

The Production of 2-Keto-L-Gulonic Acid by Different *Gluconobacter* Strains

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THE PRODUCTION OF 2-KETO-L-GULONIC ACID BY DIFFERENT *GLUCONOBACTER* STRAINS

Lana Amine Nassif

(ABSTRACT)

Vitamin C is industrially produced by the Reichstein method, which uses gluconobacters to oxidize sorbitol to sorbose then a chemical process to convert sorbose to 2-keto-L-gulonic acid (2-KLG). The establishment of a more extensive microbial process for 2-KLG production translates into a less expensive and more efficient production of vitamin C. I examined pure strains and mixed cultures for their ability to produce 2-KLG using thin layer and high performance liquid chromatography. The DSM 4027 mixed culture produced the highest yield, 25 g/L, of 2-KLG from 100 g/L of sorbose, while the gram-negative rods isolated from DSM 4027 produced 8.8 g/L, and *B. megaterium* isolated from DSM 4027 produced 1.4 g/L. Thus, the gram-negative rods in the mixed culture were the primary 2-KLG producer, but *B. megaterium* in the DSM 4027 mixture enhanced this synthesis. Authentic pure cultures of *Gluconobacter oxydans* IFO strain 3293 and ATCC strain 621 produced 3.4 g/L and 5.7 g/L, respectively. Attempts to co-culture the isolated *B. megaterium* with the isolated gram-negative rods and authentic *Gluconobacter* strains did not increase 2-KLG production, nor did growing the cultures on *B. megaterium* spent media. *Bacillus megaterium* produced an unidentified keto-compound detected on the TLC chromatograms, which suggested that *B. megaterium* converted sorbose to an intermediate that may then be converted by the gram-negative rods in DSM 4027 to 2-KLG. Limited phenotypic tests suggested that the gram-negative rods in the DSM 4027 mixture are not gluconobacters.

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TABLE OF CONTENTS

List of Figures	vi
List of Appendices	vii
INTRODUCTION	1
Gluconobacter morphology and habitat	1
Oxidative ability and metabolism.....	1
Diversity of compounds oxidized.....	2
Sorbitol-oxidizing systems that lead to vitamin C production	2
Oxidation of sorbitol to sorbose.....	2
Pathways for 2-keto-L-gulonic acid (2-KLG) production from sorbose.....	3
Pathway for 2-KLG production from D-glucose.....	4
Introduction to 2-KLG and vitamin C syntheses.....	5
Single-culture bioconversion of sorbose to 2-KLG.....	5
Two-culture bioconversion of D-glucose to 2-KLG.....	5
2-KLG production by recombinant <i>Erwinia</i> strain.....	5
2-KLG production by the mixed culture DSM 4027.....	6
Summary of microbial 2-KLG formation.....	6
Purpose of this investigation.....	6
MATERIALS AND METHODS	7
Reagents.....	7
Organisms.....	7
Glycerol stock cultures.....	7
The Fermentation Medium.....	7
Corn steep liquor (CSL) media.....	8
Isolation and maintenance of strains from the mixed culture DSM 4027.....	8
Phenotypic tests for <i>Gluconobacter</i> strain isolated from DSM 4027.....	9
Co-culture of the gram-positive rod isolated from DSM 4027 with 2-KLG producing strains.....	9
Preparation of spent medium	9
Thin layer chromatography (TLC) analysis of growth media.....	9
High performance liquid chromatography (HPLC) analysis.....	10
RESULTS	11
Growth and 2-KLG production of the mixed culture DSM 4027 incubated in the Fermentation Medium.....	11
2-KLG production by the separated gram-positive and gram-negative rods.....	15
Co-culturing the gram-positive and gram-negative rods isolated from DSM 4027.....	15
2-KLG production by authentic <i>Gluconobacter oxydans</i> strains.....	15

2-KLG production by authentic <i>G. oxydans</i> cultures when grown with the gram-positive isolate from DSM 4027.....	20
2-KLG production by various cultures when grown in spent Fermentation Medium from culturing the gram-positive isolate.....	20
Phenotypic tests on the gram-negative isolate from DSM 4027.....	24
DISCUSSION	25
Interaction between strains in the mixed culture (DSM 4027).....	25
2-KLG production by <i>G. oxydans</i> IFO strain 3293 and ATCC strain 621.....	26
The effect of co-culturing the gram-positive rods isolated from DSM 4027 with various <i>Gluconobacter</i> strains on 2-KLG production.....	26
Use of spent medium to stimulate 2-KLG production.....	27
The identification of the gram-negative rod isolated from DSM 4027.....	27
CONCLUSIONS	29
SUGGESTIONS FOR FUTURE INVESTIGATORS	30
REFERENCES	31
APPENDICES	35
CURRICULUM VITAE	44

LIST OF FIGURES

Figure 1. Thin-layer chromatographic detection of 2-keto-L-gulonic acid (2-KLG) in the Fermentation Medium after one through 10 days incubation of (a) the mixed DSM 4027 culture, (b) the gram-positive isolate from DSM 4027, and (c) the gram-negative isolate from DSM 4027	12
Figure 2. Quantitative determination of 2-keto-L-gulonic acid (2-KLG) production on day 10 of incubation for the mixed culture DSM 4027 using high performance liquid chromatography (HPLC).....	13
Figure 3. Changes in proportion of colony types of the mixed culture DSM 4027 sampled from Fermentation Medium after (a) one, (b) five, and (c) 10 days of incubation	14
Figure 4. Quantitative determination of 2-keto-L-gulonic acid (2-KLG) production on day 10 of incubation for (a) the gram-positive isolate from DSM 4027 and (b) the gram-negative isolate from DSM 4027	16
Figure 5. Thin-layer chromatography analysis of 2-keto-L-gulonic acid (2-KLG) production in the Fermentation Medium inoculated with equal quantities of the gram-negative and gram-positive isolates from DSM 4027.....	17
Figure 6. Qualitative (a) and quantitative (b) determination of 2-keto-L-gulonic acid(2-KLG) in the Fermentation Medium of cultures of <i>G. oxydans</i> IFO strain 3293.....	18
Figure 7. Qualitative (a) and quantitative (b) determination of 2-keto-L-gulonic acid (2-KLG) in the Fermentation Medium of cultures of <i>G. oxydans</i> ATCC strain 621	19
Figure 8. Thin-layer chromatography analysis of 2-keto-L-gulonic acid (2-KLG) production in the Fermentation Medium inoculated with equal quantities of <i>G. oxydans</i> IFO strain 3293 and the gram-positive isolate from DSM 4027.....	21
Figure 9. Thin-layer chromatographic detection of 2-keto-L-gulonic acid (2-KLG) in day-two spent medium from culture of the gram-positive isolate from DSM 4027 inoculated with <i>G. oxydans</i> IFO strain 3293.....	22
Figure 10. Thin-layer chromatographic detection of 2-keto-L-gulonic acid (2-KLG) in the gram-positive isolate day-two (a) and day-six (b) spent medium inoculated with the gram-negative isolate from DSM 4027.....	23

LIST OF APPENDICES

Appendix A. Thin layer chromatographic separation of 2-keto-L-gulonic acid from sorbose.....	35
Appendix B. High performance liquid chromatographic detection of 2-keto-L-gulonic acid.....	38
Appendix C. Pathways for 2-keto-L-gulonic acid production.....	42

INTRODUCTION

Gluconobacter morphology and habitat. The genus *Gluconobacter* is one of two genera called the acetic acid bacteria, and both of these genera are placed in the family *Acetobacteraceae* due to their ability to convert ethanol to acetic acid (8). The gluconobacters are gram negative, short, plump rods that occur singly or in pairs and have polar flagella if motile (8). They grow optimally at 25° to 30 °C and pH 5.5 to 6.0, and they can survive in environments where the pH is as low as 3.6 (8, 24). The natural habitats of the gluconobacters reflect their ability to generate acidic products from sugars, alcohols, and polyols, and they include flowers, fruits, honey bees, cider, beer, wine, and vinegar (8, 18). Although the gluconobacters are not pathogenic to humans and animals, they are suggested to cause pink disease in pineapples (6) and rot in apples and pears (34).

Oxidative ability and metabolism. The gluconobacters carry out a strictly respiratory type of metabolism using oxygen as the sole electron acceptor (8). They only partially oxidize their carbon and energy sources and release the incompletely oxidized product into the surrounding medium. These limited oxidations are accomplished in two ways: single-step oxidations, or more extensive oxidations (4).

Single-step oxidations are catalyzed by NAD(P) independent, plasma membrane-bound dehydrogenases (3, 7, 23). Two hydrogen atoms are removed from the primary substrate, and the electrons are passed down the electron transport chain and ultimately combine with oxygen (34). The membrane-bound dehydrogenases release the partially oxidized compound back into the surrounding medium. These rapid, single-step oxidations consume large quantities of oxygen, and the oxidation products are recovered nearly quantitatively from the medium, thereby making the gluconobacters very useful to industry (10). For example, *Gluconobacter oxydans* is used for commercial vitamin C (ascorbic acid) synthesis because of its efficient oxidation of sorbitol to sorbose, one of the precursors of ascorbic acid manufacture (14).

In addition, some strains of *Gluconobacter* carry out multiple single-step oxidations at the plasma membrane, where the product of the primary oxidation is further oxidized by secondary, and sometimes tertiary dehydrogenases. For example, through a series of oxidations, sorbose may ultimately form 2-keto-L-gulonic acid (2-KLG), the immediate precursor of vitamin C (14).

The gluconobacters are also capable of more extensive but still incomplete oxidations. The more extensive oxidation requires the transport of the substrate (the energy source) into the cell, where it is more fully oxidized, but not completely converted to carbon dioxide. Once inside the cell, soluble dehydrogenases remove two hydrogens from the energy source and transfer them to soluble hydrogen carriers such as NAD(P)⁺. These cytosolic dehydrogenases are referred to as NAD(P)-dependent, because they require NAD(P)⁺ to transport these hydrogens to the electron transport chain contained within the plasma membrane where they will eventually reduce oxygen. The oxidized substrate in the cytosol remains vulnerable to further oxidation by the soluble dehydrogenases, and the products of these incomplete oxidations (such as acetic acid) are excreted into the surrounding medium (9).

The gluconobacters are strict aerobes, yet they lack a complete tricarboxylic acid (TCA) cycle due to the absence of the succinate dehydrogenase enzyme complex (13). Therefore, it is

thought that the gluconobacters use the remaining enzymes normally associated with the TCA cycle for biosynthetic purposes such as the conversion of acetate to the amino acid glutamate (13).

The hexose monophosphate pathway is believed to be responsible for the second, more extensive type of internal, yet still incomplete, oxidations (24). Using this pathway, the gluconobacters can partially oxidize a carbon source such as glucose to carbon dioxide and glyceraldehyde-3-phosphate. The latter compound can be further oxidized to acetate by the same enzymes used in the second half of the Embden-Meyerhoff-Parnas pathway. In addition, the enzymes of the Entner-doudoroff pathway have also been demonstrated in the gluconobacters (8).

Diversity of compounds oxidized. Not only do the gluconobacters oxidize substrates and consume oxygen at very rapid rates, they also appear to oxidize many different types of substrates. In excess of one hundred different hydroxyl-containing compounds are reportedly oxidized by the gluconobacters (9). These compounds have been grouped (Edwards, M.S. thesis, 1990) into nine structural categories: aliphatic monoalcohols, aliphatic dialcohols, aliphatic polyalcohols, cyclic acids, monosaccharides, disaccharides, oligosaccharides, aldehydes, and carboxylic acids.

For those compounds with greater than one hydroxyl group, the gluconobacters follow the Bertrand-Hudson Rule (8,12). This rule states that the secondary alcohol group must be in the *cis* position with the primary alcohol group and also have the D-configuration in order to be oxidized.

Sorbitol-oxidizing systems that lead to vitamin C production. Sorbitol is one of the many polyol compounds that are oxidized efficiently to sorbose by the gluconobacters (14). Sorbitol metabolism has been extensively studied because of its potential use for industrial production of vitamin C (14). Although there are other ways of vitamin C manufacture (17), the elaborated Reichstein method (25) is believed to be the most reliable and most industrially applied. The Reichstein process chemically hydrogenates D-glucose to produce D-sorbitol, which is then dehydrogenated by *Gluconobacter oxydans* to yield L-sorbose. The L-sorbose is chemically (rather than microbiologically) converted to diacetonesorbose, which is then chemically oxidized to diacetone-2-keto-L-gulonic acid. This product finally forms the important intermediate, 2-keto-L-gulonic acid (2-KLG), which undergoes a cyclization to yield vitamin C. The conversion of D-sorbitol to L-sorbose is the only microbiological oxidation used in the Reichstein method. The establishment of an efficient microbial conversion of sorbitol to 2-KLG has long been one of the objectives of the vitamin C industry (30).

In 1962, Huang (15) described various species of *Pseudomonas*, which produce 2-KLG with yields of less than 1 g/L from media containing 20 g/L of L-sorbose. This finding was the first reported bioconversion of sorbose to 2-KLG.

In 1968, Isono *et al.* (16) studied a variety of microorganisms that produced 2-KLG from D-sorbitol and L-sorbose. They found 2-KLG production from sorbose by microorganisms classified as *Acetobacter*, *Azotobacter*, *Bacillus*, *Corynebacterium*, *Escherichia*, *Gluconobacter*, *Klebsiella*, *Pseudomonas*, and *Serratia*.

Although the focus of my research project is on the oxidation of L-sorbose to 2-KLG, a brief overview of D-sorbitol oxidation to L-sorbose will be helpful to fully understand the complete metabolism.

Oxidation of sorbitol to sorbose. In 1982, Shinagawa *et al.* (27) purified D-sorbitol dehydrogenase, the enzyme that catalyzes the conversion of D-sorbitol to L-sorbose, from the

membrane fraction of *G. suboxydans* (now called *G. oxydans*). They found that this enzyme contained three subunits: a flavoprotein (63 kDa), cytochrome c (51 kDa), and an unknown protein (17 kDa). The cytochrome c was not reduced by the addition of sorbitol unless coenzyme Q₁ is added. However, in 1990, Hoshino *et al.* (14) isolated a membrane-bound dehydrogenase from *G. melanogenus* IFO strain 3293, which oxidized both sorbitol and mannitol efficiently. This finding generated speculations on the substrate specificity of D-sorbitol dehydrogenase and the constitutive formation of the enzyme.

Pathways for 2-keto-L-gulonic acid (2-KLG) production from sorbose. To determine a metabolic pathway for catabolism of a substrate in a microorganism, it is desirable to use as many criteria as possible, such as isolation of metabolic products of the substrate from growing cultures, detection of metabolic products formed from intermediates by resting cells, analysis of catabolic enzymes, application of tracer technique using radioactive substrates, and other methods. Several of these techniques have been performed to determine the pathway used by the gluconobacters and other bacteria in the oxidation of L-sorbose to 2-KLG as described below.

A single-step oxidation in *Pseudomonas* species involves the direct oxidation of sorbose to 2-KLG was described in 1961 by Tengerdy (31) (Reaction 1, Appendix C). This pathway was proposed because no other intermediates were detected in the supernatant by paper electrophoresis when whole cells were grown on L-sorbose. In 1972, Tsukada *et al.* (32) proposed a one step reaction that oxidized the C-1 carbon of L-sorbose to 2-KLG by *Gluconobacter melanogenus* (now called *G. oxydans*) IFO strain 3293. Tsukada *et al.* (32) first grew cells in media containing either L-sorbose or glycerol then after harvest, the non-growing, whole-cell suspensions were supplied with L-sorbose for further oxidation. Although both glycerol and sorbitol-grown cells were able to consume L-sorbose, only those cells that were grown on L-sorbose converted L-sorbose to 2-KLG. These results suggested that the enzyme(s) involved in 2-KLG formation was (were) induced by L-sorbose. In addition, the enzyme system(s) was detected in the washed particulate fraction pelleted at 109,000 × g, although the presence of the soluble fraction was required for maximum activity.

The results of Tsukada *et al.* (32) raise two questions. First, is the pathway for 2-KLG formation by *G. melanogenus* IFO strain 3293 really a one-step oxidation with no intermediates? Second, is the enzyme(s) responsible for this oxidation really L-sorbose specific, or is the enzyme(s) able to react with sorbosone and/or idose?

In 1990, Yin *et al.* (35) speculated on the use of this single-step pathway by strains of *Gluconobacter* that are capable of producing 80 g/L of 2-KLG from a growth medium containing 80 g/L of sorbose. *Gluconobacter* strains were used in a co-culture with another microorganism such as *Bacillus megaterium*, or *Gluconobacter* strains were grown in a medium supplemented with rare earth metals or crude preparations from yeast cells. However, Yin *et al.* (35) did not disclose whether the *Gluconobacter* strains were ever isolated and grown in pure culture, nor did they reveal whether pure cultures of these gluconobacters produced 2-KLG in high yield. Also, I have found no published evidence for the presence of the enzyme(s) responsible for a single-step oxidation of sorbose to 2-KLG.

In 1969, Okazaki *et al.* (21) proposed the idose pathway and suggested a multi-step conversion of L-sorbose to 2-KLG by *G. melanogenus* IFO strain 3292 (Reaction 2, Appendix C). They proposed L-idose and L-idonic acid as sequential intermediates in the formation of 2-KLG from sorbose.

These authors reacted resting cell of *G. melanogenus* IFO strain 3293 with different substrates including sorbose, idose and idonic acid. Their results indicated that 2-KLG was derived from L-sorbose, L-idose, L-idonic acid and D-sorbitol. The authors concluded " ... that the formation of 2-KLG from L- sorbose is conducted through L-idose and L-idonic acid successively, although Tengerdy described 2-KLG production in *Pseudomonas* species through the oxidation of L-sorbose at C-1 ".

In 1990, Hoshino *et al.* (14) used cell fractions and artificial electron acceptors and reported on the presence of L-idonate dehydrogenase, the enzyme responsible for the conversion of L-idonic acid to 2-KLG, in the membrane fractions of cells of mutant *G. melanogenus* IFO strain 3293.

In 1975, Makover *et al.* (19) reported evidence for yet another pathway of 2-KLG production from sorbose by *G. melanogenus* IFO strain 3293 (Reaction 3, Appendix C). The sorbosone pathway was proposed, because sorbosone was found in the growth medium of cells accomplishing 2-KLG biosynthesis. These investigators attempted to locate the enzymes responsible for this two-step conversion. They failed to demonstrate the absolute requirement of NAD^+ for the conversion of L-sorbose to L-sorbosone, and this was strong evidence against the involvement of a soluble (cytosolic) L-sorbose dehydrogenase. However, they obtained evidence supporting the particulate (membrane-bound) nature of the L-sorbosone dehydrogenase responsible for the conversion of L-sorbosone to 2-KLG.

The oxidation of L-sorbose to L-sorbosone was also studied in 1975 by Kitamura *et al.* (17). Their study employed the same culture (*G. melanogenus* IFO strain 3293), and they found that growing cultures, washed cells and cell-free extracts converted L-sorbose to L-sorbosone. Factors influencing this conversion included incubation temperature and the addition of betaine, choline, and organic solvents such as toluene to the growing cultures. In addition, these authors demonstrated that NAD^+ and NADP^+ were inactive as electron acceptors in cell free extracts of *G. melanogenus* IFO 3293, and they concluded "... that the enzyme system differs from the one reported by Makover *et al.*".

In 1990, Sugisawa *et al.* (30) isolated a spontaneous mutant of *G. melanogenus* IFO strain 3293 called SPO 1 that produced 13 g/L of 2-KLG from L-sorbose. Further strain improvement was done by ultraviolet irradiation and spheroplast fusion, and they were able to achieve a production of 50 g/L of 2-KLG from media containing 100 g/L of L-sorbose.

In the same year, Hoshino *et al.* (14) studied the enzyme(s) system involved in the production of 2-KLG by the same UV mutant, and they confirmed the presence of the L-sorbosone pathway in *G. melanogenus* IFO strain 3293. They found a membrane-bound L-sorbose dehydrogenase, which catalyzed the conversion of L-sorbose to L-sorbosone, and they also described a soluble (cytosolic) L-sorbosone dehydrogenase, which catalyzed the conversion of L-sorbosone to 2-KLG and required NAD^+ or NADP^+ as a co-factor. Accordingly, they suggested that the transport of L-sorbosone into the cytosol was essential for the formation of 2-KLG. These observations contradicted the findings of Kitamura *et al.* (17) who used the parent strain (*G. melanogenus* IFO strain 3293) and reported that both of these enzymes were located in the membrane fraction.

Pathway for 2-KLG production from D-glucose. In 1982, Sonoyama *et al.* (28) reported evidence for a pathway for 2-KLG production from D-glucose in *Corynebacterium*

species (Reaction 4, Appendix C). This pathway consisted of oxidizing D-glucose to 2,5-diketogluconate (2,5-DKG), then reducing 2,5-DKG to 2-KLG.

Introduction to 2-KLG and vitamin C syntheses. The first synthesis of vitamin C was published in 1933 by Reichstein *et al.* (25). The process consists of a series of chemical oxidations, and only the conversion of sorbitol to sorbose is mediated by microorganisms. This industrial synthesis is still used worldwide for the production of vitamin C. At present, industries are trying to establish a more thorough microbial conversion of sorbose to vitamin C, because this would be more economical and more environmentally pleasing than the chemical conversion. Investigators are focusing on increasing the yield of 2-KLG, which is the immediate precursor of ascorbic acid, by microorganisms capable of oxidizing D-glucose, sorbitol, or sorbose, because a higher 2-KLG yield translates into a higher vitamin C production. Some of the microorganisms studied include *Acetobacter*, *Azotobacter*, *Bacillus*, *Escherichia*, *Gluconobacter*, *Pseudomonas*, *Erwinia*, *Serratia*, and *Corynebacterium* (16).

Single-culture bioconversion of sorbose to 2-KLG. The published literature suggests that the production of 2-KLG by the gluconobacters has been studied extensively in only one strain, *Gluconobacter oxydans* IFO strain 3293 (14). The focus of these investigations was to determine the metabolic pathway for 2-KLG production in IFO strain 3293.

Dr. Steve Stoddard (29) conducted an unpublished study at Archer Daniels Midland Inc., where he determined the yield of 2-KLG from sorbose by various *Gluconobacter* strains in pure culture. The quantity of 2-KLG produced by these *Gluconobacter* strains averaged 1.5 g/L of 2-KLG starting from 80 g/L of sorbose. Thus, the conversion of sorbose to 2-KLG by these pure *Gluconobacter* cultures was not efficient, and the prospect of using these strains for the microbial production of vitamin C did not seem promising.

Two-culture bioconversion of D-glucose to 2-KLG. In 1982, Sonoyama *et al.* (28) used a two-culture bioconversion for the biosynthesis of 2-KLG from D-glucose where these two cultures were present in a single reactor. The first step of the process was D-glucose oxidation to 2,5-diketogluconate (2,5-DKG) by an *Erwinia* species, then the second step was a reduction of 2,5-DKG to 2-KLG by a *Corynebacterium* species. At the end of the first step, *Erwinia* sp. was lysed with sodium dodecyl sulfate to decrease the number of the viable cells, then this broth containing 2,5-DKG was inoculated with *Corynebacterium* species to reduce 2,5-DKG to 2-KLG. This process had an 84 % conversion of D-glucose to 2-KLG.

In 1996, Flinn (11) reported that investigators in China have improved the two-culture bioconversion process by substituting the *Erwinia* species with a *Gluconobacter* species for the oxidation of D-glucose to 2,5-DKG, but the Chinese investigators still used the same *Corynebacterium* strain for reduction of 2,5-DKG to 2-KLG. Flinn (11) reports that Chinese industries are currently using this technology for vitamin C production instead of the well-established Reichstein procedure used almost exclusively elsewhere in the world. With this process, China has become a leading producer and exporter of vitamin C with nearly 15,000 metric tons exported in 1994.

2-KLG production by a recombinant *Erwinia* strain. Molecular biology techniques developed in the last 15 years and the possibility of gene manipulation in microbial cells, resulted in the creation of hybrids and non-conventional production of several substances in bioreactors. Gene cloning was used in many different microorganisms, showing diversity of application in a wide range of products like hormones, vaccines, enzymes, and antibiotics (22).

Based on these ideas, Anderson *et al.* (2) used gene manipulation to combine the traits of the *Erwinia* species and *Corynebacterium* species in a single cell to simplify the conversion of D-glucose to 2-KLG. Their strategy was to clone the 2,5-DKG reductase gene (gene responsible for the reduction of 2,5-DKG to 2-KLG) from *Corynebacterium* species into *Erwinia herbicola*, which was then able to oxidize D-glucose all the way to 2,5-DKG. After identification, purification and characterization, the reductase gene was cloned into *Erwinia* species, and this recombinant strain was tested for 2-KLG production. The result was a 50 % conversion of D-glucose to 2-KLG, but this made the recombinant strain less efficient in 2-KLG production than the two-culture bioconversion process.

2-KLG production by the mixed culture DSM 4027. In 1990, a U.S. patent was issued to Yin *et al.* (35) disclosing a process for 2-KLG production by a mixed culture of *Bacillus megaterium* and a *Gluconobacter* strain. This mixed culture was deposited at the Deutsche Sammlung Mikroorganismen Collection in Germany as DSM 4027, and this mixed culture reportedly accomplished 100 % sorbose conversion to 2-KLG (80 g/L of 2-KLG was formed from 80 g/L of sorbose). The patent did not reveal any information about the role of each of the two species in 2-KLG production.

Summary of microbial 2-KLG formation. Synthesis of 2-keto-L-gulonic acid (2-KLG) has been the focus of many studies by investigators in industry and universities. These investigators have tried to establish a more extensive and efficient microbial production of vitamin C to replace the Reichstein process still exclusively used worldwide. The gluconobacters and other microorganisms capable of producing 2-KLG have been investigated to increase 2-KLG synthesis, because a higher 2-KLG yield translates into a higher vitamin C production. So far, the patented DSM 4027 mixed culture of *Bacillus megaterium* and a *Gluconobacter* strain has been the highest producer of 2-KLG.

Purpose of this investigation. The primary purposes of my research were (i) to learn more about the production of 2-keto-L-gulonic acid (2-KLG) by the gluconobacters, and (ii) to investigate the roles of *Bacillus megaterium* and *Gluconobacter* strain in the patented DSM 4027 mixed culture during 2-KLG production.

MATERIALS AND METHODS

Reagents. Bacto peptone, granulated agar, yeast extract, beef extract, and urea were purchased from Difco Laboratories, Detroit, MI. Calcium carbonate, hydrated magnesium sulfate ($\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$), hydrated cupric sulfate ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$), sodium hydroxide, potassium phosphate monobasic, corn steep liquor, D(+)glucose, mannitol, and L(-)sorbose, sodium carbonate and sodium bicarbonate were obtained from Sigma Chemical Co., St. Louis, MO. In addition, Glycerol, 1-propanol, methanol, glacial acetic acid, sulfuric acid (analytical grade reagent, concentrated), potassium hydroxide, hydrated sodium-potassium tartarate ($\text{NaK-tartarate} \cdot 4 \text{H}_2\text{O}$), and o-phosphoric acid were obtained from Fisher Scientific Co., Fair Lawn, NJ. Tetrazolium blue chloride was obtained from United States Biochemical Corp., Cleveland, OH, and 95% authentic 2-keto-L-gulonic acid (2-KLG) was generously donated by Dr. Steve Stoddard at Archer Daniels Midland Co., Decatur, IL.

Organisms. The patented mixed culture designated DSM 4027 was purchased from the Deutsche Sammlung Von Mikroorganismen (DSM) Collection in Germany; this mixed culture was reported to contain a *Gluconobacter* strain and *Bacillus megaterium* and reportedly produces a high yield of 2-KLG from sorbose (35). The patent, however, did not provide any taxonomic data to confirm these species, and since I have some preliminary results, which indicated that the reported *Gluconobacter* strain in the mixed DSM 4027 culture might not be a *Gluconobacter* species, I will refer to the *Bacillus megaterium* as the gram-positive rod and to the *Gluconobacter* strain as the gram-negative rod. *Gluconobacter oxydans* IFO strain 3293 was used because Sugisawa *et al.* (30) reported that this strain has the highest productivity in oxidizing sorbose to 2-KLG among pure *Gluconobacter* strains. *G. oxydans* ATCC strain 621 was used because it has been extensively studied in our laboratory and we wanted to learn about its ability to produce 2-KLG. In addition, the gram-positive rod and the gram-negative rod were isolated from DSM 4027 and maintained separately as pure cultures.

Glycerol stock cultures. Working-stock cultures of *Gluconobacter oxydans* IFO strain 3293 and ATCC strain 621 were prepared by growing the strains in broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone until cultures reached an optical density (O.D) of about 0.9 measured with Bausch and Lomb Spectronic 20 spectrophotometer at 620 nm. One ml of this broth culture was then transferred to sterile 5 ml vials containing 2 ml of glycerol. After thorough mixing, these stock cultures were stored in the freezer at -8°C until they were used. A 0.2 ml volume of stock culture was used to inoculate 50 ml of the Fermentation Medium, and 0.8 ml was used to inoculate 200 ml of the Fermentation Medium.

The Fermentation Medium. All cultures were routinely grown in 2 L Bellco Nephelometer flasks containing 200 ml of a Fermentation Medium described by Sugisawa *et al.* (30). This medium contained (w/v) 10.5% sorbose, 0.05% glycerol, 1.5% yeast extract, 0.25% hydrated magnesium sulfate and 2.5% calcium carbonate in ultrapure water (10), and the pH of this solution was adjusted to 7.2. Since sorbose solutions are heat sensitive and caramelizes when autoclaved, a 40% (w/v) stock solution was prepared separately, adjusted to pH 7.2, filter-sterilized, and added to the other medium components after they were autoclaved to obtain a final concentration of 10.5% sorbose. When a small cell mass was needed, 500 ml Bellco Nephelometer or 500 ml erlenmeyer flasks containing 50 ml of the Fermentation Medium were prepared in a similar way.

Corn steep liquor (CSL) media. The CSL agar plates were used to monitor the change in culture types during the growth of the mixed DSM 4027 culture in the liquid Fermentation Medium, and also to isolate each strain in pure culture. The CSL medium, as described in the U.S. patent # 4,935,359, contained (w/v) 0.3% yeast extract, 0.3% beef extract, 0.3% corn steep liquor, 1% peptone, 0.1% potassium phosphate monobasic, 0.02% hydrated magnesium sulfate, 0.1% calcium carbonate, 0.1% urea, and 2% sorbose in ultrapure water. The pH of the medium was adjusted to 6.2 before the addition of the urea and sorbose solutions. To prepare this medium, stock solutions of urea and sorbose were separately prepared and sterilized then added to an autoclaved solution that contained the other components. A pH 6.2 20% urea stock solution was autoclaved separately and added to the other autoclaved medium components to get the final concentration of 0.1% urea. A 40% stock solution of sorbose was adjusted to a pH of 6.5 then filter-sterilized, and added to the rest of the medium components after they were autoclaved to get a final concentration of 2% sorbose. Agar was added at a concentration of 2% when a solid medium was needed.

During growth of DSM 4027 in the Fermentation Medium, a loopful of this mixed culture was streaked on CSL agar plates after one, five, and ten days of incubation, and the relative proportion of the colony types formed by the gram-positive rod and gram-negative rod was estimated.

Isolation and maintenance of strains from the mixed culture DSM 4027. The DSM 4027 mixed culture was received in a lyophilized form. The vial was opened using the ATCC procedure for rehydrating freeze-dried cultures (1). Cells were resuspended and rehydrated with a sterile solution containing (w/v) 1% peptone and 1% yeast extract, then the resulting suspension was transferred aseptically to an empty, sterile, glass vial, and stored in the freezer at -8 °C. The gram positive and gram negative rods from the DSM 4027 mixed culture were isolated by streaking the mixed culture on CSL agar medium. After four days of incubation at 28 °C, two colony types were observed. The Large, circular, yellow colonies were composed of gram-positive rods, and the small, circular, creamy colonies were made up of gram-negative rods. Each colony type was then streaked separately on the CSL medium and incubated for two days to isolate the two organisms and assure culture purity.

To prepare stock cultures of these two isolates, moistened, sterile swabs were used to swab each plate and inoculate test tubes containing CSL liquid medium. After two days of incubation, 0.2 ml of this liquid CSL medium was used to inoculate 500 ml Bellco Nephelometer flasks containing 50 ml of the Fermentation medium. These cultures were incubated at 28 °C in a New Brunswick model R-20 incubator with shaking at 200 reciprocations per minute. The cultures were grown until they reached an optical density of 0.9 measured with Milton Roy Spectronic spectrophotometer (model 1201) at 620 nm. Since the undissolved calcium carbonate present in the Fermentation Medium interfered with optical density measurements, one ml of culture was transferred aseptically to a sterile test tube and put in the refrigerator for a few minutes until the calcium carbonate settled to the bottom. Then, 0.1 ml was transferred from the top of the tube to a cuvette, and a few drops of 0.1 N hydrochloric acid solution were added to dissolve the remaining calcium carbonate, and 0.9 ml of sterile, ultrapure water. The optical density measurement was taken and multiplied by 10, the dilution factor.

Since these stock-cultures were going to be used as inocula in the 2-KLG production studies, the 2-KLG, which might have been formed by these two isolates when growing in the Fermentation Medium as these stock cultures were being prepared, needed to be removed from the medium by subculturing. One ml of the stock culture was placed in a sterile microcentrifuge tube

and centrifuged. The resulting supernatant fluid was discarded, and the cells were suspended in 1 ml of a sterile solution containing (w/v) 1% peptone and 1% yeast extract. This milliliter of resuspended cells was transferred aseptically to sterile 5-ml vials containing 2 ml of glycerol.

These stock cultures were stored in the freezer at -8 °C until they were used. A 0.2 ml volume of stock culture was used to inoculate 50 ml of the Fermentation Medium, and accordingly, 0.8 ml was used to inoculate 200 ml of the Fermentation Medium.

Phenotypic tests for the gram-negative rod isolated from DSM 4027. The gram-negative rod isolated from the mixed culture DSM 4027 was subjected to a few tests, which are indicative of gluconobacters. This culture was streaked onto an ethanol/calcium carbonate agar plate (5), a 10% glucose pH 4.5 agar medium (5), ATCC mannitol agar medium (1), and Dihydroxyacetone detection plate (26), and also inoculated into ATCC mannitol broth (1).

Co-culture of the gram-positive rod isolated from DSM 4027 with 2-KLG producing strains. To determine the influence of the gram-positive rods isolated from DSM 4027 on 2-KLG production by the *Gluconobacter* strains, 0.2 ml of stock-culture containing the gram-positive rods was mixed with 0.2 ml of each of the *Gluconobacter* strains (IFO 3293 and ATCC) and with the gram negative rod isolated from DSM 4027, separately. Each combination of the gram-positive rod and one of the 2-KLG producer was grown in 50 ml of the Fermentation medium for 10 days in 500 ml nephelometer flasks.

Preparation of spent medium. To determine the influence of the gram-positive spent medium on 2-KLG production by authentic *Gluconobacter* strains and the gram-negative isolate from DSM 4027, 0.2 ml of gram-positive rod stock-culture was inoculated into two 500-ml erlenmeyer flasks containing 50 ml of the Fermentation Medium and incubated at 28°C with shaking for six days. After two and six days of incubation, the cells were removed by centrifugation in a Beckman Centrifuge (model J2-21) with a JA-14 rotor at 15,300 × g for 10 minutes. The supernatant fluid was filter-sterilized using a 50 ml Nalgene Filter-Sterilizing Unit fitted with a membrane having pores of 0.2 µm diameter. This sterile spent medium was aseptically transferred to sterile 500 ml erlenmeyer flasks containing 1.25 g of oven-sterilized calcium carbonate. This separate addition of calcium carbonate was necessary because as the spent medium is filter-sterilized, the calcium carbonate is retained on the membrane. A 0.2-ml volume of stock-culture of each of the authentic *Gluconobacter* strains or the gram-negative isolate from DSM4027 was used to inoculate the sterile spent medium. These cultures were incubated at 28°C with shaking for 10 days and were sampled as before for TLC analysis.

Thin layer chromatography (TLC) analysis of growth media. Thin layer chromatography was performed to qualitatively detect the depletion of sorbose and the accumulation of 2-KLG during the ten day incubation of pure and mixed cultures. One ml samples were taken from growth flasks every day for 10 days. Cells were aseptically removed by centrifugation, and supernatant fluids were stored at 4 °C in sterile microcentrifuge tubes until all samples for each culture could be chromatographed. Five microliters of these samples were spotted in increments on a Whatman, glass-backed, silica gel, thin layer chromatogram plate (Linear-K inert pre-adsorbent, 20×20 cm, 150 Å of pore size and 250 µm thick obtained from Fisher Scientific), using a 4:1:0.1:0.01 ratio of n-propanol : water : phosphoric acid : glacial acetic acid as the mobile phase (30). Plates were pre-developed in the mobile phase then dried prior to applying the samples. Development of the spotted samples occurred at room temperature inside a filter-paper lined jar whose atmosphere was saturated with the mobile phase. Visualization of 2-KLG and sorbose spots was done by spraying the air-dried plate with an even mist of a freshly

made spray that contained equal volumes of 0.5 % (w/v) tetrazolium blue chloride dissolved in methanol and 6N sodium hydroxide (30). The glass sprayer was purchased from Supelco and was essential in disposing an evenly mist of spray reagent. and After spraying, the plates were dried for ten minutes at 110 °C, and blue spots at two different positions indicated the presence of both 2-KLG and sorbose. Under these conditions, 2-KLG had an R_f value of 0.28, and the R_f value of sorbose was 0.55. These values were determined by using (w/v) 1 % standards of authentic 2-KLG and authentic sorbose dissolved in distilled water. These R_f values did not change when these authentic standards were dissolved in the Fermentation Medium because there was no interference by this medium as no spots were observed when the medium alone was spotted in this TLC analysis.

High performance liquid chromatography (HPLC) analysis. High performance liquid chromatography was performed to quantitatively determine the yield of 2-KLG in the Fermentation Medium by the different cultures. The HPLC system was an ion-exchange Dionex model 2010i. The column used was a Phenomenex ion-exchange column (STAR-ION, A-300, 100 mm × 4.6 mm) packed with styrene divinyl benzene, a support resin that is coated with a unique quaternary amine, and which served as the stationary phase. Ion-exchange chromatography was the method of choice, because 2-KLG is an acid that produces anions, which compete with the anions of the mobile phase for the exchange site, the quaternary amine, that coats the stationary phase. The mobile phase consisted of a mixture of 70% deionized water that was degassed with helium prior to use and 30 % of an aqueous solution of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate. This water-to-buffer proportion gave the best retention time for 2-KLG (1.73 minutes), and which was achieved by using gradient programming. The flow rate of the mobile phase was 2 ml/min, and the elution of 2-KLG was followed with a suppressed conductivity electrochemical detector. The suppressor used was a 25 mM aqueous solution of sulfuric acid, and the injection volume was 10 μ l.

A standard curve of area *versus* concentration was generated using six known concentrations of authentic 2-KLG (six standards) dissolved in the Fermentation medium.

The quantity of 2-KLG formed during culture incubation was determined by injecting 10 μ l of the supernatant fluid of the different cultures sampled on days one, two, five, six, and ten into the column, and then using the standard curve to calculate the concentrations.

The ion-exchange HPLC method does not detect sorbose because sorbose is not an ionizable compound, so the depletion of sorbose during growth could not be observed on these chromatograms.

RESULTS

Growth and 2-KLG production of the mixed culture DSM 4027 incubated in the Fermentation medium. The thin Layer chromatography method used in this study had a lower detection limit of 1.5 g/L for both sorbose and 2-KLG (Appendix A). This TLC method was used to detect the ability of the patented, mixed, culture DSM 4027 to produce 2-KLG from sorbose over 10 days in the Fermentation medium (Figure 1a). This mixed culture produced a detectable amount of 2-KLG after five days of incubation, and 2-KLG continued to be accumulated through day 10 as shown by the increase in the intensity and size of the 2-KLG spots. The largest apparent surge in 2-KLG production occurred between days five and six. The concentration of sorbose in the medium appeared to decrease as 2-KLG concentrations increased, indicating that the mixed culture was converting sorbose to 2-KLG. However, sorbose was not completely depleted from the medium during the 10-day incubation (Figure 1 a). The other spots with a greater R_f value than sorbose were not identified but represent a keto-compound.

High performance liquid chromatography was used to determine the quantity of 2-KLG produced by the mixed culture in the Fermentation medium was determined after one, two, five, six and ten days of incubation. A standard curve was prepared with authentic 2-KLG that correlates the area under the 2-KLG peak with 2-KLG concentration (Appendix B). On days one and two, no 2-KLG was detected. On day five, the mixed culture produced 1.7 g/L. On day six, the medium contained 17 g/L and 25 g/L on day 10. A typical HPLC chromatogram of the Fermentation Medium after 10 days of incubation shows the 2-KLG peak eluting at approximately 1.73 minutes (Figure 2). I found, however, that the 2-KLG retention time varied a little as the concentration of 2-KLG in the medium changed. The depletion of sorbose was not detected with this HPLC method because sorbose is not an ionizable compound and cannot be observed as a peak with ion-exchange chromatography.

The change in the proportion of the two different cultures in the DSM 4027 during the 10-day incubation in the Fermentation medium was determined by streaking the culture on corn steep liquor (CSL) agar plates and gram staining each colony type. The CSL plates showed from day one through day four large, yellow colonies exclusively (Figure 3 a), and no other colony type was observed until day five (Figure 3 b). On day five, small, cream-colored colonies were detected and there were fewer of the large colonies. When the cultures were gram-stained on days one through four, only gram- positive, long rods were seen (Figures not shown). Gram-negative, short rods were not observed until day five. On day 10, almost all of the colonies on the streak plates were cream-colored ones with only few of the larger colonies found at the beginning (Figure 3 c). Gram stains of the 10-day culture showed mostly gram-negative, short rods and only a few gram-positive rods (Figure not shown).

Since TLC analysis demonstrated that 2-KLG formation was not detected until day five when the gram-negative rods were found in high numbers, it was tentatively assumed that the gram-negative rods were primarily responsible for 2-KLG synthesis.

In the patent which employed DSM 4027 for 2-KLG production, the gram- positive, long rods (large, yellow colonies) were identified as *Bacillus megaterium*, and the gram-negative, short rods (small, cream-colored colonies) were reported to be a *Gluconobacter* species. However, I found that these gram negative rods do not exhibit some phenotypic characteristics identified with the *Gluconobacter* genus. Therefore, I have been referring to the two species as the gram-positive rod and the gram-negative rod.

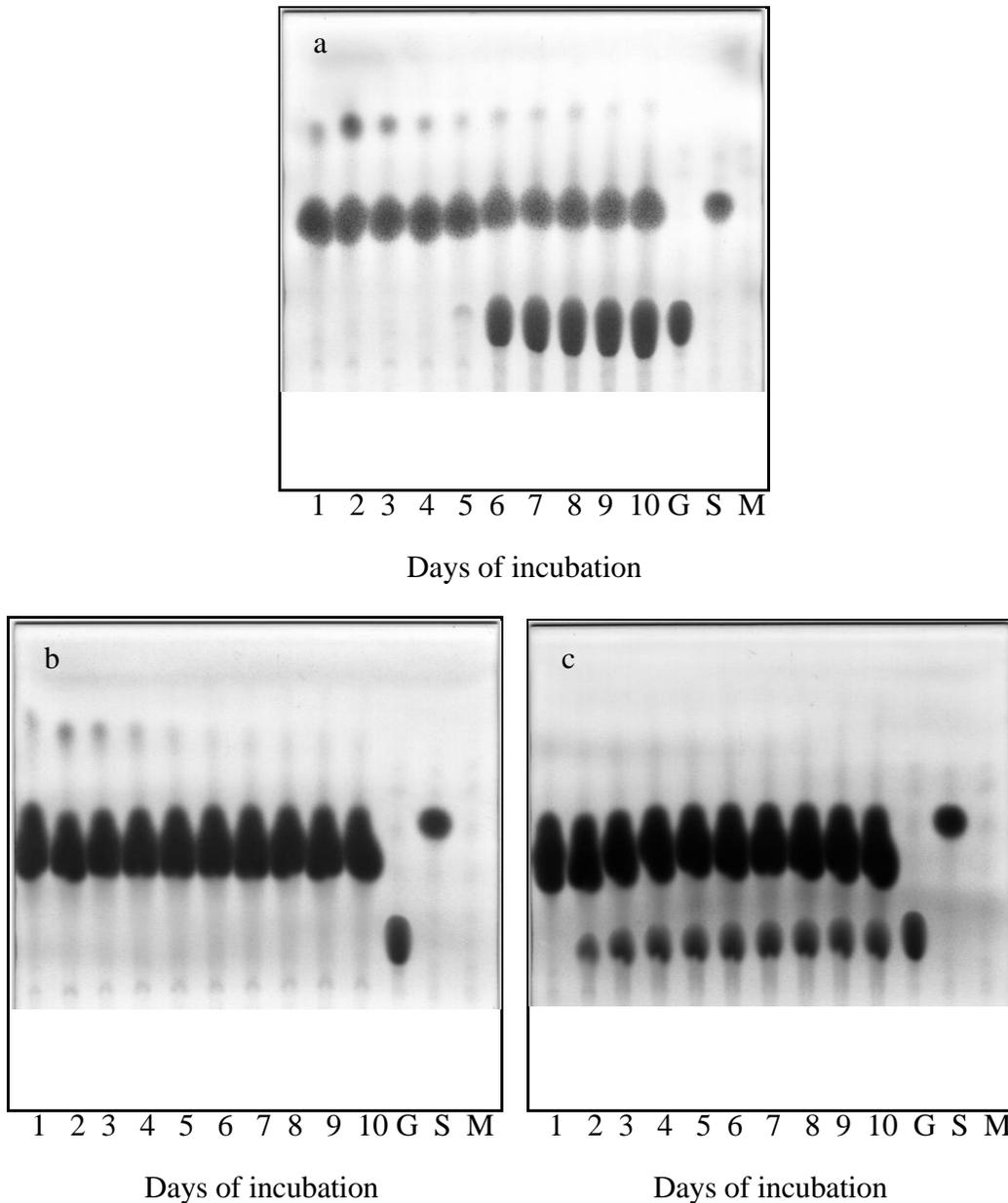


Figure 1. Thin-layer chromatographic detection of 2-keto-L-gulonic acid (2-KLG) in the Fermentation Medium after one through 10 days incubation of (a) the mixed DSM 4027 culture, (b) the gram-positive isolate from DSM 4027, and (c) the gram-negative isolate from DSM. Cultures were sampled at the designated days and spotted on thin layer chromatograms after removal of cells. A known amount of authentic sorbose (S) and 2-KLG (G) as well as sterile growth medium lacking sorbose (M) were also spotted. Chromatograms were developed using a propanol/water mixture as described in materials and methods.

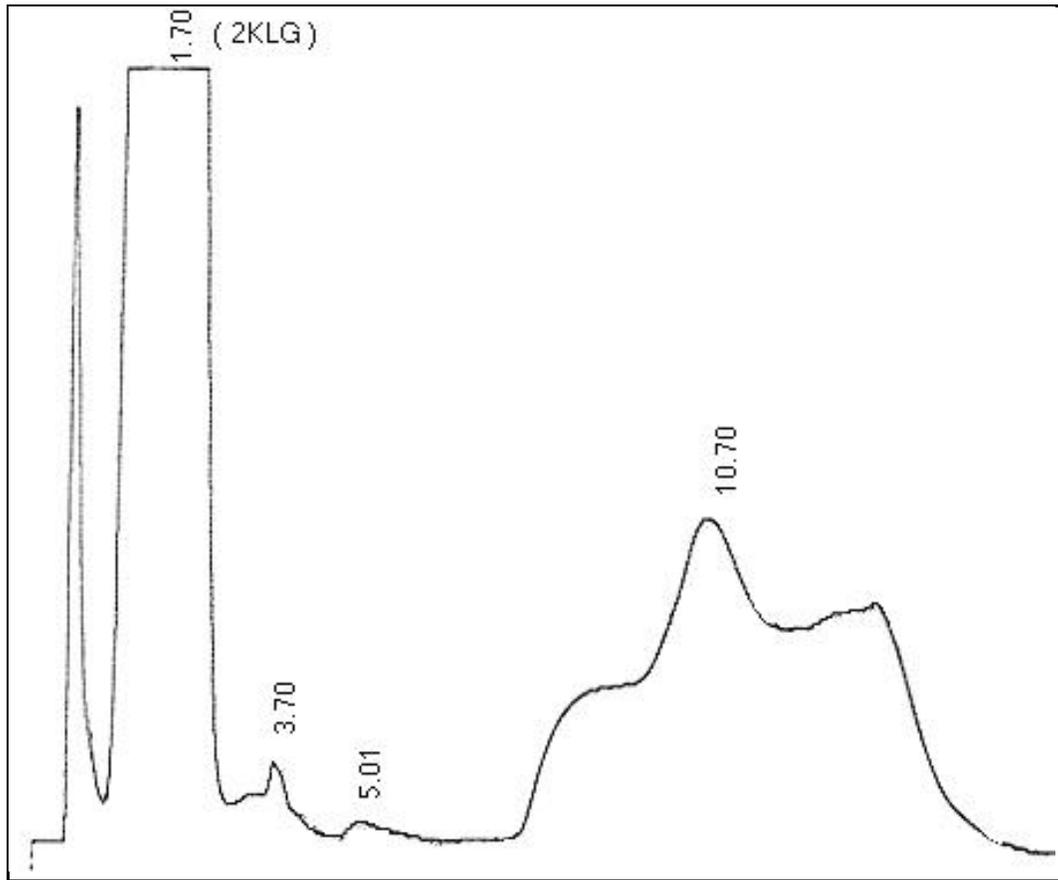


Figure 2. Quantitative determination of 2-keto-L-gulonic acid (2-KLG) production in the Fermentation Medium on day 10 of incubation for the mixed culture DSM 4027 using high performance liquid chromatography (HPLC). The HPLC chromatogram shows the elution of the 2-KLG at 1.73 minutes. The DSM 4027 mixed culture was sampled on day 10, centrifuged to remove the cells, and the supernatant fluid was injected into the ion-exchange column. A buffer containing a mixture of sodium carbonate and sodium bicarbonate was used as the mobile phase, and 2-KLG was detected using suppressed conductivity as described in materials and methods.

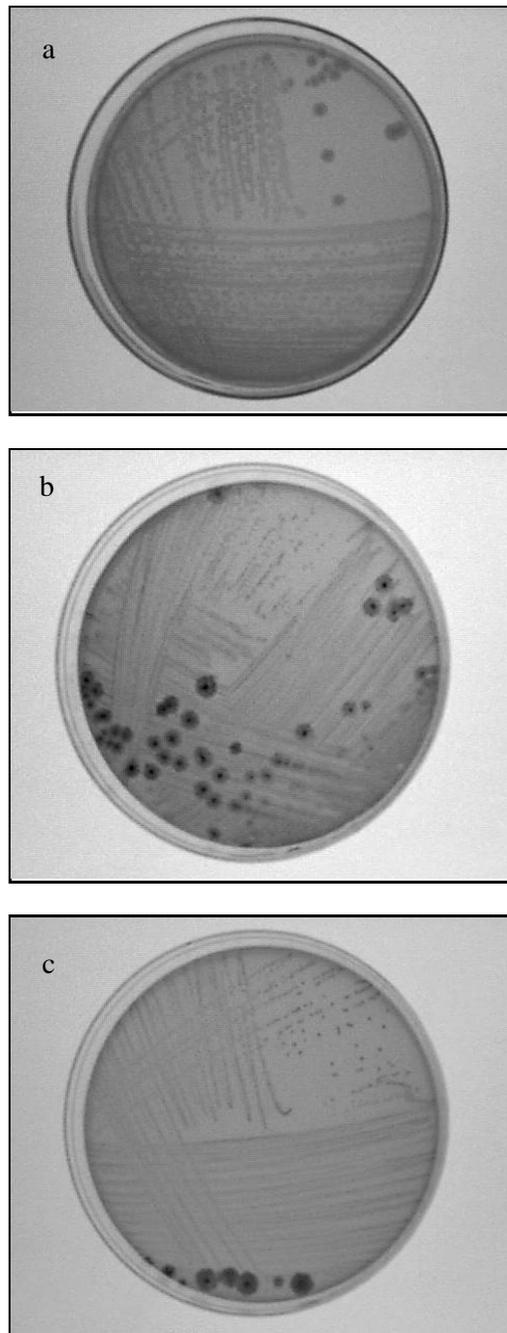


Figure 3. Changes in proportion of colony types of the mixed culture DSM 4027 sampled from Fermentation Medium after (a) one, (b) five, and (c) 10 days (c) incubation. A loopful of the culture was streaked onto corn steep liquor (CSL) agar plates. The large colonies contained only gram-positive rods, and the small colonies only gram-negative rods.

2-KLG production by the separated gram-positive and gram-negative rods.

The gram-positive rods were isolated from DSM 4027 and separately cultured in the Fermentation Medium for 10 days to determine the ability of this strain to produce 2-KLG from sorbose. Using TLC analysis, no 2-KLG was detected on any day of incubation (Figure 1 b). However, quantitative analysis using HPLC showed 1.2, 1.1, 1.2, and 1.4 g/L after two, five, six, and ten days of incubation, respectively. Figure 4a shows an example of an HPLC chromatogram representing the gram-positive culture on day 10 of incubation. This small quantity of 2-KLG was negligible compared to 25 g/L produced by the mixed culture. These results suggested that the gram-positive strain was not the sole producer of 2-KLG in this mixed culture.

The gram-negative rods were also isolated from DSM 4027 and tested for 2-KLG production in the Fermentation Medium. The TLC analysis (Figure 1 c) showed that 2-KLG was first detected on day two, instead of day five as with the mixed culture (Figure 1 a), and the detection continued through day 10. Quantitative HPLC analysis of the gram-negative culture demonstrated concentrations of 2.9, 8.0, 8.6 and 8.8 g/L from the Fermentation Medium sampled on days two, five, six and ten, respectively. Thus, the mixed culture (DSM 4027) started producing 2-KLG at a later time than the gram-negative isolate, but the mixed culture generated three-times more 2-KLG (25 g/L) by day 10 than the gram-negative isolate. These results suggested that the gram-positive strain in the mixed DSM 4027 enhanced 2-KLG production but forced the gram negative strain to produce 2-KLG at a later day in the mixed culture. An example of an HPLC chromatogram on day 10 of incubation is depicted in Figure 4b.

Co-culturing the gram-negative and gram-positive rods isolated from DSM 4027. To determine if the isolated gram-positive rod could be re-cultured with the isolated gram-negative rod and achieve the same 2-KLG production, 0.2 ml samples from each glycerol stock culture were inoculated into the Fermentation medium and incubated at 28°C with shaking. Samples of the culture medium were assayed with TLC to detect 2-KLG formation (Figure 5). Instead of 2-KLG being first detected on day five as shown with DSM 4027 (Figure 1 a), the artificially reconstituted mixed culture did not produce any detectable amount until day eight. The sizes of the 2-KLG spots were smaller for the reconstituted mixture, which indicated a lower yield of 2-KLG than that produced by the DSM 4027. However, the sizes of the 2-KLG spots for the reconstituted mixture were similar to those observed when the gram-negative isolate was cultured alone. Thus, co-culturing equal quantities of the two isolates from DSM 4027 did not appear to replicate the 2-KLG production of the original DSM 4027 mixture.

2-KLG production by authentic *Gluconobacter oxydans* strains. When *G. oxydans* IFO strain 3293 was incubated for 10 days in the Fermentation Medium, 2-KLG was detected by TLC as early as day four and continued to be present through day 10 (Figure 6 a). However, the spot sizes for all days of incubation were much smaller than those observed for the DSM 4027 mixed culture (Figure 1 a) and for the gram-negative isolate from that mixture (Figure 1 c). The HPLC analysis from cultures of IFO strain 3293 determined 2-KLG yields to be 2.3, 2.6, 3.4 g/L on day five, six, and 10, respectively. An example of an HPLC chromatogram on day 10 of incubation is shown in Figure 6b.

When pure cultures of *G. oxydans* ATCC strain 621 were grown in the Fermentation Medium and tested for 2-KLG production, TLC analysis showed the first detectable 2-KLG spot on day three with increasing concentration through day 10 (Figure 7 a). The HPLC analysis determined 2-KLG yields to be 0.62, 2.6, 5.7 g/L on days five, six, and ten, respectively. An example of an HPLC chromatogram performed on 10-day ATCC strain 621 cultures is depicted in Figure 7b.

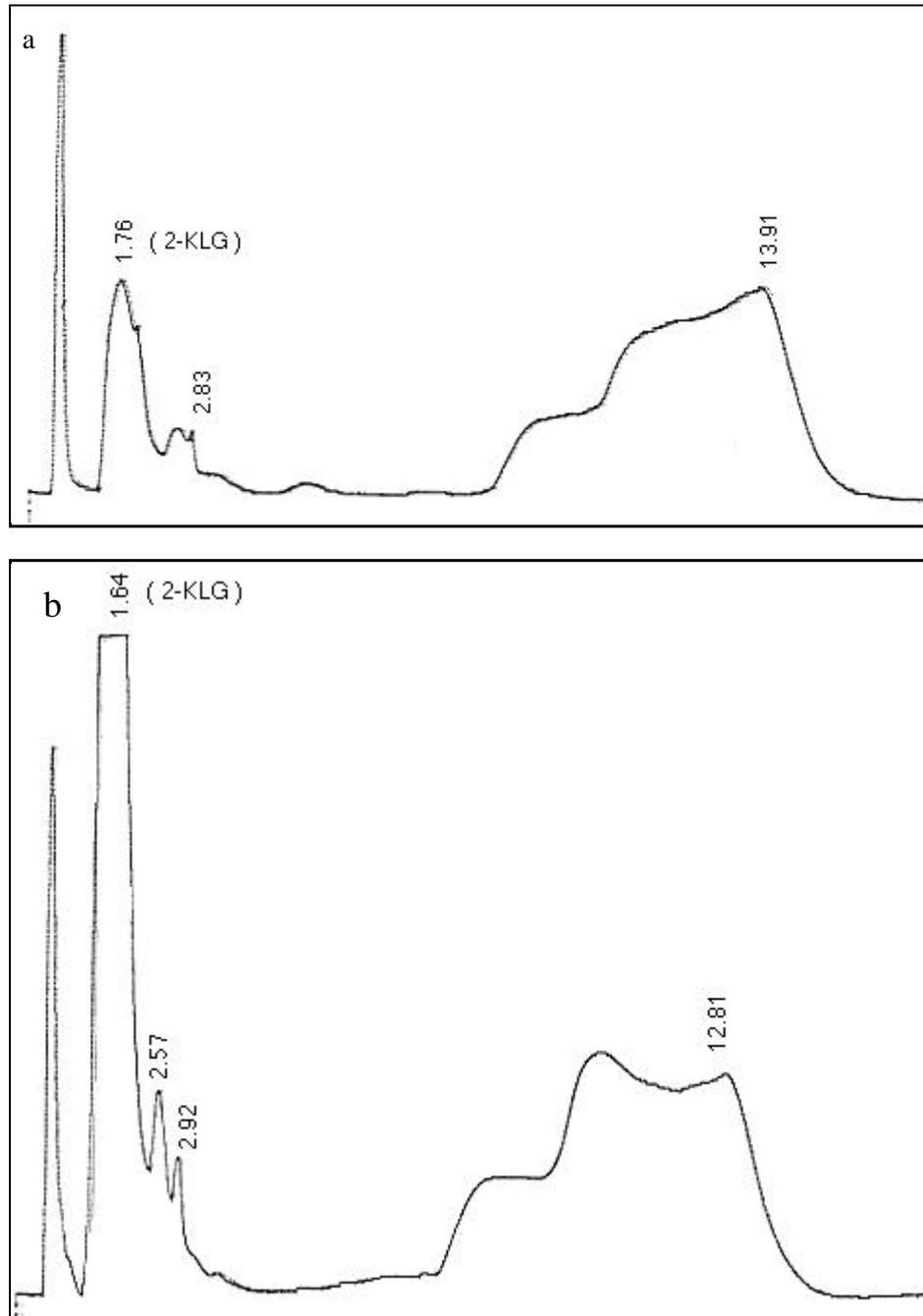


Figure 4. Quantitative determination of 2-keto-L-gulonic acid (2-KLG) production on day 10 of incubation for (a) the gram-positive isolate from DSM 4027 and (b) the gram-negative isolate from DSM 4027. The HPLC analysis was performed as described in Figure 2.

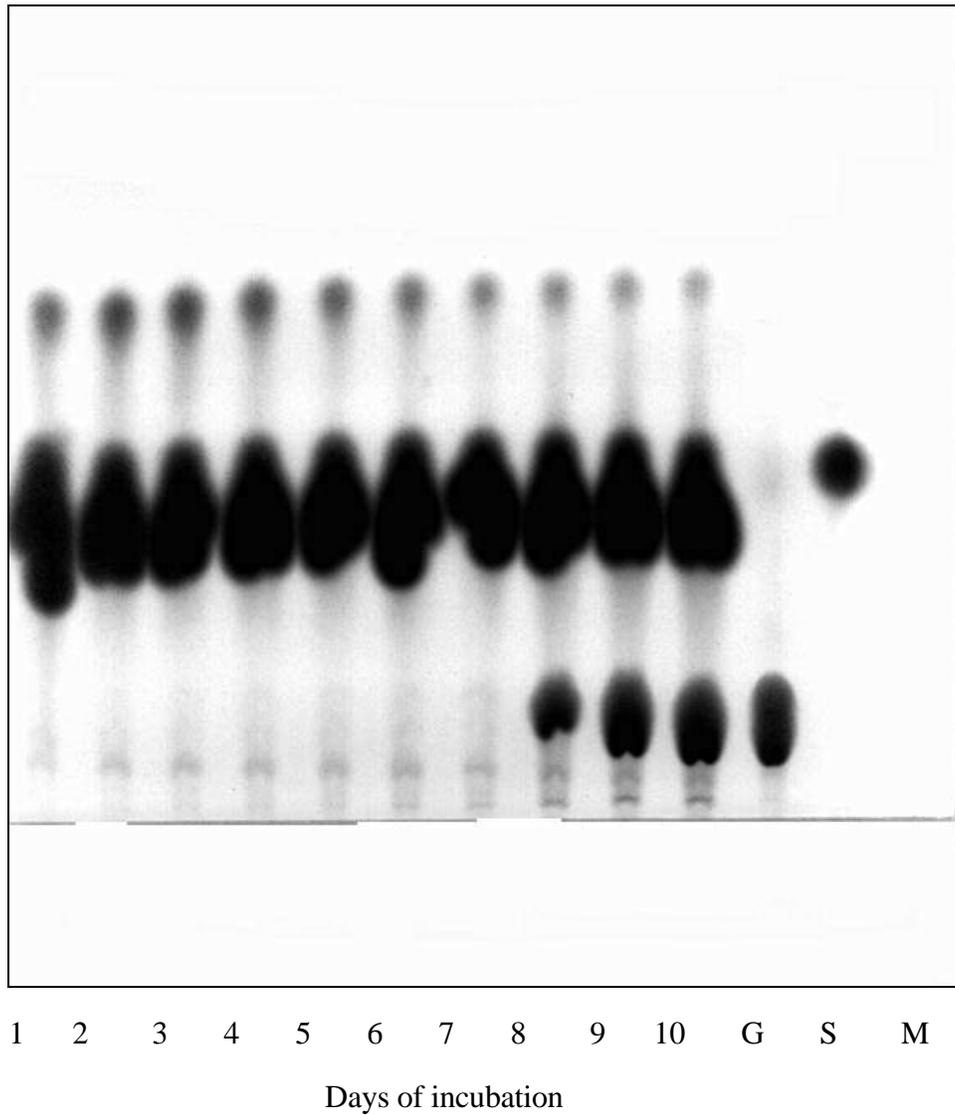


Figure 5. Thin-layer chromatography analysis of 2-keto-L-gulonic acid (2-KLG) production in the Fermentation Medium inoculated with equal quantities of the gram-negative and the gram-positive isolates from DSM 4027. Chromatograms of culture samples, authentic 2-KLG (G), sorbose (S), and sterile medium lacking sorbose (M) were developed as described in Figure 1.

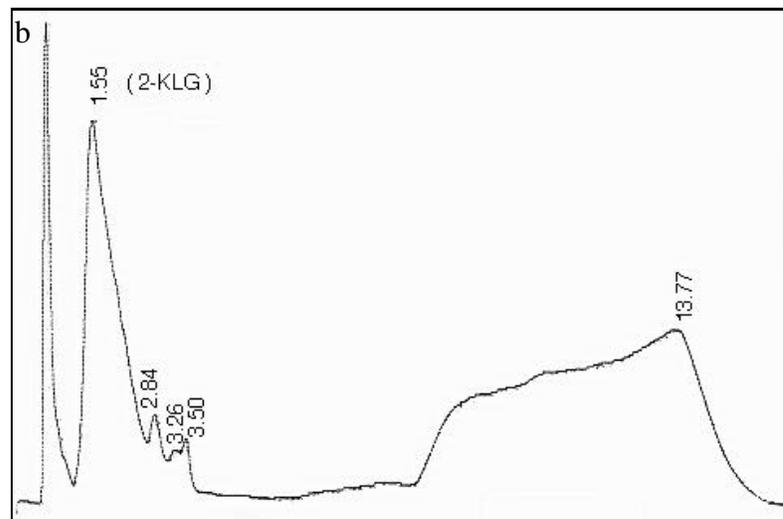
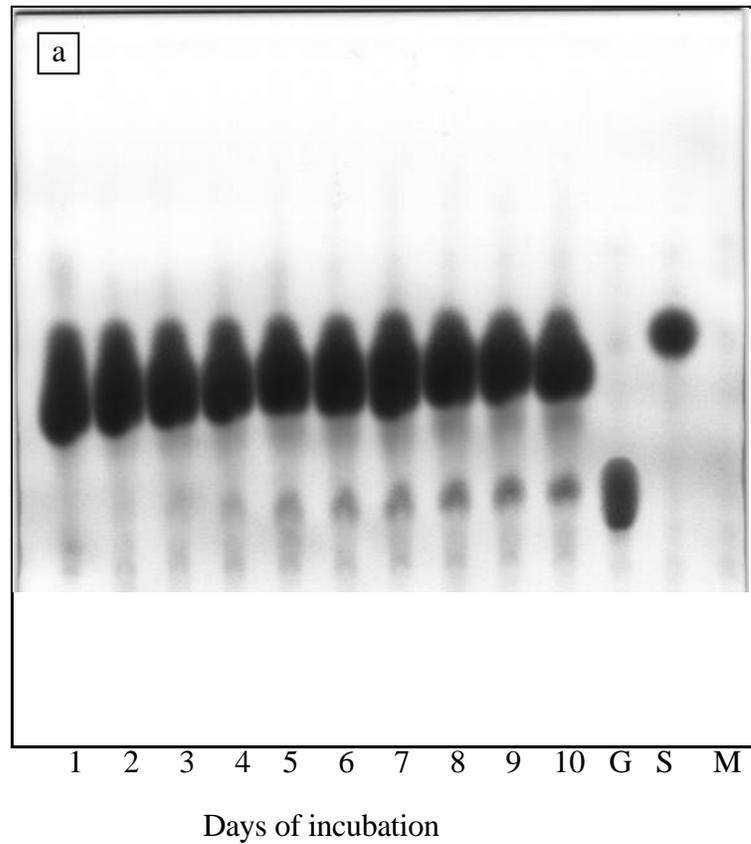


Figure 6. Qualitative and quantitative determination of 2-keto-L-gulonic acid (2-KLG) in the Fermentation Medium of cultures of *G. oxydans* IFO strain 3293. The TLC chromatogram (a) and the HPLC chromatogram (b) were prepared as described in Figures 1 and 2, respectively. Figure 6(b) is the HPLC chromatogram for the 10-day sample.

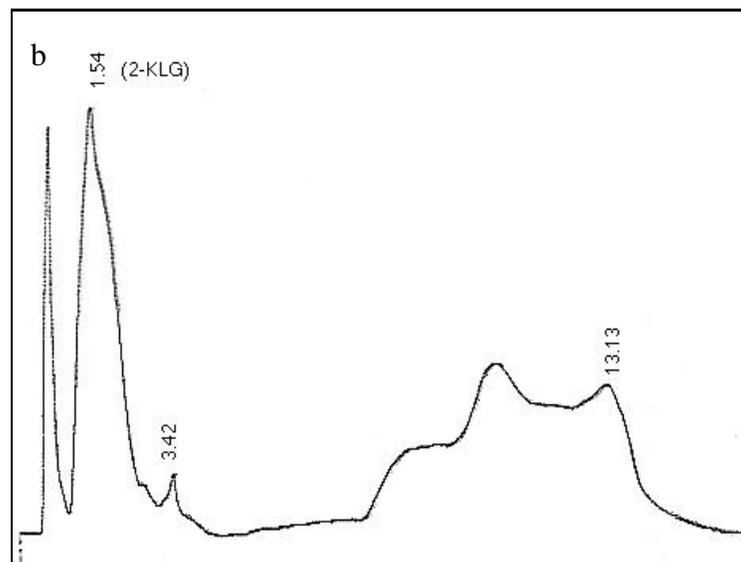
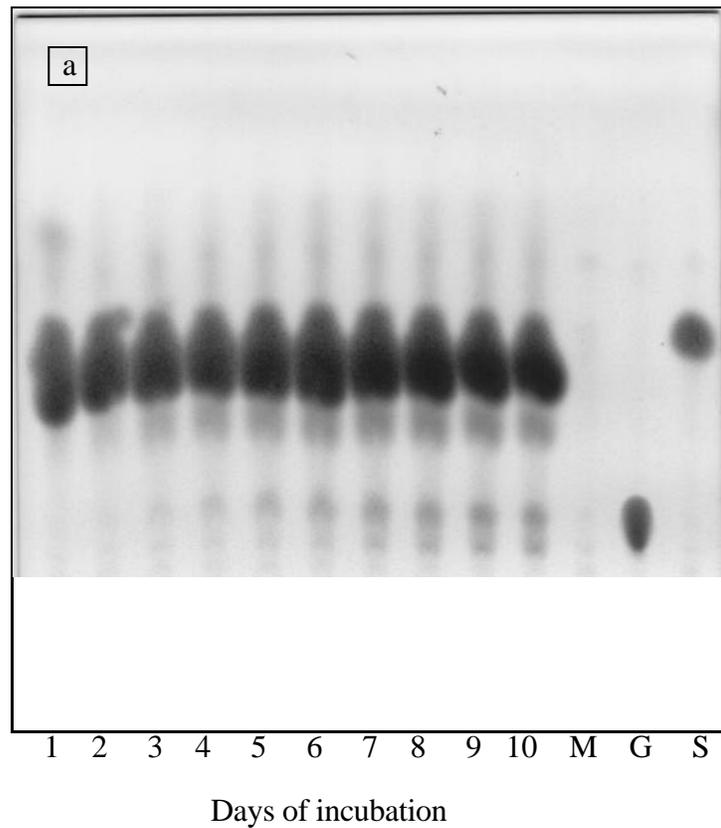


Figure 7. Qualitative and quantitative determination of 2-keto-L-gulonic acid (2-KLG) in the Fermentation Medium of cultures of *G. oxydans* ATCC strain 621. The TLC chromatogram (a) and the HPLC chromatogram (b) were prepared as described in Figures 1 and 2, respectively. Figure 7(b) is the HPLC chromatogram for the 10-day sample.

Thus, neither IFO strain 3293 nor ATCC strain 621 produced as much 2-KLG in the Fermentation Medium as the mixed culture DSM 4027 or the separately cultured gram-negative isolate from DSM 4027.

2-KLG production by authentic *G. oxydans* cultures when grown with the gram-positive isolate from DSM 4027. The role of the gram-positive rods in enhancing the production of 2-KLG by the *Gluconobacters* was investigated by growing either of the *G. oxydans* strains (IFO 3293 and ATCC 621) with the gram-positive rods in the Fermentation Medium. When IFO strain 3293 was cultured with the gram-positive rods, no 2-KLG production was detected in 10 days of incubation (Figure 8). In comparison with 2-KLG production by pure cultures of IFO strain 3293 (Figure 6 a), it appeared that co-cultivation with the gram-positive isolate from DSM 4027 inhibited 2-KLG synthesis.

Similar results were obtained when *G. oxydans* ATCC strain 621 was cultured in the Fermentation Medium for 10 days along with the gram-positive isolate from DSM 4027 (data not shown). No 2-KLG was detected through ten days of incubation even though the ATCC strain when cultured alone in this medium was able to produce it (Figure 7 a).

Thus, co-culture of the gram-positive isolate from DSM 4027 with both authentic *Gluconobacter* cultures inhibited 2-KLG synthesis.

2-KLG production by various cultures when grown in spent Fermentation Medium from culturing the gram-positive isolate. I wished to determine if the gram-positive isolate from DSM 4027 released a compound into the medium that stimulated 2-KLG production by known *Gluconobacter* strains and by the gram-negative isolate from DSM 4027 that was reported to be a *Gluconobacter* species. The gram-positive isolate from DSM 4027 was grown in the Fermentation Medium, and cells were removed from this medium by centrifugation after either two or six days of incubation. Each spent medium was filter-sterilized and then inoculated with either IFO strain 3293, or ATCC strain 621, and these cultures were incubated for 10 days. Neither *G. oxydans* IFO strain 3293 nor ATCC strain 621 produced 2-KLG in either the two-day or six-day spent medium from cultures of the gram-positive isolate. A typical TLC chromatogram is shown in Figure 9. These results indicated that the gram positive isolate did not produce a 2-KLG stimulation factor during solitary growth in the Fermentation Medium which enhanced 2-KLG synthesis in these two *G. oxydans* strains.

A similar type of experiment was performed except that the gram-negative isolate from DSM 4027 was grown in either two-day or six-day spent medium from the gram-positive isolate. The TLC analysis using the two-day spent medium demonstrated 2-KLG production after only one day of incubation of this culture, and 2-KLG concentrations appeared to increase through day 10 (Figure 10 a). When the gram-negative isolate was grown on six-day spent medium, the TLC analysis of 2-KLG production was similar to that shown with two-day spent medium, except that 2-KLG was not detected until day three (Figure 10 b). These results were different than those observed for the isolated gram-negative rod when grown in the unused Fermentation Medium (Figure 1 c). The sizes of the 2-KLG spots in these spent-media experiments were smaller, indicating a lower yield of 2-KLG in the spent medium.

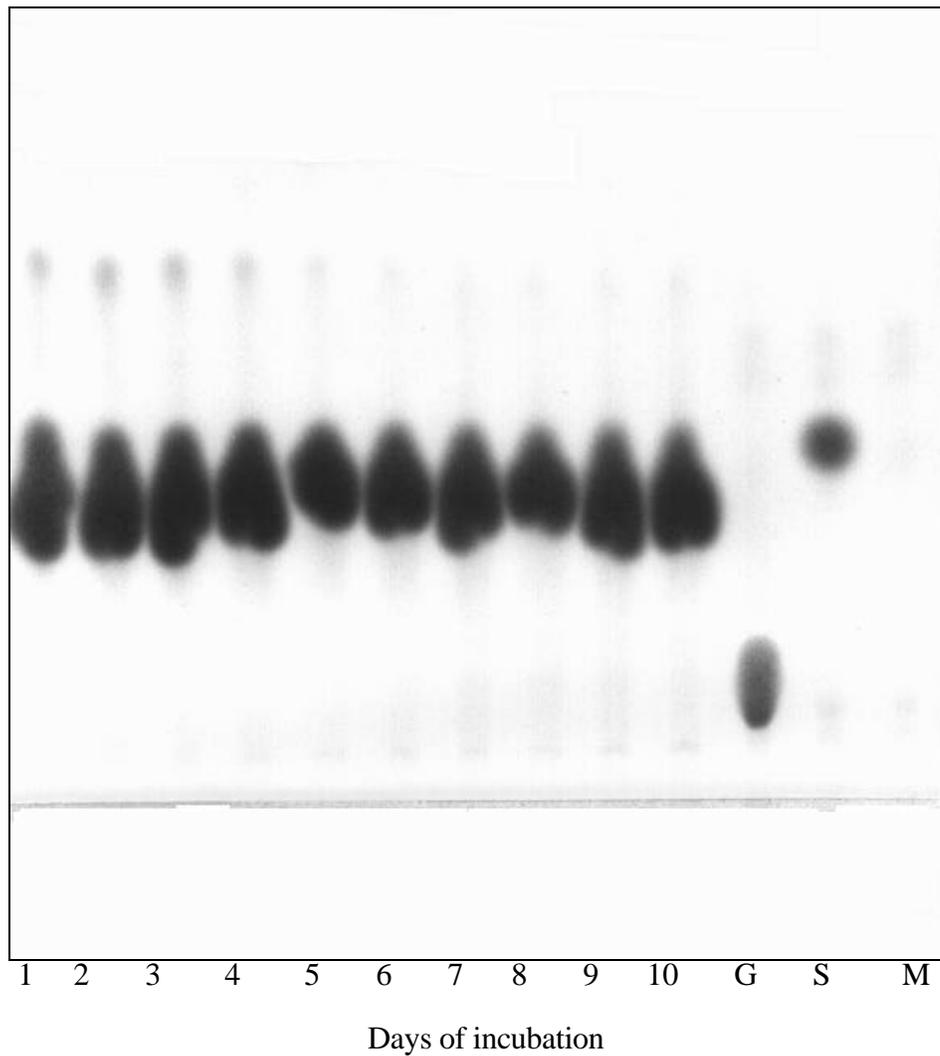


Figure 8. Thin-layer chromatography analysis of 2-keto-L-gulonic acid (2-KLG) production in the Fermentation Medium inoculated with equal quantities of *G. oxydans* IFO strain 3293 and the gram-positive isolate from DSM 4027. Chromatograms of culture samples, authentic 2-KLG (G), sorbose (S), and sterile medium lacking sorbose (M) were developed as described in Figure 1.

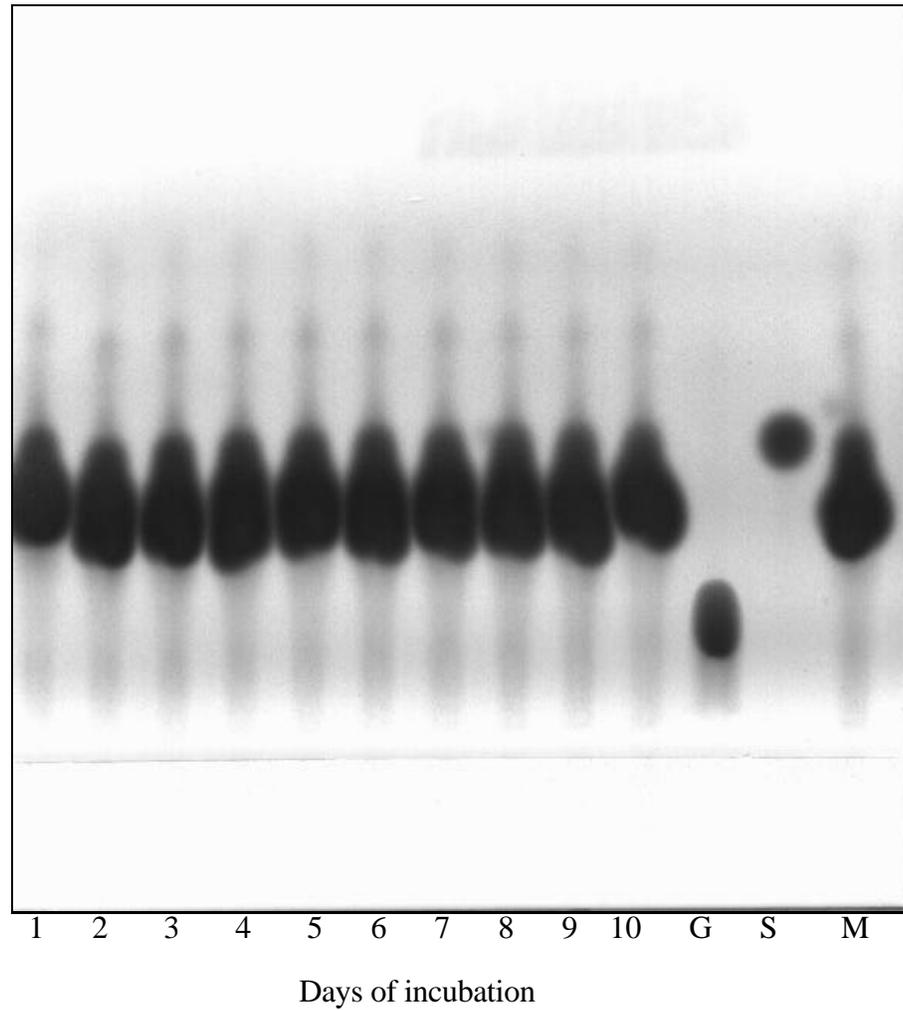


Figure 9. Thin-layer chromatographic detection of 2-keto-L-gulonic acid (2-KLG) in day-two spent medium from cultures of the gram-positive isolate from DSM 4027 inoculated with *G. oxydans* IFO strain 3293. After the gram-positive isolate was grown in the Fermentation Medium for two days, the cells were removed by centrifugation, and the spent medium was filter-sterilized, and then was inoculated with *G. oxydans* IFO strain 3293. Chromatograms of culture samples, authentic 2-KLG (G), sorbose (S), and day-two spent medium (M) were developed as described in Figure 1.

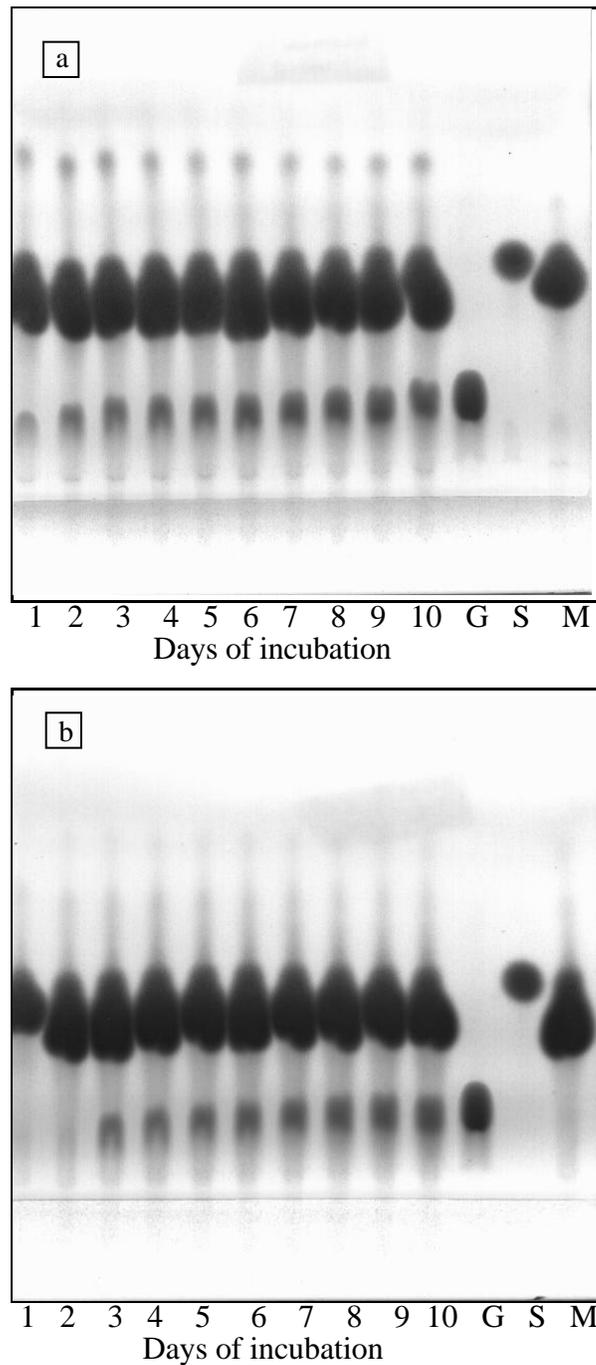


Figure 10. Thin-layer chromatographic detection of 2-keto-L-gulonic acid (2-KLG) in the gram-positive spent medium day two (a) and day six (b) inoculated with the gram-negative isolate from DSM 4027. After the gram-positive isolate was grown in the Fermentation medium for two or six days, the cells were removed by centrifugation, and the resulting spent medium was filter-sterilized, and then inoculated with the gram-negative isolate from DSM 4027. Chromatograms were developed as described in Figure 1.

Phenotypic tests on the gram-negative isolate from DSM 4027. Results obtained in the 2-KLG production studies made me question whether the gram-negative rod isolated from DSM 4027 was a *Gluconobacter* strain as reported in the U.S. patent # 4,935,359 (Yin *et al.*, 1990). This gram-negative isolate behaved differently than authentic *Gluconobacter* strains (IFO 3293 and ATCC 621) when co-cultured with the gram-positive isolate from DSM 4027. In the gram-negative isolate, 2-KLG production was not inhibited by the co-culturing as it was with *G. oxydans* IFO strain 3293 and ATCC strain 621. The synthesis of 2-KLG was also not inhibited in the gram-negative isolate when it was grown in the spent media from culturing the gram-positive isolate as it was with *G. oxydans* IFO strain 3293 and ATCC strain 621. With these major differences, this gram-negative isolate was subjected to few phenotypic tests indicative of the gluconobacters. When the isolate was streaked onto an ethanol/calcium carbonate agar plate, it did not produce any clearing of the undissolved calcium carbonate. Thus, it was assumed that this strain does not produce detectable quantities of acetic acid from ethanol, an oxidation reaction identified with the *Gluconobacter* genus. In addition, growth was not observed on plates containing 10% glucose pH 4.5, a medium only acid tolerant organisms such as the *Gluconobacters* grow on. Growth was also not observed in mannitol broth or mannitol agar. Lastly, dihydroxyacetone was not detected when the isolate was grown on plates containing glycerol.

DISCUSSION

Interaction between strains in the mixed culture (DSM 4027). The patented mixed culture (DSM 4027) produced the highest yield of 2-KLG from sorbose (25 g/L) compared to that produced by pure cultures of the gram-negative (8.8 g/L) and gram-positive rods (1.4 g/L) isolated from DSM 4027 and with that produced by the authentic cultures of *G. oxydans* IFO strain 3293 (3.4 g/L) and ATCC strain 621 (5.7 g/L). Thus, the gram-positive rods isolated from DSM 4027 produced a relatively negligible quantity of 2-KLG, hence the gram-negative rods isolated from DSM 4027 were the primary 2-KLG producers in the mixed culture. However, the isolated gram-negative rods did not produce the same quantity of 2-KLG in pure culture as they did in the mixed culture when incubated under the same conditions. Therefore, it appeared that the gram-positive rods enhanced the synthesis of 2-KLG by the gram-negative rods.

The interaction between these two species was investigated by examining the mixed culture for 10 days as it was oxidizing sorbose to 2-KLG in the Fermentation Medium. No 2-KLG was produced during the first five days of incubation when only the gram-positive rods were growing (Figure 3 a). It was not until the gram-negative rods started growing after day five that 2-KLG synthesis began (Figure 3 b), and this production increased through day ten as the growth of the gram-negative rods increased (Figure 3 c). The large difference in the 2-KLG yield between the mixed culture (DSM 4027) and the isolated gram-negative rods, when incubated under the same conditions in the Fermentation Medium, could be attributed to the presence of the gram-positive rods. The enhancement of 2-KLG production by the gram-positive rods could be explained by the ability of most *Bacillus* species (*Bacillus megaterium*) to produce and release secondary metabolites into the growth medium. These secondary metabolites could be the factors inducing the high production of 2-KLG by the gram-negative rods. Another possible explanation would be a cross-feeding between the two species. In this case, the gram-positive rods could be oxidizing sorbose to a keto-compound intermediate, which is converted by the gram-negative rods to 2-KLG.

This type of interaction between species was demonstrated in the two-step fermentation process used for the microbial production of vitamin C in China (11) where a *Corynebacterium* species is used to oxidize glucose to 2,5-diketogluconate (2,5-DKG), then a *Gluconobacter* strain is added to reduce 2,5-DKG to 2-KLG. With DSM 4027, perhaps the gram-positive rods are accomplishing a similar first step. This explanation is plausible, since the mixed culture DSM 4027 and the gram-positive rods isolated from DSM 4027 produced a compound (unidentified spots observed on the TLC chromatograms) other than 2-KLG or sorbose (Figures 1 a and 1 b). These spots were also observed in the gram-positive spent media (Figures 9 and 10) and when the gram-negative isolate (Figure 5) and *G. oxydans* IFO strain 3293 (Figure 8) and ATCC strain 621 (Figure 8) were co-cultured with the gram-positive isolate. These unidentified spots may correspond to a keto-compound produced by the gram-positive strain (*B. megaterium*) in the DSM 4027. These unidentified spots were not observed with cultures of the isolated gram-negative rods (Figure 1 c) or with pure cultures of *G. oxydans* IFO strain 3293 (Figure 6 a) and ATCC strain 621 (Figure 6 a). The HPLC analysis (Figure 4 a) indicated that the isolated gram-positive rods produced a slight amount of 2-KLG (1.4 g/L), but the ion-exchange HPLC method could not detect the depletion of sorbose from the medium, which would have supported the theory that the gram-positive rods were converting sorbose to an intermediate keto-compound.

Another possible explanation for this large difference in the yield of 2-KLG between the gram-negative isolate in mixed culture and in pure culture could be that the gram-negative isolate used a different pathway for 2-KLG production when cultured without the gram-positive isolate. Different intermediate(s), other than the keto-compound mentioned earlier, could be produced in

this different pathway, and which might not be as efficiently oxidized to 2-KLG, and thus decreasing the yield.

The synthesis of 2-KLG by the isolated gram-negative rods started on day two (Figure 1 c) instead of day five as in the mixed culture DSM 4027 (Figure 1 a). This delay in the 2-KLG production when the gram-negative rods were cultured with the gram-negative rods could be explained by the competition between these two species for the sorbose substrate.

2-KLG production by *G. oxydans* IFO strain 3293 and ATCC strain 621.

Both strains of *G. oxydans* produced 2-KLG in pure culture (Figures 6 a and 7 a) but not as much as the DSM 4027 mixed culture (Figure 1 a) or the isolated gram-negative rod from DSM 4027 (Figure 1 c). Also, the TLC chromatograms for these two authentic *Gluconobacter* strains did not show the presence of spots other than 2-KLG or sorbose. Thus, these two authentic strains might be using a different pathway for 2-KLG production than that used by the DSM mixed culture or the gram-negative rod isolated from DSM 4027. This might explain the differences in 2-KLG yield. The HPLC analysis did show a different profile for the different strains on day ten (Figures 2 , 4 a, 4 b, 6 b and 7 b), however, since ion-exchange chromatography would only elute ionizable compounds, many possible intermediates in the pathway for 2-KLG synthesis would not be detected. In other words, even if these strains had different pathways with different intermediates for 2-KLG synthesis, the selectivity of the ion-exchange column for only ionizable compounds prevents the visualization of these intermediates as peaks on the chromatogram.

The Effect of co-culturing the gram-positive rods isolated from DSM 4027 with the various *Gluconobacter* strains on 2-KLG production. The isolated gram-positive rod was added to known *Gluconobacter* strains and to the gram-negative rod isolated from DSM 4027 to determine the effect of co-culturing on 2-KLG production. Based upon TLC analysis (Figures 5 and 8), it appeared that these reconstituted mixed cultures behaved differently than the DSM 4027 mixed culture. In the artificially mixed culture of the gram-positive isolate and the gram-negative negative isolate from DSM 4027, 2-KLG production started on day eight (Figure 5) instead of day five as in the DSM mixture (Figure 1 a), and the sizes of the 2-KLG spots were smaller. This indicated an overall lower 2-KLG yield by the reconstituted mixture. However, there was no significant difference in the sizes of the 2-KLG spots when the reconstituted mixture was compared with the gram-negative rod isolated from DSM 4027 (Figure 1 c). These results indicated that there was no difference in the 2-KLG yield between the co-culture and the pure culture when co-culturing equal quantities of the gram-positive and gram-negative isolates.

The inability of the co-culture to reproduce the same amount of 2-KLG formed by DSM 4027 might be due to the ratios of the two species in the reconstituted mixture during the ten day incubation. For the reconstitution experiments, equal volumes of the two stock-cultures for each strain (approximately equal numbers) were mixed together at the time of inoculation, and the resulting mixture was incubated under the same conditions as the DSM 4027 mixture. It is possible that under the equal volume ratio condition, the gram-positive rods out-competed the gram-negative rods therefore inhibiting 2-KLG synthesis until the eighth day of incubation. It is also possible that the gram-positive rods needed to grow first and produce enough of the compound used by the gram-negative cells to produce large quantities of 2-KLG.

The different pathway theory for the inability of the reconstituted mixture to generate the same yield of 2-KLG could be eliminated because the same unidentified spots of the keto-intermediate were observed on the TLC chromatogram of the reconstituted mixture (Figure 5) as well as on the TLC chromatogram of the DSM 4027 mixed culture (Figure 1 a).

Co-culturing the gram-positive rod isolated from DSM 4027 with authentic *G. oxydans* IFO strain 3293 and ATCC strain 621 completely inhibited 2-KLG production, and this result was totally surprising. This was probably due to the competition effect as well. It is also worthy to note that the unidentified keto-intermediate spots were also observed on the TLC chromatograms of these co-cultures (Figure 8), which seems to be consistent with the presence of the gram-positive rods in the growth medium. Perhaps the gram-positive rods were converting the sorbose to a compound that could not be used by the authentic *Gluconobacter* strains, and thus inhibiting the synthesis of 2-KLG and even out-competing the gluconobacters.

The interaction between the gram-negative rods isolated from DSM 4027 and the gram-positive rods in the reconstituted mixture was different than the interaction between the authentic *G. oxydans* IFO strain 3293 and ATCC strain 621 and the gram-positive rods when co-cultured together. In the reconstituted mixture of the two isolates, 2-KLG synthesis was not completely inhibited, but 2-KLG was not produced in the same amount formed by DSM 4027. When authentic *G. oxydans* IFO strain 3293 and ATCC strain 621 were separately co-cultured with the gram-positive rods from DSM 4027, synthesis of 2-KLG by the gluconobacters was completely inhibited. These results suggested that the gram-negative rods in the mixed DSM 4027 might not be a *Gluconobacter* species as was reported by the U.S. patent #4,935,359 (35).

Use of spent medium to stimulate 2-KLG production. Spent media from culturing the gram-positive isolate from DSM 4027 was used in an attempt to stimulate 2-KLG production by the gram-negative isolate from DSM 4027 and by authentic *Gluconobacter* strains. In other words, I was interested in learning whether the presence of the gram-positive cells was needed or whether the same effect could be accomplished in the spent medium free of cells. It was reasoned that compounds produced by the gram-positive isolate might induce 2-KLG production by these strains. The data, however did not support that hypothesis.

When the gram-negative rods isolated from DSM 4027 were cultured in this spent medium (Figure 10 a and 10 b) 2-KLG production was not enhanced and even appeared to be less than that produced by the gram-negative isolate in pure culture (Figure 1c), and when the gram-positive and gram-negative isolates were co-cultured (Figure 5). When *G. oxydans* IFO strain 3293 and ATCC strain 621 were grown in spent media, no 2-KLG synthesis was detected (Figure 8). These same results were obtained when these two authentic *Gluconobacter* strains were co-cultured with the isolated gram-positive rods in the Fermentation Medium. The only time that *G. oxydans* IFO strain 3293 and ATCC strain 621 produced 2-KLG was when these strains were incubated in pure culture in the Fermentation Medium.

The results using spent media suggested that the intermediates produced by the gram-positive rods did not stimulate 2-KLG production by these authentic strains. Perhaps these two authentic *Gluconobacter* strains could not grow in spent media and were unable to oxidize the compounds produced by the gram-positive rods to 2-KLG.

The identification of the gram-negative rod isolated from DSM 4027. The patent application (35) states that the gram-negative rods in the mixed DSM 4027 culture is a *Gluconobacter* strain. The gram reaction of the isolate was negative, and the shape was that of a short plump rod. However, no growth was observed on mannitol broth and mannitol agar even though mannitol is a common substrate for gluconobacter growth. This isolate was not able to oxidize glycerol to dihydroxyacetone and gluconobacters are known to accomplish that particular oxidation (26). Also, this isolate did not exhibit growth on 10% glucose agar adjusted to pH 4.5 with acetic acid, and this medium is commonly used to isolate for gluconobacters from natural

environments (5). This isolate did not produced a zone of clearing when grown on ethanol/calcium carbonate agar plates, indicating no oxidation of ethanol to acetic acid. All of these phenotypic tests made me question the identification of the gram-negative isolate as a *Gluconobacter* species as was reported in the patent (5). More extensive molecular analysis such as rRNA sequencing and DNA-DNA homology studies should be performed to truely identify this gram-negative rod.

CONCLUSIONS

1. The DSM 4027 mixed culture of a gram-positive and a gram-negative rod, reported to be *Bacillus megaterium* and a *Gluconobacter* strain, respectively, produced the highest quantity of 2-keto-L-gulonic acid (2-KLG) from sorbose when compared with authentic *Gluconobacter* species (the *G. oxydans* cultures IFO strain 3293 and ATCC strain 621).
2. The gram-negative rod in the DSM 4027 mixed culture was the primary 2-KLG producer but the gram-positive rod in that mixed culture enhanced 2-KLG production. The stimulating factor produced by the gram-positive rod was not determined, but I propose that the gram-positive rod converted sorbose to an intermediate keto-compound, which was then converted by the gram-negative rod to 2-KLG.
3. The artificial co-culturing of equal quantities of the gram-positive rods and the gram-negative rods isolated from DSM 4027 did not produce the same high quantity of 2-KLG as the mixed culture obtained as DSM 4027. Synthesis of 2-Keto-L-gulonic acid was completely inhibited when equal quantities of the isolated gram-positive rod was co-cultured with either of the authentic *G. oxydans* cultures (IFO strain 3293 and ATCC strain 621).
4. Production of 2-Keto-L-gulonic acid was not enhanced when the different cultures were grown in the spent medium following growth of the isolated gram-positive rods. When the isolated gram-negative rod was cultured in this spent medium, synthesis of 2-KLG was lower when compared with the mixed DSM 4027, with the isolated gram-negative in pure culture, and with the co-culture of the gram-negative and gram-positive isolates. Synthesis of 2-KLG was completely inhibited in *G. oxydans* IFO strain 3293 and ATCC 621 when these authentic *Gluconobacter* strains were cultured in spent medium.
5. The gram-negative rod isolated from the mixed culture DSM 4027 was reported in the U.S. patent #4,935,359 to be a *Gluconobacter* species. However, when isolated from DSM 4027, this gram-negative rod did not exhibit phenotypic characteristics typical of *Gluconobacter* species.

SUGGESTIONS FOR FUTURE INVESTIGATORS

1. Perform high performance liquid chromatography (HPLC) to determine the quantity of 2-keto-L-gulonic acid (2-KLG) produced by the co-cultures of the gram-positive rods with the different *Gluconobacter* strains in the Fermentation Medium, and the quantity of 2-KLG produced by the various strains when grown in the spent media.
2. Co-culture the gram-positive rod with authentic *Gluconobacter* strains using different ratios of the two species, then perform thin layer chromatography (TLC) and HPLC analysis.
3. Investigate the pathway(s) for 2-KLG production by the various cultures by growing the cultures on the different intermediates and looking for 2-KLG synthesis. Also, use oxygen uptake experiments to determine the ability of these *Gluconobacter* cultures to oxidize the different pathway intermediates.
4. Investigate the different enzymes involved in 2-KLG production by examining the activities of the membrane-bound and cytosolic dehydrogenases using artificial electron acceptors.
5. Perform analyses such as rRNA sequencing and DNA-DNA homology studies to identify the gram-negative rod isolated from the DSM 4027 mixed culture.

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APPENDIX A

Thin Layer chromatographic separation of 2-keto-L-gulonic acid from sorbose

Introduction. Thin layer chromatography (TLC) analysis was performed to qualitatively detect the depletion of sorbose and accumulation of 2-KLG in the growth medium during the 10-day incubation of various cultures. The sensitivity and ability of the TLC method to separate 2-KLG from sorbose in the complex growth medium were tested prior to analyzing the various cultures for 2-KLG production.

Materials and Methods. Glass-backed silica gel thin layer chromatogram plates with Linear-K inert pre-absorbent strip (Fisher Scientific/Whatman), 20 × 20 cm, having a 0.15 μm pore size and with a 250 μm coating were used for spotting the various samples. Silica gel on the TLC plate served as the stationary phase, and the mobile phase used was a 4 : 1 : 0.1 : 0.01 ratio of n-propanol : water : phosphoric acid : glacial acetic acid (30).

The inside of the developing jar was lined with 3MM Whatman chromatography paper and saturated with the mobile phase so that the atmosphere within the jar was saturated as the plate is being developed. The mobile phase was added to the jar, and the TLC plate was placed inside and pre-developed until the solvent reached its top to sweep away any impurities. This pre-development at room temperature took approximately five hours, and the TLC plate was then air-dried overnight within a fume hood.

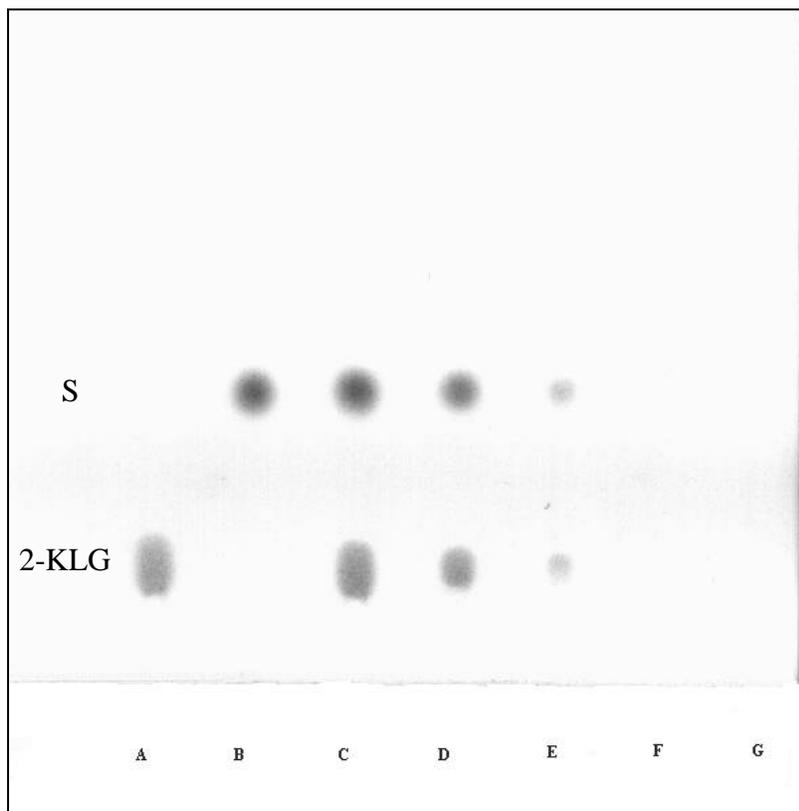
A micropipettor was used to apply five μl of each sample onto the pre-developed TLC plate in 1.5 μl, 1.5 μl, and 2 μl increments so that the size of the spot did not exceed 4 mm in diameter. The spots were air-dried after applying each increment. The spots were placed about 1.5 cm apart and 2 cm from the bottom of the plate on the inert pre-absorbent strip, which acted as an inactive blotter or staging area for rapid applications.

To test cultures for sorbose depletion and 2-KLG formation, 1 ml samples were aseptically removed from growth flasks every day for 10 days and placed in sterile 1.5 ml microcentrifuge tubes. These samples were centrifuged immediately for 10 minutes in a table top centrifuge to remove whole cells. Five μl of each fluid were then spotted along side 5 μl of an aqueous solution of 1% authentic 2-KLG and 5 μl of an aqueous solution of 1% authentic sorbose, which were used as standards. Also, 5 μl of the sterile Fermentation Medium lacking sorbose were spotted to determine if the medium alone contained any detectable compounds that might interfere with the assay. The spots were air-dried, and the plate was developed as described in the pre-development until the solvent reached its top. This development took about five hours. The plate was then air-dried as before.

To visualize the 2-KLG and the sorbose spots, TLC plates were sprayed with a fine, even mist of a spray reagent, containing equal volumes of 0.5 % tetrazolium blue chloride in methanol and 6N sodium hydroxide (30). The tetrazolium blue in this reagent detects all compounds with an alpha-keto group such as 2-KLG and sorbose. The spray reagent was made fresh each time used, because the tetrazolium undergoes an auto-reduction in approximately two hours after the reagent is

made. After spraying, plates were dried for 10 minutes at 110 °C. Both 2-KLG and sorbose were detected as blue spots on the developed plate.

Results. Standards of (w/v in dd H₂O) 1 % authentic 2-KLG, 1% authentic sorbose and mixtures of 1%, 0.5 %, 0.15%, 0.1% and 0.001% of both compounds were spotted on TLC plates to determine the retardation factors (R_f) of 2-KLG and sorbose, to test for good separation between the two compounds in a mixture and to examine the sensitivity of the qualitative TLC method. These same dilutions were also made in sterile Fermentation Medium lacking sorbose and were spotted on TLC plates to determine if other compounds in this medium could be detected or would interfere with the chromatography of sorbose or 2-KLG. No other spots were detected, and no interference occurred. This TLC method gave retardation factors (R_f) of 0.17 and 0.43 for 2-KLG and sorbose, respectively (Figure A1). The difference between these two values was high enough for a good separation between the two compounds in a mixture, as shown by the two distinct spots on the chromatogram. These R_f values did not change when the same dilutions were made in sterile Fermentation Medium lacking sorbose. The lowest concentration detected of both compounds was 0.15% (1.5 g/L) as shown in Figure A1.



Concentrations of 2-KLG and sorbose

Figure A 1. Thin layer chromatography (TLC) analysis for the separation of 2-keto-L-gulonic acid (2-KLG) from sorbose (S). The sensitivity of the TLC method and the separation of 2-KLG from sorbose were determined by spotting various concentrations of these two compounds on the TLC chromatogram. The concentrations used were the following (in % w/v of ddH₂O): (A)= 1 % 2-KLG, (B)= 1% sorbose, (C)= 1% 2-KLG and sorbose, (D)= 0.5% 2-KLG and sorbose, (E)= 0.15% 2-KLG and sorbose, (F)= 0.1% 2-KLG and sorbose, and (G)= 0.01% 2-KLG and sorbose.

APPENDIX B

High performance liquid chromatographic detection of 2-keto-L-gulonic acid

Introduction. High performance liquid chromatography (HPLC) was used to determine the yield of 2-keto-L-gulonic acid (2-KLG) in the growth medium during the 10-day incubation of various cultures. An ion-exchange method was developed and optimized for 2-KLG detection. The quantity of 2-KLG produced by various cultures was measured following a standard curve prepared by using known concentrations of authentic 2-KLG (standards), and then correlating the area under the 2-KLG peak to the concentration of 2-KLG.

Materials and Methods. The HPLC system used was an ion-exchange Dionex model 2010i. The column used was a Phenomenex ion-exchange column (STAR-ION, A-300, 100 mm × 4.6 mm) packed with styrene divinyl benzene, a support resin that is coated with a unique quaternary amine, and which served as the stationary phase. Ion-exchange chromatography was the method of choice, because 2-KLG is an ionizable acid that produces anions that compete with the anions of the mobile phase for the exchange site, the quaternary amine, that coats the stationary phase. The mobile phase consisted of a mixture of 70% deionized water, which was degassed with helium prior to use, and 30 % of an aqueous solution of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate. This water-to-buffer proportion gave the best retention time for 2-KLG and was achieved by using gradient programming. The HPLC system was programmed to test water to buffer concentrations ranging from 90:10 down to 50:50, and the concentration that gave the best retention time for 2-KLG was observed to be 70:30. The greater the ionic strength of the buffer (low water-to-buffer ratio), the more anions of the mobile phase are present, and this decreases the ability of the anions of the sample to interact with the exchange site. Thus, the sample will elute with the solvent with no retention in the column. The weaker the ionic strength of the buffer (high water-to-buffer ratio), the fewer anions of the mobile phase are present, and this will allow more interaction between the sample and the exchange site on the stationary phase, hence binding the anions strongly to the stationary phase and never eluting them from the column. Retention time is defined as the time elapsed between the sample introduction and maximum of response observed as a peak on the chromatogram. Retention time is characteristic of the sample and measures the interaction of the sample with both the mobile phase and the stationary phase. The sample partitions itself between these two phases and elutes after repetitive adsorptions back and forth between the stationary phase and the mobile phase. In the case of ion-exchange, the negatively charged anions of the solute (2-KLG) and the anions of the mobile phase (carbonate and bicarbonate) compete for the positively charged quaternary amine on the stationary phase, and movement of the solute through the column is slowed by these repetitive interactions.

The flow rate of the mobile phase was 2 ml/min, and the detector used was a suppressed conductivity electrochemical detector. The suppressor used was a 25 mM aqueous solution of sulfuric acid, and the injection volume was 10 µl.

A standard curve of area vs. concentration was generated using six known concentrations of authentic 2-KLG dissolved in the Fermentation Medium. Standard one contained (w/v) 3.638 % of 2-KLG dissolved in the Fermentation Medium. One milliliter of standard one was diluted with 9 ml and 99 ml of Fermentation Medium to achieve (w/v) 0.3638% (Standard two) and 0.03638% (Standard three) of 2-KLG. Standard four contained (w/v) 1.87% of 2-KLG dissolved

in the Fermentation Medium. One milliliter of standard four was diluted with 9 ml and 99 ml of Fermentation Medium to achieve (w/v) 0.187% (Standard five) and 0.0187 (Standard six) of 2-KLG. Ten microliters of the Fermentation Medium was injected into the column to check for background interference. Ten microliters of each known 2-KLG standard were then injected into the column, and the areas under the peaks were calculated by the HPLC system plotter.

The ion-exchange HPLC method does not detect sorbose, because sorbose is not an ionizable compound, so the depletion of sorbose during growth in the Fermentation Medium could not be detected using this method.

Results. A standard curve of area *versus* concentration (Figure B1) was prepared with six known concentrations of authentic 2-KLG, and this standard curve was used to determine the amount of 2-KLG accumulated in the Fermentation Medium.

The HPLC chromatogram of the Fermentation Medium alone (Figure B2 a) showed the complexity of the medium and the background peaks that might interfere with the elution of 2-KLG. The retention time (t_R) of 2-KLG was determined by dissolving a known amount of authentic 2-KLG in the Fermentation Medium, injecting this sample, and analyzing the chromatogram generated for the additional peak observed (Figure B2 b). The 2-KLG eluted at 1.86 minutes on the HPLC chromatogram when 10 μ l of standard two (0.3638% of 2-KLG) were injected into the column (Figure B2 b). When other 2-KLG concentrations were used, the retention time varied between 1.86 to 1.70 minutes.

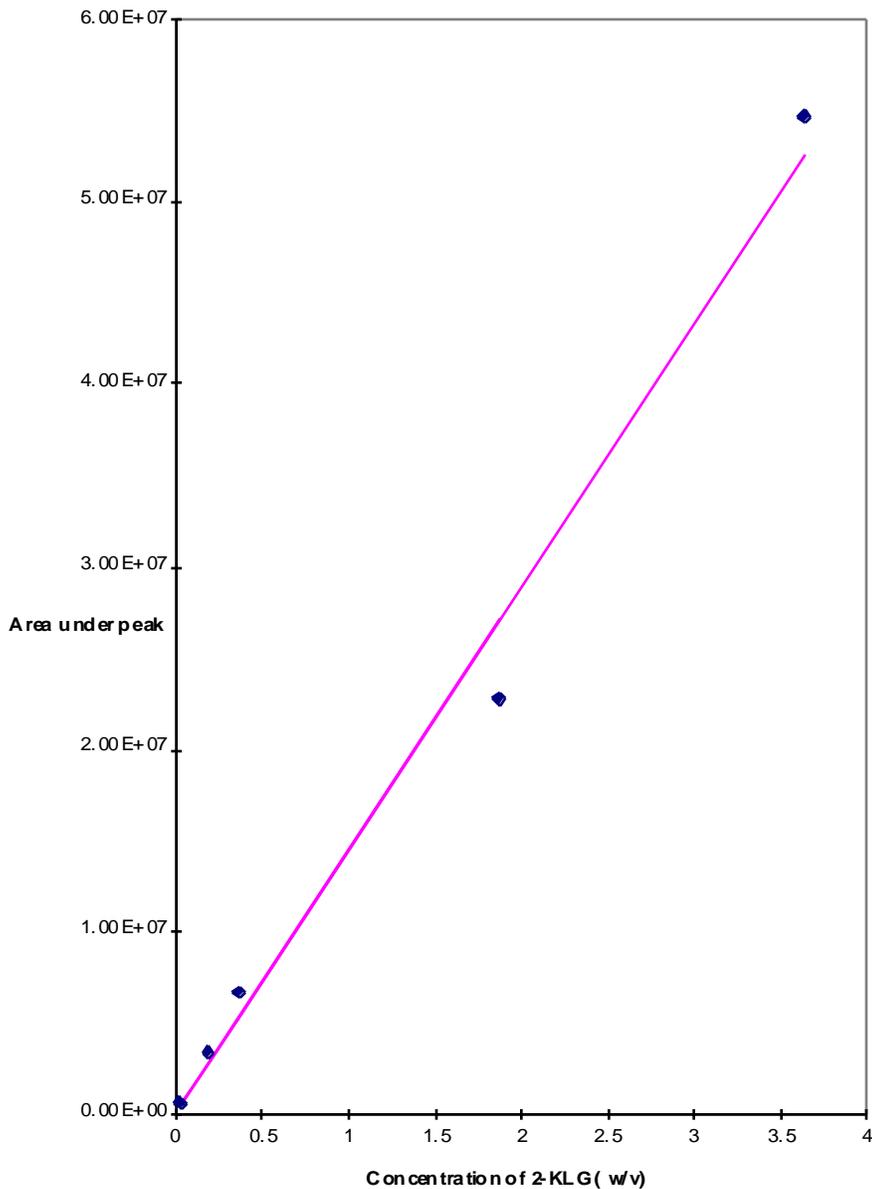


Figure B1. Standard Curve of area versus concentration prepared by using six known concentrations of authentic 2-keto-L-gulonic acid (2-KLG). Ten microliters of each standard were injected into the HPLC column, and the areas under the 2-KLG peaks were calculated by the HPLC system plotter. This standard curve was used to determine the amount of 2-KLG produced by the various *Gluconobacter* cultures.

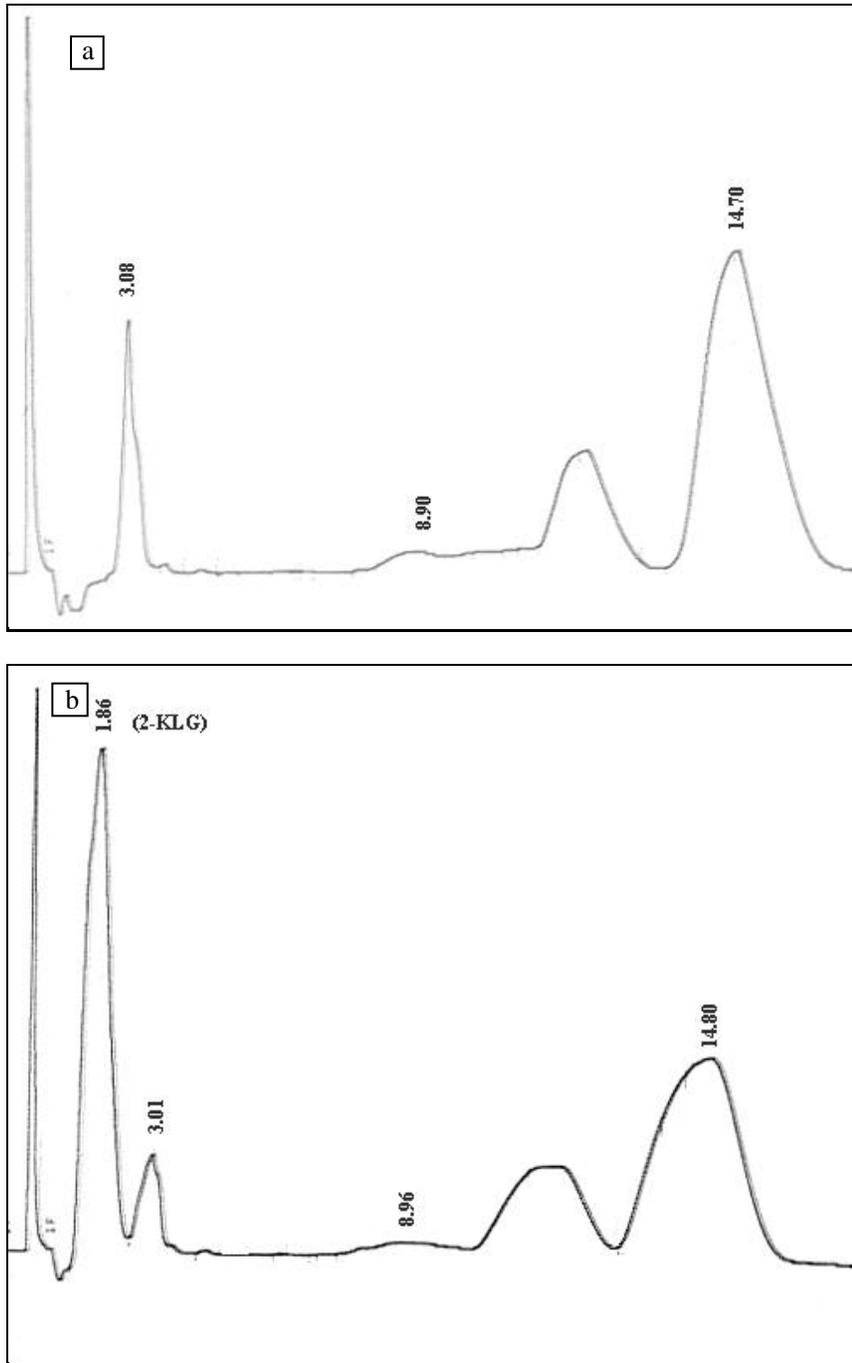
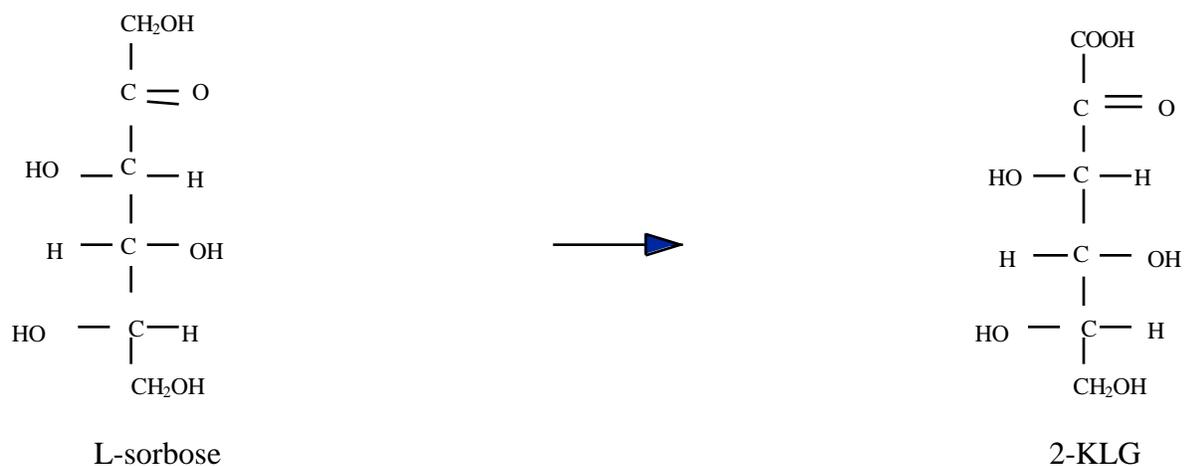


Figure B2. Detection of 2-keto-L-gulonic acid (2-KLG) in the Fermentation Medium using high performance liquid chromatography (HPLC). The HPLC chromatogram of the sterile Fermentation Medium (a) showed the peaks formed by unknown medium components. The HPLC chromatogram of (w/v) 0.3638% of 2-KLG dissolved in the Fermentation Medium (b) showed that 2-KLG eluted at 1.86 minutes.

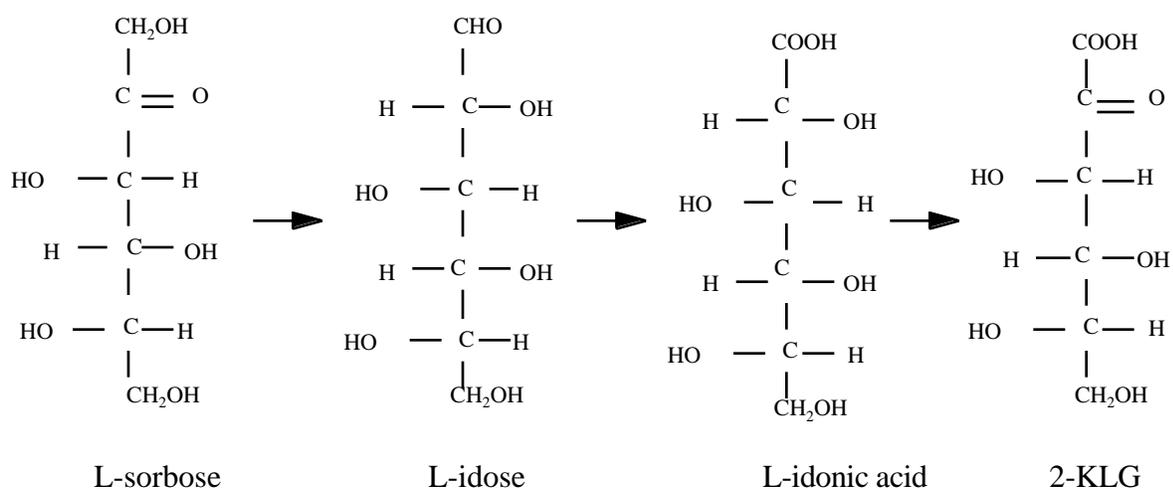
APPENDIX C

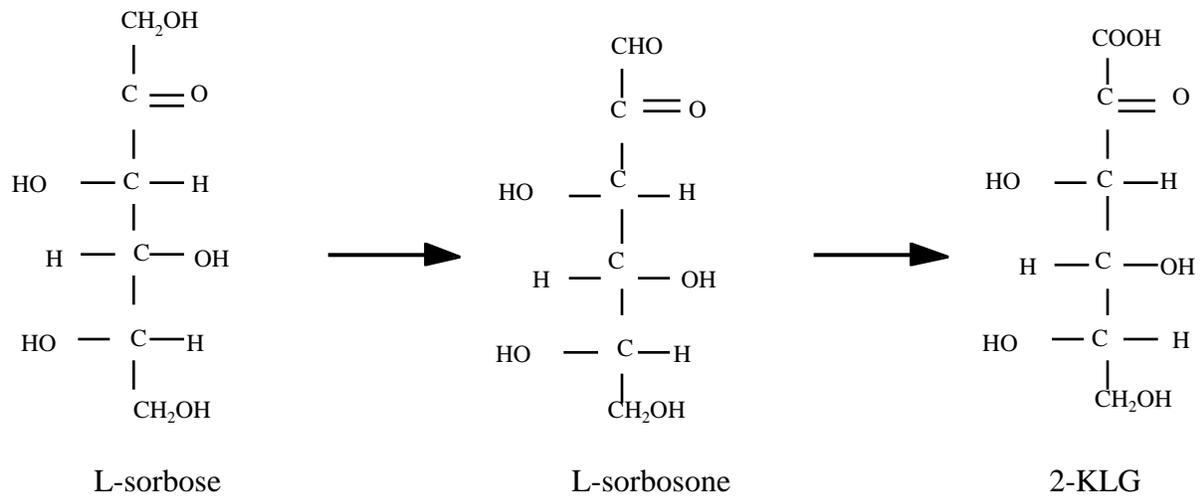
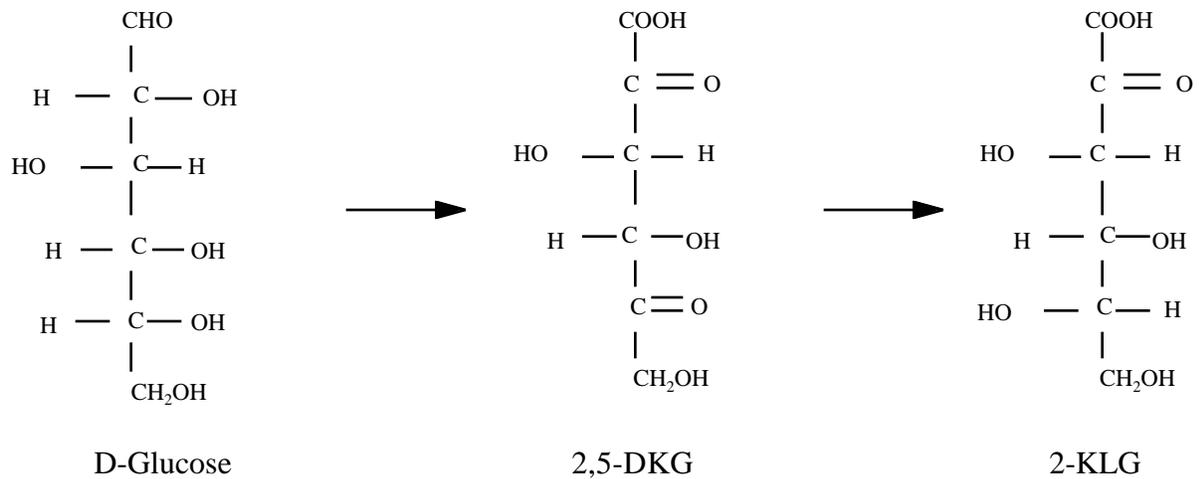
Pathways for 2-keto-L-gulonic acid production

Reaction 1: One-step conversion of L-sorbose to 2-keto-L-gulonic acid (2-KLG)



Reaction 2. Idose pathway



Reaction 3: L-sorbose pathway**Reaction 4: 2,5-Diketogluconate (2,5-DKG) pathway**

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Education:

Master of Science, Microbiology (February 1997)

Thesis : 2-Keto-L-Gulonic Acid Production by Different *Gluconobacter* Strains.

Advisor: G. William Claus

QCA: 3.96 / 4.00

Virginia Polytechnic Institute and State University
Blacksburg, VA 24061

Bachelor of Arts (May 1993, summa cum laude)

Major-Chemistry

Minor-Biology

QCA: 3.85 / 4.00

Virginia Polytechnic Institute and State University
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Research Interest(s):

Microbial physiology with industrial applications.

Related Experience:

1994 (Spring-Summer) Post-Undergraduate Research- “ The use of tetranitroblue tetrazolium assay for measuring useful biotransformations in *Gluconobacter* strains. ”

-Performed protein bioassays.

-Performed TNBT assay to detect oxidation/reduction of many different polyhydroxy compounds by *Gluconobacters* strains.

1994 (Fall- present) Graduate Research- “ Production of 2-keto-L-gulonic acid by *Gluconobacter* species as an important intermediate for commercial vitamin C synthesis. ”

- Designed experiments to detect 2-keto-L-gulonic acid (2-KLG) production by different *Gluconobacter* strains.
- Performed thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) for qualitative and quantitative detection of 2-KLG.
- Assisted in molecular biology techniques such as DNA extractions, agarose gel electrophoresis, southern blotting and transformation of *Gluconobacter* strains with plasmid DNA using electroporation.
- Maintained cultures and performed phenotypical tests on different *Gluconobacter* strains.
- Performed different biochemical tests on a wide array of microorganisms as part of GTA duties.

Teaching Experience:

Graduate Teaching Assistant

Fall Semester-1996

General Microbiology Laboratory

(Overall student evaluation rating-3.9/4.0)

Spring Semester-1996

General Microbiology Laboratory

(Overall student evaluation rating-3.9/4.0)

Fall Semester-1995

General Microbiology Laboratory

(Overall student evaluation rating-3.9)

Spring Semester-1995

General Biology Laboratory

(Overall student evaluation rating- 3.75)

Fall Semester-1994

General Biology Laboratory

(Overall student evaluation rating- 3.2)

Fall Semester-1993

General Chemistry Laboratory

-Supervised microbiological experiments and lectured about different microbial processes.

-Graded quizzes and assignments and advised students during office hours.

Publication(s):

Abstract:

Nassif, L., Y. Kazakevich, H. M. McNair, and G. W. Claus. 1996. 2-Keto-L-Gulonic Acid Production by different *Gluconobacter* Strains.

Submitted to: Abstracts for the 96th General Meeting of the American Society for Microbiology. New Orleans, Louisiana, 19-23 May 1996.

Presentations:

Poster Presentation:

“ 2-Keto-L-Gulonic Acid Production by Different *Gluconobacter* Strains ”
Presented at: 96th General Meeting of the American Society for Microbiology.
New Orleans, Louisiana, 19-23 May 1996.

Seminar (s):

“ Production of 2-Keto-L-Gulonic Acid by Different *Gluconobacter* Strains’ ”
Presented at: Annual Meeting, Virginia Branch- American Society for Microbiology. Charlottesville, VA 1-2 December, 1995.
Presented at: VPI & SU, Biology dept. Seminar, 17 April, 1996.

Research Grants Submitted:

“ Pathways for 2-Keto-L-Gulonic Acid Production by *Gluconobacters* ”
Sigma Xi Grant-In-Aid of Research
Submitted February, 1996 (not funded).

“ Formation of 2-keto-L-Gulonic acid by *Gluconobacters* as an important intermediate for commercial vitamin C synthesis ”
Sigma Xi Grant-In-Aid of Research
Submitted February, 1995 (not funded).

Meetings Attended:

96th General Meeting of the American Society for Microbiology
New Orleans, LA 19-23 May, 1996.

Annual Meeting, Virginia Branch- American Society for Microbiology
Charlottesville, VA 1-2 December, 1995.

95th General Meeting of the American Society for Microbiology
Washington, DC 21-25 May, 1995.

Annual Meeting, Virginia Branch- American Society for Microbiology
Richmond, VA 2-3 December, 1994.

Language competencies:

Fluent in English, French and Arabic due to my education in a French school in Beirut, Lebanon.

Award (s):

Undergraduate: Dean's List all semesters

Honorary Societies:

Phi Beta Kappa National Honor Society
Phi Kappa Phi National Honor Society
Phi Lambda Upsilon National Chemistry Honor Society
Phi Sigma Biology Honor Society

Professional Societies:

American Society for Microbiology
Virginia Branch- American Society for Microbiology
Virginia Academy of Science

Social\ Service Societies:

Alpha Phi Omega National Service Fraternity

Community Service:

Alpha Phi Omega: Service for Campus and Community, Fall 1992-Fall 1993.
Volunteered at Roanoke Memorial Hospital as a nurse's aid, Spring, 1992.

References:

Available upon request