

Review

Microbial Biosynthesis of Lactones: Gaps and Opportunities towards Sustainable Production

Rui Silva, Eduardo Coelho, Tatiana Q. Aguiar and Lucília Domingues *

CEB—Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal;
rsilva@ceb.uminho.pt (R.S.); e.coelho@ceb.uminho.pt (E.C.); tatiana.aguiar@deb.uminho.pt (T.Q.A.)

* Correspondence: luciliad@deb.uminho.pt

Abstract: Lactones are volatile organic compounds widely present in foods. These chemicals are applied as flavors and fragrances in the food, cosmetics and pharmaceutical industries. Recently, the potential of lactones as green solvents and fuel precursors reinforced their role as platform compounds of future bio-based economies. However, their current mode of production needs to change. Lactones are mainly obtained through chemical synthesis or microbial biotransformation of hydroxy fatty acids. The latter approach is preferred but still needs to use more sustainable substrates. Hydroxy fatty acids are non-abundant and non-sustainable substrates from environmental, health and economic points of view. Therefore, it is urgent to identify and engineer microorganisms with the rare ability to biosynthesize lactones from carbohydrates or renewable lipids. Here, we firstly address the variety and importance of lactones. Then, the current understanding of the biosynthetic pathways involved in lactone biosynthesis is presented, making use of the knowledge acquired in microorganisms and fruits. From there, we present and make the distinction between biotransformation processes and de novo biosynthesis of lactones. Finally, the opportunities and challenges towards more sustainable production in addition to the relevance of two well-known industrial microbes, the filamentous fungus *Ashbya gossypii* and the yeast *Yarrowia lipolytica*, are discussed.

Keywords: lactones; flavors; fragrances; microbial production; de novo biosynthesis; metabolic engineering; bioeconomy

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1. Introduction

Lactones are volatile organic compounds (VOCs) that are chemically classified as esters of hydroxy acids and defined by a cyclic ring. The size of the cyclic ring determines the type of lactone: α - (three atoms), β - (four atoms), γ - (five atoms) and δ - (six atoms) [1]. γ -lactones and δ -lactones are the most diverse, chemically stable and commercially appreciated (Figure 1). Derived from the lipid metabolism [2], lactones are naturally present in fruits (peaches, strawberries and mangoes), vegetables, meat, milk, butter, cheese and wine, among others, and are generally the main contributors to the aroma and flavor of these food products [1–5]. γ -lactones are more commonly found in fruits and δ -lactones in animal products [6]. These compounds have low perception thresholds (e.g., γ -nonalactone 25 $\mu\text{g/L}$, γ -decalactone 0.88–1 mg/L and γ -dodecalactone 7 $\mu\text{g/L}$) [7,8] and very powerful odor descriptors that range from peach-like and coconut to creamy and floral [9]. Generally, odor perception thresholds are inversely related to the length of the side chain of the lactone [10]. In addition, some lactones present anti-microbial and anti-inflammatory properties [11–13].

Aroma properties of chemicals are highly relevant for the manufacturing of perfumes, cosmetics and food products. The chemical properties of the different lactone rings define characteristic aromas and/or flavor notes that raise the most immediate

commercial interest in lactones as food additives and fragrances [1]. The most valuable lactone to this end, γ -decalactone, has fruity and floral aromas descriptors, fulfilling the rising consumer demand for natural compounds. Consequently, lactones reach a world market volume of dozens of million tons per year and values between ~US\$ 300–6000/kg, with a decreasing trend along the years due to the technological developments regarding their production [14,15]. Additionally, lactones have also been reported to be green solvents and fuel precursors (e.g., γ -valerolactone) [16], expanding their relevance for the transition to a bio-based sustainable economy. Some lactones are green/non-toxic solvent alternatives and their chemical properties enable their use in the production of C8 alkanes (used in jet fuel), C9 and C18–C27 alkanes (gasoline and biodiesel) and other polymers, e.g., nylon precursors [2,16]. Therefore, lactones are currently considered platform compounds for future sustainable circular bioeconomies.

Lactones are still mainly obtained through chemical synthesis, via oxidation of cyclic ketones with hydrogen peroxide and acetic acid in the presence of a strong acid catalyst. However, this raises environmental concerns due to the high use of solvents and acids, the generation of undesired racemic mixtures as a final product and the fact that it is not well accepted by consumers. In turn, microbial production is a more sustainable and efficient way of producing optically pure lactones with similar configurations and properties to those found in fruits [2]. Developed and patented biotechnological processes for lactone production have been exclusively made by biotransformation. Biotransformation refers to the microbial conversion of an immediate precursor into a lactone, and is accomplished by adding that precursor to the production medium of microorganisms with the ability to convert it into a specific lactone. For the microbial production of γ -decalactone, the yeast *Yarrowia lipolytica* has been the preferred system exploited, using hydroxy fatty acids as precursors [17]. However, this method of production holds some limitations. First, the production of a certain lactone completely depends on the availability of the corresponding precursor. It is therefore not possible to use the same medium to produce different lactones. Second, the industrial and commercial value of the precursors used are high and often competitive with that of lactones [2]. Third, hydroxy fatty acids are not an abundant nor renewable raw material and, for instance, ricinoleic acid from castor oil (used as a γ -decalactone precursor) has several health, seasonal and economic issues related to the crop of the plant from where it is extracted [18–20].

Therefore, the development of production processes that use sustainable and more abundant substrates is of the utmost importance. However, microbial species that are able to de novo biosynthesize lactones (i.e., produce lactones from sugars or other non-lipid substrates) or even perform biotransformation from non-hydroxy and more abundant fatty acids are scarce. There is also a considerable lack of research either on fruits [21,22] or microbes [23], resulting in enzymatic and genetic gaps in the biosynthetic process of lactones. Moreover, unlike other high-value chemicals, the engineering of microbial strains for lactone production was not extensively pursued, being mostly limited to the β -oxidation pathway of *Y. lipolytica* [24]. Nonetheless, in recent years, there is a growing number of studies that are addressing this problem and exploring the production of lactones from more renewable substrates. Recent examples are the identification and engineering of the filamentous fungus *Ashbya gossypii* as a microorganism able to perform de novo biosynthesis of lactones [23] and biotransformation of non-hydroxy fatty acids [10], as well as the engineering of *Y. lipolytica* to perform biotransformation of non-hydroxy fatty acids [20].

In this review, we will address the chemical diversity and applications of lactones before providing an overview of the works performed either on microbes or fruits that contributed to the current understanding of the intermediates, enzymes and pathways required for the biosynthesis of lactones. γ - and δ -lactones are the most stable and commercially valuable lactones and fungi are the most exploited microorganisms for their biotechnological production. Therefore, the identification of the most relevant studies to

the microbial production of γ - and δ -lactones in fungi either through biotransformation or by de novo biosynthesis will be presented next. Finally, we will point out some of the gaps in the research of lactones from an industrial biotechnological perspective and propose future directions. Together, these represent the challenges that should be addressed in order to drive the development of more sustainable second-generation lactone bioproduction processes.

The literature search for this narrative review was made using the PubMed and Google Scholar search engines in addition to the keywords indicated after the abstract, both in separated and conjugated forms. No time frame filters were used for the searches made, as literature addressing the de novo biosynthesis of lactones is scarce.

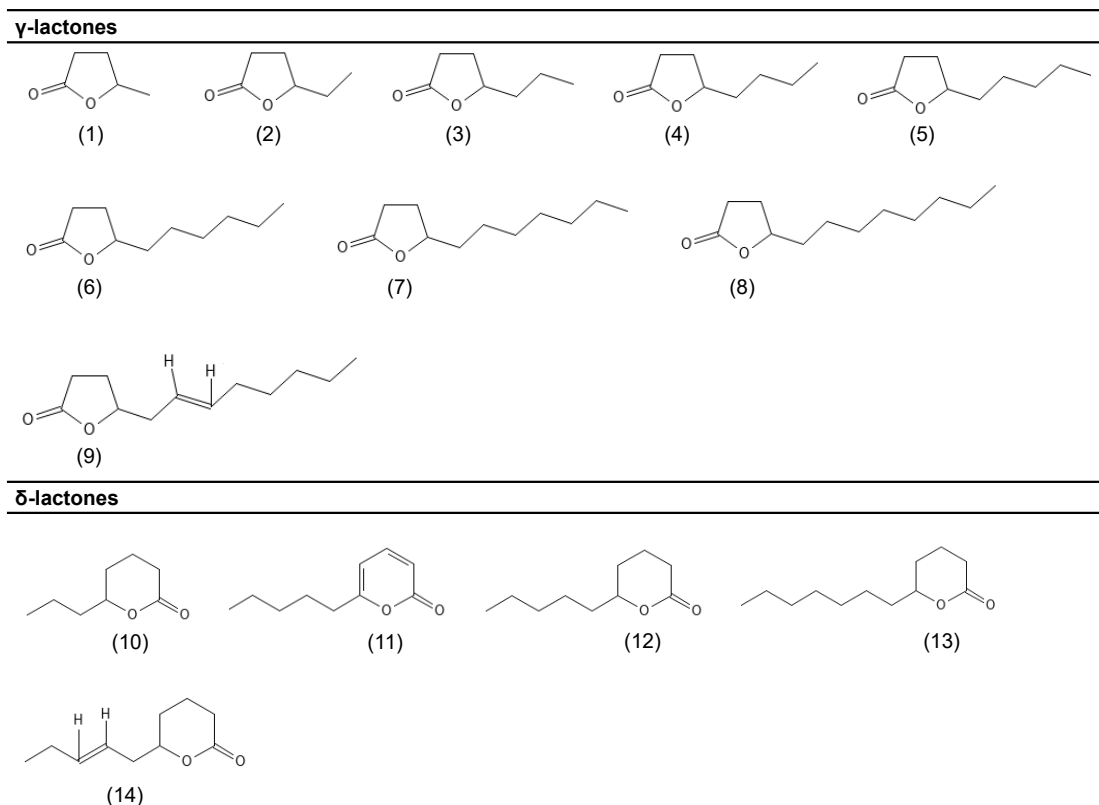


Figure 1. Chemical structures of different γ -lactones and δ -lactones produced by microbial de novo biosynthesis. Numbers refer to Table 1, where these lactones are identified and their main properties described.

2. Properties, Diversity and Applications/Chemistry and Applications

As mentioned, lactones are cyclic carboxylic esters, which can derive from intramolecular esterification of carboxylic acids. Lactones present two common structural traits: the presence of a $(-C(=O)-O-)$ carboxylic ester group and a cyclic ring organization, which can have heteroatoms, substitutions or unsaturations in the ring structure. Lactones have a variable number of carbons, and nomenclature often refers to the carboxylic acid precursor, with the total number of carbons in the molecule represented before the -lactone suffix, e.g., carbon atoms: 2-aceto, 3-propio and 4-butyro. Another key structural parameter in lactones is the number of atoms participating in the cyclic ring. This is noted in lactone nomenclature by including the prefix of α - (three atoms in the cyclic ring), β - (four atoms in the cyclic ring), γ - (five atoms in the cyclic ring) and δ - (six atoms in the cyclic ring) [1]. Among these, γ - and δ - are the more stable forms of lactones, and thus the main focus of most processes and applications [5]. For example, as reported for γ -

decalactone, its conformation is so stable that cyclization of 4-hydroxydecanoic acid occurs spontaneously at room temperature in the presence of dilute acids [25].

Lactones can be naturally found in different organisms, either in bacteria, yeast, plants and vertebrates, and are reported to be motifs used by organisms to communicate via the chemical channel [15,26]. For example, lactones are commonly found in fruits (e.g., strawberries, peaches and pineapples), where they act as signals for attracting feeders to promote pollination and seed dispersion [22]. Thus, these compounds are naturally potent flavoring agents which translate into their main application. Different lactones have different associated aroma descriptors, from which “fruity”, “peach”, “coconut” and creamy can be highlighted. Therefore, these compounds are very sought-after in food and cosmetic industries, to be used as pure fragrances and/or flavors in the most diverse formulations.

Apart from aroma applications, lactones can also be used in alternative products and commodities: biodegradable polymers for use in medical devices (e.g., polycaprolactone) [27]; green/non-toxic solvent alternatives; and drop-in agents in the production of C8 alkanes (used in jet fuel), C9 and C18–C27 alkanes (gasoline and biodiesel) and other polymers. Such is the example of γ -valerolactone, a highly promising compound for generating energy and producing carbon-based commodities, which can furthermore be sourced from sustainable and renewable resources [16,28]. Lastly, macrolide antibiotics (erythromycin, azithromycin) are also lactones, more specifically macrocyclic lactones with high structural complexity and a broad range in the management of infectious diseases [13,29].

Chemical synthesis of lactones can be promoted by different reaction mechanisms, for example from carboxylic acids by Yamaguchi esterification [30], Shiina macrolactonization [31] or Corey–Nicolaou macrolactonization [32] and from ketones by Baeyer–Villiger oxidation [33]. Most of these reactions involve the use of acids (e.g., Lewis acids), pyridines (e.g., DMAP) and/or oxidants (e.g., peroxide), either as reactants or catalysts, in addition to the precursors. Thus, utilization of unsustainable and environmentally dangerous reactants is inherent and several works have strived for greener alternatives for lactone production, either via chemical synthesis or biotechnological alternatives. The latter will be explored in more detail in this review.

3. Pathways and Enzymes Involved in the Biosynthesis of Lactones

The biosynthesis of lactones is a complex process still not fully understood at the genetic and biochemical level. First, it is important to underline the difference between de novo biosynthesis of lactones and biotransformation processes. From the current knowledge, de novo biosynthesis of lactones means that the producing microorganism does not use any immediate precursor of the final product, i.e., no fatty acid or hydroxy fatty acid derivatives are added to the production medium in order to obtain its conversion into a specific lactone. This means that in order to de novo biosynthesize lactones a microorganism should be able to: (i) use a substrate (e.g., sugars) to support the growth and biosynthesis of fatty acids; (ii) perform post-modification of these fatty acids in order to generate a derivative with a hydroxy group; (iii) shorten this hydroxy fatty acid in the β -oxidation pathway, with the reduction of two carbons in the chain per cycle; and (iv) after specific cycles of β -oxidation, generate a hydroxy acid that undergoes spontaneous lactonization under acidic conditions [21,23]. To the extent of our knowledge, only the filamentous fungi *Fusarium poae* [34], *Trichoderma viride* [35], *A. gossypii* [23] and the yeast *Sporidiobolus salmonicolor* [4] were reported to compile these traits (Table 1). Basidiomycetes, such as mushrooms (e.g., *Polyporus durus*), are known to produce several volatile compounds including γ - or δ -lactones, either through biotransformation [36] or through de novo biosynthesis [37]; however, during this review we focused on ascomycetes due to their higher relevance for industrial biotechnology. An exception was given to the basidiomycete *S. salmonicolor*, since its yeast-like growth led to deeper investigation of lactone biosynthesis in the 1990s.

Biotransformation comprises only steps (iii) and (iv), since an immediate precursor of the β -oxidation pathway is added to the production media [2,14]. Recently, there have been some advances regarding the use of non-hydroxy fatty acids in biotransformation processes for lactone production that will be discussed in Section 4. A summarized scheme of the pathways involved in lactone biosynthesis and biotransformation is depicted in Figure 2. The following subsections review the studies made on *S. salmonicolor* and on other important lactone producers (such as fruits) that contributed to the current established knowledge on lactone biosynthesis. Other microbial systems were also used to help understand the relevant enzymes and pathways involved in lactone biosynthesis, however these findings will be discussed in Section 4.2 together with the approaches taken to improve lactone biosynthesis by means of strain engineering and/or process optimization.

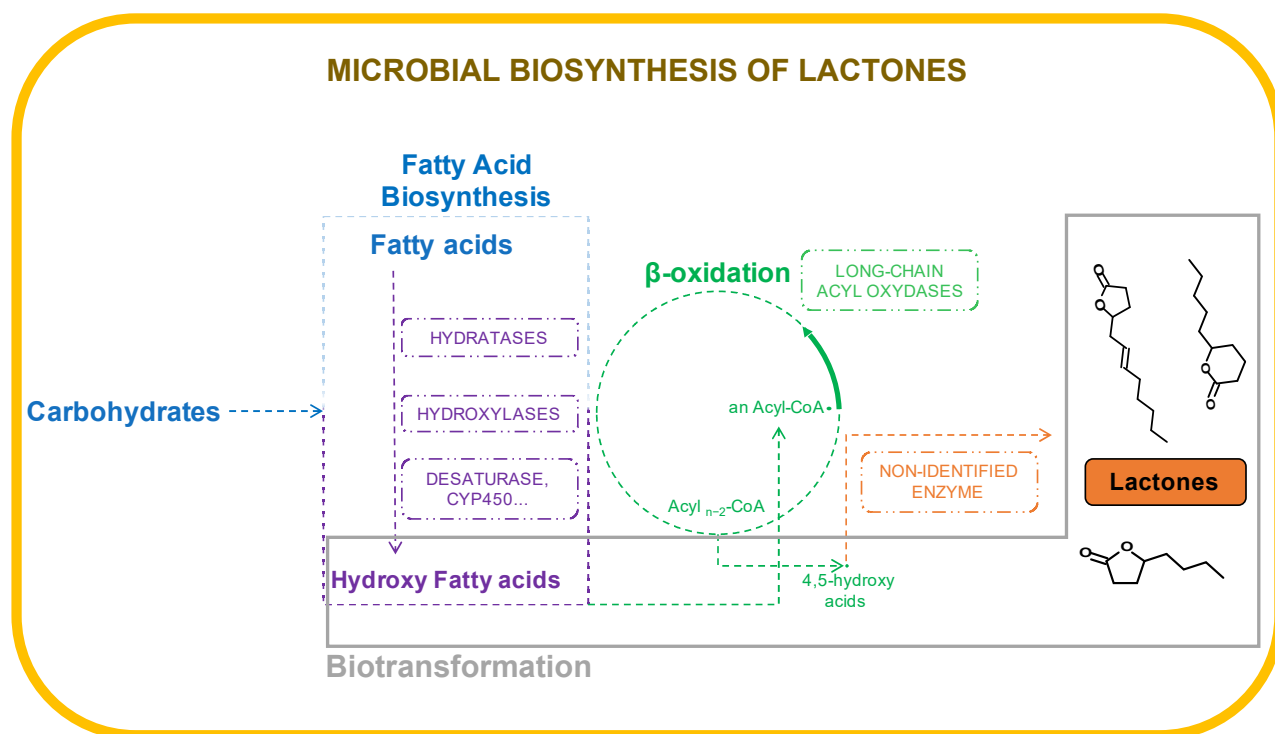


Figure 2. Schematic representation of microbial biosynthesis of γ - and δ -lactones. Overview of the different stages: biosynthesis of fatty acids (blue); formation of hydroxy fatty acids (purple); β -oxidation (green); and lactonization (orange). Traditional biotransformation processes are indicated by a grey box. Dashed boxes represent the most relevant enzymatic activities for improvement of lactones biosynthesis.

3.1. *Sporidiobolus salmonicolor*

It was in 1972 that Tahara and colleagues reported the de novo biosynthesis of γ -decalactone [4] and in 1973 of cis-6- γ -dodecenolactone [38] by *S. salmonicolor* (former *Sporobolomyces odoros*). Two years later, δ -decalactone and δ -jasmin lactone were also identified in cultures of this yeast [39]. Later on, γ -octalactone and γ -nonalactone were also detected in cultures of this yeast when the effect of media composition on the de novo biosynthesis of lactones by *S. salmonicolor* was investigated [40]. From the five γ -lactones detected in this study, the major lactones produced were cis-6- γ -dodecenolactone and γ -decalactone, while γ -octalactone and γ -nonalactone were reported for the first time [40] (Table 1).

Table 1. Lactones produced by microorganisms through de novo biosynthesis.

Compound	PubChem CID	Molecular Formula	Odor Descriptor	Microorganisms	Reference
γ -lactones					
γ -valerolactone (1)	7921	C ₅ H ₈ O ₂	Peach	<i>F. poae</i>	[34]
				<i>A. gossypii</i>	[23]
γ -caprolactone (2)	12756	C ₆ H ₁₀ O ₂	Creamy	<i>F. poae</i>	[34]
				<i>A. gossypii</i>	[23]
γ -heptalactone (3)	7742	C ₇ H ₁₂ O ₂	Creamy; vanilla; coconut	<i>F. poae</i>	[34]
γ -octalactone (4)	7704	C ₈ H ₁₄ O ₂	Coconut	<i>F. poae</i>	[34]
				<i>S. salmonicolor</i>	[40]
				<i>A. gossypii</i>	[23]
γ -nonalactone (5)	7710	C ₉ H ₁₆ O ₂	Coconut	<i>F. poae</i>	[34]
				<i>S. salmonicolor</i>	[40]
				<i>A. gossypii</i>	[23]
				<i>T. viride</i>	[12]
γ -decalactone (6)	12813	C ₁₀ H ₁₈ O ₂	Fruity, peach	<i>S. salmonicolor</i>	[38]
				<i>F. poae</i>	[34]
				<i>A. gossypii</i>	[23]
γ -undecalactone (7)	7714	C ₁₁ H ₂₀ O ₂	Fruity, peach	<i>F. poae</i>	[34]
				<i>A. gossypii</i>	[23]
				<i>T. viride</i>	[12]
γ -dodecalactone (8)	16821	C ₁₂ H ₂₂ O ₂	Peach	<i>F. poae</i>	[34]
				<i>A. gossypii</i>	[23]
				<i>S. salmonicolor</i>	[38]
				<i>T. viride</i>	[12]
<i>cis</i> -6- γ -dodecenolactone (9)	5352428	C ₁₂ H ₂₀ O ₂	Peach	<i>S. salmonicolor</i>	[38]
				<i>F. poae</i>	[34]
δ -lactones					
δ -octalactone (10)	12777	C ₈ H ₁₄ O ₂	Coconut	<i>T. viride</i>	[12]
6-pentyl- α -pyrone (11)	33960	C ₁₀ H ₁₄ O ₂	Coconut	<i>T. viride</i>	[35]
δ -decalactone (12)	12810	C ₁₀ H ₁₈ O ₂	Creamy	<i>F. poae</i>	[34]
				<i>S. salmonicolor</i>	[39]
δ -dodecalactone (13)	12844	C ₁₂ H ₂₂ O ₂	Sweet/fruity	<i>T. viride</i>	[12]
δ -jasmin lactone (14)	5352626	C ₁₀ H ₁₆ O ₂	Fruity; sweet; floral	<i>S. salmonicolor</i>	[39]

In the 1990s, to resolve the intricate pathways of lactone biosynthesis, chiro-specific analyses and enantiomer separations of the lactones produced by microorganisms were the preferred tools utilized. These works were mainly based on the study of the biotransformation of labeled fatty acids and derivatives to elucidate the precursors of particular lactones [41]. The yeast *S. salmonicolor*, beyond its ability to produce a wide range of lactones, either through de novo biosynthesis and biotransformation, was the most important study model to this end due to its diverse fatty acid metabolism. The studies with *S. salmonicolor* gave important insights concerning the de novo biosynthesis process of lactones in this yeast, mainly regarding the origin of the oxygenated derivatives of fatty acids that are the reagents of β -oxidation [40].

Many studies demonstrated the biotransformation of fatty acids and hydroxy acids into lactones in this yeast. One of the first demonstrated the conversion of labeled linoleic acid (C18:2) into δ -decalactone. This work highlighted the need for linoleic acid to undergo chemical transformations (such as hydroperoxidation) before the action of the β -

oxidation pathway in the biosynthesis of δ -decalactone [42]. This biotransformation was also studied by Haffner and colleagues [43]. In the same study, the biosynthesis of δ -jasmin lactone was demonstrated from linoleic acid (C18:3). However, the introduction of the OH group in both precursors was unclear, with the authors suggesting that it may come from the following enzymatic activities: lipoxygenases, epoxygenase/epoxyhydrolase or hydratases [43].

Subsequent work demonstrated the biotransformation of oleic acid into γ -decalactone, γ -dodecalactone and cis-6- γ -dodecenolactone in *S. salmonicolor* [44]. For γ -decalactone production, it was clear that the enzymatic hydroxylation of oleic acid (C18:1) at carbon-12 occurred before four cycles of β -oxidation and lactonization. Alternative routes for the biosynthesis of the other lactones start with the desaturation of oleic acid, leading to the formation of linoleic acid. From oleic or linoleic acid, a 10-hydratase, a 9,10-epoxygenase and/or a 10-lipoxygenase should be responsible for the hydroxy fatty acids that would generate γ -dodecalactone and cis-6- γ -dodecenolactone [44]. An epoxide pathway is also possibly present in the yeast, which for instance is able to epoxidize oleic and linoleic acid in the formation of γ -dodecalactone and cis-6- γ -dodecenolactone [45]. Indirect evidence of the presence of epoxyhydrolase in this yeast was later reported, with epoxy fatty acids derived from oleic acid being converted into γ -lactones [46].

Unlike *S. salmonicolor*, *Sporidiobolus ruinenii* is not able to de novo biosynthesize lactones. This species, together with *S. salmonicolor*, was used to study the conversion of methyl ricinoleate into γ -decalactone [47]. *S. salmonicolor* produced only γ -decalactone from this precursor, while *S. ruinenii* produced both γ -decalactone and the corresponding free acid, 4-hydroxydecanoic acid. Since lactonization is assumed to occur spontaneously in acidic conditions, the pH of the productions was controlled to exclude any interference from the media. Nonetheless, it was not demonstrated whether the free acid was a precursor or a result of the γ -decalactone catabolism. Still, two alternatives for the differences observed in methyl ricinoleate biotransformation by these species were advanced: (i) 4-hydroxydecanoic acid production by *S. ruinenii* might be the result of a thioesterification reaction of the acyl-CoA released by the β -oxidation, even though the lactonization of this free acid to γ -decalactone without acidic conditions could not be explained; (ii) the 4-hydroxydecanoic acid was the first intermediate in lactone catabolism. *S. ruinenii* would be able to open the lactone ring of γ -decalactone and circumvent its toxic effect to the cell, since lactones are more toxic than 4-hydroxydecanoic acid [47]. Both hypotheses still need further investigation.

In sum, the studies performed on *S. salmonicolor* by analyzing labeled precursors via gas chromatography and mass spectrometry allowed for the postulation of the biosynthetic routes for several lactones [45]. Overall, it can be assumed that after fatty acid biosynthesis, lactone formation starts with the hydroxylation of an unsaturated fatty acid. For instance, regarding the production of γ -lactones, key enzymatic activities were identified: hydroxylases, desaturases, hydratases, epoxygenases, lipoxygenases and monooxygenases such as P450 systems. From linoleic acid, the yeast uses a 13-lipoxygenase-peroxidase pathway for δ -decalactone production, while the same pathway is used for δ -jasmin lactone [45]. Data pointed out that these hydroxy acids were the product of unsaturated fatty acids that then entered the β -oxidation pathway before undergoing lactonization. However, the hypothesis that the oxygenated precursors could also appear from the β -oxidation pathway itself was also considered [40]. Despite the several reactions and corresponding enzymatic activities identified in these works, the identification of the genes responsible for them was not possible. This would boost the understanding of the de novo biosynthesis of lactones for industrial ends as well as the development of biotransformation processes towards more sustainable substrates.

3.2. Fruits

Fruits like peaches and strawberries are intense producers of lactones, mainly during ripening. These compounds largely contribute to the flavor and aroma of these fruits. Of the more than 100–360 volatile aroma compounds previously identified, lactones have the most pronounced effect on the fruit's aroma perception. Thus, since their genome was sequenced, these organisms have aided the elucidation of the intricate biosynthesis of lactones [21,22]. It is clear that the process starts from the fatty acid metabolism. This assumption is reinforced by the fact that in peaches, fatty acids and lactones are inversely regulated, that is, lactones increase during ripening while fatty acids decrease, indicating that the latter are the source of the former [21]. These precursors undergo some post-modifications in order to be converted into a hydroxy fatty acid before entering the β -oxidation, where the immediate precursors of lactones are formed: 4- or 5- hydroxy acids. In fruits it is also not understood whether the last step of lactonization or cyclization occurs completely by spontaneous reaction or if it is catalyzed by an enzyme. In this regard, a gene from peaches that encodes a novel type of alcohol acyl transferase was proposed as a good candidate for this last step [21].

The introduction of the hydroxy group in fruits seems to occur similarly to what happens in microorganisms. In this matter, important enzymes that can potentially catalyze the formation of hydroxy fatty acids were identified. Back in the 1990s, even when no whole genomic data was available, it was reported that in peaches (*Prunus persica* var. *nucipersica*) and strawberries (*Fragaria ananassa*) epoxidations of polyunsaturated fatty acids (e.g., linoleic or linolenic acid) were the source of oxygenated derivatives used for the biosynthesis of lactones [48]. In peaches, candidate genes for each of the following proposed reactions, which are stated to be the route from unsaturated fatty acids to hydroxy fatty acids, were identified: (i) epoxidation by an epoxidase, a peroxygenase or a cytochrome P450 (CYP) followed by the action of an epoxide hydrolase; (ii) direct hydroxylation catalyzed by a CYP or a hydroxylase; and (iii) desaturation by a desaturase followed by a hydration catalyzed by a hydratase or a CYP [21]. In strawberries, one of the most promising candidate gene is *FaFAD1* (*Fragaria* \times *ananassa*), which encodes an enzyme similar to fatty acid desaturases that catalyze the conversion of oleic acid into linoleic acid. The expression of this gene strongly correlates to the biosynthesis of γ -decalactone in ripe strawberries. Surprisingly, *FaFAD1p* conserves some crucial amino acid residues of bifunctional desaturases/hydroxylases and is also closely related to a *RcFAH12p* hydroxylase present in *Ricinus communis* castor beans—the plant that produces castor oil, which is the main source of the ricinoleic acid used for biotransformation processes [18]. Hydroxylases and desaturases share similar reaction mechanisms and therefore can be identified as homologs. This was the case of the oleate 12-hydroxylase from *R. communis* L., which is a homolog of a fatty acyl desaturase [49]. Taken together, it was proposed that γ -decalactone synthesis in strawberries goes from the conversion of oleic acid into linoleic acid via the desaturase activity of *FaFAD1p*. Linoleic acid will then be converted into a hydroxy fatty acid (ricinoleic acid or derivative) via the action of *FaFAH1p*, a CYP-like enzyme also associated with the production of this lactone. Alternatively, the hydroxylase activity of *FaFAD1p* would directly catalyze the hydroxylation of oleic acid to ricinoleic acid, generating the precursor for β -oxidation [22]. However, none of these proposed endogenous fatty acid modification enzymes have been experimentally demonstrated in fruits nor in any other model.

Beyond the convergent biosynthetic data that fruits provide to the study of lactone biosynthesis in microorganisms, the identification of these fatty-acid-modifying enzymes is a key genetic source for microbial engineering purposes.

4. Microbial Production

4.1. Biotransformation

Biotransformation processes for the production of lactones, mainly γ -decalactone, have a long history. Early in 1963, the production of lactones resulting from the catabolism of ricinoleic acid was reported for several species of the genus *Candida*, with the authors suggesting that γ -decalactone was derived from the intermediate 4-hydroxydecanoic acid [50]. Basically, the biosynthetic process is dependent on the shortening of a hydroxy fatty acid in the β -oxidation pathway and spontaneous lactonization. To produce different lactones, different fatty acids must be used, in particular with the hydroxy group in different positions. However, lactone production by biotransformation was reported from other sources, such as cheese [51]. Biotransformation of hydroxy fatty acids for the production of lactones was, since the 1990s, the chosen alternative to achieve a high-yield process. In contrast with de novo biosynthesis, microorganisms that are able to convert fatty acids into lactones are extensive. In particular, the production of γ -decalactone from castor/ricinoleic acid was the subject of intense investigation mainly in the workhorse *Y. lipolytica*, which led to several patent applications [41]. Other patents and processes covering high-yield biotransformation of lactones were related to γ -dodecalactone and γ -octalactone, for instance [6,47]. Some patents report titers above 10 g/L (e.g., 12.5 g/L) by using *Y. lipolytica* in fermenters up to 300 L [11]. The first biotransformation processes were established in the early 1980s and with the boost of novel processes, the price of lactones declined from approximately US\$ 20,000/kg to US\$ 1200/kg in the late 1990s [51]. In the beginning of the last decade at least one company (Safisis—Lessafre, France) had a commercial biotransformation process of ricinoleic acid to γ -decalactone employing *Y. lipolytica* [52].

4.1.1. *Yarrowia lipolytica*

The most well-established biotransformation process for lactone production is clearly the production of γ -decalactone from castor oil, ricinoleic acid or derivatives by *Y. lipolytica*. Castor oil is extracted from the *R. communis* plant and is very rich in ricinoleic acid [18]. These substrates are widely used but there are indications that switching castor oil for alkyl ricinoleates could help reduce the formation of foam and would be favorable for the downstream processing [6]. The biotransformation processes count with the good ability of this yeast to tolerate high concentrations of fatty acids and to perform its sequential shortening through β -oxidation (four cycles) before the formation of the immediate precursor of γ -decalactone, 4-hydroxydecanoic acid. In this process, only part of the lactonization occurs during the fermentation, being necessary the acidification and heating of the culture to achieve high yields. Normally, this process result in titers between 5–10 g/L γ -decalactone [6,11].

Pagot and colleagues started the genetic engineering work with *Y. lipolytica* to improve the biotransformation of hydroxy acids into lactones [53]. The peroxisomal β -oxidation is the pathway responsible for these production processes. It is composed of four reactions catalyzed by three different enzymes that, in a cyclic manner, remove two carbons from the chain of a fatty acid. The enzymes are an acyl-CoA oxidase, a multifunctional hydratase/dehydrogenase and a thiolase. In this work, the authors found that the deletion of one acyl-CoA oxidase encoded by *YIPOX1* increased the activity of the pathway and reduced γ -decalactone production. This lower production level was attributed to the superior consumption of this lactone or of its immediate precursor (4-hydroxydecanoic acid) by the engineered strain. This led to the assumption that the production levels can be considered an equilibrium between the production and consumption of lactones, with β -oxidation playing a relevant role in the catabolism of aromas. On the other hand, overexpression of the thiolase (*YIPOT1*) did not result in any detectable effect [53].

In regard to β -oxidation, *Y. lipolytica* seems to have a clear advantage in catabolizing different fatty-acid-derived compounds. In contrast to other yeasts that only encode 1 acyl-CoA oxidase, *Y. lipolytica* encodes five (*YIPOX1-5*) [54]. These enzymes vary from

each other regarding the chain-length-dependent activity. Concerning the two major oxidases, *YIPOX2* encodes for a long-chain oxidase with activity towards C18–C11, while *YIPOX3* encodes for a short-chain oxidase (<C11). A strain deleted in this short-chain oxidase, *YIPOX3p*, produced more than 4-fold γ -decalactone from methyl ricinoleate than the parental strain (220 vs. 50 mg/L) [55]. This significant increase could be due to the slower reconsumption of lactones or their precursors (e.g., 4-hydroxydecanoic) by the engineered strain, since β -oxidation was affected at the C10 level—its growth was clearly diminished in C10 substrates [54,56]. In order to completely block this reconsumption, no oxidase activity should be present below C10. This would only be possible with the deletion of all *YIPOX* genes except *YIPOX2*. However, the resulting strain would have serious growth defects on fatty acids [56]. In turn, *YIPOX4p*, identified as an oxidase of minor relevance when methyl oleate was used [54], was found to be essential for the biotransformation of methyl ricinoleate into γ -decalactone [55]. *YIPOX1p* does not contribute to the oxidase activity in this yeast. However, the results obtained by Pagot and Waché (1998) [53] are justified by the fact that the deletion of one *POX* gene resulted in increased activity of the other oxidases [54]. Finally, *YIPOX5*-encoded oxidase has broad substrate specificity [54].

Another strategy used to engineer this pathway involved the deletion of *YIPOX3/5* while integrating multiple copies of *YIPOX2* in the genome. In comparison with the wild-type, this strain produced more γ -decalactone from methyl ricinoleate (150 mg/L vs 71 mg/L), had a lower production of unwanted lactones (3-hydroxy- γ -decalactone and decenolides) and almost did not reconsume γ -decalactone. However, its production was slower (80 h vs. 12 h) and the strain was not able to consume all the methyl ricinoleate present in the medium (3.5 g/L in 500 mL baffled shake flasks) [57]. Later on, a similar engineering strategy was applied to an industrial *Y. lipolytica* strain (CGMCC accession number 2.1405) rather than the model strain *Y. lipolytica* W29 (ATCC 20460) [58]. This diploid strain had the two *YIPOX3* alleles deleted and multiple copies of *YIPOX2*, and was able to produce ~3.3 g/L γ -decalactone from 50 g/L of methyl ricinoleate after 110 h. This represented a 3.6-fold improvement in comparison to the parental strain, an improvement superior to that observed in the W29 strain [57]. This study highlighted the importance of screening the intraspecies (strain-level) heterogeneity of microorganisms in microbial production no matter the process or product sought. Manipulation of the *YIPOX* genes was the first and most effective strategy adopted to significantly improve biotransformation processes for the production of lactones in *Y. lipolytica*.

The lipase of *Y. lipolytica* also played a role, namely to improve the release of ricinoleic acid from castor oil. Braga and colleagues (2012) [59] tested the effect of the addition of a lipase inducer (olive oil) in baffled-shake-flask fermentations using 30 g/L of castor oil for the production of γ -decalactone. Although the maximum production level was achieved earlier in the medium with the inducer (140 h vs. 185 h), the titers obtained were lower—1.2 g/L in the medium with inducer vs. 1.6 g/L in the medium without inducer. The productivities of the two conditions tested were similar [59]. Addition of commercial lipases to hydrolyze castor oil was also attempted with similar results; when commercial lipase was present the production of γ -decalactone was faster but presented lower titers [60].

Another important factor taken into account was the toxicity of lactones towards the microorganism [24]. γ -decalactone, γ -dodecalactone and δ -decalactone were toxic for *Y. lipolytica* in the range of 150–300 mg/L, negatively impacting the lag phase or even completely blocking growth. From those lactones, γ -dodecalactone produced the more pronounced negative effects [61]. The effect on the fluidization of the cellular membrane and interaction of the lactones with its components may, at least partially, explain the mechanism of toxicity [62,63].

The optimization of process conditions for biotransformation employing *Y. lipolytica* was also frequently sought, namely the operation mode of the reactor. An intermittent fed-batch strategy for the conversion of methyl ricinoleate was tested in *Y. lipolytica*. The strategy consisted in the addition of methyl ricinoleate at specific time points to avoid

substrate-related toxicity. This approach was superior to a constant feeding strategy in terms of titers, productivity and yield. In comparison with a batch strategy, the productivity was lower but the titer and yield were superior. Overall, in this work the production of 6.8 g/L of γ -decalactone after ~168 h was achieved [64]. Again, a stepwise fed-batch was the best operation mode for the production of γ -decalactone by an engineered *Y. lipolytica* strain [65]. The MTLY40-2P strain had the *YIPOX2-5* native genes deleted and the *YIPOX2* gene overexpressed under the regulation of a promoter that allowed a decrease in catabolic repression. A cell density of 30 g/L was used for biotransformation of under 650 rpm and an aeration rate of 5.1 L/min. An initial concentration of 60 g/L of castor oil followed by two additions of more than 60 g/L was the stepwise fed-batch strategy used. A maximum titer of 7 g/L was achieved after ~80 h. This represented a 2-fold increase in comparison with the batch culture. The improvement of γ -decalactone production by the MTLY40-2P in comparison with the parental strain can be mostly justified by the elimination of the reconsumption of γ -decalactone due to the deletion of *YIPOX3* [65].

The oxidative state of the medium also influences biotransformation processes. For instance, it was shown that in a 2 L bioreactor using methyl ricinoleate as substrate the best strategy was an air flow rate of 0.9 vvm, and a pressure of 0.1 MPa (the lowest tested) in order to obtain a superior production of γ -decalactone (~300 mg/L from 10 g/L substrate). Moreover, the O_2 in the medium should be decreased when the titers of γ -decalactone were high [66]. The effect of oxygen and *k_{LA}* on lactone production by *Y. lipolytica* was a subject of other studies [67]. However, increasing the oxygen transfer rate through the agitation and aeration rate (to 650 rpm and 5.1 L/min) in a 3.7 L stirred bioreactor positively affected γ -decalactone production. The highest titers were obtained (5.4 g/L) using 60 g/L of cells and substrate (castor oil). In this case, similar productivities were observed for a batch and stepwise fed-batch (~215 mg/L/h), indicating that the first option was more suitable for the process and that at these concentrations of cells and substrate, castor oil is not toxic for *Y. lipolytica* [65].

Recently, the first step towards a more sustainable biotransformation process was given in *Y. lipolytica*. Hydroxy fatty acids are not an abundant and renewable substrate and therefore do not fulfill the needs of a circular bioeconomy. The case of ricinoleic acid present in castor oil is paradigmatic. It has been the main substrate used for biotransformation processes. However, castor oil has high toxicity associated with its extraction procedure. Castor oil is extracted from the castor beans of the native *R. communis* plant [18]. Ricin is a very lethal toxin present in this plant, its effects being particularly relevant via inhalation. Moreover, due to the presence of this toxin but also to climate adaptability issues and labor-intensive harvesting processes, it is very difficult to pursue its plantation in developed countries [19]. Therefore, Marella and colleagues engineered *Y. lipolytica* to harness this yeast with the ability to convert non-hydroxy fatty acids into lactones [20]. As a proof of concept, oleic and linoleic acid were selected as substrates due to their high presence in low-value feedstocks, such as used cooking oil or animal fats. The yeast was engineered with different bacterial hydratases: an oleate 10-hydratase from *Stenotrophomonas maltophilia* to convert oleic acid into 10-hydroxystearic acid, and a linoleate 13-hydratase from *Lactobacillus acidophilus* to convert linoleic acid into 13-hydroxyoleic acid. The heterologous expression of these enzymes allowed for the production of γ -decalactone from oleic acid and δ -decalactone from linoleic acid. Further metabolic engineering with heterologous long-chain-specific oxidases and process engineering allowed for the production of 282 mg/L γ -dodecalactone after 110 h by one of the engineered strains in the fed-batch strategy [20]. Despite the lower titers when compared to traditional biotransformation processes of hydroxy fatty acids, this is a much-needed step towards more sustainable production of lactones.

4.1.2. Other Fungi

The microbial production of lactones is dominated by the biotransformation processes employing *Y. lipolytica*. Nonetheless, there are other microorganisms that deserve attention. *Trichoderma harzianum* was used for the production of γ -decalactone from castor oil. However, the titers obtained were modest, 260 mg/L after 7 days in a 14 L stirred bioreactor that involved several process difficulties related to appropriate mixing of oil, water and mycelia [68]. *S. salmonicolor*, the model used to study the conversion of lactones, was also exploited in bioreactor biotransformation of castor oil for the production of γ -decalactone [69]. The titers obtained in this work were also low for a traditional biotransformation process, only achieving a maximum of 208 mg/L γ -decalactone after 7 days and using a stepwise fed-batch strategy with an aeration rate between 1.0 or 1.5 vvm. Other strains such as *S. ruinenii* achieved superior success in similar biotransformation processes, being able to produce 5.5 g/L γ -decalactone after ~40 days in a 7 L bioreactor, in which the medium was renewed every 10 days. These differences between the different species could be justified by a selective production of the open form of γ -decalactone, 4-hydroxydecanoic acid, which is less toxic than the lactone by *S. ruinenii*. In this case, the lactone was probably formed outside the cell by spontaneous lactonization [25]. In these yeasts, high concentrations of methyl ricinoleate led to modifications at the cellular membrane level and consequently to cell death [70], indicating a diminished tolerance to these substrates in comparison with *Y. lipolytica*.

Biotransformation of ricinoleic acid in γ -dodecalactone was also performed in *Pichia guilliermondii*, reinforcing the pathways proposed in the works with *S. salmonicolor* [71]. In this yeast, the production media did not influence the enantiomeric ratios of the lactones produced. Given the potential impact of different enantiomers on the sensory perception and aroma intensity of lactones, some works sought to decipher the production patterns of different fungi. In this context, *Y. lipolytica* and *Kodamaea (Pichia) ohmeri* were studied for their biotransformation capacity of different hydroxy fatty acids into lactones through the β -oxidation pathway [72]. These yeasts were able to convert hydroxy fatty acids derived from palmitic acid (C16:1), oleic acid (C18:1) and linoleic acid (C18:2) into γ -decalactone, γ -dodecalactone and cis-6- γ -dodecenolactone. The lactones were produced in the range of 50–150 mg/L from 1 g/L of pure or mixed hydroxy acids after 48 h. Interestingly, some differences were observed between these two yeasts. *Y. lipolytica* was able to produce γ -decalactone from the C16:1-derived hydroxy acid (monounsaturated) but not from the other two, C18:1-derived hydroxy acid (monounsaturated) and C18:2-derived hydroxy acid (polyunsaturated). This was a clear contrast with the well-known ability of this yeast to produce γ -decalactone from ricinoleic acid (C18 monounsaturated hydroxy acid). *K. ohmeri* was able to produce γ -decalactone from all the precursors studied. Regarding γ -dodecalactone and cis-6- γ -dodecenolactone, both yeasts had a similar behavior, producing these lactones from all the precursors with the exception of the C16:1-derived hydroxy acid (monounsaturated). The enantiomers of the lactones formed by these yeasts were also opposites: for the saturated γ -decalactone and γ -dodecalactone, *Y. lipolytica* predominantly produced the (S)-enantiomer and *K. ohmeri* the (R)-enantiomer, while for the unsaturated cis-6- γ -dodecenolactone, *Y. lipolytica* predominantly produced the (R)-enantiomer and *K. ohmeri* the (S)-enantiomer [72].

In the few works using the baker's yeast, the most successful process was related to the utilization of *Saccharomyces cerevisiae* to produce δ -decalactone from massoia lactones (2-decen-5-olide and 2-dodecen-5-olide) naturally present in the massoia bark oil of the Massoia tree (*Cryptocaria massoia*) [36]. Hydrogenation by *S. cerevisiae* leads to the reduction of these unsaturated lactones into δ -decalactone. Therefore, in a 2 L bioreactor several parameters were taken into account in order to optimize this biotransformation process: the presence of glucose as substrate, high oxygenation, 35 °C and an optimum pH near 6.0 resulted in high hydrogenation activity. The authors speculated that the high performance observed with glucose and high oxygenation was due to the fact that these condi-

tions favored co-factor regeneration, namely of NAD(P)H and FADH₂. Another interesting observation was related to the toxicity of both the substrate and the product towards the yeast. Increasing concentrations of both 2-decen-5-olide and δ -decalactone resulted in a significant decrease in hydrogenation activities. However, this effect was counteracted by the addition of cyclodextrin to the medium, which formed a complex with lactones, thus reducing its inhibitory effect. In practical terms, this allowed the authors to instead add all the substrate at once by stepwise addition during the fermentation, consequently reducing the time required for the process. Overall, 1.2 g/L of δ -decalactone was obtained by biotransformation of 1.7 g/L of 2-decen-5-olide after 8 h [36]. A patent application was filed concerning this subject. Several years later, Rong and colleagues optimized the biotransformation of ricinoleic acid into γ -decalactone by *S. cerevisiae* in shake-flask fermentation [73]. In this work, the yeast was able to produce ~3.48 g/L from 40 g/L of ricinoleic acid in a medium where the addition of L-carnitine had a positive effect on shortening the biotransformation period and improving the lactone production. Moreover, according to the authors, addition of another lactone to the medium (γ -octalactone) prevented the degradation of γ -decalactone by competitively inhibiting a putative lactonase. This lactonase was not identified by the authors [73]. Other studies used *S. cerevisiae* to convert 11-hydroxypalmitic acid or 3,11-dihydroxymyristic acid into δ -decalactone. Other precursors for the production of this lactone could also be the coriolic acid (13-hydroxyoctadeca-9,11-dienoic acid) obtained from the seed oil of *Coriaria nepalensis* [6].

A surprisingly good result was obtained by a specific strain of *Rhodotorula aurantiaca*, isolated in the Antarctic. This specific strain, unlike others isolated in the same region, was able to produce 5.8 g/L and 6.6 g/L of γ -decalactone from 20 g/L of castor oil in a shake flask and a bioreactor, respectively. These titers were achieved after 6/7 days of culture. Similar levels of performance can only be found in patents [74]. Subsequently, gum was added to the process in order to increase titers and to diminish the toxicity of γ -decalactone, but with limited success [75].

As mentioned above, a critical point with biotransformation processes is to obtain hydroxylated fatty acids in abundance and in an economically feasible way in order to produce lactones. Therefore, several biotransformation processes had to include an additional hydroxylation step, which normally was performed by enzymatic peroxidation of polyunsaturated fatty acids [11]. However, some attempts tried to engineer microorganisms with the ability to produce ricinoleic acid from oleic acid in order to provide a substrate for biotransformation processes. This was the case of the heterologous expression of the oleate Δ 12-hydroxylase from *Claviceps purpurea* in *Schizosaccharomyces pombe* [76]. However, this attempt was unsuccessful due to growth defects of the engineered strain, probably due to the toxicity of ricinoleic acid. In the opposite way, the heterologous expression of oleate hydratase from *Stenotrophomonas maltophilia* in *Escherichia coli* led to a high-yield strain able to convert oleic acid into 10-hydroxystearic acid at 98% (50 g/L oleic acid resulted in 49 g/L of 10-hydroxystearic acid) [77]. This work was the basis for the development of one of the most successful biotransformation processes for the production of lactones reported so far [78]. The 10-hydroxystearic acid produced by the engineered *E. coli* strain was used for the production of γ -dodecalactone by permeabilized whole cells of the oleaginous yeast *Waltomyces lipofer* [78,79]. This process was based on the conversion of the substrate into lactones by prior grown cells of *W. lipofer*. After culture conditions optimization, this biotransformation process resulted in the maximum production of 51 g/L of γ -dodecalactone using 60 g/L of substrate after 40 h, with a yield of 85% and a productivity of 1.7 mg/L/h. These production parameters are by far superior to those obtained before, namely in patents [79]. Moreover, using a different substrate, namely a commercial 12-hydroxystearic acid, *W. lipofer* was able to produce 27.6 g/L of γ -decalactone after 40 h [78]. According to the authors and to the extent of our knowledge, this is the process with the highest titers reported for a lactone produced by a microorganism. Another oleate hydratase from *Macrocooccus caseolyticus* that could be a good candidate for

this type of process, namely for the conversion of oleic acid to 10-hydroxystearic acid, was also identified [80].

Following this trend towards more sustainable production of lactones, Soares and colleagues exploited a new substrate. These authors tested the ability of *Y. lipolytica* and *Lindnera saturnus* strains to produce γ -decalactone from crude glycerol [81]. Crude glycerol is the main by-product of the biodiesel industry and contains several contaminants, including fatty acids. For this reason, and despite not being discussed in the study, it seems that the lactones produced are derived from the fatty acids present in the substrate and not from the glycerol per se. There is no report that *Y. lipolytica* can perform de novo biosynthesis of lactones [23], and it is equally unknown if *L. saturnus* can do it. Therefore, taking into account the high concentrations of crude glycerol used (100 g/L) and the maximum titer obtained in shake-flask fermentations (5.8 g/L by *L. saturnus*) [81], one can assume that this production was based on the fatty acids present in this industrial waste. Nonetheless, crude glycerol is presented here as a more sustainable and available alternative for biotransformation.

It is known that *Y. lipolytica* is not able to produce lactones from oleic acid without genetic engineering [20,23]. In turn, *A. gossypii* is able to biotransform oleic acid and other non-hydroxy fatty acids into lactones. Characterization of the fermentation broths of industrial riboflavin (vitamin B₂) production, where soybean oil is used as substrate, identified six different γ -lactones: three saturated, γ -nonalactone, γ -dodecalactone and γ -decalactone; three unsaturated, cis-6- γ -dodecenolactone, γ -jasmolactone and (Z)-5-Decen-4-olide [10]. A total of 170 mg/L of total lactones were detected, cis-6- γ -dodecenolactone being the most predominant. Soybean oil is mainly constituted of linoleic acid, oleic acid, palmitic acid and linolenic acid, indicating that the fungus is able to biotransform unsaturated fatty acids into lactones. This reinforces the idea of Silva and colleagues that *A. gossypii* may be able to insert a hydroxy group into fatty acids. Interestingly, comparing the de novo biosynthesis of lactones from sugars [23] and the biotransformation of soybean oil [10], one can see that the predominant lactone produced by the fungus is different: the former was γ -decalactone (saturated) and the latter was cis-6- γ -dodecenolactone (unsaturated). The predominant enantiomer formed of the lactones formed by *A. gossypii* assumes an (R) configuration [10]. In sum, despite the low titers, *A. gossypii* natively presents the ability that need to be engineered in *Y. lipolytica* [20].

4.2. De Novo Biosynthesis

It is known that the biosynthesis of lactones can be quite different between microorganisms, for instance regarding the types of fatty acids and hydroxy acids formed. As stated above, microbial hosts able to biosynthesize lactones from non-hydroxy fatty acids and perform de novo biosynthesis from substrates such as sugars or glycerol would enable economically competitive and more sustainable bioproduction of lactones. If these substrates were renewable or derived from industrial wastes all the biorefinery requisites towards a process contributing to a circular bioeconomy would be fulfilled. Therefore, it is urgent to identify microorganisms with the ability to de novo biosynthesize lactones or engineer that capacity in industrial models. To biosynthesize lactones a microorganism should be able to convert sugars or other non-lipid substrates into fatty acids, perform post-modifications to introduce a hydroxy group to these fatty acids and perform their chain shortening through β -oxidation. It is not therefore surprising that de novo biosynthesis of lactones is a rare natural trait among microorganisms, namely among fungi. Tables 1 and 2 display the lactones produced by de novo biosynthesis of microorganisms and the production levels obtained from different microorganisms and substrates, respectively. As for other industrial biotechnological processes, engineering of the microbial host to achieve a competitive process will always be necessary.

Some authors consider that the production of γ -decalactone by the genus *Sporidiobolus* has been known since the 1930s [47]. This yeast was able to produce γ -decalactone from different carbon sources such as glucose, sucrose, fructose and mannitol, while cis-

6- γ -dodecenolactone was produced in glucose, sucrose and fructose, but not in mannitol [38]. In liquid cultures, the production profile of γ -decalactone was also associated with the growth phase and a maximum of 4.5 mg/L was obtained from mannitol. In turn, 1.9 mg/L of cis-6- γ -dodecenolactone was detected in cultures using fructose as a carbon source [38]. The effect of media composition on lactone de novo biosynthesis by *S. salmicolor* was also investigated [40]. The main differences of the media were related to the presence or absence of amino acids and yeast extracts. Absence of these two components led to slower growth but increased lactone biosynthesis (12 mg/L vs. 3 mg/L) after 12 days of growth in liquid cultures at 25 °C. Therefore, this indicated an inversely proportional correlation between growth rate and lactone production in this yeast. From the five γ -lactones detected, γ -octalactone and γ -nonalactone were reported for the first time, but the major lactones produced were cis-6- γ -dodecenolactone (medium with amino acids and yeast extract) and γ -decalactone (medium without amino acids and yeast extract) [40].

Collins and Halim (1972) were the first to demonstrate that the characteristic coconut-like aroma in *T. viride* was due to the presence of 6-pentyl- α -pyrone, an unsaturated δ -lactone [35]. This lactone was produced from a medium based on sugars, indicating the ability of this fungus to de novo biosynthesize this compound. Moreover, its production was associated with spores rather than the mature mycelia, suggesting a production pattern linked with the growth phase [35]. Several years later, solid-state fermentation of sugarcane bagasse employing this fungus led to the production of six different lactones: 6-pentyl- α -pyrone, δ -octalactone, δ -dodecalactone, γ -nonalactone, γ -undecalactone and γ -dodecalactone. Interestingly, γ -decalactone, the major lactone produced by almost all the organisms, was not produced by this fungus. Instead, 6-pentyl- α -pyrone was the most produced lactone (3.62 mg per g of dry matter—equivalent to biomass) from a medium based on 4.5 g sugarcane bagasse after 5 days of culture [12].

The filamentous fungus *F. poae* (Peck Wollenw strain) is another of the few filamentous fungus reported to de novo biosynthesize lactones [34]. Eight saturated (γ -valero-, γ -capro-, γ -hepta-, γ -octa-, γ -nona-, γ -deca-, γ -undeca and γ -dodecalactone), and one unsaturated (cis-6- γ -dodecenolactone) γ -lactones together with one δ -lactone (δ -decalactone) were detected in liquid cultures of this fungus using malt broth as a medium. Cis-6- γ -dodecenolactone was the major lactone produced (~2 mg/L) and the main contributor to the fruity aroma felt. This lactone was even possible to detect in solid cultures, while all others were only detected in liquid cultures after 3 days of growth. Even-chain lactones were more abundant than odd-chain lactones, and aeration of the culture was crucial for production. The fungus metabolism shifted from lactone to alcohol biosynthesis when insufficient aeration conditions were provided [34]. This fungus did not produce the same enantiomers for all the lactones detected [82]. In the case of the major lactone produced, cis-6- γ -dodecenolactone, the (R)-enantiomer was predominant. However, the (S)-enantiomer was described as more intense, despite having similar odor descriptors. This is a difference to what was observed, for instance, in *P. guilliermondii* [71]. Further investigation on de novo biosynthesis of lactones by a different strain of *F. poae* demonstrated that the addition of yeast extract and amino acids increased their production [83], but the major lactone produced continued to be cis-6- γ -dodecenolactone. It was hypothesized that its synthesis started from an oxygenated derivative of linoleic acid, the major unsaturated fatty acid accumulated by the fungus. Thus, hydroperoxides, epoxides, and hydroxy derivatives from unsaturated fatty acids were considered to be the potential precursors of lactones [83]. This hypothesis was later reinforced not only by the numerous biotransformation processes developed but also by studying different organisms that are able to de novo biosynthesize lactones, such as fruits [21,22] and other fungi [23].

Penicillium roqueforti was another filamentous fungus used to study the complexity involved in the biosynthesis of lactones. Spores of this fungus were able to convert soybean fatty acids (mainly oleic and linoleic acid) predominantly into γ -dodecalactone and cis-6- γ -dodecenolactone [84]. The conversion of oleic acid into γ -dodecalactone was con-

sistent with the action of a 10-hydratase, which introduced a hydroxy group at C10. Regarding cis-6- γ -dodecenolactone, the post-modification of linoleic acid appeared to be the action of a 10-lipoxygenase. These first reactions were followed by three sequential cycles of β -oxidation. Despite these proposals, these enzymatic steps were not genetically or experimentally validated [84].

Since the 1970s there have been reports on the ability of fungi to de novo biosynthesize different lactones [4]. These works identified the ability of a species/strain to de novo biosynthesize lactones in different culture conditions and used chromatography–mass spectrometry technology to disclose the chemical configuration of the lactones produced and their precursors. Some important insights were advanced, such as the hypothesis of the lactones being produced by derivatives of unsaturated fatty acids [83], which was the basis for the development of biotransformation processes. However, those studies added scarce information about the metabolic or physiological mechanisms of the de novo biosynthesis of lactones [41]. Additionally, there was no attempt to improve the ability of the microorganisms to de novo biosynthesize lactones either by a lack of molecular/bioprocess tools or because biotransformation offered higher titers and was a more promising alternative for the development of a competitive process.

In this matter, the identification of *A. gossypii* as a fungus able to de novo biosynthesize lactones from sugars was important [23,85]. This fungus can synthesize several γ -lactones. Nine different wild strains of the fungus were able to de novo biosynthesize up to seven γ -lactones, namely γ -valero-, γ -capro-, γ -octa-, γ -nona-, γ -deca-, γ -undeca- and γ -dodecalactone [23]. From these, γ -octa-, γ -nona-, γ -deca- and γ -dodecalactone were detected at titers above their aroma perception threshold. γ -decalactone was the major lactone produced in the range of mg/L from a glucose-based medium after 2 days [23]. More importantly, the work of Silva and colleagues was the first to report the improvement of the de novo biosynthesis of lactones by metabolic engineering. Having oleic acid as the central fatty acid, the authors addressed the de novo biosynthesis of lactones by identifying two main modules for improvement: (i) the synthesis of fatty acids from carbohydrates, comprising the fatty acid biosynthetic pathway and the desaturation and elongation system, and (ii) the conversion of the fatty acids into lactones, comprising the β -oxidation pathway and the lactonization step (Figure 2). Thus, strains were engineered with the following genetic modifications: (i) overexpression of *AgDES589*, to promote the conversion of oleic acid into other derivatives, namely linoleic acid; (ii) the deletion of *AgELO624*, which encodes an elongase that catalyzes the formation of very long fatty acids (C20:0 and C22:0) that are not interesting for lactone biosynthesis; and (iii) the substitution of the *AgPOX1* gene, encoding the native acyl-CoA oxidase, for the heterologous *YIPOX2*, which encodes a specific long-chain acyl-CoA oxidase from *Y. lipolytica*, in order to fine-tune the biosynthesis of lactones towards γ -decalactone (C10). These engineered strains increased total lactone biosynthesis by more than 6-fold, specially γ -decalactone, in comparison with the parental strain in glucose-based media during cultures of 72 h [23]. This was the first work reporting the engineering of a microbial host for improving the de novo biosynthesis of lactones. This modular approach indicated that the biosynthesis of lactones can be engineered, not only by improving the conversion of the fatty acids into lactones, but also by engineering the fatty acid biosynthesis to increase the production of the desired fatty acids. In sum, by accomplishing the enhancement of microbial de novo biosynthesis of γ -lactones through engineering of the host, this work paved the way for the bioproduction of lactones from sustainable substrates.

The work of Silva and colleagues was also important in highlighting the role of a desaturase encoded by the gene *AgDES589*, which was previously characterized to be responsible for the conversion of oleic acid into linoleic acid [86]. Overexpression of *AgDES589* in *A. gossypii* led to a significant increase in the biosynthesis of lactones, mainly γ -decalactone, confirming the importance of this enzyme [23]. No orthologs of this gene are present in *S. cerevisiae* or in other post-whole-genome duplication (WGD) species of

the Saccharomycetaceae, which may justify why *A. gossypii* is the only member of the family with the distinctive trait to de novo biosynthesize lactones. Moreover, AgDES589p may possess hydroxylases in addition to its desaturase activity. Important residues are conserved between AgDES589p and hydroxylases or bifunctional hydroxylases/desaturases rather than with exclusive desaturases. This is a critical difference to *Y. lipolytica*, the main host used for γ -lactone biotransformation processes. Despite this yeast owning a similar enzyme, it does not show the level of residue conservation of AgDES589p. This may be one reason why it is unable to de novo biosynthesize lactones or natively biotransform non-hydroxy fatty acids into lactones [20,23].

The success of *Y. lipolytica* in the biotransformation processes was partly due to the availability of a good molecular understanding and toolbox for this yeast. To engineer the de novo biosynthesis of lactones a microbial host must also be amenable to genetic modifications. *A. gossypii*, used for more than 25 years for the industrial production of riboflavin, fulfills that prerequisite [87–89]. Moreover, this fungus is able to synthesize fatty acids from several carbon sources and renewable raw materials, such as lignocellulosic biomass, crude glycerol and sugarcane molasses [89], expanding the possibilities to biosynthesize lactones.

Table 2. Reported production levels of lactones obtained by de novo biosynthesis from different microorganisms and substrates. The production levels for submerged cultures are indicated per volume of culture, and for solid-state cultures per mass of cell dry weight.

Cultured Microorganisms	Lactones Produced	Substrate	Production Levels	Culture Time	Reference	
<i>A. gossypii</i>						
Non-engineered strains	γ -decalactone	Glucose	Up to 18 mg/L	2 days	[23]	
	γ -nonalactone		Up to 0.2 mg/L			
	γ -dodecalactone		Up to 82 μ g/L			
	γ -undecalactone		Up to 40 μ g/L			
	γ -octalactone		Up to 24 μ g/L			
	γ -valerolactone		Up to 12 μ g/L			
Engineered strains	γ -decalactone		Up to 31 mg/L	3 days		
	γ -octalactone		Up to 0.5 mg/L			
<i>F. poae</i>	cis-6- γ -dodecenolactone	Glucose	Up to 23 mg/L	8 days	[82]	
	γ -decalactone		Up to 1.0 mg/L			
	γ -nonalactone		Up to 0.6 mg/L			
	γ -octalactone		Up to 0.6 mg/L			
	γ -dodecalactone		Up to 0.1 mg/L			
			Up to 0.1 mg/L			
		cis-6- γ -dodecenolactone	Malt extract	2.0 mg/L	-	[34]
		γ -dodecalactone	Malt extract	-	-	[34]
		γ -decalactone				
		δ -decalactone				
	γ -caprolactone					
	γ -octalactone					
	γ -heptalactone					
	γ -nonalactone					
	γ -undecalactone					
	γ -valerolactone					
<i>S. salmonicolor</i>	cis-6- γ -dodecenolactone	Glucose	Up to 11 mg/L	12 days	[40]	
		Fructose	Up to 1.9 mg/L	8 days	[38]	
		Sucrose	1.5 mg/L		[38]	

<i>T. viride</i>	γ -decalactone	Mannitol; sucrose; fructose	Up to 4.5 mg/L		[38]
			Up to 2.7 mg/L	8 days	[38]
			Up to 1.9 mg/L		[38]
	γ -dodecalactone	Glucose	Up to 3.0 mg/L	6–9 days	[40]
			Up to 0.4 mg/L		
	γ -nonalactone	Glucose	Up to 0.3 mg/L	6–9 days	[40]
	γ -octalactone		Up to 25 μ g/L		
	δ -jasmin lactone	Fructose	-	-	[39]
	δ -decalactone				
	6-pentyl- α -pyrone	Potato dextrose	170 mg/L	3–4 days	[35]
6-pentyl- α -pyrone	Sugarcane bagasse	Up to 3.6 mg/g	5 days		
γ -nonalactone		Up to 0.3 mg/g	7 days		
γ -undecalactone		Up to 0.1 mg/g	9 days		
δ -octalactone		Up to 52 μ g/g	5 days	[12]	
δ -dodecalactone		Up to 59 μ g/g	9 days		
γ -dodecalactone		Up to 36 μ g/g	9 days		

- Not indicated.

5. Towards Sustainable Production of Lactones: Final Remarks and Perspectives

Lactone biosynthesis in natural producers, such as fungi and fruits, is a complex and multi-pathway process (Figure 2). From the current knowledge, it starts with the biosynthesis of fatty acids, specifically the biosynthesis of unsaturated fatty acids, which already involves enzymes from the desaturase system of the host [23]. Engineering the fatty acids biosynthetic pathway with the purpose to increase the biosynthesis of lactones had not been done by the date of this review. However, choosing a microbial host that possesses a good ability to synthesize fatty acids (e.g., oleaginous yeasts) and a good molecular toolbox to fine-tune the type of fatty acid that it accumulates could be the first step to create improved chassis strains. In addition, microorganisms that are able to do this in different carbon sources (glucose, glycerol, xylose, etc.), such as those present in industrial wastes or renewable raw materials (lignocellulosic biomass or crude glycerol), will be preferred.

Afterwards, three additional stages are needed to biosynthesize a lactone. The first is the introduction of a hydroxy group to the fatty acid, which can be achieved in one step or through more than one enzymatic reaction. The position of this hydroxylation in an even or odd position will be determinant to the generation of a γ -lactone or δ -lactone, respectively [6,11]. Several enzymes were proposed for this end. Until the date, the most biotechnologically promising seems to be hydratases [20,78] and desaturases/hydroxylases [23], with reported results for the production of lactones in *Y. lipolytica*, *W. lipofer* and *A. gossypii*. The source of these enzymes is diverse, from bacteria to fruits. Regardless, these sources of genetic material need to be tested in industrial microorganisms in order to study and improve microbial production of lactones. The fact that some desaturases identified in fruits and microorganisms share high homology with other fatty acid modification enzymes, such as hydroxylases and epoxidases, is also noteworthy [21,23]. Indeed, there are cases of enzymes that can catalyze both hydroxylation and desaturation, and in which the change of a few specific amino acids can alter their function [21]. Therefore, it is important to identify and characterize these bifunctional enzymes. The enzymes responsible for the chemical transformation of fatty acids before entering β -oxidation are the crucial and most complex distinguishing factor between microorganisms that perform de novo biosynthesis of lactones or only biotransformation.

The next step of chain shortening in the β -oxidation pathway is the most well-studied stage. As described above, this pathway was comprehensively studied in *Y. lipolytica* [24].

An acyl-coA oxidase from this yeast was used with success to improve lactone biosynthesis in *A. gossypii* [23]. Nonetheless, other acyl-coA oxidases should still be tested, providing better results, as is the case of plant-derived oxidases in *Y. lipolytica* [20].

Lastly, lactonization is the step that is more uncontrollable and poorly known from a biosynthetic point of view. During fermentation, it occurs with high frequency in acidic conditions, and therefore it is thought that lactonization is spontaneous rather than enzymatic, since no enzyme was identified so far. Moreover, it is unclear whether lactonization occurs completely intracellularly or if the hydroxy acid precursors are excreted and undergo spontaneous lactonization in the extracellular medium [11]. However, the identification of candidate genes in peaches opens good perspectives for the future [21]. In addition, it would be interesting to test enzymes similar to those in olefinic hydrocarbon biosynthesis, which are able to perform the conversion of β -hydroxy acids into β -lactones [90]. Beyond the elimination of short-chain acyl-CoA oxidases, genome mining of the host chosen for lactone biosynthesis in order to identify lactonase-related enzymes should be performed. These enzymes have the capacity to degrade several type of lactones, such as γ -lactones and δ -lactones [91], making the genes encoding them a clear target for deletion.

For the overall process, Adaptive Laboratory Evolution (ALE) of pre-selected strains and conditions should also be considered in order to obtain improved production phenotypes and to circumvent the toxicity that lactones may have towards the host [92]. Mimicking what was done for resolving riboflavin biosynthesis in *A. gossypii* [93], ^{13}C tracer studies would also be interesting for resolving lactone biosynthesis in this host as well as being an upgrade regarding the studies made in the 1990s on *S. salmonicolor*.

Moving from the production of lactones from hydroxy fatty acids to sugars will increase the complexity of the process and consequently the regulatory hurdles faced in the microbial host. This will lead, in an initial phase, to the reduction of titers, yields and rates when compared to traditional biotransformation processes. However, maintaining the actual mode of production of lactones from hydroxy fatty acids is not a sustainable option. Investing in new ways to produce these valuable fragrances must be pursued, at the risk of preventing lactones from being platform compounds in future sustainable and circular bioeconomies if we do not. At the moment, *Y. lipolytica* and *A. gossypii* appear to be the best hosts to deploy this work. Both microbes own the natural traits as well as the amenability and molecular toolboxes necessary to be engineered. If there is a route for second-generation bioproduction processes of lactones, which use renewable raw materials and industrial wastes as substrates, it probably should start through the systemic and systematic engineering of these microorganisms.

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