Production of 2-Keto-L-Gulonic Acid from D-Glucose by Two-Stage Fermentation

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A practical method for the production of calcium 2-keto-L-gulonate (an intermediate in the Reichstein synthesis of L-ascorbic acid) from D-glucose has been established by using a two-stage fermentation system. D-Glucose was first converted to calcium 2,5-diketo-D-gluconate by a mutant strain of Erwinia sp. in a medium containing D-glucose, corn steep liquor, (NH₄)₂HPO₄, and CaCO₃. After a 26-h cultivation, 328.6 mg of calcium 2,5-diketo-D-gluconate per ml was obtained, with a 94.5% yield from D-glucose. This broth was used directly for the next conversion without removal of cells by treatment with sodium dodecyl sulfate. The stereospecific reduction of calcium 2,5-diketo-D-gluconate to calcium 2-keto-L-gulonate was performed with a mutant strain of Corynebacterium sp. When the cell growth reached a maximum (about 16 h) in a medium containing D-glucose, corn steep liquor, NaNO₃, KH₂PO₄, and trace elements, NaNO₃ was added to the culture, and then the calcium 2,5-diketo-D-gluconate broth was fed over a period of about 50 h. Since the mutant strain requires a hydrogen donor for reduction, the calcium 2,5-diketo-D-gluconate broth was mixed with D-glucose before being fed. The results of four two-stage fermentations in 10-m³ conventional fermentors showed that an average of 106.3 mg of calcium 2-keto-L-gulonate per ml was obtained, with a 84.6% yield from D-glucose, the starting material of calcium 2,5-diketo-D-gluconate production. Calcium 2-keto-L-gulonate was stable in the broth. Neither 2-keto-D-gluconic acid nor 5-keto-D-gluconic acid was detected in the final broth.

It is well known that 2-keto-L-gulonic acid (2KLG) is readily converted to L-ascorbic acid (vitamin C) by a one-step chemical procedure in the Reichstein method (20). During the past 2 decades, considerable efforts have been made to find microbial methods for preparing 2KLG from various starting materials, including D-sorbitol, L-sorbose, L-sorbose, and L-idonic acid (2, 3, 6, 7, 9, 15, 16, 19). Available information, however, indicates that neither of these methods has yet been used for the commercial production of 2KLG. Low productivity of 2KLG or difficulty in obtaining starting materials economically seems to be the reason for their impractical use (5).

2,5-Diketo-D-gluconic acid (25DKG) is produced effectively from D-glucose by Pseudomonas albusosesamae, which was isolated by Wakisaka (17). In the course of studies on the use of 25DKG, our attention was directed toward finding microorganisms capable of converting 25DKG to 2KLG by stereospecific reduction at the C-5 position. Consequently, a number of microorganisms belonging to the coryneform bacteria were isolated from soil samples as the most effective strains for 2KLG production. This discovery encouraged us to attempt the improvement of these strains and the development of a process based on the reduction of 25DKG because 25DKG is inexpensively produced from D-glucose. Detailed information concerning these results will be subjects for future reports.

In this paper, we report a practical fermentation method, established by using 10-m³ conventional stainless-steel fermentors, for the production of calcium 2KLG (Ca-2KLG) from D-glucose via calcium 25DKG (Ca-25DKG). The process consists of a two-stage fermentation: oxidative fermentation of D-glucose to Ca-25DKG by a mutant strain of Erwinia sp., which was newly isolated from fruit, and then its reductive fermentation to Ca-2KLG by a mutant of Corynebacterium sp.

MATERIALS AND METHODS

Chemicals. Ca-25DKG and calcium 5-keto-D-gluconate were obtained from D-glucose as the oxidation products of P. albusosesamae and Acetobacter suboxydans (Shionogi), respectively, according to the meth-
Calcium l-idoate was obtained from calcium 5-keto-D-glucosionate as the reduction product of Brevibacterium ketosereductum ATCC 21914 by the method of Sonoyama et al. (unpublished data). The 2KLG was prepared from the monohydrate of diacetone gulonic acid by acid hydrolysis. Calcium 2-keto-D-glucosonate (Ca-2KDG; its free acid is referred to as 2KDG) was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). For the assay of D-glucosonic acid, glucosonate kinase, β-NADP and 6-phosphogluconate dehydrogenase, and ATP were obtained from Boehringer Mannheim-Yamanouchi Co., Ltd. (Tokyo, Japan), Oriental Yeast Co., Ltd. (Tokyo, Japan), and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively. Other chemicals used for analyses were reagent grade. All components of the media used were commercial preparations, unless otherwise noted.

Fermentation method for Ca-25DKG production. Strain SHS 6292001, a blocked mutant derived from Erwinia sp. SHS 2006 (ATCC 31626), was grown at 28°C for 24 h on agar slants containing 0.5% glycerol, 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), 0.3% peptone (Difco), 0.1% KH₂PO₄, 0.02% MgSO₄·7H₂O, and 2% agar. The preseed medium (600 ml in a 2-liter flask) consisted of 1% D-glucose, 0.5% yeast extract (Difco), 0.5% peptone (Difco), 0.1% NaNO₃, 0.1% KH₂PO₄, and 0.02% MgSO₄·7H₂O. The pH of the medium was adjusted to 7.0 before sterilization at 121°C for 15 min. A shake flask was inoculated with a fresh slant of strain SHS 752001 and incubated at 28°C for 24 h on a rotary shaker at 275 rpm. The seed medium (470 liters in a 1-m³ conventional stainless-steel fermentor) contained 1.0% D-glucose, 2.0% corn steep liquor, 0.2% NaNO₃, 0.1% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.005% p-2000 antifoam, and tap water. The pH of the medium was adjusted to 7.0 before sterilization at 121°C for 20 min. The entire contents of 10 preseed flasks were transferred into the seed medium, which was inoculated at 28°C for 24 h with an air flow rate of 0.47 m³/min (N.T.P.) and agitation at 44 rpm.

The fermentor medium consisted of (grams per liter): D-glucose, 20; corn steep liquor, 30; NaNO₃, 3.45; KH₂PO₄, 0.67; p-2000 antifoam, 0.0167; trace elements, including ZnSO₄·7H₂O (4.9 mg), MnCl₂·4H₂O (0.8 mg), thiamine hydrochloride (0.22 mg), and calcium D-pantothenate (0.17 mg); and tap water. Trace elements were filter sterilized. D-Glucose was steam sterilized at 115°C for 15 min. The rest of the medium was adjusted to a pH of 7.2 and sterilized at 121°C for 20 min. The pH of sterilized total medium was approximately 6.9. The fermentor (4.23 m³ of the medium) was inoculated with the entire contents of the 1-m³ seed fermentor and incubated at 28°C with agitation at 160 rpm, whereas air was admitted through a pipe sparger at a rate of 1.18 m³/min (N.T.P.) and a tank pressure slightly over atmosphere. When the cell growth reached a maximum (16 h in Fig. 2), 9.7 kg of a sterile solution of 48.5% (wt/wt) NaNO₃ was added to the culture, and then feeding of the Ca-25DKG broth was started as described in Results. Bacterial growth was measured by optical density in a manner similar to that of Ca-25DKG fermentation.

Analytical methods. The Ca-25DKG content in both the Ca-25DKG and Ca-2KLG fermentation broths was determined with a Technicon AutoAnalyzer by the following NH₄OH-HCl method devised by us. To 0.6 ml of suitably diluted sample (Ca-25DKG, 80 to 400 µM) of the fermentation broth, 0.6 ml of air, 0.6 ml of calcium chloride (12.0 mM), 0.32 ml of 1.5 N NH₄OH, and 0.34 ml of 4 N HCl were pumped per min and mixed in coils successively. The mixture (2.46 ml/min) was passed through a 28-ml-capacity coil (1.6-mm inside diameter) placed in a heating bath at 50°C, and then the absorbance at 460 nm was measured in the flow cell. For the assay of Ca-2KLG, direct conversion of 2KLG to L-ascorbic acid was carried out in a reaction mixture containing 1 ml of suitably diluted sample (Ca-2KLG, 5 to 28 µmol) of the fermentation broth with 3 ml of 8 N HCl. After incubation for 30 min at 90°C, the reaction was stopped by adding 4 ml of 5 N NaOH, and the resulting solution was extracted with 3 ml of isooamy alcohol. The amount of L-ascorbic acid remaining in the lower phase was determined spectrophotometrically by a modified phospho-18-tungstic acid method (1). The absorbance at 750 nm
was measured. Ca-2KDG content in the Ca-25DKG fermentation broth was estimated as follows. The fermentation broth sample was divided into two portions. One portion of the sample was used to measure the Ca-25DKG concentration with the NH₄OH-HCl method. Another portion of the sample was subjected to a treatment similar to that described above for the Ca-2KLG assay, and the absorbance at 750 nm (Ca-25DKG plus Ca-2KDG) was read. Ca-2KDG concentration was then determined by subtracting the Ca-25DKG value (750 nm), which was obtained in comparison with a standard curve of Ca-25DKG concentrations, from the sample value (750 nm) and comparing the resulting value (750 nm) with a standard curve of Ca-2KDG concentrations. To ensure the accuracies of all assay systems used here, new standard curves were made up periodically. D-Gluconic acid was determined enzymatically by the method described by Möllering and Bergmeyer (8). D-Glucose was also measured enzymatically by using the Glucose-B-Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan), based on a modified method of Sharp (22).

A paper chromatographic system, according to the method described by Wakisaka (18), was used for the identification of 2KLG, 2KD, 25DKG, 5-keto-D-gluconic acid, D-glucose, and other metabolic products. The cultures were also analyzed with a Shimazu gas chromatograph (model GC-5A) and a thermal conductivity detector for the identification of D-gluconic acid, L-idonic acid, and other metabolic products. The trimethylsilyl ester derivatives of these compounds were prepared by the method of Sweely et al. (13). A 10-μl sample was injected into a coiled glass column (2,000 by 3-mm inside diameter), packed with 5% SE-52 on 80/100 Chromosorb W AW DMCS (General Electric Co., Waterford, N.Y.). Helium served as the carrier gas. The injection port and detector temperatures were 230 and 235°C, respectively, and the initial column oven was set at 160°C, followed by a 4°C/min temperature increase to 210°C. n-Heneicosane was used as an internal standard. Peaks were identified by comparison with authentic standards.

RESULTS

Ca-25DKG production in a 10-m³ fermentor. The formation of D-gluconic acid, 2KDG, and 25DKG occurred simultaneously with D-glucose utilization during the cultivation. Figure 1 shows the results obtained when 14.4 kg of a sterile solution of 50% (wt/wt) D-glucose was fed at 4.5-min intervals over a period of 12 h. The amounts of calcium D-gluconate and Ca-2KDG decreased rapidly after completion of D-glucose feeding, whereas the accumulation of Ca-25DKG continued and reached a peak, 328.6 mg/ml at 26 h of cultivation. The yield of 94.5 mol% Ca-25DKG was obtained from the total amounts of D-glucose added. In the final broth, 2.5 mg of calcium D-gluconate per ml remained unconverted, whereas D-glucose and Ca-2KDG were not detected. The pH, which was not regulated, began at 7.1, dropped to 4.7 at 3 h of cultivation, and finally reached 3.9. The volume of the broth was increased from 2.28 to 4.2 m³ by feeding of D-glucose solution. Precipitation of calcium D-gluconate, Ca-2KDG, or Ca-25DKG was not observed during the fermentation. No 5-keto-D-gluconic acid was detected by means of paper chromatography throughout the fermentation.

Treatment of Ca-25DKG broth with sodium dodecyl sulfate. The Ca-25DKG broth (4.2 m³) was mixed with 7.27 kg of a sterile solution of 14.4% (wt/wt) sodium dodecyl sulfate and maintained at 28°C for 6 h with mild agitation at 80 rpm. By this treatment, the total viable cell population was decreased from 5 x 10⁸ to 9 x 10⁶ cells per ml. Finally, the broth was cooled and maintained at approximately 10°C to minimize the decomposition of Ca-25DKG. The Ca-25DKG broth thus treated was used directly as the source of Ca-25DKG for the next conversion, without removal of cells.

Ca-2KLG production in a 10-m³ fermentor. The mutant strain SHS 752001 requires a hydrogen donor for the reduction of 25DKG to 2KLG. Figure 2 presents the data obtained when D-glucose was used as the hydrogen donor. The Ca-25DKG broth prepared in the preceding stage was mixed with a sterile solution of 50% (wt/wt) D-glucose in the proportion of 0.2 kg of...
The feeding of the Ca-25DKG feeding, neither of 48 at 18 content was 0.01 yield below 5 um fed and was *Ca-25DKG in reverted. After D-glucose to 106.5 mg/ml (97.0 mg/ml as free acid). In the final broth, however, 1.6 mg of Ca-25DKG per ml remained unconverted. After completion of Ca-25DKG broth feeding, neither an increase nor a decrease of Ca-2KLG content in the broth was observed. The yield of Ca-2KLG from Ca-25DKG utilized was 92.5 mol%, whereas the overall yield of Ca-2KLG from D-glucose, the starting material of Ca-25DKG production, was 86.0 mol%. Total volume of the broth increased gradually throughout the period of feeding from 4.7 to 7.43 m³. The pH, which was not regulated, dropped from 8.3 to 6.1, whereas the cell population was held constant at around 22 (optical density at 660 nm).

Results of four two-stage fermentations are shown in Table 1. In all cases, D-glucose, calcium L-idenate, calcium D-gluconate, calcium 5-keto-D-gluconate, and Ca-2KD were not detected by means of paper and gas chromatography in the fermentation broth over the period of Ca-25DKG feeding.

Total accumulated Ca-2KLG in the broth was isolated by K. Tokuyama, M. Kiyokawa, and T. Sonoyama as crystals in the form of the monohydrate of its sodium salt with purities of >99% (unpublished data). A portion of these crystals was recrystallized from distilled water and submitted to physicochemical analyses by M. Sawada (unpublished data). The melting point was 145.5°C (decomposition), and the optical rotation was [α]D° = -25.6° (c = 2.0, water). Elemental analysis corresponds to C₆H₉O₇-Na·H₂O; calculated: C, 30.78; H, 4.77; Na, 9.82. Found: C, 30.80; H, 4.77; Na, 9.88. The infrared spectrum of the crystals in a KBr disk was identical to that of an authentic sample.

**DISCUSSION**

Conversion of D-glucose to 25DKG via D-gluconic acid and 2KD was first proved by Katznelson et al. (4) with *Acetobacter melanogenus* MA 6.2 and later by Wakisaka (18) with *P. albosesamae*. Recently, Stroshane and Perlman (11) reported that *A. melanogenus* ATCC 9937 converts D-glucose to 25DKG via D-gluconic acid and 5-keto-D-gluconic acid. Our strain SHS 2629001, a mutant strain derived from *Erwinia* sp., accumulated D-gluconic acid and 2KD as intermediate products (Fig. 1). No 5-keto-D-gluconic acid could be detected in the broth throughout the cultivation. This observation suggests that strain SHS 2629001 converts D-glucose to 25DKG in a pathway similar to those of *A. melanogenus* MA 6.2 and *P. albosesamae*.

The high-yield production of 25DKG from D-glucose (>90%) has already been reported with relatively low accumulation of 25DKG (about 90 mg/ml), although at shake flask scale (11, 18). In this work the production of large amounts of 25DKG was demonstrated with 10⁻³ m³ ferments.

**TABLE 1. Ca-2KLG production in 10⁻³ m³ fermenters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Accumulated Ca-2KLG (mg/ml)</th>
<th>Ca-2KLG yield from D-glucose (mol%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum</td>
<td>106.9</td>
<td>86.0</td>
</tr>
<tr>
<td>Mean</td>
<td>106.3</td>
<td>84.6</td>
</tr>
<tr>
<td>Minimum</td>
<td>105.1</td>
<td>82.9</td>
</tr>
</tbody>
</table>

a Values were obtained in four two-stage fermentations. Fermentations were carried out in the cultural conditions described in the text. The Ca-25DKG broths were fed over a period of 50 h.

D-glucose was the starting material of Ca-25DKG production.
tors. Strain SHS 2629001 converted the added d-glucose to 25DKG with a yield of 94.5 mol% and accumulated 328.6 mg of Ca-25DKG (297.6 mg/ml as free acid) per ml in low-cost corn steep liquor medium (Fig. 1); the time required for this fermentation was 26 h. This high productivity was obtained by maintaining the dissolved-oxygen concentrations in the culture broth above zero throughout the fermentation and implementing timely feeding of D-glucose.

The 25DKG is unstable, especially to heat and in alkaline solution (11). Our early attempts to isolate Ca-25DKG from the broth resulted in significant losses (more than 30%) of Ca-25DKG. This phenomenon of the instability of 25DKG was also observed by Stroshane and Perlman (11) in their studies on the isolation of 25DKG from broth. Thus, the elimination of the isolation process of Ca-25DKG, which is an additional advantage in industrial applications, was important to the success of this work. Of about 20 chemical agents tested, sodium dodecyl sulfate was selected as the most effective one having bactericidal activity against SHS 2629001 (a gram-negative rod). The use of the 25DKG broth treated with sodium dodecyl sulfate (approximately 250 μg/ml) did not inhibit the conversion of Ca-25DKG to Ca-2KLG and rather enhanced Ca-2KLG production in comparison with the use of the Ca-25DKG powder (data not shown).

The microbial reduction of 5-keto-D-gluconic acid to L-idonic acid was found first by Takagi (14) with Fusarium species and later by us (unpublished data) with B. ketsosoreductum ATCC 21914. However, the selective and stereospecific reduction of 25DKG to 2KLG by microorganisms, described here, is a new finding to our knowledge.

The mutant strain SHS 752001 could utilize various carbohydrates and low-cost molasses as hydrogen donors (data not shown). In this report we present the results obtained when d-glucose was used as the donor (Fig. 2; Table 1). All of four two-stage fermentations in 10-m³ fermentors resulted in excellent levels of Ca-2KLG production (Table 1): an average of 106.3 mg of Ca-2KLG (96.8 mg/ml as free acid) per ml was accumulated, with an 84.6 mol% yield from d-glucose, the starting material of Ca-25DKG production. The standard deviations from the mean were 0.8% for the accumulation and 1.5% for the yield. Thus, the reliability and reproducibility of the above fermentation method for Ca-2KLG production, both essential for the commercial production, were also demonstrated. Since the Ca-25DKG yield from d-glucose, the Ca-2KLG yield from Ca-25DKG utilized, and the overall yield of Ca-2KLG from d-glucose were 94.5, 92.5, and 86.0% (Fig. 1 and 2), respectively, the decomposition of Ca-25DKG during the periods of the treatment with sodium dodecyl sulfate and the storage for feeding was estimated to be about 1.5%.

Although the mutant strain SHS 752001 is strictly aerobic, low aeration rates below 0.25 vol/vol per min (N.T.P.) were suitable for Ca-2KLG production. The onset of feeding of the Ca-25DKG broth was most favorable at the early stationary phase of cell growth, indicating the optimal phase of the overflow of reducing powers such as NAD(P)H to reduction of 25DKG in the cell's metabolism.

For economic considerations, it may be noted here that these results of Ca-2KLG production were obtained with commercially feasible media for both Ca-25DKG and Ca-2KLG fermentation. In addition, the stability of Ca-2KLG allowed more flexibility in the isolation of the product. Furthermore, the absence of d-glucose, calcium d-glucionate, calcium L-idonate (the isomer of D-glucionate), calcium 5-keto-D-gluconate, and especially Ca-2KDG (the isomer of 2KLG and readily converted to isoascorbic acid in the same chemical procedure as that of L-ascorbic acid synthesis from 2KLG) in the final broth also allowed us to simplify the isolation process of 2KLG from the broth.

Similar results on the kinetics of cell growth and Ca-2KLG production were obtained with 30-liter, 1-m³, and 10-m³ fermentors (Table 2), possibly indicating easy scale-up to industrial-scale production.

In conclusion, the method described here will provide a new way of commercially producing 2KLG, an intermediate in the synthesis of L-ascorbic acid.

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**LITERATURE CITED**


