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review

Gluconic Acid: Properties, Applications and Microbial Production

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Summary

Gluconic acid is a mild organic acid derived from glucose by a simple oxidation reaction. The reaction is facilitated by the enzyme glucose oxidase (fungi) and glucose dehydrogenase (bacteria such as *Gluconobacter*). Microbial production of gluconic acid is the preferred method and it dates back to several decades. The most studied and widely used fermentation process involves the fungus *Aspergillus niger*. Gluconic acid and its derivatives, the principal being sodium gluconate, have wide applications in food and pharmaceutical industry. This article gives a review of microbial gluconic acid production, its properties and applications.

Key words: gluconic acid, glucose oxidase, microbial production, *Aspergillus niger*

Introduction

Gluconic acid (pentahydroxycaproic acid, Fig. 1) is produced from glucose through a simple dehydrogenation reaction catalysed by glucose oxidase. Oxidation of the aldehyde group on the C-1 of β-D-glucose to a carboxyl group results in the production of glucono-δ-lactone ($C_6H_{10}O_6$, Fig.1) and hydrogen peroxide. Glucono--δ-lactone is further hydrolysed to gluconic acid either spontaneously or by lactone hydrolysing enzyme, while hydrogen peroxide is decomposed to water and oxygen by peroxidase. The gluconate pathway is detailed in Fig. 2. The conversion process could be purely chemical too, but the most commonly involved method is the fermentation process. The enzymatic process could also be conducted, where the conversion takes place in the absence of cells with glucose oxidase and catalase derived from *A. niger.* Nearly 100 % of the glucose is converted to gluconic acid under the appropriate conditions. This method is an FDA approved process. Production of gluconic acid using the enzyme has the potential advantage that no product purification steps are required (*1*) if the enzyme is immobilised, *e.g.* the use of a polymer membrane adjacent to anion-exchange membrane of low--density polyethylene grafted with 4-vinylpyridine (*1*). However, this approach is not yet common in the industry, and it will not be considered in the review.

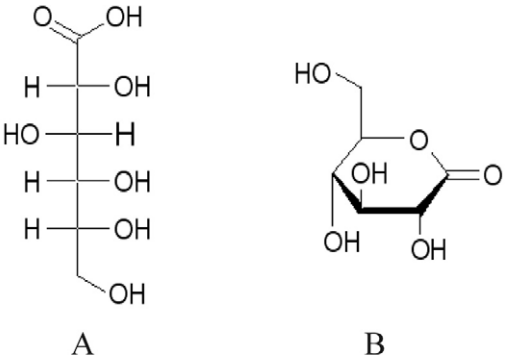

Fig. 1. Formula of gluconic acid (A) and glucono-δ-lactone (B)

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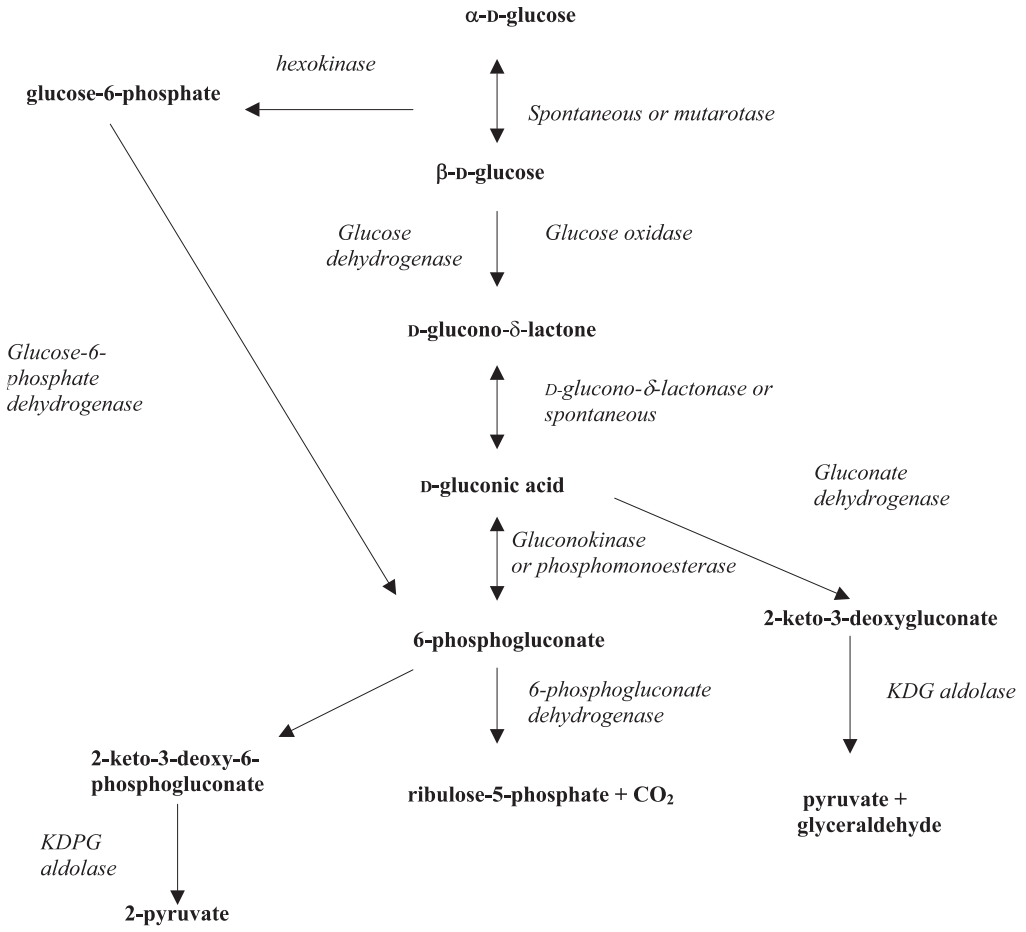

Fig. 2. General gluconate pathways

History

Gluconic acid production dates back to 1870 when Hlasiwetz and Habermann discovered gluconic acid (*2*). In 1880 Boutroux (*3*) found for the first time that acetic acid bacteria are capable of producing sugar acid. In 1922 Molliard (*4*) detected gluconic acid in the *Sterigmatocystis nigra*, now known as *Aspergillus niger*. Later, production of gluconic acid was demonstrated in bacterial species such as *Pseudomonas*, *Gluconobacter*, *Acetobacter*, and various fungal species. Studies of Bernhauer (*5–7*) showed that *A. niger* produced high yields of gluconic acid when it was neutralised by calcium carbonate and the production was found to be highly pH dependent. However, it was found that with *Penicillium* sp., the pH dependence is not as critical when compared to *A. niger*, indicating that there was some correlation between the amount and time-dependent appearance of organic acids, such as gluconic acid, citric acid, oxalic acid, which are formed under different conditions. Gluconic acid production has been extensively studied by May *et al.* (*8*), Moyer (*9*), Wells *et al.* (*10*), and Stubbs *et al.* (*11*) using *A. niger*. Using *Penicillium luteum* and *A. niger* Currie *et al.* (*12*) filed a patent employing submerged culture, giving yields of gluconic acid up to 90 % in 48–60 h. Later Moyer *et al.* (*13*) used *A. niger* in pilot plant studies and produced as high as 95 % of theoretical yields in glucose solution of 150 to 200 g/L in 24 h. Porges *et al.* (*14*) found that the process could be run semicontinuously, by the reuse of the mycelium for nine times repeatedly where the inoculum was recovered either by filtration or centrifugation. Findings of Moyer *et al.* (*13*) showed that efficiency of more than 95 % could be achieved by the addition of glucose at 250 g/L and boron compounds (1 % in solution of 250 g/L glucose) at later stages of the fungal growth with the reuse of mycelium in cycles of 24 h each.

Current commercial production of sodium gluconate uses submerged fermentation with *A. niger* and is based on the modified process developed by Blom *et al.* (*15*). It involves fed-batch cultivation with intermittent glucose feedings and the use of sodium hydroxide as neutralising agent. pH is held at 6.0–6.5 and the temperature at about 34 °C. The productivity of this process is very high, since glucose is converted at a rate of 15 g/(L·h.)

Properties

Physicochemical behaviour

Gluconic acid is a noncorrosive, nonvolatile, nontoxic, mild organic acid. It imparts a refreshing sour taste in many food items such as wine, fruit juices, *etc*. Sodium gluconate has a high sequestering power. It is a good chelator at alkaline pH; its action is comparatively

better than EDTA, NTA and other chelators. Aqueous solutions of sodium gluconate are resistant to oxidation and reduction at high temperatures. It is an efficient plasticizer and a highly efficient set retarder. It is easily biodegradable (98 % at 48 h). It has an interesting property of inhibiting bitterness in foodstuffs. Concentrated gluconic acid solution contains certain lactone structures (neutral cyclic ester) showing antiseptic property. The characterisitics are described in Table 1.

Table 1. General characteristics of gluconic acid

Gluconic acid	
Nature	Noncorrosive, mildly acidic, less irritating, nonodorous, nontoxic, easily biodegradable, nonvolatile organic acid
Relative molecular mass	196.16
Chemical formula	$C_6H_{12}O_7$
Synonym	2,3,4,5,6-pentahydroxyhexanoic acid
pKa	3.7
Melting point (50 % solution)	Lower than 12 °C
Boiling point (50 % solution)	Higher than 100 °C
Density	1.24 g/mL
Appearance	Clear to brown
Solubility	Soluble in water
Sourness	Mild, soft, refreshing taste
Degree of sourness (sourness of citric acid is regarded as 100)	29–35

In the European Parliament and Council Directive No. 95/2/EC, gluconic acid is listed as a generally permitted food additive (E 574). The US FDA (Food and Drug Administration) has assigned sodium gluconate a GRAS (generally recognized as safe) status and its use in foodstuff is permitted without limitation (*16*).

Measurement

There are several methods for the determination of D-gluconic acid and D-glucono-δ-lactone. Among them, isotachophoretic method (*17*) and hydroxamate method (*18*) are the most commonly used ones for the determination of gluconic acid. The concentration of gluconic acid is also determined by gas chromatography of their trimethylsilyl (TMS) derivatives prepared according to Laker and Mount (*19*) with inositol as internal standard.

A widely used enzymatic method (*20*) is based on the following principle: D-gluconic acid is phosphorylated to D-gluconate-6-phosphate by ATP in the presence of the enzyme gluconate kinase with the simultaneous formation of ADP. In the presence of NADP, D-gluconate-6-phosphate is oxidatively decarboxylated by 6-phosphogluconate dehydrogenase to ribulose-5-phosphate with the formation of reduced NADPH. The NADPH is stoichiometrically formed and its measurement allows direct determination of the amount of D-gluconic acid.

Occurrence

Gluconic acid is abundantly available in plants, fruits and other foodstuffs such as rice, meat, dairy products, wine (up to 0.25 %), honey (up to 1 %), and vinegar. It is produced by different microorganisms as well, which include bacteria such as *Pseudomonas ovalis* (*21*), *Acetobacter methanolicus* (*22*), *Zymomonas mobilis* (*23*), *Acetobacter diazotrophicus* (*24*), *Gluconobacter oxydans* (*25–27*), *Gluconobacter suboxydans* (*28,29*), *Azospirillum brasiliense* (*30*), fungi such as *Aspergillus niger* (*8–10*), *Penicillium funiculosum* (*31*), *P. variabile* (*32*), *P. amagasakiense* (*33*), and various other species such as *Gliocladium, Scopulariopsis, Gonatobotrys, Endomycopsis* (*34*) and yeasts such as *Aureobasidium pullulans* (formerly known as *Dematium* or *Pullularia pullulans*) (*35,36*). Ectomycorrhizal fungus *Tricholoma robustum*, which is associated with the roots of *Pinus densiflora*, was found to synthesise gluconic acid (*37*).

Applications

Gluconic acid is a mild organic acid, which finds applications in the food industry. As stated above, it is a natural constituent in fruit juices and honey and is used in the pickling of foods. Its inner ester, glucono-δ-lactone imparts an initially sweet taste which later becomes slightly acidic. It is used in meat and dairy products, particularly in baked goods as a component of leavening agent for preleavened products. It is used as a flavouring agent (for example, in sherbets) and it also finds application in reducing fat absorption in doughnuts and cones. Foodstuffs containing D-glucono-δ-lactone include bean curd, yoghurt, cottage cheese, bread, confectionery and meat.

Generally speaking, gluconic acid and its salts are used in the formulation of food, pharmaceutical and hygienic products (Table 2). They are also used as mineral

Table 2. Applications of gluconic acid and its derivatives

Components	**Applications**
Gluconic acid	Prevention of milkstone in dairy industry Cleaning of aluminium cans
Glucono-δ--lactone	Latent acid in baking powders for use in dry cakes and instantly leavened bread mixes Slow acting acidulant in meat processing such as sausages Coagulation of soybean protein in the manufacture of tofu In dairy industry for cheese curd formation and for improvement of heat stability of milk
Sodium salt of gluconic acid	Detergent in bottle washing Metallurgy (alkaline derusting) Additive in cement Derusting agent Textile (iron deposits prevention) Paper industry
Calcium salt of gluconic acid	Calcium therapy Animal nutrition
Iron salt of gluconic acid	Treatment of anaemia Foliar feed formulations in horticulture

supplements to prevent the deficiency of calcium, iron, *etc*. and as buffer salts. Different salts of gluconic acid find various applications based on their properties. Sodium salt of gluconic acid has the outstanding property to chelate calcium and other di- and trivalent metal ions. It is used in the bottle washing preparations, where it helps in the prevention of scale formation and its removal from glass. It is well suited for removing calcareous deposits from metals and other surfaces, including milk or beer scale on galvanised iron or stainless steel. Its property of sequestering iron over a wide range of pH is exploited in the textile industry, where it prevents the deposition of iron and for desizing polyester and polyamide fabrics. It is also used in metallurgy for alkaline derusting, as well as in the washing of painted walls and removal of metal carbonate precipitates without causing corrosion. It also finds application as an additive to cement, controlling the setting time and increasing the strength and water resistance of the cement. It helps in the manufacture of frost and crack resistant concretes. It is also used in the household cleaning compounds such as mouthwashes.

Calcium gluconate is used in pharmaceutical industry as a source of calcium for treating calcium deficiency by oral or intravenous administration. It also finds a place in animal nutrition. Iron gluconate and iron phosphogluconate are used in iron therapy. Zinc gluconate is used as an ingredient for treating common cold, wound healing and various diseases caused by zinc deficiencies such as delayed sexual maturation, mental lethargy, skin changes, and susceptibility to infections.

Market

Organic acids represent the third largest category after antibiotics and amino acids in the global market of fermentation. The total market value of organic acid will rise to $3 million in 2009 (*38*). Citric acid dominates the market of organic acids due to its application in various fields. The market of gluconic acid is comparatively smaller. However, 60 000 tonnes are produced worldwide annually and it is available in the market as 50 % technical grade aqueous solution (by mass).

The main product among the gluconic acid derivatives is the sodium gluconate due to its properties and applications. Manufacturers of gluconic acid and its salt in the United States are Pfizer Inc., New York, Bristol–Meyers Co., New York, Premier Malt Products Inc., Wisconsin. European gluconate producers include Roquette Frères in France, Pfizer in Ireland, Benckiser in Germany. Fujisawa and Kyowa Hakko are the manufacturers of gluconate in Japan. Calcium gluconate is also an important product among the derivatives of gluconic acid and it is available as tablets, powder, and liquid for dietary supplements.

Production of Gluconic Acid

Introduction

There are different approaches available for the production of gluconic acid, namely, chemical, electrochemical, biochemical and bioelectrochemical (*39–41*). There are several different oxidising agents available, but still the process appears to be costlier and less efficient compared to the fermentation processes. Although the conversion is a simple one-step process, the chemical method is not favoured. Thus, fermentation has been one of the efficient and dominant techniques for manufacturing gluconic acid. Among various microbial fermentation processes, the method utilising the fungus *A. niger* is one of the most widely used ones. However, the process using *G. oxydans* has also gained significant importance. Irrespective of the use of fungi or bacteria, the importance lies on the product which is produced, for example, sodium gluconate or calcium gluconate, *etc*. As the reaction leads to an acidic product, it is required that it is neutralised by the addition of neutralising agents, otherwise the acidity inactivates the glucose oxidase, resulting in the arrest of gluconic acid production. The conditions for the fermentation processes in the production of calcium gluconate and sodium gluconate differ in many aspects such as glucose concentration (initial and final) and pH control. In the process involving calcium gluconate production, the control of pH results from the addition of calcium carbonate slurry. Another important point to be noted is about the solubility of calcium gluconate in water (4 % at 30 °C). At high glucose concentration, above 15 %, supersaturation occurs, and if it exceeds the limit, the calcium salt precipitates on the mycelia and inhibits the oxygen transfer. The neutralising agent should also be sterilized separately from the glucose solution to avoid Lobry de Bruyn-van Ekenstein reaction, which alters the conformation of glucose, which results in the reduction of yield for about 30 %. On the contrary, the process for sodium gluconate is highly preferable as the glucose concentration of up to 350 g/L can be used without any such problems. pH is controlled by the automatic addition of NaOH solution. Sodium gluconate is readily soluble in water (39.6 % at 30 °C).

Gluconic acid production by filamentous fungi

Glucose oxidase

The reaction involving the conversion of glucose to gluconic acid by filamentous fungi is catalysed by the enzyme glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, E.C. 1.1.3.4). The enzyme was first isolated from a press juice obtained from *Penicillium glaucum* by Müller (*42*). The enzyme was crystallised by Kusai *et al.* (*33*) from *P. amagasakiense*. The enzyme was previously known as notatin. Glucose oxidase is a flavoprotein which contains one very tightly but noncovalently bound FAD cofactor per monomer and is a homodimer with a molecular mass of 130–320 kDa depending on the extent of glycosylation. It catalyses the reaction where glucose is dehydrated to glucono-δ-lactone, while hydrogen is transferred to FAD. The resulting $FADH_2$ is regenerated to FAD by transmission of the hydrogen to oxygen to form hydrogen peroxide (Fig. 3). Glucose oxidase is a glycoprotein. The native enzyme is glycosylated, with a carbohydrate mass percentage of 16–25 % (*43,44*). The enzyme from *A. niger* contains 10.5 % carbohydrate, which is believed to contribute to the stability without affecting the overall mechanism (*45*).

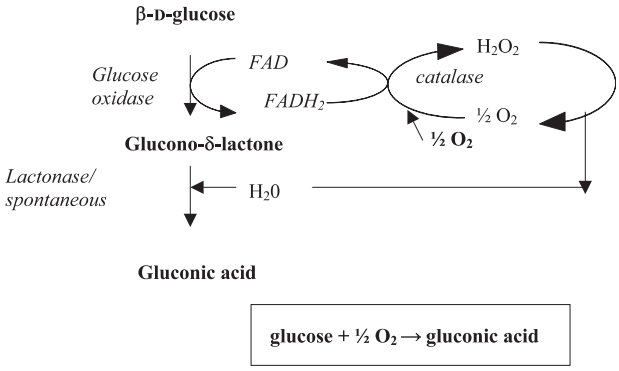

Fig. 3. Oxidation of glucose by *Aspergillus niger*

The enzyme is induced in the presence of high levels of glucose in the medium, pH around 5.5 and elevated oxygen levels. The enzyme is stable between pH= 4.0 and 6.0 at 40 °C for 2 h but is unstable above 50 °C. Liu *et al.* (*46*) conducted a study on the effects of metal ions on simultaneous production of glucose oxidase and catalase and found that calcium carbonate induced the synthesis of both enzymes. The induction of calcium carbonate was accompanied by a metabolic shift from the glycolytic pathway (EMP) to direct oxidation of glucose by the enzyme. The enzyme is found to be inhibited by hydrogen peroxide, the by-product of gluconic acid production (*47*). A study on glucose oxidase inactivation showed that only the reduced form of glucose oxidase is highly sensitive to hydrogen peroxide (*48*).

The enzyme is used in various fields such as food, clinical analysis, mainly as glucose sensor, in the quantitative determination of glucose in body fluids and urine. It is used in food processing in the removal of glucose prior to the preparation of products such as dried eggs to reduce the nonenzymatic browning. It is also used in removing residual oxygen from fruit juices, beer, and wine and also from dehydrated packaged foods.

Reports on glucose oxidase localization are ambiguous. Van Dijken and Veenhuis (*49*), and Witteveen *et al.* (*50*) reported that the enzyme of *A. niger* is intracellular and found in peroxisomes, whereas Mischak *et al.* (*51*) reported it as extracellular. There are also reports which have stated that it is intracellular prior to fungal autolysis (*52*). These varying reports on its location in the cell could be attributed to the differences of parameters and conditions adopted for the growth or due to the age of the fungal cultures. Very little is known about the mechanisms of glucose oxidase export. Zetelaki (*53*) associated export with autolysis of the fungus, whereas Mischak *et al.* (*51)* reported that the glucose oxidase of *A. niger* was excreted after synthesis.

Aspergillus niger

A. niger produces all the enzymes required for the conversion of glucose into gluconic acid, which include glucose oxidase, catalase, lactonase and mutarotase. Although crystalline glucose monohydrate, which is in the alpha form, is converted spontaneously into beta form in the solution, *A. niger* produces the enzyme mutarotase, which serves to accelerate the reaction. During the process of glucose conversion, glucose oxidase present in *A. niger* undergoes self-reduction by the removal of two hydrogens. The reduced form of the enzyme is further oxidised by the molecular oxygen, which results in the formation of hydrogen peroxide, a by-product in the reaction. *A. niger* produces catalase which acts on hydrogen peroxide releasing water and oxygen. Hydrolysis of glucono-δ-lactone to gluconic acid is facilitated by lactonase. The reaction can be carried out spontaneously as the cleavage of lactone occurs rapidly at pH near neutral, which are brought about by the addition of calcium carbonate, or sodium hydroxide. Removal of lactone from the medium is recommended as its accumulation in the media has a negative effect on the rate of glucose oxidation and the production of gluconic acid and its salt. There are reports stating that the enzyme gluconolactonase is also present in *A. niger* (*54*), which increases the rate of conversion of glucono-δ-lactone to gluconic acid.

Production of gluconic acid is directly linked with the glucose oxidase activity. Depending on the application, the fermentation broths containing sodium gluconate or calcium gluconate are produced by the addition of solutions of sodium hydroxide or calcium carbonate respectively, for neutralisation. The general optimal condition for gluconic acid production is as follows (*2*):

- Glucose at concentrations between 110–250 g/L
- Nitrogen and phosphorus sources at a very low concentration (20 mM)
- pH value of medium around 4.5 to 6.5
- Very high aeration rate by the application of elevated air pressure (4 bar).

There are two key parameters which influence the gluconic acid production. These are oxygen availability and pH of the culture medium. Oxygen is one of the key substrates in the oxidation of glucose as glucose oxidase uses molecular oxygen in the bioconversion of glucose. The concentration of oxygen gradient and the volumetric oxygen transfer coefficient are the critical factors, which monitor the availability of oxygen in the medium. These two factors highly influence the rate of the transfer of oxygen from gaseous to aqueous phase. Several reports are available on this particular aspect. The aeration rate and the speed of agitation are the two parameters which affect the availability of the oxygen in the medium. Gluconic acid production is an extremely oxygen-consuming process with a high oxygen demand for the bioconversion reaction, which is strongly influenced by the dissolved oxygen concentration. Oxygen is generally supplied in the form of atmospheric air; however, in some studies high-pressure pure oxygen has also been provided. For example, Sakurai *et al.* (*55*) supplied high-pressure oxygen at approx. 6 bar and maintained dissolved oxygen at 150 ppm. They found that immobilised mycelium of *A. niger* grown using pure oxygen produced high titres of gluconic acid in comparison with mycelium grown in air. Kapat *et al.* (*56*) found that at an agitation speed of 420 rpm and aeration of 0.25 vvm, the dissolved oxygen concentration was optimal for glucose oxidase production. The K_m value of glucose oxidase for oxygen lies in the range of air saturation in water (*57*). Lee *et al.* (*58*) obtained high volumetric productivity of gluconic acid using relatively high pressure (2–6 bar), re-

sulting in an increase in dissolved oxygen up to 150 mg/L. Generally, during the course of fungal growth, the distribution of oxygen becomes uneven, as the size of gas bubbles increases, resulting in insufficient oxygen supply (*59*). The oxygen absorption rate is also influenced by the viscosity of the culture. A rapid decrease is observed in the absorption rate of oxygen with an increase in mycelial concentration (*60*).

pH is another important parameter that influences the gluconic acid production. *A. niger* produces weak organic acids such as citric acid, gluconic acid and oxalic acid, and their accumulation depends on the pH of the nutritive medium (*61*). pH below 3.5 triggers the TCA cycle and facilitates the citric acid formation. The pH range of the fungi for the production of gluconic acid is around 4.5 to 7.0. pH=5.5 is generally considered as optimum for *Aspergillus niger* (*62*). Franke (*63*) collected some data concerning the relative activity of glucose oxidase at different pH levels and reported 5 and 35 % activity at pH=2.0 and 3.0, respectively, based on 100 % activity at pH=5.6. Report by Heinrich and Rehm (*64*) states that gluconic acid production occurs even at pH=2.5 in the presence of manganese in fixed bed and stirred bed reactors, possibly because of the difference in intracellular and extracellular pH.

Cheaper raw materials as substrates

Glucose is generally used as carbon source for microbial production of gluconic acid. However, hydrolysates of various raw materials such as agro-industrial waste have also been used as substrate. Kundu and Das (*65*) obtained a high yield of gluconic acid in media containing glucose or starch hydrolysate as the sole carbon source. Vassilev *et al.* (*66*) used hydrol (corn starch hydrolysate) as the fermentable sugar to produce gluconic acid by immobilized *A. niger*. Rao and Panda (*67*) used Indian cane molasses as a source of glucose. The cane molasses was subjected to different pre-treatments such as acid treatment, potassium ferrocyanide treatment, salt treatment, *etc*. Potassium ferrocyanide treatment gave a promising result. Gluconic acid synthesis was influenced by various metal ions such as copper, zinc, magnesium, calcium, iron, *etc*. Mukhopadhyay *et al.* (*68*) used deproteinised whey as a nutritive medium for gluconic acid production. Lactose was used as a substrate and 92 g of gluconic acid was produced from 1 L of whey containing 0.5 % glucose and 9.5 % lactose by *A. niger* immobilized on polyurethane foam. Ikeda *et al.* (*69*) used saccharified solution of waste paper with glucose concentration adjusted to 50–100 g/L for bioconversion with *A. niger*. The yields were 92 % in Erlenmeyer flasks and 60 % in repeated batch cultures in the turbine blade reactor with 800 mL of working volume. Another striking feature in the study was when xylose and cellobiose were used as the sole carbon sources, yields of gluconic acid obtained were 83 and 56 %, respectively.

Singh *et al.* (*70*) observed that grape must and banana must resulted in significant levels of gluconic acid production, *i.e.* 63 and 55 g/L respectively. The purification of grape and banana must leads to a 20–21 % increase in gluconic acid yield. They also used molasses, where the gluconate production was 12 g/L, but a significant increase in production of 60 g/L with a yield of 61 % was observed following treatment of the molasses with hexacyanoferrate. Rectified grape must appeared to be the best suited substrate, which after 144 h resulted in 73 g/L of gluconic acid with 81 % yield when compared to the value of 72 % obtained from the rectified banana must. Buzzini *et al.* (*71*) also used grape must and rectified grape must and they found that the latter substrate was better, with a production of 67 g/L and a yield of 96 % in 72 h. Citric acid was also observed as a by-product.

Use of solid-state fermentation (SSF)

SSF has been widely described for the production of industrial enzymes and organic acids (*72–76*). However, for the production of gluconic acid, there are only a few reports using SSF. Roukas (*77*) reported the production of gluconic acid by solid-state fermentation on figs. The maximal gluconic acid concentration was 490 g/kg of dry fig with 63 % yield. The addition of 6 % methanol into the substrate helped to increase the production of gluconic acid from 490 to 685 g/kg. Singh *et al.* (*78*) performed SSF by using HCl pretreated sugarcane bagasse and the highest level of gluconic acid (107 g/L) with 95 % yield was obtained. In comparison with the submerged culture, the degree of conversion was higher in SSF. The increased rate of product formation might be due to the variations of osmotic pressure, water content and dissolved oxygen. A study by Moksia *et al.* (*79*) used a two-step process, the first being the production of spores of *A. niger* by SSF on buckwheat seeds, and the second step, the bioconversion of glucose to gluconic acid by the spores recovered from the SSF medium. The interesting aspect about this work was that the spores were not allowed to germinate as the bioconversion medium did not contain any nitrogen source. The spores acted as a biocatalyst, producing 200 g/L of gluconic acid with a yield of 1.06 g per mass of glucose, very close to the stoichiometric value.

Production of gluconic acid by bacteria

Acetic acid bacteria and *Pseudomonas savastanoi* were the cultures initially observed to produce gluconic acid. Unlike in fungi, in bacteria the reaction is carried out by glucose dehydrogenase (GDH, E.C. 1.1.99.17) that oxidises glucose to gluconic acid, which is further oxidised to 2-ketogluconate by gluconic acid dehydrogenase (GADH). The final oxidation step to 2,5-diketogluconic acid (DKG) is mediated by 2-ketogluconate dehydrogenase (KGDH). The reaction steps are shown in Fig. 4. All three enzymes are localised in the membranes of the cells and are induced by high glucose concentrations (>15 mM) (*26*). GDH is an extracellular protein and has PQQ (pyrroloquinoline quinine) as a coenzyme. Also, there is an intracellular enzyme, an $NADP^+$-dependent glucose dehydrogenase, which is less involved in the gluconic acid formation when compared to the extracellular enzyme. Gluconic acid produced is exported to the cell and further catabolised *via* the reactions in pentose phosphate pathway. When the glucose concentration in the medium is greater than 15 mM, pentose phosphate pathway is repressed and thus gluconic acid accumulation takes place.

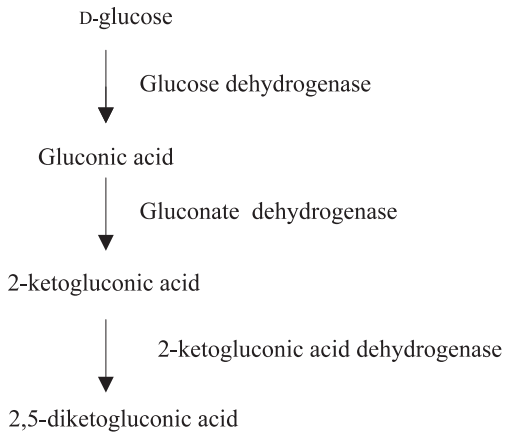

Fig. 4. Specific pathway for oxidation of glucose by *Gluconobacter*

Gluconobacter oxydans is an obligate aerobic bacterium that oxidises glucose *via* two alternative pathways. The first pathway requires an initial phosphorylation followed by oxidation *via* the pentose phosphate pathway. The second is the »direct glucose oxidation« pathway, which results in the formation of gluconic acid and ketogluconic acid (*29*). *G. oxydans* converts D-glucose into 2,5-diketogluconic acid by the action of three membrane-bound $NADP^+$-independent dehydrogenases as mentioned in Fig. 4. The acidotolerant acetic acid bacterium, *Acetobacter diazotrophicus*, exhibited high rates of gluconic acid formation. Glucose oxidation by the organism was less sensitive to low pH values than glucose oxidation by *G. oxydans.* Both the phosphorylative and direct oxidative pathways of glucose metabolism appeared to be operative. In addition to a pyridine nucleotide (strictly NAD^+)-dependent glucose dehydrogenase, *A. diazotrophicus* contained a PQQ-dependent glucose dehydrogenase, which was primarily responsible for gluconic acid formation. Bacterial gluconic acid production has limited success at industrial scale, as the oxidation proceeds with the secondary reactions leading to oxogluconic acids. The ability of *Pseudomonas* and *Gluconobacter* spp. to produce gluconolactone and gluconic acid has been exploited and the process is used commercially mainly in the production of lactone.

Acetobacter methanolicus is also used to catalyze the conversion of glucose into gluconic acid. The key advantage of using this facultatively methylotrophic microorganism as catalyst is that the gluconic acid formed is a metabolic dead-end product, and unlike in other bacterial fermentation processes, organism uses methanol, a cheap raw material as a substrate. Further in the process glucose is not assimilated or consumed for growth, so consequently the maximum theoretical yield coefficient is achieved (*80*). A patent was filed by Currie and Carter (*81*) in which the medium containing 200 g/L of glucose with other nutrients and a neutralising agent was allowed to flow through a tower packed with wood shavings or coke, which had been inoculated with *Acetobacter suboxydans*, while air was passed upwards through the packing. Tsao and Kempe (*82*), working with *Pseudomonas ovalis* found that a particular strain could convert glucose to gluconic acid with a yield of 99 %, and the rate was directly related to the efficiency of aeration.

Yeast

Research carried out by several authors (*36*,*37*,*83*,*84*) utilised *Aureobasidium pullulans*, a yeastlike form of the dimorphic fungi, for the production of gluconic acid. Various process parameters for the continuous and discontinous production of gluconic acid such as pH, oxygen, temperature and medium composition, air saturation, *etc.* were studied (*37*,*83*,*84*). The highest glucose conversion of 94 % and product yield of 87.1 % was achieved at an optimum pH of 6.5. At pH=4.5, the product selectivity and yield were very poor, reaching 67.8 and 20.7 %, respectively. Temperature range of 29 to 31 °C was found to be suitable for the production of gluconic acid by the yeast. Increase of temperature by 1 °C, namely to 32 °C, dramatically influenced the reduction in steady state concentration of biomass and product.

Immobilisation

Immobilisation techniques are involved where the biomass is immobilised onto the support and, in some cases, the enzyme isolated from the culture is immobilised. It enables repetitive use of the high biomass to carry out biochemical reactions rapidly leading to process economy and stability. Immobilisation seems to be an attractive method for accomplishing high cell densities in order to achieve rapid carbohydrate conversion to organic acids (*66*). Matrix immobilization is a simple and easy technique by which mycelia are retained on a matrix by mycelial entanglement. The type of support, cell retention, stabilization of enzyme or the mycelia and the quantum of biomass, *etc*. play important roles.

In the past, there were several investigations related to the production of gluconic acid with immobilised cells of *A. niger*. There are also reports of the immobilisation of *A. niger* pellets by flocculation with polyelectrolytes (*85*), calcium alginate (*86*), glycidyl ester copolymers (*87*) and entrapment in gels (*88*). Glass rings were used to immobilise *A. niger* for the production of gluconic acid by Heinrich and Rehm (*64*). Sakurai *et al.* (*55*) adopted a novel method for the immobilisation of *A. niger* using a support of nonwoven fabric. Vassilev *et al.* (*66*) and Mukhopadhyay *et al.* (*68*) reported the immobilisation of the same filamentous fungi on polyurethane foam. Different carriers such as calcium alginate agar, polyurethane sponge, pearlite, and activated carbon were used for the immobilisation of *Penicillium variabile* by Petruccioli *et al.* (*32*).

Free gluconic acid was continuously produced in an aerated tubular immobilized cell bioreactor using *G. oxydans* for at least 6 months, with a volumetric productivity of at least 5 g/(L·h) per 100 g/L of glucose substrate and the concentration of produced gluconic acid of about 80 g/L (*89*). Spores of *A. niger* were immobilised on sintered glass, pumice stones and polyurethane foams, and mycelia which developed on the pumice stone carrier produced high extracellular glucose oxidase (80 %) when compared to the enzyme activity on free cells (*90*).

An attempt was made by Sankpal *et al.* (*91*) to study the bioconversion of glucose to gluconic acid using *A. niger* immobilized on cellulosic fabric as a support matrix. Glucose solution (100 g/L) was made to flow through capillaries of a vertical fabric support, used for immobi-

lization, and was oxidized to gluconic acid at the interface. The system was found to run continuously for a period of 61 days utilizing the entire available glucose. The emerging broth contained a product concentration of 120–140 g/L of gluconic acid, which was higher than expected (maximum of 109 g/100 g of glucose), as a result of evaporative concentration during the downward flow. Sankpal and Kulkarni (*92*) found that the optimum biomass requirement on a porous cellulose support was 0.234 mg/cm^2 for efficient bioconversion. Increasing the quantum of biomass beyond this value resulted in an overgrown biofilm which affected productivity adversely. Morphological characteristics of immobilized *A. niger* have also been investigated.

Recovery

The recovery process depends on the method followed for broth neutralisation and the nature of carbon sources used. Generally, the downstream process is similar for the fermentation processes using fungal and bacterial species. Gluconic acid, glucono-δ-lactone, calcium gluconate, and sodium gluconate are some of the important products and their extraction process is briefly mentioned below.

For the recovery of free gluconic acid from calcium gluconate the broth is clarified, decolorized, concentrated and exposed to –10 °C in the presence or absence of alcohol. Thus the calcium salt of gluconic acid crystallizes, then it is recovered and further purified. Gluconic acid can also be obtained by precipitating the calcium gluconate from hypersaturated solutions in the cold and released subsequently by adding sulphuric acid stoichiometrically, removing the calcium as calcium sulphate. Another method of passing the solution through a column containing a strong cation exchanger is also practised where the calcium ions are absorbed.

For obtaining calcium gluconate as a product, calcium hydroxide or calcium carbonate is used as the neutralising agent. They are added to the nutritive broth accompanied by heating and vigorous stirring. The broth is concentrated to a hot supersaturated solution of calcium gluconate, followed by cooling at 20 °C, and adding water miscible solvents, which crystallises the compound. A treatment with activated carbon facilitates the crystallisation process. Finally they are centrifuged, washed several times and dried at 80 °C.

Sodium gluconate, the principal manufactured form of gluconic acid, is prepared by ion exchange. In the process developed by Blom *et al.* in 1952 (*15*), the sodium gluconate from the filtered fermented broth is concentrated to 45 % (mass per volume), followed by the addition of sodium hydroxide solution raising the pH to 7.5, and drum drying. Carbon treatment of the hot solution before drying process is practised for obtaining a refined product. Glucono-δ-lactone recovery is a very simple process. Aqueous solutions of gluconic acid are an equilibrium mixture of glucono-δ-lactone, glucono-λ-lactone and gluconic acid. At temperature between 30–70 °C the crystal which is separated from the supersaturated solution is glucono-δ-lactone. At temperature below 30 °C, gluconic acid results even above 70 °C, and the resulting product would be glucono-λ-lactone.

Molecular Biology

The molecular genetics of gluconic acid overproduction is not very well investigated. It is well known that the enzyme is actively induced by glucose concentration and high aeration and pH above 4.0. The gene encoding glucose oxidase of *A. niger* (*gox A*) has been cloned, and its amplification resulted in a 2–3-fold increase in activities (*93–95*). *A. niger* secretes multiple forms of catalases to shield itself against the arising hydrogen peroxide (*80*), among which one has been cloned and characterised (*94*). Swart *et al.* (*95*) described nine different complementation groups of glucose oxidase overproduction mutants. *Gox B*, *gox C*, and *gox F* belong to linkage group 11, *gox 1* to linkage group 111, *gox D* and *gox G* to linkage group V, *gox A* and *gox E* to linkage group VII, and the linkage of *gox H* is unknown. Their study also indicates that *gox A* overproduction is regulated by the carbon source and oxygen in an independent manner. Knowledge about gene encoding lactonase is very narrow.

The gox-encoding gene of *Penicillium variabile* P16 was isolated and characterized to identify the molecular bases of its high level of expression and in view of improving enzyme production by developing a process based on heterologous expression (*96*).

There are some works carried out on the bacterial enzyme. A Tn*5*-induced glucose dehydrogenase (GDH) deficient mutant of *Gluconobacter oxydans* IFO 3293 was characterized. DNA sequencing showed that the insertion site occurred in an open reading frame with homology to the *pqqE* gene. It was shown that acid production could be restored by addition of the coenzyme PQQ to the medium. The *pqq* cluster of *G. oxydans* ATCC 9937 was cloned and sequenced. It has five genes, *pqqA–E*. The cluster could complement the Tn*5*-induced mutation in IFO 3293. Pulsed-field gel electrophoresis suggested that the *pqq* genes are not closely linked to the *ribF* gene that produces the riboflavin cofactor for the gluconic acid dehydrogenase (*97*).

Conclusions

Although the production of gluconic acid is a simple oxidation process that can be carried out by electrochemical, biochemical or bioelectrochemical methods, production by fermentation process involving fungi and bacteria is well established commercially. Considerable progress has been made in understanding the mechanism of fermentation process by different microorganisms, and highly efficient production process, which dates back to five decades, has been developed. However, development of novel, more economical process for the conversion of glucose to gluconic acid with longer shelf life would be promising. These requirements could be met by enzymatic system. Another way of improvement is to use cheap substrates, such as methanol instead of glucose.

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