



Synthesis of Polyols and Organic Acids by Wild-Type and Metabolically Engineered *Yarrowia lipolytica* Strains

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Abstract

In the yeast *Yarrowia lipolytica*, sugar polyols and organic acids are derived from central metabolism, namely the citrate cycle and the pentose phosphate pathway. Although these metabolites have numerous applications in agro-food, chemical, and pharmaceutical industries, the main challenge is to gain productivity to obtain processes that are economically viable. *Y. lipolytica* is known for its ability to use industrial wastes or raw materials as feedstock and to grow at high cell density in large-scale bioreactor. Recent advances in metabolic engineering and synthetic biology allowed the development of efficient *Y. lipolytica*-based cell factories to bioconvert these feedstocks into added-value metabolites. This book chapter will focus on current knowledge on the synthesis of the most important polyols and organic acids in *Y. lipolytica*.

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1 Introduction

Several polyols and organic acids have been included in the top 12 platform chemical list of the US Department of Energy (Werpy and Petersen 2004). This highlights their importance in nowadays industries. Historically, most of these compounds have been produced industrially by chemical synthesis in a fossil fuel-dependent fashion. For instance, succinic acid (SA) could be produced by the catalytic hydrogenation of maleic anhydride (Ong et al. 2020) while erythritol, four-carbon sugar alcohol, could be obtained from dialdehyde starch in the presence of nickel as a catalyst at high temperatures (Carly and Fickers 2018).

In microorganisms, these compounds are also intermediates of biochemical pathways, such as SA that is involved in the Krebs cycle, or cell metabolites such as erythritol that is produced by certain osmotolerant yeasts in response to osmotic stress (Carly and Fickers 2018). Actually, there is an increasing demand from global drivers for greener and more sustainable processes to be developed in the context of circular economy. With the advent of molecular technologies such as metabolic engineering and synthetic biology, industrial microorganisms could now be engineered to bioconvert organic and industrial wastes into value-added chemicals using the so-called biorefinery concept. In this book chapter, we will focus on the non-conventional yeast *Yarrowia lipolytica* as a cell factory and detail recent advances made in strain and process engineering to produce polyols and organic acid of biotechnological interest.

2 *Yarrowia lipolytica*

Y. lipolytica is an Hemiascomycetes yeast isolated from dairy products, dry sausages, lipid-rich or alkane-containing matrix (polluted soil and sewage) or hypersaline environments such as seawater (Nicaud 2012). This yeast has specific metabolisms for alkanes and triglycerides catabolism (Fickers et al. 2005). Also, it presents other metabolic features such as the ability to sustain high osmotic pressure owing to the synthesis of a specific metabolite, namely erythritol, or the ability to synthesize and secrete enzymes (lipase, protease) with high efficiency (up to several g/L). Interest in *Y. lipolytica* started in the mid-60s for the production of single-cell proteins (SCP) from alkanes and later the yeast was a research focus for heterologous protein production. It is also considered as a model organism to study dimorphism as *Y. lipolytica* could grow either as yeast-like cell and pseudo-filamentous forms (Barth and Gaillardin 1996). *Y. lipolytica* has gained a GRAS status (Generally Recognized As Safe) that is of interest for the development of food-related applications (Groenewald et al. 2014). The complete annotated genome from strain E150 was released in 2004 (Dujon et al. 2004) and the genome of several *Yarrowia* strains are now publicly available (<http://gryc.inra.fr/>). Numerous molecular tools have been also developed such as efficient promoters and vectors for fine-tuned gene expression (Shabbir Hussain et al. 2016;

Sassi et al. 2016; Trassaert et al. 2017; Park et al. 2019), DNA assembly methods (Wong et al. 2017; Vandermies et al. 2017; Celińska et al. 2017; Larroude et al. 2019), gene disruption systems (Fickers et al. 2003), and genome editing tools including CRISPR/Cas9 (Larroude et al. 2020). All these technologies have been recently reviewed (Abdel-Mawgoud et al. 2018; Larroude et al. 2018; Bilal et al. 2020). Efficient strategies have been also developed to cultivate *Y. lipolytica* in bioreactors with the aim to maximize recombinant protein or metabolite production titers (Do et al. 2019; Vandermies and Fickers 2019). Also, review papers related to *Y. lipolytica* physiology have been published (Barth and Gaillardin 1996; Fickers et al. 2005; Nicaud 2012).

3 Organic Acid

Y. lipolytica is considered as a model organism to study the production of organic acids from different carbon sources including glycerol, glucose but also waste carbon sources (waste cooking oil, pineapple waste, olive-mill wastewater, or rapeseed oil, Table 1). Organic acids such as citric acid, iso-citric acid, succinic acid, itaconic acid, and α -ketoglutaric acid which are all intermediates of Krebs cycle will be discussed in this section. The main production data for these organic acids are summarized in Table 1.

3.1 Citric Acid

Citric acid (CA) is a tricarboxylic organic acid that plays a central role in the metabolism of all aerobic organisms. It is an intermediate of the Krebs cycle (Fig. 1). Based on its non-toxic, pH buffering, and chelating properties, CA has become an important industrial product. Applications for CA include the food, cosmetic, and pharmaceutical industries, where it is used as a flavoring agent, antioxidant, preservative, or pH buffering system. About 70% of the global total CA production is used by the food industry, 20% by detergent, and the remaining 10% by the chemical and pharmaceutical industries (Carsanba et al. 2019). The global CA production exceeded 2 million tons in 2018. The market is projected to reach a volume of nearly 3 million tons by 2024, with an expected Compound Annual Growth Rate (CAGR) of 4% between 2019 and 2024 (Fickers et al. 2020; Ciriminna et al. 2017). Historically, CA was isolated from citrus fruits, but this method was displaced by fermentation of sugars using the filamentous fungus *Aspergillus niger*. In bench-top bioreactors, a production titer of 130 kg/m³ could be obtained after five to eight days of batch cultivation (Ciriminna et al. 2017). Because of its high productivity, *A. niger* dominated the market for CA production. However, the processes developed for CA production from molasses are multi-stage, are not environmentally friendly, and are being limited by the raw material sources used (Morgunov et al. 2013).

Table 1 Some examples of organic acid produced in *Y. lipolytica*

| <i>Y. lipolytica</i> strain | Genetic modification | Medium | Organic acid, production (g/L) | References |
|-----------------------------|--|--------------------------------|--------------------------------|----------------------------|
| AJD-pADUTGut1/2 | <i>GUT1</i> ^{OE} , <i>GUT2</i> ^{OE} | Glycerol | 93, CA | Mironczuk et al. (2016) |
| SWJ-1b | Δ <i>acl1</i> , <i>ICL</i> ^{OE} | Inulin | 84, CA | Forster et al. (2007b) |
| | | Cooking oil | 80, CA | Papanikolaou et al. (2008) |
| W29 | <i>YHM2</i> ^{OE} , <i>AMPD</i> ^{OE} | Glucose | 97, CA | Liu et al. (2013) |
| NG40/UV7 | MT | Glycerol-containing wastes | 122.2, CA | Carsanba et al. (2019) |
| NCIM 3589 | WT | Pineapple waste | 202.4, CA | Wojtatowicz et al. (1991) |
| A101-1.22 | MT | Glycerol | 124.2, CA | Yuzbasheva et al. (2019) |
| A101-1.44 | MT | Glucose | 100, CA | Morgunov et al. (2013) |
| K57 | WT | Glucose | 72.1, CA | Rymowicz et al. (2010) |
| VKM Y-2723 | WT | Rapeseed oil | 70.6, ICA | Forster et al. (2007a) |
| | | Ethanol | 109.6, ICA | Kamzolova et al. (2016) |
| 704-UV4-A/NG50 | MT | Rapeseed oil | 86, ICA | Forster et al. (2007a) |
| Strain 20 | <i>ACO1</i> ^{OE} | Rapeseed oil | 72.6, ICA | Kamzolova et al. (2018) |
| PSA3.0 | EO | Glucose | 76.8, SA | Tan et al. (2013) |
| PSA02004 | WT | Sugarcane bagasse hydrolysates | 33.2, SA | Yang et al. (2017) |
| | | Fruit and vegetable waste | 140.6, SA | Li et al. (2018a) |
| H222-AZ2 | <i>Ylshd5</i> ^{OE} | Glycerol | 25, SA | Bondarenko et al. (2016) |
| PGC01003 | <i>Ylshd5</i> | YPD medium | 65.7, SA | Jost et al. (2014) |
| | | Food waste hydrolysate | 87.9, SA | Jost et al. (2014) |
| | | Glycerol | 160, SA | Yuzbashev et al. (2010) |

(continued)

Table 1 (continued)

| <i>Y. lipolytica</i> strain | Genetic modification | Medium | Organic acid, production (g/L) | References |
|-----------------------------|--|----------------|--------------------------------|------------------------------|
| PGC202 | <i>Ylshd5</i> , <i>ach</i> ; <i>PCKEO</i> , <i>SCS2^{EO}</i> | Glycerol | 110.7, SA | Cui et al. (2017) |
| | | Mix food waste | 31.7, SA | Li et al. (2016) |
| PO1f-CAD | <i>CAD^{EO}</i> | Glucose | 0.033, IA | Blazeck et al. (2015) |
| PCM-ACO-MFS | <i>MTT^{EO}</i> , <i>CAD^{EO}</i> | Glucose | 22, IA | Zhao et al. (2019) |
| H355 | WT | N-alkanes | 195, KG | Otten et al. (2015) |
| H355A | <i>IDH1^{EO}</i> , <i>PYC1^{EO}</i> | Raw glycerol | 186, KG | Chin et al. (2015) |
| VKM Y -2412 | WT | Ethanol | 172, KG | Chernyavskayaá et al. (2000) |
| | | Rapeseed oil | 102.5, KG | Yin et al. (2012) |
| RoPYC2 | <i>RoPYC2^{EO}</i> | Glycerol | 62.5, KG | Kamzolova et al. (2012) |

Abbreviations: OE: gene overexpression, WT: wild-type, MT: mutant strain (obtained by chemical and/or UV mutagenesis), ME: evolved strain, CA: citric acid, ICA: iso-citric acid, SA: succinic acid, IA: itaconic acid, KG: ketoglutarate. *GUT1*: glycerokinase, *GUT2*: glyceraldehyde-3-phosphate dehydrogenase, *ACLI*: ATP citrate lyase, *ICL*: isocitrate lyase, *YHM2*: mitochondrial citrate carrier, *YIAMPD*: adenosine monophosphate deaminase, *ACO1*: aconitase, *Ylshd5*: succinate dehydrogenase subunit 5, *ACH*: acetyl-coenzyme A hydrolase, *PCK*: phosphoenolpyruvate carboxykinase, *SCS2*: succinyl CoA synthetase, *CAD*: cis-aconitate decarboxylase, *MTT*: mitochondrial cis-aconitate transporter, *IDH1*: isocitrate dehydrogenase, *PYC1*: pyruvate carboxylase, *RoPYC2*: pyruvate carboxylase

In a study by Kamzolova and Morgunov, 43 different wild yeast isolates were evaluated for CA production (Kamzolova and Morgunov 2017). Among these, *Y. lipolytica* was found as the best CA producer on minimal medium with titer and yield of 85 g/L and 0.70 g/g, respectively (Kamzolova and Morgunov 2017). Based on this special ability and other biochemical characteristics, *Y. lipolytica* has been recognized as a potential host to produce Krebs cycle intermediates, including CA. In addition, cultivation conditions such as pH 4.5–5.0, temperature of 28 °C, and glycerol concentration in culture medium from 20 to 80 g/L were found the optimal values for CA production (Morgunov et al. 2013). The main drawback of using *Y. lipolytica* to produce CA is the co-production of iso-CA (ICA) in high amounts (Cavallo et al. 2017). However, several authors have reported that nitrogen limitation during yeast growth could alleviate this disadvantage. Indeed, nitrogen starvation activates adenosine monophosphate (AMP) deaminase (Beopoulos et al. 2009a; b). This leads to a decrease in the mitochondrial AMP concentration that inhibits the iso-citrate dehydrogenase. This finally blocks the Krebs cycle at the

GUT2 could be crucial for CA production (Mironczuk et al. 2016). With strain *Y. lipolytica* AJD pADUTGut1/2, CA titer of 63.9 g/L was obtained at pH 3.0 which corresponds to a 14.5-fold increase as compared to the wild-type strain A101 under the same experimental conditions. On glycerol-based medium, a CA titer of 93 g/L was obtained at pH 6.0 (Mironczuk et al. 2016). The purification of glycerol is an expensive process, leading to CA production process unprofitable. As an economical alternative, crude glycerol, which is a direct byproduct of biodiesel production or saponification could be used. However, the utilization of such substrates could be limited by their high content of impurities such as salts, sodium hydroxide, methanol, or other organic compounds, which could be toxic for yeasts or hinder the production process. Despite these facts, *Y. lipolytica* can easily grow on such substrates and was found to produce CA with titer of 76 g/L at pH 3 (Rzechonek et al. 2018a). Overexpression of gene *ICLI* encoding iso-citrate lyase led to a decrease in ICA production from 10–12 to 3–6% (Forster et al. 2007b). However, the overexpression of *ICLI* did not influence the total production of CA and ICA. When *ICLI* overexpression was coupled with the disruption of *ACLI* gene (*YAL10E34793g*) encoding ATP citrate lyase and overexpression of the *K. marxianus* *INU1* gene encoding inulinase, CA and ICA titers of 84.0 g/L and 1.8 g/L were obtained, respectively, from inulin (100 g/L) after 214 h of culture in a 2L scale bioreactor (Liu et al. 2013). Besides this, overexpression of genes encoding mitochondrial citrate carrier (*YHM2*, *YAL10B10736g*) and adenosine monophosphate deaminase (*AMDP*, *YAL10E11495g*) in *Y. lipolytica* wild-type strain W29, a CA titer with productivity and yield from glucose of 97.1 g/L, 0.93 g/(L h) and 0.5 g/g were obtained, respectively, during culture in bioreactor (Yuzbasheva et al. 2019).

The CA production by different *Y. lipolytica* wild-type strains has been also reported. With an acetate-negative mutant strain *Y. lipolytica* A101-1.22 grown on glycerol-containing waste from the biodiesel industry, a CA titer of 124.2 g/L was obtained in repeated batch culture (Rymowicz et al. 2010). From a screening study, strain K57 was found able to produce CA with titer of 72.1 g/L and yield of 0.77 g/g on glucose-based medium during batch culture (Carsanba et al. 2019).

Industrial wastes have been also considered as possible carbon substrates for CA production (Wojtatowicz et al. 1991; Imandi et al. 2008; Papanikolaou et al. 2008; Morgunov et al. 2013; Liu et al. 2014; Morgunov and Kamzolova 2015). Using mutant strain *Y. lipolytica* NG40/UV7 and glycerol-containing wastes, CA production was found to increase by 40.6% (122.2 g/L) as compared to that obtained with pure glycerol (Morgunov et al. 2013; Morgunov and Kamzolova 2015). Pineapple wastes were also used successfully with a production titer of 202 g/kg of waste in optimized conditions (namely, 0.34% yeast extract, 70.71% moisture content of the substrate, 0.64% KH_2PO_4 , and 0.69% Na_2HPO_4) (Imandi et al. 2008). Olive-mill wastewater (OMW) blended with glucose was used also for CA production in nitrogen-limited conditions. The titer obtained was 28.9 g/L (Papanikolaou et al. 2008). With *Y. lipolytica* SWJ-1b grown in the presence of waste cooking oil (80 g/L), a CA titer of 31.7 g/L was obtained within 336 h of culture in a 10 L bioreactor (Liu et al. 2014). The highest CA concentration,

100 g/L, was obtained by the mutant strain A-101-1.14 following the shortest growth (24 h) and production (80 h) phases from potato starch (Wojtatowicz et al. 1991).

3.2 Iso-citric Acid

Iso-citric acid (ICA) exists in the form of four stereoisomers, of which only the threo-Ds-form is an intermediate of Krebs cycle (Kamzolova et al. 2016, 2018). A promising application of ICA is sports medicine. ICA has a marked energetic and anti-hypoxic effect and can be used as a physiological stimulant of sportsmen undergoing intensive long-term physical training (Kamzolova et al. 2016). Recently, ICA has been tested as a natural prophylactic and therapeutic agent. It has been shown efficient for the treatment of iron-deficiency anemia and in the resorption of blood clots (Morgunov et al. 2019).

At present, ICA is produced via chemical synthesis. However, this results in a racemic mixture of stereoisomers that cannot be separated by chemical methods. ICA can be found also in the leaves and stems of some plants from the Crasulaceae family or in fruits such as blackberry and blackcurrant. However, the purification of ICA from plant extracts or fruit juices that contain a wide range of organic acids and other components is a complicated and very expensive technological process. Therefore, the development of biotechnological methods for ICA production is extremely important. At present, the most promising method for ICA production is considered to be microbiological synthesis (Kamzolova et al. 2016; Laptev et al. 2017).

It is known that *Y. lipolytica* accumulates ICA and CA in the culture medium when cell growth is nutrients limited. Moreover, the ICA/CA ratio greatly depends on the carbon source used for cell growth. For example, wild-type strains of *Y. lipolytica* secrete mainly CA and about 8–16% ICA on carbohydrates or glycerol while approximately 50–65% CA and 35–50% ICA on substrates like alkanes, triglycerides, ethanol, or acetate. When using ethanol as a carbon source, an ICA proportion of 35–67% was found depending on the cultivation conditions (Kamzolova et al. 2016; Holz et al. 2009). Some authors have conducted several studies to increase the ICA production titer. The recombinant *Y. lipolytica* H222-S4 strain deleted for *ICL1* gene, when grown on glycerol or glucose, showed only a smaller enhancement (by 2–5%) of ICA /CA ratio. With *Y. lipolytica* H222-S4 T5 that overexpress *ICL1* gene, the relative ICA content in the medium was as low as 5–7% of the total acids (CA and ICA) (Forster et al. 2007a, b).

Y. lipolytica VKM Y-2723 and its mutant derivative 704-UV4-A/NG50 were selected from 60 yeast strains for their ability for ICA production. Under optimal culture conditions (i.e., iron concentration of 1.2 mg/L, temperature of 29 °C, pH 6.0, pO₂ 50–55% of saturation, and 30 mM itaconic acid), *Y. lipolytica* VKM Y-2373 produced 70.6 g/L ICA and 22.4 g/L CA with an ICA/CA ratio of 1:0.32 when grown on rapeseed oil. In similar conditions, a titer of 86 g/L ICA

and 20 g/L CA with an ICA/CA ratio of 1:0.23 was obtained with *Y. lipolytica* 704-UV4-A/NG50 (Kamzolova et al. 2013). Under the above optimal culture conditions, *Y. lipolytica* VKM Y-2723 produced 90.5 g/L ICA with a yield of 0.77 g/g (Kamzolova et al. 2018). Also, catalytic activities of enzymes such as alcohol dehydrogenase, citrate synthase, aconitate hydratase, iso-citrate dehydrogenase, and iso-citrate lyase but not aldehyde dehydrogenase were found higher in a repeated-batch cultivation of 748 h than in batch cultivation. Therefore, under optimal repeated-batch culture using ethanol as substrate, *Y. lipolytica* strain VKM Y-2723 produced 109.6 g/L ICA with a production rate of 1.35 g/L/h (Morgunov et al. 2019). Besides this, overexpression of *ACO1* (*YALI0D09361g*) encoding aconitase allowed to increase ICA titer (Laptev et al. 2017; Holz et al. 2009). For the wild-type strain H222 and H222-S4 grown on sunflower oil, the ICA proportion ranged between 35 and 49%, whereas it increased up to 71% with the *ACO1* multi-copy transformant T1 without any modification in the total organic acid titer (both CA and ICA). However, ICA production was only moderately increased from 8–12% up to 13–17% with carbon sources such as glucose, sucrose, and glycerol (Holz et al. 2009). When *ACO1* was expressed in multicopy, ICA and CA titers were, respectively, of 72.6 g/L and 29.0 g/L with an ICA/CA ratio of 2.5:1 during culture in 10L bioreactor in a rapeseed oil-based medium (Laptev et al. 2017).

3.3 Succinic Acid

As an almost ubiquitous metabolite in many organisms, succinic acid (SA) is an intermediate of the Krebs cycle. Due to its numerous potential applications, SA has been recognized as one of the most important and high value-added bio-based building block chemicals by the US Department of Energy (DOE) (Beauprez et al. 2010; Ahn et al. 2016). In 2004 and 2010, the US DOE reported SA as one of the five most promising bio-based platform chemicals. SA market worldwide is forecasted to grow at a compound annual growth rate (CAGR) of 15.7% between 2020 and 2026 (Ahn et al. 2016; Tan et al. 2014, 2020a). Among all the platform chemicals, SA is currently used as a surfactant, ion chelator, additive in agricultural and food, and pharmaceutical industries. It can be used as a precursor to synthesize γ -butyrolactone, 1,4-butanedioic acid, tetrahydrofuran, and other value-added chemicals (Ahn et al. 2016; Tan et al. 2014; McKinlay et al. 2007). Currently, succinate is produced mainly from petroleum-derived maleic anhydride. However, due to near future shortages of petroleum resources, severe environmental concerns related to chemical synthesis processes and to reach an economical bio-based production of SA, extensive research works have been focusing on the development of efficient microbial strains by metabolic engineering as well as optimized fermentation and downstream processes (Ahn et al. 2016; Tan et al. 2013). Besides, bio-based SA production technologies can reduce greenhouse gas emissions by 50% and energy demand by 30–40% as compared to the chemical production process (Tan et al. 2014).

By contrast to prokaryotes, some yeasts, such as *Y. lipolytica*, are highly tolerant to low pH, rendering them attractive as an industrial host for SA synthesis (Cui et al. 2017). A *Y. lipolytica* evolved strain, named PSA3.0, which can produce SA at low pH using glucose as substrate was selected from a long-lasting culture in in situ fibrous bed bioreactor (isFBB) (Li et al. 2018a). Strain PSA3.0 produced SA with a titer of 18.4 g/L and yield of 0.23 g/g at pH 3.0. These values are, respectively, 4.8 and 4.6-fold higher than those obtained with the parental strain PSA02004 at pH 3.0. Using fed-batch culture in bioreactor, a SA titer of 76.8 g/L was obtained, which is the highest value ever achieved from a glucose-based medium at low pH (Li et al. 2018a). Furthermore, *Y. lipolytica* PSA02004 produces 33.2 g/L SA with a yield of 0.58 g/g and productivity of 0.33 g/L/h from sugarcane bagasse hydrolysates. Using glucose-rich fruit and vegetable waste (FVW) hydrolysates, SA titer of 140.6 g/L with a productivity of 0.69 g/L/h has been obtained (Li et al. 2018b; Ong et al. 2019). Under optimal conditions of repeated-batch fermentation, SA titer of 55.3 g/L and the maximal productivity 2.6 g/L/h was reached with *Y. lipolytica* strain VKPM Y3753 (Bondarenko et al. 2016).

An increase in SA titer was also achieved by replacing the native promoter of the succinate dehydrogenase by strong promoters (Cui et al. 2017; Yuzbashev et al. 2010; Jost et al. 2014; Gao et al. 2016; Li et al. 2016, 2017; Yang et al. 2017). Under oxygen limitation, *Y. lipolytica* strain H222-AZ2 obtained by exchange of the native promoter of the succinate dehydrogenase subunit 2 encoding gene by the inducible promoter *POT1*, a SA productivity of 0.152 g/L/h and titer of 25 g/L after 165 h of culture in glycerol medium were obtained (Jost et al. 2014). With *Y. lipolytica* strain PGC01003, deleted for *Ylsth5* genes encoding succinate dehydrogenase subunit 5, SA titer of 43 g/L was obtained during batch culture in 2.5 L bioreactor in medium containing crude glycerol. With a fed-batch strategy, the strain produced 160 g/L SA (Gao et al. 2016). After 21 days of evolution to enable the strain PGC01003 to catabolize glucose, the evolved strain produced 65.7 and 87.9 g/L SA using YPD medium and food waste hydrolysate, respectively (Yang et al. 2017).

Enhanced SA produced was also achieved by genetic modifications of the strains in succinate dehydrogenase or fermentation technology innovation. For example, when the native promoter of *SDH2* was replaced by an inducible promoter of *POT1*, SA titer at 25 g/L with productivity at 0.15 g/L/h was obtained by *Y. lipolytica* H222-AZ2 from glycerol under oxygen limitation (Jost et al. 2014). After inactivation of *SDH5* that encodes succinate dehydrogenase subunit 5, SA titer at 43 g/L was obtained by batch fermentation from crude glycerol. By PGC01003, and a much higher titer was achieved at 160 g/L SA via fed-batch fermentation strategy (Gao et al. 2016). Based on this, an in-situ fibrous bed bioreactor (isFBB) which could improve the initial cell density was developed, and SA titer at 198.2 g/L with average productivity of 1.46 g/L/h was achieved by PGC01003 via fed-batch strategy (Li et al. 2016). This value was further improved to 209.7 g/L when the immobilization matrix in isFBB was replaced by the more porous material (e.g., sugarcane bagasse), which is the highest value ever reported

(Li et al. 2017). However, it was reported that the accumulation of acetate was a major factor that impeded the fermentation at low pH, resulting in an obvious increase in downstream process cost (Cui et al. 2017). In order to solve this problem, the gene encoding the acetyl-coenzyme A hydrolase (*ACH1*) was deleted, and *PCK* from *S. cerevisiae* encodes phosphoenolpyruvate carboxykinase together with *SCS2* encoding endogenous succinyl CoA synthetase were overexpressed in PGC01003 to achieve the strain PGC202. As result, SA titer at 110.7 g/L with a yield of 0.53 g/g and productivity of 0.8 g/L/h was obtained by this strain via fed-batch fermentation with pH without control (final pH at 3.4) (Cui et al. 2017).

Nevertheless, all the metabolic evolution of *Y. lipolytica* for SA production has led to a partial or total loss of its ability to grow in glucose-based medium, which limits its industrial application (Yang et al. 2017). In this case, a strategy termed metabolic engineering was applied by Yang et al. to obtain a glucose-consuming *Y. lipolytica* (PSA02004) after a 21-day repeated fermentation of PGC01003 (Yang et al. 2017). As a result, the evolved strain PSA02004 could produce SA at 65.7 g/L and 87.9 g/L from the YPD medium and food waste hydrolysate at pH 6.0, respectively. Interestingly, with the help of isFBB, the pH for the cultivation of PSA02004 could be decreased to a level lower than 3.0 gradually by metabolic evolution, and the evolved strain named *Y. lipolytica* PSA3.0 that could produce SA with a titer of 19.3 g/L, productivity of 0.52 g/L/h, and yield of 0.29 g/g at pH 3.0 from YPD was achieved (Li et al. 2018a). The enzyme activity analysis demonstrated that the pathway from pyruvate to acetate was partially blocked in *Y. lipolytica* PSA3.0 after the evolution, which is beneficial to cell growth and SA production at low pH.

However, as for PGC01003, the accumulation of acetate is a limiting factor for further improvement of SA production as SA recovery request a pH adjustment leading to an increase of downstream process cost (Cui et al. 2017). To solve this issue, a new recombinant strain, named PGC202, was deleted for gene *ACH1* (*YALI0E30965g*) encoding the acetyl-coenzyme A hydrolase and overexpressing *PCK* gene from *Saccharomyces cerevisiae* encoding phosphoenolpyruvate carboxykinase together with gene *SCS2* encoding endogenous succinyl CoA synthetase was constructed. It allowed improving the SA production process through the elimination of acetic acid overflow and by-products formation. Indeed, SA titer of 110.7 g/L with a maximum yield from glycerol of 0.53 g/g and a productivity of 0.8 g/L/h were obtained during fed-batch fermentation of strain PGC202 (Cui et al. 2017). Furthermore, the strain produces 31.7 g/L SA with a yield of 0.52 g/g and productivity of 0.60 g/L/h in isFBB fermentation when using glucose-containing MFWs hydrolysate as the carbon source supplemented with 3% of tryptone (Li et al. 2019).

3.4 Itaconic Acid

Itaconic acid (IA) is naturally produced by several *Aspergillus* species (Blazek et al. 2015). The industrially versatile usability of IA and its derivatives are

reflected in the wide range of applications such as in plastics, styrene-butadiene rubber, synthetic latex, super-absorbent polymers, unsaturated polyester resins, and detergents. The field of application of these products is widespread and ranges from paint, lacquer, paper industries, hygiene, and medical products as well as in the construction sector (Kuenz and Krull 2018). Thus, IA was recognized as one of 12 value-added chemicals from biomass by the US DOE in 2004 (Blazeck et al. 2015). IA market is estimated at US\$28.4 million in the year 2020 in the USA while the world market is expected to reach \$129.3 million by 2027 due to increasing demand for bio-based chemicals (2020b). To date, two strategies have been developed to produce IA, a chemical method based on the pyrolysis of citric acid and a biosynthesis process that relies on the decarboxylation of cis-aconitic acid by microorganisms (Kuenz and Krull 2018; Zhao et al. 2019).

Although current industrial production of IA is based on *A. terreus* fermentation allowing titer higher than 80 g/L, *A. terreus* suffers from poor growth in optimal media used for IA production and is negatively affected by shear stress in the bioreactor, thus precluding fermentations under conventional conditions (Blazeck et al. 2015). Moreover, a complex and expensive downstream process has been developed at an industrial scale (Kuenz and Krull 2018; Okamoto et al. 2014; Bellasio et al. 2015; Chin et al. 2015; Otten et al. 2015). In order to address these concerns, *Y. lipolytica* was considered as an alternative IA production host as this organism can natively grow at low pH and sustains a high flux toward the citric acid cycle (Blazeck et al. 2015). There are currently two reports on engineered *Y. lipolytica* strains used to produce IA. In the study of Blazeck et al., the native cis-aconitate decarboxylase (*CAD*) gene was overexpressed, and the resulting strain produced IA with a titer of 33 mg/L (Blazeck et al. 2015). Further strain optimizations of the metabolic pathway, enzyme localization, and media optimization strategies enabled IA titer of 4.6 g/L during culture in bioreactors, representing a 140-fold improvement. A recombinant strain which overexpresses genes encoding the mitochondrial cis-aconitate transporter MTT and *CAD* allowed an IA titer of 22.0 g/L in optimized conditions (Zhao et al. 2019). This represents a 60-fold improvement over the initial titer (0.36 g/L).

3.5 α -ketoglutaric Acid

α -ketoglutaric acid (α -KG), an important dicarboxylic acid, is an intermediate of the Krebs cycle. It is also involved in amino acid metabolism and has an important role in the regulation of the balance between carbon and nitrogen metabolism in many microorganisms. α -KG is widely applied in the industrial scope, e.g., as a building block for the chemical synthesis of heterocycles, dietary supplement, component of infusion solutions, and wound healing compounds (Otto et al. 2011; Yovkova et al. 2013). Currently, α -KG is synthesized by chemical processes or biosynthesis at an industrial scale. The main chemical routes utilized succinic acid and oxalic acid diethyl esters or relied on the oxidation of glyoxylic acid with sodium glutamate using copper as a catalyst. The drawbacks of these chemical

routes are a lack of selectivity, a low yield, high risk from manipulation of harsh chemicals, and the generation of environmental hazards. These drawbacks sharply increased the downstream process cost and restricted the utilization of α -KG in food, medicine, and cosmetics applications.

Y. lipolytica is unable to synthesize the pyrimidine structure of the thiamine molecule. Thiamine is the cofactor of α -ketoglutarate dehydrogenase (*KGDH*) which is a key enzyme in the metabolism of α -KG; thus, the limitation of thiamine availability can reduce the activity of α -ketoglutarate dehydrogenase of the Krebs cycle. Under conditions of thiamine deficiency, the conversion of α -KG in the Krebs cycle is inhibited, which leads to its accumulation in the fermentation broth (Chernyavskaya et al. 2000; Kamzolova et al. 2012; Yin et al. 2012).

Currently, different strains producing α -KG from different carbon sources have been reported. *Y. lipolytica* H355 allows α -KG titer of 195 g/L with a productivity of 1.3 g/L/h in the medium containing a mixture of n-alkanes (Guo et al. 2016b). Under optimal conditions, *Y. lipolytica* VKM Y-2412 produced 172 g/L of α -KG with a yield of 0.70 g/g and 102.5 g/L α -KG with the yield of 0.95 g/g from ethanol and rapeseed oil, respectively (Kamzolova et al. 2012; Kamzolova and Morgunov 2013). However, in order to overcome the disadvantages of low yield and accumulation of byproducts, several approaches including metabolic engineering strategies and different fermentation configurations have been investigated (Guo et al. 2016a). *Y. lipolytica* H355A that overexpress the iso-citrate dehydrogenase (*IDHI*) and pyruvate carboxylase (*PYC1*) genes can produce 186 g/L α -KG from raw glycerol. This represents a 19% increased production as compared to the control strain H355. *Y. lipolytica*-RoPYC2 which overexpress *RoPYC2* gene encoding pyruvate carboxylase produced α -KG with a titer of 62.5 g/L with only 13.5 g/L pyruvate (PA). As compared to the parental strain, α -KG production in strain RoPYC2 increased by 35.3% while PA production is reduced by 69.8% (Yovkova et al. 2013; Yin et al. 2012).

4 Polyols

In some biological systems, polyols are obtained by the reduction of their keto or aldo groups into hydroxyl groups (Rice et al. 2019). As polyols, *Y. lipolytica* synthesize mainly mannitol (MAN), which is suggested to provide cofactor NADPH for fatty acid synthesis and erythritol (EOL). The latter being produced in response to osmotic stress. Derivatives of erythritol, namely erythrulose (EOSE) and threitol (TOL), are also of industrial interest and will be also discussed below (Table 2).

4.1 Mannitol

MAN is a six-carbon alcohol involved in stress tolerance in microorganisms notably in the scavenging of reactive oxygen species (ROS) (Zhang et al. 2018; Sekova et al. 2019). A possible role of MAN in fatty acid metabolism has been

Table 2 Some examples of polyols produced in *Y. lipolytica*

| <i>Y. lipolytica</i> strain | Genetic modification | Medium | Production, polyol | References |
|-----------------------------|---|---------------------------|----------------------|--------------------------------|
| AIG | <i>GUT1</i> ^{OE} (<i>YALIOF00384g</i>) | Crude glycerol Molasse | 11 g/L, MAN | Rakicka et al. (2017a, b) |
| AMM | <i>ER</i> ^{OE} (<i>YALIOF18590g</i>) | Glycerol | 44 g/L, EOL | Janek et al. (2017) |
| HCY118 | <i>ER10</i> ^{OE} (<i>YALIOD07634g</i>), <i>ER25</i> ^{OE} (<i>YALIOC13508g</i>), <i>ZWF1</i> ^{EO} (<i>YALIOE22694g</i>), <i>GND1</i> ^{EO} (<i>YALIOB15598g</i>) | Glucose | 196 g/L, EOL | Cheng et al. (2018) |
| Po1d | <i>TKL1</i> ^{OE} (<i>YALIOE06479g</i>) | Glycerol | 43 g/L, EOL | Carly et al. (2017a, b) |
| MK1 | <i>TKL1</i> ^{OE} (<i>YALIOE06479g</i>) | Glycerol | 51 g/l, EOL | Mironczuk et al. (2017) |
| MK1 | <i>TKL1</i> ^{OE} (<i>YALIOE06479g</i>), <i>TAL</i> ^{OE} (<i>YALIOF15587g</i>) | Glycerol | 46 g/l, EOL | Mironczuk et al. (2017) |
| Po1d | <i>GUT1</i> ^{OE} (<i>YALIOF00384g</i>), <i>TKL1</i> ^{OE} (<i>YALIOE06479g</i>) | Glycerol | 78 g/l, EOL | Carly et al. (2017a, b) |
| M53 | Δ <i>snf1</i> (<i>YALIOD02101g</i>) | Peanuts cake | 185 g/kg, EOL | Li et al. (2019) |
| Wratislavia K1 | WT | Glycerol | 180 g/L, EOL | Rakicka-Pustułka et al. (2020) |
| RIY210 | <i>EYD1</i> ^{OE} (<i>YALIOF01650g</i>), Δ <i>eyk1</i> (<i>YALIOF01606g</i>) | EOL | 0.12 g/gDCW, EOSE | Carly et al. (2017a, b) |
| CGMCC7326 | <i>Ss-XDH</i> ^{OEcn} | Glucose | 112 g/L, TOL | Chi et al. (2019) |

Abbreviations: MAN: mannitol, EOL: erythritol, EOSE: erythrulose, TOL: threitol, *GUT1*: glycerokinase, *ER*: erythrose reductase, *ZWF1*: glucose-6P dehydrogenase, *GND1*: 6-phosphogluconate dehydrogenase, *TKL1*: transketolase, *TAL*: transaldolase, *SNF1*: regulator of lipid accumulation, *EYD1*: erythritol dehydrogenase, *EYK1*: erythritol kinase, *XDH*: xylitol dehydrogenase

also reported (Dulermo et al. 2015). MAN production has been investigated for several *Y. lipolytica* strains and different experimental conditions. For cells grown in shake-flasks in the presence of crude glycerol, mannitol was co-produced with EOL with titers ranging from 2.6 to 14.9 g/L. During glycerol fed-batch bioreactor culture, strains A-15 and A UV'1 synthesized MAN with a titer of, respectively, 41.4 and 38.1 g/L, corresponding to the productivity of 0.29 and 0.28 g/(L h) (Tomaszewska et al. 2012). With a derivative of strain AIB overexpressing *GUT1* gene encoding glycerol kinase grown in a batch bioreactor on a mixture of molasse and crude glycerol, maximal MAN titer of 11 g/L was obtained (Rakicka et al. 2017a). Similarly, using a mixture of crude glycerol and olive oil mill wastewater, the MAN titer reached 13 g/L within 140 h with a yield from glycerol of 0.21 g/g (Sarris et al. 2019).

reported recently (Niang et al. 2020). It is first converted into erythrose by an erythritol dehydrogenase (*EYD1*, *YALIOF01650g*) and subsequently phosphorylated into L-erythrose-1P by an erythrose kinase (*EYK1*, *YALIOF01606g*). Then, L-erythrose-1P is isomerized into D-erythrose-4P by an erythrose-1P isomerase (*EYLI*, *YALIOF01584g*). Finally, erythrose-4P is generated by the activity of an erythrose-4P isomerase (*EYL2*, *YALIOF01628g*).

Several review papers focusing on EOL have been published, and only the most striking information will be reported below (Carly and Fickers 2018; Regnat et al. 2018; Rzechonek et al. 2018b). An efficient strategy for EOL synthesis consisted of constitutively expressing genes encoding erythrose reductase (ER). Overexpression of ER gene *YALIOF18590g* in *Y. lipolytica* strain AMM led to a 20% increase in EOL titer as compared to the parental strain (44.4 g/L) (Janek et al. 2017). This corresponded to the productivity of 0.77 g/(L h) and a yield from glycerol of 0.44 g/g. More recently, two additional ERs have been characterized (*YALIOD07634g* and *YALIOC13508g*) (Cheng et al. 2018). Upon overexpression of these three ERs under the control of the strong constitutive promoter hp4d, an EOL titer of 178 g/L was obtained from an initial glucose concentration of 300 g/L within 84 h. This corresponded to productivity and yield of 2.1 g/(L h) and 0.59 g/g, respectively. As ERs are NADPH-dependent enzymes, the authors engineered the co-factor metabolism by overexpressing genes *YALIOB15598g* and *YALIOE22694g* encoding 6-phosphogluconate dehydrogenase (*GND1*) and glucose-6P dehydrogenase (*ZWF1*), respectively. The corresponding enzymes are known to generate NADPH from NADP⁺. The resulting strain, HCY108, produced EOL with a titer of 190 g/L within 80 h of culture with productivity and yield from the glucose of 2.4 g/(L h) and 0.63 g/g, respectively (Cheng et al. 2018). Other genes from the PPP pathway have been also overexpressed to increase EOL titer. Overexpression of gene *YALIOE06479g* encoding transketolase (*TKLI*) in strains Po1d and MK1 yielded a 19% (43 g/L) and 51% (51 g/L) increased EOL titer, respectively. This corresponded to a productivity of 0.04 and 0.05 g/(gDCW h) (Carly et al. 2017b; Mirończuk et al. 2017). By coexpression of genes *YALIOE06479g* (*TKLI*) and *YALIOF15587g* encoding transaldolase (*TALI*), an increase in EOL titer and productivity of 45% and 46% was obtained, respectively (46.7 g/L and 0.5 g/(L h) (Mirończuk et al. 2017). Gene involved in carbon source catabolism were also overexpressed to feed the PPP pathway with precursors (i.e., fructose-6P and glyceraldehyde-3P). Overexpression of genes *YALIOF00384g* (*GUT1*) and *YALIOB02948g* (*GUT2*) encoding, respectively, glycerol kinase (GK) and glycerol-3-P dehydrogenase (G3P-DH) allowed an EOL titer of 78 g/L from an initial glycerol concentration of 100 g/L within 72 h in a 5-L bioreactor (Mirończuk et al. 2016). Besides this, overexpression of genes *GUT1* and *TKLI* in a strain disrupted for gene *EYK1* allowed an erythritol titer, productivity, and yield from glycerol of 80 g/L, 1.03 g/(L h), and 0.53 g/g, respectively (Carly et al. 2017b). By overexpressing invertase encoding gene *SUC2* from *Saccharomyces cerevisiae* and native *GUT1* gene, an EOL titer of 100 g/L with a productivity and yield of 1.1 g/(L h) and 0.67 g/g, respectively, were obtained from a mixture of raw industrial molasses (60 g/L) and crude glycerol (100 g/L)

(Rakicka et al. 2017b). Disruption of gene *SNF1* (*YALI0D02101g*) coding for a regulator of lipid accumulation allowed an EOL production of 185.4 mg/g in solid-state fermentation (SSF) using peanut press cake mixed with 40% sesame meal and 10% waste cooking oil as substrate (Li et al. 2019). EOL production in large-scale bioreactor was recently reported. Using raw glycerol feeding, strain *Y. lipolytica* Wratislavia K1 produced EOL with titer and yield from glycerol of 180 g/L and 0.53 g/g, respectively, after 144 h of cultivation in 500 L bioreactor (Rakicka-Pustułka et al. 2020). With metabolically engineered *Y. lipolytica* strain HCY118, a production titer of 196 g/L with productivity and yield from glucose of 2.51 g/L h and 0.65 g/g, respectively, were obtained in a 30 m³ bioreactor within 78 h (Wang et al. 2020).

4.3 Erythrulose

As mentioned already, erythrulose (EOSE) is an intermediate of the erythritol catabolic pathway. It has many applications in cosmetics as a sunless tanning agent and in chemistry as a precursor of several drugs such as anticancer (Bengamide E), antifungal (Tanikolide), substitute β -lactam, cytokine modulator (Cytosaxone), or cholesterol-lowering drugs (Crestor, Zetia) (Carly et al. 2018). In a *Y. lipolytica* Δ *eyk1* derivative (RIY210), the conversion of EOSE into L-erythrulose-1P is impaired. Therefore, such a mutant strain can convert EOL into EOSE. The conversion rate could be increased by the overexpression of *EYD1* encoding erythritol dehydrogenase in a Δ *eyk1* genetic background. With such a strategy, an efficient fed-batch bioreactor process was developed to convert EOL into EOSE with a conversion rate and yield of 0.116 g/g DCW·h and 0.64 g/g, respectively (Carly et al. 2017a).

4.4 Threitol

Threitol (TOL) is a diastereoisomer of erythritol that is produced naturally as an antifreeze molecule by the fungus *Armillaria mellea* as well as the Alaskan beetle *Upis ceramboides*. TOL has application as a precursor for the synthesis of anticancer drugs (treosulfan and threitol ceramide). It is also a constitutive element of oxygen-sensitive pigments incorporated in a smart plastic film used for food packaging (Chi et al. 2019). Recently, xylitol dehydrogenase (*Ss-XDH*) from *Scheffersomyces stipites* CBS6054 was found able to oxidize EOL into EOSE irreversibly and then reduce EOSE into threitol (Chi et al. 2019). By overexpression of the corresponding gene in the *Y. lipolytica* strain CGMCC7326, a good producer of EOL from glucose, a TOL production titer of 112 g/L with a yield from the glucose of 0.37 g/g has been reported (Chi et al. 2019).

5 Conclusion and Prospects

Due to its potential for organic acids and polyols production and good tolerance to low pH, *Y. lipolytica* has gradually become a cell factory for their production. However, several key steps allowing to increase strain production titer and productivity remain to be identified. Process operations are still not optimal, especially on a large scale. Further investigations must be made to obtain cost-effective processes for most of the metabolites described herein.

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