World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

AN OVERVIEW OF CITRIC ACID PRODUCTION AND PURIFICATION

Joshi Swapnil Satish*

Department of Microbiology S.S.V.P. Sanstha's Late Karmaveer Dr. P R Ghogrey Science College, Dhule-424005.

***Corresponding Author: Joshi Swapnil Satish**
Department of Microbiology S.S.V.P. Sanstha's Late Karmaveer Dr. P R Ghogrey Science College, Dhule-424005.
DOI: https://doi.org/10.56716/4/4232

Article Received on 21/09/2023 **Article Revised on 11/10/2023** **Article Accepted on 01/11/2023**

ABSTRACT

Citric acid is a major fermentation product having global market demands. It is mainly produced by *Aspergillus niger*. globally. Due to high demands, there is always a need for discovery and development of promising techniques for its production, purification and recovery. To meet its high demands generally agro waste products are preferred as raw materials for its production due their cheap costing. This article reviews the biochemistry of synthesis, types of raw materials and production techniques, microorganisms used and their production efficiency, recovery and purification of citric acid.

KEYWORDS: Citric acid, Aspergillus niger, fermentation.

INTRODUCTION

Citric acid (2-hydroxy-propane-1, 2, 3-tricarboxylic acid) derives its name from the Latin word '*citrus'*, the citrus tree, the fruit of which resembles a lemon. It is a tricarboxylic acid and a global intermediate product of kerb cycle. Citric acid is an important multifunctional organic acid with a broad range of versatile uses in household and industrial applications that has been produced industrially since the beginning of 20th century. Until microbial processes were developed, the major source of citric acid was citrus fruit i.e., lemon. The discovery of citrate accumulation by *Aspergillus niger* led to a rapid development of a fermentation process, which only a decade later accounted for a large part of the global production. According to Anastassiadis et al. (2008) 1.6 million tonnes of citric acid was produced globally in 2007 with an annual rise in demand and consumption of around 3.5-4%.

Majumder et al. (2010) reported citric acid is commonly used in food and beverages, detergents, pharmaceuticals, cosmetics, toiletries and other industries. More than 75% of citric acid is consumed in beverage and food industries, mainly as an ingredient in carbonated drinks and an acidulant. Industrially, metal finishing and cleaning accounts for the largest use of citric acid, followed by lubricants, chelating agents, animal feeds and plasticizers (Bauweleers et al., 2014). According to estimations, the market value of citric acid will continue to grow and will soon exceed $2 billion (van der Straat et al., 2014). The application of citric acid is based on three of its properties acidity and buffer capacity, taste and flavour, and chelation of metal ions. Because of its three acid groups with pKa values of 3.1, 4.7 and 6.4, citrate is able to produce a very low pH in solution, but is also useful as a buffer over a broad range of pH values (2 to 7). Citric acid has a pleasant acid taste which leaves little aftertaste. It sometimes enhances flavour, but is also able to mask sweetness, such as the aspartame taste in diet beverages. Chelation of metal ions is a very important property that has led to applications such as antioxidant and preservative. Moreover, it is a "natural" substance and fully biodegradable, economical, safe and versatile chemical for sequestering, buffering, wetting, cleaning and dispersing (Karaffa et al., 2001).

Organisms involved in the production of citric acid

Citric acid can be produced by fermentation technology using various moulds, yeasts and bacteria. Most of them, however, are not able to produce commercially acceptable yields. This fact could be explained by the fact that citric acid is a metabolite of energy metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances. Currie in 1917 discovered that some strains of black mold *Aspergillus niger* grew abundantly in a nutrient medium that had a high concentration of sugar and mineral salts and an initial pH of 2.5–3.5. While growing, these strains excreted large amounts of citric acid. This laid down the basis for industrial production of citric acid. *A niger* is normally a haploid fungus producing white septate hypha which is profusely branched. It produces black mass of conidia, which are found in chain arising from the secondary sterigmata. Citric acid (CA) is mainly produced by utilizing starchy and sugar substrates without the secretion of toxic byproducts. A large variety

of microorganisms have been recognized to be able of producing excessive amounts of citric acid under certain fermentation conditions, including fungi such as *Aspergillus aculeatus, A. carbonarius, A. awamori, A. foetidus, A. fonsecaeus, A. phoenicis* and *Penicillium janthinellum, Citromyces* and *Monilia* sp., yeasts such as *Saccharomyces sp. Candida tropicalis, C. oleophila, C. guilliermondii, Hansenula anamola, Yarrowia lipolytica* and *Pichia* sp. and bacteria such as *Arthrobacter paraffinens, Bacillus licheniformis* and *Corynebacterium* ssp (Anastassiadis et al., 2007). To ascertain the best fermentation conditions selected organism and production media should be tested for every fermentation method (Chen et al., 2014). However, *Aspergillus niger*, remains the most preferred strain for industrial production of citric acid. The main advantages of using this microorganism are: its ease of handling, its ability to ferment a variety of cheap raw materials, and high yields (Vandenberghe et al., 1999). These microorganisms have an intrinsic ability to accumulate these substances and it is generally believed that this provides the fungi with an ecological advantage, since they grow rather well at pH 3 to 5, while some species even tolerate pH values as low as 1.5. Significant optimization of citric acid production can be achieved by classical mutagenesis and genetic engineering as well.

Citric acid production

Biochemistry of citric acid production

The citric acid cycle begins when the two-carbon compound acetyl-CoA condenses with the four-carbon compound oxalacetate to form the six-carbon compound citrate. Citric acid is produced via the Embden-Meyerhof pathway and the first step of the tricarboxylic acid cycle. Citric acid is produced instead of energy when glucose is oxidized. When the cell produces citric acid; the full respiration process stops during the TCA cycle, and yields citric acid rather than energy. Regarding the process of citric acid accumulation in *A. niger*, two main metabolic pathways have involved a major role: the catabolic pathway of hexoses to pyruvate and Acetyl-Coenzyme A (Acetyl-CoA) by glycolysis and citric acid formation by TCA cycle (Alvares-Vasquez et al., 2000). As glucose is the starting carbohydrate in glycolysis for citric acid production, glucose plays an important role in citric acid production.

The success of this fermentation depends on the regulation and functioning of the glycolytic pathway and the tricarboxylic acid cycle. After the active growth phase, when the substrate level is high, citrate synthase activity increases and the activities of enzyme aconitase and isocitrate dehydrogenase decreases. Citrate synthase catalyses the reversible condensation reaction between oxaloacetate and acetyl CoA:

Acetyl-CoA + oxaloacetate↔citrate^{3+} + H^+ + CoA - SH. This equilibrium reaction favours the production of citrate (Papagianni, M 2007).

Several investigators have claimed that inactivation of a citrate degrading enzyme (e.g., aconitase or isocitrate dehydrogenases) as being essential for the accumulation of citric acid.

The essence of citric acid fermentation involves limiting the amounts of trace metals such as manganese and iron to stop *Aspergillus niger* growth at a specific point in the fermentation. A high level of citric acid production is also associated with a high intracellular concentration of fructose 2,6-biphosphate, an activator of glycolysis. Other factors contributing to high citric acid production are the inhibition of isocitrate dehydrogenase by citric acid and the low optimum pH (1.7–2.0).

Types of raw materials and production techniques

Raw Materials

Fermentation of a substrate to citric acid is directly related to the quality and quantity of the sugar source. This in turn depends on the carbon source of the substrate, as it has a marked influence on the metabolic activity of the microbial strains. Citric acid is mostly produced from starch or sucrose-based media using liquid fermentation, followed by glucose, fructose and galactose. To make citric acid production cost effective, a variety of raw materials such as molasses, several starchy materials and hydrocarbons have also been employed. Molasses is the effluent obtained from sugar industry and is the non-crystallisable residue remaining after sucrose isolation. Both, cane and beet molasses are suitable for citric acid production. However, beet molasses is preferred due to its lower content of trace metals.

Raw materials used for citric acid production can be classified in to two groups: (i) with a low ash content from which the cations could be removed by standard procedures (e.g., cane or beet sugar, dextrose syrups and crystallized dextrose); (ii) raw materials with a high ash content and high amounts of other non-sugar substances (e.g., cane and beet molasses, crude unfiltered starch hydro-lysates).

Fermentation Methods

Although chemical synthesis of citric acid is possible, it has never found commercial success. More than 90% of the citric acid produced in the world is obtained by fermentation, which has its own advantages i.e., simple and stable operation, less complicated plant with need of less sophisticated control systems, and less energy consumption. Citric acid production by fermentation can be divided in three phases, which include: (1) preparation and inoculation of the raw material, (2) fermentation, and (3) recovery of the product.

The industrial citric acid fermentation can be carried in three different ways

1. Submerged fermentation,
2. Surface fermentation and
3. Solid-state fermentation

All of these methods require raw material and inoculums preparation.

Submerged fermentation

The submerged fermentation (SmF) process is the commonly employed technique for citric acid production. It is estimated that about 80% of world production is obtained by SmF.

It is the process in which the growth and anaerobic⁄ partially anaerobic decomposition of the carbohydrates by microorganisms in liquid medium occur with plenty availability of free water. In SmF, different kinds of media are employed such as sugar and starch based media molasses and other raw materials demand pretreatment, addition of nutrients and sterilization. Inoculation is performed either by adding a suspension of fungal spores, or of pre-cultivated mycelia. When spores are used, a surfactant is added in order to disperse them completely within the medium. Submerged fermentation can be carried out in batch, fed batch or continuous systems. During fermentation, which is completed in 8 to 12 days, high amount of heat is generated, so high aeration rates are needed in order to control the temperature and to supply air to the microorganism. After fermentation, the tray contents are separated into crude fermentation fluid and mycelial mats which are washed to remove the impregnated citric acid (Soccol *et al*., 2006).

SmF presents several advantages such as higher productivity and yield, lower labour costs, and lower contamination risk.

The following main factors were found to affect citric acid fermentation: type and concentration of the carbon source, nitrogen and phosphate limitation, pH, aeration, concentration of trace elements and the morphology of the producer organism. Certain nutrients needed to be in excess (i.e., sugar, protons, and oxygen), others had to be limiting (i.e., nitrogen, phosphate) and some otherwise common feed components had to remain below defined limits (i.e., trace metals, especially manganese) (Papagianni, M 2007).

Surface fermentation

Surface culture was the method employed for large scale manufacture of microbial citric acid, employing mostly filamentous fungi. Surface fermentation is still used in industries of small and medium scale because it requires less effort in operation, installation and energy cost. The process is carried out in fermentation chambers where a large number of trays are arranged in shelves. The culture solution is held in shallow trays with capacity of 0.4 to 1.2 m^3 and the fungus develops as a mycelial mat on the surface of the medium. The fermentation chambers are provided with an effective air circulation, which passes over the surface in order to control humidity and temperature by evaporative cooling. This air is filtered through a bacteriological filter and the chambers should always be in aseptic conditions and must be conserved principally during the first two days when spores germinate. The most common contaminations are mainly caused by penicillia, aspergilli, yeasts and lactic bacteria (Swain et al., 2011).

Solid-state fermentation

In the recent times, solid state fermentation (SSF) is used as an alternative to submerged fermentation in the production of microbial metabolites. Solid-state fermentations refer to the cultivation of microorganisms in a low-water-activity environment on a non-soluble material acting as both nutrient source and physical support. The solid substrate acts as a source of carbon, nitrogen, minerals and a growth surface which absorbs the water necessary for microbial growth. In addition, the solid substrate provides anchor points for the growth and propagation of microorganisms. As microorganisms on a solid substrate are growing under conditions similar to their natural habitat, they may be able to produce certain enzymes, metabolites, proteins and spores more efficiently than in submerged fermentation.

Citric acid production by SSF (the Koji process) was first developed in Japan and is as the simplest method for its production. The absence of a liquid phase and low substrate humidity level allow facilitated aeration through the pore spaces between substrate particles, reduction of the fermentation and the liquid effluent volumes, reduced risk of bacterial contamination because of low moisture level, use of the non-sterile solid substrate in some cases, reduction in water usage and wastewater management, simplified media and utilization of agro-industrial sugar rich wastes or byproducts. The major advantages of solid-state fermentation over submerged fermentation include higher yields, low water requirement and lower operating costs (Swain et al., 2011).

Factors affecting citric acid production

Medium and its components

Carbon source

Citric acid accumulation is strongly affected by the nature of the carbon source. The presence of easily metabolized carbohydrates has been found essential for good production of citric acid. The disaccharides maltose and sucrose proofed to be better carbon sources for citric acid production than the monosaccharide glucose and fructose, whereas galactose and arabinose inhibit citric acid production. Galactose contributed to a very low growth of fungi and did not favour citric acid accumulation.

Nitrogen source

Citric acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g., urea, ammonium sulphate, ammonium chloride, peptone, malt extract, etc. Nitrogen consumption leads to pH decrease, which is very important point in citric acid fermentation. However, it is

necessary to maintain pH values in the first day of fermentation prior to a certain quantity biomass production. Urea is the safest chemical for pH control. A high nitrogen concentration increases fungal growth and the consumption of sugars, but decreases the amount of citric acid produced.

Phosphorous source

Phosphate is known to be essential for the growth and metabolism of *A. niger*. Potassium dihydrogen phosphate has been reported to be the most suitable phosphorous source. Low levels of phosphate favour citric acid production, however, the presence of excess of phosphate was shown to lead to the formation of certain sugar acids, a decrease in the fixation of CO_2, and the stimulation of growth. Phosphates acts at the level of enzyme activity and not at the level of gene expression. Different strains require distinct nitrogen and phosphorous concentrations in the medium. In fact, nitrogen and phosphorous limitation is a crucial factor in citric acid production as there is an interaction between them. Consequently, a strain with large requirements of N and P seems to be disfavoured, due to the restriction of accessibility to the nutrients in the medium.

Trace elements

A number of divalent metals such as zinc, manganese, iron, copper and magnesium have been found to affect citric acid production by *A. niger*. However, it is crucial to take into account the interdependence of medium constituents in SmF and, in SSF. Zinc favoured the production of citric acid if added with KH_2PO_4. On the other hand, the presence of manganese ions and iron and zinc (in high concentrations) could cause the reduction of citric acid yields only in phosphate free medium. SSF systems were able to overcome the adverse effects of the high concentrations of these components in the medium. As a consequence of this, the addition of chelating agents such as potassium ferrocyanide to the medium proved to be of no use. Copper was found to complement the ability of iron at optimum level; to enhance the biosynthesis of citric acid. Manganese deficiency resulted in the repression of the anaerobic and TCA cycle enzymes with the exception of citrate synthetase. This led to overflow of citric acid as an end product of glycolysis. A low level of manganese was capable to reduce the yield of citric acid by 10%. Citric acid accumulation decreased by the addition of iron, which also had some effect on mycelial growth.

Lower alcohols

Moyer (1953) first reported the effect of alcohols on the production of citric acid. Addition of lower alcohols enhances citric acid production from commercial glucose and other crude carbohydrate. Appropriate alcohols are methanol, ethanol, isopropanol or methyl acetate. The optimal amount of methanol/ethanol depends upon the strain and the composition of the medium, generally optimum range being 1-3%. It was reported that addition of ethanol resulted in two-fold increase in citrate synthetase activity and 75% decrease in aconitase activity. Whereas the activities of other TCA cycle enzymes increased slightly. Alcohols have been shown to principally act on membrane permeability in micro-organisms by affecting phospholipid composition on the cytoplasmic membrane. However, alcohols stimulate citric acid production by affecting growth and sporulation through the action not only on the cell permeability but also the spatial organization of the membrane, or changes in lipid composition of the cell wall. There are some compounds which are inhibitors of metabolism such as calcium fluoride, sodium fluoride and potassium fluoride have been found to accelerate the citric acid production. While compounds like potassium ferrocyanide have been found to decrease the yield. There are many compounds, which act in many ways to favour citric acid accumulation. Some of them are capable to impair the action of metal ions and other toxic compounds influence growth during the initial phase. Some of these are: 4- Methylumbelliferone, 3-hydroxi-2-naphtoic, benzoic acid, 2-naphtoic acid, iron cyanide, quaternary ammonium compounds, amine oximes, starch, EDTA, vermiculite, etc.

Process parameters

pH

The pH of a medium depends on the microbial metabolic activities. The most obvious reason is the secretion of organic acids such as citric, acetic or lactic acids, which will cause the pH to decrease. Changes in pH kinetics depend highly also on the micro-organism. With *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp., pH of the medium can drop very quickly until less than 3.0. Generally, a pH below 2.0 is required for optimum production of citric acid. A low initial pH has the advantage of checking contamination and inhibiting oxalic acid formation. A pH of 2.2 was reported to be optimum for the growth of the mould as well as for the production of citric acid whereas; a higher pH i.e., 5.4 and 6.0-6.5 has been found optimum for citric acid production in molasses medium.

Aeration

Aeration has been shown to have a determinant effect on citric acid fermentation. Increased aeration rates led to enhanced yields and reduced fermentation time. Varying aeration rates can have adverse effects on the fermentation performance and yield (Grewal & Kalra, 1995). Also, that stronger aeration results in increased sporulation and decreased accumulation of citric acid (Soccol et al., 2006). It is important to maintain the oxygen concentration at 25% saturation and interruptions in oxygen supply may be quite harmful. The high demand of oxygen is fulfilled by constructing appropriate aeration devices, which is also dependent on the viscosity of the fermentation broth. This is an additional reason why small compact pellets are the preferred mycelial forms of *A. niger* during fermentation. When the organism turns into filamentous developments, e.g., due to metal contamination, the dissolved oxygen

tension rapidly falls to less than 50% of its previous value, even if the dry weight has not increased by more than 5%. High aeration rates lead to high amounts of foam, especially during the growth phase. Therefore, the addition of antifoaming agents and the construction of mechanical "defoamers" are required to tackle this problem.

Fermentation temperature

The optimum temperature for citric acid formation will differ based on strain and media composition. Hence it is necessary to optimize and determine the appropriate favourable temperature before scaling up process. Filamentous fungi like *A. niger* are mesophilic, growing optimal at temperatures between 25 and 35 °C. Hence, enzyme activities as well as regulation and transport systems of microbial systems and optimal fermentation temperature must be maintained despite the large amount of heat generated by the metabolic activity of microorganisms. Cultivation at lower than optimum temperatures result in adverse growth and metabolic production and to lower metabolic activity, while higher temperatures cause enzyme denaturation and inhibition, excess moisture losses and growth arrest (Angumeenal and Venkappayya, 2012).

Recovery, purification and packaging

Product Recovery

Citric acid is recovered from fermentation broth in various ways, including the conventional classical method of acid precipitation as calcium citrate, solvent extraction, electrodialysis and ion exchange chromatography, following various steps such as fermentation, cell separation by cell filtration or centrifugation. Today citric acid is considered a commodity chemical and is available as dry crystals in the anhydrous or monohydrate form.

Precipitation Method

Precipitation is the classical method and it is performed by the addition of calcium oxide hydrate (milk of lime) to form the slightly soluble tri-calcium citrate tetrahydrate; $CaCO_3$ + Citric Acid → CO_2+ Calcium Citrate

The precipitated tri-calcium citrate is removed by filtration and washed several times with water to remove any number of contaminants. It is then treated with sulphuric acid forming calcium sulphate, which is filtered off. Finally, the liquor is concentrated in vacuum crystallizers at 20-25 °C, forming citric acid monohydrate. Crystallization at temperatures higher to this is used to prepare anhydrous citric acid. (Vandenberghe et al., 1999)**.** Depending on the purity, crude crystals are subsequently subjected to one or more recrystallization steps with purification with activated carbon before chemically pure citric acid is obtained.

Solvent Extraction method

Purification of citric acid also includes extraction from aqueous solutions with organic solvents, like butyl alcohol, acetone, and tributyl phosphate or with certain amines. Since the crystallization is inhibited because of incomplete removal of impurities and the yield is unsatisfactory, these processes have not found practical application so far due to being uneconomical.

Separation by Electrodialysis

A separation method of organic acids without requiring any addition of an alkali from fermentation broth can be done by means of an electrodialysis. The organic acids are recovered as free acids with high purity and at high yields. The fermentation medium is maintained for about 4-7 days to form citric acid and mycelia pellets in an aqueous product broth. The broth is filtered and then electro dialyzed to form a citric acid-containing aqueous broth, which is decolorized and ion-exchanged to remove colour and inorganic ions and form an aqueous citric acid solution, in which citric acid constitutes at least 98% of the product.

Ion Exchange Chromatography

A variety of ion-exchange chromatographic methods are applied for the separation of citric acid, either before or after biomass removal by filtration or centrifugation. The low molecular weight ionic impurities which are not separated from citric acid, consisting of mineral cations and anions, are eliminated by passing the liquors over cationic and anionic resins under appropriate conditions, realizing thus purification by ion exchange. The adsorbent resins must be regenerated after each adsorption cycle using alkali and organic solvents. Adsorbed impurities of cationic resin are eluted using a strong acid solution, generally hydrochloric acid and anionic resins using a strong base. Once the product has been brought to the desired purity, it would be sent to packaging and distribution.

BIBLIOGRAPHY

1. Alvarez-Vasquez, F., Gonzalez-Alcon, C. & Torres. N.V. Metabolism of citric acid production by *A. niger*: model definition, steady-state analysis and constrained optimization of citric acid production rate. *Biotechnol Bioeng*, 2000; 70(1): 82-108.
2. Anastassiadis, S., Morgunov, I.G., Kamzolova, S.V. and Finogenova, T.V. Citric Acid Production: A Patent Review. *Recent Patents on Biotechnology*, 2008; 2(2): 1-16.
3. Angumeenal, A.R. & Venkappayya, D. An overview of citric acid production. *Food sci and Tech.* 2012; 1-4.
4. Bauweleers, H. M. K., Groeseneken, D. R., & Van Peij, N. N. M. E. *U.S. Patent No. 8,637,280*. Washington, DC: U.S. Patent and Trademark Office, 2014.
5. Chen, H., He, X., Geng, H., & Liu, H. Physiological characterization of ATP-citrate lyase in Aspergillus

niger. *Journal of Industrial Microbiology and Biotechnology*, 2014; *41*(4): 721-731.
6. Currie, J.N. The citric acid fermentation of *Aspergillus niger* the citric acid fermentation article: *J. Biol. Chem*, 1917; 31: 15-37.
7. Grewal, H. S., & Kalra, K. L. Fungal production of citric acid. *Biotechnology advances*, 1995; *13*(2): 209-234.
8. Karaffa L., Sándor E., Fekete, E. & Szentirmai, A. The biochemistry of citric acid accumulation by *Aspergillus niger*. *Acta Microbiol Immunol Hung*, 2001; 48(3-4): 429-40.
9. Majumder, L., Khalil, I., Munshi, M. K., Alam, K., Rashid, H. O., Begum, R., & Alam, N. Citric acid production by Aspergillus niger using molasses and pumpkin as substrates. *European Journal of Biological Sciences*, 2010; *2*(1): 01-08.
10. MOYER, A. J. Effect of Alcohols on the Mycological Production of Citric Acid in Surface and Submerged Culture. *CULTURE*, 1953; *8*: 5-10.
11. Papagianni, M. Advances in citric acid fermentation by *Aspergillus niger:* Biochemical aspects, membrane transport and modelling. *Biotechnology Advances*, 2007; 25: 244–263.
12. Soccol, C.R., Vandenberghe, L.P.S., Rodrigues, C., Pandey, A. New perspectives for citric acid production and application. *Food Technol. Biotechnol*, 2006; 44(2): 141–149.
13. Swain, M.R., Ray, R.C. & Patra, J.K. Microbial production and applications in food and pharmaceutical industries. Dominic A. Vargas et al (Eds.), *Citric acid* (Chapter4). Nova Science Publishers, Inc., 2011.
14. Vandenberghe, L.P.S., Soccol, C.R., Pandey, A., Lebeault, J.M. Review: Microbial production of citric acid, *Braz. Arch. Biol. Technol.*, 1999; 42*:* 263–276.
15. Van der Straat, L., Vernooij, M., Lammers, M. *et al.* Expression of the *Aspergillus terreus* itaconic acid biosynthesis cluster in *Aspergillus niger*. *Microb Cell Fact*, 2014; 13(11) https://doi.org/10.1186/1475-2859-13-1.