2-Keto-L-gulonic Acid Fermentation

Part I. Paper Chromatographic Characterization of Metabolic Products from Sorbitol and L-Sorbose by Various Bacteria*

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A number of bacterial strains from type culture collections and natural sources were examined in their metabolic characteristics toward sorbitol and L-sorbose.

Paper chromatographic analyses of sorbitol and L-sorbose metabolites obtained from the cultures of various bacteria revealed that the organisms producing 2-keto-L-gulonic acid from sorbitol were merely found in the genera *Acetobacter*, *Gluconobacter* and *Pseudomonas*, whereas those producing the acid from L-sorbose were distributed in the twelve genera of bacteria: *Acetobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Bacillus*, *Escherichia*, *Gluconobacter*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Serratia* and *Xanthomonas*.

G. melanogenus, which was characterized by excellent production of 2-keto-L-gulonic acid from sorbitol, also formed several other sugars and sugar acids as the sorbitol metabolites. These compounds were identified to be D-fructose, L-sorbose, D-mannonic acid, L-idonic acid, 2-keto-D-gluconic acid and 5-keto-D-mannonic acid, respectively, by means of two-dimensional paper chromatography.

Bacteria producing 2-keto-L-gulonic acid from sorbitol were usually isolated from fruits but not from soil.

Although there have appeared several ways of the vitamin C manufacture, the elaborated Reichstein method is believed to be the most reliable one and extensively applied to the industrial scale production. As an improved modification of a process in the Reichstein method, a microbiological oxidation from L-sorbose to 2-keto-L-gulonic acid, an important intermediate in the vitamin C manufacture, was discovered by Huang.¹⁾ This finding gave a possibility of direct production of 2-keto-L-gulonic acid from sorbitol, instead of L-sorbose, from standpoint of the

structural similarity between sorbitol and L-sorbose. This consideration led us to the examination on metabolic characteristics of various bacteria toward sorbitol and L-sorbose.

The present paper deals with paper chromatographic characterization of sorbitol and L-sorbose metabolites found in cultures of various bacteria.

MATERIALS AND METHODS

Microorganisms. About five hundred strains of bacteria were provided from Institute for Fermentation, Osaka, Japan (I.F.O.), and another about five hundred strains were isolated from natural sources such as soil and fruits,

Medium used for isolating soil bacteria had the following composition; 1% of carbon source (sorbitol, L-sorbose or 2-keto-D-gluconic acid), 0.1% of NH_4NO_3 ,

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1) H. T. Huang, U. S. Patent, 3043749 (1962).

0.05% of K_2HPO_4 , 0.05% of KH_2PO_4 and 0.05% of $MgSO_4 \cdot 7H_2O$. Soil samples were added to the sorbitol-, L-sorbose- or 2-keto-D-gluconic acid-media and the mixtures were incubated at 24~25°C. When growth of bacteria had occurred in the media, pure cultures were separated by a conventional agar plate method and stored on bouillon agar slants. For the isolation of bacteria from fruits, a malt extract medium containing 3% of malt extract (Difco), 1% of ethanol and 1% of glacial acetic acid was used. After addition of fruit rinds, the malt extract media were incubated at 28~30°C for a week with shaking. When bacterial growth had occurred, the cultures were subjected to plating on an agar plate containing 3% of D-glucose, 1.5% of peptone and 0.5% of $CaCO_3$. After development of bacterial colonies surrounded with clear zones the organisms were isolated and stored as test organisms.

Cultivation of organisms. A liquid medium for cultivation consisted of the following composition; 5% of carbon source (sorbitol or L-sorbose), 0.5% of yeast extract and 2% of $CaCO_3$. A test organism was grown in a 100-ml conical flask containing 15 ml of the above liquid medium for a fortnight at 28~30°C on a rotary shaker.

Detection of metabolic products. Supernatant of the culture was treated with Amberlite IR-120 H^+ -form and subjected to one-dimensional ascending paper chromatography, in which water-saturated phenol (solvent A) or a 5:5:1:3 mixture of ethyl acetate, pyridine, acetic acid and water (solvent B) was used as the solvent. When two-dimensional chromatography was run, the solvent B was applied as the first solvent A as the second. For detecting the positions of spots on chromatograms, a triphenyltetrazolium chloride (TTC) spray²⁾ was usually applied in routine works; however, silver nitrate,³⁾ bromophenol blue,⁴⁾ *o*-phenylenediamine,⁵⁾ hydroxylamine-ferrous chloride⁶⁾ and orcinol-trichloroacetic acid (TCA)⁷⁾ were also used as the spray reagents in the detailed characterization of sugars and sugar acids.

Sugars and sugar acids. 2-Keto-D-gluconic, 5-keto-D-gluconic and 2,5-diketo-D-gluconic acids were obtained from D-glucose as the oxidation products of *Ps. fluorescens*, *G. suboxydans* and *G. melanogenus*, respectively, according to the methods of DeMoss⁸⁾ and Katznelson *et al.*⁹⁾ 5-Keto-D-fructose and 5-keto-D-mannonic acid were also prepared by microbial oxidation from D-fructose and D-mannose, respectively, according to the methods of Terada *et al.*^{10,11)} D-Mannonic acid was prepared from D-mannose by a conventional method of bromine oxidation. L-Idonic acid was provided from the reduction product of 5-keto-D-gluconic acid by eliminating D-gluconic acid according to the method of Gray.¹²⁾ Other sugars and sugar acids used were commercial preparations.

RESULTS

Paper chromatographic behaviors of sugars and sugar acids.

In order to facilitate the identification of metabolic products, chromatographic behaviors and color reactions for some sugars and sugar acids, which were anticipated to be products from sorbitol or L-sorbose, were examined by the one-dimensional ascending method using the solvents A and B. Table I shows the observed *R_f* values and the color reactions by the several spray reagents for the authentic compounds. The chromatographic resolution patterns of a mixture of these authentic compounds are also presented in Fig. 1. The resolution patterns indicated that the solvent A was useful for detection of D-fructose, L-sorbose, 5-keto-D-fructose, 2-keto-L-gulonic and 2,5-diketo-D-gluconic acids, while the solvent B was preferred for identification of hexonic acids as the corresponding lactones.

Characterization of metabolic products from sorbitol and L-sorbose by various bacteria.

About five hundred strains, including the

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8) R. D. DeMoss, "Methods in Enzymology," Vol. III, p. 232 (third printing, 1963).

9) H. Katznelson, S. W. Tanenbaum and E. L. Tatum, *J. Biol. Chem.*, **204**, 43 (1953).

10) O. Terada, K. Tomisawa, S. Suzuki and S. Kinoshita, *Nippon Nogeikagaku Kaishi*, **35**, 131 (1961).

11) O. Terada, S. Suzuki and S. Kinoshita, *ibid.*, **36**, 212 (1962).

12) B. E. Gray, U. S. Patent, 2421611 (1947).

TABLE I. *R_f* VALUES AND COLOR REACTIONS OF SOME SUGARS AND SUGAR DERIVATIVES

Compound	<i>R_f</i> value		Color reaction			
	Solvent A	Solvent B	TTC	AgNO ₃	BPB	FeCl ₃ -NH ₂ OH
Sorbitol	0.55	0.41	—	+	—	—
D-Fructose	0.55	0.45	+	+	—	—
L-Sorbose	0.45	0.44	+	+	—	—
5-Keto-D-fructose	0.29	0.40	+	+	—	—
D-Gluconic acid	0.23	0.19	—	+	+	—
D-Mannonic acid	0.24	0.20	—	+	+	—
L-Idonic acid	0.24	0.19	—	+	+	—
L-Gulonic acid	0.23	0.20	—	+	+	—
D-Gluconolactone	0.52	0.77	—	+	±	+
D-Mannonolactone	0.58	0.64	—	+	±	+
L-Idonolactone	0.51	0.76	—	+	±	+
L-Gulonolactone	0.56	0.62	—	+	±	+
2-Keto-D-gluconic acid	0.24	0.20	+	+	+	—
2-Keto-D-gulonic acid	0.19	0.19	+	+	+	—
5-Keto-D-gluconic acid	0.25	0.25	+	+	+	—
5-Keto-D-mannonic acid	0.23	0.24	+	+	+	—
2,5-Diketo-D-gluconic acid	0.09	0.16	+	+	+	—

FIG. 1. Separation of Some Sugars and Sugar Derivatives by Paper Chromatography.

Solvent A: water-saturated phenol; Solvent B: pyridine, ethyl acetate, acetic acid, water (5:5:1:3).

Compounds: 1=sorbitol, 2=D-fructose, 3=L-sorbose, 4=5-keto-D-fructose, 5=D-gluconic acid, 5'=D-gluconolactone, 6=D-mannonic acid, 6'=D-mannonolactone, 7=L-idonic acid, 7'=L-idonolactone, 8=L-gulonic acid, 8'=L-gulonolactone, 9=2-keto-D-gluconic acid, 10=2-keto-L-gulonic acid, 11=5-keto-D-gluconic acid, 12=5-keto-D-mannonic acid, 13=2,5-diketo-D-gluconic acid.

I.F.O. type cultures and the bacteria which were being preserved in our laboratory, were subjected to cultivation in the sorbitol and L-sorbose media, and the metabolic products were analyzed by ascending paper chromatography.

As shown in Table II, it was observed that many strains in the genus *Acetobacter* produced D-fructose, L-sorbose, keto sugar acids and other unidentified reducing substances from sorbitol. Table III, where the bacteria were rearranged according to the characteristics in their sorbitol and L-sorbose metabolism, indicates that the organisms producing L-sorbose from sorbitol were found in more genera of bacteria than those including D-fructose-forming bacteria from sorbitol. It is also indicated in Table III that the organisms producing keto sugar acid from sorbitol were restricted to the genera *Acetobacter*, *Gluconobacter* and *Pseudomonas*, whereas those producing the acids from L-sorbose were distributed widely in the twelve genera: *Acetobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Bacillus*, *Escherichia*, *Gluconobacter*, *Klebsiella*, *Micrococcus*, *Pseudomonas*, *Serratia* and *Xanthomonas*.

TABLE II. OXIDATION OF SORBITOL AND L-SORBOSE BY VARIOUS BACTERIA

Genus of bacteria	Number of test organisms	Sorbitol oxidation				L-Sorbose oxidation		
		Number of strains producing				Number of strains producing		
		Keto sugar acids	L-Sorbose	D-Fructose	Other reducing substances	Keto sugar acids	D-Fructose	Other reducing substance
<i>Acetobacter</i> and <i>Gluconobacter</i>	49	40	49	42	15	40	2	20
<i>Aerobacter</i>	11	0	0	0	4	3	0	7
<i>Aeromonas</i>	1	0	0	0	0	0	0	1
<i>Agrobacterium</i>	1	0	0	0	0	0	0	0
<i>Alcaligenes</i>	1	0	1	0	0	1	0	0
<i>Azotobacter</i>	4	0	0	0	1	1	0	0
<i>Bacillus</i>	81	0	16	7	10	4	2	23
<i>Bacterium</i>	4	0	2	0	1	0	0	2
<i>Brucella</i>	2	0	2	0	0	0	0	0
<i>Cellulomonas</i>	5	0	3	0	0	0	0	4
<i>Chromobacterium</i>	3	0	1	0	0	0	0	0
<i>Clostridium</i>	6	0	0	0	5	0	0	0
<i>Corynebacterium</i>	3	0	0	0	0	0	0	0
<i>Escherichia</i>	37	0	3	0	4	1	0	23
<i>Erwinia</i>	3	0	1	0	1	0	0	3
<i>Flavobacterium</i>	8	0	0	0	0	0	0	0
<i>Klebsiella</i>	1	0	1	0	0	1	0	0
<i>Lactobacillus</i>	44	0	7	1	2	0	0	0
<i>Leuconostoc</i>	7	0	0	0	0	0	0	0
<i>Micrococcus</i>	11	0	0	2	1	1	0	0
<i>Mycobacterium</i>	8	0	0	0	0	0	0	0
<i>Nocardia</i>	6	0	0	0	1	0	0	0
<i>Protaminobacter</i>	2	0	1	0	0	0	0	0
<i>Proteus</i>	7	0	1	0	0	0	0	6
<i>Pseudomonas</i>	156	2	78	5	2	136	10	9
<i>Salmonella</i>	2	0	0	0	1	0	0	0
<i>Sarcina</i>	6	0	1	0	0	0	0	0
<i>Serratia</i>	13	0	0	2	3	12	0	5
<i>Shigella</i>	1	0	0	0	0	0	0	1
<i>Streptococcus</i>	1	0	0	0	0	0	0	0
<i>Staphylococcus</i>	1	0	0	0	0	0	0	0
<i>Xanthomonas</i>	13	0	1	0	5	3	0	11

TABLE III. TAXONOMIC POSITION OF BACTERIA PRODUCING KETO SUGAR ACIDS, D-FRUCTOSE AND L-SORBOSE

Keto sugar acid-producing bacteria		D-Fructose-producing bacteria		L-Sorbose-producing bacteria
from sorbitol	from L-sorbose	from sorbitol	from L-sorbose	from sorbitol
<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Acetobacter</i>	<i>Acetobacter</i>
<i>Gluconobacter</i>	<i>Alcaligenes</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Alcaligenes</i>
<i>Pseudomonas</i>	<i>Aerobacter</i>	<i>Gluconobacter</i>	<i>Gluconobacter</i>	<i>Bacillus</i>
	<i>Azotobacter</i>	<i>Lactobacillus</i>	<i>Pseudomonas</i>	<i>Bacterium</i>
	<i>Bacillus</i>	<i>Micrococcus</i>		<i>Brucella</i>
	<i>Escherichia</i>	<i>Pseudomonas</i>		<i>Cellulomonas</i>
	<i>Gluconobacter</i>	<i>Serratia</i>		<i>Chromobacterium</i>
	<i>Klebsiella</i>			<i>Erwinia</i>
	<i>Micrococcus</i>			<i>Escherichia</i>
	<i>Pseudomonas</i>			<i>Gluconobacter</i>
	<i>Serratia</i>			<i>Klebsiella</i>
	<i>Xanthomonas</i>			<i>Lactobacillus</i>
				<i>Protaminobacter</i>
				<i>Proteus</i>
				<i>Pseudomonas</i>
				<i>Sarcina</i>
				<i>Xanthomonas</i>

TABLE IV. OXIDATION PRODUCTS FROM SORBITOL AND L-SORBOSE BY KETO SUGAR ACID-PRODUCING BACTERIA

Organism*	Sorbitol oxidation (detected substance by paper chromatography)			L-Sorbose oxidation (detected substance by paper chromatography)		
	2-Keto-L-gulonic acid	2-Keto-D-gluconic acid or 5-Keto-D-mannonic acid	D-Fructose L-Sorbose	2-Keto-L-gulonic acid	2-Keto-D-gluconic acid or 5-Keto-D-mannonic acid	D-Fructose 5-Keto-D-fructose
<i>Acetobacter acetosus</i>	++	+	+	-	-	-
<i>A. aurantium</i>	+	+	+	++	+	+
<i>A. kuetzingianus</i>	-	-	+	++	-	-
<i>A. rancens</i>	-	-	+	+	+	-
<i>A. xylinum</i>	+	+	+	++	+	-
<i>Bacterium orleanense</i>	++	+	+	+	+	+
<i>Gluconobacter albidus</i>	+	+	+	++	+	-
<i>G. capsulatus</i>	-	+	+	+	-	-
<i>G. cerinus</i>	++	+	+	++	+	+
<i>G. dioxycetonicus</i>	-	+	+	+	+	+
<i>G. gluconicus</i>	+	+	+	+	+	-
<i>G. industrius</i>	+	+	+	+	+	+
<i>G. melanogenus</i>	+++	+	+	+++	+	-
<i>G. nonoxygluconicus</i>	++	+	-	++	+	+
<i>G. oxydans</i>	+	+	++	+	+	+
<i>G. rubiginosus</i>	+++	+	++	+++	+	-
<i>G. suboxydans</i>	+	+	+	+	+	-
<i>G. suboxydans</i> var. α	+	+	+	+	+	-
<i>Aerobacter aerogenes</i>	-	-	-	+	+	±
<i>Alcaligenes faecalis</i>	-	-	+	+	-	-
<i>Azotobacter agilis</i>	-	-	-	+	-	-
<i>Bacillus subtilis</i>	-	-	-	+	-	-
<i>Escherichia coli</i>	-	-	-	+	-	-
<i>Klebsiella pneumoniae</i>	-	-	-	+	-	-
<i>Micrococcus</i> sp.	-	-	-	+	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	++	+	+
<i>Serratia marcescens</i>	-	-	-	+	+	-
<i>Xanthomonas citri</i>	-	-	±	+	+	±

* Species names of the organisms were presented according to List of Cultures, Fourth Edition, 1968 issued by Institute for Fermentation, Osaka, Japan.

The keto sugar acid-forming bacteria were further examined in their metabolic characteristics toward sorbitol and L-sorbose. The results, presented in Table IV, indicate that acetic acid bacteria comprising *Acetobacter*, *Gluconobacter* and *Bacterium* revealed resembling

patterns in the sorbitol and L-sorbose metabolisms with a few exceptions; these organisms usually produced 2-keto-L-gulonic acid with concomitant production of D-fructose, 2-keto-D-gluconic and/or 5-keto-D-mannonic acids. On the other hand, the keto sugar acid-forming

FIG. 2. Chromatographic Map of Sorbitol Metabolites of *Gluconobacter melanogenus*.

Solvent A: water-saturated phenol; Solvent B: pyridine, ethyl acetate, acetic acid, water (5:5:1:3).

Authentic compounds: 1=sorbitol, 2=D-fructose, 3=L-sorbose, 4=5-keto-D-fructose, 5=D-gluconic acid, 5'=D-gluconolactone, 6=D-mannonic acid, 6'=D-mannonolactone, 7=L-idonic acid, 7'=L-idonolactone, 8=L-gulonic acid, 8'=L-gulonolactone, 9=2-keto-D-gluconic acid, 10=2-keto-L-gulonic acid, 11=5-keto-D-gluconic acid, 12=5-keto-D-mannonic acid, 13=2,5-diketo-D-gluconic acid.

Black Spot: TTC and $\text{AgNO}_3\text{-KOH}$ positive; White spot: TTC negative, $\text{AgNO}_3\text{-KOH}$ positive.

TABLE V. COLOR REACTIONS OF METABOLIC PRODUCTS FROM SORBITOL BY *Gluconobacter melanogenus*

Compound	$\text{AgNO}_3\text{-KOH}$	TTC	$\text{NH}_2\text{OH}\cdot\text{FeCl}_3$	<i>o</i> -Phenylenediamine		Orcinol-TCA
				visible	under UV	
A	brown	red	—	greyish green	fluorescence	—
B	brown	red	—	greyish green	fluorescence	—
C	brown	red	—	greyish blue	—	—
D	brown	red	—	light brown	—	greenish yellow
E	brown	—	—	—	—	—
F	brown	red	—	light brown	—	greenish yellow
G	brown	—	brick red	—	—	—
H	brown	—	brick red	—	—	—

bacteria other than acetic acid bacteria did not practically produce any of the reducing substances other than 2-keto-L-gulonic acid.

As indicated in Table IV, *G. melanogenus* produced 2-keto-L-gulonic acid in good yields, therefore, the organism was used in the further detailed examination of sorbitol metabolism.

Characterization of metabolic products from sorbitol by *Gluconobacter melanogenus*.

To obtain a precise information of the sorbitol metabolites, the products from the culture of *G. melanogenus* were examined by the two-dimensional chromatography. The

developed chromatograms, as depicted in Fig. 2, indicate the presence of eight compounds which were tentatively termed as compounds A, B, C, D, E, F, G and H. Comparing this chromatogram with that of a mixture of the authentic sugars and sugar acids, A, B, D, E, F and G were presumed to be 2-keto-L-gulonic acid, 2-keto-D-gluconic acid, L-sorbose, sorbitol (substrate), D-fructose and D-mannonic acid, respectively. As for compounds C and H, the two-dimensional chromatography using the solvents A and B did not allow of strict identification of the compounds, however, one-

TABLE VI. OXIDATION OF SORBITOL AND L-SORBOSE BY BACTERIA ISOLATED FROM NATURAL SOURCES

Source of bacteria	Medium for isolation	Number of isolates	Sorbitol oxidation				L-Sorbose oxidation		
			Number of strains producing				Number of strains producing		
			Keto sugar acids	L-Sorbose	D-Fructose	Other reducing substances	Keto sugar acids	D-Fructose	Other reducing substances
Soil	Sorbitol medium	264	0	16	13	16	24	0	2
Soil	Sorbose medium	110	0	8	2	10	30	1	2
Soil	2-Keto-gluconate medium	108	0	2	2	8	2	0	1
Fruits	Glucose medium	30	3	7	4	3	2	1	1

dimensional chromatographies using another solvent systems permitted the determination of these two compounds; C behaved as 5-keto-D-mannonic acid with the solvent system of *n*-butanol, formic acid and water (4:1:1.5), which was effective for the differentiation of 5-keto-D-mannonic acid (*R_f*, 0.17) and 5-keto-D-gluconic acid (*R_f*, 0.24), and H behaved as the lactone of L-idonic acid with the system of *n*-butanol, acetic acid and water (4:1:1), which resolved the lactones of L-idonic acid (*R_f*, 0.23), L-gulonic acid (*R_f*, 0.31) and D-mannonic acid (*R_f*, 0.17) one another. Reliability of the chromatographic identification was further confirmed by observing several color reactions toward the respective compounds migrated on the chromatogram as indicated in Table V

Metabolic patterns of sorbitol and L-sorbose by bacteria isolated from natural sources.

About five hundred strains of bacteria isolated from soil and fruits were tested for the metabolic characteristics toward sorbitol and L-sorbose. The results, summarized in Table VI, show the distinctive evidence that the organisms producing keto sugar acid from sorbitol were isolated from fruits but not from soil, whereas those producing the acid from L-sorbose were obtained widely from both sources. Since acetic acid bacteria are usually found in nature where is abundant in plant

materials, the strains from fruits probably belong to the genus *Acetobacter* or *Gluconobacter*, although the taxonomic examinations of these organisms have not been performed.

DISCUSSION

Two types of sorbitol oxidation are known in microorganisms; one of which involves the conversion of sorbitol to D-fructose and the other to L-sorbose. Although the formation of D-fructose has been found in various bacteria such as *Acetobacter*,^{13,14)} *Azotobacter agilis*,¹⁵⁾ *Cellovibrio polytrophicus*,¹⁶⁾ *Bacillus*,^{17,18)} *Nocardia corallina*,¹⁹⁾ *Pseudomonas*,^{18,20)} and even in some yeast, *Candida utilis*,¹³⁾ the L-sorbose formation, which is generally accepted as the Bertrand type oxidation, has been discovered merely in *Acetobacter* species^{14,21)} and in *Ps. fluorescens*.²⁰⁾

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Nevertheless our results, as shown in Table III, indicate that the L-sorbose-producing organisms were distributed more widely in bacterial flora than the D-fructose-producing organisms, although the final conclusion should be given by the direct identification of the metabolite after isolation.

As for L-sorbose oxidation in microorganisms, acetic acid bacteria have been the only organisms to be examined,^{22,23)} until some pseudomonad organisms were found, by Huang,¹⁾ to convert L-sorbose to 2-keto-L-gulonic acid. Although Huang did not recognize this type of conversion in *Ps. aeruginosa*, our results reveal that the organism, among pseudomonad organisms, produced 2-keto-L-gulonic acid in better yields from L-sorbose. Moreover, it is

shown in our study that the conversion of L-sorbose to 2-keto-L-gulonic acid was effected by other bacteria belonging to the genera *Acetobacter*, *Aerobacter*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Escherichia*, *Gluconobacter*, *Klebsiella*, *Micrococcus*, *Serratia* and *Xanthomonas*.

Our finding that 2-keto-L-gulonic acid was able to be derived directly from sorbitol by the action of several acetic acid bacteria would be worth noting with respect to the Reichstein method of the vitamin C manufacture. The metabolic pathway relating to the 2-keto-L-gulonic acid production in acetic acid bacteria will be described in a forthcoming paper.

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