac taro could be hydrolyzed, it should open a new page of biotechnological applications to ever-covered area of konjac taro. However, a complete enzymatic hydrolysis of glucomannan of konjac taro remained as a tough subject of impregnable. A number of pioneering works on chemical structure determination of konjac glucomannan have been reported [1–4]. It has been confirmed that β-(1→4)-linked D-glucose and D-mannose residues form the main chain with branches through β-(1→6)-glucosyl existing for every 50–60 residues. The ratio of D-mannose to D-glucose is about 1.6 and the terminal glucosyl units to mannosyl units are about 2 [5].

It looks highly resistant to enzymatic hydrolysis with known hydrolases from commercial sources. Many trials with different digesting enzymes from various biological sources were done by many researchers. Bacterial endo-β-mannanase was applied to konjac flour to oligosaccharide production [6]. It gave only small amounts of monosaccharides even after hydrolysis at 50°C for 48 h. A purified β-mannanase from *Aspergillus terreus* was adopted to depolymerize various oligosaccharides [7]. However, it did not hydrolyze mannobiose and mannotriose, but only mannotetraose liberating D-mannose and mannotriose. Mannotriose was the predominant hydrolyzed product from locust bean gum and guar gum giving the mean yield of 16%. It was noted that the fungal enzyme liberated mannotetrasaccharide exclusively from konjac gum with 24% yield. Tsujisaka et al. [8] purified endo-β-mannanase from *Aspergillus niger* and examined the hydrolytic activity to various substances. When it was reacted with galactomannan from soy bean hull, galactomannan from guar gum, mannan from coffee bean, and glucomannan from arum root, mannooligosaccharides were found and the hydrolytic limit to those substrates was 12%, 12%, 58%, and 39%, respectively. Practical yield of D-mannose as monosaccharide is not so high. Many microbial mannanases were reviewed from various sources of bacteria and fungi [9]. However, there are few numbers of enzymes which hydrolyze glucomannan to monosaccharides with a favorable high yield. In most cases, a complete enzymatic hydrolysis of glucomannan of konjac taro looks difficult and various comparable amounts of oligosaccharides are detected after incomplete hydrolysis.

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Patent status: Yamaguchi University has applied the basic knowledge of this paper to our domestic patent office, being applied on March 17 2019. If you have any comments or question, please contact to: Mr. Isamu YOSHIMATSU, Director, IPR Center, Yamaguchi University (yosimatu@yamaguchi-u.ac.jp).A part of this paper was presented at the 2020 Annual Meeting of JSBBA in Fukuoka, programed as 3A11p09 (March 27 2020).

Why many trials of enzymatic hydrolysis to konjac taro glucomannan with mannanases become incomplete, leaving oligosaccharide as the final products [9]? In our recent works for preparing shikimic acid and its precursors from chlorogenic acid in coffee pulp or yerba mate leaves, a filamentous fungus was inoculated on the respective plant materials making coffee pulp *koji* [10,11] or yerba mate *koji* [12–14]. When the intact whole *koji* was used as the catalyst for chlorogenic acid hydrolase, caffeic acid and quinic acid were produced with higher yield. Under similar strategies, whole konjac taros were directly used to prepare respective *koji* without peeling. When commercially available konjac powder or konjac flour which had been peeled before milling was used, there were some technical difficulties to get comparable amounts of monosaccharides as the final enzymatic digestion products [6–9]. Most of them have given oligosaccharides as the final products with incomplete yield of hydrolysis. The whole taro *koji* strategy employed in this study may be well understood from another example of selective formation of α-amylase, cellulase, and hemicellulase from wheat grains [15].

In this study, preparation of taro *koji* of *Amorphophallus konjac* is dealt leading to almost complete digestion of konjac glucomannan. Plausible utilizations of konjac taro hydrolyzates to various products of biotechnological interest are discussed.

Materials and methods

Materials

Konjac taros of *A. konjac* harvested in Yamaguchi were purchased from a local farmer. They were washed, sliced without peeling, and dried for a week in a drying chamber of 30°C under 40% humidity. Since the net weight decreased to less than one-tenth of the freshly sliced taro, more than 90% of moisture was removed during the drying process and kept before use. Yeast extract for culture medium was kindly provided from Oriental Yeast Co., Ltd., Tokyo. Cane molasses was given by the courtesy of Itochu Sugar Co., Ltd. Dowex 50 × 8 and Dowex 1 x 4, products from Dow Chemical, and *n*-Butyl-Toyopearl, Tosoh, were purchased from Wako Pure Chemical Ind. Ltd., Osaka. D-Mannonic acid and 5-keto-D-mannonic acid were prepared with *Gluconacetobacter liquefaciens* NBRC 113262 [16] due to the unavailability of the authentic standard from commercial sources. A quinoprotein alcohol dehydrogenase (QADH) used for quantitative ethanol determination was prepared from *Gluconobacter suboxydans* IFO 12528 (renamed as *G. oxydans* NBRC 12528) [17]. The membrane-bound D-fructose dehydrogenase for quantitative D-fructose determination was prepared from *G. industrius* IFO 3260 (renamed as *G. japonicus* NBRC 3260) [18]. D-Fructose formation and D-mannitol consumption were measured alternatively by NADP-dependent D-mannitol dehydrogenase from *G. oxydans* NBRC 12528 [19].

Microbial strains and culture media

Twenty different fungal strains including the genera of *Mucor, Rhizopus, Monascus, Absidia, Armillaria, Aspergillus, Gibberella, Neurospora, Penicillium*, and *Trichoderma* were provided from Prof. J. Ogawa, Kyoto University. Two different strains of *Asp. oryzae* were provided from Yamaguchi Prefectural Industrial Technology Institute. All fungal strains were preserved on a malt agar slant. Czapek medium was used for fungal seed cultures. Yeast strain, *Saccharomycodes ludwigii* AKU 4400 was kindly provided from Prof. J. Ogawa and preserved on a malt agar slant. *Acetobacter aceti* IFO 3283 (renamed as *Acetobacter pasteurianus* NBRC 3283) and *A. pasteurianus* SKU 1108 (renamed as *A. pasteurianus* NBRC 101655) were preserved on the culture medium as above [17].

Fungal strain screening for taro koji preparation

Ten milliliters of water were added to several pieces of dried konjac taro slice in a petri dish and autoclaved at 120°C for 60 min. Mycelia or conidia spores were inoculated to the surface of sterilized taro slices in a petri dish. They were incubated for 2 to 4 days in a culture room of 30°C under 40% humidity. Fungal strains which extended the vegetative hyphae over taro slices within 2 days were chosen as a promising strain rather than those which could not germinate under the conditions.

Koji preparation

Several strains were grown at 30°C for 2 days in 100 mL of Czapek medium in a 500-mL Erlenmeyer flask under shaking at 200 rpm. The harvested mycelia were mixed with minced taro slices which had been steamed as above. The mixture of the mycelia and 500 g (wet wt.) of steamed taro slices were minced two times by passing through a meat chopper (Veritas Meat Chopper, Osaka). They were spread over a small cage of stainless steel or bamboo-made mimic to the traditional *koji* tray and incubated at 30°C. They were mixed upside down twice a day until the whole steamed taro slices were apparently covered with vegetative hyphae or conidia formation. *Koji* preparation from konjac taro was done with the traditional *koji* tray using 300 g (dried wt.) of konjac taro slices under the similar conditions as above.

Sugar determination in hydrolyzate of konjac taro koji

Sugar composition and sugar contents in a hydrolyzate of konjac taro *koji* were done by a conventional HPLC equipped by a Shodex Asahipak NH2P-50 4E column (φ4.6 x250 mm) with a flow rate of 0.5 mL/min of a mobile phase composing of 250 mM H_3PO_4: CH_3CN = 20:80. Elution was traced by a refractive index (RI) at 50°C of column temperature. Under the conditions, retention times of the authentic samples were recorded to be 19.27, 21.30, and 23.06 min for D-mannose, D-glucose, and D-galactose, respectively.

Alcoholic fermentation with hydrolyzate of konjac taro koji

Konjac taro *koji* hydrolyzate containing 20% sugar was prepared. The cells of *S. ludwigii* AKU 4400 grown in 500 mL of 5% cane molasses were harvested by centrifugation and washed two times with excess of cold water. Then, they were lyophilized by a lyophilizer (VD-800 F, Taitec, Tokyo) and the lyophilized yeast cells (1.3 g from 500 mL culture) were stored at −20°C before use. The dried yeast cell suspension, 60 mL, was mixed with 140 mL of konjac taro *koji* hydrolyzate. The sugar concentration was reduced to 14% in this step. The mixture was put in a cylindrical container and incubation was done in a water bath of 30°C under static conditions. Sugar contents in the culture medium were measured by a saccharometer (Atago, Tokyo). Ethanol produced was assayed with QADH [17]. To measure the absolute ethanol concentration, the end point measurement was employed [20].

Acetate fermentation with ethanol from hydrolyzate of konjac taro koji

Seed cultures of *A. pasteurianus* NBRC 3283 and *A. pasteurianus* NBRC 101655 were prepared according to Saeki [21]. Since the culture medium initially contained 2% ethanol, the final acetic acid concentration was designed to about 2% after 2 days of cultivation under shaking at 200 rpm at 30°C. When it was mixed with equal volume of a fresh medium, the initial acetic acid concentration was spontaneously adjusted to about 1%. The seed culture, 75 mL, was mixed with equal volume of 10% ethanol which had been produced by alcoholic fermentation of hydrolyzed konjac taro. Acetate fermentation was carried out in a 500-mL Erlenmeyer flask at 30°C under shaking at 200 rpm. Remaining ethanol, acetic acid formed and pH were recorded every day over the period. Acetic acid concentration was titrated with 0.5 N NaOH with phenolphthalein as the pH indicator. Remaining ethanol in the culture media was measured with QADH as above.

D-Mannose separation from hydrolyzate of konjac taro koji

D-Mannose was separated from a hydrolyzate of konjac taro *koji* according to the method described by Fischer and Hirschberger [22]. A hydrolyzate of konjac taro *koji* was weak acidic showing the pH of 3.8–4.1. It was neutralized with NaOH and decolorized by the addition of small amount of active charcoal. To 300 mL of neutralized hydrolyzate containing 17% sugar, 25 g of phenylhydrazine, and 50 mL of 30% acetic acid were added. Monosaccharides in the hydrolyzate of konjac taro *koji* were converted to the respective hydrazones. The mixture was kept overnight in a refrigerator. It was probable to regard that most of monosaccharides in hydrolyzate of konjac taro *koji* were converted to corresponding phenylhydrazones in 0.5 M acetic acid, pH 2.2. The precipitates formed were collected and washed with cold water. D-Mannose phenylhydrazone is cold water insoluble and separated easily from other species of cold water soluble phenylhydrazones. D-Mannose was liberated as free form after the phenylhydrazine was precipitated with benzaldehyde. Dark colored impurities were removed by the addition of active charcoal powders. Excess benzaldehyde in D-mannose solution was removed in an ether layer by a separating funnel. About 15 g of D-mannose was finally recovered and concentrated to a syrup, as written by Fischer and Hirschberger [23].

Oxidation of D-mannose to D-mannonic acid and 5-keto-D-mannonic acid

Two millimoles of D-mannose were incubated with 2 mL of resting cells of *Ga. liquefaciens* NBRC 113262, which had been grown for 36 h in a seed culture medium composed of 0.5% D-sorbitol and 0.3% yeast extract. In 2 mL of resting cell suspension, 250 mg of lyophilized cells was involved. Reaction was carried out at 25°C under shaking at 200 rpm reciprocally. To prepare D-mannonic acid (MNA), one reaction mixture (10 mL) was terminated after incubation for 6–8 h to remove the cells from the incubation mixture. Another reaction mixture was centrifuged after 24 h incubation. Trichloroacetic acid (TCA) was added to the supernatants to 2% and cloudy precipitate was removed by centrifugation. After confirmation of neutral pH of the supernatant, a small amount of Dowex 50 × 8 (Na^+-form) was added and mixed well. The suspension was filtered by a paper filter (5B, Advantec Toyo, Tokyo) and the filtrate was adsorbed into a column (1 x 10 cm) of Dowex 1 × 4 (acetate form). The column was washed

with water before treating it by a linear increasing concentration gradient of 500 mL of 0.5 M acetic acid and every 300 drops (= 7.8 mL) were collected. Spot test was done to confirm the eluted position of MNA and 5-keto-D-mannonic acid (KMA). MNA was detectable with bromphenol blue (BPB) reagent but not with triphenyltetrazolium chloride (TTC), while KMA was stained with BPB and TTC. The two oxidized sugars were separated mutually with chromatography by Dowex 1 × 4. Under the conditions, MNA appeared in the fractions while the column was washed with water. KMA was adsorbed to the Dowex column and eluted at the fractions from 35 to 51.

D-Mannose reduction to D-mannitol

Reduction of 1 g of D-mannose to D-mannitol was done by $NaBH_4$ under alkaline condition. Excess amounts of $NaBH_4$ of 3 to 4 equivalents to D-mannose were added and the reaction was carried out overnight in a room temperature to confirm the reduction. To consume the unreacted $NaBH_4$ in the reaction mixture, a small amount of acetone was added. The reaction mixture was adsorbed into a column of *n*-Butyl-Toyopearl (Tosoh, Tokyo), which had been treated according to the dealer's instructions. After washed the column with water, D-mannitol was eluted from the column with 50% (v/v) methanol. The fractions containing D-mannitol were collected and concentrated by a rotary evaporator at 65°C under reduced pressure. D-Mannitol concentration was preliminarily determined with a saccharometer to be 84 mg/mL. An approximate concentration of D-mannitol obtained was also determined alternatively using a membrane fraction of *G. oxydans* NBRC 3294 under essentially the same condition described by Ameyama [24].

D-Mannitol oxidation to D-fructose

D-Mannitol oxidation to D-fructose was done under essentially the same conditions as described [25]. *G. oxydans* NBRC 12528 was grown in a culture medium composed of 5% D-mannitol, 0.3% yeast extract, and 0.2% hipolypepton. Incubation was carried out for 4 days under shaking at 30°C. D-Fructose formation, D-mannitol consumption, and microbial growth measured by a Klett colorimeter were recorded periodically over the period. D-Fructose formed was quantified by two ways using D-fructose dehydrogenase (FDH) prepared from *G. japonicus* NBRC 3260 [18] and NADP-dependent D-mannitol dehydrogenase from *G. oxydans* NBRC 12528 as NADPH-dependent D-fructose reductase [19]. D-Mannitol consumption was determined with NADP-dependent D-mannitol dehydrogenase from *G. oxydans* NBRC 12528 [19].

Results and discussion

Konjac taro koji preparation process and preliminary hydrolysis of konjac taro koji

Dried konjac taro slices (Figure 1) were steamed and used for preliminary screening of fungal strain showing a potent ability to hydrolyze konjac taro polysaccharides. Fungal strains showing a better growth extending hyphae over the surface of steamed taro slices were selected (Table 1). *Asp. oryzae* Moromi, *Asp. oryzae* Hikami, *Asp. luchuensis* AKU 3302 and *Asp. sojae* AKU 3312 were chosen from the tested strains as shown in Figure 2. At this step, *Asp. luchuensis* AKU 3333 was excluded from further examination due to plenty of black conidia formation and thus smaller extent of hyphal elongation than that of the strain AKU 3302. Three strains from the genus *Mucor, Absidia lichteimi*, and *Armillaria mellea* showed no

Figure 1. Dried konjac taro slices.

Table 1. Fungal growth on konjac taro slices.

Strain	Growth extent[a]
Mucor racemosus AKU 3002	–
Mucor javanicus AKU 3009	–
Mucor fragilis AKU 3013	–
Rhizopus nigricans AKU 3105	–
Rhizopus oryzae AKU 3119	–
Monascus anka NBRC 4478	+
Monascus purpureus NBRC 4513	+
Absidia lichteimi AKU 3150	–
Armillaria mellea AKU 3670	–
Aspergillus awamori AKU 3306	+
Aspergillus oryzae Moromi	++
Aspergillus oryzae Hikami	++
Aspergilus luchuensis AKU 3302	++
Aspergillus luchuensis AKU 3333	++
Aspergillus sojae AKU 3312	++
Gibberella fujikuroi AKU 3905	+
Neurospora crassa AKU 3555	–
Penicillium chrysogenum AKU 3401	+
Penicillium notatum AKU 3406	+
Trichoderma reesei AKU 3399	+

[a]The symbols "++" and "+" mean the positive fungal growth and conidia formation after 24 h incubation. "-" indicates fungal strain requiring more than 24 h for growth initiation. Some strains marked with "-" mean no positive growth. The symbol "++" mean fungal strains showing a potent hyphal growth or a plenty of conidia formation with comparable hyphal formation.

germination. Two strains from the genus *Rhizopus* and *Neurospora crassa* showed a least germination but no further apparent growth by hyphal elongation. If a given fungus could utilize nutrients in steamed taro slices, a better growth should be observed extending filamentous hyphae. Some strains such as marked with "+" did not show hyphal elongation and soon formed conidia spores to survive themselves rather than extending vegetative hyphae. The given growth conditions were apparently unfavorable to grow for most of the tested strains. Though the growth conditions employed looked hard to grow, it may be the shortest way to choose a best strain with high probability.

Dried konjac taro slices (100 g) was steamed and divided into four portions to which fungal strain was inoculated. After 2 days of incubation at 30°C, water was added to dip the whole materials and hydrolyzed at 50°C for 12 h and 24 h. Sugar contents were measured with an aliquot of the supernatants.

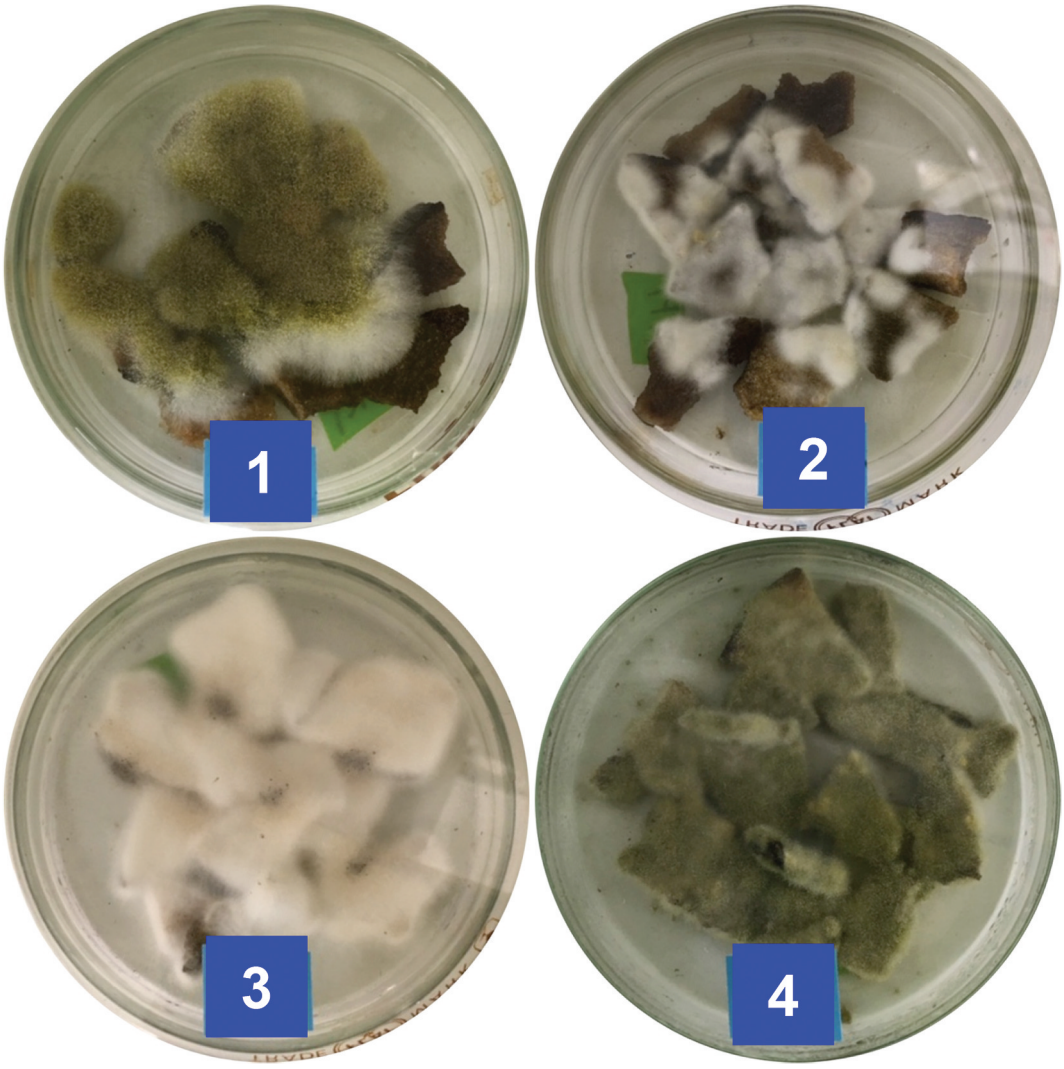

Figure 2. Fungal strains selected from screening. 1, *Asp. oryzae* Moromi; 2, *Asp. oryzae* Hikami; 3, *Asp. luchuensis* AKU 3302; 4, *Asp. sojae* AKU 3312.

Table 2. Sugar contents in hydrolyzates of konjac taro *koji* with four fungal strains.

	Incubation after	
Strain	12 h[a]	24 h[a]
Asp. oryzae Moromi	3.5%	3.5%
Asp. oryzae Hikami	3.4%	3.8%
Asp. luchuensis AKU 3302	5.5%	5.8%
Asp. sojae AKU 3312	3.8%	4.1%

[a]Dried konjac taro slices (100 g) was steamed and divided into four portions to which fungal strain was inoculated. After 2 days of incubation at 30°C, water was added to dip the whole materials and hydrolyzed at 50°C for 12 h and 24 h. Sugar contents were measured with an aliquot of the supernatants.

Preliminary data showed hydrolysis of konjac taro to some extent with the four tested strains from Table 1. As an apparent upshot, *Asp. luchuensis* AKU 3302 showed the highest hydrolytic activity among the four strains (Table 2). They were placed in a water bath of 55°C and mixed occasionally until sugar content came to a constant level after occasional checking by a saccharometer. When better hydrolytic condition was searched with konjac taro *koji* prepared with *Asp. luchuensis* AKU 3302, working temperature at 50–60°C was found optimum. Before hydrolysis, minced taro solution was solidified like the konjac food by the addition of some alkali, whereas a hydrolyzed solution did not solidify any more.

Optimal condition for konjac taro koji preparation

The taro slices (200 g dried wt.) were dipped in minimum amount of tap water and steamed in an autoclave. They were minced mechanically and mixed with 50 g wet wt. mycelia of *Asp. luchuensis* AKU 3302 which had been grown for 2 days in 500 mL of Czapek medium. They were minced again giving about 500 g of apparent wet weight at the initial stage before *koji* incubation. They were divided into four portions and incubated separately in the same culturing chamber. One portion was harvested every 24 h interval for 4 days and stored at −20°C before use. They were dipped in 300 mL tap water and hydrolyzed at 55°C. As suggested by the data in Table 3, incubation of konjac taro *koji* preparation for 3 days seemed to be enough to give the best konjac taro *koji* judging from sugar contents found in the hydrolyzed solutions. It was concluded that, under these conditions, nearly 50 g of monosaccharides was recovered from 100 g of dried konjac taro slices. An apparent amount of filter cake after filtration of hydrolyzed solution appeared to be minimal after 3 to 4 days of *koji* cultivation. Visible white hyphae extended over the minced steamed konjac taro, similar to that of "*Haze-Komi*" in rice *koji* preparation in which hyphae grew into the inner part of rice grains, was observed, as shown in Figure 3. They were covered with white hyphae over the steamed konjac taro and some hyphae extended into the inner part, though the image was not shown clearly in Figure 3.

Table 3. Sugar contents in hydrolyzates of konjac taro *koji* prepared by *Asp. luchuensis* AKU 3302.

	Sugar found after hydrolysis for	
Koji incubation (day)[a]	12 h[b]	24 h[b]
1	5.1%	6.0%
2	7.1%	8.9%
3	9.5%	11.0%
4	9.5%	10.5%

[a]Steamed dried kojac taro slices (200 g) were minced with 50 g (wet wt.) of mycelia of *Asp. luchuensis* AKU 3302. They were divided into four portions and incubated at 30°C. Incubation was terminated by transferring each portion to a deep freezer at −20°C every one day interval. [b]The total volume of each portion was adjusted to 250 mL with water and their sugar contents were measured after hydrolysis for 12 h and 24 h at 55°C.

Sugar contents in hydrolyzates prepared by the selected four fungal strains

Sugar contents in hydrolyzates prepared by the selected four fungal strains are shown in Table 4. The highest sugar content was found with the hydrolyzate prepared by *Asp. luchuensis* AKU 3302. It is possible to use it to alcoholic fermentation, because both D-glucose and D-mannose are well known to be fermented with alcoholic yeasts. Once ethanol becomes possible to produce, ethanol can be further oxidized to vinegar. HPLC-profiles also support the data above, as displayed in Figure 4. The HPLC profile obtained with *Asp. luchuensis* AKU 3302 shows scarce amounts of oligosaccharides which appear before the peak of D-mannose. It is clearly different from the data that showed a lot of oligosaccharides of mannotetraose or mannopentaose [7]. It is uncertain whether hydrolysis of konjac taro with *Asp. luchuensis* AKU 3302 is complete or not. However, considering from the small peaks before D-mannose corresponding to oligosaccharides, *Asp. luchuensis* AKU 3302 looks a promising strain leading enzymatic hydrolysis of konjac taro to monosaccharides with the highest yield. *Asp. oryzae* used extensively for rice *koji* for sake brewing was not so effective for the purposes. Thus, *Asp. oryzae* may be highly effective to hydrolyze α-amylose, in which α-(1→4) linkage of D-glucose is predominated. It may be reasonable to suggest that monosaccharide formation by *Asp. luchuensis* AKU 3302 was realized as the result of enzymatic cooperation of various enzymes, but not with the catalytic activity by a single enzyme. It is important to clarify the differences in distribution of enzymes and strength in individual enzyme activities between a common strain of *Asp. oryzae* and *Asp. luchuensis* AKU 3302, when they are grown

Figure 3. Konjac taro *koji* prepared under the optimal condition. *Koji* preparation from konjac taro was done using the traditional *koji* tray from 300 g (dried wt.) of konjac taro slices under the similar conditions as above.

Table 4. Sugar contents in hydrolyzates measured by HPLC system employed.

	D-Mannose (%)[a]	D-Glucose (%)[a]
Original steamed taro[b]	-[c]	-[c]
Asp. oryzae Moromi	1.12	1.20
Asp. oryzae Hikami	-[c]	1.20
Asp. luchuensis AKU 3302	8.62	8.23
Asp. sojae AKU 3312	2.51	3.06

[a]Sugar contents in individual samples were automatically calculated from HPLC system employed.
[b]Abiotic control.
[c]Less than 0.01% or undetectable.

on konjac taro. As suggested by selected production of cellulase and hemicaellulase from *Asp. oyzae* using starch-free wheat bran [15], variety of enzymes and enzyme activities formed may be different in innermost part from outermost part of konjac taro. From these future activities, it can be expected to understand why *Asp. luchuensis* AKU 3302 is specific for hydrolysis of konjac taro.

Expected practical applications of hydrolyzate from konjac taro koji

Since comparable amounts of monosaccharides can be obtained by the action of *Asp. luchuensis* AKU 3302 to steamed konjac taros, plausible uses of many varieties of applications of the monosaccharides are coming up. Some of them are shown in Figure 5 with possible explanations itemized below.

(1) Alcoholic fermentation with hydrolyzates of konjac taro koji

Alcoholic fermentation with hydrolyzates of konjac taro *koji* was examined with *S. ludwigii* AKU 4400. As shown in Figure 6, ethanol accumulation increased to about 7% after 6 days of incubation, when the sugar contents of 14% were initially used. D-Mannose and D-glucose are known to be well fermented to ethanol by most of alcoholic yeasts. Thus, both of D-mannose

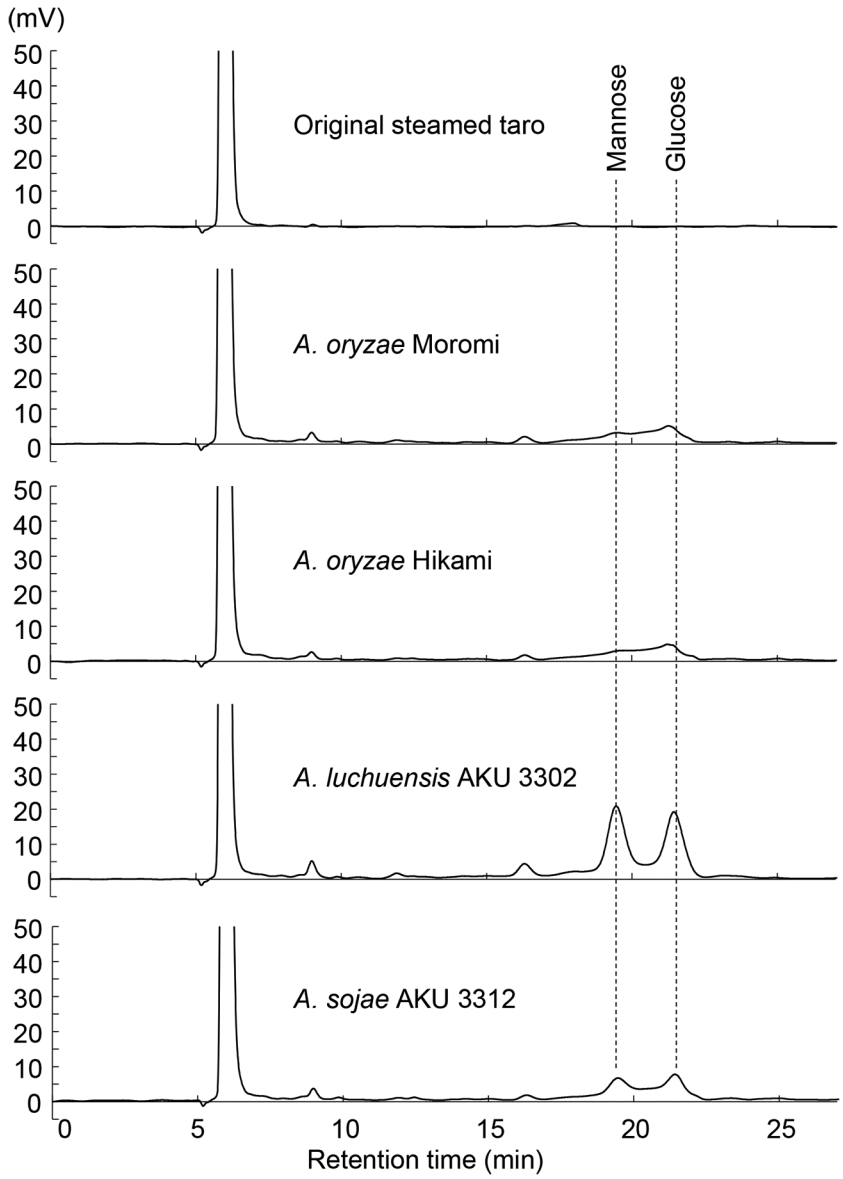

Figure 4. HPLC profiles of hydrolyzates of konjac taro *koji* with four fungal strains.

and D-glucose found in the hydrolyzate of konjac taro were almost converted equally to ethanol under the conditions. It is very much promising to use hydrolyzate of konjac taro *koji* producing alcoholic beverages. It can be further available to produce distilled liquors such as our traditional "*Shochu*" or spirits. The reason why *S. ludwigii* AKU 4400 was used in this study came from the physiological characteristics of the strain. As reported by Saeki [21], the strain is highly acid tolerant and able to ferment sugars under acidic conditions. In the presence of common organic acids such as acetic, glycolic, succinic, lactic, malic, citric, and tartaric as high as 2%, alcoholic fermentation by *S. ludwigii* was not affected. On the other hand, a common alcohol-producing yeast *Saccharomyces cerevisiae* hardly produced ethanol in the presence of 2% acetic, citric, and tartaric acids. Vinegar production from konjac taro by a mixed culture of acid-tolerant yeast and acetic acid bacteria may be promising innovation in future. Most hydrolyzates from konjac taro *koji* showed some acidic that may be caused by organic acid formation such as citric acid. Since alcoholic fermentation with *S. ludwigii* AKU 4400 was not affected under some acidic conditions, it could be possible to construct a novel system for vinegar production by combination of alcoholic fermentation and vinegar fermentation. Checking a variety of organic acid formation is one of the forthcoming research subjects.

(2) Vinegar (acetic acid) fermentation with ethanol obtained from konjac taro

Ethanol produced as above was diluted two times with seed culture of *A. pasteurianus* NBRC 3283 and *A. pasteurianus* NBRC 101655, both of which had been designed to accumulate 2% acetic acid in the seed cultures. The strain *A. pasteurianus* NBRC 3283 is one of the most traditional vinegar-producing strain

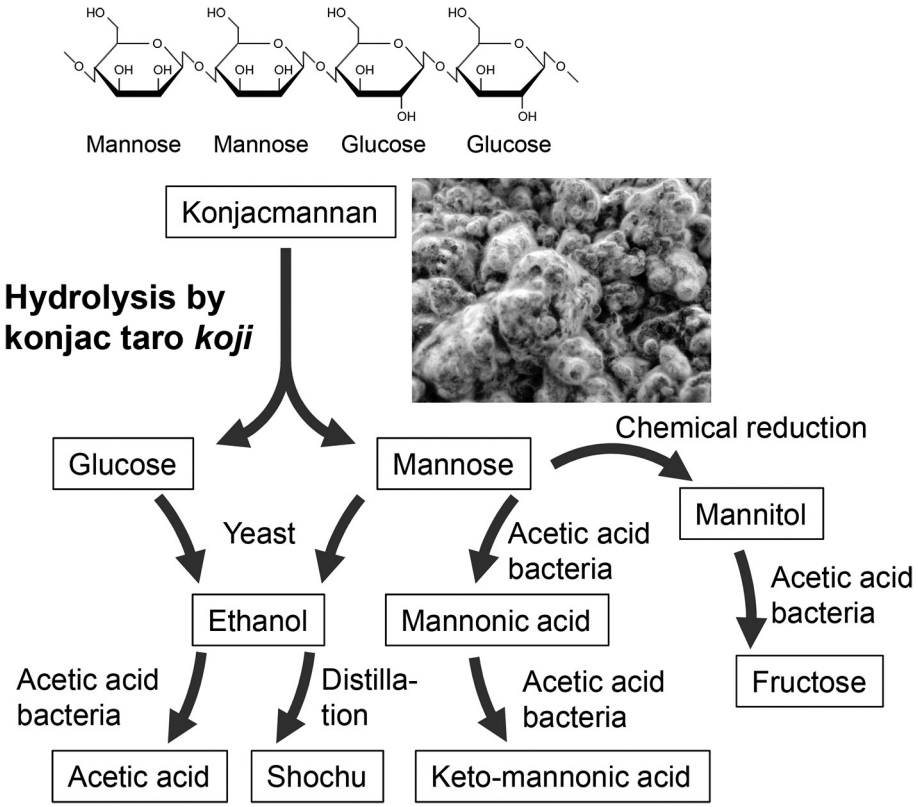

Figure 5. Plausible applications of konjac mannan after hydrolysis with konjac taro *koji*.

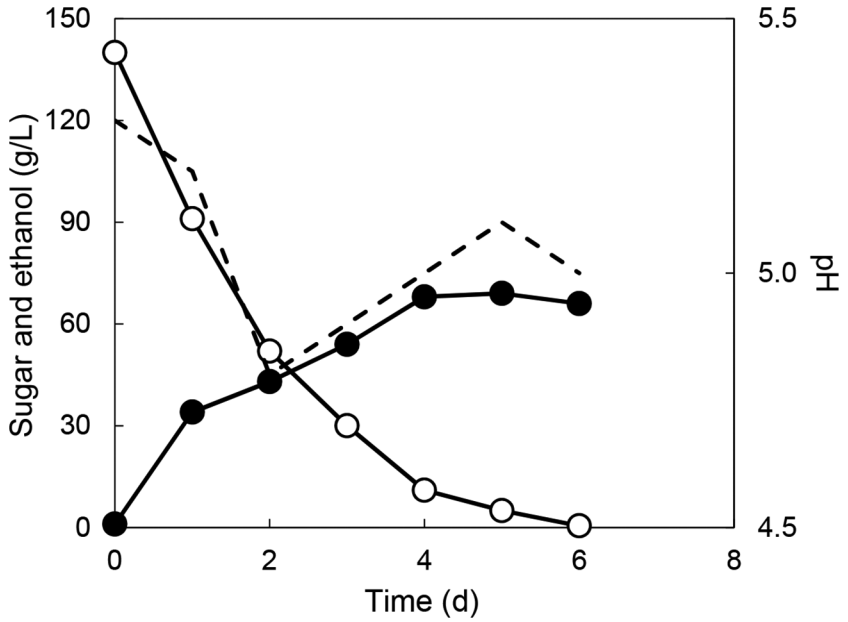

Figure 6. Alcoholic fermentation with hydrolyzate from konjac taro *koji*. Yeast cell suspension, 60 mL, was mixed with 140 mL of hydrolyzate from konjac taro *koji* containing 20% sugar in a cylindrical container. It was stood in a water bath of 30°C. Ethanol formation (black circle), sugar consumption (white circle), and pH (broken line) were periodically measured for the period as indicated.

deposited by Prof. K. Kondo in 1946 to the Institute for Fermentation, Osaka (IFO). *A. pasteurianus* NBRC 101655 was isolated in Thailand as characterized to be a typical thermotolerant strain [26]. As shown in Figure 7, acetate fermentation started after 1day lag time and came to end after 7 days of incubation. Increase in acidity was observed inversely proportional to decrease in ethanol concentration. In both cases, the final pH of the culture broths came down to 3.2. Vinegar thus produced was colored in brown coming from konjac taro. This was undertaken with the intent of making vinegar from konjac taro, which may be expected to give a somewhat different taste and color to the vinegar produced.

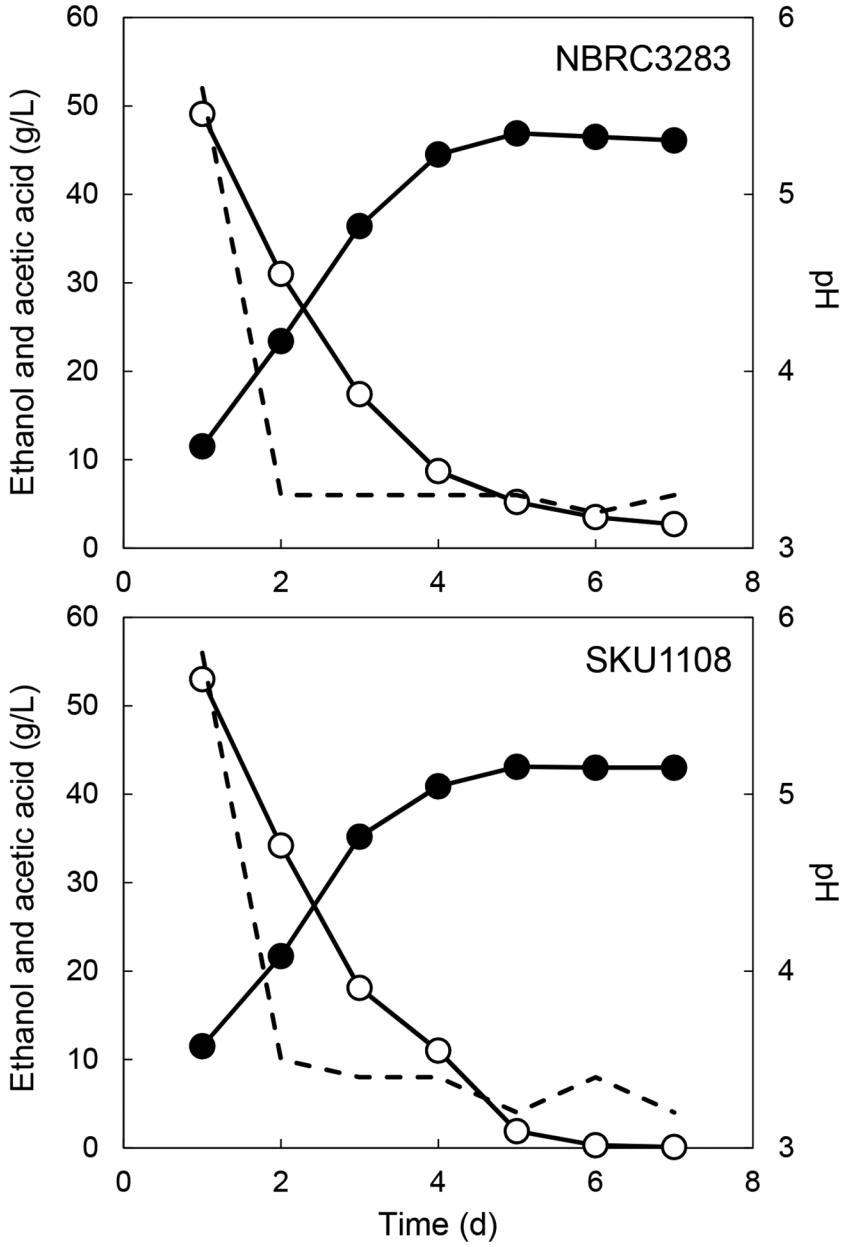

Figure 7. Vinegar production with alcohol from konjac taro. Two seed cultures, 75 mL, of acetic acid bacteria as indicated were mixed separately with 75 mL of 10% ethanol obtained after alcoholic fermentation of hydrolyzate from konjac taro *koji*. They were incubated at 30°C under shaking at 200 rpm. Remaining ethanol (white circle), acetic acid formed (black circle) and pH (broken line) were measured periodically as indicated.

(3) D-Mannose separation from hydrolyzed konjac taro koji

D-Mannose was separated from a hydrolyzate of konjac taro *koji* according to the method described by Fischer and Hirschberger [22,23]. Most of monosaccharides in a hydrolyzed konjac taro *koji* solution were converted to phenylhydrazones. It is quite advantageous to apply the fact established by Fischer and Hirschberger for D-mannose separation from a starch hydrolyzed solution. D-Mannose phenylhydrazone was separated from other osazones, because it was insoluble in cold water. D-Mannose was liberated as free form after the phenylhydrazine moiety was precipitated with benzaldehyde. About 15 g of D-mannose from hydrolyzate containing 50 g of monosaccharide was recovered in finally concentrated syrup. There were some difficulties to calculate the initial contents of D-mannose in the original konjac taro and the final recovery of D-mannose after liberation of phenylhydrazine from D-mannose osazone. Thus, in this study, D-mannose was separated exclusively from hydrolyzate of konjac taro *koji*, though there were still some technical difficulties to recover D-mannose with higher yield. More convenient and effective methods to separate D-mannose are required in future.

(4) D-Mannose oxidation to D-mannonic acid and 5-keto-D-mannonic acid

Regarding descriptions of D-mannonic acid (MNA) and 5-keto-D-mannonic acid (KMA), Terada et al. reported formation of MNA and KMA in a series of D-fructose oxidation with *G. cerinus* var. *ammoniacus* Asai IFO 3267 [27–29]. They suggested that isomerization of D-fructose to D-mannose should be the critical step. Isono et al. [30] found and identified them in the culture medium of *G. melanogenus*, an excellent producer of 2-keto-L-gulonic acid, when grown on D-sorbitol medium as well as L-sorbose medium. D-Mannose oxidation looks ubiquitous with many strains of acetic acid bacteria over the genera of *Gluconobacter* and *Gluconacetobacter*. MNA and KMA were formed from D-mannose with resting cells of *Ga. liquefaciens* NBRC 113262 [16]. Since the authentic MNA and KMA were commercially unavailable, chromatographic behaviors and color intensity of the compounds were displayed tentatively with the authentic D-gluconic acid (GA), authentic 5-keto-D-gluconic (5KGA) and authentic 2-keto-D-gluconic acid (2KGA) as a tentative standard. The supernatant obtained after 6–8 h incubation was mainly occupied by MNA when checked by a spot test. Since MNA possesses no intramolecular ketone, it was negative to staining by TTC but positive by BPB. When the reaction mixture after 24 h incubation was treated as above, the fractions eluted from 35 to 51 were strong with TTC suggesting existence of oxidized sugar acid involving intramolecular ketone (Figure 8). Production of MNA and KMA has not been extensively conducted in industrial scale and their plausible utilities also have not been developed yet. However, if D-mannose could be supplied from konjac taro, practical utilization of the compound may open a new page, similar to GA as concrete admixture. GA is also practically significant in food technology as a seasoning.

(5) D-Fructose production from D-mannitol

D-Mannose was reduced to D-mannitol by chemical reduction under laboratory scale with $NaBH_4$. D-Mannitol provided initially in the reaction mixture was completely oxidized to D-fructose with an apparent yield of nearly 100%, as shown in Figure 9. It was exemplified with growing cells of *G. oxydans* NBRC 12528. D-Fructose production from D-mannitol by the oxidative fermentation of acetic acid bacteria is

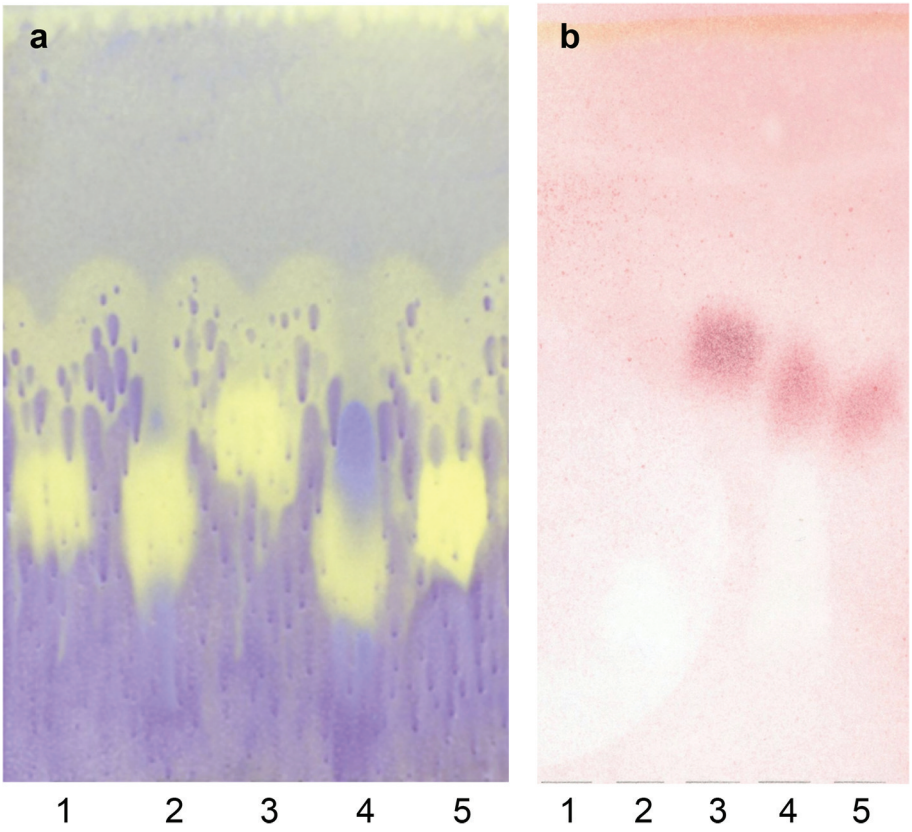

Figure 8. D-Mannose oxidation to D-mannonic acid and 5-keto-D-mannonic acid. D-Mannonic acid (MNA) and 5-keto-D-mannonic acid (KMA) obtained as the final preparations from Dowex 1 × 4 chromatography were developed together with authentic gluconic acid (GA), authentic 5KGA, and authentic 2KGA. A solvent mixture of *t*-butanol: formic acid: water = 4: 1: 1.5 was used. (a) One set was developed by a thin-layer cellulose plate (TLC cellulose of analytical, Merck, Darmstadt, Germany) and sprayed with BPB. (b) Another set was developed by a paper of analytical (51-A, Advantec Toyo) and stained by spraying alkaline TTC. Line 1; authentic GA, line 2; MNA, line 3; authentic 5KGA, line 4; KMA, line 5; authentic 2KGA.

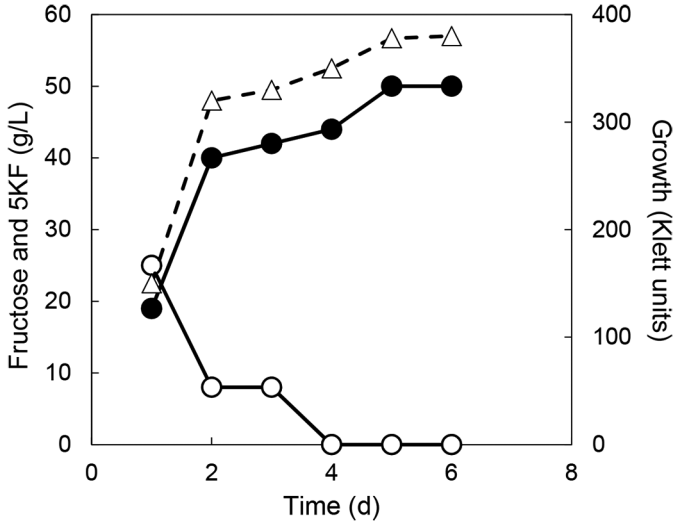

Figure 9. D-Fructose formation from D-mannitol with *G. oxydans* NBRC 12528. *G. oxydans* NBRC 12528 was inoculated in 100 mL medium containing 5% D-mannitol. Incubation was carried out at 30°C under shaking at 200 rpm. D-Mannitol remaining (white circle), D-fructose formed (black circle), and microbial growth (white triangle) were measured periodically as indicated.

highly superior to the currently performed technology of D-fructose production from D-glucose by the action of D-xylose isomerase [31]. The reaction of D-xylose isomerase comes to the reaction equilibrium when D-fructose formation comes to about 40%. Thus, there are some technical difficulties to increase D-fructose concentration beyond the reaction equilibrium by the expense of higher cost. The final product is sold as "glucose-fructose syrup" when D-glucose concentration is higher than D-fructose, or "fructose-glucose syrup" in the reverse case. In the oxidative fermentation of sugar by acetic acid bacteria, on the other hand, substrate given is oxidized almost completely to the corresponding oxidation product. D-Fructose production from D-mannitol can be performed with higher yield [32]. In case of D-mannitol oxidation, there are two possibilities of occurrence of oxidation within the molecule of D-mannitol at the position of C2 and C5. Both cases give D-fructose after the reaction. It is different from the case of D-gluconic acid oxidation giving 2KGA or 5KGA, when the position of C2 is oxidized and C5 is oxidized, respectively.

The expected utilizations of konjac taro are exemplified preliminarily as above. The data presented here potently indicate promising outcomes of practical applications of konjac taro, most of which have never been proposed so far. D-Glucose has been the central importance in carbohydrate metabolism as well as in industrial technology. D-Mannose may play a minor part in carbohydrate chemistry. In this study, several numbers of D-mannose utilizations are proposed, though the significance of D-glucose would not be influenced. Nevertheless, it should be noticed that there are many possibilities of D-mannose utilizations, if D-mannose resource like konjac taro become ubiquitous.

In the last 30 years, konjac taro agriculture in Japan has declined by less than half. Many factors leading to the present status can be indicated, as itemized below: (a) konjac taros are required for at least 3 years before harvesting. It is apparent that the labor cost invested in konjac taro farming rewards with smaller incomes than those of rice farming or other agriculture. Rice harvesting is available every year with higher market price than konjac taro. (b) Most farming fields used for konjac taro are limited to mountainous areas where rice farming is inadequate and thus a hard labor is required for less economic effect. (c) An increased rate of elderly farmers has accelerated the decline of konjac taro farming. (d) As an additional factor leading decrease in agriculture, farmers in such rural places have to protect their crops from wild animal attacks by wild boar, monkey, and deer. As one of the counter measures, some local governments have recommended farmers to plant *A. konjac* which is unfavorable feeds to wild animals. Thus, planting *A. konjac* is one idea to keep farmers getting some incomes. However, the total income from konjac taro farming may not be effective when considered the current market price of 100–150 yen/kg raw konjac taro (as of 2015 governmental statistics). It may disadvantageous when compared to other agricultural crops that yield annual income with higher market price. If a novel value-added innovation with konjac taro become possible, better incomes to konjac taro farmers could expect differently from the traditional konjac food production.

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Disclosure statement

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Author contributions

OA, NK, KM, and TY designed the study. OA performed main part of the experiments. YA was concerned with D-mannose separation from hydrolysate of konjac taro *koji* and D-mannose reduction to D-mannitol. OA wrote the manuscript in consultation with RAH, NK, KM, YA, and TY. HA, RT, and JT joined to this study with their expertized techniques in traditional sake brewing. RT was responsible for HPLC analysis. RAH contributed with his technical advice as an expert of *Aspergillus kawachi*.

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