Overview of citric acid production from Aspergillus

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Overview of citric acid production from *Aspergillus niger*

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Citic acid has high economic potential owing to its numerous applications. It is mostly produced by microbial fermentation using *Aspergillus niger*. In view of surges in demand and growing markets, there is always a need for the discovery and development of better production techniques and solutions to improve production yields and the efficiency of product recovery. To support the enormous scale of production, it is necessary and important for the production process to be environmentally friendly by utilizing readily available and inexpensive agro-industrial waste products, while maintaining high production yields. This article reviews the biochemistry of citric acid formation, choices of citric-acid producing microorganisms and raw materials, fermentation strategies, the effects of various fermentation conditions, citric acid recovery options and the numerous applications of citric acid, based on information drawn from the literature over the past 10 years.

**Keywords:** *Aspergillus niger*; citric acid; fermentation; glucose; microbial

**Introduction**

Citric acid, or 2-hydroxy-propane-1,2,3-tricarboxylic acid (C₆H₈O₇·H₂O) (Figure 1), is a naturally occurring weak organic acid found in all citrus fruits. The name of this organic acid is derived from Latin word *citrus*, which refers to trees of the genus *Citrus*, including lemon trees. Citric acid in its pure form is readily soluble in water and colourless (Angumeenal & Venkappayya 2013). It is solid at room temperature. Citric acid has a melting point of 153°C and it decomposes at higher temperatures. Citric acid has a molecular weight of 210.14 g/mol and possesses three functional groups of carboxylic acid in its structure (Papagianni 2007).

Citric acid can be derived from natural sources (e.g. lemon, lime and orange) or synthetic sources (e.g. chemical reaction and microbial fermentation). The method of extracting citric acid from lemon juice was pioneered by a Swedish chemist, Karl Wilhelm Scheele (1742–1786), in 1784. This method was adopted in England around 1826 for the commercial production of citric acid using lemons imported from Italy. The method maintained its monopoly as the only commercial source for citric acid production until the late nineteenth century, when a German botanist, Wehmer, in 1893, first observed the feasibility of obtaining citric acid through the fermentation of a sugar medium containing inorganic salts with *Penicillium glaucum* (Soccol et al. 2006). Two years after this discovery, Wehmer successfully isolated two strains which were able to produce citric acid. These strains were later named *Citromyces* spp. (*Penicillium*). However, the production of citric acid using *Citromyces* spp. did not gain much popularity in industrial practice because of contamination problems and the long fermentation process time (Bauweleurs et al. 2014).

In 1916, a study conducted by James Currie made a breakthrough for successful economic industrial production of citric acid from *Aspergillus niger*. He discovered that significant amounts of citric acid could be obtained from various strains of *A. niger*. The most important findings were the ability of *A. niger* to grow at a pH of around 2.5–3.5, which curbed the formation of gluconic and oxalic acid, and the increase in citric acid production with increasing sugar concentration. This single piece of research laid the foundation for present-day industrial citric acid production, which was established in the USA by the pharmaceutical company Pfizer in 1923 (Dashen et al. 2014).

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Over the years, a great variety of microorganisms has been studied, ranging from fungi and bacteria to yeasts. However, the microbial conversion of organic materials to citric acid is a complex biochemical reaction which requires careful control and specifically tailored operating conditions. Factors affecting the fermentation process include the concentration and type of carbon source, the pH of the fermentation medium, phosphate and nitrogen limitations, aeration, the morphology of the citric-acid producing microorganism and the concentrations of trace elements. Some of the nutrients, such as trace metals like manganese, phosphate and nitrogen, must be below defined limits to have a positive effect on the fermentation process. However, other elements, such as oxygen and sugar, are required in excess (Soccol et al. 2006; Max et al. 2010).

\textit{Aspergillus niger} is superior to other microorganisms for the commercial synthesis of citric acid because of its better production yield. It is easy to handle, can ferment various cheap raw materials and delivers high yields. As such, strains of this microorganism can be improved to create industrial strains for use in commercial production, and mutagenesis and strain selection have been carried out for such improvement. Different mutagens, including radiation, such as ultraviolet, X-rays and gamma-rays, and chemicals, such as ethyl methane sulphonate and diethyl sulphonate, have been used to induce the mutation of \textit{A. niger}.

Citric acid can also be produced by purely chemical reactions. Citric acid was first synthesized chemically in 1880 by Grimaux and Adam, using glycerol as a starting material, but this method was not economically competitive enough compared to other production routes such as fermentation. Therefore, microbial fermentation became the choice for commercial citric acid fermentation as it was more successful than chemical means. Citric acid is multifunctional and vital in many industries. Its vast and extensive range of versatile applications in various industries since the beginning of the twentieth century has created a continuous high demand. The worldwide production of citric acid in 2007 was 1.6 million tonnes, with an annual increase in demand and consumption of 3.5–4.0% (Anastassiadis et al. 2008). Citric acid is commonly used in food and beverages, detergents, pharmaceuticals, cosmetics, toiletries and other industries (Majumder et al. 2010). The beverage and food industries account for about 75% of the world’s citric acid consumption, 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).

Consumption of citric acid is expected to multiply owing to its low prices and numerous applications. The competitive pricing of citric acid is mainly caused by suppliers from China being willing to sell it at the lowest price possible, and this phenomenon has led to extremely tough competition for European suppliers (Gruben et al. 2014). ADM and Tate & Lyle have had to shut down their citric acid plants in Ireland and the UK owing to fierce competition from China (Weyda et al. 2014). In 2012, China was responsible for 59% of the global production of citric acid (Angumeenal & Venkappayya 2013). 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).

Based on market trends, it is apparent that there will be a surge in the global citric acid demand. Hence, it is crucial to optimize citric acid production by looking for alternatives that are more economical, more environmentally friendly and have a higher production yield than current methods. In light of this, this review discusses the biochemistry of citric acid production, raw material choices, selection of citric-acid producing microorganisms, production methods and strategies, the effects of various fermentation conditions and recovery options. The aim is to provide a thorough and comprehensive review, compared to other reviews that focus on specific areas (Pazouki & Panda 1998; Anastassiadis et al. 2008; Dhillon et al. 2011a).

\textbf{Biochemistry of citric acid production by \textit{Aspergillus niger}}

\textbf{Formation of citric acid}

The biochemistry of citric acid formation is complex. The production of citric acid is caused by the collective effect of different nutritional conditions in the medium (Kristiansen & Sinclair 1979). Nutritional conditions synergistically affect the production yield of citric acid, including carbon source concentration, dissolved oxygen, hydrogen ions, and suboptimal concentrations of phosphate and trace metals. Some studies report that limitation of the nitrogen source and deficiency of manganese or phosphate in the fermentation medium will inhibit the anabolism of \textit{A. niger}, resulting in protein degradation which, in turn, leads to a higher concentration of ammonium ions (Rohr & Kubicek 1981).

Excessive citrate can be produced by \textit{A. niger} through an active glycolytic pathway (Figure 2). Citrate is a well-known inhibitor of glycolysis, thus making it an area of

![Figure 1. Structural formula of citric acid, characterized by three –COOH groups.](image-url)
research interest. Under specific conditions, citrate inhibition can be curbed as a result of build-up of different positive effectors of the phosphofructokinase gene (PFK) (Käppeli et al. 1978; Kristiansen & Sinclair 1979). Protein breakdown occurs as a result of manganese deficiency and this leads to an increase in intracellular NH₄⁺ concentration (Habison et al. 1983). The phenomenon is known as the ‘ammonium pool’ and it inhibits the enzyme phosphofructokinase, which is vital for the conversion of fructose and glucose to pyruvate. As a result, there is a flux through glycolysis and citric acid formation. High glucose and NH₄⁺ concentrations will strongly limit 2-oxoglutarate dehydrogenase formation and eventually inhibit the catabolism of the citric acid present in the tricarboxylic acid (TCA) cycle (Rohr & Kubicek 1981).

Results from a study by Papagianni et al. (2005) on the early stages of accumulation of citric acid by *A. niger* contradicted those from the studies by Rohr and Kubicek (1981) and Habison et al. (1983), who reported that the presence of an intracellular ammonium pool does not account for the inhibition of phosphofructokinase. Ammonium ions enter cells to form glucosamine by combining with glucose, rather than just being simply deposited inside the cell (Boddy et al. 1993).

Thus, inhibition of the phosphofructokinase enzyme was found not to be a result of the ammonium pool. This is caused by several factors such as the release of synthesized glucosamine into the fermentation broth, the very low concentration of intracellular ammonium ions (about one-hundredth of the external pH) and the intracellular pH. Papagianni et al. (2005) recommend further investigation into the relationship of the enzymes phosphofructokinase, 2-oxoglutarate dehydrogenase and glucosamine synthase with the high levels of glucose and ammonium ions within the TCA cycle. The enzymes of *A. niger* act as the driving force in citric acid formation. These enzymes include invertase (a membrane-bound enzyme), which hydrolyses sucrose into glucose and fructose, which are transported into the cell as the reaction pathway from sucrose to citric acid begins outside the cell (Rubio & Maldonado 1995). Hexokinase is more abundant than other enzymes in *A. niger*, and it has an affinity for glucose over fructose in a ratio of 1000:1. Citrate inhibits glucokinase non-competitively (Steinböck et al. 1994). Schreierl-Kunar et al. (1989) showed that mutation can be used to produce an *A. niger* strain with higher affinity for sucrose growth.

Glucose oxidase stimulates *A. niger* to oxidize glucose to gluconic acid (Hayashi & Nakamura 1981). The enzyme is most potent under a high concentration of glucose, with strong aeration and low concentrations of other nutrients (typical conditions for citric acid fermentation) (Mischak et al. 1985; Dronawat et al. 1995). The effect of glucose oxidase is limited, however, as it is deactivated at pH lower than 3.5. The pH is reduced as a result of the accumulation of protons in the fermentation broth (Roukas & Harvey 1988). It is not known whether the gluconic acid will be used later in the citric acid formation during fermentation. Phosphofructokinases (PFK1 and PFK2) are responsible for the phosphorylation of fructose-6-phosphate. PFK1 phosphorylates at the C₁ position to produce fructose-1:6-bisphosphate. High concentrations of ATP, citrate and manganese inhibit PFK1, but it can be activated by the products of the PFK2 reaction, such as Zn²⁺, Mg²⁺, NH₄⁺ and fructose-2,6-bisphosphate (Kubicek-Pranz et al. 1990).

Well-known studies by Martin and Wilson (1951) and Cleland and Johnson (1954) revealed that citric acid was formed via the glycolytic reactions pathway. The glycolytic and pentose phosphate reaction pathways act as a channel for *Aspergillus* species to utilize glucose and other carbohydrates for biosynthesis and maintenance. Pyruvate kinase was previously found to be an essential regulatory stage in citric acid synthesis, but a study showed that the enzyme in its pure form was only marginally affected by the metabolic levels of inhibitors (Meixner-Monori et al. 1984). Citrate synthase is a catalyst for the reversible condensation reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetate. This reaction favours citrate production and, as a result, thioester hydrolysis occurs during the reaction (Mischak et al. 1985). The equation is shown below:

\[
\text{Acetyl CoA} + \text{Oxaloacetate} \leftrightarrow \text{Citrate}^3+ + \text{H}^+ + \text{CoA} - \text{SH}
\]

Three different isocitrate dehydrogenase isoenzymes exist in *A. niger*, which can be differentiated from each
Accumulation of citric acid

In general, the build-up of citric acid involves deactivation of aconitase and/or isocitrate dehydrogenase. Activity in the Krebs cycle produces intermediates necessary for biomass formation during the formation of citric acid (Jernejc et al. 1992; Aghdam & Taherzadeh 2008). The Krebs cycle is a series of eight reactions that take place in the mitochondrion. These reactions take a two-carbon molecule (acetate) and completely oxidize it to carbon dioxide. The cycle is summarized in the following chemical equation:

$$\text{Acetyl CoA} + 3\text{NAD} + \text{FAD} + \text{ADP} + \text{HPO}_4^{-2} \rightarrow 2\text{CO}_2 + \text{CoA} + 3\text{NADH}^+ + \text{FADH}^+ + \text{ATP}$$

Therefore, the accumulation of citric acid probably results from enhanced (deregulated) biosynthesis instead of inhibited degradation (Punekar et al. 1984).

Kubicek-Pranz et al. (1990) explained the accumulation of citric acid by associating it with the tricarboxylate transporter competing for citric acid with aconitase. Under conditions where the tricarboxylate transporter has a greater affinity for citric acid than for aconitase, the enzyme removes citric acid from the mitochondria of the cell without inhibiting the enzymes present in the cycle.

Glutamine synthase is linked with the effects of pH, ammonia and manganese ions on the accumulation of citric acid (Punekar et al. 1984). The low pH value during the
production phase (pH < 2) reduces the risk of contamination by other microorganisms and inhibits the production of unwanted organic acids (gluconic and oxalic acids), which facilitates recovery of the product. The presence of Mn^{2+} or Mg^{2+} ions in the enzyme structure determines its form. The isozymes of isocitrate dehydrogenase in A. niger, which are present in the mitochondria as well as the cytoplasm, require Mg^{2+} or Mn^{2+} for reaction, and their inhibition by α-ketoglutaric and citric acid ensures citric acid accumulation (Ratledge & Kristiansen 2001).

Isocitrate undergoes a two-phase conversion process to become α-ketoglutarate. First, protons are detached to form oxalosuccinate. Secondly, carbon dioxide is formed and released, completing the conversion. The metal ions Mg^{2+} and Mn^{2+} are essential for the reaction, as they are attached to the active sites by these enzymes. They are responsible for the stabilization of oxalosuccinate (intermediate reaction product) and the substrate–enzyme binding. Either Mg^{2+} or Mn^{2+} can be utilized by the enzymes without adverse effects on their activity. Citric acid accumulation is made possible as citrate and α-ketoglutarate inhibit the NADP^+-dependent form of the enzyme (Mattey 1977).

**Microbial production of citric acid via fermentation**

**Microorganisms**

Species of Aspergillus such as A. wenti, A. foetidus, A. aculeatus, A. awamori, A. fumigatus, A. phoenicis and A. carbonaries, as well as Trichoderma viride and Mucor pyriformis, have been found to produce significant amounts of citric acid (Berovic & Legisa 2007).

Besides fungi and bacteria, yeast species such as Candida tropicais (Legiša & Mattey 1986), Candida oleophila (Küppeli et al. 1978), Candida guillermontdi (Angumeenal et al. 2003), Yarrowia lipolytica (Angumeenal & Venkappayya 2013), Torulopsis, Hansenula, Debaromyces, Torula, Pichia, Kloekera, Saccharomyces and Zygosaccharomyces are capable of producing citric acid from n-alkanes and carbohydrates (Weyda et al. 2014). The drawback of using yeast is that it produces large quantities of isocitric acid, which is an undesirable by-product; therefore, mutant strains that have low aconitase activity are required. In addition, the increasing cost of oil makes it less feasible economically as oils are now used as the principal carbon source, in a manner analogous to the previous use of alkanes (Mazinanian et al. 2015).

Aspergillus niger has so far maintained its place in citric acid production as it has advantages over other bacterial microorganisms such as Arthrobacter paraffinm, Bacillus licheniformis, Bacillus subtilis, Brevibacterium flavum, Corynebacterium spp. and Penicillium janthinellum (Ikram-ul et al. 2004). It is easy to handle, can ferment a broad range of low-cost raw materials and provides high yields (Themelis & Tzanavaras 2001). Mutagenesis has been used in recent years to improve the citric-acid producing strains so that they can be used in industrial applications. The most common methods include the use of mutagens to induce mutations on the parental strains. The mutagens utilized for improvements are gamma radiation, ultraviolet radiation and often chemical mutagens. For hyper-producer strains, a hybrid method that combines ultraviolet and chemical mutagens is used (Ratledge & Kristiansen 2001). The selection methods are the passage and single-sporo methods. The passage method is preferred, as the single-sporo method has a disadvantage in that organic acids (oxalic and gluconic acids) and mineral acids simulate the presence of citric acid (Soccol et al. 2006).

The fermentation technique also affects the citric acid yield. As an example, a strain may have a good yield in the submerged fermentation process but a poor yield in the solid-state fermentation process. Therefore, the producer strains need to be tested in each of the fermentation methods as well as the industrial substrates to ascertain the best fermentation method (Chen et al. 2014). Microorganisms for citric production have to be inoculated by spores, which are transferred to the fermentation medium. The various transfer media include air, and can be in the form of a suspension which is then introduced into bottles containing the substrate. Ideally, for high yields, an incubation time of 7 days is required for A. niger. However, after the 7 days of incubation, the capacity for germination tends to reduce with time (Vergano et al. 1996).

**Substrates**

A wide range of substrates is utilized in the fermentation process of the microorganisms. Materials such as hydrocarbons, molasses and starchy materials are commonly used. The review by Soccol et al. (2006) mentions examples such as beet molasses, black strap molasses, cane molasses, carob pod extract, n-paraffin, glycerol, corn starch, hydrolysate starch, yam bean starch, wood hemicellulose, olive oil, rapeseed oil, palm oil and soya bean oil.

Owing to the need to use less expensive substrates with the aim of reducing the production costs of citric acid and making it more environmentally sustainable, the non-crystallizable effluents (molasses) after sucrose isolation from sugar refineries may be used. Molasses offers reduced cost and a high sugar content of 40–55% in the form of fructose, glucose and sucrose (Dronawat et al. 1995). The quality of molasses varies according to its source. Therefore, it requires pretreatment [e.g. mixing with K_{2}Fe(CN)_{6} at pH 4.5, 90°C for 15 min and then removal of the precipitate by filtration] to make it suitable for fermentation. This variation in the molasses is usually a result of the different varieties of sugar cane/beet, cultivation methods and storage conditions (Li et al. 2014).
Studies have shown that beet molasses produces a higher yield than cane molasses; this can be attributed to the relatively high content of trace metals such as magnesium, zinc, manganese, calcium and iron in cane molasses, which are all detrimental to the synthesis of citric acid (Chen et al. 2014). Attempts have also been made to improve the citric acid yield of molasses by the addition of yield-increasing materials. Lu et al. (1998) used a plant constituent (phytate) to supplement the molasses at the beginning of incubation, thereby tripling the citric acid accumulation (Themelis & Tzanavaras 2001). Ambati and Ayyanna (2001) used a palmyra jiggery, which is sugar syrup extracted from the palmyra palm. Wojtawicz et al. (1993) supplemented beet molasses with natural oil with a high unsaturated fatty acid content, and observed an increased yield. In recent years, as a result of increasing demand for citric acid and increasing environmental concerns, there has been more interest in feasible novel substrates for citric acid production. Various residues and by-products from the agricultural industry have been used as substrates for different fermentation systems, as shown in Table 1.

Some substrates lack necessary ingredients such as nitrogen and phosphate that are required to be at optimum levels for optimum citrate formation. In situations where these nutrients are unavailable, nitrogen and phosphate are supplied by the addition of KNO₃ or NH₄NO₃ and K₂HPO₄ or KH₂PO₄, respectively (Angumeenal & Venkappayya 2013).

### Table 1. Raw materials used for citric acid fermentation.

<table>
<thead>
<tr>
<th>Type of fermentation</th>
<th>Raw materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>Brewery waste, carob sugar, cotton waste, tubes of <em>Asphodelus aestivus</em>, turnip whey (enriched with molasses)</td>
</tr>
<tr>
<td>Submerged</td>
<td>Bagasse hydrolysates, beet molasses, blackstrap molasses, brewery wastes, cane molasses, carob pod extract, coconut oil, corn starch, date syrup, glycerol, hydrolysate starch, n-paraffin, palm oil, olive oil, soya bean oil, rapeseed oil, spent grain liquor, wood hemicellulose, whey permeate, xylan hydrolysate, yam bean starch</td>
</tr>
<tr>
<td>Solid state</td>
<td>Apple pomace, carob pod, carrot waste, cassava bagasse, cellulose hydrolysate and sugar cane, coffee husk, corn cob, deoiled rice bran, grape pomace, kiwi fruit peel, kumara, molasses, mussel processing wastes, okara, orange waste, pineapple waste, rice bran, sucrose, sugarcane-pressmud, wheat bran</td>
</tr>
</tbody>
</table>

### Fermentation strategies in citric acid production

Citric acid production by fermentation has become established as the most widely used and economical process to obtain citric acid. Over 90% of the citric acid used around the globe today is produced from fermentation. This method offers advantages such as having simple, stable and less complicated operations; requiring less complex control systems and lower technical skill; consuming less energy; and not being critically affected by frequent plant power failure (Soccol et al. 2006). In general, all fermentation processes, irrespective of the type of fermentation, have three phases: preparation and inoculation, fermentation, and recovery of the citric acid.

Over the years, citric acid fermentation has undergone a series of developments. In the 1910s, production was limited to species of *Penicillium* and *Aspergillus* utilizing surface or stationary culture conditions. In the 1940s, Shu and Johnson (1948a) developed submerged fermentation from *Aspergillus*. This research provided the basis for submerged fermentation.

Various types of fermenter have been used for the fermentation to produce citric acid. These include trays, glass incubators, Erlenmeyer conical flasks, rotating and horizontal drum bioreactors, and packed-bed single-layer packed-bed and multi-layer packed-bed column bioreactors.

### Surface fermentation

Surface fermentation, also known as liquid surface culture, was the original citric acid industrial production technique. Even though in recent years submerged fermentation has gained popularity, there are still small- and medium-scale industries that make use of this method (Bauweleers et al. 2014). Surface fermentation offers advantages such as lower installation and energy costs (as it does not require energy for aeration and agitation), and is also foam free. However, it is labour intensive (Drysdale & McKay 1995) and sensitive to changes in composition of the media (Benghazi et al. 2014). This method consists of two phases, both of which are characterized by a rapid uptake of carbohydrates. The first phase is the development of the fungus as mycelial mat on the surface of the medium and the second phase utilizes carbohydrates by converting them to citric acid (Kiel et al. 1981). The process is conventionally performed in fermentation chambers, using trays made from materials such as special-grade steel, high-purity aluminium or polyethylene. However, stainless steel trays are preferred, as they are resistant to deformation with prolonged use (Bauweleers et al. 2014).

Since the chamber needs to be effectively ventilated, sterile air is aseptically passed continuously over the medium surface through a bacteriological filter. The air supplies the required oxygen demand of the microorganisms due to the highly aerobic nature of the
process; it also controls the humidity and temperature (via evaporative cooling). The air also serves to remove carbon dioxide, which is an inhibitor of citric acid production at concentrations higher than 10% (Soccol et al. 2006). Contamination is also an important issue in surface cultures. Yeasts, penicillia, lactic bacteria and species of Aspergillus are the most common sources of contamination (Soccol et al. 2006). After completion of the second phase, the citric acid is recovered by washing the mycelial mats and the impregnated citric acid is extracted (Max et al. 2010).

Other research on surface culture includes Sakurai and colleagues’ kinetics study of A. niger citric acid production by surface culture and their research on surface liquid fermentation of banana waste (Sakurai et al. 1991).

**Submerged fermentation**

This is the most widely used fermentation technique in the world today. Eighty per cent of the world’s production is estimated to be from the submerged method (Roukas 1991). Submerged fermentation was developed after surface fermentation. It requires more sophisticated installation, higher energy cost and rigorous control, and there is formation of foam (which can be resolved using anti-foaming agents), but it provides higher productivity and yields, has reduced capital, maintenance and labour costs, and carries lower contamination risks. In addition, it is less sensitive to change in the medium composition, providing a wider range of substrates and better control of substrates; this advantage makes molasses usable as a medium for citric acid production (Max et al. 2010). Submerged fermentation is mostly operated as a batch system (Kishore et al. 2008); however, continuous systems are possible and are used in practice. Submerged fermentation also includes the shake flask technique, which is usually used for the optimization of fermentation conditions (Ikram-ul et al. 2004). This is basically an Erlenmeyer flask which is placed on a shaker and stirred continuously throughout the fermentation process.

Darouneh et al. (2009) performed a comparative study between surface culture and submerged culture techniques. The outcome from this study was that surface fermentation is superior to submerged fermentation in terms of yield and productivity of citric acid. Ali et al. (2002a) studied citric acid production utilizing cane molasses in a stirred fermenter (Ikram-ul et al. 2004). Selected mutants of A. niger producing citric acid from cane molasses were also investigated (Majumder et al. 2010). Soccol et al. (2006) studied the influence of metal-complexing agents on the mycelial growth, conidial germination and morphology of A. niger. Roukas (1991) demonstrated the ability of beet molasses to produce citric acid using shake flasks. Anastassiadis et al. (2008) studied the oxygen requirements for citric acid production using Yarrowia lipolytica. The construction materials (e.g. austenitic stainless steel, type 316L stainless steel) for a submerged fermenter must be able to withstand low pH (acidic) conditions. Both stainless steel and ordinary steel will degrade under acidic conditions and inhibit citric acid formation. A thorough study on molasses and pumpkin as substrates, using A. niger 14/20 and 79/20 strands, showed that both substrates can be used for the production of citric acid, but a mixed substrate gives better overall results (Majumder et al. 2010).

**Solid-state fermentation**

The solid-state process, or ‘Koji’ fermentation, originates from Japan, which has an abundance of agro-industrial residues/wastes (Kareem et al. 2010). This process involves the cultivation of microorganisms in the absence of free liquid on moist solid materials (Käppeli et al. 1978). The solid materials act as a physical support and source of nutrients for the microorganism. Under optimal conditions, the process should be completed in 4 days (Drysdale & McKay 1995). The main advantage of solid-state fermentation is its superior yield and the ability to utilize inexpensive and widely available agro-industrial residues as substrates for bio-production, making it more environmentally friendly than submerged fermentation (Falony et al. 2006). It requires less water and has lower operating costs, and does not require complex equipment. There is no need for pretreatment as the system is less sensitive to the presence of trace elements compared to submerged fermentation (Berovic & Legisa 2007).

One limitation of this method is that it does not completely utilize available nutrients owing to poor heat and oxygen transfer in the substrate (Sangsurasak & Mitchell 1995). There is also a limited pool of viable microorganisms, and strains with large nitrogen and phosphorus requirements cannot be used. Agro-industrial wastes that have been utilized include banana peel (Max et al. 2010), cotton waste (Kiel et al. 1981), kiwi fruit peel (Hang et al. 1987), date pulp (Assadi & Nikkhah 2002), apple pomace ultrafiltration sludge and solid waste (Kareem et al. 2010), orange peel waste (Torrado et al. 2011), pineapple waste (Kareem et al. 2010), wheat bran and soya bean meal (Sauer et al. 2008), fresh kumara (Ipomoea batatas), potato (Solanum tuberosum) and taro (Colocasia esculenta) (Arshad et al. 2014).

**Factors affecting citric acid fermentation**

**Carbon source**

Studies over several decades have shown that the carbon source affects the citric acid yield directly. Monosaccharides and disaccharides are the preferred carbon source as they are more rapidly metabolized by the fungus than polysaccharides, thus producing higher yield (Mattey 1992). Polysaccharides are not suitable as the raw material as the decomposition process takes too long to meet...
the rate of sugar catabolism necessary for the production of citric acid. The slow rate of polysaccharide hydrolysis is due to reduced enzymatic activity, which affects the pH in the fermentation medium (Hossain et al. 1984; Xu et al. 1989; Papagianni et al. 2005). Sucrose is superior to glucose, fructose and lactose, in order of decreasing citric acid yield (Angumeenal & Venkappayya 2013). The superiority of sucrose has been attributed to the strong extracellular mycelium-bound invertase of A. niger, which rapidly hydrolyses sucrose at low pH (Kubícek-Pranz et al. 1990).

Pure sucrose or glucose may not be economically feasible on an industrial scale. Therefore, low-grade carbon sources such as wastes from sugar refineries, for example cane and beet molasses are used. Owing to the various sources from which these raw materials are obtained, there may be a need for pretreatment. Cations are generally the major contaminants, and the commonly used method of pretreatment is by precipitation using potassium ferrocyanide or cation-exchange resins (Angumeenal & Venkappayya 2013a). The concentration of the carbon source is as critical to the success of citric acid production as the type of carbon source. Since citric acid production relates directly to sugar concentration, as the concentration increases so does the amount of citric acid produced (Xu et al. 1989). However, results showed that the maximum concentration can be obtained with 14–22% of sugar. A study by Xu et al. (1989) using sucrose, glucose, fructose, mannose and maltose obtained the maximum yield at a sugar concentration of 10% (w/v), except for glucose, where a 7.5% (w/v) maximum yield was obtained (Amenaghawon & Asien 2012).

Nitrogen limitation

The concentration of nitrogen has been found to have a strong effect on the production of citric acid, as nitrogen is not only part of a cell’s proteins, but also necessary for cellular metabolism (Ali et al. 2002b). Molasses and other industrial media are usually nitrogen rich, whereas laboratory media require additional ammonium salts as supplements. The type of nitrogen source affects the synthesis of citric acid as well as the fungal growth. Ammonium nitrate promotes reduced vegetative growth, while ammonium sulphate promotes a longer period of vegetative growth. Nitrogen limitation is necessary, because at a concentration greater than 0.25%, oxalic acid accumulates and it will decrease the citric acid yield (Gupta et al. 1975). A high nitrogen concentration increases the consumption of sugar and fungal growth, while decreasing the amount of citric acid produced (Hang et al. 1977). Ammonium sulphate is the preferred choice of salt as it does not produce the unwanted oxalic acid (Nigam 2009), while reducing the pH of the medium as the salt is consumed. Kristiansen and Sinclair (1979) also discovered that nitrogen limitation is required for a good yield of citric acid in a continuous system.

The maximum citric acid yield in a laboratory-scale stirred fermenter was achieved with a concentration of ammonium nitrate kept at 0.2% (Ali et al. 2002a). Kareem et al. (2010) supplemented the fermentation medium containing pineapple waste with 0.25% (w/v) of methanol and observed a 17.6% increase in the production of citric acid. The biomass increased as well. Further increases or decreases in concentration led to the distortion of fungal growth and lower citric acid production.

Phosphorus source

Along with nitrogen and the carbon source, phosphate has also been shown to be a critical factor. For fungal growth, a phosphorus concentration of 0.5–5 g/l is required for citric acid production (Shu & Johnson 1948b). The addition of phosphorus has only a slight effect on the accumulation of citric acid and mycelial growth (Hang et al. 1987). Kubícek and Röhr (1977) deduced that citric acid accumulates with limited phosphate, even when nitrogen is not limited.

pH of culture medium

The pH of the medium changes continuously as a result of microbial metabolic activities, largely because of the secretion of organic acids such as citric acid, and the unwanted gluconic and oxalic acid. The metabolic activities of microbes such as Aspergillus, Rhizopus and Penicillium species are able to reduce the pH quickly to below 3, while other fungi such as Sporotrichum and Pleurotus species produce a more stable pH between 4 and 5 (del Campo et al. 2006). The pH of the fermentation medium is most important during the sporulation and production phase. In the germination stage, the germinating spores absorb ammonia and release protons, thereby increasing the acidity of the medium and favouring the production of citric acid. At low pH of about less than 2, the formation of unwanted products such as oxalic and gluconic acid is inhibited, and the possibility of contamination by other microorganisms is also reduced, making recovery of citric acid easier (Max et al. 2010). The initial pH of a medium must be optimized and defined to suit the microorganism, substrate and production technique. Dashen and colleagues showed that the substrate and production technique influences the pH kinetics, which is the complete pH-rate profile showing the pH changes in difference substrates (Dashen et al. 2014).

Concentration of alcohols

Moyer (1953) first reported the effect of alcohols on the production of citric acid. He found out that methanol had a profound positive effect on the formation of citric acid.
from different carbon sources, such as crude carbohydrate sources as well as commercial glucose (Moyer 1953). It was further concluded that the composition of the medium and the strain of microorganism used determine the optimum amount of methanol/ethanol required, which should mostly lie between 1% and 3%.

Moyer (1953) also studied the effect of ethanol on citric acid accumulation, it concluded that the addition of ethanol doubles the citrate synthetase activity, decreases theaconitase activity by 75% and slightly increases the activities of other TCA cycle enzymes. These effects of the alcohol result in increased citric acid accumulation, which is attributed to the slow degradation of the citric acid resulting from reduced aconitase activity. Acting as a carbon source, ethanol also increases the availability of carbon in the citric acid cycle. Roukas and Harvey (1988) obtained maximum citric acid production at a methanol concentration of 2%, whereas a concentration higher than that led to a decrease in citric acid production. It was further concluded that the inductive effect of methanol was a result of its inhibitory effect on metal ions (Kiel et al. 1981). This result shows that the amount of methanol has to be limited for optimum citric acid production.

### Trace elements

Studies on divalent metal ions including manganese, zinc, copper, magnesium and iron have shown that they have effects on citric acid production (Käppeli et al. 1978; Dronawat et al. 1995). Tomlinson et al. (1950) concluded that the optimum concentrations of iron and zinc are 1.3 and 0.3 ppm, respectively. These authors further explained the importance of manganese for cell function, sporulation and production of secondary metabolites, and mainly in cell wall synthesis. Manganese deficiency affects the anabolism of A. niger, causing a high intracellular ammonium concentration. A decrease in the accumulation of citric acid with iron has been observed, as well as changes in mycelial growth (Mischak et al. 1985). Grewal and Kalra (1995) deduced that at high zinc concentrations, the fungi maintained growth without accumulation of citric acid (Drysdale & McKay 1995). Nickel, molybdenum and cobalt are some other trace metals reported to affect the citric acid accumulation of A. niger (Habison et al. 1983). The independence of medium constituents has to be taken into account as it is crucial to citric acid production; therefore, robust control of these trace elements is required for the optimum production of citric acid.

### Aeration

The highly aerobic nature of the bio-production of citric acid makes the amount of oxygen supplied a critical factor. Therefore, varying aeration rates can have adverse effects on the fermentation performance and yield (Grewal & Kalra 1995). At high aeration rates, there is reduced partial pressure of the dissolved carbon dioxide in the medium. Carbon dioxide is a substrate for pyruvate carboxylase as it replaces the supply of oxaloacetate for citrate synthase. The reaction catalyzed by pyruvate decarboxylase produces carbon dioxide, but extreme aeration can incur some losses. Increased levels of carbon dioxide are damaging to the final biomass and concentrations of citrate (Angumeenal & Venkappayya 2013).

However, carbon dioxide has been shown to have a positive effect on the synthesis of citric acid (Vandenberghe et al. 1999). The high partial pressure of carbon dioxide impedes the liberation of spores of the filamentous fungi, thereby enhancing citric acid accumulation. However, high aeration rates result in foaming in the medium, mainly during the growth phase, requiring the use of antifoaming agents and/or mechanical defoamers. Economically, it is better to gradually increase the aeration rate, but Li et al. (2014) recommend that aeration should be performed evenly across the medium with similar intensity. This is in line with the study by Kubicek et al. (1980), which revealed that a disruption process (i.e., interruptions in aeration) up to 20 min during idiophase irreversibly destroyed the organism’s ability to synthesize and accumulate citric acid, although the viability of the organism was maintained. The role of low aeration is meant to curb the respiratory activity of A. niger, thereby shifting the metabolism away from biomass production to the synthesis of citric acid. It was also concluded that stronger aeration results in increased sporulation and decreased accumulation of citric acid (Soccol et al. 2006).

### Other factors

Other factors that have effects on citric acid production include lipids, such as groundnut oil (Millis et al. 1963; Kumar & Ethiraj 1976; Souza et al. 2014), and sodium monofluoroacetate (Meixner-Monori et al. 1984). Millis et al. (1963) showed that lipid will improve the yield of citric acid with no effect of the dry weight of mycelium. Kareem et al. (2010) showed the effects of calcium fluoride, sodium fluoride and potassium fluoride on the industrial production of citric acid. The main factors that affect citric acid production are shown in Table 2.

### Recovery of citric acid

Recovery of citric acid can be achieved by three major unit operations, namely precipitation, extraction and adsorption (mainly using ion-exchange resin). It is possible to use crystallization but it carries a risk of contamination as a result of unwanted materials from the raw material and autolysis of the microbial cells (Drysdale & McKay 1995). Precipitation has been the most used method for the recovery of citric acid as it is applicable to all processes, but it requires the removal of the micelles from the fungus, fermentation broth and suspended material by filtration...
Table 2. Factors affecting citric acid production.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon source</td>
<td>Positive: sucrose, glucose, fructose, galactose</td>
</tr>
<tr>
<td></td>
<td>Negative: starch, xylose, arabinose, sorbitol, pyruvic acid</td>
</tr>
<tr>
<td>Phosphorus source</td>
<td>Positive: potassium dihydrogen phosphate (0.5–5.0 g/l)</td>
</tr>
<tr>
<td>Nitrogen source</td>
<td>Positive: ammonium nitrate, ammonium sulphate, peptone, malt extract, urea (0.1–0.4 gN/l)</td>
</tr>
<tr>
<td></td>
<td>Negative: high concentrations lead to biomass production</td>
</tr>
<tr>
<td>pH</td>
<td>Positive: &lt; 3 (depends on species)</td>
</tr>
<tr>
<td>Trace elements</td>
<td>Positive: zinc, copper, magnesium sulphate (low levels)</td>
</tr>
<tr>
<td>Lower alcohols</td>
<td>Positive: methanol, ethanol, n-propanol, iso-propanol, methylacetate (1–4% v/w)</td>
</tr>
</tbody>
</table>

Calcium oxide hydrate (milk lime) is added to the medium and the lime is then precipitated into tricalcium citrate tetrahydrate. Then, the precipitate is filtered and washed with water. Thereafter, it is treated in an acidulator with calcium sulphate, a sulphuric-acid forming gypsum. Next, it is filtered and the mother liquor is treated with active carbon and passed through anion and cation exchangers. It is further concentrated by evaporation in a vacuum at 40°C. Lastly, crystals of citric acid monohydrate are formed in a vacuum crystallizer at a temperature of 20–25°C and anhydrous citric acid is formed at crystallization temperatures above 36.5°C (Kubicek 1986; Grewal & Kalra 1995). The wastes from this process include calcium sulphate, and microorganism residues containing amino acids, sugar, colloid, pigment and inorganic matter, which could be supplied to cement factories and dried for use in feed factories, respectively (Dhillon et al. 2011b).

Solvent extraction is an alternative to the purification and crystallization of citric acid, as it has reduced environmental impacts compared with the precipitation method, by making use of n-octyl alcohol and tridodecylamine for citric acid extraction, thereby eliminating the production of gypsum. This method was also recommended by the US Food and Drug Administration for use in food and drug applications (Grewal & Kalra 1995). Electrodialysis makes use of electrically charged membranes, utilizing the electrical potential difference between the ionic and aqueous solution to separate them. This technique has been proven to be economical for clear fermentation media. However, it

Table 3. Applications of citric acid.

<table>
<thead>
<tr>
<th>Applications</th>
<th>Industry</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food</td>
<td>Animal feed</td>
<td>Feed complement</td>
</tr>
<tr>
<td>Candy</td>
<td></td>
<td>Acts as acidulant. Prevention of sucrose crystallization provides dark colour in candies, sucrose inversion. Provides tartness</td>
</tr>
<tr>
<td>Dairy products</td>
<td></td>
<td>Emulsifier (ice creams and processed cheese), antioxidant and acidifying agent (cheese products)</td>
</tr>
<tr>
<td>Fats and oils</td>
<td></td>
<td>Stabilizing action. Synergist for other antioxidants as a sequestrant</td>
</tr>
<tr>
<td>Frozen fruits</td>
<td></td>
<td>Inactivates trace metals to protect ascorbic acid. Lowers pH to inactivate oxidative enzymes. Neutralizes the residual lye</td>
</tr>
<tr>
<td>Jellies and jams</td>
<td></td>
<td>Adjusts pH (to the range where pectin acts as a gelling agent). Acts as an acidulant. Provides tartness, tang and flavour. Increases the effectiveness of antimicrobial preservatives</td>
</tr>
<tr>
<td>Beverages</td>
<td>Fruit and vegetable juices</td>
<td>Stabilizer in commercially prepared juices</td>
</tr>
<tr>
<td></td>
<td>Soft drinks and syrups</td>
<td>Simulates fruit flavour and tartness. As an acidulant in carbonated and sucrose-based beverages</td>
</tr>
<tr>
<td></td>
<td>Wines and ciders</td>
<td>Browning prevention in some white wines. Turbidity prevention. Oxidation inhibition. pH adjustment</td>
</tr>
<tr>
<td>Pharmaceutics</td>
<td>Pharmaceuticals</td>
<td>Provides rapid dissolution of active ingredients. Acidulant in mild astringent formulations, anticoagulant. Effervescent in powders and tablets in combination with bicarbonates, solubilization action for cathartics, antioxidant in vitamin preparations</td>
</tr>
<tr>
<td>Cosmetics and toiletries</td>
<td></td>
<td>Metallic-ion chelator and buffering agent. Adjusts pH. Antioxidant</td>
</tr>
<tr>
<td>Other</td>
<td>Other</td>
<td>Buffering agent, sequesters metal ions, neutralizes bases</td>
</tr>
<tr>
<td></td>
<td>Metal oxide removal from ferrous and non-ferrous metal surfaces, operational cleaning of iron and copper oxides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In electroplating, copper plating, metal cleaning, leather tanning, printing inks, bottle washing compounds, floor cement, textiles, photographic reagents, concrete, plaster, adhesives, paper, polymers, tobacco, waste treatment, chemical conditioner on teeth surface, ion complexation in ceramic manufacture, etc.</td>
<td></td>
</tr>
</tbody>
</table>
is 50% more expensive than other recovery processes such as precipitation (Amenaghawon & Aisien 2012). In laboratory analyses, the determination and quantification of citric acid are done using spectrophotometry (Themelis & Tzanavaras 2001; Jham et al. 2002), gas chromatography (Jham et al. 2002), high-performance liquid chromatography (Jham et al. 2002), high-resolution nuclear magnetic resonance spectroscopy (del Campo et al. 2006) and the traditional method of titration (Williams 1984).

Applications of citric acid
The versatility and non-toxicity of citric acid are its main positive characteristics. It is accepted globally as ‘generally recognized as safe’ (GRAS) and has been endorsed by the FAO/WHO committee on food additives. It cuts across various industries for various applications. Examples of citric acid applications are shown in Table 3 (Grewal & Kalra 1995).

Conclusions
This review focuses on the details of citric acid production. The economic potential, biochemistry, choices of microorganism employed, raw materials, types of fermentation technique, factors affecting citric acid production, quantification techniques and recovery techniques are discussed. Currently, citric acid is the most produced organic acid in the world. Global production is expected to increase further with increasing demand. Although citric acid production using A. niger provides satisfactory performance at the moment, there is still room for greater improvements in increasing yield and minimizing waste by developing novel fermentation techniques and the optimization of A. niger using genetic manipulation. Microbial strains which are capable of fermenting any substrates have been develop ed to maximize the utilization of agro-industrial waste and provide a more efficient and environmentally friendly production process.

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