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FUNGAL CITRIC ACID PRODUCTION USING WASTE MATERIALS: A MINI-REVIEW

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ABSTRACT

Citric acid is one of the extensively used organic acids in many industries. There is a worldwide demand for citric acid consumption due to their many industrial applications. It is also considered as a Generally Recognized As Safe (GRAS) compound. In this review, current developments in microbial fermentation processes for citric acid production have been discussed. In food, beverages, milk and sugar processing several biodegradable organic waste materials are generated in large quantities. These include sugarcane bagasse, grape pomace, apple pomace, pineapple pomace, vegetables, tapioca, coconut husk, banana peels, citrus peels, whey and decaying fruits are found to be potential substrates for citric acid production. A significant effect of substrate concentration, inorganic salts, initial sugar concentration, moisture and additives etc on citric acid production have been highlighted for further improvement in fermentation process. Recent developments in upstream and downstream processes for citric acid production are also deeply discussed. This review gives insights for future possibilities of cost effective fermentation process for citric acid production from several cheap raw materials. Formation of citric acid as an industrial byproduct will help to tackle waste disposal issue and also reduce the dependency of industry over other citric acid producers. Thus, the industry would be benefitted ecologically and economically.

Keywords: Citric acid, GRAS, Fermentation, Substrates, Waste, Fungi

INTRODUCTION

Citric acid (2-hydroxy propane 1, 2, 3-tricarboxylic acid) is one of the most important natural organic acids. Its name is derived from the Latin word 'Citrus' and obtained naturally by metabolic pathways which are performed in living cells via tri-carboxylic acid cycle (**Swain *et al.*, 2012**). Citric acid is one of the natural organic acids known to be non-toxic and pleasant with sour taste. It is also considered as a Generally Recognized As Safe (GRAS) compound. It is a naturally occurring compound in citrus fruits like oranges, berries, limes, lemons, tangerines and grapes. Citric acid is commonly used as a preservative, acidulant, antioxidant, emulsifier and buffer in food industry. For these reasons, citric acid is continuously used as a common ingredient in variety of food products. Thus, all over the world such beneficial compound is of a high demand for their daily consumptions (**Soccol *et al.*, 2006; Radwan *et al.*, 2010; Vasanthabharathi *et al.*, 2013**). Citric acid has several applications in food, chemical and pharmaceutical industries. It is reported that 70% of citric acid produced by industries is used in food industries only and the remaining 30% is used in the chemical, pharmaceutical, medical and other industries (**Soccol *et al.*, 2003**). Food industry is the largest consumer of citric acid due to their several advantageous properties (**El-Hussein *et al.*, 2009; Yalcin *et al.*, 2010**). Citric acid occupies a key position in the global market due to their heavy usage in many industries (**Ali *et al.*, 2016**).

Production of citric acid was carried out by several physical and chemical methods. However, such conventional methods are found to be an expensive, complex and not eco-friendly (**Gupta *et al.*, 2015; Yin *et al.*, 2017; Yu *et al.*, 2018**). Moreover, annual production rate of citric acid is low while the rate of demand is high. Hence there is a need to obtain continuous production of citric acid to fulfil the market demand. Production of citric acid by using microorganisms is much more feasible when compared with plant and animal sources. There are several microorganisms with the ability to produce citric acid such as *Bacillus subtilis*, *B. licheniformis*, *Corynebacterium* spp., *Aspergillus niger*, *A. flavus*, *Mucor piriformis*, *Trichoderma viride*, *Penicillium janthinellum*, *Candida tropicalis*, *C. lipolytica* and *C. intermedia* etc (**Kapoor *et al.*, 1983; Papagianni, 2007**). Literature survey has revealed that *A. niger* is found to be a potential producer of citric acid (**Selvankumar *et al.*, 2014; Singh *et al.*, 2016; Alnassar *et al.*, 2016**). Commercial production of citric acid has been mainly carried out by microbial fermentation process, with increasing production by improving the environmental parameters and genetic manipulation of microorganisms which are used (**Ali *et al.*, 2016**). Microorganisms have the ability to utilize cheap raw waste materials and convert them into value added products such as organic acid (**Singh *et al.*, 2016**). Microbial fermentation may be carried out by using three main methods: 1) Submerged fermentation 2) Surface fermentation and 3) Solid substrate fermentation (**Gupta *et al.*, 2015**). Solid surface fermentation is found to be a better alternative process to conventional submerged fermentation. In this process, microorganisms utilize cheap waste materials as substrates. The waste materials generated in food industries are used as substrates by microorganisms for citric acid production. In food, beverages, milk and sugar processing several biodegradable organic waste materials are generated in large quantities. These include sugarcane bagasse, grape pomace, apple pomace, pineapple pomace, vegetables, tapioca, coconut husk, banana peels, citrus peels, whey and decaying fruits that can be used as fodder and feedstock for biogas production (**Hang and Woodams, 1984; Hang, 1998; Murad *et al.*, 2003; Kumar *et al.*, 2003; Hamdy, 2013; Kareem and Rehman, 2011**). Such nutrient rich and cheap wastes can be utilized as substrates by microorganisms for the production of citric acid as value-added product. In this review, overview of bioconversion of different wastes into citric acid by microorganisms is highlighted.

HISTORY OF CITRIC ACID PRODUCTION

In 1784, citric acid was extracted from lemon fruits for the first time by Karls Scheels in England. The lemon juice was treated with calcium hydroxide to form calcium citrate. It was treated with sulfuric acid to obtain citric acid. The industrial production of citric acid was started in 1890 and the industrial process was used until 1919. At that time, citric acid was sold at high cost due to Italian monopoly. In 1917, James Currie was the first to observe that some strains of *A. niger* have the ability to produce citric acid and can be used for commercial purpose. He investigated that these strains were able to grow well in acidic

environment (pH 2.5-3.5) containing high sugar concentration. The environmental conditions aforementioned were found to stimulate the citric acid production (**Kristiansen and Sinclair, 1979**). Around 1929, Pfizer started commercial production of citric acid with surface fermentation techniques. In 1948, technologies got developed where molasses was used as a cheap raw substrate instead of sucrose for citric acid production. Around late 60's, n-alkanes were effectively used as substrate by many bacteria and yeasts for citric acid production (**Anastassiadis *et al.*, 2008**). All over the world, about 80% of citric acid production was performed by submerged fermentation process (**Yalcin *et al.*, 2010**). Citric acid production by submerged fermentation was strongly influenced by several physico-chemical parameters such as aeration, pH, temperature, trace elements, morphology of microorganisms, initial carbon and nitrogen concentrations (**Dhulappa, 2016**).

Currently, molasses is used as substrate for production of citric acid. In this process, there is a higher chance of high level of cations contamination and the needs continuous control on process parameters. These cations are usually originated from insoluble residues formed by precipitation with potassium ferrocyanide. Therefore, due to the complexity of such pretreatment process results into reduction of citric acid production. Thus, the search continues for new potential alternatives. Solid substrate fermentation (SSF) is one of the methods that can be used for industrial fermentation. There are several advantages of SSF such as high productivities, low production costs, low water consuming, extended stability of products and eco-friendly etc (**Hölker and Lenz, 2005**). The citric acid is a commercially valuable product. The worldwide demand for citric acid is around 3 million tons. The annual production of citric acid was reported as 700 thousand tonnes in 1993, 1.4 million tonnes in 2004, 1.6 million tonnes in 2008 and 1.8 million tonnes in 2010 (**Yalcin *et al.*, 2010; Addo *et al.*, 2016**). The consumption of citric acid was 75, 10 and 15 % by food industries, pharmaceuticals and other industries, respectively (**Ali *et al.*, 2016**). United States was the largest producer of citric acid in the world. Chinese production volume of citric acid has surpassed the production in United States from 1995 due to the introduction of cheap raw materials as substrate for citric acid production that reduced their cost. All over the world, the production rate of citric acid has been increasing at the rate of 3.5 - 4 % per annum (**Amenaghawon *et al.*, 2015**) due to their several applications (figure 1).

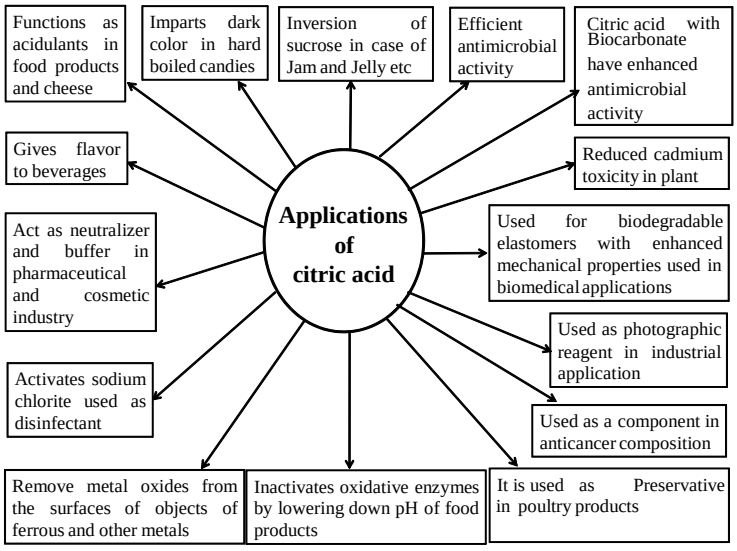

Figure 1 Different applications of citric acid (**Zimmermann and Wausau, 1940; Leveskis, 1979; Soccol *et al.*, 2006; Anastassiadis *et al.*, 2008;Glevitzky *et al.*, 2009; EI-Sheikh *et al.*, 2010; Javanmard and Endan, 2010; Swain *et al.*, 2012; Ioannou and Ghoul, 2013; Brima and Abbas, 2014; Singh *et al.*, 2016**).

STRAIN IMPROVEMENT PROGRAM FOR CITRIC ACID PRODUCTION

Different types of yeasts, molds and bacteria are being used in industry for commercial production of citric acid including *C. tropicalis, C. oleophila, C. guilliermondii, C. citroformans, Hansenula anamola, Yarrowia lipolytica, Saccharomicopsis lipolytica, . niger, A. carbonarius, A. aculeatus, A. awamori, , A. fonsecaeus, A. foetidus, A. phoenicis, Corynebacterium ssp., P. janthinelium, P. restrictum, Talaromyces* sp., *T. viride, Ustulinavulgaris, B. licheniformis, Arthrobacter paraffinens*. However, several commercially developed strains of *A. niger* have been most ideally used for citric acid production (**Sardinas, 1972; Ikeno *et al.*, 1975; Kapelli *et al.*, 1978; Grewal and Kalra, 1995; Lu *et al.*, 1997; Abbas *et al.*, 2016a; Alnassar *et al.*, 2016**).

A. niger is a mold with asexual reproduction belonging to the class Deuteromycetes. It gives rise to black colored erect conidiophores with globose columella at the tip (**Singh *et al.*, 2016**). It has been reported that the mycelium form of fungus has a key role to obtain final yield of citric acid (**Kristiansen and Sinclair, 1979**). *A. niger* has been widely used in several industrial fermentation processes due to its ability to convert cheap substrates into value-added products with higher yields. Being a dominant filamentous fungus, there are reduced chances of contamination and complications in handling it. Immobilization of *A. niger* is found to be useful method for continuous production of citric acid (**Garg and Sharma, 1992**). *A. niger* is a commonly occurring natural flora of soil and many edible products like bread, onions, banana and wheat bran etc. Serial dilution of soil samples is carried out and allowed to grow on potato dextrose agar (PDA) (**Selvankumar *et al.*, 2014; Abbas *et al.*, 2016a**). Based on growth and distinct morphological characteristics, *A. niger* colonies are identified and allowed to grow on newly prepared PDA plates. Screening of citric acid producing strains of *A. niger* is identified by growing on Czapeck dox agar plates containing bromocresol green. Yellow colored zone appears around fungal colonies which have potential of citric acid production. All fungal isolates are then preserved on PDA slants under refrigeration (4˚C). The isolates may also be stored in glycerol solution (30% V/V) at -76˚C (**Selvankumar *et al.*, 2014**).

Pure culture of *A. niger* is inoculated on sterile slants of PDA and incubated at 25-32˚C for 5-7 days. The inocula are obtained in 10 ml of sterile distilled water containing two drops of 0.1% tween 80 solutions. A small sterile inoculation needle is used to scrap the spores of fungi with proper care to avoid contamination and without disturbing the agar surface. Usually, ≈ $(1\text{-}2)\times10^7$ spores are suspended into the sterile medium (**Selvankumar *et al.*, 2014; Alnassar *et al.*, 2016**). The number of spores is counted by using haemocytometer. The prepared inocula should be used within 7 days because older spores have tendency of consuming citric acid which is initially produced before completion of fermentation. The starter culture can be prepared by using sucrose salt media. The culture is incubated at 30°C for 2-3 days and further used as per need (**Alnassar *et al.*, 2016; Abbas *et al.*, 2016 a,b**).

Strain improvement program is one of the approaches to increase the yield of citric acid. It can be enhanced by modifying metabolism of the fungus, *A. niger* (**Swain *et al.*, 2012**). Strain improvement can be achieved by mutagenesis process. Various mutant strains of *A. niger* are obtained and used for commercial production of citric acid (**Jialong *et al.*, 2000**). Various parameters need to be taken into accounts while selecting an improved strain which are as follows : 1) Strains should have stable physiological and biochemical characters 2) Citric acid should not be used by strains for any other purpose 3) Strains should not produce other metabolic acids such as oxalic, gluconic and malic acid etc. (**Yalcin *et al.*, 2010**). Mutagenesis can be achieved by either physical, chemical means and by gene cloning. The most common physical mutagens used are gamma and UV radiations. Chemicals such as diethyl sulfonate (DES), N-Methyl-N-Nitrosoguanidine, ethidium bromide etc., are well known chemical mutagens (**Zia *et al.*, 2010**). Enzyme diffusion zone analysis is a specific method for screening and identification of mutated improved strain which is based on enzymatic reaction on plate media (**Prasad *et al.*, 2014**). Other two methods such as Single Spore Technique (SST) and Passage Method (PM) are well known alternative methods for selection of improved strains (**Soccol *et al.*, 2006**). Strains with robust citric acid production capacity would be possible by identifying molecular mechanisms involved in citric acid production.

FERMENTATION PROCESSES FOR CITRIC ACID PRODUCTION

Liquid substrate fermentation

In liquid substrate fermentation process, a substrate used is soluble in liquid media. In this process, a lot of free flowing liquid is used without any physical support to the microorganisms. This process can be performed by two different methods a) Submerged fermentation and b) Surface fermentation (**Reddy, 2002**) as shown in the Figure 2.

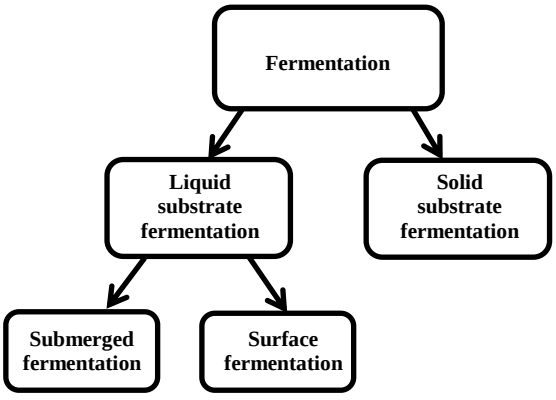

Figure 2 Different fermentation processes for citric acid production

Submerged fermentation process is commonly used for citric acid production. They have advantages like higher yields and lower labour costs make it more suitable for citric acid production (**Rohr *et al.*, 1983**). In this process, anaerobic or partially anaerobic decomposition of carbohydrates is carried out by microorganisms in liquid medium containing a lot of freely available water (**Swain *et al.*, 2012**). The nutrient substratum is in liquid form which is also called broth and the organism grows inside the substratum. Spargers and impeller blades are used to maintain the culture conditions (**Reddy, 2002**). Aseptic and uniform conditions are maintained in the specialized vessels known as bioreactors which are required for fermentation. The performance of a bioreactor is dependent on different parameters such as biomass concentration, aseptic conditions, heat transfer and operation at optimum process conditions. Stirred tank bioreactor and airlift bioreactor are commonly preferred for submerged fermentation process. Stirred tank bioreactors have several advantages such as high oxygen transfer rates, high biomass productivity, low investment and low operating costs for aerobic fermentation process. There are several complex medium ingredients such as corn steep liquor, molasses and soybean flour which are used as inexpensive nutrition source in fermentation (**Kristiansen and Sinclair, 1979; Swain *et al.*, 2012; Alnasaar *et al.*, 2016**). Airlift reactor can be advantageous if fermentations have low shear and energy requirements. In this reactor, high pressure sterilized air is provided from the bottom of the bioreactor for uniform mixing and oxygenation of medium. It also has disadvantages like inadequate sterilization, higher capital investment, aeration requirements and high power requirements (**Pometto *et al.*, 2005**).

Surface fermentation is a stationary batch fermentation process in which microorganisms grow on the substratum and derive nutrients from it. Nutrient media like cane molasses, beet molasses, wheat bran, potato starch and glucose syrup are commonly used (**Kristiansen and Sinclair, 1979; Alnasaar *et al.*, 2016**). Prepared media is placed in thin layers in shallow trays (1-2.5 cm depth) and inoculated with desired microorganism. Continuous aeration is necessary to supplant the CO_2 generated. Approximately 40-60% humidity is maintained to prevent moisture loss from the surface of the medium. Once the fermentation process is over, crude fermentation fluid and mycelial mats are separated. Further, they need washing in order to remove any impregnated citric acid. The disadvantages of this process are unequal distribution of air and nutrients, high labour requirements and inefficient use of floor space (**Reddy, 2002**). This process is still used in small and medium scale industries due to their some advantages such as low energy cost, low installation cost and less efforts for operation (**Swain *et al.*, 2012**).

Solid substrate fermentation

Solid substrate fermentation in which fermentation process is carried out on a non-soluble material which acts as physical support as well as a source of nutrients instead of free flowing liquid (**Pandey, 1992**). This has been practiced from ancient times, common examples including application of *A. oryzae* in koji process for fermentation of rice and cheese production by *P. roquefortii*. In Koji process, the mould (*A. oryzae*) is grown on a steamed rice medium. The rice is a major source polysaccharide starch. The starch present in medium can not utilize by yeast as an energy source. The enzymes such as glucoamylase and alpha amylase used in Koji process are responsible for conversion of rice starch into glucose. Thus, glucose can be easily utilized as an energy source by yeast during the sake brewing process. Thus, this process is also known as saccharification (**Furukawa, 2012**). Solid substrate fermentation can also be useful to obtain variety of chemicals and value added products like enzymes, biologically active secondary metabolites, amino acids, single cell proteins, mushroom and organic acids etc. Recently, solid substrate fermentation is used for the production of flavor compounds, enzymes, colorants, biopesticides and other industrially important products. This process have some advantages such as low energy requirements, high product yields, low risk of contamination, better product characteristics, less efforts in downstream processing, less effluent generation and simple operation as compared to submerged fermentation. It was found that the economic efficiency is 100 folds as compared to submerged fermentation. Solid substrate fermentation serves to give anchorage to filamentous fungi which results in good growth and high productivity. Thus, different solid nutrient rich agronomic wastes are effective and economic for solid substrate fermentation. This process may have some drawbacks such as difficulties to scale up, low amenability of the process to standardization, difficult control of process parameters and problems with heat buildup (**Tengerdy, 1996; Holker and Lenz, 2005; Kapilan, 2015; Handa *et al.*, 2016**).

WASTE MATERIALS UTILIZED FOR CITRIC ACID PRODUCTION

A variety of wastes are continuously produced from different types of industrial processes. Such wastes can be cost effective substrates for microorganisms to obtain value added products like citric acid listed in Table 1.

Table 1 Waste materials utilized as substrates by fungi for citric acid production

Microorganisms	Substrate/Media	Citric acid production (g/l)	References
A. niger (Van Tieghem MTCC 281)	Waste apple pomace	4.6 g/100 gram of pomace	**Kumar *et al.*, 2010**
Y. lipolytica (NRRL Y-1095)	Glycerol	59.00	**Avila-Neto *et al.*, 2014**
A. niger (FUO2)	Cassava peel malted sorghum	1.93	**Adeoye *et al.*, 2015**
A. niger ($FUO1_{10}$)	Cassava peel malted sorghum	9.40	**Adeoye *et al.*, 2015**
A. niger	Synthetic medium	14.68	**Alsudani *et al.*, 2015**
A. niger (GCB 117)	Cane molasses	14.17	**Iqbal *et al.*, 2015**
Y. lipolytica (K-168)	Carrot juice-based medium	80.53	**Urak *et al.*, 2015**
A. niger (GMCC 5751)	Liquefied corn medium	151.67	**Wang *et al.*, 2015**
A. niger (ATCC 9142)	Cocoyam starch	108.00	**Amenaghawon *et al.*, 2015**
Y. lipolytica (SJW-1b)	Corn steep liquor	27.5	**Liu *et al.*, 2015**
Y. lipolytica CBS 2073)	Crude glycerol	10.00	**Ferreira *et al.*, 2016**
Y. lipolytica W29 (ATCC 20460)	Crude glycerol	10.00	**Ferreira *et al.*, 2016**
A. niger (KA88)	Corn cobs solid substrate	138.24	**Addo *et al.*, 2016**
A. niger	Mango peels	7.52	
	Sweet orange peels	11.01	**Abbas *et al.*, 2016b**

However, such substrates need pre-treatments prior their utilization as substrate. Dried wastes like coconut husk, coconut cake, sunflower cake and corn cobs etc could be cost effective substrates (**Lingappa *et al.*, 2009; Addo *et al.*, 2016**). Some of the wastes collected may have high moisture content which becomes susceptible for contamination with undesirable microorganisms result into uncontrolled microbial growth. These wastes include decaying fruits, pomaces of grape, apple, orange, sweet-orange, pineapple, vegetables, tapioca, spoiled coconut and banana peels etc. Therefore, such wastes need to be dried until moisture content comes down to the desired level (**Kumar *et al.*, 2010; Abbas *et al.*, 2016a; Ali *et al.*, 2016**). Drying of wastes is commonly performed at 60˚C in mechanical driers. Period of drying varies depending upon the nature of wastes. For example, decayed fruits need 2 days for proper drying while banana or citrus peels may require 8 h. These are then pulverized and sieved through suitable sieve prior to use as substrate. Banana peels are supplemented with various nitrogenous compounds such as ammonium phosphate and peptone (**Kareem and Rehman, 2011**). Corn cobs also need $(NH_4)_2HPO_4$ as a preferred nitrogen source (**Addo *et al.*, 2016**). Such pulverized medium is added with sufficient amount of water and autoclaved at 121˚C for 1 h. Such properly cooked media is useful for increasing the amenability of the media to microorganisms. Other wastes such as dairy wastewater, cane molasses, rice straw and sugarcane bagasse need prior treatment before their use as substrate. Dairy wastewater has 2.5 % reducing sugars in the form of lactose which are concentrated to get 5% of lactose. The proteins and lipid content may get precipitated during sterilization process. Therefore, the mixture needs to be heated at 90˚C and maintain their optimum pH 4.3. The separated proteins and lipids should be removed by centrifugation process (**Kim *et al.*, 2002**). Cheese whey need fortification of lactose, NH_4NO_3, KH_2PO_4, ZnSO4, K_4 $[Fe(CN)_6]$ before their use as substrates (**Alnassar *et al.*, 2016**). Rice straw are washed and dried at 70˚C overnight then pretreated with NaOH at room temperature for 1 h. Further, they are washed with water, neutralized and kept for drying at 70˚C overnight. Straws are then pulverized and added with pretreated molasses (**Ali *et al.*, 2011**). Sugarcane bagasse is dried, cut down into small pieces and grinded to obtain desired particle size. These particles are moistened with 15-20% of sucrose solution or molasses solution. Such substrates are fortified with NH_4NO_3, KH_2PO_4, $MgSO_4$, and $CuSO_4$ before their use as substrates (**Kumar *et al.*, 2002**). Sugarcane bagasse extract may be processed for their use in submerged fermentation. Bagasse is sun-dried and grinded to obtain particle size of 1.2-1.6 mm. It is soaked in distilled water and the extract is obtained after filtration process. Cane molasses

need to be diluted to obtain 15% of sugar level and the pH of medium is adjusted using HCl (**El-Hussein *et al.*, 2009**). Orange peels have also been reported for their use in submerged conditions. Orange peels are initially washed properly, cut into small size, dried, powdered, sieved and fortified with glucose, sucrose, NH_4NO_3, $MgSO_4$ and $(NH_4)_3PO_4$ (**Singh *et al.*, 2016**). Moisture of orange peel is maintained at desired level and autoclaved at 121˚C for 15-60 minutes. Precaution is needed to be taken so that the substrate selected is easily available and of same quality for every batch of production. Table 2 showed different types of microorganisms having ability to produce citric acid. However, literature survey revealed that fungi and yeasts are most commonly used for citric than bacteria. Table 2 clearly indicates that fungi and yeasts are comparatively highest citric acid producers than the bacterial system.

Table 2 Citric acid production (g/L) by different microorganisms

Microorganisms	Citric acid yield (g/L)	Substrate	References
Fungi			
Penicillium oxalicum	530.00	Potato dextrose liquid medium	**Li *et al.*, 2016**
A. niger	173.20	Corn flour liquefied	**Wang *et al.*, 2017**
A. niger ATCC12846	162.70	Wheat bran extract	**Yu *et al.*, 2018**
A. niger H915-1	157.00	Corn steep liquor and corn starch	**Yin *et al.*, 2017**
A. niger MTCC 282	46.50	Colocassia antiquorum (10% w/v)	**Ganne *et al.*, 2008**
Yeast			
Y. lipolytica Wratislavia 1.31	155.20	Crude glycerol (86% wt/wt)	**Rywińska *et al.*, 2010**
Saccharomycopsis lipolytica	152.30	Canola oil	**Good *et al.*, 1985**
Y. lipolytica 1.31	144.50	Crude glycerol(25% wt/wt)	**Rymowicz *et al.*, 2005**
Y. lipolytica NG40/UV5	140.00	Rrapeseed oil	**Morgunov *et al.*, 2018**
Candida lipolytica	47.00	n-paraffins	**Crolla and Kennedy, 2001**
Bacteria			
Bacillus licheniformis	42.00	Glucose	**Kapoor *et al.*, 1983**
Cornybacterium sp	40.00	Dodecanerice	**Kapoor *et al.*, 1983**
Acetobacter xylinum	2.20	Saccharificate medium	**Lu *et al.*, 2016**

Table 3 shows citric acid yield (g/L) in different fermentation processes like solid substrate fermentation, surface fermentation and submerged fermentation. Table 3 showed that yield of citric acid was higher in soild substarte fermentation process when compared with surface and submerged fermentation.

Table 3 Citric acid yield (g/L) in different fermentation processes

Citric acid fermentation processes	Microorganisms	Substrates/media	Yield of citric acid (g/Kg) or (g/L)	References
Solid Substrate Fermentation	*A.niger* NRRL2001	Apple pomace	766.00	**Hang and Woodams, 1984**
	A. niger MTCC 281	Coconut cake	108.90	**Lingappa *et al.*, 2002**
	A. niger UABN 210	Banana peels	82.12	**Kareem and Rahman, 2011**
	A. niger	Oat bran	62.00	**Rao and Reddy, 2013**
	A. niger	Rice straw	50.20	**Ali *et al.*, 2012**
Surface Fermentation	*A. niger* RCNM 17	Sugar	24.37	**Narayanamurthy *et al.*, 2008**
	A. niger ATCC 9142	Grain liquor	19.00	**Roukas and Kotzekidou, 1986**
	A. niger ATCC 9142	Corn starch hydrolysate	2.70	**Andrew and Corresponding, 2012**
Submerged Fermentation	*A. niger*	Corn flour liquefied	173.20	**Wang *et al.*, 2017**
	A. niger ATCC12846	Synthetic medium combined with wheat bran extract	162.7	**Yu *et al.*, 2018**
	A. niger MO-25	Molasses and chicken feather	68.8	**Ozdal and Kurbanoglu, 2018**
	Native *A. niger*	Banana peels	51.68	**Abbas *et al.*, 2016a**

INFLUENCE OF DIFFERENT PARAMETERS ON CITRIC ACID PRODUCTION PROCESSES

Inoculum is added into media and kept for incubation at 30˚C for the predetermined period. Various tests are carried out for process control. The yield of final product is dependent on various parameters that are described below:

Initial Sugar Concentration

The major waste is generated by food industry during fruits and beverages processing. Such waste is in the form of pomaces, peels and trimmed portions etc. The pomace is the solid material obtained after extraction of fruits. It contains skin, pulp, seeds and stems of fruits. This pomace is considered as promising substrate for citric acid production. They are found rich in carbohydrates but low in proteins. It was observed that apple and grape pomace containing about 30% of reducing sugars. For such substrates, supplementation of any particular nutrients is not needed since they are found to be in optimum concentration. Other substrates such as citrus fruit peels, rice straw and sugarcane bagasse have fewer amounts of fermentable sugars and therefore moistened with sucrose solution to increase the level of sugar (**Kumar *et al.*, 2002; Ali *et al.*, 2011**). Sweet lime peel is supplemented with sucrose solution to achieve sugar concentration of 31.8g/100g dry solid. Other substrates such as corn cob, orange peel and pomegranate peel is similarly treated (**Hamdy, 2013; Addo *et al.*, 2016; Prabha and Subbarangaiah, 2016**). The reducing sugar content of dairy and cheese wastewater is increased to 5% and 15%, respectively (**Kim *et al.*, 2002; Murad *et al.*, 2003**).

Moisture, process additives and pH

In case of solid substrate fermentation process, the moisture content is maintained in between 50-80%. Increase in the moisture content beyond 70% results into decreased sugar consumption and citric acid production. Addition of methanol in media increases the required moisture content. In case of pineapple pomace, the highest yield (11.3g/100g) was achieved at 4% of methanol and 70% of moisture content. In the absence of methanol, yield was found highest (9.7g/100g) at 60% moisture content (**Kumar *et al.*, 2003**). Apple pomace shows similar result at 4% methanol and 73% moisture content with the yield of 883 g/kg of sugar. In the absence of methanol, yield was reduced to 259 g/kg of sugar even at same moisture content (**Hang and Woodams, 1984**). In case of grape pomace, the highest yield was 600g/kg of sugar consumed in presence of 3% methanol and 65.4% moisture content. It was noticed that yield was reduced to 275 g/kg of sugar consumed in the absence of methanol (**Hang, 1998**). When banana peel is used as substrate and optimum moisture content is maintained which gives yield of 52.08 g/L (**Abbas *et al.*, 2016**). Orange peel substrate gives yield of 640 g/kg at 65% w/v moisture content. However, the yield also depends on the strain of microorganism used. In case of sugarcane bagasse, the highest yield 19.8 g/100g was obtained after treatment with clarified molasses and 4% methanol (**Kumar *et al.*, 2002**). The yield obtained was 5.023 g/100g when rice straw was used as substrate in optimized condition (**Ali *et al.*, 2011**). There is no exact information of the effect induced by methanol. However, it is seen that various trace elements such as Fe^{2+}, Mn^{2+} and Zn^{2+} are responsible for suppression. Methanol has dehydrating action on the substrates and also intensifies the outbound permeability of cellular walls for secretion of citric acid (**Yadegary *et al.*, 2013**). In addition, other lower alcohols such as ethanol and n-propanol may also induce the same effect (**Kareem and Rehman, 2011**). There are several substrates such

as dairy wastewater, cane molasses and cheese whey that are used in submerged fermentation processes. The yield obtained was about 4 g/L when dairy wastewater was used as substrate with 5% of sugar concentration without any particular additions (**Kim *et al.*, 2002**). On the other hand, various additions such as methanol, ethanol, EDTA, and combination of methanol and EDTA were tested when cane molasses was used as substrate. It was noticed that in the presence of 3% of methanol, yield obtained was 61.62 g/L and was reduced to 48.43 g/L when methanol was replaced by 4% of ethanol. When EDTA alone was added at the rate of 250 ppm, yield was about 59.74 g/L. Maximum yield of ≈92.86 g/L obtained when whey was used as substrate supplemented with 15% of sugar. However, addition of riboflavin (10 mg/L) or 4% tri-calcium phosphate can reduce the required sugar addition significantly to 10% but may also affect citric acid production (**Murad *et al.*, 2003**). The experimental conditions for citric acid production by *A. niger* was optimized and the highest citric acid yield of 19.447 g/l was reported when the rice husks filtrate medium was used (**Alsudani *et al.*, 2015**). Several mutants of *A. niger* were obtained and tested for citric acid production. Amongst the wild strains, a strain (FUO 2) has been reported for the highest citric acid production (1.93 g/l) when grown on hydrolyzed cassava peel medium. The mutant strain $FUO1_{10}$ was developed after exposure to UV-radiation and gave highest yield of 9.4 g/l. Thus, mutation showed improvement of 4.87 fold in citric acid production. The process optimization was done by using the mutant strain (FUO 110) on the input parameters such as substrate concentration, process time, inoculum size and initial pH. The highest yield of citric acid was 88.73 g/l and thus, showed improvement of 45.97 fold over the best wild strain (**Adeoye *et al.*, 2015**). The high level of ATP showed a strong inhibitory effect on citric acid production by *A. niger*. Thus, increasing NADH oxidation and reducing the level of intracellular ATP can accelerate glycolysis and the TCA cycle to enhance citric acid production (**Wang *et al.*, 2015**). The solid substrate such as corn cobs was used for citric acid production by using *A. niger* KA88. One-factor-at-a-time (OFAT) model was employed for optimization of the fermentation media to obtain the maximum yield of citric acid. The final OFAT fermentation media gave 138.24 g citric acid/kg dry corn cob. Thus, corn cob proved to be a highly promising solid substrate for production of citric acid (**Addo *et al*., 2016**). Similarly, sugarcane molasses was also used as carbon source for maximum citric acid production (**Iqbal *et al.*, 2015**). Additives like low molecular weight alcohols, trace metals, phytate, lipids etc have been reported to stimulate citric acid production by *A. niger* on cocoyam starch. A three variable, three-level Box-Behnken design (BBD) was used to develop a statistical model to test effect of Zinc (II), Iron (III) and methanol on citric acid production. Response surface methodology (RSM) was also used for optimization of such stimulants. It was seen that citric acid production was enhanced with increasing concentration of zinc and methanol. The maximum citric acid production was 108 g/L (**Amenaghawon *et al.*, 2015**). Mango peels and sweet orange as novel substrates were used for citric acid production by *A. niger* and the fermentation parameters were optimized. Maximum citric acid production was obtained by using mango peel 11%, sucrose concentration 5%, inoculum 2%, potassium dihydrogen phosphate and ammonium nitrate at pH 5 and 32°C after 8 days. In sweet orange peel fermentation process maximum citric acid was obtained by using 11% sweet orange peel, disodium hydrogen phosphate, inoculum 2%, sucrose concentration 25% at 32° C and pH 4 after 6 days (**Abbas *et al.*, 2016a**). Citric acid production was maximum by *A. niger* using banana peel 20%, inoculum 5%, potassium dihydrogen phosphate and ammonium nitrate at pH 5 and 32ºC after 8 days of fermentation (**Abbas *et al.*, 2016b**). Generally, initial pH for citric acid production is adjusted below 4.5 because higher pH favors production of gluconic and oxalic acid.

Effect of trace elements, phosphate source and surface area to volume ratio

The trace elements are one of the most important factors in citric acid production by *A. niger* (**Hang and Woodams, 1998**). In particular, metal ions like zinc, manganese, iron, copper and magnesium are found to be crucial elements in citric facid production. Therefore, it is necessary to consider the interdependence of medium components. The yield of citric acid can be increased when the level of the trace elements is kept optimum, mostly in case of submerged fermentation (**Soccol *et al.*, 2006**). It was clearly noticed that the presence of phosphate in production medium had a great impact on citric acid yield. The low concentration of phosphate has a positive impact on citric acid production. The phosphate enhances the enzyme activity and do not affect at the level of gene expression. The presence of an excess amount of phosphate source in medium can decrease the carbon dioxide fixation which results into certain sugar acids formations and the stimulation of growth (**Grewal and Kalra, 1995; Vandenberghe *et al.*, 1999; Soccol *et al.*, 2006**). It was observed that a strain of *A. niger* produced a low amount of citric acids in submerged fermentation conditions. It was seen that higher yield of citric acid was obtained under surface culture conditions when compared with submerged conditions. This clearly revealed that citric acid production is dependent on an appropriate supply of oxygen and surface area to volume ratio. It was seen that addition surface area provided to growth of microorganisms, thus resulting in high yield of citric acid production (**Lakshminarayana *et al.*, 1975**). The surface area to volume ratio is one of the most influencing parameter observed in citric acid production. It was seen that increasing the surface area to volume ratio from 0.25-2 cm^2/mL showed increased citric acid production from 0-21 g/L (**Mazinanian *et al.*, 2015**).

BIOREACTOR DESIGNS FOR CITRIC ACID PRODUCTION

Bioreactor is a vessel in which a biological reaction or process is performed. It is facilitated with accessories required to maintain suitable environment for growth and activity of microorganism. Liquid substrates such as whey and molasses can be typically fermented by using the conventional Fermenter with sophisticated automations in case of submerged fermentation process (**Lingappa *et al.*, 2009**). In case of solid surface fermentation, some limited options of bioreactors and process controlling systems are available (**Rodriguez and Sanroman, 2005**). To perform solid surface fermentation effectively at industrial level following designs have been proposed as follow:

Tray

This consists of flat trays to be incubated in a room provided with proper ventilation and temperature control systems through circulation of humidified air. The depth of shallow trays should not be more than 7 cm. Similarly, depth of the solid medium should not be more than 5 cm for proper air circulation into the solid medium (**Okafor, 2007**). However, this design is not found attractive due to requirement of a large number of trays, labor and extra floor space.

Packed bed

It comprises of a column mostly made of glass or plastic. It has a perforated base where substrate is retained. Humidified air is continuously passed with pressure through perforations. Temperature is maintained via water circulation by using jacket. However, recovering of final product from this bioreactor is difficult due to non-uniform microbial growth. Further, inadequate removal of heat from bioreactor might be harmful to fermentation process (**Rodriguez *et al.*, 2000**).

Horizontal drum

Such rotating bioreactors are found to be useful for proper mixing and aeration with minimal damage to inoculum or formed products. Paddles and baffles are used for this purpose. Only drawback of this bioreactor is that substrate has to be filled only up to 30 % of the total capacity of vessel for proper mixing and aeration (**Domniguez *et al.*, 2001**).

Fluidized bed

In this bioreactor, there is constant flow of air provided with pressure to prevent coalescence of particles. Such bioreactors have several advantages such as higher mass, heat transfer, proper aeration, agitation and higher heat buildup due to shear forces. However, harm to inoculum may lower the rate of productivity (**Mitchell *et al.*, 2003**). The above described bioreactors are of basic designs. The lab scale design for bioreactors could not be entirely useful for scale up process. Initially, scale up process was characterized by thumb rules mainly including heat and mass transfer phenomena. Later on, researchers also began to develop quantitative approaches towards mass transfer, mathematical modeling and analysis of process for development of scale up process (**Durand, 2003**). Microbes were studied for better understanding of behavioral pattern under various environmental conditions.

TECHNIQUES FOR RECOVERY OF CITRIC ACID

After the desired incubation period, the fermentation process is stopped by the action of heat. The end product (citric acid) may contain mycelium and varying amounts of impurities such as mineral salts, other organic acids and proteins etc. The method used for product recovery depends on the raw materials used in fermentation (**Grewal and Kalra, 1995**). In solid substrate fermentation method, the contents of the bioreactor are dried in oven at 50˚C for 2 h. Distilled water is then added in excess and thoroughly mixed by using mechanical shakers. After desired mixing, the contents are filtered. In the submerged fermentation, the mycelium biomass is heated up to 70˚C for 15 minutes which results into partial coagulation of proteins. The oxalic acid formed in the fermentation process is removed by increasing the pH with calcium hydroxide at 72-75˚C. Calcium oxalate is further precipitated and eliminated by centrifugation or filtration process. Citric acid remains in solution in the form of calcium salt (mono-calcium citrate). Further, extraction of citric acid is carried out by various techniques such as precipitation, solvent extraction, adsorption/absorption on ion exchange resin, electro-dialysis, ultra/nano-filtration or liquid membranes.

Precipitation

It is one of the conventional methods used for the recovery of citric acid. Equivalent amount of lime is added to the solution of mono-calcium citrate in

order to precipitate it as tri-calcium citrate. The efficiency of the process depends on several parameters such as citric acid concentration, pH, temperature and rate of lime addition. Pure and large crystals are formed after milk of lime (180-250 kg/m^3) is added gradually. Temperature needs to be maintained at or around 90˚C with pH 6. The process requires presence of more than 15% of citric acid concentration within the solution. Due to the solubility of citric acid, the minimal expected loss is ≈ 4-5% (**Soccol *et al.*, 2006**). The precipitated calcium citrate is washed with hot water (90ºC) to remove impurities such as saccharides, chlorides and colored substances. Further, calcium citrate is treated with sulfuric acid and calcium sulfate precipitate formed (gypsum) is eliminated. After elimination of precipitate, remaining solution contains ≈ 25-30% of citric acid. Further, purification is carried out by using activated charcoal or ion exchange columns. Thus, purified solution is then concentrated in vacuum evaporator below 40˚C to avoid caramelization and finally dried to obtain citric acid crystals. Two forms of citric acid can be obtained during crystallization process. Citric acid anhydrate form is obtained when temperature dependent crystallization process is performed above 36.5˚C. However, citric acid monohydrate form is obtained when crystallization is carried out below 36.5˚C. The optimum temperature to obtain 100 % yield of citric acid was 50°C for 20 minutes. The citric acid need not undergo any phase transition and the product obtained is of high purity (**Li *et al.*, 2016**). Disadvantage of precipitation process is that a large quantity of waste is generated per ton of citric acid produced (**Pazouki and Panda, 1998**).

Solvent extraction

Recovery of citric acid by solvent extraction is also commonly used. It is an extraction process in which solvent used with very low or no solubility in aqueous phase. The solvent used should have maximum solubility with citric acid and fewer amounts of impurities. Each solvent used for extraction is characterized by its equilibrium distribution coefficient which is defined as the ratio of acid concentration of the extract to the acid concentration of the aqueous phase (**Kristiansen and Sinclair, 1979**). Solvent systems such as mixture of *n*-octyl alcohol, tridodecylamine and isoalkane (**Soccol *et al.*, 2006**), Alanine 336 in heptane or xylene (**Sirman *et al.*, 1990**), mixture of butylacetate and N, N-disubstituted alkylamide (**Yi *et al.*, 1987**) have been used. Amine extraction has been found to be a prospective method of separation of carboxylic or hydroxycarboxylic acid from aqueous solution. The citric acid is recovered by distilling the solvent or by washing off the extract with water. From the aqueous solution, purified citric acid is subsequently crystallized and concentrated. Compressed CO_2 is passed through the concentrated citric acid solution in acetone in order to avail the anti-solvent effect of CO_2 for removal of the residual impurities. Finally, food grade citric acid is obtained by simple decolourization and crystallization (**Shishikura *et al.*, 1992**). The advantage of the solvent extraction method is to prevent the use of lime and H_2SO_4 (**Pazouki and Panda, 1998**). Such process is a highly efficient one and requires less amount of energy.

Adsorption and ion exchange techniques

These techniques are also employed for extraction of citric acid. Such techniques have a good selectivity in extraction process. This technique requires lesser energy and also no phase transition occur. Two categories of resin are commonly employed such as macro-porous adsorption and ion exchange. The polymeric adsorbent to be used should be neutral, non-ionogenic, micro-reticular and water insoluble styrene-based polymers. Better selectivity and higher capacity of the adsorbent may be achieved by using weakly basic anionic exchange resins which is impregnated with tertiary amine or pyridine or strongly basic anion exchange resins. The adsorption process is carried out by using dense compact fixed bed with alternate contact to the feed mixture and desorbent. Macro-porous resins are dependent on hydrogen bonding, Van der Waal's forces and dipole ion interaction for process of separation. The pH of the feed solution is maintained below the first ionization constant of citric acid for the separation. The use of simulated moving bed counter current flow system is used as an efficient technique of ion-exchange separation for continuous operations of adsorption and desorption. The exchange capacity of ion exchange resins is dependent on the particle size, ions and degree of cross linking. These processes are advantageous due to their quick recovery, high capacity, specificity and low regeneration consumption. In this process, no co-product of calcium sulphate is produced. The disadvantage of this process is a large requirement of desorbent causing dilution of the resultant citric acid solution and formation of waste liquor in large quantities (**Kristiansen and Sinclair, 1979; Li *et al.*, 2016**).

Membrane Separation

In this method, the membrane used is essentially a thin, artificial or natural barrier which allows selective mass transport of solute or solvent across barrier to achieve the physical separation and enrichment process. It involves techniques such as electrodialysis, reverse osmosis, nano-filtration, ultra-filtration and micro-filtration. Such techniques have selectivity and adoptability. Electro-dialysis had been used for separation of citric acid in the early 1970's. These processes are found highly efficient in separation but more energy consuming. The membranes used in this technique are also expensive (**Li *et al.*, 2016**).

Methods of citric acid estimation

The amount of citric acid in the filtrate was estimated by traditional method of titration. In this method, citric acid was measured by titration with 0.1 N NaOH against phenolphthalin as an indicator. The end point in this method was pink color. The yield percentage of citric acid was further calculated (**Priede and Latvian, 2005**).**T**he citric acid was also determined by spectrophotometrically by the acetic anhydride-pyridine method and (**Miller, 1958**), gas chromatography (**Jham *et al.*, 2002**), high-resolution nuclear magnetic resonance spectroscopy (**del Campo *et al.*, 2006**), high performance liquid chromatography (HPLC) method (**Wang *et al.*, 2017**).

CONCLUSION

Citric acid is one of the extensively used organic acids in many industries. Biodegradable wastes such as mango peel, orange peels, glycerol, cane molasses, coconut cake, canola oil and banana peel etc are found to be potential substrates for citric acid production. Formation of citric acid as a byproduct will reduce waste disposal problems and also reduce the dependency of industry over other citric acid producers. The biomass generated during citric acid fermentation can be effectively utilized for biogas production and also in fertilizers preparation. Thus, the industry would be benefitted ecologically and economically. Some strains of *A. niger* are found to be potential organisms for citric acid fermentation. Both solid state fermentation and submerged fermentation can be employed for citric acid production based on the substrate used. Only constraint which the industry needs to overcome is studying the actual mechanisms involved in *A. niger* for citric acid production. Therefore, designing of appropriate bioreactors with precise control over process parameters is thus of high importance. Significant efforts are needed towards improvements in scale up process. Elimination of such obstacles will be economically advantageous to the industry.

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