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NOTE

5-Keto-D-fructose production from sugar alcohol by isolated wild strain *Gluconobacter frateurii* CHM 43

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ABSTRACT

Gluconobacter frateurii CHM 43 have D-mannitol dehydrogenase (quinoprotein glycerol dehydrogenase) and flavoprotein D-fructose dehydrogenase in the membranes. When the two enzymes are functional, D-mannitol is converted to 5-keto-D-fructose with 65% yield when cultivated on D-mannitol. 5-Keto-D-fructose production with almost 100% yield was realized with the resting cells. The method proposed here should give a smart strategy for 5-keto-D-fructose production.

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Regarding to 5-keto-D-fructose (5KF) production by acetic acid bacteria, the first report was done in 1960 by Terada *et al.* [1–4]. They identified the unknown substance from D-fructose oxidation to be 5KF. Formation and utilization of 5KF were reported with *Gluconobacter cerinus* IFO 3267 [5]. Thereafter, a membrane-bound D-fructose dehydrogenase (FDH, EC 1.1.99.11) catalyzing 5KF formation from D-fructose was purified and characterized as a flavoprotein dehydrogenase-cytochrome complex from the membrane fraction of *Gluconobacter industrius* IFO 3260 (renamed as *Gluconobacter japonicus* NBRC 3260) [6]. Later, the genes encoding FDH of NBRC 3260 was identified as *fdhSCL* and its high expression strain was also constructed [7]. Since the first description of 5KF production by acetic acid bacteria, many efforts have been done to create a strain for better 5KF production [8–11]. 5KF has been evaluated as a natural sweetener with low calorie [1,12,13] that can be substituted with D-glucose preventing metabolic diseases caused by high blood glucose level. Deppenmiere's group reported 5KF production from D-fructose with genetically modified *Gluconobacter oxydans* strains where they used plasmid-based *fdh* of *G. japonicus* NBRC 3260 [8] or *G. japonicus* LMG 1281 [9]. Later, Battling *et al.* [11] reported 5KF production from D-fructose with a novel plasmid-free strain of *G. oxydans*. The two groups of German scientists elaborated bacterial strains for 5KF production but the principle has remained within the same scope of Terada *et al.* [1–4] who used D-fructose as the starting material for 5KF formation. Deppenmiere's group also reported 5KF production from sucrose, instead of D-fructose, with *G. oxydans* strain having chromosome-integrated *fdh* of *G. japonicas* NBRC 3260 and invertase gene of *G. japonicus* LMG 1417 [10]. In this case, sucrose was efficiently converted to D-fructose and D-glucose, then the D-fructose to 5KF efficiently, but the D-glucose remained in the culture medium.

We tried to look for alternative convenient substrates for 5KF production as well as a better 5KF producing strain among isolated wild strains. D-Mannitol was chosen as the most promising substrates. The reason came from the metabolic characteristics of acetic acid bacteria. In general cases of the oxidative fermentation catalyzed by the cytoplasmic system of acetic acid bacteria, D-sorbitol is oxidized predominantly to L-sorbose and D-mannitol to D-fructose. Crude membrane fraction of *Gluconobacter frateurii* CHM 43 showed a strong FDH activity (0.8–1.0 unit/mg protein) comparable to that of *G. japonicus* NBRC 3260 (0.7–1.0 unit/mg) [6]. When *G. frateurii* CHM 43 was grown in D-mannitol medium, D-mannitol initially added was consumed, then D-fructose accumulation started within 3 days. Thereafter, 5KF accumulation came to the maximum level while D-fructose disappeared rapidly (Figure 1(a)). The pH of the culture medium gradually decreased to 4.0. The bacterial growth showed a biphasic (Figure 1(a)). The first phase was brought by D-mannitol oxidation and the second by D-fructose oxidation. In Figure 1(b), an aliquot of the culture supernatant along the cultivation was spotted on a TLC sheet. After it was developed and dried well, the TLC sheet was sprayed by an alkaline-triphenyl tetrazolium chloride (TTC) solution. D-Fructose and 5KF reacted with TTC but not D-mannitol. Since one ketone moiety is found in the

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This paper is dedicated to 50th Anniversary of the Laboratory of Applied Microbiology, Yamaguchi University, founded in 1969 by the late Professor Emeritus Minoru Ameyama for studying the oxidative fermentation and biotechnological applications of acetic acid bacteria.

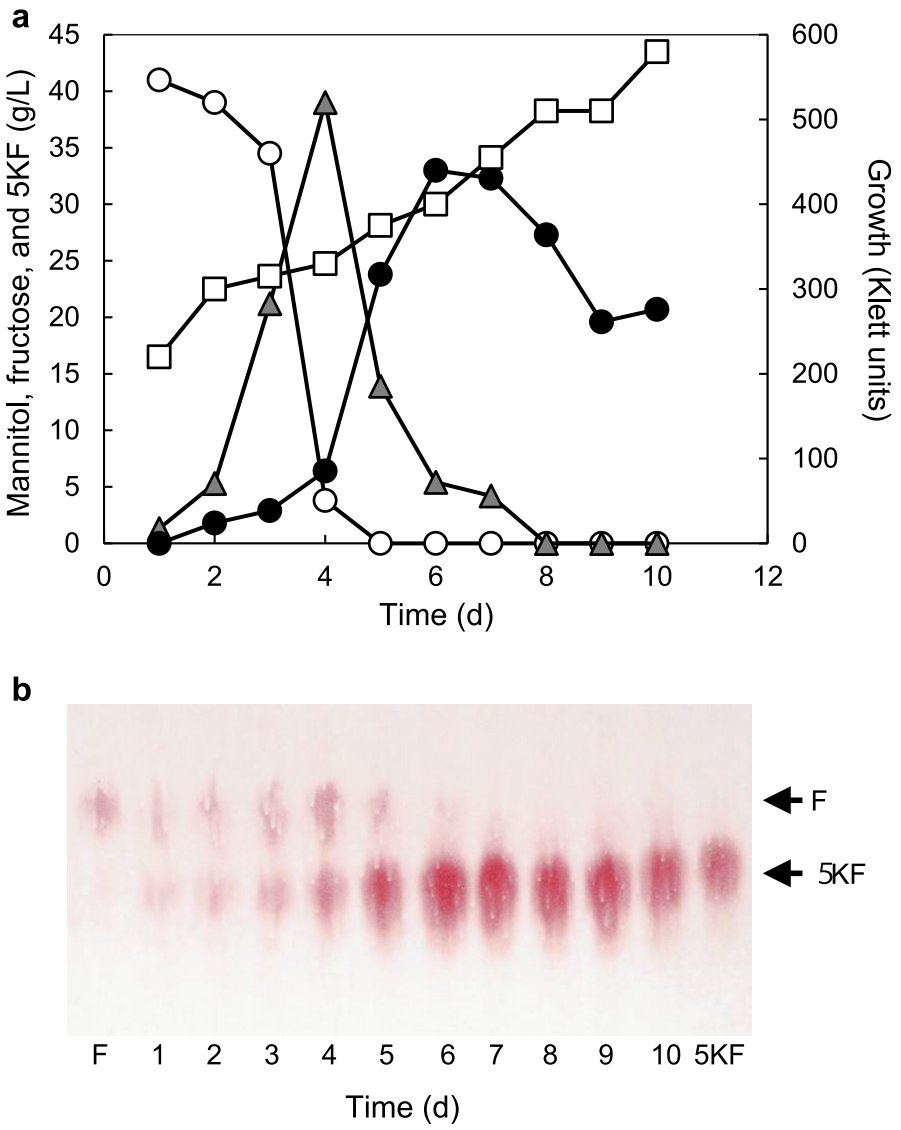

Figure 1. Production of 5-keto-D-fructose during cultivation of *G. frateurii* CHM 43. (a) The organism was cultured on a medium containing 5% D-mannitol, 0.3% yeast extract (Oriental Yeast Co., Ltd, Tokyo), and 0.3% highpolypepton. The medium (150 mL) was put in an Erlenmeyer flask of 500 mL volume with a sided arm. The cultivation was done at 30°C under shaking at 200 rpm. The bacterial growth (□) was recorded by a Klett Summerson colorimeter through the side arm without taking the cotton stopper off. Incubation was carried out for the period as indicated. D-Mannitol (○) was measured by reading increase of optical density at 340 nm caused by NADPH when assayed with NADP-MLDH. D-Fructose (▲) was measured by two ways [1]: with FDH using potassium ferricyanide as electron acceptor and [2], reduction of D-fructose to D-mannitol by reading decrease of optical density of NADPH at 340 nm. 5KF (●) was measured by reading decrease of optical density of NADPH at 340 nm. The amounts of D-mannitol (○), D-fructose (▲), and 5KF (●) in the culture medium were measured as described in the text. (b) An aliquot of the culture medium was spotted on a thin-layer cellulose plate (TLC cellulose of analytical, Merck KGaA, Darmstadt, Germany) and developed with a solvent of *t*-butanol: formic acid: water = 4: 1: 1.5. TLC plate was sprayed by a mixture of triphenyltetrazolum chloride (TTC) and KOH. Sugar acids having intramolecular ketone are stained as a deep pink spot with TTC. F and 5KF mean the standard D-fructose and 5-keto-D-fructose, respectively.

molecule of D-fructose and two in 5KF, TTC reacted stronger with 5KF than D-fructose. When D-fructose formation during cultivation was assayed enzymatically, D-fructose accumulated as high as 82% to D-mannitol initially added, though D-fructose looked small from TLC chromatography. 5KF accumulated in the culture medium was finally measured to be 65% to the amount of D-mannitol initially added as shown in Figure 1.

The feature of D-mannitol utilization by *G. frateurii* CHM 43 was different from that of *G. suboxydans* IFO 12528 (renamed as *G. oxydans* NBRC 12528) which showed almost 100% D-fructose accumulation due to the absence of FDH, as shown previously [14]. D-Mannitol was measured by NADP-dependent D-mannitol dehydrogenase (NADP-MLDH) from *G. oxydans* NBRC 12528 [15]. D-Fructose was measured by two ways with the membrane-bound FDH from *G. japonicus* NBRC 3260 [6] and NAD-dependent D-mannitol dehydrogenase from *G. oxydans* NBRC 12528 [15]. 5KF was measured by NADPH-dependent 5KF reductase (KFR) which can be prepared from one of the following strains: *G. japonicus* NBRC 3260, *G. frateurii* CHM 43, or *G. oxydans* NBRC 3257 [16].

5KF production from D-mannitol was examined by resting cells of *G. frateurii* CHM 43 (Table 1), where 5KF was rapidly produced *via* D-fructose. No

Table 1. 5-Keto-D-fructose (5KF) production from D-mannitol with resting cells of *G. frateurii* CHM 43.

Incubation (h)	D-Mannitol[a] (g/L)	D-Fructose[a] (g/L)	5KF[a] (g/L)
0	50.0	0	0
3	27.4	38.8	11.2
6	tr.[b]	10.5	26.7
12	tr.	tr.	50.0

[a]The resting cells were prepared with the cells of *G. frateurii* CHM 43 after cultivation for 36 h on D-mannitol with the same composition as in Figure 1. The cells were collected and washed two times with 5 mM acetate buffer, pH 5.0. The cell suspension of 240 mg wet wt./mL was used. The reaction mixture (1 mL) containing 0.4 mL of cells (96 mg wet cells), 50 mg of D-mannitol, and 0.5 mL of 0.2 M acetate buffer, pH 5.0, was incubated. The reaction was shaken at 150 rpm at 25°C and the reaction was terminated periodically as indicated by the addition of trichloroacetic acid to 2%. After the reaction mixture was spun down by a table top centrifuge, an aliquot of the supernatant was used. D-Mannitol, D-fructose, and 5KF were measured under the same methods as indicated in Figure 1.

[b]tr., trace amount less than 0.01 g/L.

D-mannitol and D-fructose was detected by the enzymatic method described above at the end of the reaction, when the incubation was carried out in acetate buffer, pH 5.0, at 25°C for 12 h. Thus, the resting cells of *G. frateurii* CHM 43 were shown to be a potent catalyst for 5KF production from D-mannitol with almost 100% yield within a short period. D-Fructose behaved as a metabolic intermediate with a short lifetime. The reaction conditions employed seemed to be not far from the optimum condition, though it was not examined exactly.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contribution

OA, TMN, NK, KM, YA, and TY designed the study. OA and TMN performed the experiments. OA wrote the manuscript in consultation with RAH, NK, KM, and TY.

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