Selective, High Conversion of d-Glucose to 5-Keto-d-gluconate by *Gluconobacter suboxydans*

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Selective, high-yield production of 5-keto-d-gluconate (5KGA) from d-glucose by *Gluconobacter* was achieved without genetic modification. 5KGA production by *Gluconobacter* suffers byproduct formation of 2-keto-d-gluconate (2KGA). By controlling the medium pH strictly in a range of pH 3.5–4.0, 5KGA was accumulated with 87% conversion yield from d-glucose. The pH dependency of 5KGA formation appeared to be related to that of gluconate oxidizing activity.

**Key words:** acetic acid bacteria; *Gluconobacter*; oxidative fermentation; 5-keto-d-gluconate; quinoprotein glycerol dehydrogenase

5-Keto-d-gluconate (5KGA) is an attractive compound with a number of potential applications in various industries but industrial production of 5KGA has not been established because all known methods for 5KGA preparation are based on fermentation by *Gluconobacter*, the production mechanism of which has been unclear. Recently, we have shown that 5KGA formation is done by a membrane-bound quinoprotein, glycerol dehydrogenase (GLDH). 2, 3

*Gluconobacter* oxidizes d-glucose to d-gluconate with a membrane-bound quinoprotein, glucose dehydrogenase (GDH), and then is converted to two keto-d-gluconates, 5KGA and 2-keto-d-gluconate (2KGA), by reaction of GLDH and FAD-dependent gluconate dehydrogenase (GADH) respectively. These reactions are done through electron transport from the dehydrogenases to terminal ubiquinol oxidases in the respiratory chain. To achieve selective production of 5KGA, optimum conditions for 5KGA production by *Gluconobacter* species, such as strains, carbon sources and salt condition, were examined previously, in which *G. suboxydans* IFO 12528 was selected as an excellent 5KGA producer, and 1% d-glucose and 1% sodium d-gluconate were selected as the best carbon sources for production.

In this study, to improve cost performance for industrial production of 5KGA, we examined the best conditions of 5KGA production in a medium containing d-glucose as sole initial substrate. It was found that the pH of the culture medium is the most important factor for selective production. 5KGA was produced by controlling the medium pH in a range of 3.5–4.0. This provided 87% conversion from glucose without any genetic modification.

*G. suboxydans* IFO 12528 was grown at 30°C on 3 L of G-GA medium, including 1% d-glucose and 1% sodium d-gluconate as carbon source (initial substrate concentration, 101 mM) (see the legend to Fig. 1), which is the best medium composition for 5KGA production defined previously, and on glucose medium, containing 2% d-glucose as sole carbon source (111 mM) in a 5-L jar fermentor. As shown in Fig. 1, in G-GA medium, the bacterium grew to a Klett unit of 170 concomitantly with decreases in the medium pH to about 3.8, and accumulated 5KGA at up to 86 mM (85% conversion), whereas in glucose medium, cell growth was a little reduced, to a Klett unit of 135, and the conversion rate to 5KGA was also reduced to 59% (final 65 mM 5KGA). Under these conditions, although 5KGA was selectively produced, d-gluconate accumulated in the medium. This impairment was probably due to the remarkable pH drop during growth to around pH 3.0. Therefore, to improve the medium pH, the culture in the glucose medium was controlled at pH 5.0, a condition favorable to 5KGA formation (panel C, solid squares), but 2KGA formation is also very much stimulated (panel B, open squares). Thus, it can be tentatively concluded that (i) adjustment of the medium pH to nearly 3.0 also lead to reduced production because of d-gluconate accumulation (panel...
C, solid diamonds), and thus (iii) high-yield production of 5KGA can be achieved from D-glucose by controlling the medium pH between 3.0 and 5.0, as expected from the pH behavior in G-GA medium (panel A, solid diamonds).

To examine the effects of pH control on the conversion of D-glucose to 5KGA via gluconate, the medium pH in glucose culture was controlled to a range of pH 3.0–5.0 by alkaline titration without adjusting the initial pH. *G. suboxydans* IFO 12528 was grown on 3 L of glucose medium (initially at pH 6.5) in a 5-L jar fermentor. The medium pH of the glucose medium decreased to about pH 3.0, as shown in Fig. 1, due to the oxidation of D-glucose to D-gluconic acid. We tried to lower than pH 5.0, pH 4.5, pH 4.0, and pH 3.5 by alkaline titration, and the actual minimum values were at pH 4.6, 4.3, 3.8, and 3.1 respectively (Fig. 2, panel A). In these cultures, the concentration of 5KGA reached 96 mM and 89 mM at 170 h-cultivation respectively when the pH was adjusted to 3.5 and 4.0. On the other hand, when the pH was adjusted to 4.5 and to 5.0, 5KGA accumulation reached a maximum (about 70 mM) at 80 h-cultivation, and then decreased with further cultivation. Concomitantly with the decreased 5KGA concentration in the medium, the pH of the culture medium increased again (due to an absence of adjustment with acid), and cell growth also increased. These results suggest that accumulated 5KGA is taken up into the cells and is utilized by the assimilative pathway. This phenomenon is different from the case in which the pH is controlled at pH 5.0, as shown in Fig. 1, where 5KGA was formed and remained in the culture medium, while in the case of Fig. 2, 5KGA assimilation occurs when a...
rise in the medium pH is not controlled. Although the reason is not clear at this moment, it can be surmised that the medium pH regulates expression of the 5KGA transporter. As for 2KGA production, although it was low under all conditions, 2KGA accumulation was much lower at pH 3.5 and 4.0 (3.2 and 7.6 mM respectively) than at pH 4.5 and 5.0 (12.0 and 15.0 mM respectively). The results clearly indicate the importance of pH control in 5KGA production from glucose.

We have examined the effects of pH on 5KGA and 2KGA oxidation in resting cells, and found that the optimum pH for 5KGA production was in a very acidic range, pH 2.5–3.0, while the production of 2KGA is in a more broad pH range around pH 5.0.7 These pH profiles for 5KGA production by the resting cells confirm the pH dependency of growing cells observed in this study.

To understand the pH dependency of d-gluconate oxidation leading to 5KGA and 2KGA formation multilaterally, d-gluconate oxidizing activity was confirmed with GLDH- or GADH-deleted mutants, which were constructed as described previously.3,5 Although such genetic mutations can affect the expression of many other genes, this is thought not to be serious for the pH profile of ketogluconate production, because d-glucuronate oxidizing activity reflects only two components, membrane-bound dehydrogenases (GLDH and GADH) and terminal ubiquinol oxidase, located in the cytoplasmic membrane.

To prepare the cell suspension for the measurement, *G. suboxydans* wild type and the mutant strains were first cultured in G-GA medium, but the cell yield was very low under these growth conditions, and d-gluconate oxidizing activities were not compared because the GLDH mutant had undetectable activity. Therefore, these experiments were performed with cells grown on SG-G medium, containing 0.5% d-glucose, 2% sodium d-gluconate, and 0.5% glycerol as carbon source, which has been known to lead to relatively high 2KGA production as compared with G-GA medium.7 The wild-type cells accumulated 13.5 mM 5KGA and 9.75 mM 2KGA in the medium at the same time (data not shown), whereas a GADH disruptant (ΔgndG) accumulated 5KGA (43.9 mM) but not any 2KGA, as expected, and the GLDH disruptant (ΔsldBA) exhibited relatively high 2KGA production (21.3 mM) but no 5KGA production (data not shown). These 2KGA and 5KGA productivities were higher in each enzyme-disrupted mutants than those of the wild-type strain.

Using mutants defective in GLDH or in GADH, the pH profiles of d-gluconate oxidizing activity were compared (Fig. 3). The wild-type cells showed activity in a broad pH range around pH 3.0 to 6.0, whereas ΔgndG having only GLDH and ΔsldBA having GADH only exhibited higher d-gluconate oxidase activity at around pH 3.0 to 4.5 and around pH 4.0 to 6.0 respectively. These pH optima were consistent with the results for pH effect on selective 5KGA production in growing cells as described above (Fig. 2), where 5KGA production occurred at about pH 3 to 4, but decreased at above pH 4.5.

The present study indicates that selective, high conversion of 5KGA from d-glucose can be achieved by control of medium pH (Fig. 2). The data suggest that the effect of pH on the productivity of keto-d-glucuronates is correlated with the pH dependency of keto-d-glucuronate formation with two different enzymes, 2KGA-yielding GADH and 5KGA-forming GLDH (Fig. 3).

5KGA is a useful intermediate that can be utilized in various fields such as the food industry. For industrial production of 5KGA, many attempts at improvement of 5KGA production have been done using genetically modified strains.8–11 In our previous study, 92% conversion from d-glucose to 5KGA was achieved in G-GA medium,7 and in this study 5KGA was produced at an 87% conversion rate from d-glucose as sole substrate using a wild-type *Gluconobacter* strain. Thus *Gluconobacter* without gene modification can be useful for high 5KGA conversion from d-glucose by a culture with controlled medium pH.

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### References


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**Fig. 3.** pH Profiles of d-Glucuronate Oxidizing Activity in Wild and Mutant Strains of *G. suboxydans* IFO 12528.

*Gluconobacter* strains were cultured in 100 mL of SG-G medium in a 500-mL Erlenmeyer flask at 30 °C until the late exponential phase. After harvesting of the cells, a cell suspension was prepared with 10 mM KPB (pH 6.0) after once washing with the same buffer. The d-glucuronate oxidizing activity of the wild-type cells (●), ΔgndG cells (▲), and ΔsldBA cells (▲) was measured polarographically with an oxygraph equipped with a Clark-type oxygen electrode at 25 °C. The reaction mixture (1.5 mL) contained McIlvaine buffer (pH 2.5–8.0), 100 mM sodium d-gluconate, and the cell suspension. The oxygen uptake rate was defined in terms of an enzyme unit that oxidizes 1 μmol of substrate per min, in which 1 μatom O is calculated by assuming that the buffer contains 498 natom O/mL.