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Substrate Selectivity of *Gluconobacter oxydans* for Production of 2,5-Diketo-D-Gluconic Acid and Synthesis of 2-Keto-L-Gulonic Acid in a Multienzyme System

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Abstract

Substrate selectivity of *Gluconobacter oxydans* (ATCC 9937) for 2,5-diketo-D-gluconic acid (2,5-DKG) production was investigated with glucose, gluconic acid, and gluconolactone in different concentrations using a resting-cell system. The results show that gluconic acid was utilized favorably by *G. oxydans* as substrate to produce 2,5-DKG. The strain was coupled with glucose dehydrogenase (GDH) and 2,5-DKG reductase for synthesis of 2-keto-L-gulonic acid (2-KLG), a direct precursor of L-ascorbic acid, from glucose. NADP and NADPH were regenerated between GDH and 2,5-DKG reductase. The mole yield of 2-KLG of this multienzyme system was 16.8%. There are three advantages for using the resting cells of *G. oxydans* to connect GDH with 2,5-DKG reductase for production of 2-KLG: gluconate produced by GDH may immediately be transformed into 2,5-DKG so that a series of problems generally caused by the accumulation of gluconate would be avoided; 2,5-DKG is supplied directly and continuously for 2,5-DKG reductase, so it is unnecessary to take special measures to deal with this unstable substrate as it was in Sonoyama's tandem fermentation process; and NADP(H) was regenerated within the system without any other components or systems.

Index Entries: 2-Keto-L-gulonic acid; *Gluconobacter oxydans*; glucose dehydrogenase; 2,5-diketo-D-gluconic acid reductase; 2,5-diketo-D-gluconic acid; L-ascorbic acid; glucose; gluconic acid; NADP(H) regeneration.

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Introduction

Gluconobacter oxydans can convert both glucose and gluconic acid into 2,5-diketo-D-gluconic acid (2,5-DKG) *(1)*. However, there is no report about which one is used to predominantly produce 2,5-DKG. In this study, we investigated substrate selectivity of *G. oxydans* for 2,5-DKG production using glucose, gluconic acid, and gluconolactone as substrates in different concentrations. A multienzyme system of 2-keto-L-gulonic acid (2-KLG) synthesis was set up by coupling the resting cells of *G. oxydans* with glucose dehydrogenase (GDH) and 2,5-DKG reductase.

2-KLG is a direct precursor of L-ascorbic acid. Currently, L-ascorbic acid is produced mainly by the Reichstein-Grussner process *(2)* or by a modified Reichstein process, e.g., a two-stage fermentation process *(3)*. There are six bacterial fermentation processes for L-ascorbic acid production *(2)*, all having 2-KLG as a direct precursor of L-ascorbic acid. Among these pathways, however, only the 2,5-DKG and 2-KLG pathways have the potential to be developed into new processes to compete with the Reichstein procedure. Nevertheless, neither of these two new processes has been used in industry *(4)*.

Biosynthetically, the 2,5-DKG and the 2-KLG pathways are composed of the same enzymatic system for the biotransformation from glucose to 2-KLG *(5)*. This enzymatic system includes GDH (EC 1.1.99.17), gluconate dehydrogenase, 2-keto-D-gluconate dehydrogenase (EC 1.1.99.4), and 2,5-DKG reductase.

The first three enzymes are membrane-bound enzymes, which are electron transport–linked dehydrogenases that convert D-glucose to 2,5-DKG in the periplasmic space *(5)*. The last enzyme, 2,5-DKG reductase, which is a single polypeptide with NADPH as a cofactor, completely and stereoselectively reduces 2,5-DKG into 2-KLG.

According to the enzymatic mechanism and considering the necessity of NADPH regeneration, an enzymatic procedure was designed to realize the conversion from glucose to 2-KLG (Fig. 1). In this system, NADP-dependent GDH (EC 1.1.1.47) was used instead of the quino-protein GDH (EC 1.1.99.17) in the periplasmic space for NADPH regeneration and gluconate production. NADP(H) was regenerated between NADP-dependent GDH and 2,5-DKG reductase.

Resting cells of *G. oxydans* were used to combine the NADP-dependent GDH and 2,5-DKG reductase by converting the gluconic acid, the product of NADP-dependent GDH, into 2,5-DKG, the substrate of 2,5-DKG reductase. Glucose, gluconic acid, and gluconolactone are in this multienzyme system. The critical question is, Which substrate is used predominantly for production of 2,5-DKG by *G. oxydans*? If glucose were used prior to gluconate, the multienzyme system of 2-KLG synthesis would not function, because the subsystem of NADP/NADPH regeneration would not be achieved in this case and there would be no NADPH supply for 2,5-DKG reductase. Presumably, if gluconate were used prior to glucose, the reaction system would work. It is also necessary to investigate the effect of

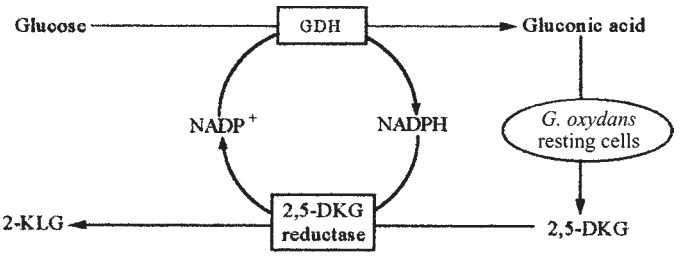

Fig. 1. Schematic view of the multienzyme system of 2-KLG synthesis with NADP(H) regeneration.

gluconolactone in the system, although it could be easily hydrolyzed into gluconic acid.

Materials and Methods

Enzyme and Microorganisms

G. oxydans (ATCC 9937) and *Corynebacterium* sp. (ATCC 31090) were obtained from the American Type Culture Collection. Glucose dehydrogenase (EC 1.1.1.47) was purchased from Sigma. The 2,5-DKG reductase was isolated and purified from the cells of *Corynebacterium* sp. ATCC 31090 according to Miller et al. *(6)*.

Chemicals

L-Ascorbic acid and calcium 2-KLG were provided by Jinan Pharmaceutical (Jinan, China). Calcium 2,5-DKG was produced using *G. oxydans* as described by Qazi et al. *(7)* and isolated from fermentation broth as described by Wakisaka *(1)*. All other chemicals used were reagent grade. All components of the media used were commercial preparations, unless otherwise noted.

Analysis of Glucose, 2,5-DKG, and 2-KLG

Glucose was analyzed by glucose oxidase; 2,5-DKG was determined by the following NH_4OH-HCl method devised by Sonoyama et al. *(8)* and modified by Yin et al. *(9)*. To a 1.0-mL broth sample, 1.0 mL of 12.0 m*M* $CaCl_2$, 0.55 mL of 1.5 *M* NH_4OH, and 0.6 mL of 4 *M* HCl were added successively and mixed in an 18-mL test tube. The mixture was placed in a heating bath at 50°C for 12 min. Then, the absorbance at 460 nm was measured. For the assay of 2-KLG, a direct conversion of 2-KLG into L-ascorbic acid was completed in a reaction mixture containing 5 mL of 2-KLG solution and 5 mL of 7 *M* sulfuric acid. After incubation for 30 min at 100°C, the reaction was stopped by adding 100 mL of cold distilled water *(10)*. The amount of L-ascorbic acid formed was determined by iodine titration *(11)*.

Preparation of Resting Cells

The strain of *G. oxydans* was grown at 28°C for 24 h on an agar slant containing 0.5% yeast extract, 0.3% peptone, 2.5% mannitol, and 2.0% agar powder. The preseed medium (200 mL in ten 100-mL flasks) and the seed medium (1800 mL in ten 1-L flasks) consisted of 2.4% mannitol, 0.1% glucose, 0.3% peptone, 0.5% yeast extract, and tap water. These media were adjusted to pH 6.8 with 0.1 *M* NaOH or 0.1 *M* HCl before sterilization at 115°C for 20 min. The preseed inoculum was prepared by inoculating a fresh agar slant of *G. oxydans* in the sterile medium and incubating at 28°C for 15 h on a rotary shaker at 280 rpm. The entire contents of the preseed flasks were added to previously sterilized seed medium and incubated at 28°C for 24 h on a rotary shaker at 280 rpm.

G. oxydans cells were harvested by centrifuging at 6000*g* for 20 min. The pellet was washed three times with physiologic saline and recovered by centrifugation. The cell paste was stored at 4°C for more than 24 h before use.

2,5-DKG Production by Resting Cells of G. oxydans

A series of solutions including glucose, gluconate, gluconolactone, glucose and gluconate (1:1 [w/w]), glucose and gluconolactone (1:1 [w/w]), and gluconate and gluconolactone (1:1 [w/w]) were prepared with 0.1 *M* Tris-HCl buffer (pH 7.0) at concentrations of 2.5, 5.0, and 10.0% containing 0.1% $CaCO_3$, respectively, and sterilized at 121°C for 20 min.

Biotransformation experiments were performed in 200-mL Erlenmeyer flasks. Each flask contained 2 g of *G. oxydans* resting cells (wet weight) and 40 mL of substrate solution. The mixture was incubated at 28°C on a rotary shaker at 150 rpm. The broth samples were collected at 8-h intervals for assay of 2,5-DKG production and glucose consumption.

Assay of GDH (12)

GDH (30 mg of crude powder) (Sigma) was dissolved in a 10-mL 20 m*M* Tris-HCl buffer (pH 7.0). Routine assay mixtures contained 2.6 mL of 0.1 *M* Tris-HCl buffer (pH 7.0), 0.1 mL of 1.5 m*M* NADP, 0.2 mL of 1 *M* glucose, and enzyme solution in a final volume of 3 mL. The reaction was monitored for the initial linear increase in absorbance at 340 nm. One unit of GDH activity corresponds to the production of 1 µmol of NADPH/min at 25°C.

Assay of 2,5-DKG Reductase (6)

All spectrophotometric measurements were carried out in cuvets of 1-cm optical path using either a VIS-NIR-3100 (Shimadzu graphicord) or UV-240 (Shimadzu) at 25°C, unless noted otherwise. Routine assay mixtures contained 2.6 mL of 0.1 *M* Tris-HCl buffer (pH 7.0). 0.2 mL of calcium 2,5-DKG (9 µmol), 0.2 mL of 1 m*M* NADPH, and enzyme solution

in a final volume of 3 mL. The reaction was monitored for the initial linear decrease in absorbance at 340 nm. Activity was proportional to the amount of 2,5-DKG reductase added. One unit of activity corresponds to the production of 1 µmol of NADP/min at 25°C. Protein was determined by the method of Folin-phenol *(13)* with bovine serum albumin as the standard.

Purification of 2,5-DKG Reductase

Corynebacterium sp. (ATCC 31090) was grown in a 10-L stainless-steal fermentor (B. Braon, Germany) as described by Sonoyama and Kobayashi *(14)*. The cells were harvested by centrifugation using a Sorvall RC28S refrigerated centrifuge at 6000*g* for 20 min. Cell paste was stored at –20°C.

The 2,5-DKG reductase was isolated and purified from the cells according to Miller et al. *(6)*. The specific activity of the enzyme was 0.0195 U/mg of protein.

Multienzyme Reaction System

Coupling of 2,5-DKG Reductase with GDH

A 3.0-mL quartz cuvet was used to mix 0.2 mL of GDH (0.00542 U), 0.2 mL of 2,5-DKG (3.2 mg/mL), 0.2 mL of 1 *M* glucose (10 mg/mL), 0.04 mL of 20 m*M* NADP, and 2.2. mL of 0.1 *M* Tris-HCl buffer (pH 7.0). The mixture was incubated at 25°C and monitored for the increase in absorbance at 340 nm. When the absorbance reached 0.3 (about half the amount of NADP reduced to NADPH), 0.2 mL of 2,5-DKG reductase (0.00542 U) was added. The reaction was kept for 24 h at 28°C, and then the amount of 2-KLG produced in the cuvet was determined by the method as described above.

Coupling of 2,5-DKG Reductase and GDH with Resting Cells of *G. oxydans*

To produce 2,5-DKG, 4 g (wet wt) of *G. oxydans* cells was added to 40 mL of 5% gluconate in a 500-mL flask and fermented at 28°C on a rotary shaker at 160 rpm. After 24 h, 4 mL of GDH (0.1084 U), 4 mL of 2,5-DKG reductase (0.1084 U), 0.48 mL of 10 m*M* NADP, and 40 mL of 5% glucose were added. The mixture was incubated at 28°C for 72 h on a rotary shaker at 160 rpm. A 5-mL sample of the mixture was examined at 6-h intervals for the determination of 2-KLG. The turnover number of regeneration of Co II was calculated according to the amount of 2-KLG produced.

Results and Discussion

Glucose Consumption

The results of glucose consumption by *G. oxydans* resting cells are shown in Fig. 2. Obviously, the concentration of glucose was decreased although the yield of 2,5-DKG was very low. Glucose is a principal source of energy for adenosine triphosphate production *(15)* and one of the best

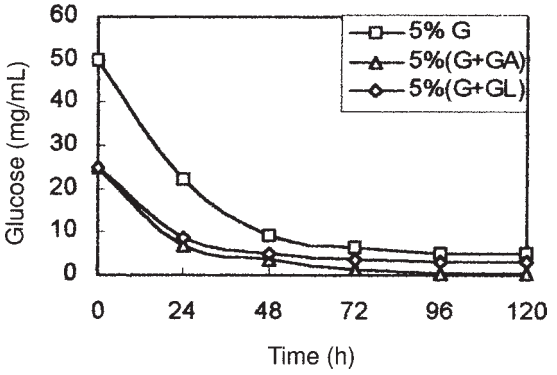

Fig. 2. Time course of glucose consumption by *G. oxydans* resting cells. G, glucose; GA, gluconic acid; GL, gluconolactone.

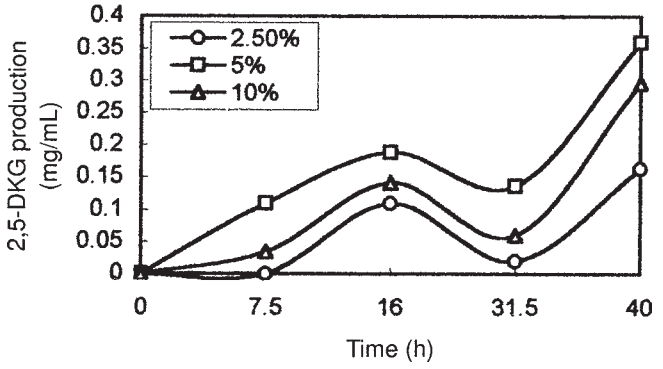

Fig. 3. Time course of 2,5-DKG production by *G. oxydans* resting cells with different concentrations of glucose as substrate.

carbon sources for most aerobic bacteria and facultative aerobe *(16)*. There are several pathways of metabolism of glucose in bacteria, such as the Embden-Meyerhof-Parnas pathway, the hexose monophosphate pathway, the Entner-Doudoroff pathway, and PK pathway *(17)*. It seems that most of the glucose is not used for 2,5-DKG production but consumed through other pathways.

2,5-DKG Production by Free Cells of G. oxydans

The results of 2,5-DKG production with different substrates in different concentrations are presented in Figs. 3–8. As depicted, the concentration of substrate at 5.0% was suitable for 2,5-DKG production for both single and mixed substrates.

Among all the different substrates, the highest yield of 2,5-DKG was observed with 5.0% gluconolactone as substrate, and gluconic acid ranked second. The yield of 2,5-DKG using gluconolactone and gluconic acid as substrate was higher than that of glucose (Fig. 9).

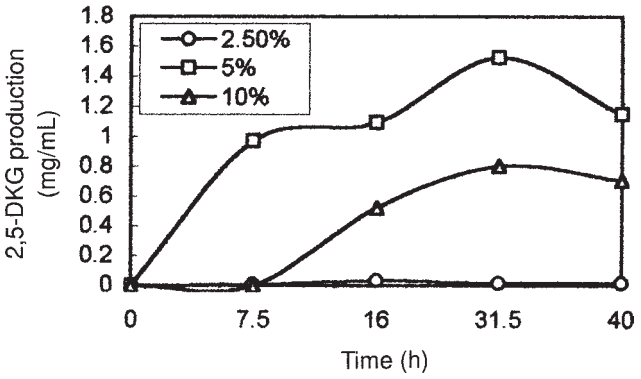

Fig. 4. Time course of 2,5-DKG production by free cells of *G. oxydans* with different concentrations of gluconic acid as substrate.

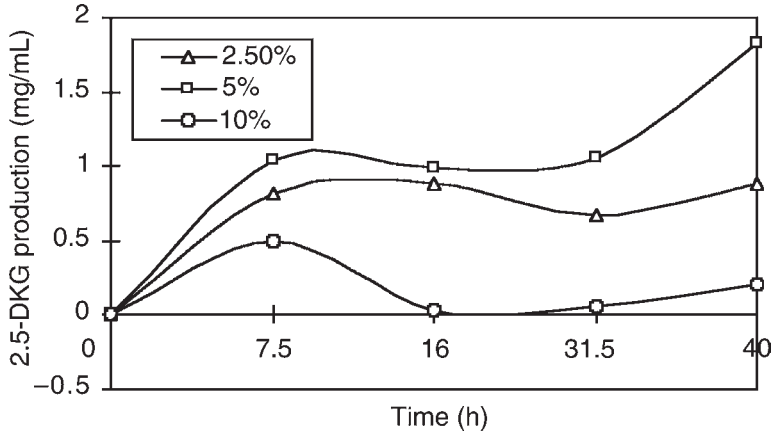

Fig. 5. Time course of 2,5-DKG production by free cells of *G. oxydans* with different concentrations of gluconolactone as substrates.

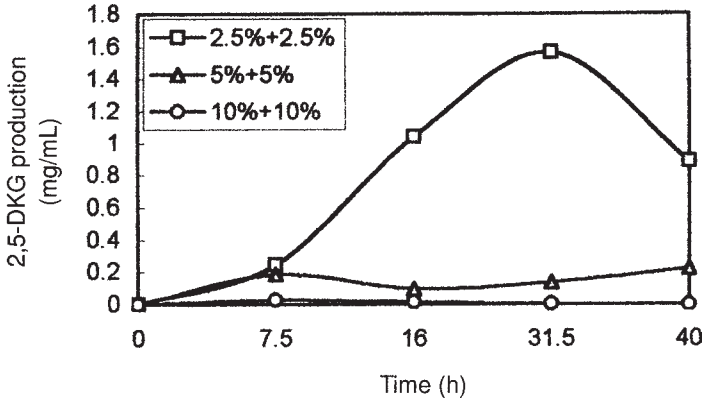

Fig. 6. Time course of 2,5-DKG production by *G. oxydans* resting cells with different concentrations of glucose and gluconic acid as substrates.

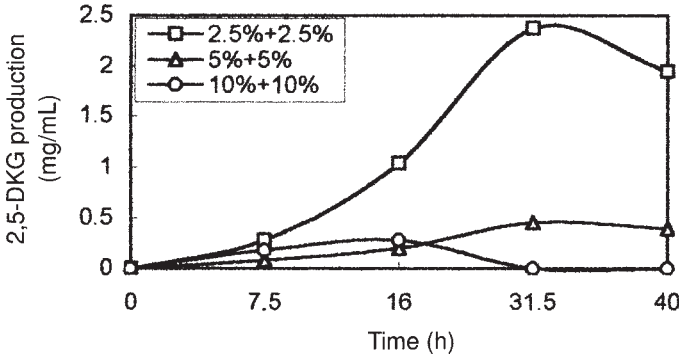

Fig. 7. Time course of 2,5-DKG production by *G. oxydans* resting cells with different concentrations of glucose and gluconolactone as substrates.

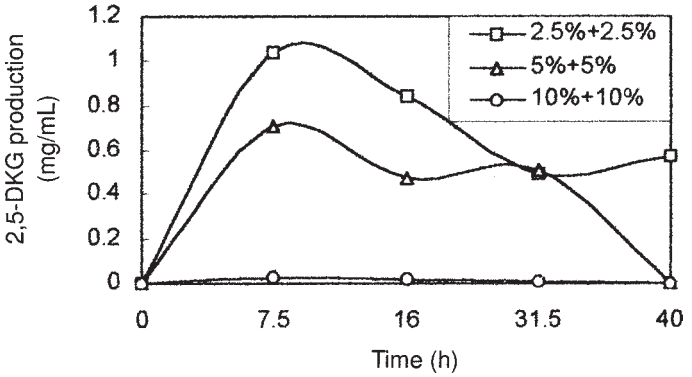

Fig. 8. Time course of 2,5-DKG production by *G. oxydans* resting cells with different concentrations of gluconic acid and gluconolactone as substrates.

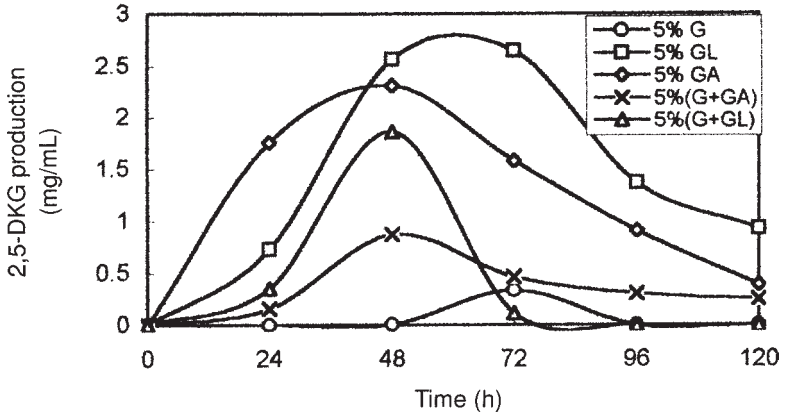

Fig. 9. Time course of 2,5-DKG production by *G. oxydans* resting cells with 5% of different substrates. G, glucose; GL, gluconolactone; GA, gluconic acid.

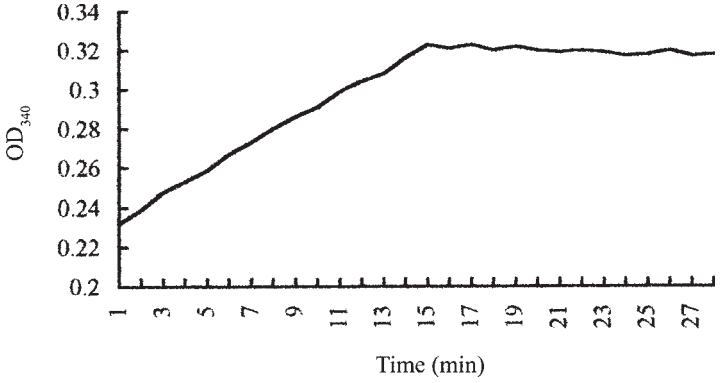

Fig. 10. Regeneration of Co II by coupling GDH with 2,5-DKG reductase (absorbance of NADPH at 340 nm).

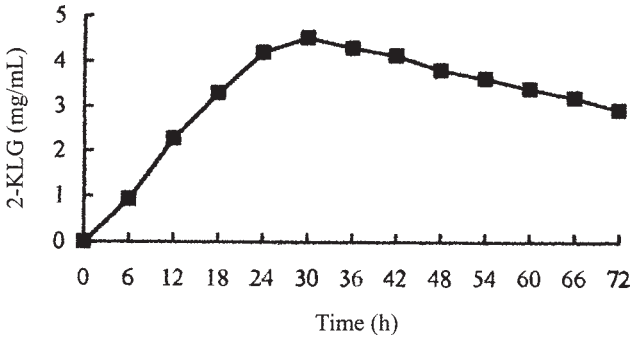

Fig. 11. 2-KLG production of the multienzyme system of *G. oxydans* cells coupled with GDH and 2,5-DKG reductase.

Coupling of 2,5-DKG Reductase with GDH

Before 2,5-DKG reductase was added to the reaction system, the absorbance at 340 nm had increased steadily. This proves that NADP had been reduced into NADPH. After 2,5-DKG reductase was added to the system, the increase in absorbance at 340 nm stopped and remained at the same level during the period of measurement (Fig. 10). This demonstrates that NADP(H) was regenerated in this system.

Production of 2-KLG by Multienzyme System

The amount of 2-KLG produced in the multienzyme and resting-cell system is shown in Fig. 11. The mole yield of 2-KLG was 16.8% within 30 h. The corresponding turnover number of NADP(H) was 388.

Conclusion

The objective of this research was to clarify which substrate, glucose or gluconate, was utilized predominantly by *G. oxydans* cells to produce

2,5-DKG. The results show that gluconate is preferred as the substrate rather than glucose in 2,5-DKG production. Since there are more metabolic pathways of glucose than of gluconate in bacteria, when glucose is used as the substrate, there will be more choices for it other than the 2,5-DKG pathway; thus, the probability of 2,5-DKG synthesis was largely decreased. Gluconate is an intermediate of the 2,5-DKG pathway. When gluconate is used for 2,5-DKG synthesis, the steps of 2,5-DKG synthesis are fewer and the consumption of energy is less than when glucose is used. Probably these are the reasons that gluconate is preferred rather than glucose to produce 2,5-DKG.

The multienzyme system of 2-KLG synthesis with Co II regeneration was mainly composed of two coupling subsystems: 2,5-DKG reductase coupled with GDH and this subsystem coupled with *G. oxydans* resting cells. The key enzyme for the NADP(H) regeneration subsystem is 2,5-DKG reductase. The specificity to 2,5-DKG and the stability of 2,5-DKG reductase made the reaction from 2,5-DKG to 2-KLG work well in this complex system, even with a crude enzyme. However, this subsystem needs a continuous supply of 2,5-DKG and a decrease in gluconate. *G. oxydans* can use gluconate predominantly to produce 2,5-DKG. When *G. oxydans* is coupled with GDH and 2,5-DKG reductase, the problem is resolved naturally and the multienzyme system of 2-KLG synthesis from glucose is formed.

There are at least three advantages to using *G. oxydans* resting cells to connect GDH with 2,5-DKG reductase for production of 2-KLG from glucose. First, gluconate produced by GDH will be transformed into 2,5-DKG immediately, so that a series of problems generally caused by the accumulation of gluconate will be avoided. Second, 2,5-DKG is supplied directly and continuously for 2,5-DKG reductase. 2,5-DKG is unstable in the reaction condition, which is one of the main problems in the tandem fermentation process developed by Sonoyama et al. *(8)*. In our system, it is unnecessary to take special measures to deal with 2,5-DKG. Third, NADP(H) is regenerated within the system without any other components or systems.

The total turnover number of coenzyme II regeneration and the yield of 2-KLG were very low with this batch-type reaction system. If this system is operated continuously, the situation will be largely improved. The question of a continuously operated process is, How can coenzyme II be retained in the reactor so that it can continue to separate the product from the system? Several possibilities are known to retain cofactors and increase the total turnover number *(18–20)*. It seems that nanofiltration is the best choice for this system, because, on one hand, the molecular weight of NADP(H) is larger than 700 Daltons. The molecular weight of substrates, products, and buffer compounds in the system are <200 Daltons. On the other hand, NADP(H) is chemically unstable, so it has no advantage to conjugates of NADP(H) with water-soluble polymer as a method for retaining it in the reactor *(19)*.

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