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Metabolic and process engineering for producing the peach-like aroma compound γ-decalactone in *Yarrowia lipolytica*

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Abstract

Due to its strong and unique peach-like aroma, γ-decalactone is widely used in dairy products and other foods or beverages. The oleaginous yeast *Yarrowia lipolytica*, which is generally regarded as safe, has shown great potential in the production of this flavor compound. Recently, the development of metabolic and process engineering has enabled the application of *Y. lipolytica* for the production of γ-decalactone. This review summarizes the relevant biosynthesis and degradation pathways of *Y. lipolytica*, after which the related metabolic engineering strategies to increase the accumulation of γ-decalactone are summarized. In addition, the factors affecting γ-decalactone accumulation in *Y. lipolytica* are introduced and corresponding process optimization strategies are discussed. Finally, the current research needs are analyzed to prospect remaining challenges and future directions in this field.

Keywords：*Yarrowia lipolytica*, Metabolic engineering, Process engineering, Flavor, γ-Decalactone, β-Oxidation

1. Introduction

Flavors and fragrances are based on specific volatile compounds with a pleasant and comforting aroma. They are high-value-added fine chemical products that are widely used for the perfuming of products in the food, tobacco, alcohol, household chemical and cosmetic industries.[1] At present, the flavor and fragrance industry accounts for more than a quarter of the food additive market, reaching global sales of 28.1 billion US dollars in 2019, and also showing a robust growth trend.[2]

With the improvement of global living standards, consumers pay increasing attention to the sources, types and quality of flavors and fragrances. Consumers are inclined to perceive natural compounds as superior to chemically identical synthetic alternatives, leading to increased focus on flavor and fragrance biosynthesis..[3] According to US and European Union regulations (e. g. CFR 1990 and EEC 1334/2008), natural compounds are defined as substances isolated from natural resources or obtained through microbial transformation or enzymatic reactions, including precursor substances isolated from nature. In the past, the most common methods for producing flavor compounds were plant extraction and chemical synthesis. However, flavors and fragrances produced by chemical synthesis are often racemic mixtures of isomers, the production process is complicated, while the process conditions are harsh and often generate toxic waste. And it cannot be equivalent to natural compounds. Therefore, natural flavors and fragrances extracted from plants methods are favored by consumers due to their safety. However, plant extraction still has many disadvantages, such as low product concentrations, high prices, seasonal

environmental influences, and the presence of toxins in plants.[4-5] By contrast, biotechnologically produced flavors and fragrances are considered natural products, while the production methods are more economical and environmentally friendly. Therefore, the use of biological methods to produce flavors and fragrances has become a focus of research and an industrial development around the world.[6]

Notably, a number of aroma compounds are lactones. which are cyclic carboxylic acid esters formed by intramolecular esterification of hydroxy fatty acid molecules.[6-7] According to the position of the hydroxyl group, they can be classified as γ-, δ-, ω-lactones, etc.,[8] most of which have fruity or dairy-like aromas, and exhibit oily characteristics. Among them, the biological production pathway of γ-decalactone, with a peach- or coconut-like aroma, was discovered first.[9] Due to the pleasant flavor and good safety of γ-decalactone, it was certified as a safe food and pharmaceutical additive by the US Food and Drug Administration (FDA) in 1969[10]. Since its early introduction, γ-decalactone has been widely used in the perfume industry because of its attractive fragrance and low fragrance threshold characteristics, with hundreds of tons marketed each year.[11-12] Many microorganisms have shown the potential to produce γ-decalactone, including *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, as well as species of *Candida*, *Pseudomonas*, *Sporobolomyces*, and *Rhodotorula* (Table 1). Notably, *Y. lipolytica* was found to have the strongest γ-decalactone production capacity[13-15] *Y. lipolytica* can effectively use the hydrophobic substrates as carbon sources for growth and product synthesis, with a strong ability to metabolize glycerides and alkanes.[16-18] This yeast is a Generally Recognized as Safe (GRAS)

organism, its genome has been sequenced and annotated, its gene expression regulation mechanism is relatively clear, and it has a well-developed genetic toolkit, including CRISPR/Cas9, EasyClone YALI, Golden Gate, and many other methods.[19-24] In addition, this yeast has high tolerance to acidity, osmotic pressure and fermentation inhibitors. Based on these advantages, *Y. lipolytica* has become a valuable cell factory for the production of various natural and non-natural compounds, including recombinant proteins, terpenes, organic acids, single cell oils, and fatty acid lactones.[8, 25-36] As mentioned above, *Y. lipolytica* exhibits higher γ-decalactone production capacity than other microbes, and with the continuous development of metabolic engineering tools, *Y. lipolytica* is expected to become the most competitive host for the industrial production of γ-decalactone.

This review firstly summarizes the metabolic pathways for the production of γ-decalactone in *Y. lipolytica*, after which it introduces metabolic and process engineering strategies that can improve the yield. Among the metabolic engineering strategies, modifications of the hydroxylation pathway and the β-oxidation pathway are described in detail. The current factors affecting the production of γ-decalactone, including substrates, oxygen, lipase, bioreactor configurations, separation systems, etc., are also introduced. Finally, we also present some new insights, mainly focusing on novel renewable feedstocks for the production of γ-decalactone.

2. Metabolic pathways for γ-decalactone accumulation in *Y. lipolytica*

2.1 Gamma-decalactone biosynthesis pathway

Most studies in *Y. lipolytica* used the pathway from ricinoleic acid to γ-

decalactone. The substrate ricinoleic acid is activated by acyl-CoA synthase to produce ricinoleoyl-CoA. Subsequently, 4-hydroxydecanoyl-CoA is produced by four steps of β-oxidation in peroxisomes, and then undergoes lactonization to produce γ-decalactone.[37-38] The β-oxidation process includes four steps, catalyzed by acyl-CoA oxidase, 3-ketoacyl-CoA thiolase and a bifunctional enzyme, which finally produce one acetyl-CoA and one acyl-CoA that is shorter by two carbons.[39] In this pathway, acyl-CoA oxidase catalyzes the first reaction, which is the rate-limiting step of β-oxidation, and has a direct relationship with the production of γ-decalactone.[40-41] *Y. lipolytica* has six acyl-CoA oxidases (Pox1-6) with different chain-length preferences.[41-43] Among them, Pox1 and Pox6 did not exhibit significant activity in this pathway,[38] but it was also reported that inactivation of Pox1 would lead to enhanced β-oxidation activity, while the content of γ-decalactone was reduced.[44] Pox2 is long-chain-specific and can oxidize fatty acids from C18 to C10, while Pox3 is short-chain-specific and can degrade fatty acids from C10 to C4. At the same time, both enzymes show high oxidation activity.[45-47] Although Pox4 and Pox5 do not show the same high oxidative activity as Pox2 and Pox3, they still have certain activity on the full spectrum of unbranched fatty acids, from C18 to C4.[43, 48-50] A thorough study of the β-oxidation pathway and the activity of different acyl-CoA oxidases is a prerequisite to construct different *Y. lipolytica* strains with increased production of γ-decalactone (Figure 1).

Silva et al. developed a metabolic engineering strategy for fatty acid biosynthesis and β-oxidation in *Ashbya gossypii*, deciphering the *de novo* biosynthesis of lactones

from glucose, and fine-tuned the ratio of lactones to increase γ-decalactone biosynthesis to more than 99% of the relative yield.[51] In a different approach, Marella et al. constructed a hydroxylation module and a carbon chain shortening module in *Y. lipolytica* to synthesize γ-dodecalactone and δ-decalactone using oleic acid and linoleic acid as substrates, respectively. By optimizing the reaction conditions, the titer was increased 4-fold.[52] Although there are no reports on the *de novo* biosynthesis of γ-decalactone in *Y. lipolytica*, there have been reports on the high production of oleic acid, *de novo* synthesis of ricinoleic acid, and the use of ricinoleic acid to produce γ-decalactone by *Y. lipolytica.* Combining these reports and utilizing the metabolic engineering strategies and modular construction methods adopted in the above studies will be of great help in the *de novo* synthesis of γ-decalactone from glucose in *Y. lipolytica*.

2.2 Gamma-decalactone degradation pathway

In the process of using microorganisms to produce γ-decalactone, the product titer will reach a peak and then gradually decrease due to degradation through multiple pathways. Early studies emphasized the role of key enzymes involved in β-oxidation, especially acyl-CoA oxidase. As mentioned above, Pox3 with short-chain specificity can degrade γ-decalactone to acetyl-CoA. Similarly, Pox4 and Pox5 can also degrade γ-decalactone, although their degradation ability is not as strong as that of Pox3.[36, 46, 48, 53] In addition, γ-lactonase can open the ring of γ-decalactone, and the cell can then oxidatively degrade the ring-opened product through β-oxidation.[54] In addition, the ω-oxidation of lactones results in dicarboxylic acids, but the strains that

produce this diacid are not capable of β-oxidation.[55] In this context, eliminating the influence of degradation factors will be an effective means to increase the yield of γ-decalactone.

3. Engineering strategies to increase γ-decalactone accumulation in *Y. lipolytica*

3.1 Engineering the hydroxylation pathway

According to the existing research, γ-decalactone production is usually based on using a hydroxy fatty acid (ricinoleic acid) as the substrate, after shortening the carbon chain through β-oxidation pathway, and then forming γ-decalactone through a lactonization reaction (Figure 2). However, the synthesis of hydroxy fatty acids remains a challenge. This is because hydroxylation is usually the most difficult compared to other metabolic pathways. The hydroxylation process can be catalyzed by four different enzymes, including hydratase, hydroxylase, lipoxygenase and epoxy-hydrolase.[56-57] Although they all form hydroxy fatty acids, the hydroxylation reactions catalyzed by the four enzymes are quite different. Hydrating enzymes can usually directly convert free fatty acids that need to be hydroxylated.[58-59] Fatty acids need to pass through the Kennedy pathway before they can be converted by hydroxylases.[60] Finally, lipoxygenase and epoxy-hydrolase can directly act on free fatty acids, but they will form unstable intermediates.[57, 61] In a pioneering study, Holic et al. introduced the Δ12 hydroxylase derived from *Claviceps purpurea* (*Cp*Fah12) into *Schizosaccharomyces pombe* to convert oleic acid into ricinoleic acid. After optimizing the fermentation conditions, the ricinoleic acid titer reached 0.14 g/L, accounting for 52.6% of total fatty acids.[62] Beopoulos et al. first heterologously

160 expressed *Ricinus communis* Δ12 hydroxylase (*Rc*Fah12) in *Y. lipolytica*, which
161 resulted in ricinoleic acid accumulation up to 7% of total lipids.[63] After comparing the
162 effects of *Rc*Fah12 and *Cp*Fah12, the more effective *Cp*Fah12 was selected for
163 multicopy expression to increase the ricinoleic acid titer to 35% of total lipids. After
164 additional metabolic engineering transformations, the final strain accumulated
165 ricinoleic acid up to 43% of total lipids, corresponding to 63 mg/g dry cell weight.
166 This is by far the most efficient production of ricinoleic acid reported in *Y. lipolytica*.
167 In subsequent studies, Meesapyodsuk et al. co-expressed *Cp*Fah12 and *Cp*Dgat1
168 (diacylglycerol acyltransferase derived from *Claviceps purpurea*) in *Pichia pastoris*,
169 which resulted in higher lipid content and ricinoleic acid levels than expressing
170 *Cp*Fah12 alone.[64] Recently, Marella et al. expressed the oleate 10-hydratase derived
171 from *Stenotrophomonas maltophilia* (*Sm*Ohy) in *Y. lipolytica*, which catalyzes the
172 production of 10-hydroxystearic acid from oleic acid. In addition, linoleate 13-
173 hydratase derived from *Lactobacillus acidophilus* (*La*Lhy) was also expressed to
174 catalyze the production of 13-hydroxyoleic acid from linoleic acid, providing
175 precursors for the production of lactones.[52] Notably, it is highly promising to obtain
176 the precursors of γ-decalactone by finding efficient hydroxylases.

177 **3.2 Engineering the β-oxidation pathway**

178 In order to increase the production of γ-decalactone, researchers have focused on
179 metabolic engineering to modify the β-oxidation pathway of *Y. lipolytica*. As
180 mentioned above, β-oxidation in *Y. lipolytica* is catalyzed by three enzymes, including
181 acyl-CoA oxidase, 3-ketoacyl-CoA thiolase and a bifunctional enzyme. Pagot et al.

overexpressed the gene encoding thiolase in *Y. lipolytica*, but this modification had no effect on the production of γ-decalactone.[44] As the rate-limiting enzyme that catalyzes the first step of the reaction, acyl-CoA oxidase has received great attention.

To test the effects of the six acyl-CoA oxidases Pox1 to Pox6 on the production of γ-decalactone in *Y. lipolytica*, researchers constructed *Y. lipolytica* strains with single or multiple gene deletions, and the ability of these strains to produce γ-decalactone was tested.[43, 49] Pagot et al. found that the activity of β-oxidation was increased after knocking out the *POX1* gene, but the production of γ-decalactone decreased.[44] Waché et al. knocked out the *POX2*, *POX3*, and *POX5* genes, and then introduced multiple copies of *POX2*, which increased the production of γ-decalactone while reducing the accumulation of 3-hydroxy-γ-decalactone, dec-2-en-4-olide, and dec-3-en-4-olide.[65] Similarly, Groguenin et al. found that Pox4 exhibits minimal activity on the substrate ricinoleic acid and participates in the degradation of γ-decalactone, so they knocked out the *POX4* gene in the Δ*pox2*Δ*pox3*Δ*pox5* mutant and re-expressed the same *POX2* gene to construct a new strain (Δ*pox2*-Δ*pox5*, pPox2-Pox2). It was found that the content of γ-decalactone produced by this strain within 48 hours was 10 times that of the wild-type strain.[48] Guo et al. used homologous recombination to disrupt the *POX3* gene by integrating the copper-resistance gene *CRF1* at its locus in the genome of *Y. lipolytica*, which increased the output of γ-decalactone 2.9 times, from 0.19 to 0.53 g/L.[53] Since then, they obtained a null mutant Tpp-11 which possesses multiple copies of *POX2* and disrupted *POX3* gene on two chromosomes. By this approach, the yields of γ-decalactone were

succeeded to increase from 0.9 g/L to 3.3 g/L.[45] By knocking out the *POX2-POX5* genes and ectopically overexpressing the *POX2* gene, Braga et al. constructed a new strain MTLY40-2P, which minimized the degradation of γ-decalactone. On this basis, the *LIP2* gene was overexpressed to increase the productivity of γ-decalactone.[66] Finally, the concentration of γ-decalactone reached 7 g/L by applying a gradual feeding strategy (Table 2). With further in-depth study, the emergence of new metabolic engineering strategies for optimizing β-oxidation may further enhance the production of γ-decalactone in the future.

3.3 Engineering the lactonization reaction

Lactonization generally occurs after the β-oxidation pathway. The hydroxyl and carboxyl groups are condensed with the concomitant loss of a water molecule, whereby the hydroxy fatty acid is cyclized. Different types of lactones are distinguished according to the position of the hydroxyl group. The first type is generated intracellularly by the action of 4-hydroxydecanoate cyclase, while the second type is generated through acidification and heating of the medium to promote spontaneous cyclization after 4-hydroxydecanoic acid accumulates extracellularly. Notably, the two mechanisms are not completely independent.

In microorganisms, fatty acids are not metabolized in their free form, but β-oxidation proceeds in the form of acyl-CoA. Therefore, intracellular esterification of 4-hydroxydecanoyl-CoA requires a thioesterase, which generates free fatty acids for lactonization by the corresponding cyclases.[67] In a pioneering study, Peng et al. compared the activity of alcohol acyltransferases (*Pp*Aat1) in peach cultivars (*Prunus*

persica) with high and low aroma, and evaluated their effects on the production of γ-decalactone. They found that *Pp*Aat1 derived from highly aromatic peaches is effective in catalyzing the conversion of 4-hydroxydecanoyl-CoA into γ-decalactone.[68] In addition, Peng et al. also isolated five *AAT* genes from the peach genome and expressed them in *Y. lipolytica*, which revealed that only *Pp*Aat1 can generate γ-decalactone. The strain expressing only *Pp*Aat1 produced a γ-decalactone titer of 2.2 g/L, which was 8 times that of the wild-type strain.[69] These studies thus provide a new direction to strengthen the lactonization pathway and improve the production of γ-decalactone.

4. Factors affecting γ-decalactone accumulation and related process optimization strategies

4.1 Role of the substrate

Y. lipolytica can use a variety of different substrates to produce γ-decalactone. Arguably the most suitable substrate is castor oil, which contains 86% ricinoleic acid, [12] which can be easily transformed into γ-decalactone via the β-oxidation pathway. Gomes et al. found that 30 g/L is the best concentration of castor oil in the medium, resulting in a maximal γ-decalactone concentration of 1.8 g/L.[70] In addition to castor oil, the authors also used methyl ricinoleate as a substrate. In shake flask fermentations, 30 g/L methyl ricinoleate resulted in the highest productivity (15 $mgL^{-1}h^{-1}$).[71] Because the source of castor oil is a toxic plant, Soares et al. attempted to use crude glycerol as an alternative substrate for the production of γ-decalactone.[72] Crude glycerol is a residual by-product of biodiesel manufacturing that is widely available

and cost-effective. When 10% crude glycerol was added to the medium, *Y. lipolytica* CCMA0357 produced 2.3 g/L γ-decalactone after 120 hours of fermentation. Although the yield was lower than with castor oil, the study demonstrated that it is feasible to use crude glycerol to produce γ-decalactone.

However, castor oil, ricinoleic acid, and methyl ricinoleate all have the same drawbacks when used as substrates, since all the three substances are poorly soluble in water, leading to the aggregation of oil droplets and poor dispersion in the medium. Therefore, the material transfer of these substances into *Y. lipolytica* cells is limited, reducing the production of γ-decalactone. Strategies to solve this problem include adding emulsifiers (Tween 80, etc.) to enhance the dispersion of substrates in the fermentation broth and increase the transport rate of substrates into cells.[73] In addition, phase transfer catalysts, such as L-carnitine, can also be added to facilitate the dissolution of the substrate in the aqueous phase or to accelerate the entry of the substrate into cells.[74]

4.2 Role of lipases

The most common direct substrate for the production of γ-decalactone is ricinoleic acid, which can be obtained by hydrolyzing castor oil or methyl ricinoleate using lipases.[67, 70-71] Many studies have shown that adding lipases to induce the pre-hydrolysis of the substrate can reduce the time for γ-decalactone to reach the maximum production. Braga et al. reported that *Y. lipolytica* W29 is the best strain for lipase production, and pre-induction of lipase expression decreased the lag phase of γ-decalactone production.[75] Additionally, Gomes et al. investigated the effect of

different commercial enzymes on the hydrolysis of castor oil.[70] They found that Lipozyme TL IM was the best commercial enzyme, and the optimal hydrolysis conditions were pH 8 and 27°C, which advanced the peak of γ-decalactone production by nearly 50 hours. In order to save costs and improve efficiency in industrial production, Braga et al. constructed *Y. lipolytica* JMY3010 by overexpressing the *LIP2* gene, which enables the strain to produce more lipase and more rapidly hydrolyze castor oil, thereby accelerating the production of γ-decalactone.[66] Although extracellular lipases can have a very good effect on exogenous substrates, intracellular lipases are needed to construct a *de novo* pathway for γ-decalactone synthesis. Dulermo et al. reported that two intracellular lipases of *Y. lipolytica*, Tgl3 and Tgl4, can effectively release ricinoleic acid from TAGs. Among them, Tgl4 is the main lipase responsible for TAG degradation, and Tgl3 is a positive regulator of Tgl4.[76]

4.3 Role of oxygen

The β-oxidation reaction is an essential step in the production of γ-decalactone by *Y. lipolytica*, and it depends on the level of dissolved oxygen in the biotransformation system. When the oxygen mass transfer rate increases, the respiration of microorganisms is enhanced. Therefore, increasing the dissolved oxygen in the fermentation system during biotransformation can improve the β-oxidation capacity of yeast cells, leading to faster substrate conversion and improved product yields.

Aguedo et al. used methyl ricinoleate as substrate for the production of γ-decalactone in *Y. lipolytica*. After increasing the oxygen solubility, they observed that

292 the activity of 3-hydroxy-CoA dehydrogenase was increased, resulting in a higher
293 yield of 3-hydroxy-γ-decalactone, which reached 0.26 g/L.[77] At the same time, the
294 cell growth rate was accelerated, but the yield of γ-decalactone decreased. Later,
295 García et al. used Doehlert factorial design to study the effect of aeration on lactone
296 accumulation in *Y. lipolytica*.[78] It was observed that under low aeration conditions, the
297 yeast cells can accumulate a higher amount of γ-decalactone, while under high
298 aeration conditions, the accumulation of 3-hydroxy-γ-decalactone is higher. By
299 contrast, the accumulation of dec-2-en-4-olide and dec-3-en-4-olide was not related to
300 the aeration conditions. Reis et al. used a small bioreactor with an air shock system,
301 which halved the time period for γ-decalactone to reach the maximum concentration
302 by increasing the oxygen mass transfer rate in the system.[79] Gomes et al. evaluated the
303 influence of oxygen volumetric mass transfer coefficient (K_La) on the production of γ-
304 decalactone from methyl ricinoleate in a two-phase medium, and observed that K_La is
305 an important factor in the production of γ-decalactone.[80] The maximum product yield
306 reached 0.14 g/L at a K_La value of 70 h^{-1}. In order to obtain a high yield of 3-hydroxy-
307 γ-decalactone, García et al. focused on the effect of β-oxidation pathway, which is
308 strongly affected by oxygen and pH. Very low oxygen levels can inhibit the activity
309 of acyl-CoA oxidase, limit the activity of β-oxidation, and increase the accumulation
310 of γ-decalactone.[81] By continuously increasing the aeration, the regeneration of
311 NADH is affected, thereby affecting the activity of 3-hydroxyacyl-CoA
312 dehydrogenase, so that the production of 3-hydroxy-γ-decalactone is gradually
313 increased, and only a few intermediates are produced. In addition, they added the

surface-active plastic material polymethyl methacrylate (PMMA) to the reactor. By improving the aeration conditions, the hydrophobicity of *Y. lipolytica* was improved, and an adhesive cell film was formed, which resulted in higher yield of 3-hydroxy-γ-decalactone, reaching 1.7 g/L.[82] Similarly, Braga et al. further studied the effect of oxygen transfer rate (OTR) on the production of γ-decalactone by *Y. lipolytica* W29, and observed that a low oxidation rate can increase the product concentration.[83] However, a higher OTR can shorten the time needed to reach the production peak, leading to higher production efficiency, increasing the titer of γ-decalactone to 5.4 g/L. Further research revealed that the mass transfer rate of oxygen was higher when a reactor with higher agitation power was used to produce γ-decalactone, which further improved the product yield.[84]

4.4 Role of substrate and product toxicity

Although genetic modification can greatly increase the yield of γ-decalactone, there are still many factors that limit its industrial production, such as the toxicity of substrates and products.

In a pioneering study, Lin et al. found that the method of adding ricinoleic acid at intervals during the cultivation process can greatly accelerate the production of γ-decalactone, but the number of living cells of the production strain will be significantly reduced with the continuous increase of ricinoleic acid concentration, which in turn reduced the γ-decalactone yield. Furthermore, lactones are toxic to the cell itself.[85] When the lactone in the fermentation broth accumulates to a certain concentration, it will inhibit cell growth, resulting in a decrease of product synthesis.[86]

In 2003, Aguedo et al. used FTIR experiments to reveal that the toxicity of γ-decalactone to *Y. lipolytica* may be due to the strong hydrophobic interaction between the molecule and membrane phospholipids, which changed the fluidity of the cell membrane.[87]

More recently, the use of microbial cell immobilization for reducing the toxic effects of substrates and products in the process of biotransformation and synthesis has become a research hotspot. Lee et al. immobilized *Sporidiobolus salmonicolor* CCRC21975 in k-carrageenan, chitosan, agarose or calcium alginate. A comparison of the immobilized biocatalysts revealed that calcium alginate is the most suitable for the production of γ-decalactone.[88] Lin et al. immobilized *Y. lipolytica* in a mixture of PVA and carrageenan, increasing the production of γ-decalactone to 0.48 g/L.[89] Zhao et al. obtained immobilized yeast cells by mixing 2% sodium alginate and 2% attapulgite with *Y. lipolytica*, and the yield of γ-decalactone reached 4.17 g/L.[90] Moreover, the yield of γ-decalactone remained as high as 3.87 g/L after three cycles of reuse. Braga et al. studied the immobilization of *Y. lipolytica* strains that can be used to produce γ-decalactone and found that immobilization on polymethacrylate and DumpUM® can reduce the toxicity of substrates and increase the production of γ-decalactone. Among them, DumpUM® was the most effective and increased the yield of γ-decalactone from 0.95 to 1.60 g/L.[91] Notably, Zhao et al. used attapulgite (ATG) to immobilize *Y. lipolytica* G3-3.21 in a cosolvent system containing an ionic liquid (IL).[92] After optimizing the conditions, a γ-decalactone yield of 8.05 g/L was obtained, and after reuse for 10 times, the yield of γ-decalactone could still reach 7.51

g/L, which is equivalent to 93.3% of the yield in the first batch. It is suggested that a good reusability and potential for industrial application. Recently, Rong et al. used a porous starch delivery system to reduce cell damage during the production of γ-decalactone from ricinoleic acid, and obtained a product titer of 3.36 g/L.[93] Similalry, Zhang et al. used bacterial cellulose-alginate (BC-ALG) composite beads as cell carriers for *Y. lipolytica*, which increased the yield of γ-decalactone to 8.37 g/L.[94] Thus, immobilized cells are significantly more resistant to toxic components in the fermentation system than free cells. Accordingly, the production capacity of strains can be significantly improved by using immobilized cells for microbial fermentation.

4.5 Process optimization

The production of γ-decalactone is influenced by many process-related factors, including both culture conditions and product separation (Figure 3). Notably, a suitable culture pH is beneficial to the production of γ-decalactone. García et al. found that the optimal pH for the production of γ-decalactone under low aeration conditions is 6, while under high aeration conditions, the optimal pH is 4.[78] Gomes et al. used empirical modeling to optimize the production of lactones. When *Y. lipolytica* was used for the biotransformation of ricinoleic acid to produce γ-decalactone, the optimal pH was 6.17 and the optimal dissolved oxygen concentration (DO) was 44.4%.[95] In addition, when they were studying the effect of lipase on the hydrolysis of castor oil, they found that after adding exogenous lipase, the optimal hydrolysis conditions were pH 8 and 27℃, which increased the production rate of γ-decalactone.[70] Furthermore, García et al. increased the cell concentration 2-fold in medium containing 30 g/L

castor oil, which increased the lactone yield 2.2 times, reaching 2.7 ± 0.4 g/L.[78]

Scale-up from shake-flask to fermenter culture is a very important step for large-scale production of γ-decalactone. Rabenhorst et al. compared the ability of *Y. lipolytica* strain HR 145 (DSM 12397) to produce γ-decalactone in a 10 liter and 300 liter bioreactor. Under the optimal stirring and aeration conditions, 12.5 g/L of γ-decalactone was obtained.[96] Recent studies have shown that fed-batch fermentation is an effective method to increase the production of γ-decalactone because it can avoid the degradation of γ-decalactone while reducing the toxicity and inhibitory effect of the substrate on cells. Gomes et al. studied the effects of batch and fed-batch culture on the accumulation of γ-decalactone, and found that higher productivity of γ-decalactone (168 mg L^{-1} h^{-1}) could be obtained under batch cultivation, but in terms of output, the effect of intermittent batch feeding was better, resulting in 6.8 g/L of γ-decalactone.[97] In addition, Braga et al. found that both batch and stepwise fed-batch culture of *Y. lipolytica* at an initial cell density of 60 g/L resulted in a γ-decalactone productivity of 215 ± 19 mg L^{-1} h^{-1}. However, the best γ-decalactone concentration of 5.4 ± 0.5 g/L was obtained in batch culture. This shows that these two strategies are suitable for industrial production.[84] Notably, the authors also studied the accumulation of γ-decalactone in tradition stirred tanks (STR) and airlift bioreactors, and found that compared with STR, the concentration of γ-decalactone in airlift bioreactors increased 2 times, reaching approximately 3 g/L.[84] Guan et al. used the expanded vermiculite delivery system to improve the biotransformation efficiency of ricinoleic acid to γ-decalactone, and the product titer was increased to 6.2 g/L.[98]

At present, the separation and purification of volatile fragrance products remains a complicated and challenging step. Accordingly, researchers investigated novel techniques to separate γ-decalactone. *In situ* separation technology (ISPR) is an extraction method that can quickly remove without harming cells, so as to prevent the final products from inhibiting cell growth. The advantages of using ISPR in the process of biotransformation include (1) mitigating the toxicity of the product, so that the target product can continue to accumulate and reach a high level, (2) reducing product loss due to degradation or uncontrolled leakage (such as evaporation), as well as (3) simplifying the downstream separation and purification steps. In order to further eliminate the toxic effect of the product and improve γ-decalactone production, reaction separation coupling technology has been applied to biotransformation. Reaction separation coupling technology refers to the simultaneous completion of both reaction and separation processes in a single set of equipment, integrating the separator and reactor in a system. The main media used for the separation of γ-decalactone include activated carbon, resins and organic solvents. Some studies have found that the separation of γ-decalactone by activated carbon or hydrophobic resin can alleviate product inhibition, but the production of γ-decalactone is reduced due to the presence of the adsorption medium.[99] Yu et al. found that by adding 7.5% AB-8 resin, the toxicity of high concentrations of γ-decalactone to yeast can be reduced. Consequently, the product yield increased by 36%, reaching 2.17 g/L.[100] Alchihab et al. added natural tragacanth gum during the production of γ-decalactone, and the product yield was increased to 6.5 g/L.[86] In

addition, the authors also studied the adsorption of γ-decalactone by three Macronet resins, and found that MN-202 can adsorb γ-decalactone more effectively than the other two resins, with an adsorption rate of 85%. Moreover, the resin was found to be easy to regenerate, indicating its potential for the industrial production of γ-decalactone.[101] Li et al.[101] found that adding the organic solvents isopropyl myristate or isopropyl hexadecanoate for phase transfer of γ-decalactone from the fermentation broth can improve the product yield compared to an aqueous single-phase system and the purity can reach more than 98.3%.Recently, Kothari et al. used a continuous stirred tank reactor (CSTR) to produce γ-decalactone, achieving the highest production rate of 200 mg/L/h in 10 hours.[102] Furthermore, the produced γ-decalactone was isolated and purified from the biotransformation medium using a combination of chromatography and liquid-liquid extraction, resulting in 80% product purity and 85% overall recovery. Małajowicz et al. compared the effectiveness of liquid-liquid extraction, hydrodistillation and adsorption onto porous materials (zeolite, vermiculite and Amberlite XAD-4 resin).[103] According to the results, Amberlite XAD-4 was the best medium, capable of adsorbing 80% γ-decalactone (Table 3). This study provides effective guidance for the isolation of γ-decalactone from biotransformation media. In conclusion, coupled separation technology can be used to continuously separate the produced γ-decalactone from yeast cells, effectively reducing product toxicity and degradation, greatly improving the final yield.

5. Summary and future directions

Based on current research, the substrates for the production of γ-decalactone by

Y. lipolytica are mostly castor oil, ricinoleic acid and methyl ricinoleate, while most metabolic engineering studies focused on the modification of the β-oxidation pathway. However, there are still many problems to be solved before *Y. lipolytica* can become a commercially viable industrial host for the production of this important aroma compound.

First of all, the castor oil is extracted from the castor bean, which is the seed of the plant *Ricinus communis*. Although castor oil has many applications as an important industrial raw material, castor beans contain extremely toxic ricin and ricinine, with lethal doses as low as 7 mg. Therefore, seeking new substrates is an important strategy for the industrially viable production of γ-decalactone. As a monounsaturated fatty acid, oleic acid accounts for 37.23% of the total fatty acids of wild-type *Y. lipolytica*. In addition to its high value in industry, medicine and other fields, oleic acid can be used as a platform compound for the production of other fatty acids. Recently, Beopoulos et al. expressed the Δ12 hydroxylase derived from *Claviceps purpurea* in *Y. lipolytica* to achieve high production of ricinoleic acid using oleic acid as the substrate.[63] As discussed above, ricinoleic acid can be used as a direct precursor for the synthesis of γ-decalactone. Furthermore, Marella et al. used oleic acid and linoleic acid as substrates to produce γ-dodecalactone and δ-decalactone by expressing different hydratase enzymes.[52] Therefore, the use of oleic acid as a substrate to produce γ-decalactone is feasible and needs further research, especially focusing on the screening of different hydroxylases, hydratases and acyl-CoA oxidases. However, Oleic acid production from high oleic plant oils is limited by

long growing cycles and climate. In addition, global demand for high oleic plant oils drives deforestation of tropical forests, leading to rainforest destruction, climate change, and biodiversity loss[113]. The source of ricinoleic acid, castor, is highly toxic, and its widespread cultivation is hindered. The supply of ricinoleic acid depends on the import of castor oil from developing countries. The economic instability of the production area often leads to fluctuations in the supply, price and quality of castor oil. Therefore, the use of oleic acid, ricinoleic acid and other hydroxy fatty acids as substrates to produce γ-decalactone is not a sustainable choice. Therefore, we must seek more novel methods to produce γ-decalactone as a sustainable fragrance compound for the future bio-economy, which can be more competitive in the market. The biological production route of *Y. lipolytica* using renewable raw materials and industrial wastes (such as glucose, glycerol, etc.) as substrates to produce γ-decalactone will be the focus of future research.

Due to its peach-like fragrance and low aroma threshold, γ-decalactone has broad application prospects in the flavor and fragrance industries. With the development of metabolic and process engineering, the use of the biomanufacturing to produce γ-decalactone has greater advantages compared with traditional plant extraction and chemical synthesis methods. In this review, we summarized strategies for increasing the yield of γ-decalactone in *Y. lipolytica*, including the metabolic engineering of the hydroxylation pathway, β-oxidation pathway, and lactonization reaction, as well as fermentation optimization for process engineering strategies. The use of renewable raw materials to produce γ-decalactone is a strong future trend, even though the

current titer, production rate and yield (TRY) values are still unsatisfactory.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 1 Comparison different microbes that can produce γ-decalactone

Strains	Substrates	cultivation conditions	Titer of γ-decalactone (g/L)	Productivity (g/L/hr)	References
Candida sp.	Ricinoleic acid	Flask	0.13	0.005	9
Sporidiobolus sp.	Ricinoleic acid	Flask	0.14	-	104
Sporobolomyces odorus	Castor oil	Bioreactor	0.21	-	105
Trichoderma harzianum	Castor oil	Bioreactor	0.26	0.001	106
Pichia guilliermondii	Ricinoleic acid	Agitated inverted T tubes	0.71	0.002	107
Rhodotorula glutinis	Castor oil	Bioreactor	0.85	0.007	14
Saccharomyces cerevisiae	Ricinoleic acid	Flask	3.48	0.058	74
Lindnera saturnus	Castor oil	Flask	5.80	0.048	72
Rhodotorula aurantiaca	Castor oil	Bioreactor	6.60	0.046	108
Mucor sp.	Ethyl decanoate	Bioreactor	10.50	0.247	109
Y. lipolytica	Castor oil	Bioreactor	12.50	0.178	96

Table 2 Metabolic engineering of *Y. lipolytica* for efficient production of γ-decalactone

Strains	Substrates	Engineering strategies	cultivation conditions	γ-Decalactone (g/L)	Productivity (g/L/hr)	References
Y. lipolytica Po1d	Methyl ricinoleate	*POX1* disrupted	Flask	0.08	0.002	44
Y. lipolytica JMY185	Methyl ricinoleate	*POX2* overexpressed and *POX3, POX5* disrupted	Flask	0.15	0.002	65
Y. lipolytica MTLY40-2P	Methyl ricinoleate	*POX2* overexpressed and *POX3-5* disrupted	Flask	0.40	0.008	48
Y. lipolytica As2.1045	Methyl ricinoleate	*POX3* disrupted and copper-resistance gene *CRF1* integrated	Flask	0.53	0.008	53
Y. lipolytica As2.1045	Methyl ricinoleate	Multiple *POX2* overexpressed and *POX3* disrupted in the diploid strain	Fermentation tube	3.3	0.033	45
Y. lipolytica MTLY40-2P	Castor oil	*POX2* overexpressed and *POX3-5* disrupted, Step-wise fed-batch culture	Bioreactor	7.00	0.1	66
Y. lipolytica Po1g	4-Hydroxydecanoyl-CoA	*Pp*Aat1 overexpressed	Flask	2.20	-	69

Table 3 Process engineering to enhance the production of γ-decalactone

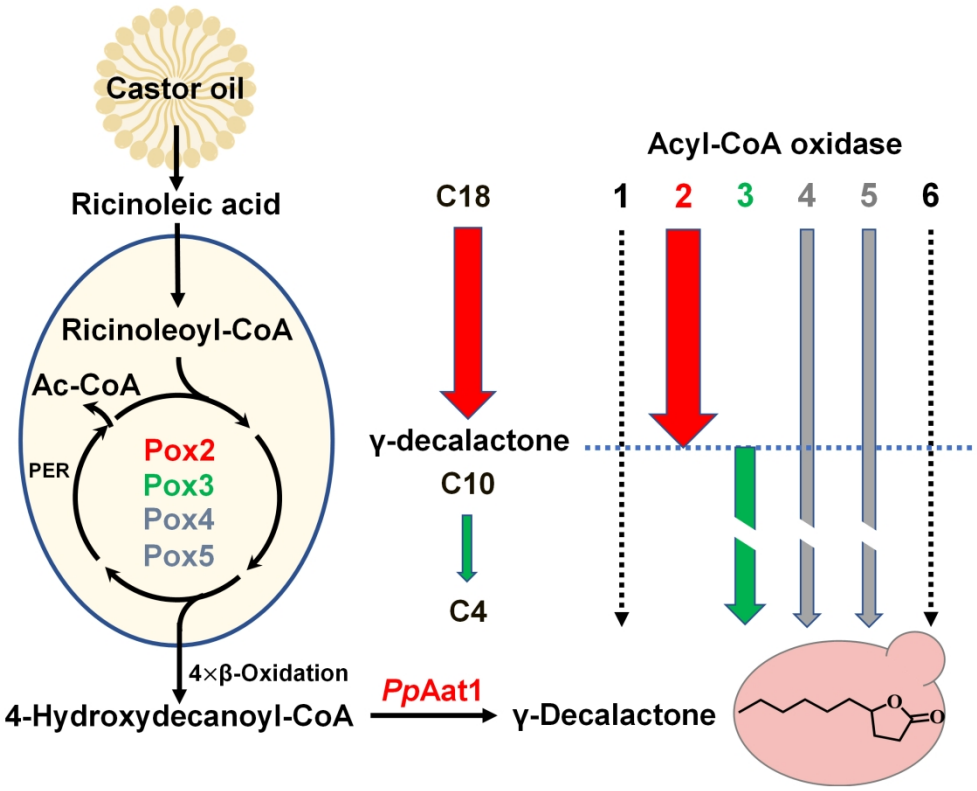
Strains	Substrates	Operation modes	Fermentation time (h)	Titer of γ-decalactone (g/L)	References
Y. lipolytica AS2.1405	Castor oil	Immobilization using the mixture of PVA and carrageenin	24	0.48	89
Y. lipolytica W29	Methyl ricinoleate	Batch	-	0.68	95
Y. lipolytica W29	Methyl ricinoleate	Fed-batch	173	6.80	97
Yarrowia sp. CGMCC2.1405	Castor oil	Immobilization using sodium alginate compounded with attapulgite	48	4.17	90
Y. lipolytica W29	Castor oil	Batch	160	1.84	70
Y. lipolytica DSM 3286	Castor oil	Fed-batch	70	0.22	110
Y. lipolytica W29	Castor oil	Immobilization using DupUM®	264	1.60	91
Y. lipolytica W29	Methyl ricinoleate	Airlift biofilm	60	1.70	82
Y. lipolytica W29	Castor oil	Airlift	104	2.90	84
Y. lipolytica W29	Castor oil	Fed-batch	25	5.40	83

Y. lipolytica G3-3.21	Castor oil	Immobilization using attapulgite	-	8.05	92
Y. lipolytica CGMCC 2.2087	Ricinoleic acid	Batch (Porous-Starch Delivery System)	48	3.36	93
Y. lipolytica CGMCC 2.2087	Ricinoleic acid	Batch (Expanded Vermiculite Delivery System)	60	6.20	98
Y. lipolytica CGMCC 2.2087	Ricinoleic acid	Immobilization using Bacterial Cellulose-Alginate Composite Beads	48	8.37	94
Y. lipolytica MTLY40-2p	Castor oil	Batch	168	5.50	111

Figure 1. The metabolic pathway from ricinoleic acid to γ-decalactone and related activities of six acyl-CoA oxidases of *Yarrowia lipolytica* (Pox1-Pox6). Adapted from Groguenin et al.[48]Activity of 6 acyl-CoA oxidases: Pox1 and Pox6 have no detectable activity, Pox2 and Pox3 have high activity, and Pox4 and Pox5 have low activity. Pox2 has long-chain specificity, Pox3 has short-chain specificity, while Pox4 and Pox5 have measurable activity on the full spectrum of unbranched fatty acids. The red, green and gray arrows represent different engineering strategies, the red arrow indicates gene overexpression, the truncated green and gray arrows indicate gene knockout.

Figure 2. Metabolic pathway for *de novo* biosynthesis of γ-decalactone and the related engineering strategies. The red and green letters represent different engineering strategies, red enzyme names indicate gene overexpression or heterologous expression, green enzyme names indicate gene knockout. References a-c are: a[112]; b[63]; c[68-69, 83].LB, lipid body; ER, endoplasmic reticulum; PER, peroxisome; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; G3P, glycerol-3-phosphate; Pyr, pyruvate; Ac-CoA, acetyl-CoA; CIT, citrate; OAA, oxaloacetate; ICT, isocitrate; α-KG, α-ketoglutarate; Mal-CoA, malonyl-CoA; Acl1, ATP-citrate lyase 1; Acl2, ATP-citrate lyase 2; Acc1, acetyl-CoA carboxylase; FAS, fatty acid synthase (including FAS1 and FAS2); Scd, stearoyl-CoA desaturase or Δ9 desaturase; *Pg*Scd, *Puccinia graminis* Δ9 desaturase; Elo1, fatty acid elongase 1; *Ma*Elo2, *Mortierella alpina* fatty acid elongase 2; Fad2, Δ12 desaturase; *Cp*Fah12, *Claviceps purpurea* hydroxylase; Dga1, Acyl-CoA diacylglycerol acyltransferase 1; Dga2, Acyl-CoA diacylglycerol acyltransferase 2; Lro1, phospholipid diacylglycerol acyltransferase; Pox1-6, acyl-CoA oxidase; *Pp*Aat1, *Prunus persica* alcohol acyltransferases, FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide.

Figure 3. Metabolic and process engineering strategies for producing the peach-like aroma compound γ-decalactone in *Y. lipolytica*. **a**. engineering the hydroxylation pathway, β-oxidation pathway and lactone synthesis reaction to produce γ-decalactone. **b**. choosing an appropriate substrate to increase the yield. **c**. expressing lipase to accelerate the hydrolysis of castor oil to ricinoleic acid. **d**. optimizing the dissolved oxygen concentration is beneficial to eliminate the influence of by-products. **e**. immobilizing cells to reduce the toxicity of substrates and products. **f**. expanding the fermentation system to increase the production of γ-decalactone. **g**. using separation systems to increase product purity effectively.



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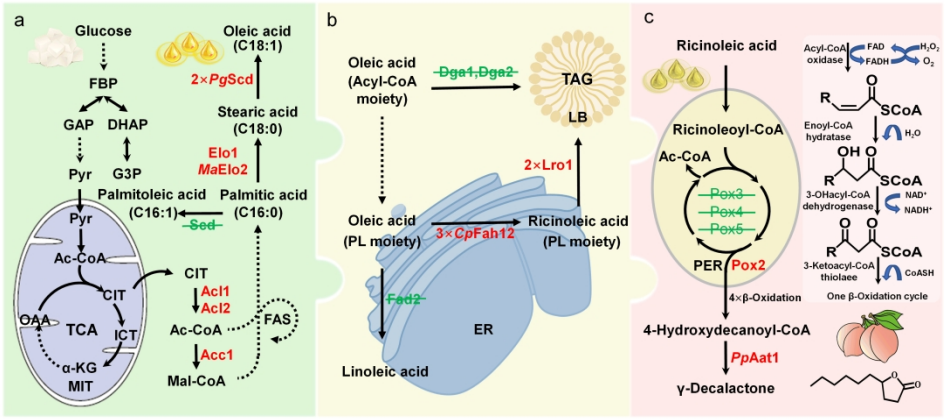

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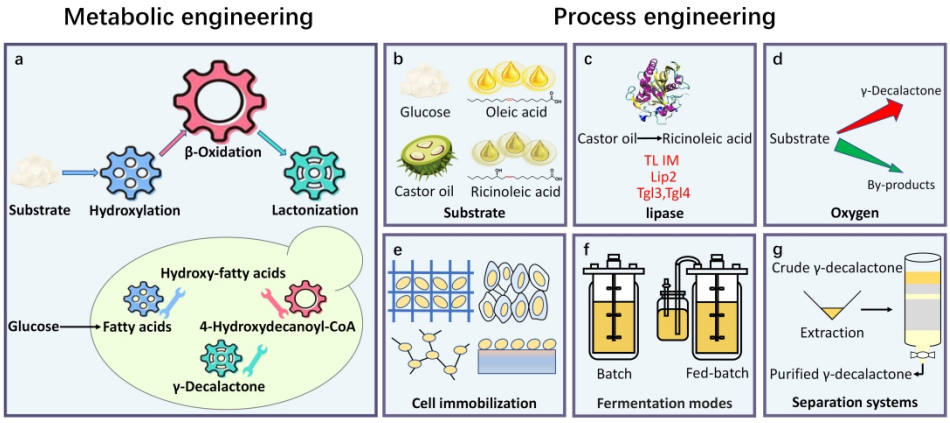

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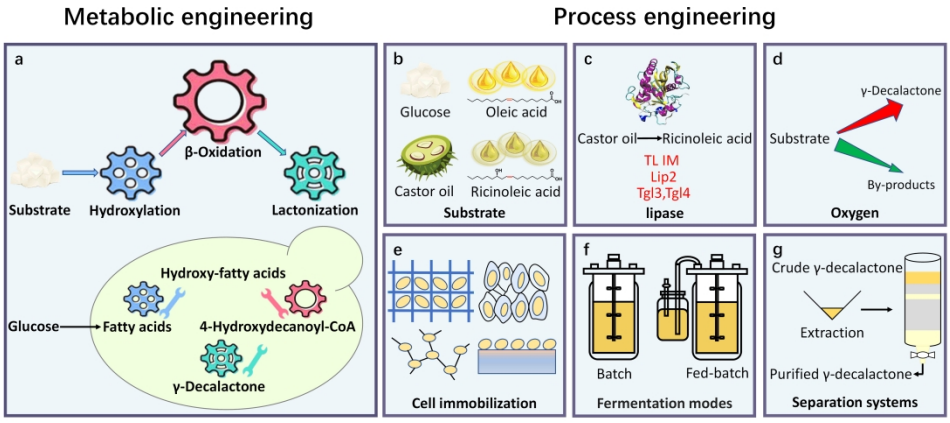

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