review

Microbial processes for ascorbic acid biosynthesis: a review

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L-Ascorbic acid is an important product currently made using the Reichstein process, which is mainly chemical. Recently, bacteria have been identified that are able to transform in a very efficient way glucose to 2,5-keto-D-gluconic acid and this product to 2-keto-L-idonic acid, precursor of L-ascorbic acid. When the corresponding strains are used toegther, it is possible to get 2-keto-L-idonic acid directly from glucose. Moreover, new strains have been constructed by introducing a gene from a strain responsible for the second step into a strain responsible for the first step. By using one of the new strains, the transformation can be performed in a single step with only one strain. However, the classical process still remains the most competitive.

Keywords: L-Ascorbic acid; fermentation; bacteria; yeast; genetic engineering; enzyme

Introduction

L-Ascorbic acid finds its use mainly in the food industry, being a vitamin as well as possessing antioxidizing characteristics. During the last 20 years this product has steadily increased (the doubling time is about 6 years) and in 1984 world production reached 35,000 tons.[1] L-Ascorbic acid, initially isolated from citrus, was first chemically synthesized by the Reichstein procedure.[2] Some processes using bioconversions have been described,[3] but until now, due to low yields obtained, they have not been developed. One of the most recent processes involves a mixed fermentation with two steps including the application of genetic engineering. These latter techniques suggest the development of promising new processes able to compete with the Reichstein procedure.

The Reichstein process

In this process, the substrate (glucose) is transformed in five steps as follows (*Figure 1*):

1. The reduction of glucose to sorbitol using a nickel catalyst.
2. The oxidation of L-sorbitol to L-sorbose, which may be performed by *Bacterium xylinum* (also named *Acetobacter xylinum*) or by *Acetobacter suboxydans*.
3. The production of diacetone-sorbose or 2,3 : 4,6-di-isopropylidene-L-xylo-2-hexofuranose after treatment with acetone and sulfuric acid.
4. An oxidation of this product to 2-keto-L-gulonic acid using platinium as catalyst.
5. Enolization and internal lactonization of this 2-keto-L-gulonic acid to L-ascorbic acid.

The first industrial trials using this five-step process allowed a yield of 15–18% conversion of sorbitol to L-ascorbic acid. Until now, this has remained the method of choice due to the cheap availability of the glucose substrate, the chemical stability of the intermediates (especially diacetone-sorbitol), and the improvements introduced into the procedure.

Theoretically, one molecule of glucose (MW 180) produces one molecule of L-ascorbic acid (MW 176). In the most favorable hypothesis, the yield can reach 98%. In fact, because of molecular rearrangement, it is observed that three hydrogen atoms and one to four oxygen atoms cannot be derived from the glucose molecule. Thus, the maximum yield cannot be more than 87%.

At the moment, with the acquired improvements, the practical yield of this chemical process is thought to be around 50%.

Bacterial fermentation processes

At present there are six bacterial fermentation processes for vitamin C production. However, all of these processes give as a direct precursor of L-ascorbic acid, 2-keto-L-gulonic acid, which is also called 2-keto-L-

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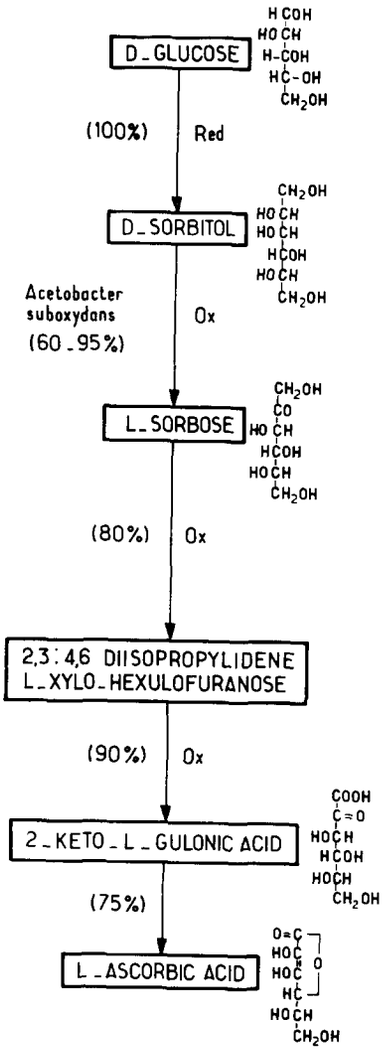

Figure 1 Scheme indicating the reactions used in the synthesis of L-ascorbic acid according to the Reichstein process. This figure indicates the formulae of the main intermediate products, the type of transformation, either oxidation (Ox) or reduction (Red), and the yields obtained

idonic acid. The different pathways, named after one of their main metabolic intermediates, are the following:

1. Sorbitol pathway;
2. L-idonic acid pathway;
3. L-gulonic acid pathway;
4. 2-keto-D-gluconic acid pathway;
5. 2-5-diketo-D-gluconic acid pathway;
6. 2-keto-L-gulonic acid pathway.

These are summarized in *Figure 2* and explained below.

Sorbitol pathway

This biosynthesis was described for the first time by Motizuki *et al.*[4] Sorbitol is transformed by fermentation to 2-keto-L-gulonic acid. Transformation is performed by several strains of the genus *Pseudomonas* and *Acetobacter*, but details of the metabolic pathway are not known.

The yield of transformation of sorbitol to 2-keto-gulonic acid does not normally exceed 10%, although a yield of 70% (since disputed[5]) has been recorded for one strain of *Acetobacter cerenusote*.

This transformation has also been described by several other authors.[6,7] Beside 2-keto-L-gulonic acid, some other products are also formed.[8,9] Okazaki *et al.*[9] suggested the following biosynthesis pathway:

sorbitol
↓
L-sorbose
↓
L-idose
↓
L-idonic acid
↓
2-keto-L-gulonic acid

L-Idonic pathway

The biosynthesis using L-idonic acid as an intermediate is a multistep transformation. The actual known metabolic intermediates are D-gluconic acid, 5-keto-D-gluconic (or 5-keto-L-idonic) acid, L-idonic acid, and 2-keto-L-idonic (or 2-keto-L-gulonic) acid.

The first oxidation step, transformation of D-glucose to D-gluconic acid, is not detailed here, and only information concerning the three following reaction steps is given.

Oxidation of D-gluconic acid. The first known oxidation of D-gluconic acid to 5-keto-D-gluconic acid was performed by Boutroux[10–12] using *Bacterium oblungus*. This transformation has also been carried out using *Acetobacter melanogenum*,[13] *Acetobacter suboxydans*,[14–30] *Bacterium* spp.,[31–34] *Pseudomonas* spp.,[35] and others.[17,36] Among the cited microorganisms, *Acetobacter suboxydans* appears to be the most efficient, with transformation yields of glucose to 5-keto-D-gluconic acid of about 90%.[20,30,37]

Reduction of 5-keto-D-gluconic acid. Several processes allowing the reduction of 5-keto-D-gluconic acid to L-idonic acid have been described.[38,39,42] Note that the process proposed by Sonoyama *et al.*[39] mentions the extraction of an enzymatic system from *Fusarium oxysporum* able to perform this transformation with a yield of 80%. However, this reaction is not completely direct. There is a loss of efficiency due to recycling of 5-keto-D-gluconic acid to D-gluconic acid.[43,44] The series of reactions is summarized below.

(glucose)
↓
D-gluconic acid
↓
5-keto-D-gluconic acid
↓
L-idonic acid
↓
2-keto-L-idonic acid

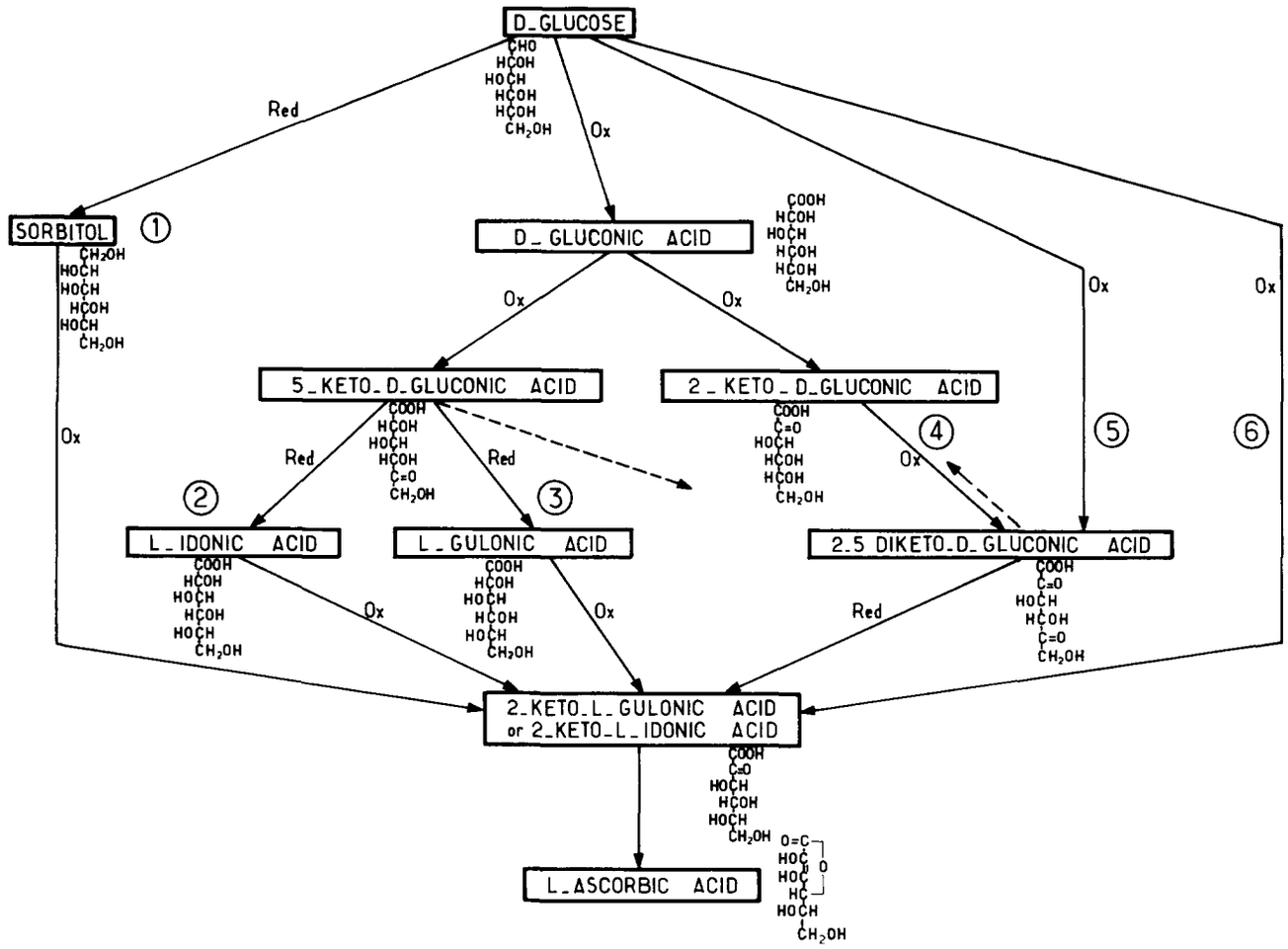

Figure 2 Diagram representing the different pathways for the transformation of glucose to 2-keto-L-gulonic acid also named 2-keto-L-idonic acid, direct precursor of L-ascorbic acid. This figure indicates the formulae of the intermediate products, and the types of transformation, either oxidation (Ox) or reduction (Red). Dotted lines indicate minor reactions

This transformation is also performed by *Acetobacter suboxydans*,[45] *Bacterium glucunicum*,[31,32] *Pseudomonas* spp.,[35] and *Acetobacter* spp.

Oxidation of L-idonic acid. The first microorganism known to perform this transformation is *Pseudomonas mildenbergii*, with a measured yield of 80%.[43] Other microorganisms having that property were also identified: *Acetobacter* spp.,[46] *Cyanococcus chromospirans*,[47] *Micrococcus auriantacus*,[48] *Pseudomonas aeruginosa*,[49] *Ps. fluorescens*,[50–56] and *Ps. 2-ketogulonicum*.[57] With *Ps. fluorescens*, transformation yields of 90% have been mentioned.[51–54] High yield values have also been found by Liebster *et al.*[58] using *Pseudomonas chromospirans* and by Fujisawa *et al.*[59] using *Pseudomonas fluorescens*.

L-*Gulonic acid pathway (oxidation of* L-*gulonic acid)*

The L-gulonic acid pathway has in common with the L-idonic pathway the two first steps of that pathway (oxidation of D-gluconic acid and reduction of 5-keto-D-gluconic acid). But this last reaction leads to the formation of L-gulonic acid, the precursor of 2-keto-L-idonic acid (*Figure 2*). The first two steps have already been described. Details concerning the oxidation of L-gulonic acid are given by Kita.[60] According to Kita, the transformation of 5-keto-D-gluconic acid can be performed using *Xanthomonas transluscens*, with a yield of 90% and a final concentration of 100 g l^{-1},[1] or *Xanthomonas trifolii* (*Erwinia lahyri*).[61,62]

*2-Keto-*D-*gluconic acid pathway*

Three main steps constitute this pathway:

Oxidation of D-gluconic acid. A large number of microorganisms can transform D-gluconic acid using this pathway.[3] The transformation of D-glucose to 2,5-diketo-gulonic acid was identified first by Katznelson *et al.*[63] in *Acetobacter melanogenum* and later in *Pseudomonas albosesamae*.

It should be also noted that *Acetobacter suboxy-*

dans, known for synthetizing 5-keto-D-gluconic acid from D-gluconic acid, can synthetize 2-keto-D-gluconic acid.[16,18,64,65]

In addition, in 1982, Sonoyama *et al.*[66] mentioned for the first time the ability of *Erwinia* spp. to accumulate D-gluconic and 2-keto-D-gluconic acids.

Oxidation of 2-keto-D-gluconic acid. This oxidation may be carried out by *Bacterium hoshigaki*[67] and *Bacterium glucunicum*[16,17,68] with 2,5-D-diketo-D-gluconic acid as product. This is also biosynthetized by *Acetobacter* spp. especially *A. melanogenum*.[32,67,69–71] This illustrates the bioconversion of glucose with D-gluconic and 2-keto-D-gluconic acids as intermediates.[72] A process that directly transforms D-glucose into 2,5-diketo-D-gluconic acid using either *Acetobacter fragum*[73] or *Acetomonas albosesamae*[74–75] has also been published.

Reduction of 2,5-diketo-D-gluconic acid. This is the step that has been the most studied. In 1975, Sonoyama *et al.* described a process producing 2-keto-L-gulonic acid from 2,5-kieto-D-gluconic acid.[76,77] The mentioned strains are of the genus *Brevibacterium, Arthrobacter, Micrococcus, Staphylococcus, Pseudomonas,* and *Bacillus*.

With *Brevibacterium ketosporum* the yield can reach 15% even when initial substrate concentration is 50 g l^{-1}. With the other microorganisms, yields are not more than 1%.

The use of *Corynebacterium* has been suggested since 1976, first assays giving a 2-keto-L-gulonic acid production yield of about 10%. Present-day performances are reported to be near 80%.[76]

Another process should also be mentioned that employs *Citrobacter* strains. However, these strains catalyse only the transformation of 2-5-diketo-D-gulonic acid, and one preliminary step performed with *Acetobacter cerenus* is necessary. With such microorganisms, yields of about 30% are mentioned, with initial substrate concentration of 100 g l^{-1}.[78]

2,5-Diketo-D-gluconic acid pathway

Processes allowing the production of 2,5-diketo-D-gluconic acid in a single step have been set up. The genus *Erwinia* already mentioned (see Oxidation of D-gluconic acid) is also noted for this transformation.[79] The given descriptions mention a concentration in glucose of 200 g l^{-1} and fermentation time of 20 h producing yields of about 75%.

Moreover, it has been noted that interesting performances have been obtained simply by maintaining contact of glucose with cell debris. Fermentation procedures using *Acetobacter cerenus* allowing yields of about 90% have been described.[80–81]

2-Keto-L-gulonic acid pathway

As shown in *Figure 2*, this pathway is apparently direct, and allows the production of 2-keto-L-gulonic acid, direct precursor of L-ascorbic acid from glucose. However, this pathway has been made possible only with the advent of recent developments. Among these, the main ones are:

1. Two-stage or mixed cultures;
2. Mutant selection;
3. Isolation of 2,5-diketo-D-gluconic acid reductase in *Corynebacterium;*
4. Gene transfer of 2,5-diketo-D-gluconic acid reductase of *Corynebacterium* into *Erwinia*.

Two-stage and mixed cultures. The realization of two-stage cultures for the preparation of 2-keto-L-gulonic acid was first described by Sonoyama.[82] This progress resulted from the observation that *Erwinia* has the ability to transform glucose into 2,5-diketo-D-gluconic acid,[66] while *Brevibacterium* transforms this latter product to 2-keto-L-gulonic acid.[76,83]

Mixed staged cultures were also first described by Sonoyama *et al.*[84] These allow the production of 2-keto-L-gulonic acid from glucose. Two strains are used. The first strain is: *Acetomonas, Acetobacter, Gluconobacter,* or *Erwinia*[85]; the second is *Brevibacterium*. The first strain transforms glucose into 2,5-diketo-D-gluconic acid, and the second transforms this product into 2-keto-L-gulonic acid.

This two-step process may support certain variations, but generally the initial glucose concentration is such that the 2,5-diketo-D-gulonic acid concentration in the medium reaches about 25 g l^{-1} (with yields of about 10–15%).

This process is interesting because 2-keto-D-gluconic acid is not present in the medium. In fact, this last product, which is metabolized by the first strain, is normally difficult to separate from its optical isomer, which is the desired product.

Mixed staged cultures using mutants. The improvement of this process has been initiated with mutants of *Corynebacterium* replacing *Brevibacterium* as the second strain.[86] The mutants employed have the following characteristics:

First, they are unable to metabolize 5-keto-D-gluconic acid from 2,5-diketo-D-gluconic acid (this being a normal characteristic of the *Corynebacterium* genus).

Second, the reduction of 2,5-diketo-D-gluconic acid to 2-keto-L-gulonic acid is made more efficient in the presence of hydrogen donors or nitrate salts which permit the use of carbohydrates or organic acids as hydrogen donors.

Another aspect of this process is the use of a surfactant, such as sodium dodecyl sulfate, which kills the first strain at the end of the first phase of growth without inhibiting growth of the second. With such improvements, yields of transformation of 2,5-diketo-D-gluconic acid to 2-keto-L-idonic acid can reach 93%.

Thus, the transformation of glucose to 2-keto-L-gulonic acid using a mixed staged culture with *Acetobacter* and *Corynebacterium* mutants can reach values of 80%. These values are quite compatible with those theoretically obtainable with the Reischtein process,

indicating the growing importance of biotechnological processes.

Existence of the 2,5-diketo-D-reductase activity in *Corynebacterium*. The enzyme responsible for the reduction of 2,5-diketo-D-gluconic acid to 2-keto-L-gulonic acid by *Corynebacterium* has been identified and characterized by Sonoyama *et al.*[87] This enzyme is intracellular and requires a cofactor. Its optimum pH is 6–7, its molecular weight is 29,000 and its isoelectric point is 4.4. Oxalate ions and glyceraldehyde are potent inhibitors. Beside the previous reaction, this enzyme also catalyses the reduction of 5-keto-fructose to L-sorbose.

Application of genetic engineering. The transformation of glucose to 2-keto-L-gulonic acid is performed in two steps, a fermentation followed by a biotransformation. The use of genetic engineering techniques allows the insertion of the gene responsible for the biosynthesis of the enzyme catalysing the second transformation into a microorganism performing the initial transformation.[88,89] For such ends, a vector containing the enzyme gene has been constructed and introduced into *Erwinia herbicola* allowing the expression of 2,5-diketo-D-gulonic reductase of *Corynebacterium*. Thus, using such engineered microorganisms, it is possible now to transform glucose directly into the precursor of L-ascorbic acid, 2-keto-L-gulonic acid. Transformation yields of glucose can reach 30%, values that seem to be much less than those obtained with the two-step assays.

Preparation of ascorbic acid

Each of the previous pathways leads to 2-keto-L-idonic acid (also called 2-keto-L-gulonic acid; see *Figures 1* and *2*), the stable direct precursor of L-ascorbic acid. To date, the transformation of the first product into the second is chemically performed in very acidic and alcoholic conditions. For example, one process involves methyl-2-keto-L-idonate.[90] Generally, the yields from transformation reach values of about 75%.[91–93]

Yeast fermentation process

Yeasts are known to exhibit two main characteristics as regards L-ascorbic acid biosynthesis.

First, yeasts constitutively produce the enzyme L-galacto-γ-lactone oxidase, which is able to catalyse the transformation of L-gulono-γ-lactone into L-ascorbic acid.[94–96] Yeasts can also produce enediol analogs of L-ascorbic acid from D-glucose.[97]

There is doubt as to the usefulness of yeast for a process in which bacteria are well established. The main problem is that yeast can catalyse only one of the seven steps of biotransformation required. This particular series of reactions is not encountered in bacteria but might be present in plant and animal liver cells. This series is summarized below:

glucose
↓
glucose-6-phosphate
↓
UDP-glucose
↓
UDP-D-glucuronic acid
↓
D-glucuronic acid
↓
L-gulonic acid
↓
L-gulono-γ-lactone
↓
L-ascorbic acid

Second, yeasts produce D-erythroascorbic acid, analog of L-ascorbic acid, from sucrose, hexoses, and pentoses.[98–100] The enzyme responsible for this transformation is located in mitochondria.[100] Many species are mentioned: *Candida, Hansenula, Klyuveromyces, Torulopsis,* etc. Among these, *Candida* appears to be the most proficient, especially if selected after mutation as a function of their acid production.

Discussion

The overall pathways and reactions allowing the preparation of L-ascorbic acid from glucose have been described.

Yeasts and eukaryotic cells have not been studied extensively, and for the moment they were found constitutive in only one reaction of the seven-step pathway.

Bacteria appear to be the most analyzed microorganisms, and apparently are the most efficient. This biotransformation requires five reactions and there are six possible pathways. One, two, or four intermediates may be necessary, with 2-keto-L-gulonic acid being the direct precursor of L-ascorbic acid. The transformation of this last compound was up to now chemically performed. These pathways are summarized in *Figure 3*, in which the most representative microorganisms performing the different steps with corresponding yields of transformation have been indicated.

The main characteristics of these bacterial transformations are the following:

The sorbitol pathway has sorbitol as the unique intermediate. Its yield does not exceed 8%.

The L-idonic acid pathway utilizes three microorganisms (*Acetobacter suboxydans, Fusarium oxysporum, Pseudomonas* spp.). With such a transformation, the overall yield can reach 65%.

The L-gulonic acid pathway is analog to the previous. However, the transformation of 5-keto-D-gluconic acid into L-gulonic acid does not appear to occur.

The 2-keto-D-gluconic acid pathway requires three steps, each performed preferentially the first with *Erwinia*, the second with *Acetobacter*, and the third with *Brevibacterium* or *Corynebacterium*. In this case, the overall yield cannot exceed 65%. The 2,5-diketo-D-gluconic acid pathway is performed in two steps, the

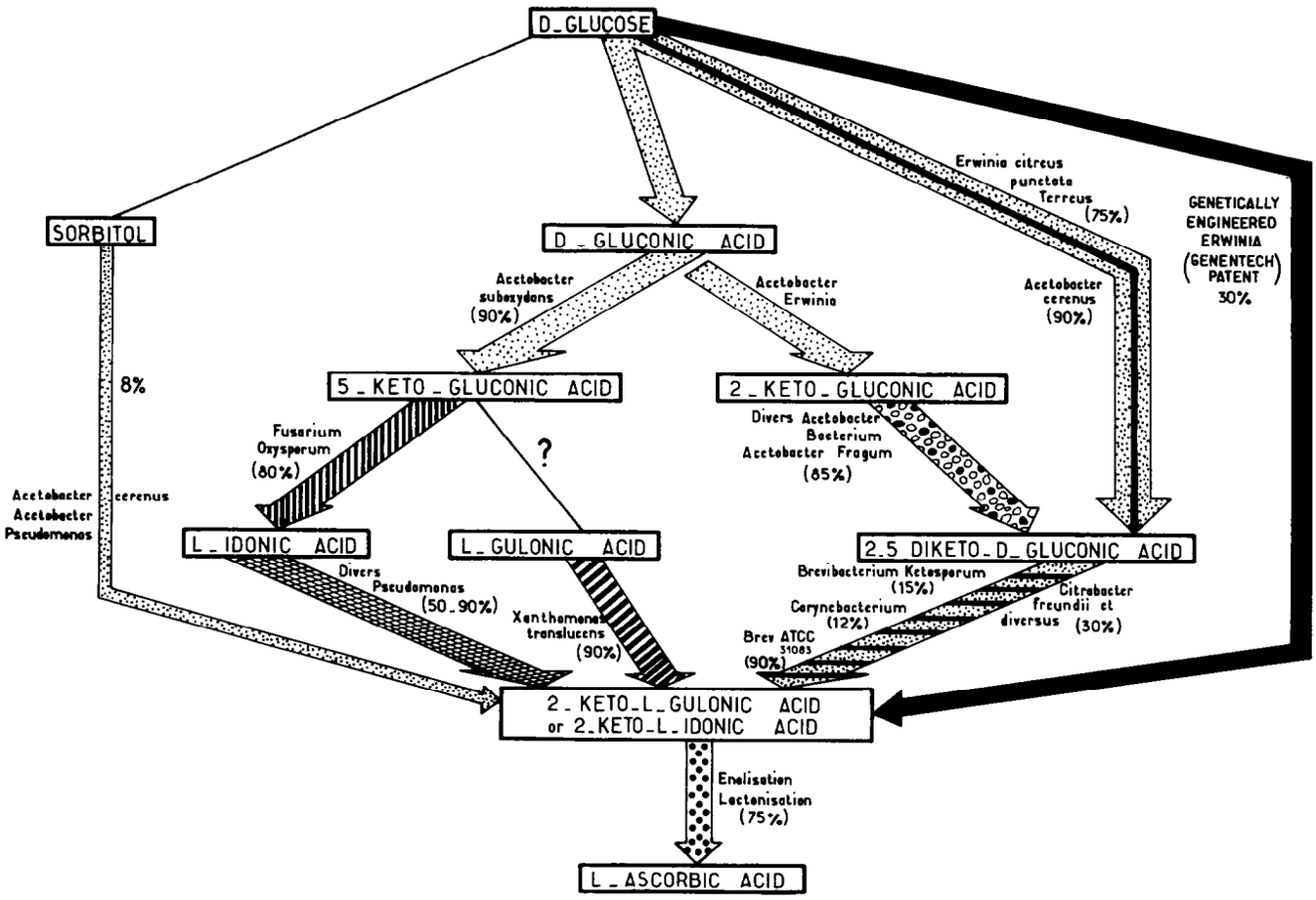

Figure 3 Diagram representing the different pathways for the transformation of glucose to 2-keto-L-gulonic acid, also called 2-keto-L-idonic acid, direct precursor of L-ascorbic acid. This figure shows the main microorganisms which are known to perform these transformations. Percentage values in parentheses are transformation yields obtained with the indicated microorganism

first by *Erwinia* or *Acetobacter cerenus*, the second by *Brevibacterium* or *Corynebacterium*. The overall yield can reach 75%.

The 2-keto-L-gulonic acid pathway involves a two-stage process or a mixed culture fermentation of *Erwinia* with *Corynebacterium* or *Brevibacterium* mutants. The yield now reaches 90%. With the use of genetic engineering techniques, this pathway can be performed in one step but apparently with lower performances (about 30% yield).

During recent years, available information indicates that L-ascorbic acid has been produced essentially by the Reichstein method. It is known that in this process four steps, one biochemical and three chemical, are necessary to transform sorbitol into ascorbic acid (see *Figure 1*). If the hydrogenation of glucose, necessary for obtaining sorbitol, is included, there are thus five steps and the overall yield is now around 50%.

The most significant developments in the use of microorganisms for the production of L-ascorbic acid are those of Sonoyama *et al.*[82–86] and of Estell *et al.*[87–88] The former, using mixed staged cultures with special mutants, allow global yields of the same order of magnitude as those obtained with the Reichstein process; the latter, using genetically engineered microorganisms, reach performances of 22%. However, information on optimization of genetic modification of the bacteria host is at this time incomplete or unpublished.

It would seem that the most promising biochemical process competitive with the Reichstein procedure would have only one biotransformation step. Thus, it is necessary that the bacterial pathway includes the last transformation (2-keto-L-gulonic acid to L-ascorbic acid). This implies further genetic modifications of the engineered bacterial cell. It has been seen that the last step allows the conversion of the formula $C_6H_{10}O_7$ (2-keto-L-gulonic acid) to the formula $C_6H_8O_6$ (L-ascorbic acid, *Figure 2*). Thus it corresponds to one reduction and one oxidoreduction. At least two enzymes are required, signifying the transfer of at least two more genes into the bacterial host. In order to get a single-step fermentation process able to produce L-ascorbic acid from glucose, it is necessary to introduce a total of at least six enzymatic activities into bacteria, while according to recent progress, only four might be present.

Conclusion

In summary, in order to obtain in one step L-ascorbic acid from glucose, an ideal bacterium requires at least six enzymatic activities. Compared with what is present in yeast (seven enzymes), this number appears not very different. Although until now, most work has been carried out using prokaryotic cells, it is not evident that bacteria are the best hosts for this kind of fermentation. At present, genetically modified bacteria with four enzyme activities have been produced as compared to the single activity known to be carried by certain yeasts. It is this comparison which makes bacteria apparently preferable. However, only additional research will allow determination of the best host cell. In any case, to have a successful direct biological process for L-ascorbic acid production, research will lead to a multi-genetically engineered cell. As for L-ascorbic acid preparation, up to now bacteria remain the best candidate.

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Note added in proof: In a recent paper, Grindley *et al.*[1] proposed the utilization of *Erwinia citreus* as the host strain and the modification of fermentation conditions and of the promoter used for the expression of the *Corynebacterium* reductase. Direct conversion of glucose into 2-keto-L-gulonate with a yield of 49% and a final concentration of about 20 g/l was obtained after 72 hours of fermentation.

1. Grindley, J. F., Payton, M. A., Van de Pol, H. and Hardy, G. *Appl. Environ. Microbiol.* 1988, **54,** 1770–1775.