

Citric acid production by yeasts: Fermentation conditions, process optimization and strain improvement

Seda Karasu Yalcin¹, M. Tijen Bozdemir² and Z. Yesim Ozbas^{3*}

¹Department of Food Engineering, Faculty of Engineering and Architecture, Abant Izzet Baysal University, Golkoy 14280, Bolu, Turkey.

²Department of Chemical Engineering, Faculty of Engineering, Hacettepe University, Beytepe 06532, Ankara, Turkey.

³Department of Food Engineering, Faculty of Engineering, Hacettepe University, Beytepe 06532, Ankara, Turkey.

*Corresponding author: e-mail: yesim@hacettepe.edu.tr, Phone: +90 312 297 71 12, Fax: +90 312 299 21 23

Citric acid is the most important organic acid produced by fermentation, widely used in food, pharmaceutical and chemical industries. Although *Aspergillus niger* is the traditional producer of citric acid, during the last 30 years the interest of researchers has been attracted by the use of yeasts for citric acid fermentation processes. Among the yeast species, *Yarrowia lipolytica* is known as a potential producer of citric acid. Environmental factors that have been shown to exert an effect on citric acid production are the type and concentration of carbon source of the fermentation medium, nitrogen and phosphate limitations, aeration, trace elements, initial pH and temperature. Besides the regulation of product formation by environmental conditions, strain selection and improvement has become the important factor. The improvement of citric acid producing yeast strains has been carried out by mutagenesis and selection. Because of annual growths in demand of citric acid, using alternative processes and strains for its production are in progress.

Keywords citric acid; yeasts; *Yarrowia lipolytica*; fermentation parameters

1. Citric acid production by yeasts

Citric acid is a commercially valuable organic acid, widely used in food, pharmaceutical and beverage industries [1]. It is the main additive used in the food industry. Citric acid is widely used to impart a pleasant, tart flavour to foods and beverages. It also contributes to the formulation of many foods as an acidulant, antioxidant, emulsifier or preservative [2, 3]. Among the uses of citric acid, about 70% is used in the food industry, and 10% in the cosmetics and pharmaceuticals [4]. There is a great worldwide demand for citric acid consumption due to its low toxicity when compared with other acidulants [3]. It is reported that the supply of natural citric acid is very limited and the demand can only be satisfied by biotechnological fermentation processes. Citric acid is known as the most important organic acid produced in tonnage by fermentation and is the most exploited biochemical product [3, 5]. The annual production of citric acid was reported as 700 thousand tons in 1993 [6], 1.4 million tons in 2004 [3], and 1.6 million tons in 2008 [7, 8].

A large number of microorganisms have been employed for citric acid production, but a few of them can produce citric acid in industrial scale [3]. It is reported that *Aspergillus niger* is almost exclusively used for industrial scale production of citric acid [5], but during the last 30 years the interest of researchers has been attracted by the use of yeasts as citric acid producers [9]. A number of different strains, mostly belonging to the *Candida* (*Yarrowia*) genus have been used for citric acid production, mainly in conventional batch processes, but also in continuous culture, and with immobilized cells [10]. The yeast species which were reported to produce citric acid are; *Candida* (*Yarrowia*) *lipolytica*, *Candida guilliermondii*, *Candida oleophila*, *Candida intermedia*, *Candida paratropicalis*, *Candida zeylanoides*, *Candida catenulata*, *Candida parapsilosis*, *Pichia anomala*, and some *Rhodotorula* species [6, 11]. Among the yeast species, *Yarrowia lipolytica* is known as a potential producer of citric acid [1, 12] and has been developed as a microbial cell factory for citric acid production in recent years [7]. The main advantages of using yeasts are mentioned as follows: Yeasts are characterized by greater resistance to high substrate concentrations than fungi, with comparable conversion rates and have greater tolerance to metal ions that allows the use of less refined substrates. Using yeasts also gives a better process control due to their unicellular nature [13, 14]. It was reported that citric acid production by yeast could be in the future an alternative to *A. niger* one, especially if the yeast biomass became an additive to animal food and not a by-product [15]. However, the major disadvantage of using yeasts is the simultaneous production of citric and isocitric acids [13, 16]. It is reported that the ratio of citric:isocitric acid can vary between 1:1 to 20:1 according to the yeast strain, carbon source and micronutrient concentration [13]. Selection of a yeast strain with high citric acid production and giving high citric acid:isocitric acid ratios has been reported as the principal step of a citric acid production process.

It is reported that more than 90% of the citric acid produced in the world is obtained by fermentation. The industrial citric acid production can be carried out in three different ways: by submerged fermentation, surface fermentation and solid-state fermentation or “Koji” process [3]. It is estimated that about 80% of the world citric acid production is obtained by submerged fermentation in stirred tanks of 40-200 m³ or larger airlift fermentors of 200-900 m³ capacity [17]. Submerged fermentation can be carried out in batch, fed batch or continuous systems, although the batch mode is

more frequently used [3]. Citric acid production by yeasts is exclusively carried out by submerged cultivation. The submerged fermentation process is desirable because of its higher efficacy due to higher susceptibility to automatization [18]. Solid-state fermentation is commonly used for *A. niger*. However, there have also been reports for yeasts [3].

Fermentative production of citric acid arises from a primary energy metabolism although it is non-growth associated [19]. Citric acid is a metabolite of energy metabolism, the concentration of which will only rise to appreciable amounts under conditions of metabolic imbalances [5, 20]. The first stages of citric acid formation in the cell involve the breakdown of hexoses to pyruvate in glycolysis, followed by its decarboxylation to produce acetyl – CoA. The CO₂ released during this reaction is not lost, but is recycled by pyruvate carboxylase in the anaplerotic formation of oxaloacetate. Normally, oxaloacetate would largely be supplied through the completion of tricarboxylic acid (TCA) cycle, allowing recommencement of the cycle by condensing with acetyl – CoA to form citrate, catalysed by citrate synthase. However, in order to accumulate citrate, its onward metabolism must be blocked. This is achieved by inhibiting aconitase, the enzyme catalysing the next step in the TCA cycle. Inhibition is accomplished by the regulation of environmental conditions, such as removal of iron, an activator of aconitase. Consequently, during citrate accumulation, the TCA cycle is largely inoperative beyond citrate formation, hence the importance of the anaplerotic routes of oxaloacetate formation [17]. It is reported in some studies that citric acid production by yeasts starts only after the nitrogen source depletes, that is, at the end of the exponential growth phase and with a production medium devoid of nitrogen source [10, 21]. Citric acid accumulation is a very complex process affected by fermentation conditions, during which various metabolic and morphological changes take place in a complex form [21].

2. Effects of fermentation conditions on citric acid production by yeasts

It is reported that citric acid production rates and yields are highly dependent on the type of microorganism, the type of substrate and the culture conditions [10]. Factors that have been shown to exert an effect on citric acid production are the type and concentration of carbon source of the fermentation medium, nitrogen and phosphate limitations, aeration, trace elements, initial pH, and temperature [5, 20].

2.1. Effects of carbon sources

Citric acid accumulation is strongly influenced by the type and concentration of carbon source. The type of the carbon source can be varied according to the microorganism used. Substrate profiles of *A. niger* and yeasts used for citric acid production can be extremely different from each other. For *A. niger*, sucrose is the most favourable substrate among the easily metabolized pure carbohydrates, followed by glucose, fructose and galactose. Molasses is often used as raw material for citric acid production by *A. niger*. However, in citric acid production processes of yeasts; many distinct substrates could be used besides carbohydrates. Some yeasts are known to be able to produce citric acid from a wider range of carbon sources than fungi do. The invention of citric acid production by yeasts dates back to investigations on their abilities to grow on *n*-alkanes as carbon sources and to produce various valuable substances such as citric acid. Various fractions of straight-chain paraffins were the preferred substrates according to the studies in 1970s [6]. However, the world oil crisis of 1973/74 almost entirely ended the exploitation of *n*-alkanes as feedstock for the industrial production of citric acid. It was then discovered that certain yeasts could also produce citric acid from carbohydrates, especially from glucose as carbon source. Many yeasts that grow on carbohydrate substrates have the ability to accumulate high concentrations of citric acid during tricarboxylic acid cycle respiration. However, *Y. lipolytica* is the only species known for its capability of maximizing citric acid production [22]. *Y. lipolytica* and other *Candida* strains are able to produce citric acid from various carbohydrates, whereby glucose has generated increasing interest [21]. Other carbon sources such as edible oils, ethanol, molasses, starch hydrolysates and pure or raw glycerol have been used to produce citric acid from *Y. lipolytica* [12, 16, 23]. It was also reported that lower isocitric acid concentrations were obtained in media containing glucose than those obtained when *n*-alkanes were used.

Although fructose assimilation levels of some yeasts are reported as low, molasses or invert sugar mixtures can also be used for citric acid production by yeasts [24]. In a study performed with *C. lipolytica*, it was reported that high citric acid yields were obtained when glucose, fructose and glycerol were used as substrates [25]. In a study carried out by Karasu-Yalcin et al. [26], growth and citric acid production characteristics of a novel endogenic strain *Y. lipolytica* 57 were investigated in comparison with a citric acid producer strain, *Y. lipolytica* NBRC 1658, in glucose and fructose media in a batch system. The best results for citric acid production were obtained when initial substrate concentrations were above 100 g/L. The ratio of citric:isocitric acid was changed between 11.20-16.62 in the examined media. It was also reported that the highest citric acid production rates were obtained with the endogenic strain by using fructose as substrate. Maximum citric acid concentration was obtained as 65.1 g/L in a medium containing 200 g/L of fructose with the novel strain. The changes in the maximum specific citric acid production rates and specific growth rates with the initial fructose concentration of the medium for two *Y. lipolytica* strains are shown in Table 1. Maximum specific growth rate was obtained at 50 g/L initial fructose concentration for both strains. Specific citric acid production rate was maximum at 100 and 150 g/L initial fructose concentrations for the endogenic and NBRC strains, respectively. Table 1 also represents the difference between the production characteristics of the two strains in the same medium and

conditions. Concentration of carbon source used for citric acid production should be very high (100 to over 200 g/L), which seems logical when a bulk product is produced economically [18]. High substrate concentration (120-250 g/L) is given as a factor enhancing microbial citric acid production [10]. It was reported that very low amounts of citric acid was produced at sugar concentrations below 50 g/L [18], which can also be observed from Table 1. In a study performed by Antonucci et al. [10], it was reported that both the specific rate of product formation and rate of substrate consumption increased at high substrate concentration. With respect to the biochemical basis for the relationship between citric acid accumulation and sugar concentration, it was shown that a high sugar concentration induces an additional glucose transport system. It is stated that the increased uptake of glucose under conditions of high sugar supply will counteract the inhibition of hexokinase by trehalose 6-phosphate [18].

Table 1 Variations of maximum specific citric acid production rate and specific growth rate with initial fructose concentration of the medium for two *Y. lipolytica* strains at initial pH 5.2 and 30°C [26].

S_{F_0} (g/L)	v_m (g citric acid/g mo.h)		μ (h ⁻¹)	
	<i>Y. lipolytica</i> 57	<i>Y. lipolytica</i> NBRC 1658	<i>Y. lipolytica</i> 57	<i>Y. lipolytica</i> NBRC 1658
0	0	0	0.003	0.003
20	6.7×10^{-4}	7.8×10^{-4}	0.012	0.096
50	0.0012	0.0026	0.029	0.122
100	0.0179	0.0042	0.024	0.106
150	0.0158	0.0055	0.025	0.063
200	0.0160	0.0048	0.024	0.063

S_{F_0} : Initial fructose concentration, v_m : Maximum specific citric acid production rate, μ : Specific growth rate

In another study by Karasu-Yalcin et al. [27], mannitol and glycerol were used for citric acid production by two *Y. lipolytica* strains and satisfactory results were obtained in both media. Mannitol was determined as a novel potential polyalcohol for citric acid production, suggesting the use of raw materials containing mannitol. Ethyl alcohol is also another substrate choice for citric acid production by yeasts in recent years [9, 28, 29]. Arzumanov et al. [30], reported citric acid biosynthesis by *Y. lipolytica* repeat-batch culture on ethanol. The highest citric acid concentration (105 g/L) and product yield (88.3%) were determined with 50% feed every three day, with ethanol concentration not exceeding 1.2 g/L, at pH 4.5 and 28°C.

Some yeasts which assimilate *n*-alkanes, can also assimilate some hydrophobic substrates such as fatty acids, triglycerites, oils and fats [31, 32]. *Y. lipolytica*, *C. tropicalis* and some *Rhodotorula* strains can produce citric acid by using edible oils [33]. For this purpose, sunflower, soybean, olive and rapeseed oil could be used [3]. Kamzolova et al. [2], reported that maximum citric acid concentration was 135 g/L when rapeseed oil was used in a batch system and its concentration was kept above 5 g/L. In another study, besides rapeseed oil, sunflower oil was suggested as a potential substrate for citric acid production by *Y. lipolytica* [14]. Maximum citric acid concentration obtained by *Y. lipolytica* 704 was reported as 40 g/L in a medium containing sunflower oil in fed-batch culture at pH 6.0. It was reported that the hydrolysis of vegetable oil by yeast lipase occurred with the production of two types of substrates, glycerol and fatty acids. Kamzolova et al. [14], demonstrated that *Y. lipolytica* 704 simultaneously utilize fatty acids and glycerol in the presence of vegetable oils.

For both increasing production yields and process economy, many natural substrate sources could be used for citric acid production. Table 2 represents various raw materials as well as pure substrates used for citric acid production by yeasts. The utilization of relatively impure raw materials such as crude, unfiltered starch hydrolyzates as well as raw glycerol marks a significant improvement in industrial citric acid production [6, 34]. In recent years, considerable interest has been developed in utilization of agricultural wastes including date seeds, whey [35-37], molasses, apple pomace, grape pomace, carob pod [38, 39], ram horn hydrolyzate [40, 41], and olive-mill waste water [42] for citric acid production. In a study performed by Wojtatowicz et al. [24], glucose hydrol was used as a substrate and 80 g/L citric acid was obtained at 100 g/L initial glucose concentration with a mutant strain of *Y. lipolytica*. Raw glycerol, by-product of bio-diesel production process, was also used as carbon source in citric acid production [42]. It was reported that during the manufacture of 10 kg of biodiesel by esterification of rapeseed oil, 1 kg of raw glycerol was also produced. In a study performed with acetate mutants of *Y. lipolytica*, a maximum citric acid concentration of 124.5 g/L was reached by using raw glycerol at an initial concentration of 200 g/L [22]. Whey, a significant by-product of dairy industry, can also be used as a natural fermentation medium for citric acid production. Sometimes, whey can be used after addition of some nutrients such as sugars or nitrogen sources. In a study by Abou-Zeid et al. [35], whey was used for citric acid production by *Y. lipolytica*, after addition of glucose, maltose, saccharose and date-seed hydrolysate at different concentrations. It was suggested that whey supplemented with date seed hydrolysate could be a potential cheap raw material for citric acid production. In another study, citric acid production characteristics of two *Y. lipolytica* strains were investigated in whey supplemented with glucose, fructose and some nitrogen nutrients. Maximum citric acid

concentration was obtained as 49 g/L in whey supplemented by fructose, with the use of a novel endogenic *Y. lipolytica* strain [43]. Grape must, naturally containing glucose and fructose as substrates (Table 2) was a potential medium supporting yeast growth and also citric acid production. The same authors also suggested that grape must could be used as a novel natural substrate for citric acid production by *Y. lipolytica* and concluded that enzyme activities related to substrate consumption of the strains could be affected by the composition of the grape must, indicating the complexity of natural media [43]. It is well known that the citric acid fermentation is greatly affected by the presence of some trace metals, so various techniques have been used to remove metallic inhibitory substances from the raw materials. Heavy metals can inhibit the growth of microorganisms, influence the ionic strength and pH of the medium, and are involved in the inactivation of enzymes associated with citric acid metabolism in the TCA cycle. Deionization or some chemical pretreatment methods should be applied to raw materials before using them as substrates for citric acid production [44].

2.2. Effects of the other fermentation parameters

The pH of fermentation medium is known as one of the most critical parameter for citric acid production processes by yeasts. It is reported that initial pH of the fermentation medium must be very well defined and optimized depending on the microorganism, substrate and production technique. When working with yeasts, initial pH values above 5 should be used since citric acid production is adversely affected below pH 5 [45]. It is reported that citric acid concentration decreases below pH 5 due to the accumulation of some polyalcohols like erythritol, arabitol and mannitol, instead of citric acid [6, 45]. Adverse effect of low pH is also explained by inhibition of citrate production in the cell and transport of citrate from cell membrane. In a study performed with *C. oleophila*, effect of pH on citric acid release by specific active transport system was investigated in a continuous system [46]. It was demonstrated that active citric acid transport system was a pH-dependent mechanism. In the same study, it was also reported that growth of the yeast, composition of biomass and citric acid release were directly affected by pH, and maximum citric acid concentration was obtained at pH 5. Karasu-Yalcin et al. [47], reported that maximum citric acid concentrations and citric acid yields obtained with *Y. lipolytica* 57 and *Y. lipolytica* NBRC 1658 were maximum between the initial pH range of 5.2-7.0 in a fermentation medium containing glucose. In the same study, maximum specific growth rates were obtained at pH 5.2 and 6.0 for the strain 57 and NBRC 1658, respectively. However, Kamzolova et al. [48], reported that citric acid production, and ratio of citric acid to isocitric acid depended on pH of the medium, and *Y. lipolytica* produced almost equal amounts of citric and isocitric acids at pH 4.5 while predominantly accumulated co-product isocitric acid at pH 6.0. A list of some data in the literature on citric acid production by several yeast strains at various fermentation conditions were presented in Table 2.

It is known that optimum temperatures for growth of cells and product formation may be different in some fermentation processes [49]. It is also quiteyly deliberated that determination of the optimum temperature for a batch process is necessary before scaling-up the process [50, 51]. Optimum temperature for citric acid production may change relative to the used strain and medium conditions. Effects of temperature on growth and citric acid productions of different strains were submitted in a number of studies. Temperature reported in various researches on citric acid production by yeasts was between 22-35°C. The optimal temperature range for both citric acid production and biomass models of *C. lipolytica* was stated as 26-30°C [50]. Rane and Sims [13], determined the optimal temperature for growth of *C. lipolytica* Y 1095 as 27°C. In another study, growth and citrate production of *C. lipolytica* were reported even at 35°C by using a medium containing glucose [52]. Karasu-Yalcin et al. [47], obtained that citric acid productions of *Y. lipolytica* NBRC 1658 and *Y. lipolytica* 57 both decreased when temperature was increased from 30 to 35°C. Although the maxima of cell dry mass were reported at 20°C for both strains, the best results for citric acid production were observed at 30°C in the same study.

Citric acid production is directly influenced by the concentration and nature of the nitrogen source in the fermentation medium [3, 54]. Corn-steep liquor, urea and ammonium salts have all been used as nitrogen sources in citric acid production by yeasts. The most suitable organic and inorganic nitrogen sources for citric acid production by *Y. lipolytica*, *C. paratropicalis* and *C. guilliermondii* have been reported to be yeast extract and ammonium chloride, respectively [19]. It was reported that citric acid production began after depletion of nitrogen source in the medium [55]. For this reason, the source of nitrogen and its concentration has a primary role on citric acid production, and high concentrations of nitrogen compounds may have negative effects on citric acid production rate. In a research performed by *C. lipolytica*, effects of different nitrogen sources on citric acid production were investigated and the best results were obtained with ammonium chloride [25]. In the same study, it was reported that the experiments were carried out with using 0 to 4 g/L of ammonium chloride, and the highest citric acid production was obtained when ammonium chloride concentration was 1.5 g/L. Imandi et al. [12], investigated citric acid production of *Y. lipolytica* from raw glycerol by using yeast extract as a nitrogen source with an optimum concentration of 0.2682 g/L. Karasu-Yalcin et al. [47], investigated the effect of ammonium chloride concentration on citric acid production by *Y. lipolytica* strains in the range of 0-6 g/L. It was reported that citric acid production increased by increasing ammonium chloride concentration from 0 to 2 g/L in a defined fermentation medium containing glucose. However, addition of this nitrogen source to whey at a concentration of 2 g/L extremely decreased citric acid production by approximately 50% [43]. It should be noted that the effect of nitrogen sources is mainly observed in chemically defined media, as no further nitrogen is necessary when some raw materials are used as carbon source [18].

Table 2 Citric acid production by several yeast strains at different fermentation conditions

Yeast strain	Fermentation type	Substrate	S ₀	pH & T	C _{c m}	Y _{p/s} (g/g)	Ref
<i>Y. lipolytica</i> N1	Submerged, continuous	Ethanol	0.01-1.0 g/L	pH 4.5 28°C	14.4-19.2 g/L	n	[28]
<i>Y. lipolytica</i> NCIM 3589	Batch	Raw glycerol	54.4 g/L	pH n 30°C	77.39 g/L	n	[12]
<i>C. lipolytica</i> Y 1095	Submerged, batch	Glucose	50-150 g/L	pH 5.5 27°C	13.6-78.5 g/L	0.50-0.79	[13]
<i>Y. lipolytica</i> A-101	Repeated batch	Glucose	92 g/L	pH 5.5 29°C	34.3 g/L	0.84	[53]
<i>C. lipolytica</i> Y 1095	Batch	<i>n</i> -paraffin	100-150 g/L	pH n 26-30°C	9.8 g/L	n	[50]
<i>Y. lipolytica</i> NCIM 3589	Solid state	Pineapple waste	n	pH n 30°C	202.35 g/kg	n	[1]
<i>C. oleophila</i> ATCC 20177	Submerged, continuous	Glucose	250 g/L	pH 5.0 30°C	57.8 g/L	n	[46]
<i>Y. lipolytica</i> A 101-1.14	Submerged, batch	Glucose hydrol (39.9% glucose)	400 mL/L	pH 5.5 30°C	> 80 g/L	0.93	[24]
<i>Y. lipolytica</i> UOFS Y-1701	Batch	Sunflower oil	30 g/L	pH 5.8 26°C	18.7 g/L	n	[33]
<i>Y. lipolytica</i> 1.31	Submerged, batch	Raw glycerol	200 g/L	pH 5.5 30°C	124.5 g/L	0.62	[22]
<i>Y. lipolytica</i> 187/1	Submerged, batch	Rapeseed oil	> 5 g/L	pH 5.0 28°C	135 g/L	1.55	[2]
<i>Y. lipolytica</i> ACA-DC 50109	Batch	Glucose in OMW-based medium	65 g/L	pH 5.0-6.0 28°C	28.9 g/L	0.82	[42]
<i>Y. lipolytica</i> NRRL YB-423	Batch	Pure glycerol	40 g/L	pH 6.0 28°C	21.6 g/L	0.54	[16]
<i>Y. lipolytica</i> LGAM S(7)1	Batch	Raw glycerol	80-120 g/L	pH>5 28°C	33-35 g/L	0.42-0.44	[23]
<i>Y. lipolytica</i> NBRC 1658	Batch	Mannitol	120 g/L	pH 5.2 30°C	20.25 g/L	0.17	[27]
<i>Y. lipolytica</i> 57	Batch	Pure glycerol	160 g/L	pH 5.2 30°C	32.80 g/L	0.21	[27]
<i>Y. lipolytica</i> NBRC 1658	Batch	Glucose in whey-based medium	100 g/L	pH 5.2 30°C	38.88 g/L	0.38	[43]
<i>Y. lipolytica</i> 57	Batch	Fructose in whey-based medium	150 g/L	pH 5.2 30°C	49.23 g/L	0.33	[43]
<i>Y. lipolytica</i> 57	Batch	Grape must	S _{G0} = 78.30 g/L and S _{F0} = 85.16 g/L	pH 5.2 30°C	32.09 g/L	0.48	[43]

S₀: Initial substrate concentration, S_{F0}: Initial fructose concentration of the grape must, S_{G0}: Initial glucose concentration of the grape must, T: Temperature, C_{c m}: Maximum citric acid concentration, Y_{p/s}: Citric acid yield based on consumed substrate, n: Not indicated, OMW: Olive-mill waste water

The effects of trace metal ions have been known for a long time and had been the key to the establishment of successful fermentation processes, although the effect is much more pronounced in the submerged fermentation [18]. Different explanations have been offered regarding the biochemical mechanism of action of trace metals [6]. Divalent metal ions such as zinc, manganese, iron, copper and magnesium have been found to affect citric acid production [3]. It

was reported that fermentation medium should contain metal ions especially manganese, iron and zinc at required amounts for inducing cell growth to obtain high citric acid yields [18]. 20% decrease in citric acid accumulation was reported when concentration of manganese exceeded 2 µg/L. The concentrations of metal ions below which citric acid is accumulated in high amounts are not absolute, and they depend on their relative proportion to other nutrients, particularly phosphate. It was reported that some researchers have claimed a particularly strong influence of iron on citric acid accumulation, which is, however, not supported by others. Iron limitation has been claimed to lead to an inactivation of aconitase, the enzyme catalysing further degradation of citric acid within the tricarboxylic acid cycle [18]. In a study performed by Anastassiadis and Rehm [46], the addition of iron has been found to enhance biomass formation and to affect continuous citric acid production significantly, for yeast *C. oleophila* growing on glucose. Finogenova et al. [28], reported that the main factor determining citric acid production in *Y. lipolytica* was growth limitation by nitrogen, whereas zinc or iron limitation conditions resulted in insignificant cell growth without citric acid production. Addition of zinc to the medium alleviated the zinc deficiency symptoms and increased the production of citric acid. In the same study, it was also indicated that the intensive citric acid production in *Y. lipolytica* required high intracellular iron amounts in the range of 0.2-2.5 mg/g, in conditions of nitrogen limitation growth of cells, grown on ethanol. At an intracellular iron content of 7.0 mg/g, citric acid production was completely inhibited. Karasu-Yalcin et al. [47], reported the effects of different mineral salts on citric acid production by two *Y. lipolytica* strains. It was found that iron and copper inhibited citric acid production for the used concentrations, while cell growth was positively affected by the addition of these minerals. By the addition of zinc sulphate into the fermentation medium, citric acid production of the two yeast strains were differently affected. When compared with the medium without the zinc sulphate, maximum citric acid concentration obtained by *Y. lipolytica* NBRC 1658 was decreased in the media containing the salt. The highest value of the maximum citric acid concentration analyzed was 41.63 g/L for the domestic *Y. lipolytica* strain 57 in the medium containing 0.008 g/L of the same zinc salt.

Since citric acid production is an aerobic process, accumulation of high amounts of citric acid is also dependent on strong aeration [18, 56]. The role of oxygen as a factor influencing citric acid production has been investigated with yeasts as well as with *A. niger* grown on various substrates [57, 58]. The necessity of high oxygen uptake is evident from the metabolic balance of citrate formation and the high sugar concentration used in the fermentation media [6]. Dissolved oxygen concentration influences the citric acid formation directly. The high demand of oxygen is reached by constructing appropriate aeration devices, which is also dependent on the viscosity of the fermentation medium [3]. In hydrocarbon fermentations, both aeration and agitation affect the extent of dissolved oxygen and available substrate in the medium [59, 60]. It is known that agitation increases the area available for oxygen transfer by dispersing air and insoluble substrate in the culture fluid in the form of fine bubbles. However, increased agitation can impose shear stress on cell walls as well as the cell-insoluble substrate interface. Therefore, an optimum agitation speed is required to maximize product production [59]. It was reported that the concentration of citric acid produced by *C. tropicalis* increased with an increase in dissolved oxygen concentration, whereas the isocitric acid concentration decreased at the same time. In another study, it was determined that citric acid production of *Y. lipolytica* 704 was completely inhibited by decreasing dissolved oxygen concentration from 60-95% to 28-30% saturation [58]. However, Kamzolova et al. [58], reported that oxygen requirements of *Y. lipolytica* N1 for growth and citric acid synthesis depended on the iron concentration in the medium. At relatively low dissolved oxygen concentrations and a high iron concentration, citric acid accumulation was as high as 120 g/L in continuous culture.

The optimization of fermentation conditions are of primary importance in the development of any fermentation process owing to their impact on the economy and practicability of the process [5]. Work to improve fermentative production of citric acid is continually in progress. Different techniques of production are continuously studied showing new perspectives for the production of this organic acid [3].

3. Strain selection and improvement for overproduction

By using conventional techniques of fermentation in stirred vessels and modifying fermentation parameters, citric acid productivity can be achieved up to certain level [4]. It is known that citric acid production characteristics of yeasts are highly dependent on strain diversity. This phenomenon was demonstrated in several strain-screening studies [2, 16, 61]. Basically, selection of strains begins with isolation from natural habitats according to common microbiological methods, followed by screening for their citric acid production capabilities. Several of such strains have been incorporated in governmental or industrial culture collections [6]. Besides, finding novel potential yeast strains to be used in citric acid production processes is interest of researchers in recent years.

To improve the process productivity and yield of citric acid, either physical or biological parameters require modification. In this respect, strain improvement has become the important activity [4]. The improvement of citric acid producing strains has been carried out by mutagenesis and selection. The most employed technique has been by inducing mutations in parental strains using mutagens. Among mutagens; γ -irradiation, UV irradiation and chemical mutagens are often used. It is reported that, UV treatment can frequently be combined with some chemical mutagens [3]. Successive treatments with physical and chemical mutagens followed by testing a large number of colonies will be necessary before strains with improved performance can be isolated. In selecting strains or mutants for large-scale

production, several important factors need consideration. These include stability of strains without undergoing physiological or biochemical degeneration upon subculture for mass propagation, non-utilization of the acid formed and non-formation of the other metabolic acids like gluconic, oxalic, and malic acid [4]. In a reported study, *C. lipolytica* Y-1095 was treated with two different mutagens, UV-irradiation and N-methyl-NT-nitro-N-nitrosoguanidine (NTG). It was determined that the UV-irradiation was better in inducing more productive isolates. Four mutants, each of them giving a yield of about 75-80% of citric acid more than the original parent, were selected [62]. In a study carried out with four commercial strains and two mutants of *Y. lipolytica*, a UV-induced mutant was found to be the most suitable for citric acid production from glucose hydrol [24]. In another study by Finogenova et al. [63], mutants of *Y. lipolytica* VKM Y-2373 with increased ability to synthesize citric acid were obtained by using UV- irradiation and NTG. It was reported that three mutants that displayed higher biosynthetic ability as compared with the initial strain, were selected after the treatments of UV irradiation or NTG. Additionally, three mutants were generated from the combined action of UV and NTG, and their biosynthetic activity exceeded that of the initial strain by 43.9%. In another research, a citrate nonutilizing strain which had an improved (by twofold) citric to isocitric acid ratio was isolated after mutagenesis of *Saccharomycopsis lipolytica* ATCC 20228 with NTG [64]. It was reported that a combination of mutagenesis and medium development had been shown to be complementary methods for improvement of citric acid production from canola oil by *S. lipolytica*.

Most strains of *Y. lipolytica* grow very efficiently on acetate as sole carbon source. Several mutants have been isolated and characterized, which were blocked in the utilization of acetate. It was reported that utilization of acetate is related to induction of glyoxylate pathway, which has a major role in citric acid metabolism. Mutants blocked in the activity of acetyl-coenzyme A synthetase were characterized. Acetyl-coenzyme A is needed for the induction of the glyoxylate cycle, which is not induced in acetyl-coenzyme A deficient mutants [65]. Rymowicz et al. [22], used three acetate-negative mutants of *Y. lipolytica* for citric acid production from raw glycerol and maximum citric acid concentration was obtained as 124.5 g/L with the strain 1.31.

For improving citric acid production characteristics of *Y. lipolytica*, some genetic engineering studies other than mutation were also reported. To extend the substrate spectrum of *Y. lipolytica* to sucrose-containing mixtures like molasses, the *S. cerevisiae* *SUC2* gene, expressed with the *XPR2* promoter and signal sequence, was introduced in different strains. Such Suc⁺-transformants were able to grow on sucrose and citric acid could be produced from molasses [31].

4. Conclusions

Today, yeasts are potential producers of citric acid and strains of *Y. lipolytica* are used in the industrial productions [22]. It was reported that the consumption of citric acid has an increase of 5% per year [2]. Because of the large and ever-increasing demand for citric acid, alternative cultivation processes involving yeast strains are used for its production [22]. In this respect, utilization of some renewable substrates such as agricultural wastes has been the interest of researchers in recent years [1]. Besides process optimization, there is a great interest in the possibility of obtaining different strains of yeasts giving high yields of citric acid. Improvement of citric acid producing strains can be achieved by mutation and selection studies. Strains with superior characters, such as enhanced citric acid production and increased rate of fermentation can be selected after subjecting the genetic material to physical or chemical mutagenic agents [66]. In order to make citric acid production by yeasts industrially more feasible, further investigations should be devoted to selecting a high citrate-producing strain and to optimizing its metabolic pathways and the operating conditions [10].

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