Microbial Production of 2-Keto-L-Gulonic Acid from L-Sorbose and D-Sorbitol by *Gluconobacter melanogenus*[†]

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The strain SPO1 producing 13 g of 2-keto-L-gulonic acid (2KGA) per liter was isolated as a spontaneous mutant of *Gluconobacter melanogenus* IFO 3293. For the enhancement of 2KGA productivity, we did further strain improvement studies of the mutant.

As a result, the mutant U13, producing about 60 g of 2KGA per liter from 100 g of L-sorbose per liter, was obtained. In addition, the mutant Z84, producing about 60 g of 2KGA per liter from 100 g of D-sorbitol per liter, was also obtained.

During the fermentation from L-sorbose and D-sorbitol, 5 to 10 g of L-idonic acid per liter was produced as a by-product, but L-idonic acid was converted to 2KGA before the end of fermentation.

Vitamin C is industrially produced by the Reichstein method.¹⁾ D-Glucose is chemically hydrogenated to produce D-sorbitol and the product is then biochemically dehydrogenated to yield L-sorbose by *Acetobacter xylinum*. This L-sorbose is then chemically converted to diacetonesorbose and its product, diacetone-sorbose, is chemically oxidized to diacetone-2-keto-L-gulonic acid, which is a precursor of vitamin C. Only the process to convert D-sorbitol to L-sorbose is a biochemical step. The establishment of an efficient process for microbial production of 2KGA has long been one of the objectives in the vitamin C industry.

According to the literature, microbial production of 2KGA may be done by several different processes, which can be classified into two groups: one is the direct production of 2KGA from L-sorbose or D-sorbitol by a single microorganism or mixed culture, the other is a two-step fermentation of 2KGA from D-glucose using two different microorganisms.

Direct Production of 2KGA

In 1962 Huang described various species of *Pseudomonas* as producers of 2KGA with a yield of less than 1 g per liter from 20 g L-sorbose per liter.²⁾ This was the first occurrence of 2KGA production by fermentation.

Isono and his colleagues studied a variety of microorganisms for the fermentation of 2KGA from D-sorbitol and L-sorbose.³⁾ They found that microorganisms classified into twelve genera namely, *Acetobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Bacillus*, *Escherichia*, *Gluconobacter*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Serratia*, and *Xanthomonas*, produced the keto acid from L-sorbose. Their results also indicated that only members of the genera *Acetobacter*, *Gluconobacter*, and *Pseudomonas* produced 2KGA from D-sorbitol. According to the patent specification of Motizuki *et al.*,⁴⁾ the highest yield (6.5 g of 2KGA per liter) was obtained from *Gluconobacter melanogenus* IFO 3292 with 50 g of

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D-sorbitol per liter.

Chibata *et al.* also disclosed a process for the production of 2KGA by members of the genera *Serratia*, *Acromobacter*, and *Alcaligenes*.⁵⁾ The yield of 2KGA in the culture, however, was less than 2 g per liter.

Direct production methods to convert L-sorbose into 2KGA with a mixed culture have also been reported by Lin *et al.*⁶⁾ and Zinsheng *et al.*⁷⁾; 30 and 37 g of 2KGA per liter were produced from 70 g and 100 g of L-sorbose per liter of mixed culture broth. These two microorganisms were identified as *Pseudomonas striata* and *Gluconobacter oxydans*.

A similar process had already been reported by Perlman and his group who described the conversion of L-sorbose to 2KGA by mixtures of immobilized cells of *Gluconobacter melanogenus* and *Pseudomonas* species.⁸⁾

Two-step fermentation of 2KGA

Sonoyama *et al.*⁹⁾ reported a two-step fermentation method for the production of calcium-2KGA from D-glucose *via* calcium 2,5-diketo-D-gluconic acid. The process consists of a two-step fermentation: (1) an oxidative fermentation of D-glucose to 2,5-diketo-D-gluconic acid by a mutant strain of *Erwinia* sp., and (2) a reductive fermentation of 2,5-diketo-D-gluconic acid to 2KGA by a mutant of *Corynebacterium* sp. An 84.6% yield, 106.3 g of calcium-2KGA per liter, was produced from D-glucose. Furthermore, Anderson *et al.*¹⁰⁾ reported that the gene which codes for 2,5-diketo-D-gluconic acid reductase (converting 2,5-diketo-D-gluconic acid into 2KGA), in *Corynebacterium* sp was cloned and expressed in *Erwinia herbicola*. The resultant recombinant organism converted D-glucose into 2KGA yielding 1 g per liter in a single fermentation.

Other studies describing methods for vitamin C synthesis were comprehensively described by Kulhanek.¹¹⁾

However, the fermentation method to produce 2KGA from L-sorbose, D-sorbitol, or D-glucose have not yet been established as practical, and vitamin C is still produced by

Reichstein's method worldwide. If the problem of direct oxidation of L-sorbose or D-sorbitol to 2KGA could be solved, Reichstein's method would be further simplified.

Therefore, we compared the productivity of several strains of *G. melanogenus* which were reported to convert L-sorbose or D-sorbitol into 2KGA. As a result, higher 2KGA yields from L-sorbose were obtained from *G. melanogenus* IFO 3293 than from all other strains tested. Accordingly, it was selected as the parent strain, and the strain improvement by mutagenesis and spheroplast fusion was done to increase 2KGA productivity based on the following three objectives.

First, we wanted to improve 2KGA productivity from more than 7% of L-sorbose and then improve 2KGA productivity from D-sorbitol because D-sorbitol is cheaper than L-sorbose made from D-sorbitol by the fermentation method. We also wanted to replace yeast extract with corn steep liquor as a nitrogen source in the production medium because corn steep liquor is cheaper than yeast extract.

The 2KGA productivity of mutants were evaluated by tube culture as a primary screening and by flask culture as the second screening. The selected mutants by the above mentioned screenings were finally evaluated in 3-l jar fermentors for their 2KGA productivity.

Here we describe a strain improvement study in which the mutants produced about 60 g of 2KGA per liter either from 100 g of L-sorbose or D-sorbitol per liter in a medium containing corn steep liquor as a nitrogen source.

Materials and Methods

Chemicals. L-Sorbose and 2KGA were generously supplied by Hoffmann-La Roche Inc., Nutley. The other chemicals used for analyses were reagent grade. All components of the media used were commercial preparations, unless otherwise noted.

Microorganisms. *Gluconobacter melanogenus* IFO 3292, IFO 3293, IFO 3294, IFO 12257, and IFO 12258 were purchased from the Institute for Fermentation, Osaka

(IFO). These microorganisms were maintained on mannitol agar slant medium having the following composition per liter: 5.0 g of yeast extract (Difco), 3.0 g of polypeptone (Daigo), 25.0 g of D-mannitol (Wako), 15.0 g of agar, and 15.0 g of CaCO_3 . Various mutants were maintained on potato-agar or plates containing: 1.0% yeast extract (Oriental), 1.0% polypeptone (Daigo), 2.0% glycerol, 0.5% glucose, and 1.5% agar in 100 ml of potato extract filtrate which was prepared with cheesecloth after extracting 1.0 kg of peeled and sliced potatoes in 5 l of deionized water for 40 min at 121°C.

Media. The composition of the medium No. 5 (Y.E.) is: 1.5% yeast extract, 0.25% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% glycerol, 1.5% CaCO_3 , 0.15% antifoam, and 8 to 10% L-sorbose or D-sorbitol (sterilized separately). The composition of the medium No. 5 (C.S.L.) is the same as No. 5 (Y.E.) except that 2% corn steep liquor was used instead of 1.5% yeast extract. In pH-controlled jar fermentations, CaCO_3 was omitted from the above medium.

Preparation of Seed Culture. One loopful of agar culture of a microorganism was transferred into 5 ml of the medium No. 5 (Y.E.) including 8% L-sorbose or D-sorbitol in a test tube (diameter, 21 mm) and incubated at 30°C for 2 days. One ml of this culture was transferred into 50 ml of the same medium in a 500 ml Erlenmeyer flask and incubated at 30°C for 2 days. This culture was used to inoculate a flask or a jar fermentor.

Flask Fermentation. One ml of seed culture was used to inoculate 50 ml of the medium No. 5 (Y.E.) or the medium No. 5 (C.S.L.) containing 10% L-sorbose or D-sorbitol in a 500-ml Erlenmeyer flask. The flasks were incubated at 30°C on a rotary shaker at 180 rpm for 4 days.

Jar Fermentation. A glass jar fermentor, type MB-C 2000 (Iwashita, Tokyo, Japan) was used. A fermentor has a total volume of 3 l with a top drive system and temperature, pH, D. O., and exhaust gas monitors. Its working volume was 2 l and the fermentation was done at 30°C. Fermentation was usually started with an inoculum size of 2.5% (50 ml for 2 l of the medium).

Analysis. The concentrations of 2KGA, L-sorbose, and D-sorbitol were measured by high-performance liquid chromatography (HPLC). All 2KGA values shown in this paper pertain to its free form.

L-Sorbose and D-sorbitol concentrations were measured using a carbohydrate column (ID of 3.9 mm by 39 cm, Waters). The mobile phase was acetonitrile- H_2O (82:18, v/v) with a flow rate at 2.0 ml per minute. Detection was monitored by the change in refractive index.

The concentration of L-idonic acid was measured by a Shimadzu GC-4CM gas chromatograph (GC) equipped with a hydrogen flame ionization detector. The liquid phase was OV-17 on Carbowax and was packed in a

capillary column (fused silica, 0.24 mm ID $\times$ 50 m, Chromato Packing Center Co.). Nitrogen was used as a carrier gas at a flow-rate of 40 ml per minute. The temperatures at the detector and the injection port were set at 280°C. The column temperature was set at 135°C.

One-quarter ml of sample solution or culture filtrate, centrifuged at 8,000 rpm to remove cells, was freeze-dried for 17 hr. To this lyophilized sample in a test tube were added 0.5 ml of pyridine dried on NaOH, 10 mg of *o*-methylhydroxylamine (E. Merck) and 0.1% (volume/volume) each of *n*-tetradecane and hexadecane. This sample was left for 17 hr in a desiccator at room temperature. Then 0.5 ml of a 1:1 mixture of pyridine and the reagent liquid, which is a 99:1 mixture of bis (trimethylsilyl)-trifluoro-acetamide and trimethylchlorosilane, was added. After the sample was dissolved by shaking, it was kept at 80°C for 1 hr. This sample was used for GC analysis.

Thin layer chromatography (TLC) was used to follow the production of 2KGA and consumption of L-sorbose or D-sorbitol, and was used to detect the formation of by-products. Silica gel (Kiesel gel 60 F₂₅₄, 0.25 mm, Merck) TLC plates and *n*-propanol- H_2O -1% H_3PO_4 -HCOOH (400:100:10:1) as its solvent system were used.

2KGA, L-sorbose, and other compounds containing alpha-keto group were detected by blue tetrazolium, and other compounds including D-sorbitol without an alpha-keto group were detected by tetrabase. The plates were sprayed with blue tetrazolium reagent containing equal volumes of 0.5% blue tetrazolium in methanol and 6 N NaOH and heated to produce violet spots. For the detection of compounds by tetrabase reagent, the plates are sprayed with 0.5% KIO_4 solution and then sprayed with the mixture of an equal volume of tetrabase-saturated 2 N CH_3COOH and 15% MnSO_4 solution. White spots appeared against a blue background.

Cell growth was monitored at an optical density of 550 nm after dissolving the remaining calcium carbonate with hydrochloride.

All the values were expressed as the concentration against the starting volume of cultivation.

Results

2KGA production by various *gluconobacters*

The five strains of *G. melanogenus* described in Materials and Methods were cultivated in the medium containing 2.5% L-sorbose, 0.5% glycerol, 0.5% yeast extract, 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5% CaCO_3 . One loopful of agar slant culture of each strain was inoculated into 5 ml of sterilized medium in a test tube and cultivated at 27°C on a tube shaker. After 7 days the culture was assayed for 2KGA

Table I. 2-KETO-L-GULONIC ACID PRODUCTION
BY VARIOUS *G. melanogenus* STRAINS

	2-KGA productivity ^a (g per liter)
<i>G. melanogenus</i> IFO 3292	0.08
IFO 3293	2.77
IFO 3294	0.59
IFO 12257	0.16
IFO 12258	0.21

^a From 25 g of L-sorbose per liter.

production. As shown in Table I, all of the strains formed 2KGA to some extent. Among them, *G. melanogenus* IFO 3293 was the best. The yield obtained in this experiment is comparable to the yields already reported.²⁻⁵⁾ *G. melanogenus* IFO 3293 was selected as the parent strain for further improvement.

Strain improvement by single colony isolation

The first trial for strain improvement was done by single-colony isolation. The slant culture of *G. melanogenus* IFO 3293 was suspended in sterilized water and spread on mannitol agar plates, with a composition the same as that of the agar slant. Colonies formed on the plates after incubation at 27°C for 4 days were picked up and maintained on mannitol agar medium. Each colony was inoculated into a test tube containing 5 ml of the medium containing 5% L-sorbose, 0.5% glycerol, 0.5% yeast extract, 0.5% MgSO₄·7H₂O, and 0.5% CaCO₃ and cultured on a test tube shaker at 27°C for 6 days. The cultured broth was centrifuged and the supernatant was analyzed for 2KGA and remaining L-sorbose on TLC and HPLC.

As a result, the isolated strains showed three different characteristics in terms of 2KGA production and remaining L-sorbose. They were 2KGA high producers (about 13 g per liter), middle producers (about 4.3 g per liter), and non-producers. The strains SPO1 and SPO3 isolated as 2KGA high producers produced 13 g of 2KGA per liter from 50 g of L-sorbose per liter. The yield of 2KGA of strain SPO1 against consumed L-sorbose was

thought to be higher than that of strain SPO3 estimated from the amount of remaining L-sorbose. Therefore, the strain SPO1 was selected as the best producer of 2KGA and was used for further studies.

Strain improvement by mutagenesis

Further strain improvement was done to obtain mutants producing 2KGA more efficiently than the mutant SPO1 from L-sorbose and D-sorbitol by mutagenesis and spheroplast fusion.

For mutagenesis, the agar slant culture of the strain to be mutated was inoculated into 5 ml of medium No. 5 (Y.E.) or No. 5 (C.S.L.) in a test tube and incubated at 30°C for 2 days on a tube shaker. The cells were collected by centrifugation and washed twice with 50 mM potassium phosphate buffer, pH 6.0 to 8.0, and suspended in the same buffer.

For UV irradiation, two ml of the cell suspension was placed on a glass plate and kept under a UV light (15 W) at a distance of 30 cm. Samples were taken out at intervals and kept in dark for 60 min before spread on agar plates.

For various mutagen treatments, the cell suspension was distributed into test tubes, to which 0.005 to 1.0 mg per ml of a mutagen was added and incubated at 30°C for 30 min on a tube shaker. The treated cells were washed with the above buffer twice to rinse out the reagent and spread on agar plates with an appropriate dilution. They were then incubated at 30°C for 4 days. The colonies were transferred by streaking onto agar plates.

For spheroplast fusion, the spheroplasts were prepared by the method of Ameyama *et al.*¹²⁾ with some modifications.¹³⁾ Cells were suspended in the lysis buffer containing 100 mM Tris·HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 20 mM MgCl₂·6H₂O, and 20 mM CaCl₂·2H₂O, and incubated with 500 µg of lysozyme per ml at 33°C for 2.5 hr. The spheroplast suspension was washed with HiMCP medium containing 0.2 g of MgSO₄·7H₂O, 0.5 g of KH₂PO₄, 0.34 g of K₂HPO₄, 10 g of (NH₄)₂SO₄, 10 mg of NaCl, 10 mg of

Table II. 2KGA PRODUCED FROM L-SORBOSE AND D-SORBITOL BY VARIOUS MUTANTS IN FLASK CULTURE

Mutant No.	2KGA Produced (g per liter) (range)				
	Substrate	L-Sorbose		D-Sorbitol	
		Basal medium	No. 5 (Y.E.)	No. 5 (C.S.L.)	No. 5 (Y.E.)
UV10		30.0	25.0	25.5	25.0
s66-23		40-45	—	28.0	—
E1		55.1	—	—	—
H2		62.8	—	—	—
L8		61.9	—	51.3	—
N44-1		56-61	46-50	47-50	—
U13		59-66	2-3 ^a	46-50	—
			(+ trp: 52-54)		
R117		54-55	—	54-57	—
V11		30-40	—	57-61	38-45
W5		60-64	3-4 ^b	56-59	4-5
X48		58-60	51-52	58.0	46-52
Z84		—	—	60.4	54-57
Z29		—	—	62.5	54-59

7% L-Sorbose or D-sorbitol was used for the mutant UV10 and s66-23 and the other mutants were cultivated in the medium containing 10% L-sorbose or D-sorbitol.

^{a, b} The mutants U13 and W5 were tryptophan (trp) auxotrophs and the addition of 0.1-0.2 g per liter of tryptophan restored 2KGA production.

MnSO₄·4H₂O, 10 mg of FeSO₄·7H₂O, 5 mg of Ca·pantothenic acid, 0.2 mg of *p*-amino benzoic acid, 0.2 mg of nicotinic acid, 10 mg of casamino acids, 10 g of mannitol, 90 g of D-sorbitol, and 15 g of polyvinylpyrrolidone¹⁴⁾ in one liter of distilled water, and resuspended in the same medium. Spheroplast suspensions of two mutants, A6 and s14-23, were mixed together and centrifuged at 1,000 × *g* for 10 min. The spheroplast mixture was resuspended in HiMCP medium followed by the addition of 40% polyethyleneglycol 1,000, and incubated at 20°C for 10 min. The fused suspension was diluted with HiMCP medium, centrifuged, resuspended in HiMCP medium, and plated on HiMCP agar medium containing 1.8% agar. After incubation at 27.5°C for 6 days, regenerants were obtained.

The 2KGA productivity of the mutants selected through this study was evaluated by flask fermentation and the results are summarized in Table II.

The mutants shown in the table are classified into the following 4 groups according to our strain improvement strategies taken up in this

study:

1. 2KGA-high producers in the medium No. 5 (Y.E.) containing L-sorbose—ex. UV10, s66-23, H2, *etc.*

2. 2KGA-high producers in the medium No. 5 (C.S.L.) containing L-sorbose—ex. N44-1, U13, W5, and X48.

3. 2KGA-high producers in the medium No. 5 (Y.E.) containing D-sorbitol—ex. L8, N44-1, U13, R117, *etc.*

4. 2KGA-high producers in the medium No. 5 (C.S.L.) containing D-sorbitol—ex. V11, X48, Z84, and Z29.

(Figure 1 shows the summary of the steps of mutations and the mutants obtained.)

The mutant U13 and its mutant W5 produced 59 to 66 g of 2KGA per liter in medium No. 5 (Y.E.) containing 10% L-sorbose. However, because they were tryptophan auxotrophs (data not shown), they produced only 2 to 4 g of 2KGA per liter in the medium No. 5 (C.S.L.). The low 2KGA productivity of these mutants was recovered by supplementation with 0.1 to 0.2 g of tryptophan per liter as shown in Table II. The mutant X48 was a

Fig. 1. Induction of 2KGA High Producing Mutants. Mutation was done by the conventional method. Many of these methods have been described in various publications.¹⁵⁾

Abbreviations: UV irradiation (UV), *N*-methyl-*n*-nitro-*N*-nitrosoguanidine (NTG), Acridine orange (AO), 2-Methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino]acridinedihydrochloride (ICR-170), 2-Methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]acridinedihydrochloride (ICR-191), 4-Nitroquinoline-*N*-oxide (4NQO), single colony isolation (SCI).

revertant of such auxotrophy derived from W5.

The mutant R117, derived from the mutant N44-1, was selected as a 2KGA-high producer (54–57 g per liter) from 10% D-sorbitol in the medium of No. 5 (Y.E.). Through further mutagenesis using the medium No. 5 (C.S.L.) containing 10% D-sorbitol, we obtained mutants Z29 and Z84, which produced 54 to 59 g of 2KGA per liter.

Comparison of 2KGA productivity in 3-l jar fermentor

For the further evaluation of 2KGA productivity of the selected mutants, 3-l jar fermentations were done.

The mutants selected as 2KGA-high producers from L-sorbose were cultivated in medium No. 5 (Y.E.) or No. 5 (C.S.L.) containing 10% L-sorbose and for some the pH was

Fig. 2. Course of 2KGA Production from D-Sorbitol by Mutant V11 Using 3-l Jar Fermentor.

No. 5 (C.S.L.) medium containing 9% D-sorbitol was used as the production medium, and the culture conditions were as follows; agitation speed 500 rpm, aeration speed 0.75 vvm, temperature 30°C, working volume 2.0 l, and a controlled pH of 6.0 with 2N Na₂CO₃.

(—○—), D-sorbitol; (—●—), 2KGA; (—■—), L-sorbose; (—△—), O.D.; (—▲—), L-idonic acid; (—▼—), pH; (—◇—), 2N Na₂CO₃ consumed.

controlled and for others it was not. The mutants selected as 2KGA-high producers from D-sorbitol was cultivated under the conditions described above.

For the best yields of 2KGA from L-sorbose, the mutant U13 produced 60 g of 2KGA per liter from 100 g of L-sorbose per liter in medium No. 5 (Y.E.) while the pH was not controlled, and the mutant X48 produced 50.6 g of 2KGA per liter from 100 g of L-sorbose per liter in medium No. 5 (C.S.L.) while the pH was controlled. From D-sorbitol, the mutant Z84 produced 60.3 g of 2KGA per liter from 100 g of D-sorbitol per liter in the medium No. 5 (C.S.L.) while the pH was controlled. (Table III) On the other hand, the mutant UV10 produced 33.2 and 25.5 g of 2KGA per liter, at best, from 100 g of L-sorbose and D-sorbitol per liter, respectively.

Thus, about a two-fold increase in the pro-

Table III. COMPARISON OF 2KGA PRODUCTIVITY BY SELECTED MUTANTS IN 3-l JAR FERMENTOR

Mutant No.	Basal medium	2KGA Produced (g per liter)		
		pH Non-Control	pH Control	(pH value)
Substrate: 10% L-Sorbose				
UV10	No. 5 (Y.E.)	33.2	23.4	(5.5)
	No. 5 (C.S.L.)	25.4	—	
L8	No. 5 (Y.E.)	58.0	—	
N44-1	No. 5 (Y.E.)	56.5	52.2	(5.5)
	No. 5 (C.S.L.)	44.7	45.4	(6.0)
U13	No. 5 (Y.E.)	60.0	47.3	(5.5)
W5	No. 5 (Y.E.)	55.0	53.5	(5.5)
X48	No. 5 (Y.E.)	54.2	53.2	(5.5)
	No. 5 (C.S.L.)	50.1	50.6	(6.0)

Substrate: 10% D-Sorbitol				
UV10	No. 5 (Y.E.)	25.5	—	
R117	No. 5 (Y.E.)	53.2	49.6	(5.5)
V11	No. 5 (Y.E.)	59.8	49.9	(5.0)
	No. 5 (C.S.L.)	—	55.5	(6.0)
Z29	No. 5 (C.S.L.)	53.5	57.2	(6.0)
Z84	No. 5 (C.S.L.)	55.8	60.3	(6.0)

In fermentation without pH control, 1.5% CaCO_3 was added in the medium. During pH-controlled fermentation, Na_2CO_3 or NaOH was used as a neutralizing agent.

ductivity of 2KGA was attained by our strain improvement study.

Course of 2KGA production by the mutant V11

The mutant V11 was grown in a 3-l jar fermentor to observe the course of 2KGA production. The fermentation was done in medium No. 5 (C.S.L.) containing 90 g of D-sorbitol per liter and the pH was controlled so that it did not drop below 6.0. As shown in Fig. 2, D-sorbitol was almost completely consumed in about 30 hr of fermentation, and L-sorbose was formed accordingly. The L-sorbose level reached its maximum at 30 hr after fermentation, and then decreased to zero after another 35 hr of fermentation. The formation of 2KGA started about 10 hr after the start of fermentation. During the 2KGA fermentation, the accumulation of L-idonic acid was noticed. L-Idonic acid accumulated in accordance with the formation of 2KGA until L-sorbose was completely dissimilated, and then it gradually decreased. The formation of 2KGA, after complete dissimilation of L-sorbose, was due to that derived from L-idonic

acid. Rapid CO_2 evolution was observed at the early stage of fermentation, but not during the stage of conversion from L-idonic acid to 2KGA. The total amount of CO_2 that was evolved during the fermentation was equivalent to about 25 to 30% of L-sorbose added as a carbon source.

Discussion

We have succeeded in breeding mutants producing 60 g of 2KGA per liter either from 100 g of L-sorbose or D-sorbitol per liter. The molar yield calculated against the initial substrate concentration was about 60%. To lower the 2KGA production cost, 2KGA high producing mutants in a medium containing corn steep liquor instead of yeast extract as a nitrogen source were obtained. Significant increases in the production of 2KGA helped to improve the cost-effectiveness of vitamin C production by Reichstein's method relative to the energy input involved. Despite the excellent yields of 2KGA obtained under the culture conditions used, the feasibility of large-

scale commercial production by the mutant is limited at present. The cost-effectiveness of this operation must be totally evaluated as an improved Reichstein's method in terms of the cost of the energy input for optimal aeration, the fermentation apparatus, and the harvesting facilities. In addition, the labor intensiveness of the purification of 2KGA must be considered. Advances in biotechnological applications, including cell immobilization technologies and supercritical extraction procedures, may reduce the labor intensiveness of the purification processes, but these advances add further burdens in terms of the equipment and energy input involved.

On the other hand, the exact mechanism of 2KGA fermentation by our mutants remains to be elucidated for further improvement of 2KGA productivity.

The following three pathways for the biosynthesis of 2KGA have been proposed: (1) the L-sorbose pathway suggested by Makover *et al.*,¹⁶⁾ (2) the one step reaction involving the oxidation of the C-1 position of L-sorbose to 2KGA described by Tengerdy,¹⁷⁾ (3) a multi-step conversion of L-sorbose to 2KGA in which L-idose and L-idonic acid were sequential intermediates described by Okazaki *et al.*¹⁸⁻²⁰⁾

In our 2KGA fermentation from L-sorbose or D-sorbitol, the accumulation of L-idonic acid was observed and then L-idonic acid was converted to 2KGA as shown in Fig. 2. L-Idonic acid is suggested to be one of the intermediates for 2KGA. Kitamura *et al.*²¹⁾ reported that L-sorbose, 2KGA, and L-idonic acid were formed from L-sorbose using a cell-free extract of *Gluconobacter melanogenus* IFO 3293 and proposed the following metabolic pathway for the formation of 2KGA from L-sorbose:

Our growing cultures also formed L-sorbose, 2KGA, and L-idonic acid from L-

sorbose in agreement with the results of Kitamura.

However, the characterization of the enzymes concerned with these reactions has not been elucidated. In view of 2KGA fermentation, we are interested in explaining why the 2KGA productivity of our mutants increase dramatically.

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References

- 1) T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chem. Acta*, **17**, 311 (1934).
- 2) H. T. Huang, U. S. Patent, 3,043,749 (July 10, 1962).
- 3) M. Isono, I. Nakanishi, K. Sasajima, K. Motizuki, T. Kanzaki, H. Okazaki and H. Yoshino, *Agric. Biol. Chem.*, **32**, 424 (1968).
- 4) K. Motizuki, T. Kanzaki, H. Okazaki, H. Yoshino, K. Nara, M. Isono, I. Nakanishi and K. Sasajima, Japan Patent Publication, No. 51-40154 (Nov. 1, 1976).
- 5) I. Chibata, S. Yamada and S. Ishikawa, Japan Patent Publication, No. 49-39838 (Oct. 29, 1974).
- 6) Y. G. Lin, Y. L. Hua, D. J. Liu, T. Z. Xin, W. D. Si and Y. Z. Zheng, *Acta Microbiologica Sinica*, **20**, 246 (1980).
- 7) Y. Zinsheng, *Acta Microbiologica Sinica*, **21**, 185 (1981).
- 8) C. K. A. Martin and D. Perlman, *Eur. J. Appl. Microbiol.*, **3**, 9195 (1976).
- 9) T. Sonoyama, H. Tani, K. Matsuda, B. Kaseyama, M. Tanimoto, K. Kobayashi, S. Yage, H. Kyotani and K. Mitsushima, *Appl. Environ. Microbiol.*, **43**, 1064 (1982).
- 10) S. Anderson, C. B. Marks, R. Lazorus, J. Miller, K. Stafford, J. Seymour, D. Light, W. Rastetter and D. Estell, *Science*, **230**, 144 (1985).
- 11) M. Kulhanek, *Adv. Appl. Microbiol.*, **12**, 11 (1970).
- 12) K. Matsushita, E. Shinagawa, O. Adachi and M. Ameyama, *Agric. Biol. Chem.*, **45**, 1515 (1981).
- 13) Y. Setoguchi, M. Shinjoh, S. Someha, T. Hoshino and A. Fujiwara, Abstracts of Papers, the Annual Meeting of the Society of Fermentation Technology, Japan, Osaka, October, 1987, p. 77.
- 14) T. Akamatsu and J. Sekiguchi, *Agric. Biol. Chem.*, **45**, 2887 (1981).

- 15) Y. Tajima, T. Yoshida and T. Kada, "Chemical Mutagens," Kodansha Scientific Inc., Tokyo, 1973.
 - 16) S. Makover, G. B. Ramsey, F. M. Vane, C. G. Witt and R. B. Wright, *Biotech. Bioeng.*, **17**, 1485 (1975).
 - 17) R. P. Tengerdy, *J. Biochem. Microbiol. Technol. Eng.*, **3**, 241 (1961).
 - 18) H. Okazaki, T. Kanzaki, M. Doi, K. Nara and K. Motizuki, *Agric. Biol. Chem.*, **32**, 1250 (1968).
 - 19) H. Okazaki, T. Kanzaki, K. Sasajima and Y. Terada, *Agric. Biol. Chem.*, **33**, 207 (1969).
 - 20) T. Kanzaki and H. Okazaki, *Agric. Biol. Chem.*, **34**, 432 (1970).
 - 21) I. Kitamura and D. Perlman, *Eur. J. Appl. Microbiol.*, **2**, 1 (1975).
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