Citric Acid Production of Yeasts: An Overview

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Abstract

Background

Citric acid, an intermediate product of the Krebs cycle, has a wide usage area in the food industry since it has some functions such as acidulant, flavouring agent, preservative and antioxidant. Although molds are the most commonly used microorganisms in the citric acid production, it is known that there are significant advantages of using yeasts.

Purpose and scope

The microbial citric acid production mechanism needs to be well understood to make production more efficient. In this study, the yeasts used in the production, fermentation types and the factors affecting production were reviewed with studies.

Methodology

Although production of citric acid can be produced by chemical synthesis, the fermentation is preferred because of its low cost and ease of use. More than 90% of citric acid produced in the world is obtained by fermentation.

Results

Yarrowia lipolytica, *Candida zeylanoides* and *Candida oleophila* are evaluated for citric acid production with substrates such as molasses, glucose, sucrose and glycerol. On the other hand, there is great interest in developing processes with new substrates and/or microorganisms.

Conclusion

Although the microbial strain is an important factor, the factors such as carbon, phosphorus and nitrogen sources, aeration, the presence of trace elements and pH are also parameters affecting the production.

Keywords: citric acid, yeast, fermentation, *Yarrowia*, *Candida*

Introduction

Citric acid is an intermediate product that is colorless, odorless, readily soluble in water and alcohol, solid at room temperature and melts at 153°C. It occurs when carbohydrates are oxidized to carbon dioxide in the Krebs cycle (1). Its molecular weight is 192.12 g/mol, which has three different values of pKa (3.13, 4.76 and 6.39) (2). Citric acid is the primary metabolic product formed in the Krebs cycle used by all aerobic organisms (3). The citric acid cycle is shown schematically in Figure 1.

Citric acid exists widely in nature and is present in fruits such as lemon, orange, pineapple, plum, peas, peach and also in bones, muscles and blood of animals (5). It was first isolated from lemon juice by the Swedish chemist Carl Scheele in 1784 (6). However, the first commercial citric acid was produced from calcium citrate in England in 1860 (7). In addition, it was reported that Wehmer was the first researcher who demonstrated that *Penicillium* species can produce citric acid in the medium containing sugar and inorganic salts in 1893 (8). In 1917, American food chemist James Currie discovered that certain

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Figure 1. The citric acid cycle (4).

strains of *Aspergillus niger* can be used for the citric acid production, and Pfizer company started industrial production using this process (9).

Citric acid is accepted as GRAS by the JECFA (9) and is widely used in the food and pharmaceutical industries (10). 70% of the citric acid produced is used in the food industry, 12% in the pharmaceutical and the remaining 18% in other industries (11,12). This organic acid has a wide area of application in the food industry due to its properties such as pleasant flavor, non-toxic agent, antioxidant (13), acidulant, flavouring agent (13–15), preservative and also emulsifier (14). It has also been used as a crosslinker in the production of detergent co-builder and biodegradable polymers in dishwasher cleaners over the past decade (16). The areas of usage of citric acid in the food industry and its functions are presented in Table 1. Citric acid production is considered a complex process that is affected by many metabolic and morphological changes (17). All the yeasts, molds and bacteria contain the citric acid cycle, but only some of them can increase citric acid production (18). It is reported that the annual production of citric acid is approximately 1.600.000 metric tons and that the biggest producer in the world is China with a share of about 40% (12). It was reported that the global volume of citric acid is over two million tons and its production has increased by 5% annually (19). The global market for citric acid is estimated to reach 3.6 billion dollars by 2020. Moreover, an annual growth rate of 5.5% from 2015 to 2020 is also expected (20). Global demand continues to increase due to its applications in nanotechnology

and tissue engineering (21).

Citric Acid Production from Yeasts

Citric acid is described as the most important organic acid produced in tonnage by microbial processes (27–29). In the world, more than 90% of citric acid production is obtained through fermentation (6,10,23).

Candida, *Hansenula*, *Pichia*, *Debaryomyces*, *Torulopsis*, *Kloeckera*, *Trichosporon*, *Torula*, *Rhodotorula*, *Sporobolomyces*, *Endomyces*, *Nocardia*, *Nematospora*, *Saccharomyces*, and *Zygosaccharomyces* are potential producers for citric acid (30). Nevertheless, some *Candida* species such as *C. lipolytica*, *C. tropicalis*, *C. oleophila*, *C. intermedia*, *C. guilliermondii* (30,31), *C. paratropicalis*, *C. zeylanoides*, *C. catenulata*, *C. parapsilosis* (14), *C. citroformans* (32), *C. fibrae*, *C. subtropicalis* (31), *C. albicans*, *C. atmospherica*, *C. brumptii*, *C. chalmersii*, *C. hitachinica*, *C. krusei*, *C. melibiosa*, *C. mycoderma*, *C. pelliculosa*, *C. petrophilum*, *C. pulcherrima* and *C. rugosa* (13) can be regarded to be among them.

At the end of the 1960s, it was determined that *Yarrowia lipolytica* showed quite high yields when n-alkanes were used as a substrate in citric acid production (33). However, it was also reported that glucose-containing media should be preferred rather than n-alkanes due to the low production of isocitric acid (34). Using yeasts in the production has some advantages such as using many carbon sources due to the metabolic versatility and growth (2). Some studies about citric acid production by yeasts (strains, substrates, some fermentation conditions and

Table 1. Areas of usage of citric acid in the food industry and its functions

Industry	Functions	References
Wines and ciders	<ul style="list-style-type: none"> - Prevention of browning in some white wines - Prevention of turbidity in wines and ciders - pH adjustment 	(10,22,23)
Soft drinks and syrups	<ul style="list-style-type: none"> - Provides tartness - Enhancing the natural fruit flavor - Acid regulation in carbonated and sucrose based beverages 	(10)
Jellies, jams and preservatives	<ul style="list-style-type: none"> - pH adjustment - Acid regulation - Providing flavor, tang and tartness - Increasing the efficiency of antimicrobial preservatives 	
Animal feed	- Feed complementation	(10,23)
Gelatin desserts	- pH adjustment	(22)
Animal fats and oils	- Producing synergetic effect with other antioxidants	
Frozen fruits	<ul style="list-style-type: none"> - Inactivation of oxidative enzymes - Decreasing pH - Ascorbic acid protection 	
Meat products	- Antioxidant	(24)
	- Prevention of coagulation or clotting of fresh blood in slaughterhouses	(25)
Seafoods	- Prevention of discoloration and the development of off odors and flavors by chelating trace metals	
	<ul style="list-style-type: none"> - Maintaining the stability and flavor by inactivating endogenous enzymes - Enhancing the action of antioxidants 	(26)
Candies	<ul style="list-style-type: none"> - Provides tartness - Reducing sucrose inversion - Producing dark color in hard candies - Acid regulation 	(27)
Dairy products	<ul style="list-style-type: none"> - Emulsifier in ice creams and processed cheeses - Acidifying agent in many cheese products - Antioxidant 	(22,27)

the maximum amounts of citric acid) are presented in Table 2. Traditionally, *Aspergillus niger* has been used in the commercial production of citric acid from molasses, sucrose or glucose. Moreover, there is a great interest in various yeasts which have production capacity of citric acid from various carbon sources (35). The reasons for the preference of yeasts in the production are use of a variety of carbon sources, tolerance to certain conditions such as high concentrations of substrates, metal ions (36) and low oxygen levels (37) and fewer health hazards (38). Besides, the use of less refined substrates, the reduction in substrate and waste treatment, as well as the cost of product recovery (36) and easier genetic modifications by molecular techniques (39) are other important reasons for their preference. On the other hand, the biggest problem in the use of yeasts for production is considered to be the formation of isocitric acid (36,37,40). This undesired by-product can not be easily obtained from plant tissue or by chemical synthesis (37). Isoc-

itric acid was first found at growth of *Y. lipolytica* strains on n-alkanes in 1966 (13). After citric acid synthesis, isocitric acid is produced by the Krebs cycle, in a step catalyzed by aconitate hydratase (2). Isocitric acid is the structural isomer of citric acid. Excess synthesis of isocitric acid produced from cis-aconitate by the aconitase enzyme in the presence of iron ions occurs with high aconitase and citrate synthase activities and low isocitrate lyase and isocitrate dehydrogenase activities (41). The formation of this product varies depending on the strain used, substrate and current production conditions (36,37). For this reason, it is emphasized that strains should be selected from those with the low isocitric acid capacity (42). It has been stated that wild-type strains can produce about 8-16% isocitric acid from carbohydrates or glycerol as the sole carbon source and 35-50% isocitric acid on the gluconeogenic substrates alkanes and renewable triglycerides, ethanol or acetate and also 35-67% isocitric acid on ethanol as the carbon source depending on

cultivation conditions (37)

Citric acid is produced in the stationary phase of growth. The accumulation and release of citric acid in the cell are realized by different mechanisms (43). However, in a study carried out with *C. oleophila*, it was reported that there was a specific active transport mechanism for the earlier passage of citric acid through the cell membrane than isocitric acid (44).

Fermentation Types Used in Citric Acid Production **Submerged Fermentation (SmF)**

SmF is one of the methods used for commercial citric acid production (32). While SmF can be applied in batch, fed-batch or continuous systems, the batch system is the most preferred method (6,27). Stirred tanks with 40-200 m³ capacity or larger airlift fermenters with 200-900 m³ capacity can be used for SmF (15). The bioreactors to be used for production should be acid-resistant because of the corrosive effect of citric acid and the decreasing pH value during fermentation (32). Tower fermenters are mainly preferred in terms of price, size and operation. Fermenters are also equipped with an aeration system to maintain the high dissolved oxygen level. Cooling can be performed by an external water film (27). This fermentation takes 6-8 days depending on growth of microorganism (31).

Anastassiadis and Rehm (44) researched citric acid production by *C. oleophila* ATCC 20177 under submerged continuous system using glucose. As a result, maximum citric acid concentration and citric/isocitric acid ratio were determined as 57.8 g/L and 15.6 respectively at pH 5.0. In addition, Kamzolova et al. (45) investigated citric acid production capacity of *Y. lipolytica* 187/1 with submerged batch fermentation. The strain was cultivated in a fermentor. They determined that 135 g/L citric acid was obtained when rapeseed-oil was used (under nitrogen limitation conditions). In another study conducted on an acetate-negative mutant of *Y. lipolytica* Wratislavia K1, highest citric acid production (110 g/L) was determined after 168 h of fed-batch cultivation with the total glycerol concentration of 250 g/L (46).

SmF requires dilution of carbon source, pre-treatment of the appropriate nutrients and sterilization in line or in the bioreactor. This method offers advantages such as less space (32), sophisticated control mechanism (25), high productivity and yield, lower labour costs (6,25,47) and lower contamination risk (6). On the other hand, the factors such as high-cost medium, sensitivity to metal inhibition, contamination risk and high amount of post-recovery wastewater generation are considered as disadvantages for this method (25).

Solid State Fermentation (SSF)

SSF fermentation is considered as an alternative method in which agro-industrial wastes can be evaluated for citric acid production (27,47). This type of fermentation has an advantage about bioconversion of organic solid wastes through the production of biologically active metabolites both at the laboratory and industrial scale (48). Nevertheless, this fermentation type is a small-scale operation (15). Moreover, it is considered to be

the simplest method for citric acid production (6,10,27). This method was primarily applied through raw materials such as fruit wastes and rice bran (6,10). The Erlenmeyer conical flasks, glass incubators, trays, rotating and horizontal drum bioreactors, packed-bed column bioreactor, single-layer packed-bed and multi-layer packed-bed systems are used for production with SSF (23). In this fermentation, the solid substrate is adjusted to 70% moisture level, the initial pH of the medium and the incubation temperature are adjusted to about 4.5-6.0 and 28-30°C respectively (depending on the microorganism used). Fermentation lasts for 96 h under optimum conditions (10). Although *A. niger* is the most commonly used microorganism in this method, it has been stated that some yeasts species such as *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* could also be used in this type of production (6). Imandi et al. (49) used the statistical experimental design for the optimization of citric acid production by *Y. lipolytica* NCIM 3589 in SSF using pineapple waste. Finally, the optimum conditions were found to be yeast extract 0.34 (w/w%), moisture content of the substrate 70.71 (%), KH₂PO₄ 0.64 (w/w%) and Na₂HPO₄ 0.69 (w/w%). Finally, they determined that citric acid concentration at these optimum conditions was 202.35 g/kg ds.

The use of yeast with high nitrogen and phosphorus requirements is not appropriate due to the lower diffusion rate of nutrients and metabolites that occur at lower water activities in the SSF (10). This method involves difficulties in control of process parameters such as pH, moisture, temperature, nutrients. Other disadvantage is higher recovery product costs due to the use of products with a higher impurity. On the other hand, this method has many advantages such as the use of simple technology, higher yield, wider range of low cost medium resembling natural habitat for various microorganisms, better oxygen circulation, less susceptibility to the inhibition of trace elements. Use of lower energy and cost requirements, lower risk of bacterial contamination and less post-recovery waste are other advantages of this method (25). It is also among the advantages that trace elements do not affect citric acid production as much as in SmF (10). As well as the advantages mentioned above, SSF requires less water usage in the upstream process and produces less waste water formation during the downstream process. On the other hand, it does not require the addition of some nutrients because of the use of agro-industrial residues (23).

Factors Affecting the Production

Temperature

Although the optimum temperature for citric acid production varies depending on the organism and equipment used, it generally varies between 26 and 35°C (18). Previous studies about the production of citric acid have indicated that 28 and 30°C were usually selected as a fermentation temperature (Table 2). Moeller et al. (50) reported that the maximum citric acid concentration (41 g/L) was obtained at 30°C. They found that the citric acid concentration decreased rapidly at 34°C compared to at 32°C. In another study, maximum citric acid was achieved at 28°C by glycerol-grown *Y. lipolytica* NG40/UV7 and citric

acid concentration decreased gradually with temperature alteration (51). Anastassiadis and Rehm (52) studied the influence of temperature on citric acid production by *C. oleophila* and they determined that the maximum citric acid concentration (63.5 g/L) and citric/isocitric acid ratio (28.8) were achieved at 30°C.

Carbon Sources

The type and concentration of the carbon source are important for the process (10,23) and yeasts can metabolize various carbon sources such as hydrocarbons, molasses, ethanol, vegetable oils, glycerol, galactose and glucose etc. (13). The presence of carbohydrates that are fermented more quickly by the micro-organism is essential for production of citric acid (10,23). The yields from glucose (g/g) decrease significantly at the concentration below 100 g/L, and that only small amounts of citric acid are produced at sugar concentration below 50 g/L (53). Karasu-Yalçın et al. (54) reported that maximum citric acid concentration and productivity were achieved at 160 g/L initial glycerol concentration, the highest product yield was obtained at 120 g/L initial glycerol concentration for the *Y. lipolytica* 57 at the end of the 418 h. Rane and Sims (55) detected that citric acid production by *C. lipolytica* Y 1095 depend on the initial glucose concentration and fermentation time. They obtained 13.6 g/L citric acid with 50 g/L initial glucose concentration at 27 h. Additionally, citric acid concentration was increased to 78.5 g/L with 150 g/L initial glucose concentration at the end of the 128 h. In a study conducted on the citric acid production by *Y. lipolytica* PR32, it was reported that when glucose concentration in the medium was 50 g/L, citric acid concentration formed was 24.76 g/L. However, when glucose concentration in the medium was 120 g/L, citric acid concentration was increased to 64.58 g/L with a yield of 0.72 g/g and a productivity of 0.45 g/L/h (56).

The fact that many natural substrates containing carbohydrate can be used more efficiently and afford in citric acid production is important (42). It has been indicated that the yeasts such as *Y. lipolytica*, *C. guilliermondii* and *C. oleophila* have the possibility to use a wider carbon source than fungi (especially, n-alkanes, glucose, raw glycerol, ethanol and galactose). Furthermore, yeasts can use less refined substrates (45). Hattori and Suzuki (57) used 10% (w/v) n-alkane as substrate in a fermentor, and the citric acid concentration was determined as 87 mg/ml at pH 5.5 after 100 h for *C. zeylanoides* KY6166. It has been stated that molasses and invert sugar mixtures may also be used for citric acid production by yeast, although fructose assimilation levels of some yeasts are low (58). Glucose syrups from starch hydrolysis, sugar beet molasses and low-quality sugarcane by-products are the most preferred substrates in industrial productions. The yield of citric acid production increases depending on the initial sugar concentration in batch processes or glucose feeding rate in the chemostat process. It is reported that the highest productivities are usually obtained when sugar is used at the level of 14-22%, since it was stated that this level leads to the suppression of ketoglutarate

dehydrogenase (8). In a study, *C. tropicalis* ATCC 20115, *C. oleophila* ATCC 20373 ve ATCC 20177, *C. zeylanoides* ATCC 20367, *Y. lipolytica* ATCC 20237, ATCC 20346, ATCC 46330 and *Saccharomycopsis lipolytica* IFO1658 were examined for citric acid production. Fermentation was carried out in shake flasks. As a result, when sugarcane molasses was used as a substrate 91.4 g/L of citric acid was achieved with *C. zeylanoides* ATCC 20367 (59).

As can be seen in Table 2, in previous studies, ethanol, canola oil, sunflower oil, rapeseed oil, glycerol, molasses, n-paraffin, fructose, sucrose, glucose and glucose hydrol were used as carbon sources for citric acid production by yeasts.

Nitrogen and Phosphorus Sources

Citric acid production is influenced by the concentration and nature of the nitrogen source (10,23). Substances such as ammonium sulphate, ammonium chloride, malt extract, peptone, urea (47), yeast extract, beef extract, dry corn-steep, soybean (60), ammonium, sodium and potassium nitrate (22) are used as nitrogen source. However, Darvishi et al. (29) reported that urea was the best nitrogen source for citric acid production. On the other hand, some media such as molasses may not need additional nitrogen sources because they are rich in nitrogen (8). Ammonium nitrate at a concentration higher than 0.25% has been found to cause oxalic acid accumulation. But, it is stated that ammonium sulphate increases citric acid production without causing oxalic acid accumulation (22).

It was determined that citric acid production by yeasts starts after depletion of the nitrogen source (58). Nitrogen restriction coupled with a high concentration of glucose is very important for the citric acid production by yeasts. Citric acid is released by a specific energy-dependent transport system induced by intracellular nitrogen restriction (61). Because citrate synthase is negatively affected by the presence of ammonium ions (41). Souza et al. (62) determined that the citric acid production began after the stationary growth phase, when extracellular nitrogen was a limiting factor for yeast growth. Anastassiadis et al. (17) calculated citric acid concentration as 80 g/L in fed-batch fermentation with *Candida oleophila* ATCC 20177. They remarked that ammonium nitrogen was the limiting factor for citrate formation. It was also stated that nitrogen content of *C. oleophila* biomass decreased during the production phase. According to obtained results, low intracellular nitrogen content and NH₄⁺ concentration were found effective for the production.

Good adjustment of the high C/N in production with yeasts plays an important role in citric acid biosynthesis. Nitrogen deficiency in the culture substrate leads to a rapid decrease in intracellular AMP. The excessive decrease of AMP concentration in the cell reduces the isocitrate dehydrogenase activity which converts isocitric acid to α -ketoglutaric acid. Such conditions lead to intracellular accumulation of large quantities of isocitrate (63). Carsanba et al. (64) studied for the citric acid production by *Y. lipolytica* K57 in batch bioreactor. Finally, maximum citric acid yield (0.77 g/g glucose), titre (72.3 g/L

Table 2. Maximum citric acid production by several yeast strains at different fermentation conditions

Strain	Substrate	ISC (g/L)	N and P sources	Agitation rate (rpm)	Cult.	Time	pH	T (°C)	CA _{max} (g/L)	ICA (g/L)	References
¹ KY 6166	n-paraffin	%10	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄	600	F	100	5.5	30	87	-	(57)
² NR-RL-Y-1095		166♦	KH ₂ PO ₄ , Fe(NO ₃) ₃ ·9H ₂ O	1000		168	-	26	12		(71)
		157♦	Urea, KH ₂ PO ₄ , Fe(NO ₃) ₃ ·9H ₂ O	200	S	120	-	9.8	(18)		
¹ ATCC 20367	Molasses	%18	Molasses + KH ₂ PO ₄			1000	F	144	6.5	30	91.4
³ ATCC 20177	Glucose	120	NH ₄ Cl, KH ₂ PO ₄	139.5	-			50.1	1.8		(17)
		240		58	5.0	74.2	3.9	(79)			
² H222		100	NH ₄ NO ₃ , KH ₂ PO ₄	92	6.0	41	3.05	(50)			
² D 1805				80	4.7	95	10	(80)			
² Y 1095		150	YE, NH ₄ Cl, KH ₂ PO ₄	800	S	128	5.5	27	78.5	7	(55)
²				120		168	7.0	30	22.8	(1)	
² NCIM 3472		100	NaNO ₃ , KH ₂ PO ₄	160	-	5.5	8.4	-	(75)		
² PR32		Sucrose	60	YE, CSL, KH ₂ PO ₄ , K ₂ HPO ₄	200	F	240	6.5	28	111.1	(56)
² VKM Y-2373			30	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(NO ₃) ₂	-		144	6.0	30	85	4.7
² K57	110		YE, (NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , Na ₂ HPO ₄	800	-	6.0	28	72.12	(64)		
² ACA-DC 50109	149.5			-	555	6.5		42.9	-	(65)	
² M1	100			200	144	6.0	29	27	(82)		
² H222	Glycerol	150	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄	-	F	171	30	132.6	16.4	(83)	
² H222-S4 (p67ICL1) T5		100	Proteose peptone, NH ₄ Cl, KH ₂ PO ₄			191				6.8	140
² A-101-1.14		Glucose hydrol	400♦	700	80	5.5		100	-	(34)	
² 1.31		Glycerol	200	-	S	132		124.5	3.9	(84)	
² 57			160			418		5.2	32.8	-	(54)
² Wratislavia AWG7			200	600	F	550		5.5	97.8	5.1	(85)
² Wratislavia 1.31			150	800		-			92.8	-	(72)
² Wratislavia AWG7						85.7			3.1	(86)	
² A-101-B56-5						100			72	6.8	57.15
² NG40/UV7	20		(NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(NO ₃) ₂		192	5.0	28	115	4.6	(51)	

Strain	Substrate	ISC (g/L)	N and P sources	Agitation rate (rpm)	Cult.	Time	pH	T (°C)	CA _{max} (g/L)	ICA (g/L)	References	
² LGAM S(7)1	Glycerol	120	YE, (NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , Na ₂ HPO ₄	185	S	236	5-6	28	35.1	-	(88)	
² ACA-DC 50109		164		180		600	6.1		62.5		(89)	
² NRRL YB-423		40		200		240	6.0		21.6	1.9	(90)	
² ACA-DC 50109		104.9		200-250	F				33.55	-	(91)	
² ACA-YC 5033		120	180	S	375	50.1	(92)					
² NCIM 3589		54.4	YE, KH ₂ PO ₄ , Na ₂ HPO ₄	150	F	72	5-6	30	77.4		-	(93)
² Wratislavia K1		150	YE, NH ₄ Cl, KH ₂ PO ₄	-		168	5.5		110	3.1		(46)
² A-101-1.22		125	800	1129		4.5	124.2		7.2	(94)		
² N15		170		YE, (NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(NO ₃) ₂			144	98	3.3	(95)		
² VKM Y-2373	Ethanol	<1.2	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(NO ₃) ₂ , yeast autolysate	-	F	145	4.5	28	116.8	-	(76)	
² N1		<1	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , yeast autolysate			-	21.6		6.4	(74)		
² VKM Y-2373	Canola oil	<10	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(NO ₃) ₂ .4H2O	800		144	5.0	29	22.6	90.5	(68)	
² NTG9		15	NH ₄ Cl, KH ₂ PO ₄ , K ₂ HPO ₄			216		30	137.5	49.2	(78)	
² NG40/UV7		Rapeseed oil	20		YE, (NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(NO ₃) ₂	168	4.5	28	175	5.6	(35)	
² 187/1	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(NO ₃) ₂			-	144	5.0	135		-	(45)		
² N 15		4.5	150			(96)						
² UOFS Y-1701	Sunflower oil	30	YE, NH ₄ Cl, K ₂ HPO ₄	160	S	240	5.8	26	18.7	46.8	(97)	
² TEMYL3		100	(NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , K ₂ HPO ₄ , Ca(-NO ₃) ₂ .4H ₂ O	150		408	5.5	27	66.2		(98)	
² SWJ-1b	Waste cooking oil	80	-	250	F	336	-	28	31.7	6.5	(66)	
² B9	Whey+ a	20a	YE, NH ₄ Cl, K ₂ HPO ₄	150	S	120	5.5	20	33.3	4.9	(99)	
² 57	Whey+ b	150b		-		290	5.2	30	49.23	2.57	(100)	
	Fructose	200				191			65.1	5.58	(101)	
² ACA-DC 50109	OMW + c	65c	-	180		-	5-6	28	28.9	-	(102)	
² ACA-YC 5033		35c	YE, (NH ₄) ₂ SO ₄ , KH ₂ PO ₄ , Na ₂ HPO ₄		144	6.0	18.9		(103)			
		80c			384	5-6	52		(104)			

¹: *Candida zeylanoides*, ²: *Yarrowia lipolytica*, ³: *Candida oleophila*,

ISC: Initial substrate concentration, N: Nitrogen, P: Phosphorus, Cult: Cultivation, S: Shake flask, F: Fermenter, Time: Fermentation time, h: hours, T: Fermentation temperature, CA_{max}: Maximum citric acid production, ICA: Isocitric acid, -: unspecified, ♦: ml/L

YE: Yeast extract, CSL: Corn steep liquor, OMW: Olive mill wastewater

^a: Lactose, ^b: Fructose, ^c: Glucose

citric acid) and productivity (0.04 g/g h) were obtained with C/N ratio of 367. Papanikolaou et al. (65) examined the citric acid production of *Y. lipolytica* ACA-DC 50109 using different C/N ratios (110, 138, 172 and 500 mol/mol). It was determined that when C/N ratio and initial glucose concentration were increased, biomass and citric acid concentrations were increased also. Maximum citric acid concentration (42.9 g/L) and biomass (7.2 g/L) were calculated with 149.5 initial glucose concentration and 500 C/N ratio. Additionally, Anastassiadis et al. (44) pointed out that biomass-specific nitrogen feed rate was the most effective factor influencing continuous citric acid production at yeast-based productions.

It was determined that the presence of phosphate in the medium has a significant effect on citric acid yield. Low phosphate levels have an enhancing effect on citric acid production and this effect is effective at the level of enzyme activity. Moreover, it has been stated that excess of phosphate leads to a decrease in carbon dioxide fixation and causes the formation of some sugar acids and the stimulation of growth (10). Additionally, production of citric and isocitric acid has usually been actualized under the restriction of yeast growth by nitrogen or other mineral components, such as sulphur, phosphorus and magnesium (45). Liu et al. (66) obtained citric acid from waste cooking oil using *Y. lipolytica* SWJ-1b and they indicated that the optimal concentration of ammonium sulphate added for the production was 0.2 g/L. In this condition, it was reported that 21.4 g/L citric and 5.8 g/L isocitric acid were obtained respectively. It was also found that extra ammonium sulphate was needed when waste cooking oil was used for citric acid production. Previous studies about the production of citric acid have shown that ammonium sulphate, ammonium chloride, yeast extract, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were commonly used as nitrogen and phosphorus source (Table 2).

pH

The initial pH value should be adjusted and optimized according to the microorganism, substrate and production technique (23). There are many studies showing that the initial pH value of the medium for yeasts generally varies between 5.0 and 6.5 (Table 2). It is indicated that pH is an effective factor for the productions by *Y. lipolytica* and neutral pH values are favored for citric acid productions (41). Förster et al. (67) obtained citric acid by *Y. lipolytica* H222-S4 (p67ICL1) T5 strain using sucrose in the fermentation medium. Fermentation was carried out in a bioreactor. According to the data obtained, when the production medium was adjusted at values of pH 5.0, 6.0 and 6.8, citric acid concentrations were calculated as 87, 127 and 140 g/L respectively. Moeller et al. (50) investigated the effect of pH value on the citric acid production on *Y. lipolytica* H222. As a result of the study, maximum citric acid (24.91 g/L), selectivity of the production (89.87%), yield (0.22 gCA/gglucose) and volumetric citric acid productivity (0.27 gCA/L h) were calculated at pH 6.0. Kamzolova et al. (68) examined the role of different pH values on the production of citric acid by *Y. lipolytica*

VKM Y-2373 strain. According to obtained results, they stated that lowest (12.1 g/L) and the highest (40.15 g/L) citric acid concentration were obtained at pH 7.0 and pH 4.5, respectively.

Aeration

Oxygen is a very important parameter for microbial growth and metabolite production (69). It is reported that the formation of all organic acids increases under high aeration and that the high oxygen requirement results from the metabolic balance of citrate formation and the high sugar concentration used (70). The dissolved oxygen concentration also affects the concentration and type of organic compounds produced by yeasts (69). Since citric acid production is also an aerobic process, the oxygen plays an important role in production (10,22,23). Citric acid yield increases depending on the increase in aeration rate, while the fermentation time is shortened (22). The interruption in aeration during batch fermentation was found to be quite harmful (10). It is very important to determine an optimal agitation rate for good production (100-180 g/L). On the other hand, high levels of agitation (1000 and 1200 rpm) can result in a shear stress on the cell-insoluble substrate interface on cell walls. Furthermore, it may lead to less cell-substrate interaction or decrease the cell viability (71). Another disadvantage of very high aeration rate (>1.0 vvm) is the low partial pressure of dissolved CO₂ in the broth. CO₂ is important as a substrate for pyruvate carboxylase, but excessive aeration may cause some losses. Furthermore, it was emphasized that high CO₂ levels were not found useful for the final concentrations of citrate and biomass (8). The aeration of the medium is applied at the same concentration throughout the fermentation process. It is found economically favorable to choose low oxygen levels at the beginning of fermentation. However, the combination of oxygen with air in the SmF increases citric acid production, but this is economically unviable. During the growth phase, high aeration levels may cause a large amount of foam formation. For this reason, it is emphasized that antifoaming agents should be added and mechanical defoamers should be used to solve this problem (23).

Rywińska et al. (72) used glycerol as substrate for citric acid production by *Y. lipolytica* Wratislavia AWG7 and *Y. lipolytica* Wratislavia 1.31. As an important result it was found that an increase in agitation and aeration rate had not any effect on dissolved oxygen concentration and citric acid production. Considering the research results, maximum citric acid concentration (92.8 g/L) and yield (0.63 g/g) were calculated by *Y. lipolytica* Wratislavia 1.31 at 0.24 vvm. Crolla and Kennedy (71) investigated the effect of fermentor agitation on citric acid production by *C. lipolytica* using n-paraffin. Eventually, it was determined that maximum citric acid concentration (12 g/L) was achieved using 1000 rpm at 7 days with fed-batch fermentation. Sabra et al. (73) proved that control of dissolved oxygen concentration enhanced the citric acid production by *Y. lipolytica* in cultures grown on glucose in mono or dual substrate fermentations. Moreover, it was determined that dissolved oxygen concentration had no significant effect on the production

which performed using glycerol.

As can be seen in Table 2, for citric acid production, agitation rates range from 120 to 200 rpm for shake flask cultivation and 200 to 1000 rpm for fermenter type.

Alcohols

It has been reported that ethyl alcohol, as well as carbohydrates, can be used in citric acid production and thus, high yields can be obtained (74). Lower alcohols such as methanol, ethanol, n-propanol, isopropanol and methyl acetate inhibit citric acid production when they are used in pure form, but if added as crude carbohydrates increase the production. The alcohols have been reported to neutralize the negative effects of metals in citric acid production (generally in amounts about 1 to 5%) (10,23). The use of methanol and ethanol in the optimal amount varies depending on the strain and composition of the medium. It was stated that alcohols are effective on membrane permeability in microorganisms by affecting the phospholipid composition (10,47). Pazouki et al. (75) reported that citric acid production by *C. lipolytica* NCIM 3472 was decreased with methanol addition to glucose based medium. On the other side, it was determined that using molasses (35 kg/m³) with methanol (3% v/v) increased the citric acid concentration. Arzumanov et al. (76) studied citric acid production by mutant strain *Y. lipolytica* VKM Y-2373 with using ethanol as the sole carbon source. Maximum citric acid concentration was calculated as 116.8 g/L in the batch system.

Trace Elements

Metal ions such as zinc, iron, copper, manganese and magnesium affect citric acid production (10). Some important integral components of metalloenzymes such as zinc and iron are involved in the yeast metabolism. Zinc is present as a component of dehydrogenases, aldolases, polymerases and proteases. It was determined that the need of zinc ions for yeasts increased in processes in which ethanol was used as a substrate. Moreover, it was indicated that NAD-dependent ADH activity under zinc restriction decreases significantly in different yeast species. Zinc is important because it acts as a stabilizer of the cell structure and some organelles (74).

Iron ions have been found to be effective on the activities of enzymes such as AIDH, AH, catalase, peroxidases and the components of the mitochondrial electron transfer chain. On the other hand, it was stated that iron ions that are present at intracellular concentrations determine yeast requirements for oxygen (74). It has also been reported that iron restriction may cause the inactivation of aconitase in the tricarboxylic acid cycle (15). Additionally, the high efficiency of citric acid may be provided with a small excess of copper ions (10).

Finogenova et al. (74) examined the citric acid production by mutant *Yarrowia lipolytica* N1 using ethanol. It was proved that the production of citric and isocitric acid from ethanol required high concentrations of zinc and iron ions. At the same time, it was stated that intracellular iron concentration determined whether citric acid or isocitric acid was predominantly

formed. In another study, it was proved that iron and copper salts had inhibition effects on citric acid production. It was reported that addition of manganese salt caused a decrease for the citric acid concentration. Maximum citric acid concentration (41.63 g/L) was achieved by *Y. lipolytica* 57 by addition of 0.008 g/L of zinc sulphate (77).

Manganese significantly affects the idiophase metabolism. While cell growth increases in the presence of manganese, sugar consumption and acidogenesis decrease drastically. Manganese deficiency results in the suppression of anaerobic and Krebs cycle enzymes, except for citrate synthase and this event leads to an overflow of citric acid. On the other hand, magnesium has been described as a metal involved both in citric acid production and cell growth (22).

Good et al. (78) studied the effect of micronutrients on citric acid production by *S. lipolytica* NTG9 using canola oil. As a result, it was determined that iron and zinc were found to have inhibitory effects on the production. Additionally, it was found that manganese stimulated citric acid synthesis. Liu et al. (66) investigated the effects of different magnesium amounts on the citric acid production obtained by *Y. lipolytica* SWJ1b using waste cooking oil. They reported that best concentration for the production was determined as 1 g/L under non-optimized conditions.

Conclusion

The fact that the demand for citric acid with the developing industry is increasing from year to year makes citric acid production more important. The mechanism of microbial citric acid production needs to be well understood to meet the demand and to make production more affordable and efficient. The most species widely used in citric acid production is *A. niger*, however, yeasts play a prominent role in citric acid production. Yeasts can be preferred in the production because of some advantages such as high conversion ratios, tolerance to high substrate concentrations and less susceptibility to metal ion impurities and the tolerance to low oxygen levels. *Y. lipolytica* is a good producer in citric acid production, it is also thought that various yeast species, including *C. zeylanoides* and *C. oleophila* can be used in citric acid production.

Abbreviations

NAD: Nicotinamide adenine dinucleotide; FAD: Flavin adenine dinucleotide; GTP: Guanosine triphosphate; GDP: Guanosine diphosphate; CO₂: Carbon dioxide; GRAS: Generally Recognized as Safe; JECFA: Joint FAO/WHO Expert Committee on Food Additives; SmF: Submerged Fermentation; SSF: Solid State Fermentation; C/N: Carbon to Nitrogen ratio; AMP: Adenosine monophosphate; AH: Aconitate hydratase; ADH: Alcohol dehydrogenase; AIDH: Aldehyde dehydrogenase.

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Conflict of interest

The authors declare that they have no competing interests.

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