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Minireview

MINIREVIEW

Yarrowia lipolytica: a model yeast for citric acid production

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One sentence summary: The easier cultivation, higher productivity, lower requirements on C sources used, simpler strain engineering over molds and its classification as GRAS make *Y. lipolytica*-mediated processes very promising for CA production.

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ABSTRACT

Every year more than 2 million tons of citric acid (CA) are produced around the world for industrial uses. Although initially extracted from citrus, the low profitability of the process and the increasing demand soon stimulated the search for more efficient methods to produce CA. Currently, most world CA demand (99%) is satisfied by fermentations with microorganisms, especially filamentous fungi and yeasts. CA production with yeasts has certain advantages over molds (e.g. higher productivity and easier cultivation), which in the last two decades have triggered a clear increase in publications and patents devoted to the use of yeasts in this field. *Yarrowia lipolytica* has become a model yeast that proved to be successful in different production systems. Considering the current interest evidenced in the literature, the most significant information on CA production using *Y. lipolytica* is summarized. The relevance on CA yields of key factors such as strains, media formulation, environmental conditions and production regimes is thoroughly discussed, with particular focus on increasing CA productivity. Besides, the possibility of tuning the mentioned variables to reduce concomitant isocitric acid production—the biggest disadvantage of using yeasts—is analyzed. Available methods for CA purification/quantification are also discussed.

Keywords: citric acid; yeast; *Yarrowia lipolytica*; production conditions; industry

INTRODUCTION

Citric acid (CA), the most commonly used organic acid in industry, is the 2-hydroxypropane-1, 2, 3 tricarboxilic acid, an intermediate organic compound in the tricarboxylic acid (TCA) cycle. For this reason, CA is not only found in citrus and other fruits, but it is also produced by microorganisms.

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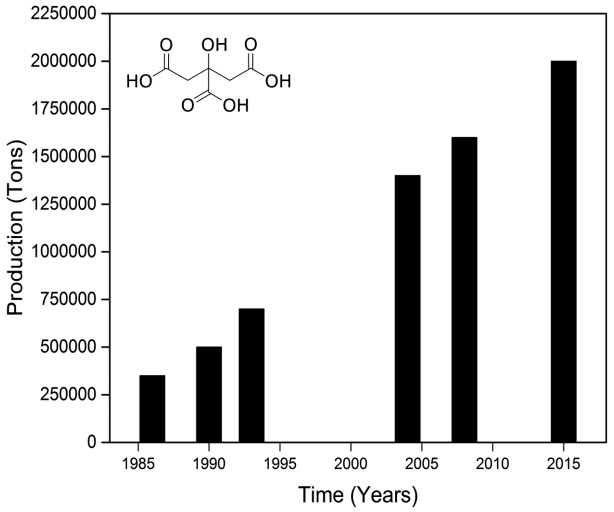

Figure 1. Total annual production of CA in tons. Information compiled from the published literature (Kubicek and Rohr 1986; Bu'Lock 1990; Roehr, Kubicek and Kominek 1993; Soccol, Vandenberghe and Rodrigues 2006; Sauer *et al.* 2008; Ciriminna *et al.* 2017). Inset: Molecular formula of CA.

CA is a weak organic acid, highly soluble in water (147.76 g 100 mL^{-1} at 20°C), with a molecular weight of 192.12 $g{\cdot}mol^{-1}$. It has three carboxyl groups, with three dissociation constants: $pKa_1 = 3.13$, $pKa_2 = 4.76$ and $pKa_3 = 6.39$. When CA loses a proton in aqueous solutions, citrate ions are produced, which can form salts with many metals. CA can crystallize as anhydrous or monohydrate form: if the process occurs at a temperature above 36.6°C the anhydride is produced, while the monohydrate is obtained at lower temperatures (Milsom 1987). Once crystallized, the monohydrate can be converted to the anhydrous form by heating over 74°C. Due to its characteristics as carboxylic acid, CA melts in the range of 135°C–152°C, and it is completely decomposed by 248°C (Wyrzykowski *et al.* 2011; Apelblat 2015).

Since it was first produced employing fungus in the 19th century, the world annual production of CA has been increasing due to its wide range of applications. With an expected annual growth of 3.7% till 2020 (Sauer *et al.* 2008), CA is becoming the most industrially produced chemical of microbial origin and the most used organic acid. Figure 1 shows the growing commercial production of CA since 1980s. The importance of CA mainly relies in the food industry, where it is employed as an additive against oxidative deterioration in flavor or color. This is because CA has long been accepted as Generally Recognized as Safe (GRAS) and approved by the Joint FAO/WHO Expert Committee on Food Additives (Merritt and Bouchard 1979).

CA is commonly used in soft drinks and wines, desserts, jams, jellies, candies, preserved fruits, frozen fruits and vegetable juices, fats, animal or vegetable oils and fish. Moreover, some products as fruits, vegetables or cheese are treated with CA solution before freezing or during emulsification, thus reducing enzymatic browning and loss of vitamin C. Due to its classification as GRAS, CA is also broadly used in pharmaceutics, where the free acid is used in combination with bicarbonates in effervescent products or as an acidulant in mildly astringent formulations or as excipient in tablets (Abou-Zeid and Ashy 1984; Soccol, Vandenberghe and Rodrigues 2006; Garcia-Fernandez *et al.* 2016). CA can also be used against several viruses (Ciriminna *et al.* 2017) and as antimicrobial agent in CA-coated manganese ferrite nanoparticles (Lopez-Abarrategui *et al.* 2016). Citrate is also involved in clinics, where it can be employed as an anticoagulant in blood transfusion. On the other hand, CA market is expanding because of the advances in biomedicine applications such as in nanomedicine, tissue engineering and drug delivery. In those fields, CA is gaining worldwide attention as an innocuous molecule due to many properties such as biodegradability, biocompatibility and non-toxicity (Dhillon *et al.* 2011).

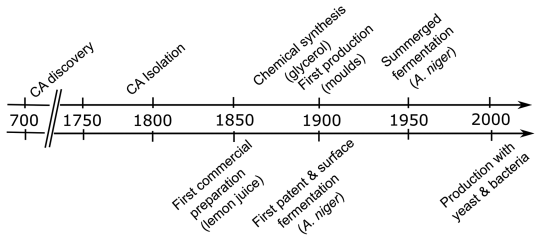

Figure 2. Time line for most important events in CA history.

Besides its use in food and pharmaceutical industries, CA has many other applications such as leather tanning, inks and dyeing and—taking advantage of its capacity of complexing heavy metals—in electroplating, metal extraction and cleaning (Dhillon *et al.* 2011, 2017). CA can be also employed in agriculture as micronutrient, for enhancing phosphorus availability in plants (Soccol, Vandenberghe and Rodrigues 2006) and against phytopathogens (Morgunov *et al.* 2017). Among other applications, CA has recently been reported as an emerging cross-linker and for environmental remediation uses (Awadhiya, Kumar and Verma 2016; Ciriminna *et al.* 2017).

CITRIC ACID PRODUCTION: FROM FRUITS TO FUNGI

Because of its wide range of applications, there has been a great interest in obtaining CA in industrial quantities along the years. CA history began in the eighth century, when it was discovered by the alchemist Abu Musa Jabir Ibn Hayyan. In 1784, Carl W. Scheele isolated CA from lemon juice and crystallized it, although the first commercial preparation dates from 1860 when CA was precipitated with calcium salts (Abou-Zeid and Ashy 1984). This method was employed up to 1920 (Milsom 1987). Meanwhile, in 1880 Adams and Grimaux synthesized CA from glycerol, but this chemical method did not result commercially competitive, and for that reason, alternative processes were required (Adam and Grimaux 1880; Max *et al.* 2010). A decade later, Wehmer observed CA could be produced as a by-product in oxalic acid production from *Penicillium glaucum* (Wehmer 1893), and in 1894, the first industrial fermentation plant was built. The site was closed years later due to the large and polluting process used (Wehmer 1894).

In 1913, Zahorsky obtained a patent for CA production using *Sterigmatoocystis nigra* (*Aspergillus niger*) (Zahorsky 1913). After that, many *A. niger* strains were found suitable CA producers and different production systems using this fungus were developed. In the first half of the 20th century, Currie developed CA fermentation in surface culture (Currie 1916, 1917). However, in 1950, submerged fermentation was successfully employed, which meant an improvement because it requires less space, it is less labor intensive and gives higher production rate (Grewal and Kalra 1995). The most important events related to CA discovery and production are summarized in Fig. 2. For more information on CA history and system methods with *A. niger*, Abou-Zeid and Ashy (1984); Kubicek and Roehr (1986); Milsom (1987); Soccol, Vandenberghe and Rodrigues (2006); Anastassiadis et al. (2008); and Apelblat (2015) can be consulted.

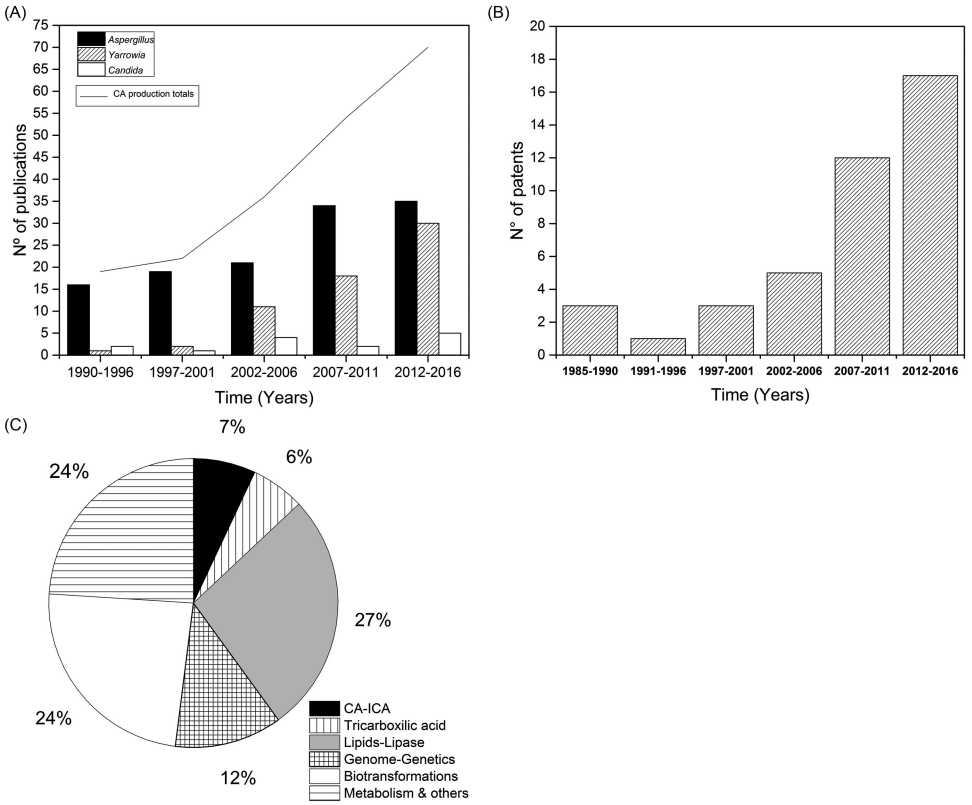

Figure 3. Academic and patent publications related to CA production. (**a**) Time evolution of CA production with microorganisms in academic publications, 1990–2016: solid black bars account for publications with the keywords 'citric acid + *Aspergillus* + production' in their title; solid white bars stand for contributions including 'citric acid + *Yarrowia* + production', and cross pattern bars result from the search 'citric acid + *Candida* + production'. The line over the bars collects results from all three publication searches. Source: Pubmed NCBI; (**b**) Time evolution of the number of patents alluding to CA production with *Y. lipolytica*, 1985–2016. Patent search was performed using the Thomson Innovation Database, a collaboration platform for searching and analyzing global patents integrated with analytics and workflow tools, which allow access to more than 40 databases from different countries. Descriptors used in the search: 'citric acid' + '*Yarrowia*' (checked in the title, abstract and claims). Only the first published documents of each family were considered. (**c**) Relative topic distribution of academic publications containing the words '*Yarrowia lipolytica*' in their title. Source: Pubmed NCBI.

Despite the wide use of *A. niger* in CA production, the traditional process from molasses has some disadvantages, e.g. it requires many stages, it is limited by raw materials sources and it is usually dangerous for the environment (Morgunov, Kamzolova and Lunina 2013), producing accumulation of significant amounts of solid and liquid wastes (Kamzolova, Lunina and Morgunov 2011). For that reason, bacteria and yeasts have been also investigated as alternatives for CA production. Since 1960s many researchers have been working specially with yeasts as potential producers (Abou-Zeid and Ashy 1984; Mattey 1992; Karasu Yalcin, Bozdemir and Ozbas 2010a), including *Candida* species (*C. guilliermondii, C. oleophila, C. intermedia, C. tropicalis, C. parapsilosis, C. fibriae, C. zeylanoides, C. catenulate, C. parapsilosis*), *Brettanomyces*, *Debaromyces, Endomyces, Endomycopsis, Pichia*, *Rhodotorula*, *Hansenula, Torula, Torulopsis, Trichosporon, Kloekera, Saccharomyces, Zygosaccharomyces* species and *Y. lipolytica* (Grewal and Kalra 1995; Roehr, Kubicek and Komínek 1996; Papagianni 2007; Anastassiadis et al. 2008; Max *et al.* 2010; Souza, Schwan and Dias 2014). Bacteria have also been proposed, e.g. *Arthrobacter, Alkaligensis, Achromobacter, Aerobacter, Bacillus, Brevibacterium, Corynebacterium, Klebsiella, Micrococcus, Nocardia,* and *Pseudomonas spp.* (Grewal and Kalra 1995; Soccol, Vandenberghe and Rodrigues 2006; Anastassiadis et al. 2008).

The interest in using yeasts over molds is due to a number of advantages, such as their resistance to high substrate concentrations and tolerance to metal ions, thus allowing the use of cheaper and raw substrates; and mainly to the fact they have higher fermentation rates, they are easier to cultivate than filamentous fungi, and they appear as better candidates for continuous fermentation (Grewal and Kalra 1995). Moreover, yeasts can be easily genetically modified (Fu *et al.* 2016), so strains can be engineered to increase CA production. On the other hand, the concomitant production of isocitric acid (ICA) is the major disadvantages of yeasts over molds.

In order to illustrate the academic interest in CA production with molds and yeasts registered during the last decades, and particularly the relevance and evolution of the genus *Aspergillus*, *Candida* and *Yarrowia*; Fig. 3a shows the number of publications related to their CA production as a function of time. Before 1990, publications on CA production were mostly related to the genus *Aspergillus*, and only around 15% of the publications contained the word '*Candida*' in its title. During 1990s, few works related to *Candida* and *Yarrowia* were published. However, since then, an ever-increasing interest in CA production with yeast—especially with *Y. lipolytica*—has been registered, justified by its simpler cultivation and manipulation compared to filamentous fungi (Fig. 3a).

The interest in CA production with *Y. lipolytica* is also illustrated in the industrial field by the increasing number of patents published during the last 30 years. In Fig. 3b, the evolution of this technology over time is shown. Patent search resulted in a universe of more than 90 patent families, which were manually reviewed and filtered to retain 41 documents related to CA production by *Y. lipolytica* (Please refer to Supporting

Table 1. Most cited patents alluding to CA production with *Y. lipolytica*. Descriptors used are the same as those detailed in Fig. 3b. The citation number includes all the patents in the family, in order to consider that the earliest document may not necessarily be the most cited one.

Publication number	Title	Assignee	Inventor	Publication year	Count of citing patents
WO2006009434A1	METABOLIC ENGINEERING OF XYLOSE FERMENTING EUKARYOTIC CELLS	DSM IP ASSETS MANAGE, DSM IP ASSETS BV, DELFT UNI of TECH, DE LAAT W T A M, KUYPER S M, PRONK J T, VAN DIJKEN J P, WINKLER A A	DE LAAT W T A, KUYPER S M, PRONK J T, VAN DIJKEN J P, WINKLER A A	2006	81
WO2008041840A1	METABOLIC ENGINEERING OF ARABINOSE-FERMENTING YEAST CELLS	DSM IP ASSETS MANAGE, DSM IP ASSETS NV, DSM IP ASSETS BV, DE WINDE H, PRONK J T, VAN DIJKEN J P, VAN MARIS A J A, WINKLER A A, WISSELINK H W	DE WINDE H, PRONK J T, VAN DIJKEN J P, VAN MARIS A J A, WINKLER A A, WISSELINK H W	2008	61
WO2010074577A1	XYLOSE ISOMERASE GENES AND THEIR USE IN FERMENTATION OF PENTOSE SUGARS	DSM IP ASSETS MANAGEM, DSM IP ASSETS BV, C5 YEAST CO BV, C5 YEAST CO LTD, DE BONT J A M, ROYAL NEDALCO BV, TEUNISSEN A W R H	DE BONT J A M, TEUNISSEN A W R H	2010	47
US5071764A	PROCESS FOR TRANSFORMATION OF YARROWIA LIPOLYTICA	PFIZER INC	DAVIDOW L S, DEZEEUW J R	1991	45
WO2009011591A2	NOVEL ARABINOSE-FERMENTING EUKARYOTIC CELLS	DE BONT J A M, ROYAL NEDALCO BV	DE BONT J A M	2009	43
WO2009109633A1	A PENTOSE SUGAR FERMENTING CELL	DSM IP ASSETS MANAGEM, DSM IP ASSETS BV	GIELESEN B E M, KLAASSEN P, VAN DER LAAN J M, VAN SUYLEKOM G P	2009	42
WO2009109631A1	A PENTOSE SUGAR FERMENTING CELL	DSM IP ASSETS BV	GIELESEN B E M, KLAASSEN P, VAN DER LAAN J M, VAN SUYLEKOM G P	2009	22
WO2004048559A1	METABOLICALLY ENGINEERED MICRO-ORGANISMS HAVING REDUCED PRODUCTION OF UNDESIRED METABOLIC PRODUCTS	BRO C, FLUXOME SCI AS, NIELSEN J, REGENBERG B	BRO C, NIELSEN J, REGENBERG B	2004	17

information for complete data on the mentioned patents, Table S1). Since 1991 this technological area has experienced a constant growth, with an average of 0.4 patents filled per year in 1990s, 1.4 in the first decade of the 21st century, and 3.0 since 2010. From this general perspective, it can be inferred that CA production with *Y. lipolytica* is currently in a developmental stage, with the highest growth within the last 10 years (Fig. 3b). In terms of owners (assignees), the distribution of patents evidence that most of the members of this group are manufacturing companies (roughly 80% of patent belong to profit institutions), indicating that this technology mostly belongs to the industrial sector. The greater patent owner is DSM IP ASSETS, with almost 29% of all filled applications. A second group includes Akad Wissenschaften DDR and Organo Balance GMBH, with almost 5% each, followed by 26 other entities (including companies, universities and mixed assignees) that share 2.4% of the total number of applications each. On the other hand, Delft Uni of Tech, Uni of Dresden Tech, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Centre national de la recherche scientifique (CNRS) and L'Institut National de la Recherche Agronomique (INRA) are among the academic institutions that own patents on this field.

To further illustrate the more relevant entities involved in CA production with *Y. lipolytica*, data from the most cited patents on the topic have been summarized in Table 1. Up to the present, the one from DSM IP ASSETS and Delft Uni of Tech is the one that has received the highest number of citations, suggesting its importance within the industrial field.

YARROWIA LIPOLYTICA

Yarrowia lipolytica belongs to the *Hemiascomycetes* family (Barth and Gaillardin 1997), and it is a 'non-conventional yeast', phylogenetically distant from *Saccharomyces cerevisiae* or other well-studied yeast species (Spencer, Ragout de Spencer and Laluce 2002; Barth 2013). *Yarrowia lipolytica* is considered a non-pathogenic microorganism and has been classified as GRAS by the American Food and Drug Administration (FDA). *Yarrowia lipolytica* first belonged to the *Candida* genus, because no sexual state had been described until the middle 1960s, when the perfect form was identified with two mating types (A and B) (Barth and Gaillardin 1997; Beopoulos *et al.* 2009). The genus name *Yarrowia* was proposed by van der Walt and von Arx (van der Walt and von Arx 1980) in the acknowledgement of David

Yarrow from Delft Microbiology Laboratory (Yarrow 1972). The species name '*lipolytica*' originates from its lipid hydrolyzing ability.

In terms of morphology, *Y. lipolytica* presents dimorphism, which means that this fungus is able to form yeast cells, pseudohyphae and septate hyphae (van der Walt and von Arx 1980). The predominant cell forms depend not only on the strain used, but also on some environmental conditions (Barth and Gaillardin 1996, 1997). In this respect, some carbon sources can induce mycelium development, as oleic acid, oleic alcohol or linoleic acid, together with some nitrogen sources such as meat extract (Ota *et al.* 1984; Barth and Gaillardin 1997). Strains and growth conditions can also determine different colony morphologies, from smooth and glistening to heavily convoluted and mat (Barth and Gaillardin 1997). For more information about *Y. lipolytica* physiology and genetics, Barth and Gaillardin (1997); Spencer, de Spencer and Laluce (2002); Beopoulos *et al.* (2009); Nicaud (2012) and Barth (2013) can be consulted.

In reference to its natural occurrence, *Yarrowia* is commonly isolated from consumables products such as cheese (e.g. Camembert, Livarot and Rokpol), yoghurts and sausages (Barth and Gaillardin 1996; Fickers *et al.* 2005b; Groenewald *et al.* 2014). An important character of this microorganism is its auxotrophy in thiamine, because it is not able to synthesize the pyrimidine backbone. However, *Y. lipolytica* can grow in many environments, such as lipid-rich (sewage and oil polluted media), marine or hypersaline media and a wide range of substrates (e.g. hydrocarbons, fatty acids, alcohols and acetate) may be employed in media composition (Bankar, Kumar and Zinjarde 2009; Beopoulos *et al.* 2010; Coelho, Amaral and Belo 2010; Nicaud 2012; Zinjarde *et al.* 2014; Liu, Ji and Huang 2015; Sekova, Isakova and Deryabina 2015). This adaptability allows *Y. lipolytica* to have plenty of applications in different fields. For example, the capability of growing on n-alkanes and 1-alkenes positions this yeast as a good candidate for bioremediation of hydrocarbon-contaminated soils and aquatic environments.

In the past decades, *Y. lipolytica* has also been studied as a model for physiological and genetic research (genes involved in the yeast-to-hyphae transition and cell cycle, mitochondrial functioning, protein secretion, lipid biogenesis and hydrophobic substrate utilization, among others) (Domínguez, Fermiñán and Gaillardin 2000; Fickers *et al.* 2005a; Nicaud 2012; Barth 2013; Liu, Ji and Huang 2015), and several uses in recombinant DNA have been developed (plasmids, VLPs and expression systems) (Bankar, Kumar and Zinjarde 2009; Barth 2013; Zhu and Jackson 2015).

In reference to recent application fields of *Y. lipolytica*, one-fourth of the corresponding academic contributions of the last five years have been related to metabolism (sugar consumption, biochemistry, stress and others; Fig. 3c), whereas approximately 12% of the publications dealing with *Y. lipolytica* have been related to genomics and genetics. *Yarrowia lipolytica* is an excellent model for obtaining many biotechnological products. For example, *Y. lipolytica* produces economically valued metabolites such as pyruvic acid and TCA intermediates (CA, ICA, α-ketoglutarate and succinic acid); aminoacids as lysine; proteins and enzymes as RNases, phosphatases, esterases, lipases and alkaline and acid proteases. In this regard, Fig. 3c shows that 27% of the publications of *Y. lipolytica* between 2012 and 2016 were related to lipids and lipase, 7% dealt with CA/ICA production, and 6% with the production of other TCA. The remaining percentage of recent publications related to *Y. lipolytica* involve other biotechnological transformations.

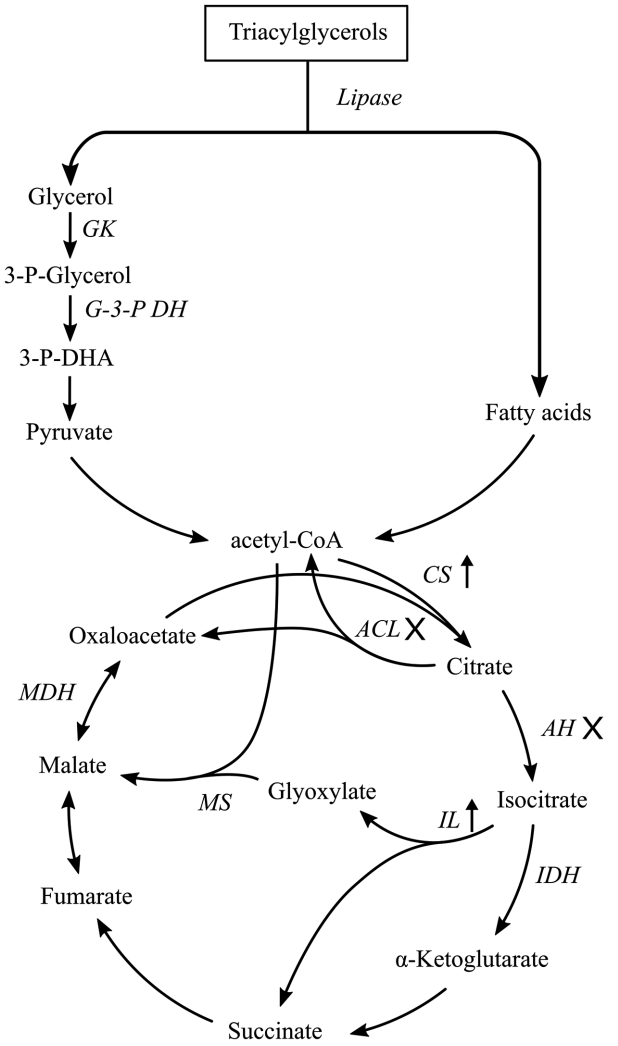

Figure 4. Triacylglycerols metabolic pathway; TCA and glyoxylate cycles. GK glycerol kinase; G-3-P DH glycerol-3-phosphate dehydrogenase; 3-P-DHA dihydroxyacetone-3-phosphate; CS citrate synthase; AH aconitate hydratase; IDH isocitrate dehydrogenase; IL isocitrate lyase; MS malate synthase; MDH malate dehydrogenase; ACL ATP-citrate lyase. Strategies to affect positively the intracellular levels of CA: the arrows indicate overexpression of CS and IL, while cross marks indicate disruption of genes encoding AH and ACL. Figure adapted from: Journal of the American Oil Chemists' Society, Biochemistry of citric acid production from rapeseed oil by *Yarrowia lipolytica* yeast, 88, 2011, 1965–1976, Kamzolova, Svetlana V. Lunina, Julia N. Morgunov, Igor G. Reprinted with permission of Springer.

CA SYNTHESIS IN *Y. LIPOLYTICA*

CA is produced in living cells as an intermediate of the TCA cycle. However, it can be accumulated in some bacteria and fungi by an induced abnormality in the cycle. The process is called Gaden type II fermentation, in which CA is produced from primary metabolism but it is not growth associated (Gaden 1959).

The most important requirement for CA accumulation in yeast is the deficiency in nitrogen source, because CA production starts when available nitrogen has been consumed (Karasu Yalcin, Bozdemir and Ozbas 2010a; Morgunov, Kamzolova and Lunina 2013). Citrate synthase (CS), the enzyme that converts CA from oxaloacetate and acetyl-CoA (Fig. 4), is negatively modulated by the concentration of ammonium ion in the medium (Il'chenko *et al.* 2002). For that reason, it is important to

ensure a high C/N ratio, so the excess of carbon is redirected to produce CA in the stationary growth phase. Moreover, isocitrate dehydrogenase (NAD-IDH), the enzyme that produces α-ketoglutarate from ICA, is allosterically activated by adenosine monophosphate (AMP). When nitrogen is limited, the enzyme AMP-deaminase cleaves AMP and produces $NH4^+$ ions, which in turn regulates negatively NAD-IDH. In that way, both CA and ICA accumulate in the mitochondria (Papanikolaou and Aggelis 2009; Papanikolaou *et al.* 2008a, 2013).

For CA production, high CS activity and low activities of other TCA cycle enzymes are required to avoid further metabolism of CA through the cycle (Finogenova *et al.* 1991, 2002; Kamzolova, Finogenova and Morgunov 2008; Kamzolova *et al.* 2011). This is usually confirmed by the overexpression or the disruption of the genes encoding the mentioned enzymes. For example, in Förster *et al.* (2007), the higher levels of expression of isocitratelyase (IL)-encoding gene ICL1 provoked a change in the CA/ICA ratio, favoring CA production. Several works with recombinant *Y. lipolytica* dealing with the specific effect of the TCA enzymes in CA production and accumulation can be found in the literature (Förster *et al.* 2007; Holz *et al.* 2009, 2011; Fu *et al.* 2016; Tan *et al.* 2016). Fig. 4 shows target enzymes that may be genetically engineered to increase CA synthesis.

Due to its metabolic versatility and growth, many carbon sources can be employed to produce CA from *Y. lipolytica*. Among them, substrates of increasing interest are oils, especially waste or cheap ones, because of their low cost and concomitant lipase production during their metabolism (Kamzolova *et al.* 2007; Kamzolova, Lunina and Morgunov 2011; Liu *et al.* 2015a). Metabolic events in oil consumption start with the hydrolysis by lipase, which occurs outside the cell. The enzymatic reaction yields glycerol and fatty acids (Fig. 4). Glycerol then enters into the cells and it is phosphorylated, via glycerol kinase (GK), and it is further metabolized mainly via glycolysis and TCA cycle (Fig. 4; Makri, Fakas and Aggelis 2010; Kamzolova, Lunina and Morgunov 2011). On the other hand, fatty acids activate the glyoxylate cycle (Fickers *et al.* 2005a). When lipids are oxidized, high amounts of acetyl-CoA are produced, suppressing the oxidation of pyruvic acid and stops the functioning of TCA cycle. However, key enzymes of the glyoxylate cycle (IL and malate synthase) actively catalyze the breakdown of ICA to succinic and glyoxylic acids with the formation of the final product malate, an intermediate of TCA cycle (Kamzolova, Lunina and Morgunov 2011).

In oleaginous yeasts, an important enzyme that changes intracellular levels of CA is the ATP-citrate lyase (ACL) that mediates the conversion of CA into oxaloacetate and acetyl-CoA, a precursor for fatty acids biosynthesis (Papanikolaou and Aggelis 2009). In fact, ACL is the key enzyme of lipid accumulation, because the excess of acetyl-CoA is essential for *de novo* synthesis of lipids (Fig. 4; Gonçalves, Colen and Takahashi 2014).

ICA is produced by the TCA cycle, right after CA synthesis, in a step catalyzed by aconitate hydratase (AH). For that reason, it is co-produced with CA in wild yeasts (Abe and Tabuchi 1968; Kamzolova *et al.* 2015a), and it is the major secondary metabolite that can decrease CA yields. ICA is considered the most undesirable by-product of highly pure CA, because the CA/ICA ratio affects the crystallization of the final product (Aurich *et al.* 2012; Rywińska *et al.* 2013). Moreover, separation of both acids is difficult, since CA and ICA are chiral compounds (Heretsch *et al.* 2008). Strains and media design can be tested for a high CA/ICA ratio, and different strategies can be employed. For example, CA synthesis can be favored over ICA by nitrogen deprivation, but not by phosphorus or sulfur deprivation (Kamzolova, Lunina and Morgunov 2011). Kamzolova, Finogenova and Morgunov (2008) reported the dependence of CA/ICA ratio with the pH of the medium. Low pH values stimulated CA transport through cell membrane, while ICA transport showed to be pH independent (Kamzolova, Finogenova and Morgunov 2008). Moreover, Finogenova *et al.* (2002) reported that an increase of the iron concentration promoted the formation of ICA. Another strategy to control CA/ICA production is to employ *Y. lipolytica* mutant strains: both AH genes disruption and the overexpression of IL can conduce to a decrease in ICA synthesis and the increase CA levels (Förster *et al.* 2007). On the contrary, AH overexpression provokes more production of ICA over CA (Holz *et al.* 2009). In fact, it is possible to suppress CA synthesis using inhibitors of the glyoxylate cycle, switching the process to ICA (Finogenova *et al.* 2005).

CA PRODUCTION WITH *Y. LIPOLITYCA*: KEY FACTORS AFFECTING THE FERMENTATION PROCESS

Wild type and mutant strains

One important factor in CA production, if not the most influencing, is the strain employed (Kamzolova *et al.* 2005; Levinson, Kurtzman and Kuo 2007; Karasu Yalcin, Bozdemir and Ozbas 2010a). Many *Y. lipolytica* strains are available in different collections (e.g. NRRL, ATCC, W, VKM, NCIM, UFLA, NCYC, LGAM, NBRC and ACA-YC). Several inbred lines have been obtained by different groups originating from German (H222), French (W29) and American (CBS6124-2) strains (Barth and Gaillardin 1996; Nicaud 2012). Although several strains can excrete CA as a response to a metabolic imbalance in nitrogen deficiency conditions, improvements in CA production are usually achieved by mutagenesis. Commonly, acetate or aconitase mutants or overexpressing IL are obtained increasing the CA/ICA ratio without changing the total acid amount (CA + ICA) (Förster *et al.* 2007; Holz *et al.* 2009, 2011; Karasu Yalcin, Bozdemir and Ozbas 2010b; Kamzolova *et al.* 2015b). The increased capacity of mutants in CA production compared with wild-type strains is illustrated in several works (Hamissa, Abou-Zeid and Redwan 1981; Wojtatowicz, Rymowicz and Kautola 1991; Anastassiadis et al. 2008; Rywińska *et al.* 2010). Actually, wild-type *Y. lipolytica* strains generally synthesize CA in tenths g L^{-1}, whereas mutant strains reach values above 100 g L^{-1}. Several techniques involving mutagenesis with chemical or physical agents and subsequent selection have been investigated (Kubicek and Karaffa 2001; Soccol, Vandenberghe and Rodrigues 2006). Table 2, Section a, briefly illustrates the differences in CA production using wild-type *Y. lipolytica* strains. In Table 2, Section b, the increased CA production of mutants over wild-type *Y. lipolytica* strains is exemplified.

Production media: carbon sources and other components

Carbon sources

For yeasts, and particularly for *Y. lipolytica*, one of the first substrates assayed as carbon sources were n-alkanes, which is correlated with the wide distribution and low prices of petroleum in 1960s, when yeasts began to be employed for CA production (Crolla and Kennedy 2004; Finogenova *et al.* 2005; Berovic and Legisa 2007). Nevertheless, not only the rise in petroleum price, but also the difficulties associated with the low solubility in water and the low resulting CA/ICA ratio, provoked the progressive disuse of n-alkanes as carbon sources (Berovic and

Table 2. Comparison of different parameters affecting CA productivity and CA/ICA ratio with *Y. lipolytica* in batch systems.

Variable	*Y. lipolytica* strain	Carbon source	Initial substrate concentration ($g\ L^{-1}$)	Time (h)	Final CA concentration ($g\ L^{-1}$)	CA yield ($g\ g^{-1}$)	CA/ICA ratio	Final productivity ($g\ L^{-1}\ h^{-1}$)	Reference
(a) Wild type strains on glucose	NRRL—Y1095	Glucose	150	96	50	0.61	7	0.54	Rane and Sims (1993)
	ACA-YC 5031	Glucose	30	72	1.9	0.11	n.a.	0.03**	Papanikolaou *et al.* (2009)
	ACA-YC 5028	Glucose	30	98	8.2	0.29	n.a.	0.08**	Papanikolaou *et al.* (2009)
	W29	Glucose	30	142	18	0.62	n.a.	0.13**	Papanikolaou *et al.* (2009)
	H222	Glucose	160	42	62	0.37	10.1	1.48	Moeller *et al.* (2010)
(b) Effect of mutations (Wild type = WT; corresponding mutant strain = M)	VKM 2373 (WT)	Sunflower oil	20	144	68	0.64	1.2	1.05	Kamzolova, Finogenova and Morgunov (2008)
	N15 (M)	Sunflower oil	20	144	150	1.32	30	1.56	Kamzolova, Finogenova and Morgunov (2008)
	WT A-101 (WT)	Pure glycerol	150	100	66.5	0.44	3.7	0.65	Rywińska *et al.* (2010)
	W 1.31 (M)	Pure glycerol	150	100	82	0.53	24	0.79	Rywińska *et al.* (2010)
	AWG7 (M)	Pure glycerol	150	100	82.9	0.53	27	0.66	Rywińska *et al.* (2010)
(c) Glycerol, effect of carbon source's purity	W 1.31	Pure glycerol	150	100	82	0.53	24	0.79	Rywińska *et al.* (2010)
	W 1.31	Raw glycerol	150	100	63.9	0.41	20	0.65	Rywińska *et al.* (2010)
	N15	Pure glycerol	170	144	98	0.7	30	1.14	Kamzolova *et al.* (2011)
	N15	Raw Glycerol	100	144	71	0.9	12.7	0.89	Kamzolova *et al.* (2011)
	NG40/UV7	Pure glycerol	20	192	115	0.64	25	0.6**	Morgunov, Kamzolova and Lunina (2013)
	NG40/UV7	Raw glycerol	20	192	112	0.9	21	0.58**	Morgunov, Kamzolova and Lunina (2013)
(d) Glucose and raw glycerol, effect of substrate concentration	LGAM (S)7	Glucose	34	134	10.5	0.32*	n.a.	0.08**	Papanikolaou *et al.* (2006)
	LGAM (S)7	Glucose	42	140	15	0.39*	n.a.	0.11**	Papanikolaou *et al.* (2006)
	LGAM (S)7	Glucose	52	220	20.1	0.41	n.a.	0.09**	Papanikolaou *et al.* (2006)
	LGAM (S)7	Glucose	150	555	42.9	0.57	n.a.	0.08**	Papanikolaou *et al.* (2006)
	ACA-YC 5033	Raw glycerol	70	187	28	0.42	n.a.	0.15**	André *et al.* (2009)
	ACA-YC 5033	Raw glycerol	90	220	31.2	0.43	n.a.	0.14**	André *et al.* (2009)
	ACA-YC 5033	Raw glycerol	120	275	50.1	0.44	n.a.	0.18**	André *et al.* (2009)

*:Yield values not informed in the references were calculated directly as the ratio between the final CA concentration and carbon source consumed; **: productivity values not informed were calculated directly as the ratio between the final CA concentration and fermentation time. n.a.: not available. In section (b) mutant *Y. lipolytica* N15 was derived from the wild strain VKM Y-2373, treated with N-methyl-N´-nitro-N-nitrosoguanidine and UV radiation, selected as incapable of CA utilization; strains W 1.31 and AWG7 are both acetate negative mutants derived from wild type strain A-101. In section (c) strain NG40/UV7 is another mutant of VKM Y-2373, also obtained with a combination of the mutagenic agents mentioned above.

Legisa 2007). Since that moment, different substrates have been investigated, starting with glucose and then moving to alternative and cheaper materials, especially due to the fact that *Y. lipolytica* is usually resistant to high concentrations of contaminants as metal ions or high sugar levels. Actually, *Y. lipolytica* is considered a bioremediating microorganism that is capable of consuming raw and unpurified materials, including wastewaters (Bankar, Kumar and Zinjarde 2009).

Figure 5 summarizes the carbon sources that have been most used for CA production with *Y. lipolytica*. Besides CA yield and productivity, the carbon source can also significantly affect the CA/ICA ratio (Fickers *et al.* 2005a). The use of oils, alcohols, glucose, *glucose hydrol*, starch and cellulose hydrolysates, molasses, invert sugar mixtures, dates and agroindustrial wastewaters have been proposed (Wojtatowicz, Rymowicz and Kautola 1991; Abou-Zeid and Khoja 1993; Shah *et al.* 1993; Arzumanov, Shishkanova and Finogenova 2000; Antonucci *et al.* 2001; Zarowska *et al.* 2001; Finogenova *et al.* 2002; Il'chenko *et al.* 2002; Aurich, Förster and Mauersberger 2003; Kamzolova *et al.* 2005; Moeller *et al.* 2007; Papanikolaou *et al.* 2008b; Kamzolova,

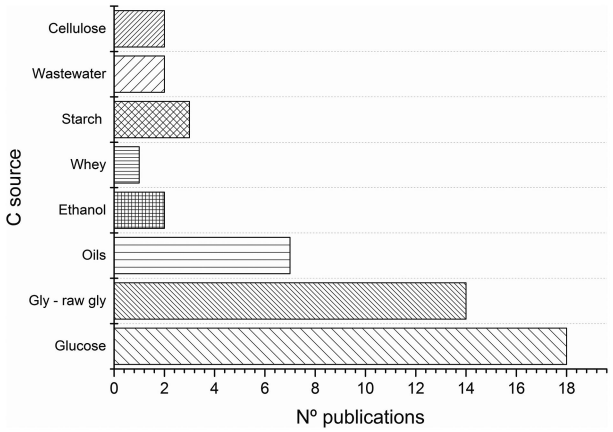

Figure 5. Publications related to CA production with *Y. lipolytica* using different carbon (C) sources of the last two decades. Keywords used in the search (title): 'carbon source + *Y. lipolytica* + CA'; source: Pubmed NCBI.

Finogenova and Morgunov 2008; Darvishi *et al.* 2009; Karasu Yalcin, Bozdemir and Ozbas 2009a; Mafakher *et al.* 2010; Liu *et al.* 2014).

Nowadays, one of the most promising substrates is glycerol (Papanikolaou *et al.* 2002; Rymowicz *et al.* 2010; Rywińska and Rymowicz 2010; Rywińska, Rymowicz and Marcinkiewicz 2010; Rywińska *et al.* 2011, 2012, 2013), since it is a by-product of the boom economy of biodiesel: approximately 10% of the biodiesel produced during the oil transesterification step with methanol results in residual (raw) glycerol (Zhou *et al.* 2008; Samul, Leja and Grajek 2014). For that reason, there is a growing interest in giving glycerol a high-added value use, thus reducing biodiesel production costs and preventing environmental problems (Papanikolaou and Aggelis 2009). Figure 5 illustrates the interest deserved by glycerol for CA production, with a publications number only exceeded by glucose. Many of the carbon sources described in academic articles are also mentioned in the patent literature (Supporting information, Table S1), suggesting their use within the industrial field. On the other hand, sugars as arabinose and xylose have deserved high attention in the patents of the last 10 years, probably due to the interest in valorizing lignocellulosic sources.

Among the plenty of contributions that have studied the use of glycerol (Rymowicz *et al.* 2006; Rywińska *et al.* 2009, 2010; Imandi *et al.* 2007; Papanikolaou and Aggelis 2009; Da Silva *et al.* 2012; Kamzolova *et al.* 2015b), some works have compared the utilization of pure against raw glycerol (Rywińska *et al.* 2009, 2010). According to Rywińska *et al.* (2009, 2010), the best results in CA production were obtained with pure substrates compared with residual glycerol (Table 2, Section c). Impurities present in the glycerol may contain iron, which can activate AH, thus consuming CA and decreasing yields. Similar results were obtained in Levinson, Kurtzman and Kuo (2007) and Kamzolova *et al.* (2011). On the contrary, in Morgunov, Kamzolova and Lunina (2013) CA production was very similar for both raw and pure glycerol, suggesting the importance of the presence of traces micronutrients or contaminants that could positively or negatively affect the growth of the microorganism in residual glycerol. In any case, in industrial processes the use of raw glycerol would make the process more profitable. In the last years, other alternatives and non- or low-cost carbon sources—some of them only regionally abundant—have been proposed (Karasu Yalcin, Bozdemir and Ozbas 2009a; Wang *et al.* 2013; Liu et al. 2015a; Arslan, Aydogan and Taskin 2016).

Carbon source concentration

High substrate concentrations generally favored CA synthesis (Rane and Sims 1993; Papanikolaou *et al.* 2002; Karasu Yalcin, Bozdemir and Ozbas 2010a). In this respect, it was observed that high sugar concentration induces an additional glucose transport system in cells, which in turn increases CA production (Kubicek and Karaffa 2001). For example, using glucose as carbon source, Antonucci *et al.* (2001) showed that high substrate concentrations at the initial production phase (85–250 g L^{-1} of glucose assayed) increased CA production and yield. Similar results were obtained in Papanikolaou *et al.* (2006) with glucose concentrations between 34 and 150 g L^{-1}. Moreover, CA production has shown to be low when sugar concentration was below 50 g L^{-1} (Kubicek and Roehr 1986; Karasu Yalcin, Bozdemir and Ozbas 2009b).

However, substrate inhibition was observed by Karasu Yalcin, Bozdemir and Ozbas (2009b), where a non-competitive model was found to be suitable for glucose and fructose above 150 g L^{-1}. In this case, the highest CA concentration was found for 150 g L^{-1} of both carbon sources, above which CA concentration decreased. Substrate inhibition was also reported by other authors when using glucose as carbon source (Moresi 1994; Moeller *et al.* 2007). Table 2, Section d, illustrates the effect of substrate concentration when using glucose and glycerol as carbon sources.

Yarrowia lipolytica mutant strains are commonly employed with higher substrate concentrations, probably because they can be more tolerant to these conditions than wild type strains (Rymowicz *et al.* 2006; Levinson, Kurtzman and Kuo 2007). The concentration of the carbon source used is also related with the regimen system employed to produce CA. According to Rywińska, Rymowicz and Marcinkiewicz (2010) in batch culture the carbon source is generally used in concentrations between 10% and 15%, but other systems as fed batch or repeated batch (RB) may use higher concentrated media.

Utilization of two substrates

Some authors have studied the simultaneous consumption of two carbon sources. In Papanikolaou *et al.* (2002) and later in Workman, Holt and Thykaer (2013), glucose and glycerol were provided together in equivalent concentrations. Remarkably, *Y. lipolytica* preferred glycerol to glucose when producing CA, since glycerol was taken in higher doses than glucose. A possible explanation for the observed behavior is that the energy excess produced by glycerol (C3) decreased the activity of C6 pathway during glycolysis, since glycerol could not block C6 transporters because their carriers are different (Papanikolaou *et al.* 2002). According to Workman, Holt and Thykaer (2013) three transporter genes were found in BLAST search, while for hexoses there is only one transporter. On the other hand, in Kamzolova, Lunina and Morgunov (2011), when glycerol and fatty acids (rapeseed oil) were provided, they were both consumed at the same time, suggesting no catabolism suppression. The utilization of two substrates simultaneously can increase carbon conversion efficiency, thus affecting the economy of the process due to the higher yields obtained (Babel 1990, 2009).

Nitrogen sources

As it was mentioned earlier, *Y. lipolytica* produces CA only in nitrogen deprivation conditions. For this reason, nitrogen is normally maintained at very low or null concentrations; commonly, 0.1–0.4 g L^{-1} is required for CA synthesis (Gonçalves, Colen and Takahashi 2014). However, some authors have suggested that the importance in nitrogen deprivation is not its concentration but, instead, the C/N ratio, which has also shown to be determinant for the resulting CA/ICA ratio produced (Papanikolaou *et al.* 2002; Levinson, Kurtzman and Kuo 2007; Levinson, Kurtzman and Kuo 2007; Ochoa-Estopier and Guillouet 2014).

Regarding the nature of the nitrogen source, there is a preference for ammonium salts, although urea, peptones, and malt extract are also used (Gonçalves, Colen and Takahashi 2014). Some authors have determined that $(NH_4)_2SO_4$ or NH_4Cl are the best sources for CA production, but usually with the addition of yeast extract (YE) (Abou-Zeid and Ashy 1984; Rane and Sims 1996; Da Silva *et al.* 2012). More recently, Liu et al. (2015b) replaced YE with corn steep liquor (CSL) with good results, suggesting CSL as an alternative and cheaper nitrogen source.

Other macrocomponents

Besides nitrogen deprivation, deficiencies in other macrocomponents such as sulfur and phosphorus, can also lead to CA synthesis by *Y. lipolytica*. In Rywińska, Wojtatowicz and Rymowicz (2006), authors assayed different limiting

conditions in the production of CA. Interestingly, *Y. lipolytica* produced CA efficiently in nitrogen and sulfur deficiencies (Y p/s = 0.69 and 0.74 g g^{-1}, respectively), although biomass yields were higher in sulfur deprivation. Besides, Kamzolova *et al.* (2011) reported that in *Y. lipolytica* N15, VKM-2373 and 212, phosphorus and sulfur limitations can be involved in the co-production of ICA together with CA (Kamzolova *et al.* 2011). However, while nitrogen exhaustion triggers secretion of CA and ICA, thiamine limitation condition causes secretion of mainly 2-ketoglutaric acid and pyruvic acid instead of CA (Finogenova *et al.* 2005; Vong, Au Yang and Liu 2016).

On the other hand, and despite yeasts are more resistant to metals presence than filamentous fungi, metal concentrations need to be controlled. Finogenova *et al.* (2002) studied the effect of iron under nitrogen limitation, and found that increasing intracellular iron up to 2.5 mg g^{-1} resulted in increasing CA production. Above 2.5 mg g^{-1}, a decrease in CA synthesis was observed, with inhibition starting from 7 mg g^{-1}. According to Gonçalves, Colen and Takahashi (2014) iron activates the production of acetyl coenzyme A, a CA precursor. However, the excess of iron activates AH, leading to ICA synthesis (Crolla and Kennedy 2001). Similarly, Finogenova *et al.* (2002) reported that in zinc limitation conditions (below 0.2 mg g^{-1}), *Y. lipolytica* N1 biomass production was low and the CA production was repressed. Above 0.2 mg g^{-1} of zinc, CA production was limited by nitrogen. In this condition, the increase in zinc concentration up to 1 mg g^{-1} caused an increase in CA production.

Additives used to enhance CA production

A number of additives have been recommended for enhancing CA production. Among them, oils and fats have been reported to increase CA production and additionally control foam formation (Grewal and Kalra 1995; Soccol, Vandenberghe and Rodrigues 2006). However, since those compounds can be consumed by *Y. lipolytica*, their concentration may be difficult to control. Another option is to add surfactants that affect cells permeability and thus CA synthesis. In Mirbagheri *et al.* (2011), the addition of Triton X-100 between 1% and 2%, increased the production of CA in 1.4–1.8-folds. However, above 2% of Triton, the surfactant could cause cellular lysis.

On the other hand, enhancement of CA production can be achieved by the addition of substances that inhibit TCA enzymes. This is the case of monofluoracetate, which converts to monofluorocitrate and inhibits AH, improving the CA/ICA ratio (Barth and Gaillardin 1997; Spencer, de Spencer and Laluce 2002). Nowadays, it is safer and easier to obtain AH mutants than employing monofluoracetate in culture media. Mutants with very low AH activity may be selected using monofluoroacetate resistance as criterion (Kubicek, Punt and Visser 2010).

Another strategy that has been reported to enhance CA production, involves the addition of foreign CA. Remarkably, CA could enhance its own production, although the mechanism is not known. In 2013, Morgunov *et al.* found that adding foreign CA into the culture media of a mutant could stimulate endogenous CA production. Similar findings were early reported for the strain ATCC 9773 (*Candida lipolytica*) (Fried 1972), although the beneficial effect of CA addition seems to depend on the strain used (Moeller *et al.* 2007).

Environmental conditions

Any microbiological process is governed by the environmental conditions in the cultivation medium. In the following paragraphs the importance of controlling temperature, pH, agitation rates and oxygen concentration, depending on *Y. lipolytica* requirements is exemplified.

Temperature

Yarrowia lipolytica can normally grow at temperatures below 32°C–34°C (Spencer, de Spencer and Laluce 2002; Beopoulos *et al.* 2009; Nicaud 2012), but the optimum temperature is usually between 26°C and 30°C. However, the optimum temperature for metabolites production may not be necessarily the same than the optimum temperature for growth, and it may vary with the strain (Shuler, Kargi and Kargi 2002; Karasu Yalcin, Bozdemir and Ozbas 2010a). The previous was observed by Moeller *et al.* (2007), who found that *Y. lipolytica* H222 grew optimally in the 30°C–34°C range, while the highest levels of CA production were obtained at 30°C. Similar results were observed by Karasu Yalcin, Bozdemir and Ozbas (2010b). Among the literature reviewed in this contribution, the temperature employed for CA production has been mostly in the 28°C–30°C interval.

pH

Other environmental factor that has to be controlled during CA production is extracellular pH. In yeast, optimum pH range is higher than for filamentous fungi (i.e. 2–3). This is because pH values below 4.5 may affect the permeability of yeast cell membranes, resulting in low transport of both substrate and products (Crolla and Kennedy 2004; Morgunov, Kamzolova and Lunina 2013). Moreover, at pH values lower than 5.5, polyols as mannitol and erythritol have been reported to be preferentially produced, instead of CA (Mattey 1992). Literature review indicates that optimal pH range may vary with the strain employed, although highest CA production is generally observed in the 4.5 – 7 interval (Morgunov, Kamzolova and Lunina 2013; Kamzolova *et al.* 2011; Moeller *et al.* 2007).

There is also evidence that pH can influence the CA/ICA ratio, especially with wild-type strains. In Kamzolova, Finogenova and Morgunov (2008), authors observed that *Y. lipolytica* VKM Y-2373 produced almost the same quantity of both acids at pH 4.5, whereas ICA was predominantly produced at pH 6. On the other hand, as it was seen for temperature, the optimum pH for production is not necessarily the same as that for growing (Karasu Yalcin, Bozdemir and Ozbas 2010b). In Timoumi *et al.* (2017), a detailed study of the stress response to pH perturbation on *Y. lipolytica* W29 is available.

Aeration and oxygen requirements

Yarrowia lipolytica is a strictly aerobic yeast, so aeration is a fundamental factor which greatly affects CA production. In general terms, aeration is critical for heat dissipation and mass transfer and it contributes to regulating the temperature of the fermentation media, water vapor and humidity, and volatile compounds produced during process (Shojaosadati and Babaeipour 2002; Dhillon *et al.* 2011). An increase in the availability of dissolved oxygen (DO) often results in improving yields of secondary metabolites (Suresh, Srivastava and Mishra 2009; Gonçalves, Colen and Takahashi 2014). Moreover, high DO levels often increase the proportion of yeast form in *Y. lipolytica* compared with mycelia or pseudomycelia forms (Rywińska *et al.* 2012; Bellou *et al.* 2014). Contrarily, an insufficient oxygen supply can lead to the non-functioning of the CS enzyme, thus directly affecting CA production (Il'chenko *et al.* 2002; Rywińska *et al.* 2012). In reference to the effect of DO on CA/ICA ratio, some authors have reported a positive effect, favoring CA synthesis (Okoshi *et al.* 1987; Rywińska *et al.* 2012), although other authors have found no influence (Finogenova *et al.* 1991; Rane and Sims 1994).

Table 3. Different systems for CA production using pure and crude glycerol.

Strain	Carbon source	System	CA (g L^{-1})	Q CA (g $L^{-1}h^{-1}$)	Reference
K1	Glycerol 200 g L^{-1}	Fed batch (begining)	72	0.72*	Rymowicz, Rywińska and Gładkowski (2008)
K1	Glycerol 200 g L^{-1}	Fed batch (48 h)	83	0.6 *	Rymowicz, Rywińska and Gładkowski (2008)
K1	Glycerol 200 g L^{-1}	Fed batch (48 & 97 h)	110	1.5	Rymowicz, Rywińska and Gładkowski (2008)
W 1.31	Glycerol 200 g L^{-1}	Fed batch (pulses)	126	1.05	Rywińska, Rymowicz and Marcinkiewicz (2010)
W 1.31	Glycerol 200 g L^{-1}	Fed batch (constant feeding)	155.2	0.6	Rywińska, Rymowicz and Marcinkiewicz (2010)
AWG7	Glycerol 200 g L^{-1}	Fed batch (pulses)	113.5	0.94	Rywińska, Rymowicz and Marcinkiewicz (2010)
AWG7	Glycerol 200 g L^{-1}	Fed batch (constant feeding)	157.5	0.6	Rywińska, Rymowicz and Marcinkiewicz (2010)
A-101–1.22	Raw glycerol 150 g L^{-1}	Batch	112	0.71	Rymowicz *et al.* (2010)
A-101–1.22	Raw glycerol 187.5 g L^{-1}	CR	96–107	1.42	Rymowicz *et al.* (2010)
A-101–1.22	Raw glycerol 250 g L^{-1}	RB	124.2	0.85	Rymowicz *et al.* (2010)

*Productivity values not informed in the references were calculated directly as the ratio between the final CA concentration and fermentation time. n.a.: not available.

CA production has generally been reported to be optimal within the 50%–80% saturation range (Rane and Sims 1994; Rywińska *et al.* 2012; Morgunov, Kamzolova and Lunina 2013). According to Rywińska *et al.* (2012), this DO saturation can be reached controlling agitation in the 800–900 rpm interval and aeration between 0.24 and 0.36 vvm. Higher agitation or aeration rates may affect CA productivities, probably for some undesired effects of shear stress (Moresi 1994; Makri, Fakas and Aggelis 2010). In this respect, iron complemented media could be an alternative for industrial production in order to decrease the need for high oxygen concentrations, and their associated costs (Finogenova *et al.* 2002; Kamzolova *et al.* 2003).

Production systems

Once strain and culture medium have been settled, depending on the purpose of the process the type of production system must be chosen. In the following paragraphs, different regimes that have been used for CA production are mentioned. An interesting overview of systems employed for CA production using pure and raw glycerol can be found in Rywińska *et al.* (2013).

Although most research on CA production has been carried out in discontinuous systems, modifications of batch cultures–as fed-batch and RB–or the employment of continuous cultures may enhance CA yield and productivity. Moreover, one of the advantages when using regimes different from batch is the possibility to increase substrate concentration. Regarding fed batch operation, intermittent or continuous feeding of nutrients can be employed, which may influence productivity and CA concentration.

Several works have studied CA production in fed batch systems, employing mainly glycerol as a carbon source. In Rymowicz, Rywińska and Gładkowski (2008), both CA and erythritol were obtained from raw glycerol, studding three types of feeding: from the beginning of the process; after 48 h; or twice after 48 and 97 h (Table 3). The greatest productivity (1.5 g $L^{-1}h^{-1}$) and CA concentration (110 g L^{-1}) were obtained using the last method. Later, in Rywińska, Rymowicz and Marcinkiewicz (2010), two fed batch systems were studied. In the first one 200 g L^{-1} of total glycerol were added in pulses, while in the second 300 g L^{-1} were supplemented at a constant rate. Higher productivity was achieved with the first system, although higher CA concentration was obtained with the second configuration (Table 3). In Morgunov, Kamzolova and Lunina (2013), a different technique for feeding was implemented: pulses of glycerol were added when the respiratory activity decreased due to the carbon source consumption (pO_2 changed by 10%). In this case, productivities around 0.8 g $L^{-1}h^{-1}$ were obtained.

Aiming to increase CA productivity, RB and cell recycle (CR) systems have also been studied. In Rymowicz *et al.* (2010) batch configuration led to the lowest productivity, whereas CR led to the highest one (Table 3). Repeated fed-batch (Moeller *et al.* 2010; Kamzolova *et al.* 2015b) and continuous cultivation systems have also been proposed for metabolic studies in *Y. lipolytica* (Rywińska *et al.* 2011; Ochoa-Estopier and Guillouet 2014). On the other hand, although the commonest regime in yeasts is submerged cultivation, some works reported the use of immobilized cells in RB or continuous air-lift fermenters (Kautola *et al.* 1991; Rymowicz *et al.* 1993). The previous is also illustrated in the contribution of Arslan, Aydogan and Taskin (2016), where the possibility of using non-sterile deproteinized whey in immobilized culture has been reported. In this respect, new technologies can still be developed to make CA production more economically sustainable.

DOWNSTREAM PROCESSES

Recovery and purification of CA

In industrial CA production, chemical purification of products generally consists in the precipitation of CA with calcium salt ($CaCO_3$) or $Ca(OH)_2$ at high temperatures (85°C–90°C), yielding calcium citrate. The precipitate is then washed with hot distilled water to remove sugars and media remnants. Then, it is subsequently treated with H_2SO_4, giving CA and $CaSO_4$ (gypsum) as a by-product. Since $CaSO_4$ is not soluble at room temperature, it can be separated easily by filtration. CA can then be decolorized with activated carbon and further purified using ion exchange chromatography or other techniques (Bjorn, Linden and Mattey 2002; Anastassiadis et al. 2008). Additional steps devoted to the concentration of CA by evaporation, crystallization or drying are usually implemented (Abou-Zeid and Ashy 1984; Pazouki and Panda 1998; Dhillon *et al.* 2011). In Wang *et al.* (2013), the purification method is thoroughly detailed and the crystals obtained are shown. Some authors have highlighted the difficulty to precipitate CA when ICA is present (Rywińska *et al.* 2010; Aurich *et al.* 2012). In this respect, the importance of the calcium proportion used for precipitation has been mentioned: adding 3.3 parts of $Ca(OH)_2$ per culture media induces CA precipitation, whereas adding two more parts of $Ca(OH)_2$ ICA precipitation is induced (Garibay, Ramírez and Canales 2004).

As an alternative to classical CA precipitation involving $CaCO_3/H_2SO_4$ steps, Kamzolova *et al.* (2015b) have recently produced technical grade sodium citrate from glycerol-containing biodiesel waste, in a process with several steps involving the use of activated charcoal, hydrochloric acid, alkalization and

Table 4. Advantages and disadvantages of methods used for CA purification and detection/quantification.

	Method	Advantages	Disadvantages
Purification	Precipitation (traditional method)	Cheap reagents	Unsatisfactory yields; difficulty to crystallize CA when impurities are present; large amounts of by-products salts and water wastes; high energy costs related with evaporation.
	Solvent extraction	Fast; variety of solvents; eco-friendly and relative cheap reagents	Coefficient of distribution of solvents; solvents disposal. Only extraction with tertiary amine method commercially viable
	Electrodialysis	Reduced effluent amount Continuous process; reduced product inhibition; improved yields and productivity	Special equipment; high energy costs Resin shelf-life; disposal; regeneration capacity. ISPR not fully developed
Identification & quantification	HPLC	Quantitative; high accuracy and precision; different methods; can differentiate between CA and ICA	HPLC equipment required; HPLC grade reagents
	Pentabromoacetone	Quantitative; different methods to produce pentabromoacetone	Many steps (time consuming) and several expensive reagents involved; interference with proteins and ICA
	Pyridine-acetic anhydride	Quantitative; need of few reagents; relative fast and simple	Pyridine toxicity; water interference; ICA interference
	TLC	Fast and simple; many systems available	Only qualitative; many interferences
	FT-IR	Rapid and accurate for samples such as wine, juices	Quality analysis depends on composition of the sample; many interferences
	Capillary electrophoresis	Rapid and simple; can differentiate between CA and ICA	Capillary electrophoresis equipment required; usually used in soft drinks, wine and juice
	Enzymatic	Quantitative; can differentiate between CA and ICA	High costs; instability of reagents

rotary evaporation. In order to avoid problems related to crystallization, such as inhibition caused by impurities and unsatisfactory yields and for reducing residues amount (including high amounts of water), solvent extraction has also been studied (Dhillon *et al.* 2011). Some works have been developed using different kinds of organic solvents, although amine extraction has been found to be the most promising method (Bauer *et al.* 1989; Thakre *et al.* 2016). Solvent extraction methods can be found in Pazouki and Panda (1998).

Aiming to reduce contaminant effluents generation at industrial scale, alternative methods of CA purification in the electrodialysis (ED) field have been proposed, including laboratory ED, electrodeionization, bipolar membranes and two-phase electroelectrodialysis (Pinacci and Radaelli 2002; Luo *et al.* 2004, 2017; Widiasa, Sutrisna and Wenten 2004; Nikbakht, Sadrzadeh and Mohammadi 2007; Sun, Lu and Wang 2017). Other alternative methods or a combination of techniques involving the use of weakly basic or ion exchange resins (Jianlong, Xianghua and Ding 2000; Peng 2002), or moving beds to facilitate crystallization (Wu *et al.* 2009; Teixeira *et al.* 2012) have also been proposed. These methods can function integrated with the fermentation, allowing continuous processes. In this respect, in situ product recovery (ISPR) has also been investigated, which involves a template added to the fermentation culture over whose surface CA crystallization occurs (Stark and von Stockar 2003; Dhillon *et al.* 2011). Table 4 summarizes advantages and disadvantages of the mentioned CA purification techniques.

Identification and quantification of CA

Nowadays, HPLC is the most used technique for CA detection (Apelblat 2015), but depending on the specific needs (accurate, quantification or simple detection) other methods can be employed.

Since the 20th century, different spectrophotometric methods for CA determination have been developed involving CA neutralization, oxidation, complexation, esterification and other types of reactions or their combinations (Apelblat 2015). In methods involving oxidation to pentabromoacetone, CA is measured spectrophotometrically using either sodium sulfide, sodium iodide, thiourea, potassium iodide or pyridine (Camp and Farmer 1967). Although the technique involving thiourea was originally developed for a rapid determination in blood, nowadays it is still used for fermentations samples (Wang *et al.* 2013; Liu et al. 2015b). Another spectrophotometric method still used today relays on the pyridine-acetic anhydride determination (Marier and Boulet 1958). It is important to note that water interferes in the reaction between CA and the reagents, decreasing the absorbance measurement and producing a nonlinear effect in the calibration curve (Odland 1971). However, 40 year later, some works still carry on assays with aqueous samples without no previous drying.

On the other hand, when the purpose is only to determine whether CA is present in a definite sample (no quantification being required), TLC methods can be employed. In Sočič and Gaberc-Porekar (1981), TLC was developed using p-dimethylaminobenzaldehyde and acetic acid anhydride for using in fermentation media samples. With this purpose, several other chromatographic systems have been proposed. For example, Lee, So and Heo (2001) developed a layer chromatography for detecting the presence of several organic acids (CA but also lactic, acetic, propionic, butyric and succinic acids), using acetone, water, chloroform, ethanol and ammonium hydroxide as eluent solvents. A system composed of formic acids, methyl ethyl ketone, acetone and water or formic acid, cineole and n-propanol was also early proposed (Fried 1972).

In the last years, FT-IR and capillary electrophoresis have been used increasingly for detection and quantification of CA, especially in soft drinks, wine or juice (Saavedra, García and Barbas 2000; Patz *et al.* 2004; Regmi, Palma and Barroso 2012). Enzymatic methods are also employed for both CA and ICA

quantification. These methods are based on the colorimetric detection of the NADH consumed when NAD^+ is produced during enzymatic reactions. In the case of CA, the acid is transformed into oxaloacetate by the ACL. Then, oxaloacetate can be converted into pyruvate or react to produce malate. Pyruvate can also react and produce lactate, and both reactions yield concomitantly transformation of NADH into NAD^+, which can be measured spectrophotometrically. Table 4 summarizes advantages and disadvantages of different techniques mentioned.

CONCLUSIONS AND PERSPECTIVES

Pharmaceutical, food and chemical industries utilize CA extensively because of its recognition as safe, pleasant acid taste, high water solubility and chelating and buffering capacities, among others. The wide range and new emerging applications of CA trigger the annual growth of its global production, making microbial processes a field of continuous research to satisfy the ever-increasing demand observed.

In the current contribution, the most relevant information on a model yeast for CA production such as *Y. lipolytica* has been summarized. The key effects on CA production of the strain, medium formulation, environmental conditions and production regimes used have been thoroughly discussed, with particular focus on available opportunities to increase CA productivity and control the CA/ICA ratio.

The review of published literature on CA production with *Y. lipolytica* strains evidences a great effort devoted to replace expensive substrates with cheaper sources. Particularly, for CA production with *Y. lipolytica*, non-expensive raw materials including agroindustrial by-products and residues such as glycerol or plant oils are promising substrates to be used as carbon sources, but quality and availability of the media components often depends on the location of the CA production site. Moreover, the use of pentoses (e.g. xylose and arabinose) derived from hemicelluloses offers an interesting opportunity, in view of the abundance of lignocellulosic material and the increasing interest on the valorization of by-products remaining from cellulose isolation. As it is extensively illustrated in the current review, the selection of any substrate should be further accompanied by the optimization of environmental conditions, which play a role not only on CA productivity, but also on the relative importance of concomitant ICA production. In terms of fermentation regimen possibilities, fed and RB seem the more promising options to increase productivity.

Despite large-scale microbial production of CA is still mainly carried out with *A. niger*, the easier cultivation and higher productivity reported for *Y. lipolytica* over molds, the lower requirements on the purity/quality of the carbon sources used, the classification of *Y. lipolytica* as GRAS, and the possibility of simpler strain engineering due to its unicellular nature; all contribute to making yeast processes very promising for CA production. The previous is illustrated by the positive evolution of published patents during the last years, as well as by the preponderant presence of profit institutions and companies among their owners. Finally, although conversion of the well-established technology of CA production with molds into yeast processes would imply important investments, the effort may be compensated if CA production becomes part of a biorefinery structure, in which enzymes, unicellular protein and single cell oils are concomitantly produced, making the process more profitable and environmentally friendly.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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