Microbial production of Organic acids

A large number of organic acids with actual or potential uses are produced by microorganisms. Citric, itaconic, lactic, malic, tartaric, gluconic, mevalonic, salicylic, gibberellic, diaminopimelic, and propionic acids are some of the acids whose microbial production have been patented.

i. Citric Acid

Citric acid is a tribasic acid with the structure shown in Fig.1.

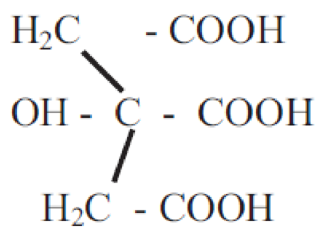

Fig. 1. Structure of Citric Acid

It crystallizes with the large rhombic crystals containing one molecule of water of crystallization, which is lost when it is heated to 130°C. At temperatures as high as 175°C it is converted to itaconic acid, aconitic acid, and other compounds.

Uses of Citric Acid

Citric acid is used in the food industry, in medicine, pharmacy and in various other industries.

Uses in the food industry

(i) Citric acid is the major food acidulant used in the manufacture of jellies, jams, sweets, and soft drinks.

(ii) It is used for artificial flavoring in various foods including soft drinks.

(iii) Sodium citrate is employed in processed cheese manufacture.

Uses in medicine and pharmacy

(iv) Sodium citrate is used in blood transfusion and bacteriology for the prevention of blood clotting.

(v) The acid is used in effervescent powders which depend for their effervescence on the CO_2 produced from the reaction between citric acid and sodium bicarbonate.

(vi) Since it is almost universally present in living things, it is rapidly and completely metabolized in the human body and can therefore serve as a source of energy.

Uses in the cosmetic industry

(vii) It is used in astringent lotions such as aftershave lotions because of its low pH.
(viii) Citric acid is used in hair rinses and hair and wig setting fluids.

Miscellaneous uses in industry
(ix) In neutral or low pH conditions the acid has a strong tendency to form complexes, hence it is widely used in electroplating, leather tanning, and in the removal of iron clogging the pores of the sand face in old oil wells.
(x) Citric acid has recently formed the basis of manufacture of detergents in place of phosphates, because the presence of the latter in effluents gives rise to eutrophication (an increase in nutrients which encourages aquatic flora development).

Biochemical Basis of the Production of Citric Acid

Citric acid is an intermediate in the citric acid cycle (TCA) (Fig.2). The acid can therefore be caused to accumulate by one of the following methods:

(a) By mutation—giving rise to mutant organisms which may only use part of a metabolic pathway, or regulatory mutants; that is using a mutant lacking an enzyme of the cycle.

(b) By inhibiting the free-flow of the cycle through altering the environmental conditions, e.g. temperature, pH, medium composition (especially the elimination of ions and cofactors considered essential for particular enzymes). The following are some of such environmental conditions which are applied to increase citric acid production:

(i) The concentrations of iron, manganese, magnesium, zinc, and phosphate must be limited. To ensure their removal the medium is treated with ferro-cyanide or by ion exchange resins. These metal ions are required as prosthetic groups in the following enzymes of the TCA: Mn^{+2} or Mg^{+2} by oxalosuccinic decarboxylase, Fe^{+3} is required for succinic dehydrogenase, while phosphate is required for the conversion of GDP to GTP (Fig. 2).
(ii) The dehydrogenases, especially isocitrate dehydrogenase, are inhibited by anaerobiosis, hence limited aeration is done on the fermentation so as to increase the yield of citric acid.

(iii) Low pH and especially the presence of citric acid itself inhibits the TCA and hence encourages the production of more citric acid; the pH of the fermentation must therefore be kept low throughout the fermentation by preventing the precipitation of the citric acid formed.

(iv) Many of the enzymes of the TCA can be directly inhibited by various compounds and this phenomenon is exploited to increase citric acid production. Thus, isocitric dehydrogenase is inhibited by ferrocyanide as well as citric acid; aconitase is inhibited by fluorocitrate and succinic dehydrogenase by malonate. These enzyme antagonists may be added to the fermentation.

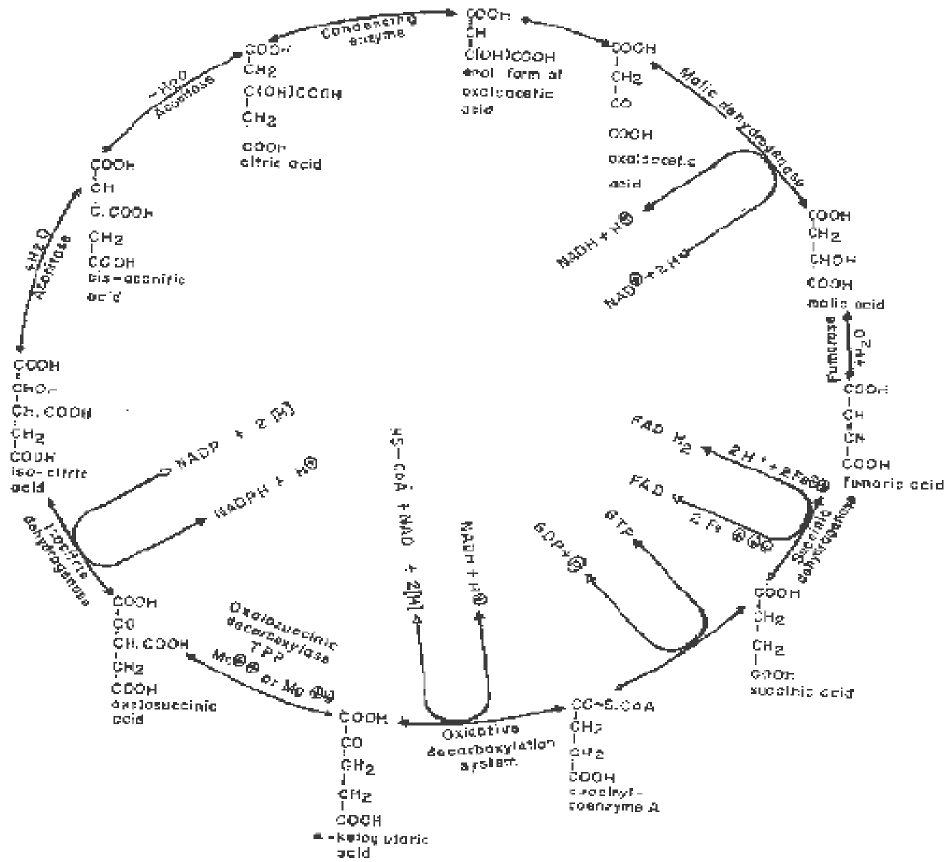

Fig. 2. The Tricarboxylic Acid Cycle. Citric acid can be caused to accumulate by using a mutant lacking an enzyme of the cycle or by inhibiting the low of the cycle.

Fermentation for Citric Acid Production

For a long time the production of citric acid has been based on the use of molasses and various strains of *Aspergillus niger* and occasionally *Asp. wenti*. In recent times, yeasts, especially *Candida* spp. (including *Candida guilliermondii*), have been used to produce the acid from sugar.

Paraffins became used as substrate from about 1970. In the processes described mainly by Japanese workers, bacteria and yeasts have been used. Among the bacteria were *Arthrobacter paraffineus* and corynebacteria; the yeasts included *Candida lipolytica* and *Candida oleiphila.*

Fermentation with molasses and other sugar sources can be either surface or submerged. Fermentation with paraffins however is submerged.

(a) *Surface fermentation*: Surface fermentation using *Aspergillus niger* may be done on rice bran as is the case in Japan or in liquid solution in flat aluminum or stainless steel pans. Special strains of *Asp. niger* which can produce citric acid despite the high content of trace metals in rice bran are used. The citric acid is extracted from the bran by leaching and is then precipitated from the resulting solution as calcium citrate.

(b) *Submerged fermentation*: As in all other processes where citric acid is made by fermentation, the fermenter is made of acid-resistant materials such as stainless steel. The carbohydrate sources are molasses decationized by ion exchange, sucrose or glucose. $MgSO_4 .7H_2O$ and KH_2PO_4 at about 1% and 0.05–2% respectively are added (in submerged fermentation phosphate restriction is not necessary). The pH is never allowed higher than 3.5. Copper is used at up to 500 ppm as an antagonist of the enzyme aconitase. Addition of 1–5% of methanol, isopranol, or ethanol to fermentations containing unpurified materials increase the yield; the yields are reduced in media with purified materials.

As high aeration is deleterious to citric acid production, mechanical agitation is not necessary and air may be bubbled through. Anti-foam is added. The fungus occurs as a uniform dispersal of pellets in the medium. The fermentation lasts for five to fourteen days.

Extraction

The broth is filtered until clear. Calcium citrate is precipitated by the addition of magnesium-free $Ca(OH)_2$. Since magnesium is more soluble than calcium, some acid may be lost in the solution as magnesium citrate if magnesium is added. Calcium citrate is filtered and the filter cake is treated with sulfuric acid to precipitate the calcium. The dilute solution containing citric acid is purified by treatment with activated carbon and passing through ion exchange beds. The purified dilute acid is evaporated to yield crystals of citric acid. Further purification may be required to meet pharmaceutical stipulations.

ii. Lactic Acid

Lactic acid is produced by many organisms: animals including man produce the acid in muscle during work.

Properties and chemical reactions of lactic acid

(i) Lactic acid is a three carbon organic acid: one terminal carbon atom is part of an acid or carboxyl group; the other terminal carbon atom is part of a methyl or hydrocarbon group; and a central carbon atom having an alcohol carbon group. Lactic acid exists in two optically active isomeric forms (Fig. 3).

(ii) Lactic acid is soluble in water and water miscible organic solvents but insoluble in other organic solvents.

(iii) It exhibits low volatility. Other properties of lactic acid are summarized in Table1.

(iv) The various reactions characteristic of an alcohol which lactic acid (or its esters or amides) may undergo are xanthation with carbon bisulphide, esterification with organic acids and dehydrogenation or oxygenation to form pyruvic acid or its derivatives.

(v) The acid reactions of lactic acid are those that form salts and undergo esterification with various alcohols.

(vi) Liquid chromatography and its various techniques can be used for quantitative analysis and separation of its optical isomers.

Technical grade lactic acid is used as an acidulant in vegetable and leather tanning industries. Various textile finishing operations and acid dyeing of food require low cost technical grade lactic acid to compete with cheaper inorganic acid. Lactic acid is being used in many small scale applications like pH adjustment, hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyl resin modifier, solder flux, lithographic and textile printing developers, adhesive formulations, electroplating and electro-polishing baths, detergent builders.

Lactic acid was among the earliest materials to be produced commercially by fermentation and the first organic acid to be produced by fermentation. Chemical processing has offered and continues to offer stiff competition to fermentation lactic acid. Very few firms around the world produce it fermentatively, but this could change when the hydrocarbon-based raw material, lactonitrile, used in the chemical preparation becomes too expensive because of the increase in petroleum prices.

Fig. 14.3 Optical Isomers of Lactic Acid

Lactic acid exists in two forms, the D-form and the L-form. When the symbols (+) or (–) are used, they refer to the optical rotation. However optical rotation in lactic acid is difficult to determine because the pure acid has low optical properties. The acid also spontaneously polymerizes in aqueous solutions; furthermore, salts, esters, and polymers have rotational properties opposite to that of the pure acid from which they are derived. These make it difficult to use optical rotation for characterizing lactic acid.

Many organisms produce either the D- or the L-form of the acid. However, a few organisms such as *Lactobacillus plantarum* produce both. When both the D- and L-form of lactic acid are mixed it is a racemic mixture. The DL form which is optically inactive is the form in which lactic acid is commercially marketed.

Uses of lactic acid

(i) It is used in the baking industry. Originally fermentation lactic acid was produced to replace tartarates in baking powder with calcium lactate. Later it was used to produce calcium stearyl-2-lactylate, a bread additive.

(ii) In medicine it is sometimes used to introduce calcium in to the body in the form of calcium lactate, in diseases of calcium deficiency.

(iii) Esters of lactic acid are also used in the food industry as emulsifiers.

(iv) Lactic acid is used in the manufacture of rye bread.

(v) It is used in the manufacture of plastics.

(vi) Lactic acid is used as acidulant/flavoring/pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods. In contrast to other foods, it has the advantage of having a mild acidic taste.

(vii) It is non-volatile odorless and is classified as GRAS (generally regarded as safe) by the FDA.

(viii) It is a very good preservative and pickling agent. Addition of lactic acid aqueous solution to the packaging of poultry and fish increases their shelf life.

(ix) The esters of lactic acid are used as emulsifying agents in baking foods (stearoyl-2- lactylate, glyceryl lactostearate, glyceryl lactopalmitate). The manufacture of these emulsifiers requires heat stable lactic acid, hence only the synthetic or the heat stable fermentation grades can be used for this application.

(x) Lactic acid has many pharmaceutical and cosmetic applications and formulations in topical ointments, lotions, anti-acne solutions, humectants, parenteral solutions and dialysis applications, as an anti-caries agent.

(xi) Calcium lactate can be used for calcium deficiency therapy and as anti caries agent.

(xii) Its biodegradable polymer has medical applications as sutures, orthopedic implants, controlled drug release, etc.

(xiii) Polymers of lactic acids are biodegradable thermoplastics. These polymers are transparent and their degradation can be controlled by adjusting the composition, and the molecular weight. Their properties approach those of petroleum derived plastics.

(xiv) Lactic acid esters like ethyl/butyl lactate can be used as environment-friendly solvents. They are high boiling, non-toxic and degradable components.

(xv) Poly L-lactic acid with low degree of polymerization can help in controlled release or degradable mulch films for large-scale agricultural applications.

Fermentation for lactic acid

Although many organisms can produce lactic acid, the amounts produced are small; the organisms which produce adequate amounts and are therefore used in industry are the homofermentative lactic acid bacteria, *Lactobacillus* spp., especially *L. delbrueckii*. In recent times, *Rhizopus oryzae* has been used. Both organisms produce the L-form of the acid, but

Rhizopus fermentation has the advantage of being much shorter in duration; furthermore, the isolation of the acid is much easier when the fungus is used.

Lactic acid is very corrosive and the fermenter, which is usually between 25,000 and 110,000 liters in capacity is made of wood. Alternatively, special stainless steel (type 316) may be used. They are sterilized by steaming before the introduction of the broth as contamination with thermophilic clostridia yielding butanol and butyric acid is common. Such contamination drastically reduces the value of the product.

During the step-wise preparation of the inoculum, which forms about 5% of the total beer, calcium carbonate is added to the medium to maintain the pH at around 5.5–6.5. The carbon sources used in the broth have varied widely and have included whey, sugars in potato and corn hydrolysates, sulfite liquor, and molasses. However, because of the problems of recovery for high quality lactic acid, purified sugar and a minimum of other nutrients are used.

Lactobacillus requires the addition of vitamins and growth factors for growth. These requirements along with that of nitrogen are often met with ground vegetable materials such as ground malt sprouts or malt rootlets. To aid recovery the initial sugar content of the broth is not more than 12% to enable its exhaustion at the end of 72 hours. Fermentation with *Lactobacillus delbrueckii* is usually for 5 to 10 days whereas with *Rhizopus oryzae*, it is about two days.

Although lactic fermentation is anaerobic, the organisms involved are facultative and while air is excluded as much as possible, complete anaerobiosis is not necessary. The temperature of the fermentation is high in comparison with other fermentations and is around 45°C. Contamination is therefore not a problem, except by thermophilic clostridia.

Extraction

The main problem in lactic acid production is not fermentation but the recovery of the acid. Lactic acid is crystallized with great difficulty and in low yield. The purest forms are usually colorless syrups which readily absorb water.

At the end of the fermentation when the sugar content is about 0.1%, the beer is pumped into settling tanks. Calcium hydroxide at pH 10 is mixed in and the mixture is allowed to settle. The clear calcium lactate is decanted off and combined with the filtrate from the slurry. It is then treated with sodium sulfide, decolorized by adsorption with activated charcoal, acidified to pH 6.2 with lactic acid, and filtered. The calcium lactate liquor may then be spray-dried.

For *technical grade* lactic acid, the calcium is precipitated as CaSO4.2H2O which is filtered off. It has 44–45% total acidity. *Food grade* acid has a total acidity of about 50%. It is made from the fermentation of higher grade sugar and bleached with activated carbon. Metals especially iron and copper are removed by treatment with ferrocyanide. It is then filtered. *Plastic grade* is obtained by esterification with methanol after concentration. High-grade lactic acid is made by various methods: steam distillation under high vacuum, solvent extraction, etc.