Microbial production of citric acid

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ABSTRACT

Citric acid is the most important organic acid produced in tonnage and is extensively used in food and pharmaceutical industries. It is produced mainly by submerged fermentation using *Aspergillus niger* or *Candida* sp. from different sources of carbohydrates, such as molasses and starch based media. However, other fermentation techniques, e.g. solid state fermentation and surface fermentation, and alternative sources of carbon such as agro-industrial residues have been intensively studied showing great perspective to its production. This paper reviews recent developments on citric acid production by presenting a brief summary of the subject, describing micro-organisms, production techniques, and substrates, etc.

Keywords: Citric acid, *Aspergillus niger*, submerged fermentation, solid state fermentation, substrates

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RESUMO

O ácido cítrico é o ácido mais produzido em termos de tonagem e é extensivamente utilizado pelas indústrias alimentícia e farmacêutica. É produzido principalmente por fermentação submersa utilizando o fungo *Aspergillus niger* e leveduras do gênero *Candida* sp. à partir de diferentes fontes de carbono, como a glicose e meios à base de amido. No entanto, outras técnicas de fermentação, e.g. fermentação no estado sólido e em superfície, e fontes alternativas de carbono tem sido intensamente estudadas mostrando grande perspectivas para o processo. O presente trabalho apresenta um resumo dos últimos avanços sobre a produção do ácido cítrico, descrevendo de maneira sucinta os trabalhos mais recentes, descrevendo microrganismos, técnicas de produção e substratos empregados, etc.

INTRODUCTION

Citric acid ($C_6H_8O_7$, 2 - hydroxy - 1,2,3 - propane tricarboxylic acid), a natural constituent and common metabolite of plants and animals, is the most versatile and widely used organic acid in the field of food (60%) and pharmaceuticals (10%). It has got several other applications in various other fields. Currently, the global production of citric acid is estimated to be around 736000 tones/year (Química e Derivados, 1997), and the entire production is carried out by fermentation. In Brazil, almost the entire demand of citric acid is met through imports. There is constant increase (3.5-4%) each year in its consumption, showing the need of finding new alternatives for its manufacture.

Historical developments

Citric acid was first isolated by Karls Scheels in 1874, in England, from the lemon juice imported from Italy. Italian manufacturers had monopoly for its production for almost 100 years, and it was sold at high cost. This led extensive attempts all over the world to find alternatives way for its production, which included chemical and microbial techniques. In 1923, Wehmer observed the presence of citric acid as a by-product of calcium oxalate produced by a culture of *Penicillium glaucum*. Other investigations showed the isolation of two varieties of fungi belonging to genus *Citromyces* (namely *Penicillium*). However, industrial trials did not succeed due to contamination problems and long duration of fermentation (Rohr et al., 1983). The industrial process was first open by Currie, in 1917, who found that *Aspergillus niger* had the capacity to accumulate significant amounts of citric acid in sugar based medium. He also showed that high concentrations of sugar favoured its production, which occurred under limitation of growth. In the thirties, some units were implanted in England, in Soviet Union, and in Germany for the commercial production. However, the biochemical basis was only cleared in the fifties with the discovery of the glycolytic pathway and the tricarboxylic acid cycle (TCA). Consequently, an improved process employing submerged fermentation was developed in United States (Aboud-Zeid and Ashy, 1984).

Although methods were well developed to synthesis citric acid using chemical means also, better successes were achieved using microbial fermentations, and over the period of time, this technique has become the method of ultimate choice for its commercial production, mainly due to economic advantage of biological production over chemical synthesis (Mattey, 1992). Much attention has been paid on research to improve the microbial strains, and to maintain their production capacity.

Applications of citric acid

Citric acid is mainly used in food industry because of its pleasant acid taste an its high solubility in water. It is worldwide accepted as "GRAS" (generally recognized as safe), approved by the Joint FAO/WHO Expert Committee on Food Additives. The pharmaceutical and cosmetic industries retain 10% of its

utilization and the remainder is used for various other purposes. Table 1 presents main applications of citric acid.

Table 1. Applications of citric acid

Industry	Applications
Beverages	Provides tartness and complements fruits and berries flavors. Increases the effectiveness of antimicrobial preservatives. Used in pH adjustment to provide uniform acidity.
Jellies, Jams and Preserves	Provides tartness. pH adjustment.
Candy	Provides tartness. Minimizes sucrose inversion. Produces dark color in hard candies. Acts as acidulant.
Frozen fruit	Lowers pH to inactivate oxidative enzymes. Protects ascorbic acid by inactivating trace metals
Dairy products	As emulsifier in ice creams and processed cheese; acidifying agent in many cheese products and as an antioxidant.
Fats and oils	Synergist for other antioxidants, as sequestrant.
Pharmaceuticals	As effervescent in powders and tablets in combination with bicarbonates. Provides rapid dissolution of active ingredients. Acidulant in mild astringent formulation. Anticoagulant.
Cosmetics and toiletries	pH adjustment, antioxidant as a metallic–ion chelator, buffering agent.
Industrial applications	Sequestrant of metal ions, neutralizant, buffer agent
Metal cleaning	Removes metal oxides from surface of ferrous and nonferrous metals, for preperational and operational cleaning of iron and copper oxides
Others	In electroplating, copper plating, metal cleaning, leather tanning, printing inks, bottle washing compounds, floor cement, textiles, photographic reagents, concrete, plaster, refractories and moulds, adhesives, paper, polymers, tobacco, waste treatment, etc.

MICRO-ORGANISMS USED FOR CITRIC ACIC PRODUCTION

A large number of micro-organisms including bacteria, fungi and yeasts have been employed to produce citric acid. 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. Kubicek and Rohr (1986) reviewed the strains reported to produce citric acid. Table 2 shows the micro-organisms used to produce citric acid. Among these, only *A. niger* and certain yeasts such as *Saccharomycopsis* sp. are employed for commercial production. However, the fungus *A. niger* has remained the organism of choice for commercial production. The main advantages of using this micro-organism are: (a) its ease of handling, (b) its ability to ferment a variety of cheap raw materials, and (c) high yields.

Table 2. Micro-organisms employed for citric acid production

Micro-organisms	References
Fungi	
Aspergillus niger	Hang & Woodams 1984, 1985, 1987, Roukas 1991, Garg & Hang 1995, Lu et al. 1997, Pintado et al. 1998, Vandenberghe et al., 1999b, c
A. aculeatus	El Dein & Emaish, 1979
A. awamori	Grewal & Kalra, 1995
A. carbonarius	El Dein & Emaish, 1979
A. wentii	Karow & Waksman, 1947
A. foetidus	Chen, 1994; Tran et al., 1998
Penicillium janthinelum	Grewal & Kalra, 1995
Yeasts	
Saccahromicopsis lipolytica	Ikeno et al., 1975; Maddox et al., 1985; Kautola et al., 1992 Wojtatowicz et al., 1993; Rane & Sims, 1993
Candida tropicalis	Kapelli et al., 1978
C. oleophila	Ishi et al., 1972
C. guilliermondii	Miall & Parker, 1975; Gutierrez et al., 1993
C. parapsilosis	Omar & Rehm, 1980
C. citroformans	Uchio et al., 1975
Hansenula anamola	Oh et al., 1973
Bacteria	
Bacillus licheniformis	Sardinas, 1972
Arthrobacter paraffinens	Kroya Fermentation Industry, 1970
Corynebacterium sp.	Fukuda et al., 1970

Strains selection and improvement

The two principal methods of selecting populations, namely, "the single-spore technique" and the "passage method" have been used for selecting citric acid producing micro-organisms The single-spore technique has the disadvantage that mineral acid or organic acids (gluconic acid, oxalic acid) simulate the presence of citric acid. Rohr et al. (1979) improved this method by incorporating a specific stain for citric acid (para-di-methylamino benzaldehyde), instead of using the indicator.

The most employed technique to improve citric acid producing strains has been by inducing mutations in parental strains using mutagens. Among physical mutagens, g-radiation (Bonatelli and Azevedo, 1983 ; Gunde-Cimerman, 1986 ; Islam et al., 1986) and UV-radiation (Pelechova et al., 1990) have often used. To obtain hyper-producer strains, frequently UV treatment could be combined with some chemical mutagens, e.g. aziridine, N-nitroso-N-methylurea or ethyl methane-sulfonate (Musilkova et al., 1983). By using a suitable selection technique on model medium with non-specific carbon sources, a strain yielding high amounts of citric acid from unusual substrates can be obtained from the mutants produced.

Another approach for strain improvement has been the para-sexual cycle, as first described by Pontecorvo et al. (1953). According to Das and Roy (1978), diploids displayed higher citric acid yields

compared to their parent haploids, but tended to be less stable (Bonatelli and Azevedo, 1983). Protoplast fusion appeared to be a promising tool to extend the range of genetic manipulation of *A. niger* with respect to citric acid production. Kirimura et al. (1988a) studied protoplast fusion of production strains. They were able to obtain fusants with acid production capacities exceeding those of the parent strains in solid state fermentation, but not in submerged fermentation. Some other aspects of strain improvement could be the resistance to detrimental constituents of fermentation raw materials, capability of utilizing raw materials (starch, cellulose, pectin containing and other waste materials). However, there is no single effective technique to achieve hyper-producing mutants and much remains to be done in this area.

PRODUCTION TECHNIQUES AND RAW MATERIALS

Although citric acid is mostly produced from starch or sucrose based media using liquid fermentation, a variety of raw materials such as molasses, several starchy materials and hydrocarbons have also been employed. Rohr et al. (1983) classified raw materials used for citric acid production 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).

Several attempts have been made to produce citric acid using molasses, which is preferred due its low cost and high sugar content (40-55%). The composition of molasses depends on various factors, e.g. the kind of beet and cane, methods of cultivation of crops and fertilizers and pesticides applied during cultivation, conditions of storage and handling (e.g. transport, temperature variations), production procedures, etc. Both, cane and beet molasses are suitable for citric acid production. However, beet molasses is preferred due to its lower content of trace metals. Generally, cane molasses contains calcium, magnesium, manganese, iron and zinc, which have a retarding effect on the synthesis of citric acid. Consequently, some pre-treatment is required for the removal/reduction of trace metals. Despite that, cane molasses posses difficulties in achieving good fermentation yields.

Various other agro-industrial residues such as apple pomace, cassava bagasse, coffee husk, wheat straw, pineapple waste, sugar beet cosset, kiwi fruit peel, etc. have been investigated with solid state fermentation techniques for their potential to be used as substrates for citric acid production (Pandey and Soccol, 1998, Pandey et al. 1999, Vandenberghe et al., 1999a,b, c). In fact, these residues are very well adapted to solid-state cultures due to their cellulosic and starchy nature. However, despite the fact that these solid residues provide rich nutrients to the micro-organisms, and are good substrates for growth and activity of micro-organisms, much remains to be done for developing commercially feasible process utilizing these residues (Pandey 1992, 1994, Pandey and Soccol 1998).

Liquid fermentation

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. Several advantages such as higher yields and productivity and lower labour costs are the main reasons for this. Two types of fermenters, conventional stirred fermenters and tower fermenters are employed, although the latter is preferred due to the advantages it offers on price, size and operation (Rohr et al., 1983). Preferentially, fermenters are made of high-grade steel and require provision of aeration system, which can maintain a high dissolved oxygen level. Fermenters for citric acid production do not have to be built as pressure vessels since sterilization is performed by simply steaming without applying pressure. Cooling can be done by an external water film over the entire outside wall of the fermenter.

In SmF, different kinds of media are employed such as sugar and starch based media (Table 3). Molasses and other raw materials demand pre-treatment, addition of nutrients and sterilization. Inoculation is performed either by adding a suspension of spores, or of pre-cultivated mycelia. When spores are used, a surfactant is added in order to disperse them in the medium. For pre-cultivated mycelia, an inoculum size of 10% of fresh medium is generally required. Normally, submerged fermentation is concluded in 5 to 10 days depending on the process conditions. It can be carried out in batch, continuous or fed batch systems, although the batch mode more frequently used.

Table 3. Raw materials employed in submerged fermentation for citric acid production

Raw material	Strain	Citric acid	Yield, %	References
Brewery wastes	*A. niger* ATTC 9142	19 g/L	78.5	Roukas & Kotzekidou, 1986
Beet molasses	*A.niger* ATTC 9142	109 g/L	-	Ogawa & Fazeli, 1976
	Yarrow lipolytica A101	54 g/L	68.7 [a]	Kautola et al., 1992
Cane molasses	*A. niger* T 55	-	65	Kundu et al, 1984
Wood Hemicellulose	*A. niger* IMI- 41874	27 g/L	45 [a]	Maddox et al., 1985
	S. lipolytica IFO 1658	9 g/L	41	Maddox et al., 1985
Date syrup	*A. niger* ATTC 9142	-	50	Roukas & Kotzekidou, 1997
Corn starch	*A. niger* IM-155	-	62	Nguyen et al., 1992
Starch hydrolysate	*Y. lipolytica* DS-1	-	-	Shah et al., 1993
	Y. lipolytica A-101	-	75	Wojtatowicz et al., 1993
Rapeseed oil	*Y. lipolytica* A-101	-	57	Wojtatowicz et al., 1993
Soybean oil	*Y. lipolytica* A-101	-	63	Wojtatowicz et al., 1993
Coconut oil	*C.lipolytica* N-5704	-	99.6 [b]	Ikeno et al., 1975
Palm oil	*C.lipolytica* N-5704	-	155 [b]	Ikeno et al., 1975
Olive oil	*C.lipolytica* N-5704	-	119 [b]	Ikeno et al., 1975
Soybean oil	*C.lipolytica* N-5704	-	115 [b]	Ikeno et al., 1975
Glycerol	*C.lipolytica* N-5704	-	58.8 [b]	Ikeno et al., 1975
n-Paraffin	*C.lipolytica* N-5704	-	161 [b]	Ikeno et al., 1975

[a] *based on sugar consumed;* [b] *based on oils and fatty acids*

Surface fermentation: The first individual process for citric acid production was the liquid surface culture (LSC), which was introduced in 1919 by Société des Produits Organiques in Belgium, and in 1923 by Chas Pfizer & Co. in US. After that, other methods of fermentation, such as submerged fermentation

were developed. Although this technique is more sophisticated, surface method required less effort in operation and installation and energy cost (Grewal and Kalra, 1995).

In the classical process for citric acid manu-facture, the culture solution is held in shallow trays (capacity of 50-100 L) and the fungus develops as a mycelial mat on the surface of the medium. The trays are made of high purity aluminium or special grade steel and are mounted one over another in stable racks. The fermentation chambers are provided with an effective air circulation in order to control temperature and humidity. Fermentation chambers are always in aseptic conditions, which might be conserved principally during the first two days when spores germinate. Frequent contamination are mainly caused by Penicilia, other Aspergilli, yeast and lactic bacteria (Rohr et al, 1983; Morgant, 1988). Refined or crude sucrose, cane syrup or beet molasses are generally used as sources of carbon. When applied, molasses is diluted to 15-20% and is treated with hexacyanoferrate (HFC).

Solid-state fermentation

Solid-state fermentation (SSF) has been termed as an alternative method to produce citric acid from agro-industrial residues (Pandey 1991, 1992, 1994, Soccol 1994, Pandey and Soccol 1998). Citric acid production by SSF (the Koji process) was first developed in Japan and is as the simplest method for its production. SSF can be carried out using several raw materials (Table 4). Generally, the substrate is moistened to about 70% moisture depending on the substrate absorption capacity. The initial pH is normally adjusted to 4.5-6.0 and the temperature of incubation can vary from 28 to 30°C. The most commonly organism is *A. niger.* However there also have been reports with yeasts (Maddox and Kingston, 1983; Tisnadjaja et al., 1996). One of the important advantages of SSF process is that the presence of trace elements may not affect citric acid production so harmfully as it does in SmF. Consequently, substrate pre-treatment is not required.

Table 4. Raw materials employed in solid state fermentation for citric acid production.

Raw material	Strain	Citric acid	Yield(%)	Reference
Apple pomace	*A.niger* NRRL2001	766 g/kg [a]		Hang & Woodams, 1984
	NRRL 2270	816 g/kg [a]		
	NRRL 599	771 g/kg [a]		
	NRRL 328	798 g/kg [a]		
	NRRL 567	883 g/kg [a]		
Grape pomace	*A.niger* NRRL2001	413 g/kg [a]	88	Hang & Woodams, 1985
	NRRL 2270	511 g/kg [a]		
	NRRL 599	498 g/kg [a]		
	NRRL 328	523 g/kg [a]		
	NRRL 567	600 g/kg [a]		
Kiwifruit peel	*A .niger* NRRL 567	100 g/kg [a]		Hang & Woodams, 1987
Cellulose hydrolysate and Sugar cane	*A. niger*	29 g/kg	44	Mannomani & Sreekantiah, 1987
Orange waste	*A. niger*	46 g/kg		Aravantinos-Zafiris et al., 1994
Beet molasses (Ca-alginate gel)	*A.niger* ATCC 9142	35 g/L		Roukas, 1991
Saccharose (Sugar cane bagasse)	*A. niger* CFTRI 30	174 g/kg [b]		Shankaranand & Lonsane, 1993
Coffee husk	*A. niger* CFTRI 30	150 g/kg [b]		Shankaranand & Lonsane, 1994
Carrot waste	*A.niger* NRRL 2270	29 g/kg [b]	36	Garg & Hang, 1995
Okara (soy residue)	*A. niger*	96 g/kg [b]		Khare et al., 1995
Pineapple waste	*A.niger* ATCC 1015	132 g/kg [b]		Lima et al., 1995
	A.niger ACM 4942	194 g/kg [b]	74	Tran et al., 1998
Glucose (Sugar cane bagasse)	*A.niger* CBS733.88	21.24 g/L		Pallares et al., 1996
Kumara (starch containing)	*A. niger* Yang no 2	103 g/kg [b]		Lu et al., 1997
Mussel processing wastes (polyurethane foams)	*A. niger*	300 g/kg		Pintado et al., 1998
Cassava bagasse	*A. niger* LPB-21	347 g/kg [b]	67	Vandenberghe et al., 1999c

[a] *based on sugar consumed;* [b] *based on dry matter*

Different types of fermenters such as conical flasks, glass incubators and trays, etc. have been used for citric acid fermentation in SSF. Vandenberghe et al. (1999a,b) used Erlen-meyer flasks and glass columns for the production of citric acid from gelatinized cassava bagasse. Higher yields were obtained in flasks without any aeration, and very little sporulation was observed. The same yields were found in column reactors only with variable aeration. This showed great perspective to use SSF process for citric acid production in simple tray type fermenters.

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. Hossain et al. (1984) showed that sucrose was the most favourable carbon source followed by glucose, fructose and galactose. Galactose contributed to a very low growth of fungi and did not favour citric acid accumulation.Other sources of carbon such as sorbose, ethanol, cellulose, manitol, lactic, malic and a -acetoglutaric acid, allow a limited growth and low production. Starch, pentoses (xyloses and

arabinoses), sorbitol and pyruvic acid slow down growth, though the production is minimal (Yokoya, 1992).

According to Kovats (1960), initial sugar concentration was critical for citric acid production and other organic acids produced by *A. niger*. Xu et al. (1989) reported that *A.niger* strains needed an initial sugar concentration of 10-14% as optimal; no citric acid was produced at sugar concentration of less than 2.5%. Honecker et al. (1989) showed that immobilized cells of *A.niger* needed lower concentrations of sucrose than free cells culture, in order to obtain high yields (200 g of citric acid/L for free cells culture, and 120 g/L for immobilized cells). Maddox et al. (1985) reported the influence of different sources of carbon on citric acid production by *A. niger* and *Saccharomycopsis lipolytica*. Glucose, maltose, galactose, xylose and arabinose were tested. Fermentation was carried out in 8 and 4 days, respectively, at 30°C and 180 rpm. Better results were found for *A. niger* with 0.45 g of citric acid/ g of glucose corresponding to 27 g/L. *S. lipolytica* produced 0.41 g/g of glucose or 9 g/L which was not so bad.

As presented previously, several raw materials can be employed successfully for citric acid production. There are some critical factors (costs, need of pretreatment), which should be considered for substrate determination. One another aspect is the presence of trace elements, which can act as inhibitors or stimulants. Consequently, sometimes it is necessary to conduce a pre-treatment, e.g.; precipitation of trace metals of molasses by potassium ferrocyanide.

Nitrogen source: Citric acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g. urea, ammonium sulfate, ammonium chlorure, peptone, malt extract, etc. Nitrogen consumption leads to pH decrease, which is very important point in citric acid fermentation (Rohr et al., 1983, Kubicek and Rohr, 1986). However, it is necessary to maintain pH values in the first day of fermentation prior to a certain quantity biomass production. Urea has a tampon effect, which assures pH control (Raimbault, 1980). The concentration of nitrogen source required for citric acid fermentation is 0.1 to 0.4 N /liter. A high nitrogen concentration increases fungal growth and the consumption of sugars, but decreases the amount of citric acid produced (Hang et al., 1977).

Phosphorous source: Presence of phosphate in the medium has a great effect on the yield of citric acid. Potassium dihydrogen phosphate has been reported to be the most suitable phosphorous source. Shu and Johnson (1948) reported that phosphorous at concentration of 0.5 to 5.0 g/L was required by the fungus in a chemically defined medium for maximum production of citric acid. Phosphate is known to be essential for the growth and metabolism of *A. niger* (Shankaranand and Lonsane, 1994). 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 (Kubicek et al., 1979). It is interesting to note that 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, the study of their combined effect is necessary (Pintado et al., 1993; Chen, 1994). Pintado et al. (1998) reported how the culturing modality conditions the behavior of the micro-organisms referring to the tendencies of production as a function of the levels of N and P. The author used as first order an empirical model based on rotatable

design to study the effect of both nutrients. As expected, for the two studied strains, a similar behavior was noticed, showing an improvement towards low levels of N and P in submerged culture, and toward high levels in solid state culture, and with superior productions for the last one. Shankaranand and Lonsane (1994) affirmed that the specificity of solid state culture is largely due to a lower diffusion rate of nutrients and metabolites, which occurs in low water activity conditions. Consequently, strains with large requirements of N and P seems to be disfavored, due to the restriction of accessibility to the nutrients in the medium.

Trace elements: Trace element nutrition is probably the main factor influencing the yield of citric acid. 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, probably, 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. Shankaranand and Lonsane (1994) noticed that there were few differences in the response of *A. niger* to metal ions and minerals in SSF and in SmF systems. 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 (Kubicek and Rohr, 1978). A low level of manganese (ppm) 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. Benuzzi and Segovia (1996) reported that the presence of different copper concentrations in the pellet formation medium was very important in order to enhance a suitable structure, related to cellular physiology, for citric acid production. The optimal initial $CuSO_4.5H_2O$ concentration was 78 mg/L.

Magnesium is required both for growth as well as for citric acid production. Optimal concentration of magnesium sulfate was found in the range of 0.02-0.025% (Kapoor et al., 1983).

Lower alcohols: Addition of lower alcohols enhances citric acid production from commercial glucose and other crude carbohydrate. Appropriate alcohols are methanol, ethanol, iso-propanol 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%. The effect of methanol or ethanol have been extensively studied by many authors (Hamissa, 1978; Mannomani and Sreekantiah, 1988; Georgieva et al., 1992; Dasgupta et al., 1994).

Mannomani and Sreekantiah (1987) 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. They also found that coconut oil influenced citric acid production in a sucrose medium when added at 3% (v/w). Alcohols have been shown to principally act on membrane

permeability in micro-organisms by affecting phospholipid composition on the cytoplasmatic membrane (Orthofer et al., 1979). However Meixner et al. (1985) argued against a role of membrane permeability in citric acid accumulation. Ingram and Buttke (1984) found that 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.

Miscellaneous: 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, potassium ferrocyanide has 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-Methyl-umbelliferone, 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 culture may change in response to 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 can drop very quickly until less than 3.0. For other groups of fungi such as *Trichoderma*, *Sporotrichum*, *Pleurotus* sp., pH is more stable (between 4 and 5). Besides, the nature of the substrate also influences pH kinetics (Raimbault et al., 1997).

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 (Srivastava and De, 1980) whereas, a higher pH i.e. 5.4 and 6.0-6.5 has been found optimum for citric acid production in molasses medium (Roukosu and Anenih, 1980).

Aeration: Aeration has been shown to have a determinant effect on citric acid fermentation (Rohr et al., 1983; Dawson et al., 1986). Increased aeration rates led to enhanced yields and reduced fermentation time (Grewal and Kalra, 1995).

The influence of dissolved oxygen concentration on citric acid formation has been examined. It is important to maintain the oxygen concentration above 25% saturation and interruptions in oxygen supply may be quite harmful (Kubicek et al., 1980). 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 (Kubicek and Rohr, 1986). 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%. Aeration is performed during the whole fermentation with the same intensity through the medium at a rate of 0.5 to 1.5 vvm. However, because of economic reasons, it's usually preferred to start with a low aeration rate (0.1 to 0.4 vvm). 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.

PRODUCT RECOVERY

The recovery of citric acid from liquid fermentation is generally accomplished by three basic procedures, precipitation, extraction, and adsorption and absorption (mainly using ion exchange resins). Citric acid extraction has been described by the Food and Drug Administration (1975) of the United States and by Colin (1960,1962). Citric acid extracted by this method has been recommended suitable for use in food and drugs. 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. The precipitated tri-calcium citrate is removed by filtration and washed several times with water. It is then treated with sulphuric acid forming calcium sulphate, which is filtered off. Mother liquor containing citric acid is treated with active carbon and passed through cation and anion exchangers. Several anion-exchange resins are commercially available. Finally, the liquor is concentrated in vacuum crystallizers at 20-25°C, forming citric acid monohydrate. Crystalization at temperatures higher to this is used to prepare anhydrous citric acid.

CONCLUSIONS AND PERSPECTIVES

Since the beginning of this century, citric acid production has been intensively studied and great alternatives to this process have been found to follow its great demand. The use of alternative raw materials to produce citric acid by SmF, LSC, and SSF seems to be a suitable possibility. However, it is necessary to adapt the right type of raw material to the right technique e.g. cassava bagasse employed as substrate in SSF, or cellulose hydrolysate used in SmF. The need of some pre-treatment of raw materials may enhance the fermentation efficiency. One area, which needs attention is the development of continuous culture techniques which have been attempted but only at the laboratory scale. Another area is the strain improvement with improved substrate utilization efficiency.