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Microbial Production of Citric Acid and other Organic Acids

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Abstract

The first individual process for citric acid production was the liquid surface culture which was introduced in 1919 in Belgium, and in 1923 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. The main aim of this review is to detail the microbial community during this fermentation, raw materials, recovery methods and type of reactor they use. Organic acids such as citric, malic and pyruvic are used extensively as acidulants in the food industry. Inulin, a fructose polymer found as a carbohydrate reserve in plants, was investigated as a possible substrate for organic acid production. Aeration was found to have a significant effect on acid production. Submerged fermentation of inulin produced a higher yield of citric acid than surface fermentation with optimum acid production occurring at a concentration of 14%. An investigation into the enzymes produced during citric acid fermentation by *Aspergillus niger* revealed that both extracellular and cell lysate invertase and inulinase were produced during fermentation. Citric acid cannot be recovered directly from the fermented liquor by crystallization because of excess of impurities. 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, and the entire production is carried out by fermentation.

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Introduction

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 and 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 (Max *et al.*, 2010; Vandenberghe *et al.*, 1983). Organic acids such as citric, malic and pyruvic are used extensively as acidulants in the food industry. Inulin, a fructose

polymer found as a carbohydrate reserve in plants, was investigated as a possible substrate for organic acid production. *Aspergillus niger* produced citric acid on sucrose and inulin during surface fermentation (Drysedale, 2018; Vandenberghe *et al.*, 1983). Aeration was found to have a significant effect on acid production. Submerged fermentation of inulin produced a higher yield of citric acid than surface fermentation with optimum acid production occurring at a concentration of 14%. An investigation into the enzymes produced during citric acid fermentation by *Aspergillus niger* revealed that both extracellular and cell lysate invertase and inulinase were produced during fermentation (Rogers *et*

al., 2006). The yeast-like fungus *Aureobasidium pullulans* was shown to produce poly- α -L-malic during submerged fermentation of sucrose, glucose, galactose, maltose, inulin, xylose, fructose, and an equimolar mixture of glucose and galactose. The polymer was characterised by hydrolysis and analysis of constituent acids. Screening of 107 yeast strains identified pyruvic acid production by several strains of *Kluyveromyces marxianus* when grown on sucrose, fructose, maltose, glucose, inulin and an equimolar mixture of glucose and galactose (Drysdale, 2018; Feng *et al.*, 2018). 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 (Show *et al.*, 2015). 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 (Vandenberghe *et al.*, 1999).

Substrates used for citric acid production

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. (Mischak *et al.*, 1984) 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) (Vandenberghe *et al.*, 1983).

Responsible microorganisms for organic acid production

Microbial production of organic acids has become a fast-moving field due to the increasing role of these compounds as platform chemicals. In recent years, the portfolio of specialty fermentation-derived carboxylic acids has increased considerably, including the production of glyceric, glucaric, succinic, butyric, xylonic, fumaric, malic, itaconic, lactobionic, propionic and adipic acid through innovative fermentation strategies (Alonso *et al.*, 2015). This review summarizes recent trends in the use of novel microbial platforms as well as renewable and waste materials for efficient and

cost-effective bio-based production of emerging high-value organic acids. Advances in the development of robust and efficient microbial bioprocesses for producing carboxylic acids from low-cost feedstocks are also discussed. The industrial market scenario is also reviewed, including the latest information on the stage of development for producing these emerging bio-products via large-scale fermentation (Alonso *et al.*, 2015).

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 (Vandenberghe *et al.*, 1983).

Although mainly acid production process, other strains of fungi apart *A. niger* *A. niger* has been used in the citric from, various kinds of yeast and some bacteria are known to accumulate citric acid in the medium (32). The reason for choosing citrate producing organism are: cheap raw materials *A. niger* over other potential (molasses) used as substrate; high consistent yields (Yigitoglu, 1992).

Reactor type (fermentor and type of 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. 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 (Krishna, 2005; Vandenberghe *et al.*, 1983). 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 (Vandenberghe *et al.*, 1983) (Table 1).

The submerged process is the most commonly used either in stirred tank reactors or tower fermentors. The process is run in batch. Fermentors are equipped with aeration systems capable of maintaining high dissolved oxygen levels, which is critical for high citric acid production (Sanchez-Riera, 2010).

In SmF, different kinds of media are employed such as sugar and starch based media. 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 (Vandenberghe *et al.*, 1983).

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 and 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 (Vandenberghe *et al.*, 1983). In the classical process for citric acid manufacture, 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 (Max *et al.*, 2010). 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 is mainly caused by *Penicilia*, other *Aspergilli*, yeast and lactic bacteria. 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) (Ranjan, 2012; Vandenberghe *et al.*, 1983).

Solid-state fermentation

Solid-state fermentation (SSF) has been termed as an alternative method to produce citric acid from agro-industrial residues. 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. One of the important advantages of SSF process is that the presence of trace elements may not affect citric acid production as harmfully as it does in SmF. Consequently, substrate pre-treatment is not required. 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.*, 1999) used Erlenmeyer 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 (Vandenberghe *et al.*, 1999).

Appropriate product recovery techniques (downstream process)

Citric acid cannot be recovered directly from the fermented liquor by crystallization because of excess of impurities. In the classic protocol, citric acid is precipitated as calcium citrate by the addition of lime. The washed precipitate is treated in aqueous suspension with H₂SO₄, yielding gypsum as a by-product. The citric acid solution is concentrated by vacuum evaporation and crystallized at low temperatures (Sanchez-Riera, 2010).

Solvent extraction can also be applied with the advantage of avoiding the formation of gypsum. Different solvents can be used; the extraction step is usually carried out at low temperature and the solvent is stripped with hot

water. A mixture of n-octyl alcohol and tridodecylamine has been recommended for citric acid used in food and drug applications (Sanchez-Riera, 2010; Kumar *et al.*, 2017).

Table.1 Raw materials employed in submerged fermentation for citric acid production(Vandenberghe *et al.*, 1983)

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

^a based on sugar consumed; ^b based on oils and fatty acids

Table.2 Micro-organisms employed for citric acid production(Vandenberghe *et al.*, 1983)

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

Table.3 Raw materials employed in submerged fermentation for citric acid production (Vandenberghe *et al.*, 1983)

Raw material	Strain	Citric acid	Yield, %	References
Brewery wastes	<i>A. niger</i> ATTC 9142	19 g/L	78.5	Roukas & Kotzekidou, 1986
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	<i>Y. lipolytica</i> A-101	-	75	Wojtatowicz <i>et al.</i> , 1993
Rapeseed oil	<i>Y. lipolytica</i> A-101	-	57	Wojtatowicz <i>et al.</i> , 1993
Soybean oil	<i>Y. lipolytica</i> A-101	-	63	Wojtatowicz <i>et al.</i> , 1993
Coconut oil	<i>C.lipolytica</i> N-5704	-	99.6 ^b	Ikeno <i>et al.</i> , 1975
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Olive oil	<i>C.lipolytica</i> N-5704	-	119 ^b	Ikeno <i>et al.</i> , 1975
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Glycerol	<i>C.lipolytica</i> N-5704	-	58.8 ^b	Ikeno <i>et al.</i> , 1975
n-Paraffin	<i>C.lipolytica</i> N-5704	-	161 ^b	Ikeno <i>et al.</i> , 1975

^a based on sugar consumed; ^b based on oils and fatty acids

Table.4 Raw materials employed in solid state fermentation for citric acid production (Vandenberghe *et al.*, 1983)

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

^a based on sugar consumed; ^b based on dry matter

Table.5 Applications of citric acid (Vandenberghe *et al.*, 1983)

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.

Some other recovery techniques have been studied and patented. They include the combination of membrane filtration with adsorption resins, the use of different anionic exchange resins and the application of electrodialysis (Sanchez-Riera, 2010).

Current market share of the product worldwide

Citric acid (C₆H₈O₇, 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 (Vandenberghe *et al.*, 1983).

Citric acid is a 6-carbon containing tricarboxylic acid which was first isolated from lemon juice. It is a natural

component of many citrus fruits, and was crystallized from lemon juice by Scheele in 1874. Approximately 70% of citric acid produced is used in the food and beverage industry for various purposes, 12% in pharmaceuticals and about 18% for other industrial uses. The estimated world production of citric acid was reported as 350.000 tons/year in 1986. It however was recently reported that the world market requirement of citric acid is around 500.000 tons/year (Yigitoglu, 1992).

Citric acid is mainly used in food industry because of its pleasant acid taste and 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 5 presents main applications of citric acid (Swain *et al.*, 2012; Vandenberghe *et al.*, 1983).

In conclusion, microbial production of organic acid is a promising technology to meet the demand of the World. Production of those organic acids may not be costly because it can use wastes as raw materials during the production. Therefore, science society should give

concerns to this technology since it has no side effect on the environment.

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