Biotechnologically relevant features of gluconic acid production by acetic acid bacteria

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Abstract

The many uses of gluconic acid and some of its salts are arousing increasing interest in these compounds and in their production levels. Although gluconic acid and gluconates can be obtained chemically, they are currently almost exclusively biotechnologically produced, mostly by fungus based methods. There is, however, an ongoing search for alternative microorganisms to avoid the problems of using fungi for this purpose and to improve the productivity of the process. Especially promising in this respect are acetic acid bacteria, particularly *Gluconobacter* strains. This paper discusses the main variables and operating conditions to be considered in optimizing gluconic acid production by *Gluconobacter*.

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Introduction

Gluconic acid (GA) and some of its salts are widely used by various industries among which food, building, textile and pharmaceutical ones can be highlighted.[1] The fact that they are listed among the most produced chemicals[2] testifies to their importance. All the products in this list have been the subject to careful study in order to avoid potential risks for humans and the environment, at present, GA and its salts are classified as low-hazard substances.[3]

A total amount of 65,000 to 100,000 metric tons of GA and gluconates is produced each year,[3,4] virtually exclusively via fermentation processes. The production and uses of GA and its salts are described in detail elsewhere.[1,5-11] Briefly, these substances are used as ingredients of personal care products, pharmaceuticals, chelating agents (*e.g.*, for calcium in the building industry), industrial cleaners and metal surface treatments[8] as well as stabilizing agents for textile bleachers.[3] Additionally, because of their prebiotic properties, GA and its major derivatives are being used as additives in foods and drinks and are considered as key components in the development of new beverages.[1,12-14] Despite their flexibility and the large amounts used, no potential hazards justifying further investigation of their safety have so far been identified.

The most common biotechnological process for GA production is based on fungi and uses *Aspergillus niger*. This microorganism yields the GA via D-glucono-δ-lactone as intermediate reaction product. Specifically, the enzyme glucose oxidase catalyses the conversion of glucose to the lactone, which subsequently gives GA (fast at either acidic or alkaline conditions and slow at neutral pH; also the hydrolisis of glucono-δ-lactone is enzymatically facilitated by lactonase, specially required at neutral pH). The process requires carefully adjusting the pH of the medium and ensuring an adequate supply of oxygen.[8] In fact, the high oxygen demand of the biotransformation process, and the typical difficulties of homogenizing a highly viscous medium such as one with fungal mycelia, considerably raise operational costs and hinder homogeneous distribution of dissolved oxygen in the culture

medium.[15] Also, the pH of the medium should be controlled in the range 4.5-7.0 because a lower value triggers the tricarboxylic acid cycle and leads to citric rather than GA.[8] These difficulties have boosted a search for alternative microorganisms capable of producing GA[1] (particularly acetic acid bacteria).

Acetic acid bacteria

Belonging to the family *Acetobacteraceae*, acetic acid bacteria (AAB) comprise 19 genera and 87 species,[16] among which representatives of G*luconobacter* are especially prominent with regard to GA production.[11,17-22] Similarly to fungi - albeit with different enzymes -, these bacteria oxidize glucose virtually stoichiometrically to GA via glucono-δ-lactone.[21] The oxidation reaction occurs in the bacterial periplasm and is effected by a membrane-bound pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) located in the outer side of the cytoplasmic membrane; glucose and the resulting GA may undergo other biotransformation reactions depending on the particular operating conditions.[21,23-34]

One of the most remarkable features of *Gluconobacter* spp is their incomplete oxidation metabolism, by which a large amount of glucose can be rapidly oxidized to GA for energy generation while simultaneously acidifying the medium;[28,34,35] GA thus formed can be further converted into various ketoacids.[36] Although glucose, gluconates and ketogluconates can be used by the microorganism[25,35,37] (Figure 1), all these pathways are strongly influenced by some variables such as pH, the glucose concentration in the culture medium, the amount of dissolved oxygen and the presence of calcium ions. It is therefore essential to carefully examine the influence of these variables in order to optimize the operating conditions for optimal conversion of glucose to GA and protection of the product from further reaction.

Optimum conditions for gluconic acid production

Influence of pH and the initial concentration of substrate

Olijve and Kok[25] examined the influence of pH on GA production by *Gluconobacter oxydans* (ATCC 621 H) in two different culture media and found the assimilative pentose phosphate pathway to be virtually completely inhibited below pH 3.5-4. Since the formation of GA as the fermentation process develops decreases the pH of the medium, it seems reasonable to either control this variable or keep it below the inhibitory level. This is logical if one considered the metabolic features of the genus *Gluconobacter* and the action of its enzymes - some of which are illustrated in Figure 1. Such enzymes[36-38] are either dehydrogenases bound to the cytoplasmic membrane or soluble NAD(P)-dependent cytosolic oxidoreductases. The former are usually highly active at acid pH (especially in the range 3.0-6.0), whereas the activity of the latter is more prominent in alkaline media (particularly at pH 8.0–11.0). Olijve and Kok[25] also found the initial concentration of glucose in the medium to be strongly influential on the oxidation of glucose and gluconate through the pentose phosphate pathway; specifically, this pathway was considerably less important - and led to GA rapidly accumulating in the medium - at glucose concentrations

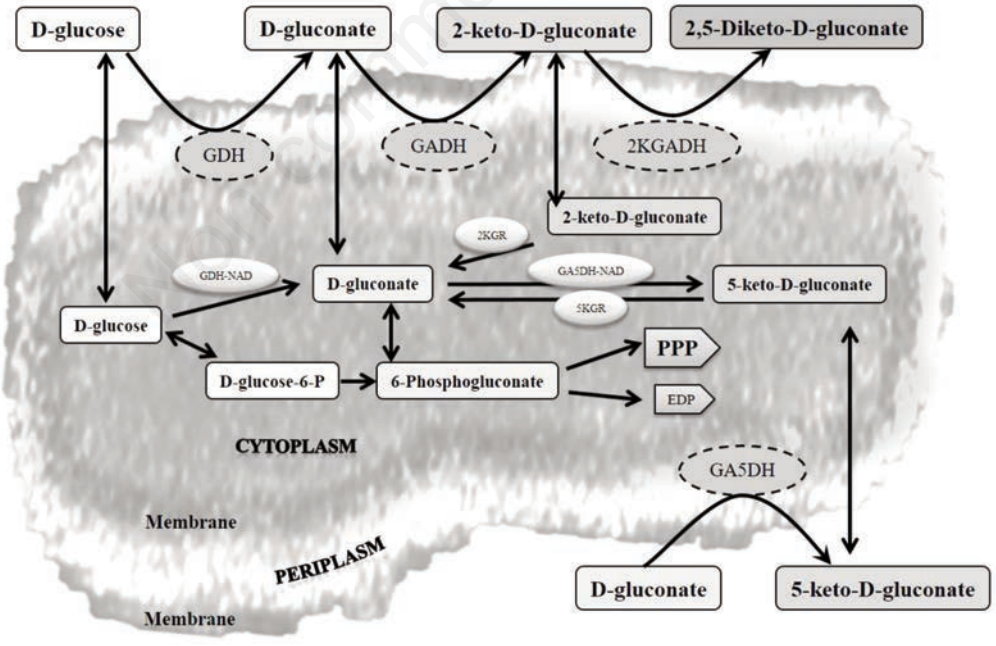

Figure 1. Glucose metabolism in *Gluconobacter*. GDH, PQQ-dependent D-glucose dehydrogenase (in the cytoplasmic membrane, EC 1.1.5.2) and (GDH-NAD) NADP-dependent D-glucose dehydrogenase (in the cytoplasm EC 1.1.1.47); GADH: FAD-dependent D-gluconate 2-dehydrogenase (EC 1.1.99.3); 2KGADH: FAD-dependent 2-keto-D-gluconate dehydrogenase (EC 1.1.99.4); GA5DH, PPQ-dependent D-gluconate 5-dehydrogenase (in cytoplasmic membrane) and (GA5DH-NAD) NADP-dependent D-gluconate 5-dehydrogenase (in cytoplasm EC 1.1.1.69); 2KGR: 2-keto-D-gluconate reductase (EC 1.1.99.3); 5KGR: 5-keto-D-gluconate reductase (EC 1.1.1.69). PPP, Pentose Phospate Pathway. EDP, Entner Doudoroff Pathway.

above 0.9–2.7 g/L (5-15 mM); at lower concentrations, the share of the uptake pathway is more prominent. This microbial behaviour, which is closely related to substrate availability in the medium, is seemingly used by AAB to compete with other microorganisms.[28,34,35] In fact, if AAB are present in an environment rich in carbon sources that can be rapidly depleted by other competing microorganisms, they cause the fast, imcomplete oxidation of such sources (*e.g.*, by converting sugars into sugar acids, which are less readily used by many other microorganims, and facilitating their accumulation as carbon sources for subsequent use). Simultaneously, AAB lower the pH and increase the inhibitory effects of the accumulated acids on other microorganisms. Obviously, when the fermentation medium contains little glucose as substrate, AAB must inevitably assimilate a significant part of it for biomass synthesis. Therefore, ensuring efficient GA production with AAB entails using relatively high initial concentrations of glucose - above the previously mentioned concentration range in any case.

Due to the activity of the membrane bound dehydrogenases forming GA from higher concentrations of glucose, in the absence of pH control during the fermentation process, the potential decrease in pH will be directly related to the initial glucose concentration. Accordingly, the higher is the substrate concentration, the higher can be the amount of GA formed and the lower the final pH as a result. However, the concentration of glucose by itselt also seems to inhibit in some extent the activity of *G. oxydans*. Thus, Velizarov and Beschkow[31,32] used *G. oxydans* NBIMCC 1043 grown on a synthetic medium containing variable concentrations of glucose from 5 to 250 g/L and found the maximum specific growth rate (μ_m) and the final GA yield ($Y_{GA/G}$) to be unequivocally related to the initial concentration of glucose. In fact, increasing glucose concentration up to 90 g/L led to increased μ_m values up to a maximun of 0.39 h^{-1} with $Y_{GA/G}$ = 90%; however, higher glucose concentrations inhibited its conversion, as was reflected by long lag phases, and diminished μ_m and $Y_{GA/G}$ values. The longer lag phases can be ascribed only to the presence of high glucose levels and, in the absence of pH control, the diminished values for the maximum specific growth rates and final GA yields to the synergic effect between the high concentrations of glucose and the inhibition due to the GA by the decrease of the pH. Cell growth and GA formation were virtualy stopped altogether below pH 2.0.

It is important to remind the growth behaviour of this microorganim; *G. oxydans*[35] is believed to involve two growth stages depending on whether incomplete oxidations or the assimilation of glucose and gluconate prevails. The first pathway involves fast, incomplete oxidation of glucose and hence results in low growth yield of cells on glucose. In the second stage, once glucose has been virtually exhausted, incompletely oxidized substrates from the previous stage are assimilated; although slower than the previous stage, this one leads to higher substrate-based growth yields and hence from this perspective to more efficient use of the substrate by the microorganism. As a rule, cell growth is directly related to glucose uptake and GA accumulation during the first stage.[13,39]

Velizarov and Beschkow[32] examined the potential inhibitory effect of GA on cell growth and its own production by adding different amounts of the acid from 2.8 to 138 g/L to the initial medium; they immediately identified the occurrence of lag phases the duration of which increased with increasing concentration of GA added to the medium. However, the inhibitory effect was seemingly the result of the acid medium - the pH decreased with increasing amount of GA added.

Also, Krajewski *et al.*[40] demonstrated strong negative effects of gluconate (and ketogluconate) formation from glucose by the analysis of mutant strains of *G. oxydans* N44-1 whose gene for membrane-bound glucose dehydrogenase was inactivated. This mutant no longer produced GA or ketogluconates from glucose, but revealed increased carbon dioxide formation and increased growth rate and growth yield. If additionally the gene for cytoplasmic glucose dehydrogenase was knocked out, the growth rate and growth yield was even further increased by up to 78% and 271%, respectively, compared to the parental strain.

Influence of the formation of ketoacids

As can be seen from Figure 1, GA can be further oxidized to ketogluconates in a rapid manner in the periplasm. Specifically, GA can be converted into 2-ketogluconate (2-KGA) or 5-ketogluconate (5-KGA) competitively by two types of oxidizing enzymes bound to the cytoplasmic membrane, namely:[35,36] FAD-dependent D-gluconate 2-dehydrogenase (FAD-GADH)[41,42] for 2-KGA and PQQ-dependent D-gluconate 5-dehydrogenase (PQQ-GLDH)[38] for 5-KGA. Some strains allow 2-KGA to be further converted into 2,5-diketogluconic acid (2,5-DKGA) by FAD-dependent 2-keto-D-gluconate dehydrogenase (FAD-2KGADH). Also, under favourable conditions, 2-KGA and 5-KGA in the medium can be assimilated by the microorganism[35,38] and converted to gluconate in the cytoplasm. Ketoacids may thus be part of the above-described microbial strategy of AAB involving incomplete oxidation of the substrate. Perhaps the ketoacids are not readily utilized by other microorganisms sharing their habitat with *Gluconobacter* spp., which would imply that (in addition to the energetic benefit they have) the oxidation of glucose to ketoacids may be a strategy of the AAB to withdraw the glucose from competing organisms.

The extent to which gluconates are oxidized to ketogluconates is difficult to assess because it depends on the particular strain and operating conditions. In any case, in the next two sections the main variables afecting this issue will be discussed.

Influence of pH and the presence of $CaCO_3$

In experiments with *G. oxydans* ATCC 621H and no pH control - which led to a rapid drop below pH 3 - or adjustment to pH 2.5 from the start, Olijve and Kok[25] and Weenk *et al.*[43] found glucose to be rapidly oxidized virtually quantitatively to GA without formation of any ketoacids. On the other hand, using a fixed pH of 5.5 or adding $CaCO_3$ to the medium led to the formation of 2-KGA and 5-KGA once virtually all glucose had been used. All eleven *Gluconobacter* strains studied behaved identically except for slight differences among the results.[43]

In continuous operation experimens with *G. oxydans* ATCC 621 and *G. oxydans* IFO 3290 under no pH control - which led to a pH of about 2.5 in the steady state -, Seiskari *et al.*[44] succeeded in obtaining GA as the main oxidation product over a period of at least 6 months. However, raising the glucose concentration in the medium to ca. 200 g/L inhibited to some extent GA formation - which is consistent with the results of Velizarov and Beschkov.[31] Again, a decreased concentration of glucose in the steady state favoured the formation of 2-KDG and 5-KGA at the expense of GA. Qazi *et al.*[45] used *G. oxydans* ATCC 9937 and, unlike Weenk *et al.*,[43] found the best acidity conditions for 2,5-DKGA to be pH < 3.5 and the effect of pH to be even more marked than that of the glucose concentration. Also, they recommended using a slightly higher pH at the beginning in order to facilitate microbial growth and oxidation of glucose to GA, and then allow it to evolve freely in order to favour oxidation to 2-KGA as an intermediate product and subsequent formation of 2,5-DKGA. These authors, however, used a medium containing 10 g $CaCO_3$/L.

Using *G. oxydans* NBIMCC 1043, Velizarov and Beschkov[31] and Beschkov *et al.*[33] concluded that the formation of ketoglu-

conates may be completely inhibited when working without pH control. These results contradict those of Qazi *et al.*,[45] but are in agreement with those previously obtained by Weenk *et al.*[43] and other authors.[13,14] Beschkov *et al.*[33] examined the effects of setting a constant pH of 5.5 and adding $CaCO_3$ at the start. Controlling the pH of the medium resulted in a ketogluconate yield of only 15% as compared to 80% obtained by Weenk *et al.*[43] The difference may have resulted from the fact that Beschkov *et al.*[33] used an initial glucose concentration of 90 g/L, which was approximately twice that used by Weenk *et al.*[43] Consistent with the results of previous studies,[43,46] the presence of $CaCO_3$ in the culture medium considerably favoured ketoacids production by effect of the low solubility of their calcium salts shifting the chemical equilibria to formation of the acids.

Silberbach *et al.*[47] examined the performance of *G. oxydans* strains DSM 3503 and NCIMB 8084 cultured in an orbital shaker under identical conditions in the presence of $CaCO_3$. While the first strain gave GA virtually as the sole product and only very small amounts of 2-KGA and 5-KGA, the second strain produced substantial amounts of ketogluconic acids. Carefully designed experiments with strain NCIMB 8084 conducted in fermentation vessels to ensure better control of pH and aeration exposed the importance of changing the pH for optimal performance of the enzymes acting in each stage. Thus, the optimum pH for GA formation was 4.8 and that for 2,5-DKGA 3.15. Also, maximizing the 2,5-DKGA yield required precise control of the pH by addition of NaOH relative to the addition of $CaCO_3$ to the culture medium.

Influence of dissolved oxygen

The formation of ketogluconates is known to be influenced by the concentration of dissolved oxygen (DO) in the culture medium. Thus, Buse *et al.*[29] found DO control to have a strong impact on the formation of, for example, 2,5-DKGA, under the action of *G. melanogenum* ATCC 9937. Thus, keeping the DO concentration at 50 or 100% the saturation level during the overflow metabolism stage led to a 2,5-DKGA yield of 80% in both cases - with some kinetic differences, however. On the other hand, using a DO concentration of 200% the saturation level had an adverse impact on 2,5-DKGA formation in terms of both kinetics and yield. In the absence of DO control, the concentration of dissolved oxygen rapidly dropped to 2% and had a highly adverse effect on 2,5-DKGA production. These results were seemingly the consequence of activity changes in the enzyme gluconic dehydrogenase, which catalyses the first stage in the formation of ketoacids from GA. In fact, Buse *et al.*[29] found the enzyme production pattern to differ markedly depending on the degree of oxygen availability at the initial fermenation stage: a low oxygen level delayed production. Therefore, maximizing 2,5-DKGA production seemingly requires keeping the DO concentration in the medium at at least 30% the saturation level. It should be noted that these authors used a medium containing a high concentration of $CaCO_3$ (10 g/L). In any case, oxygen availability is much less influential than other variables such as pH, the initial glucose concentration or the presence of calcium ions, provided the DO concentration exceeds a minimum level.[14]

Some of the previous results, particularly those of the influence of the initial glucose concentration, pH and calcium content, obtained using synthetic well defined media, have been confirmed also working with complex natural media such as strawberry purée.[13,14,48] For instance, *G. japonicus* CECT 8443 transformed selectively glucose into GA without pH control (changing from 3.3 to 2.4). The preservation of the acid it was directly related with the initial glucose content; when a medium with a low initial glucose concentration (about 17 g/L) was used, the formed GA was subsequently transformed into ketogluconates, nevertheless, high initial glucose concentrations (about 70 g/L) resulted in no further transformation of GA throughout the process.

Conclusions and future prospects

This paper is an overview of the influence of the main factors affecting the production of GA, an important compound used in numerous industrial applications, by AAB, namely: pH, initial concentration of glucose in the medium, concentration of $CaCO_3$ added and dissolved oxygen level, in decreasing order of importance.

If the aim is to maximize GA production, then the pH should be kept below 3.5-4 by either allowing it to evolve freely from the start or using a lower initial value slightly greater than 2.5 in any case. As regards the initial amount of glucose, concentrations in the range 0.9-90 g/L facilitate formation and accumulation of GA in the medium; however, higher concentrations inhibit glucose conversion. The presence of $CaCO_3$ facilitates the formation of ketoacids from previously formed GA; this requires mimizing its concentration in the culture medium. Finally, the dissolved oxygen concentration should be kept at 20–30% the saturation level for optimum results.

Although the previous conditions may differ for each specific strain and culture medium, the variables studied here appear to have a similar influence with different *Gluconobacter* spp. Therefore, modelling studies for optimizing GA production based on these microorganisms, entail setting the best possible value or range of values for each of the previous variables.

While the results examined here apply to wild strains, advances in the sequencing and targeted molecular engineering of some strains of AAB may open up avenues for obtaning new strains with increased capacities and selectivity for a number of incomplete oxidations, while overcoming some of the restrictions imposed by wild strains.

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