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MINI-REVIEW

Chemical vs. biotechnological synthesis of C_{13}-apocarotenoids: current methods, applications and perspectives

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Abstract Apocarotenoids are natural compounds derived from the oxidative cleavage of carotenoids. Particularly, C_{13}-apocarotenoids are volatile compounds that contribute to the aromas of different flowers and fruits and are highly valued by the Flavor and Fragrance industry. So far, the chemical synthesis of these terpenoids has dominated the industry. Nonetheless, the increasing consumer demand for more natural and sustainable processes raises an interesting opportunity for bio-production alternatives. In this regard, enzymatic biocatalysis and metabolically engineered microorganisms emerge as attractive biotechnological options. The present review summarizes promising bioengineering approaches with regard to chemical production methods for the synthesis of two families of C_{13}-apocarotenoids: ionones/dihydroionones and damascones/damascenone. We discuss each method and its applicability, with a thorough comparative analysis for ionones, focusing on the production process, regulatory aspects, and sustainability.

Keywords Apocarotenoid · Terpenoid · Ionone · Aroma · Chemical synthesis · Bioflavor · Biotechnological synthesis

Introduction

Aroma compounds are employed both as flavoring ingredients and in fragrance formulations. Products that contain these types of compounds are used daily and include personal care and household products, beverages, and packaged foods. The total market for flavors and fragrances (F & F) was estimated at US$ 27.5 billion in 2014 with an annual growth of around 4 % (Leffingwell and Associates 2015). Chemically defined aromas have the advantage over complex preparations (e.g., vegetable and fruit extracts) to allow for more accurate formulations and flavor profiles. Many aroma compounds found in nature are traditionally produced via chemical synthesis. However, there is an increasing interest in natural aroma compounds, particularly in the areas of flavoring and cosmetics.

Natural aroma compounds can be extracted from their primary sources and further purified. However, natural sources often contain low concentrations of the desired compounds, making the extraction expensive and cumbersome. In this regard, biotechnology is a promissory alternative to produce more sustainable and natural aroma compounds (Berger 2009).

Apocarotenoids are natural compounds derived from—mostly enzymatic—oxidative cleavage of C_{40}-carotenoids. In plants, this extensive group of compounds acts as pigments, scents, and signal and regulatory molecules (Walter et al. 2010). The length of their aliphatic chain and degree of oxidation largely determine their properties and functions. Larger apocarotenoids, such as bixin (C_{24}), are generally pigments, with important applications in foods and cosmetics (Bouvier et al. 2003). The shorter molecule, abscisic acid (C_{15}), is a non-volatile plant hormone with high oxygen content, which plays a regulatory role in various physiological processes in plants (Cutler et al. 2010). By contrast, C_{13}-apocarotenoids—traditionally known as C_{13}-norisoprenoids in the food and beverage field—have lower oxygen content and are volatile. Moreover, several of these compounds have been identified as key contributors to the aroma of flowers and fruits (see e.g., Ristic et al. 2010).

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The most researched C_{13}-apocarotenoids are ionones (α, β, and γ isomers), dihydroionones (α and β isomers), damascones (α and β isomers), and β-damascenone. These compounds are derived from and have been identified in various plants and products and have a pleasant fruity and flowery aroma (Burdock 2009). The discovery of ionones by Tiemann and Krüger (1893) immediately promoted the idea of possible applications of these compounds in the flavor and fragrance industry. Additionally, the use of β-ionone as an intermediate for the chemical synthesis of β-carotene and vitamin A boosted the development of industrial methods for these and other C_{13}-apocarotenoids.

The development of biotechnological methods for the synthesis of ionones from carotenes began in the late 1990s by employing non-specific enzymes. Later, carotenoid cleavage dioxygenases (CCDs), enzymes that catalyze the oxidative cleavage of carotenoids, were discovered (Schwartz et al. 1997, 2001). This enabled the development of improved biotechnological methods for preparing ionones. Currently, these methodologies have only been implemented at the laboratory scale for the synthesis of α- and β-ionones. Considering the low concentration of C_{13}-apocarotenoids in plant sources, biotechnological methods are emerging as the most promising alternative for the sustainable production of these compounds. Besides, these strategies have potential applications in the synthesis of optically active C_{13}-apocarotenoids that are difficult to synthesize chemically.

This review focuses on the current biotechnological methods for the synthesis of ionones and discusses their advantages and disadvantages compared to industrial chemical synthesis. Additionally, dihydroionones, damascones, and β-damascenone are also included, in order to evaluate their potential biotechnological synthesis in the future.

C_{13}-apocarotenoids and its applications

The most studied C_{13}-apocarotenoids, as well as those with more applications in the F&F industry, are ionones, dihydroionones, damascones, and β-damascenone. These compounds have extremely low odor thresholds, in the order of nanogram per liter of air (Serra 2015). Structurally, these apocarotenoids share the same megastigmane carbon skeleton (Fig. 1). The difference between them is the position of the carbonyl group and the number of double bonds in the cyclohexane ring and butanone side chain. Ionones, dihydroionones, and damascones have structural isomers, which differ in the double bond position of the cyclohexane ring. These isomers are designated by prefixes α, β, and γ (Fig. 1). Others, like γ-damascone and ε-damascone, have not been reported in nature, even though they are used in the fragrance industry for their pleasant smell. The α-ionone, γ-ionone, dihydro-α-ionone, and α-damascone have a chiral carbon at the 6 position; they are, therefore, optically active molecules, which can be found as two enantiomeric states (R and S).

The natural occurrence of α- and β-ionone—as well as its dihydro forms—has been identified in various flower scents, fruits, and leaves, contributing significantly to the perception of their aromas (Winterhalter and Rouseff 2001). Ionone aroma is commonly described as fruity, floral, and violet-like (Burdock 2009). The α- and β-ionones are widely used in fragrances of several personal care products, such as perfumes, body lotions, shampoos, and deodorants (Lalko et al. 2007a; Lalko et al. 2007d). Their applications are also widely distributed in the food industry, as flavoring substances in several foods, with a total annual consumption of at least 2 t per year in the USA (Burdock 2009). However, it is worth mentioning that most of the industrially synthesized β-ionone is employed as an intermediate for the chemical synthesis of β-carotene and vitamin A, as well as other aroma chemicals. Dihydroionones are utilized with less frequency than ionones (Lalko et al. 2007b; Lalko et al. 2007c; Burdock 2009), yet their appreciation in fine fragrance is increasing.

Damascones and β-damascenone are sometimes grouped under the name of rose ketones due to their important contribution to the aroma of this flower oil (Pickenhagen 1999). These compounds are also added to fragrances of many personal care products (Lapczynski et al. 2007a; Lapczynski et al. 2007b; Lapczynski et al. 2007c) and employed as food flavoring agents (Surburg and Panten 2006), although to a much lesser extent than ionones. Damascones and β-damascenone have been described in processed foods and leaves, beverages, and essential oils but have been scarcely found in fresh products (Werkhoff et al. 1991; Sefton et al. 2011). This strongly suggests a non-enzymatic generation from apocarotenoid precursors that can be promoted by processing conditions.

Table 1 summarizes the main C_{13}-apocarotenoids, their natural occurrence, enantiomeric composition in nature, and sensory description. Remarkable odor differences have been described between enantiomers of chiral apocarotenoids. Additionally, α-ionone is found in nature as nearly enantiopure (R) form.

Biosynthesis of apocarotenoids

The similarity of the chemical structure of some volatile compounds and carotenoid counterparts probably allowed for the identification of apocarotenoids as derivative compounds generated by the oxidative cleavage of carotenoids. For example, β-ionone, a widely distributed aroma compound in plants, derives from the cleavage of β-carotene.

Carotenoids are C_{40}-isoprenoid compounds produced by phototrophic organisms, such as algae and plants, and certain

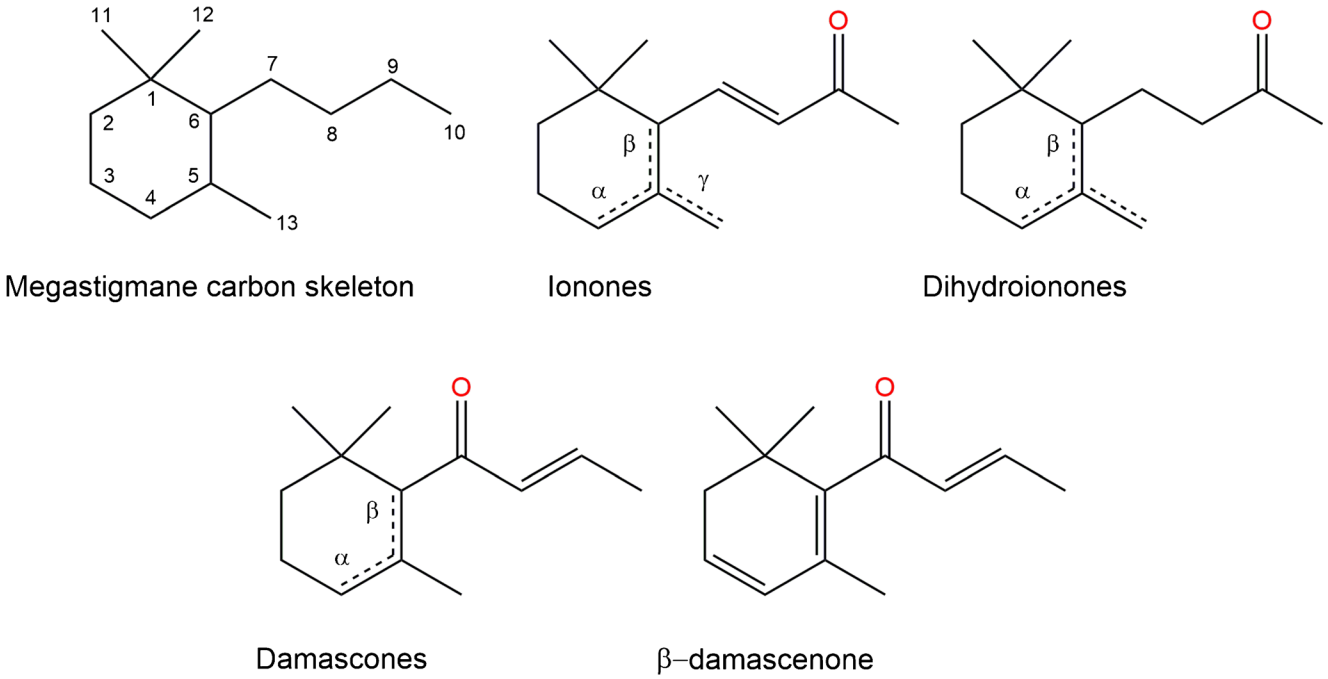

Fig. 1 Chemical structures of natural C_{13}-apocarotenoids. *Dashed lines* represent the position of the double bond in each isomer (α, β, γ)

fungi and bacteria, as well. Their biosynthesis has been extensively reviewed (Hirschberg 2001; Rosati et al. 2009; Kirby and Keasling 2009; Cazzonelli 2011; Walter and Strack 2011). Briefly, the synthesis of carotenoids in almost all photosynthetic organisms depends on the supply of the two C_5 universal isoprene-building units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), through the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway. Some microorganisms, such as bacteria and fungi, which do not contain the DXP pathway, synthesize the same precursors via the mevalonic acid pathway (MVA). In plants, both pathways are present, but in distinct, cross-talking localizations. The MVA pathway is located in the cytosol, while DXP enzymes are present in plastids. Regardless of the organism, the sequential condensation of three IPP molecules and one DMAPP molecule results in the formation of geranylgeranyl diphosphate (GGPP), the building block for the carotenoid biosynthesis. The first step in the carotenoid pathway is the condensation of two GGPP molecules to form 15-*cis*-phytoene. Subsequent desaturation steps lead to the synthesis of *cis*-lycopene, via ζ-carotene, which is then converted to *all-trans*-lycopene. The cyclization of lycopene bifurcates the pathway into two branches: one branch leads to β-carotene and its xanthophylls derivatives, such as neoxanthin, and the other produces α-carotene and corresponding xanthophylls derivatives, such as lutein. Further modifications to the 40-carbon skeleton account for the broad diversity of carotenoids.

Although in nature the formation of some apocarotenoids can occur non-enzymatically, e.g., by photochemical oxidation of carotenoids (Walter and Strack 2011), the predominant mechanism is via enzymatic oxidative cleavage catalyzed by CCDs, a non-heme iron-dependent family of enzymes (Harrison and Bugg 2014).

Since the characterization of the maize *Vp14* gene, which encodes a 9′-*cis*-epoxycarotenoid dioxygenase necessary for abscisic acid (ABA) biosynthesis (Schwartz et al. 1997), other biosynthetic apocarotenoid enzymes have been identified, including CCD1, CCD4, CCD7, and CCD8, as well as several putative CCDs across plants, microorganisms, and mammals (Harrison and Bugg 2014). The preponderance in C_{13}-apocarotenoids among many scent compounds indicates a bias for the cleavage in the 9-10 and/or 9′-10′ bond. Cytosolic CCD1 is considered the major responsible enzyme for the synthesis of volatile C_{13}-apocarotenoids, since its transcript levels correlate with the release of aromas from flowers, such as *Petunia hybrida* (Simkin et al. 2004), *Osmanthus fragrans* (Baldermann et al. 2010), and *Rosa damascena* (Huang et al. 2009a). Nevertheless, knockdown of this gene resulted in an abundance of a C_{27} intermediate, instead of C_{40} precursors, suggesting the participation of other CCD enzymes in the formation of these volatile apocarotenoids (Floss et al. 2008). For example, the cloning and expression of CCD7 and CCD4 enzymes in carotene-accumulating *Escherichia coli* cells resulted in the formation of β-ionone by specific cleavage at the 9,10 double bond of β-carotene (Schwartz et al. 2004; Huang et al. 2009b). Additionally, CCD4 expression is correlated with high levels of β-carotene and emission of β-ionone in *Crocus sativa*, suggesting its contribution to the production of this apocarotenoid (Rubio et al. 2008). The wide variety of apocarotenoids in nature can result from the

Table 1 Natural C_{13}-apocarotenoids and its odor descriptions

Compound	Natural occurrence	Natural enantiomeric composition	Odor description	References
α-Ionone	Several flowers and fruits (e.g., violet and *Acacia farnesiana* flowers, raspberry, blackberry)	(R)-enantiomer (%): Violet, 99.9 Raspberry, 99.9 Carrot, 95 Vanilla, 97.1	(R): violet-like, fruity, raspberry-like, flowery, strong impact (S): woody, cedar wood-like, raspberry-like	(Werkhoff et al. 1991) (Fehr and Guntern 1992) (Burdock 2009)
β-Ionone	Several flowers and fruits (very similar to α-ionone)	Achiral	Floral, violet-like, more woody and fruity than α-ionone	(Burdock 2009)
γ-Ionone	Rose and *Gongora* orchid flowers, tomato and ambergris tincture	Not investigated	(R): weak green, fruity, pineapple-like odor with metallic aspects (S): linear, very pleasant, floral, green, woody odor with a very natural violet tonality	(Brenna et al. 2002) (Burdock 2009) (Awano et al. 2005)
Dihydro-α-ionone	Often found together with α- and β-ionones	Only has been isolated the (R)-enantiomer from costus root and violet flowers	(R): floral, violet-type odor with slightly fruity aspects. Less pronounced woody side than dihydro-β-ionone (S): floral orris-type odor, with woody aspects and a distinct honey note	(Brenna et al. 2002) (Burdock 2009)
Dihydro-β-ionone	Often found together with α- and β-ionone	Achiral	β-ionone-type odor of floral-woody, orris-type tonality, with green, earthy undertones	(Brenna et al. 2002) (Burdock 2009)
α-Damascone	Rose flowers, tea and tobacco	(R)-enantiomer (%): Black tea, 53 tobacco extract, 52.8	(R): Reminiscent of rose petals. More pronounced apple and fruity notes (S): Reminiscent of rose petals. More pronounced and fresher floral note	(Werkhoff et al. 1991) (Fehr and Galindo 1991) (Burdock 2009)
β-Damascone	Rose and *Osmanthus fragrans* flowers, tea and tobacco	Achiral	Fruity and floral notes. Reminiscent of blackcurrant, plum, rose honey and tobacco	(Sell 2014)
β-Damascenone	Rose flowers and fruits (e.g., blackberry, lychee, mango, tomato)	Achiral	Natural sweet fruity. Rose, plum, grape, raspberry	(Sefton et al. 2011) (Burdock 2009)

large number of carotenoid precursors, variable sites of carotenoids cleavage, and further modifications after cleavage (Schwartz et al. 2001).

Chemical synthesis of C_{13}-apocarotenoids

Chemical synthesis of ionones

Almost all commercial ionones are currently produced by chemical synthesis. Figure 2 summarizes the industrial reactions involved in the production of these compounds. The α and β-ionones are traditionally prepared from citral and acetone, a well-known process for more than a century (Tiemann and Krüger 1893; Tiemann 1896; Chuit and Bachofen 1902). This method, called Tiemann synthesis, is performed in two steps. In the first instance, aldol condensation of citral with acetone is carried out in alkaline conditions to give pseudoionone. Then, cyclization of pseudoionone to ionone is catalyzed by inorganic acids (Hibbert and Cannon 1924). An alternative source of pseudoionone is the Saucy-Marbet reaction that involves (R/S)-dehydrolinalool and isopropenyl methyl ether in mild alkaline conditions (Saucy and Marbet 1967). The ratio of different ionone isomers obtained in the cyclization reaction depends on the type of acid employed and its concentration. Thus, concentrated sulfuric acid produces mainly β-ionone, although phosphoric acid produces preferentially α-ionone (Royals 1946); and to obtain γ-ionone, a Lewis acid, such as BF_3, is required (Ohloff and Schade 1963). Moreover, the isomeric purity reached in the cyclization reactions is over 90 % for the α-isomer and about 96 % for the β isomer. In the case of γ-ionone, the reaction has a limited regioselectivity of about 60 %. Furthermore, the preparation of γ-ionone by photochemical isomerization of α-isomer has also been reported, achieving 83 % yield and 96 % isomeric purity (Serra et al. 2007). Finally, dihydro-α- and dihydro-β-ionone are synthesized by partial catalytic hydrogenation of the corresponding ionone regioisomers (Bauer et al. 1997; Chapuis and Jacoby 2001).

The company BASF is one of the most important producers of chemical β-ionone. It sells β-ionone for fragrance applications and also uses it for in-house synthesis of vitamin A. BASF employs the Tiemann reaction to synthesize the pseudoionone. Required citral is obtained in a continuous process from isobutene and formaldehyde in the presence of oxygen (Wegner et al. 2008). Other companies, like Hoffmann-La Roche and Adisseo, have different pseudoionone synthesis methods (Schaefer 2014), but they only use this precursor

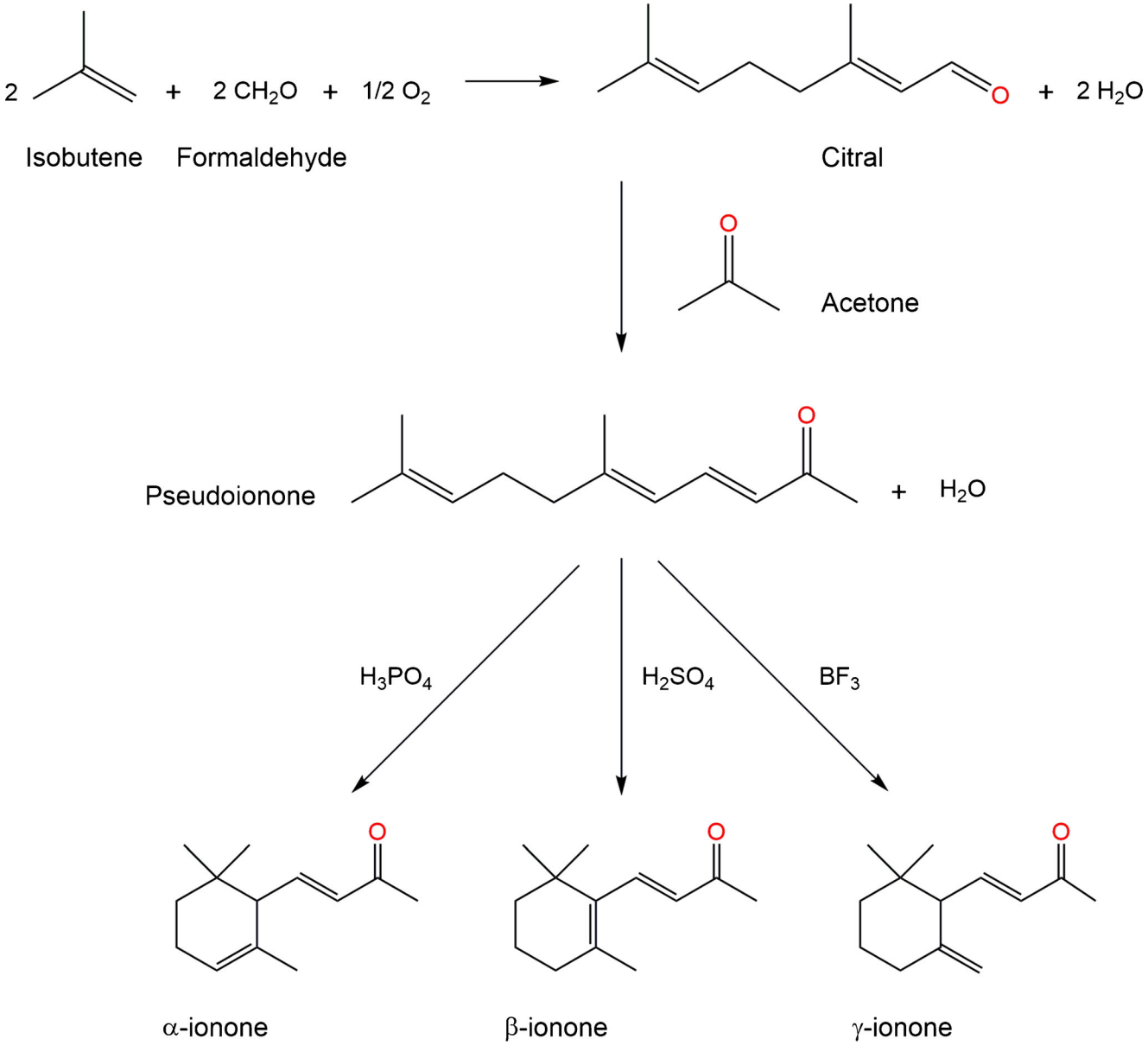

Fig. 2 Ionone chemical synthesis. Condensation of citral and acetone yield pseudoionone. The subsequent cyclisation of pseudoionone produces ionones. Isomer distribution depends on the acid and concentration used. BASF synthesizes the citral from isobutene and formaldehyde

internally to produce vitamin A and do not commercialize β-ionone. Most companies producing α-, β-, and γ-ionones for fragrance and flavoring applications are located in China, where they probably synthesize these molecules from citral and acetone as starting materials.

The α- and γ-ionones are both chiral compounds. The corresponding synthetic chemical methods described above result in racemic mixtures, which is the case for all the ionones currently available on the market. Nevertheless, for certain applications, especially in fine perfumery, it could be of interest to have enantiomerically pure or optically enriched products. Few strategies using only chemical synthesis to preferentially obtain a specific enantiomer of ionones have been proposed, and their industrial application is remote. The (R)-α-ionone is the major natural enantiomer and its fragrance has been described as more floral and fruity than the corresponding (S) enantiomer (Table 1), transforming the former into a much more attractive candidate for flavoring and perfumery applications than the latter. The first approach to get enantiomerically enriched α-ionone was through an optical resolution of the racemic sample, using fractional crystallization of menthylhydrazone derivatives (Sobotka et al. 1943). This procedure, as well as other classic, subsequently developed, racemic resolution methods is rather laborious and cumbersome. Later, different synthetic chemical methods from near enantiopure starting materials were developed. These

methods do not need resolution steps because starting materials are obtained in enantioselective syntheses from achiral compounds. Thereby, (R)- and (S)-α-ionones can be prepared with an enantiomeric excess (ee) ≥ 98–99 %, from either (R)- and (S)-α-damascones (Fehr and Guntern 1992), or from (R)- and (S)-α-cyclogeraniols (Bovolenta et al. 2004), respectively. Alternative procedures that use (S)-phorenol (Pfander and Semadeni 1995) or enantiopure 2-iodocycloalkyl phosphates (Soorukram and Knochel 2004) as starting materials to synthesize (R)-α-ionone in 85 and 97 % ee, respectively, have also been described. The latter method has also been applied to the synthesis of (R)-dihydro-α-ionone with 98 % ee. Although these approaches are still quite laborious, they represent an improvement with respect to the tedious, classic resolution procedures.

In the case of γ-ionone, efforts have focused on the preparation of the (S) enantiomer, the most powerful aroma among all ionones (Brenna et al. 2002). Enantioenriched (S)-γ-ionone was first prepared from (S)-α-cyclogeranic acid, which in turn was obtained by optical resolution of racemate (Oritani and Yamashita 1987). More recently, (S)-γ-ionone was synthesized from enantioenriched building blocks prepared by asymmetric synthesis, without resolution techniques. Thus, (2S,6R)-*cis*-2-hydroxy-γ-cyclogeraniate (Beszant et al. 2002) or (S)-α-cyclogeraniol (Bugoni et al. 2015) was used to achieve (S)-γ-ionone with 88 and 95 % ee, respectively.

Chemical synthesis of damascones and β-damascenone

The chemical synthesis of damascones and β-damascenone was developed more than seven decades after the synthesis of ionones. The first approach to synthesize β-damascone was developed by Firmenich. They utilized the addition of propenylmagnesium bromide or propynyllithium to β-cyclocitral and the subsequent oxidation with manganese dioxide (Demole et al. 1970). Similarly, α-damascone can be obtained using α-cyclocitral. Subsequently, Firmenich developed another industrial method, a Grignard reaction between methyl α- or β-cyclogeraniate and allylmagnesium chloride, in the presence of lithium diisopropylamide (LDA), followed by isomerization of the double bond in an acidic environment to give racemic α- or β-damascone (Fehr and Galindo 1986). A modification of this protocol is the enantioselective protonation of the enolate intermediate using ephedrine derivatives to give (S)-α-damascone with a 93 % ee (Fehr and Galindo 1988, 1994). Alternatively, the same method indicated above for the synthesis of (S)-α-ionone was successfully applied to the synthesis of (S)-α-damascone (Bovolenta et al. 2004). This enantiomer has a fresher and more flowery aroma profile than the (R) enantiomer (Table 1), so could be desirable in some perfumery preparations.

Finally, β-damascenone can be synthesized following the same β-damascone production strategy. This involves a Grignard reaction but, in this case, the starting material is methyl-β-safranate (Fehr and Galindo 1986). Another synthetic approach, also employing β-damascone as the starting material, generates a new double bond in the cyclohexane motif, through dehydrogenation of epoxy-, hydroxy-, or bromo- damascone derivatives (Demole et al. 1970; Schulte-Elte et al. 1973; Azzari et al. 1990). More recently, the synthesis of β-damascenone from β-ionone through a retro-α-ionol intermediate (Campagnole and Delmond 2007), or myrcene-derived γ-pyronene, was reported (Boulina et al. 2007).

Biotechnological synthesis of C_{13}-apocarotenoids

The biotechnological production of aromas and flavors can be divided in two main categories: enzymatic biocatalysis and whole cell biocatalysis. In general, enzymatic biocatalysis is employed for simple transformations that require few steps (e.g., in vitro oxidative cleavage of β-carotene by CCD enzymes to yield β-ionone). Alternatively, whole cell biocatalysis is more useful in complex conversions, mixtures of products, transformations of simpler substrates involving a large number of reactions to obtain the final product or biosynthesis where the regeneration of expensive cofactors is required (Krings and Berger 1998). Whole cell biocatalysis is generally performed by microorganisms and can be separated into two approaches: de novo biosynthesis involving the catabolism of simple precursors, such as carbohydrates, lipids, or amino acids/proteins, to obtain target compounds derived from the microorganism's secondary metabolism (e.g., β-ionone synthesis from glucose in metabolically engineered yeast) and bioconversion, which takes advantage of the capacity of microorganisms to convert a substrate into a chemically related compound in few steps (e.g., degradation of β-carotene to β-ionone by peroxidase-secreting fungi).

Enzymatic biocatalysis

Characterization of the enzymes that catalyze the oxidative cleavage of carotenoids enabled the possibility to biotechnologically synthesize natural C_{13}-apocarotenoids. Lipoxygenases (LOX) were the first described enzymes capable of oxidatively cleaving carotenoids to obtain C_{13}-apocarotenoids (Belin et al. 1998). These co-oxidation reactions initially involve the generation of lipidic peroxyl radicals by oxidation of unsaturated fatty acids catalyzed by LOXs. Then, radicals promote the oxidative cleavage of carotenes. The cleavage of β-carotene by this method is non-specific and generates volatile products, such as β-ionone, β-cyclocitral, and a variety of non-volatile compounds, i.e., apo-, 5,6-epoxy-, 5,8-epoxy- products, as well as unoxidized compounds (Wu et al. 1999). Although an industrial application of this method for α- and β-ionones was

proposed (Belin et al. 1998), the low yields and limited specificity achieved probably made this method commercially unfeasible. However, it is particularly advantageous for the synthesis of α-ionone from natural α-carotene, because the naturally occurring (R) enantiomer is essentially obtained, due to the 6′R-chirality of the substrate (Britton et al. 2004). Also, Bosser and Belin (1994) described an analogous method of β-carotene co-oxidation with aldehydes using xanthine oxidase (XO). In this case, the radical intermediates are inorganic reactive oxygen species (ROS). With this strategy, various volatiles, such as β-ionone (≈1 mol%), epoxy-β-ionone, β-cyclocitral, and pseudoionone were obtained. Unfortunately, XO-based methods are limited by the low solubility of the substrate in water, whereas the hydrophilic ROS generated by XO are inactive in organic solvents. To improve the accessibility of ROS to β-carotene, surfactants have been used to promote the formation of water-dispersed β-carotene micelles (Waché et al. 2006). Another improvement of the XO-based procedure was the further development of a multiphasic system to recover β-ionone in the reaction medium. In this system, ROS-mediated degradation of the product was reduced, and the yield was significantly increased (34 mol%) (Ly et al. 2008).

Fungal peroxidases are another class of enzymes utilized to obtain β-ionone from β-carotene. Culture supernatants of the fungus *Lepista irina* can transform carotene into various volatiles: β-ionone (13 mol%), β-cyclocitral, dihydroactinidiolide, and 2-hydroxy-2,6,6-trimethylcyclohexanone (Zorn et al. 2003b). The key enzyme in this reaction was identified as a peroxidase. The enzyme requires hydrogen peroxide, which can be generated by O_2 reduction, catalyzed by another enzyme present in the culture supernatant. Nevertheless, apparently, only traces of hydrogen peroxide that could be generated by autoxidation are necessary. Also, the MsP1 and MsP2 peroxidases of *Marasmius scorodonius* have been recently purified (Scheibner et al. 2008), and MsP2 was expressed in *E. coli* and *Saccharomyces cerevisiae* (Zelena et al. 2009b). These enzymes are useful for the oxidative breakdown of various carotenes and xanthophylls. The composition of the volatile fraction after the oxidation of β-carotene with these enzymes was similar to the one obtained with the peroxidases from *L. irina*; although a lower yield of β-ionone was achieved (7.9 mol%) (Zelena et al. 2009a).

The discovery of CCD enzymes opened a new possibility for a more specific synthesis of ionones from carotenes. These enzymes can accept a wide variety of carotenoids, either carotenes or xanthophylls (Harrison and Bugg 2014). The symmetrical oxidative cleavage of β-carotene (i.e., cleavage on both sides) can be catalyzed by CCD1s from different plants (e.g., Schwartz et al. 2001; Vogel et al. 2008). The resulting stoichiometry is 2 mol of β-ionone for 1 mol of β-carotene, with C_{14}-aldehyde as a by-product (Fig. 3). Similarly, heterologously expressed CCD4 from different plants can produce β-ionone from β-carotene both in vitro and in vivo (Huang et al. 2009b; Bruno et al. 2015). However, a recent study indicates that at least the CCD4 from potato catalyzes only the asymmetric cleavage on one side of the β-carotene molecule; thus, the products obtained are β-ionone and C_{27}-aldehyde (10′-apo-β-carotenal) (Bruno et al. 2015). The asymmetric cleavage of β-carotene has also been reported for CCD7 (Schwartz et al. 2004; Auldridge et al. 2006). There is only one report on the development of a biocatalytical process for the synthesis of β-ionone mediated by CCDs (Nacke et al. 2012). In this study, the enzymatic cleavage of a micellar preparation of β-carotene is carried out by cell lysate of *E. coli* expressing a recombinant CCD1 from *Arabidopsis thaliana*. The reaction proceeds in an enzymatic reactor coupled to a continuously organophilic pervaporation system for in situ separation of β-ionone. This approach achieves a substrate conversion close to 60 %.

There are three potential substrates that produce α-ionone by CCDs: α-carotene, δ-carotene, and ε-carotene. In the case of α-carotene, the predicted volatile products are α-ionone and β-ionone; for δ-carotene, α-ionone, and pseudoionone; and for ε-carotene, 2 mol of α-ionone. To date, only the biocatalytic generation of α-ionone with δ-carotene as a substrate, employing a CCD1 from corn (Vogel et al. 2008) and melon (Ibdah et al. 2006) has been demonstrated. In the case of corn CCD1, 6-methyl-5-hepten-2-one is obtained as an additional by-product, as a result of 5,6-cleavage activity. The broad natural occurrence of (R)-α-ionone (Table 1) is probably determined by the 6′R enantiopurity of the ε-ring in precursor carotenes (Britton et al. 2004).

Enzymatic biocatalysis can also be advantageous in a combined approach with traditional chemical synthesis. This could be particularly helpful for the synthesis of enantiomerically enriched C_{13}-apocarotenoids, using the enantioselectivity of certain enzymes (Brenna et al. 2002; Brenna et al. 2011; Serra 2015). The first chemical-enzymatic strategies were developed for the optical resolution of α- and γ-ionone racemic mixtures (Brenna et al. 1998; Aleu et al. 1999; Fuganti et al. 2000). These methods are based on the resolution of alcohol derivatives of these C_{13}-apocarotenoids, where the key steps are the enantioselective acetylation mediated by a lipase from *Pseudomonas cepacia*. The same enzyme has been employed for the asymmetric synthesis of 3,4-dehydro-γ-ionone, α- and γ-damascone enantiomeric forms, starting from racemic α-ionone (Serra et al. 2006; Serra and Fuganti 2006). A similar procedure was developed by Takasago International for producing optically active α-ionone, based in the hydrolysis of α-ionol ester by a *Candida antartica* lipase (Yamamoto et al. 2009). Although these methods provide very high enantioselectivity (>97 % ee), they still require fractional crystallization or chromatographic separations as a procedure step.

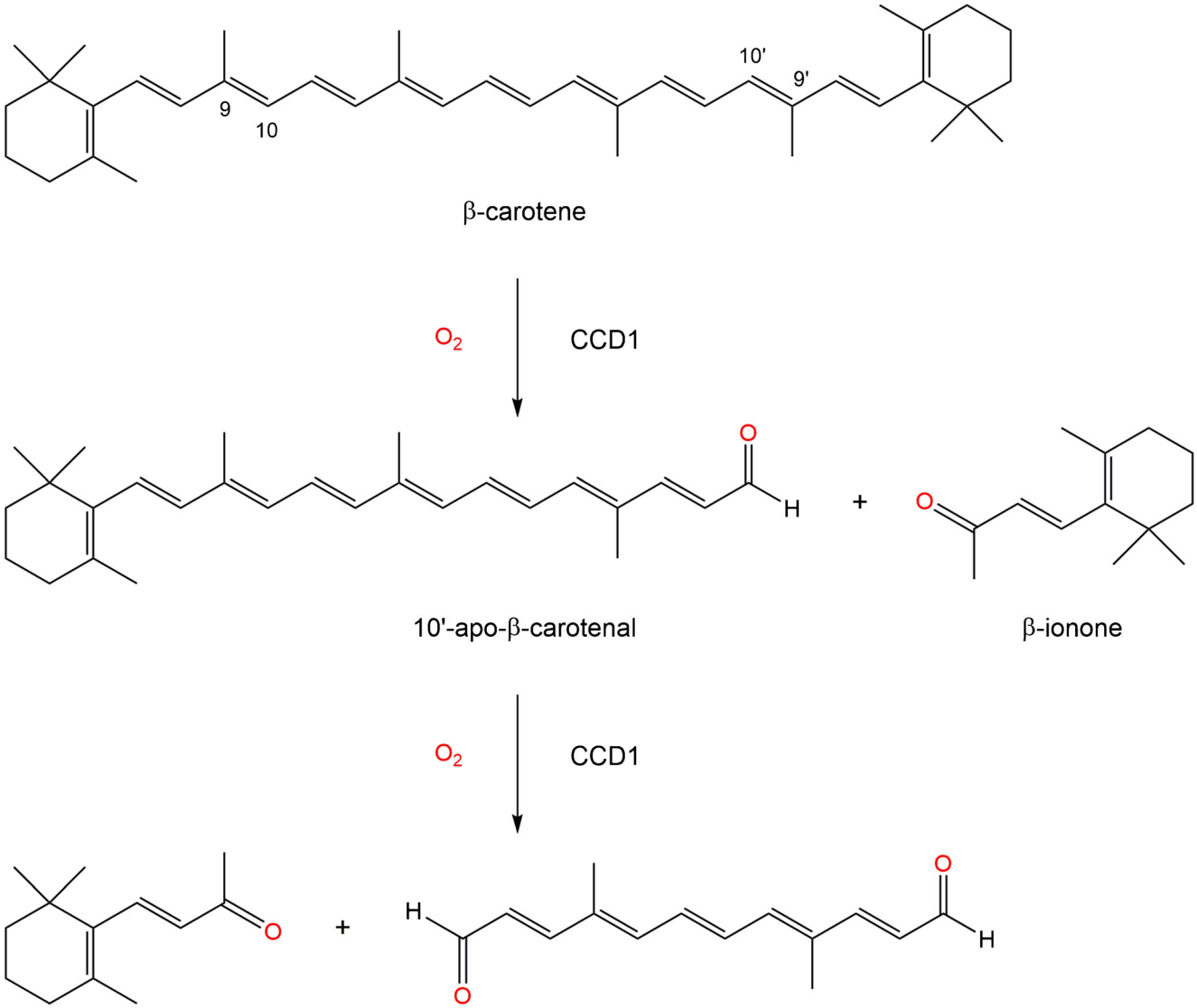

Fig. 3 Biocatalytic synthesis of β-ionone from β-carotene. CCD1 catalyzes the oxidative cleavage of β-carotene in the 9′-10 and 9-10 bonds, symmetrically in two steps. This reaction is analogous for other carotenoid substrates. Asymmetrical cleavage on one side of β-carotene has been described for CCD4 and CCD7

Whole cell bioconversion

The simplest whole cell strategy is bioconversion. A variety of microorganisms, especially bacteria and fungi, have the natural ability to bioconvert carotenoids into volatile C_{13}-apocarotenoids (Rodríguez-Bustamante and Sánchez 2007). Considering that most natural carotenoids are expensive, the industrial use of these microorganisms to produce carotenoid-derived aroma compounds seems commercially unattractive. However, in the particular case of β-carotene, which can be easily extracted from plants, the production of natural β-ionone and its reduced derivatives by whole cell bioconversion could be feasible. Surprisingly, submerged cultivation of several β-carotene-degrading fungi showed that only some were able to produce volatile compounds, and only dihydroactinidiolide was identified as conversion product (Zorn et al. 2003a). By contrast, mycelium-free culture supernatants of some fungal cultures were able to convert β-carotene into β-ionone (8–10 mol%), β-cyclocitral, dihydroactinidiolide, and 2-hydroxy-2,6,6-trimethylcyclohexanone. These results strongly suggest that these primary products are produced by extracellular β-carotene degradative enzymes and further metabolized intracellularly.

Another example is the bioconversion of the xanthophyll lutein to tobacco-like aromas by a mixed culture of *Paenibacillus amylolyticus* and *Trichosporon asahii* isolated

from marigold flower (Sánchez-Contreras et al. 2000; Rodríguez-Bustamante et al. 2005). The resulting volatile fraction contained β-ionone (9.4 %) and its reduced derivatives dihydro-β-ionol (84.2 %), dihydro-β-ionone (3.5 %), and 3-hydroxy-β-ionone (2.9 %). Interestingly, *T. asahii* was found to be responsible for dehydroxylating and cleaving lutein to produce β-ionone; and *P. amylolyticus* transformed this product into reduced forms (Maldonado-Robledo et al. 2003). Further optimization of the culture medium and design of a recovery system were developed to maximize aroma production (Rodríguez-Bustamante et al. 2005; Rodríguez-Bustamante et al. 2006).

De novo synthesis by metabolic engineering

De novo synthesis through metabolic engineering is the strategy with the greatest potential, although a longer time could be needed to maximize product yield and productivity from simple carbon sources, like glucose. This approach requires the inclusion of new metabolic pathways into the microbial cell, enabling it to synthesize these aroma compounds from an intermediate of the endogenous metabolic pathways. Most studies related with CCD characterization have cloned and expressed these enzymes in *E. coli* strains containing the carotenoid biosynthetic genes (e.g., Schwartz et al. 2001; Simkin et al. 2004; Rubio et al. 2008; Vogel et al. 2008). The latter, together with the previously reported synthesis of β-carotene in *S. cerevisiae* (Verwaal et al. 2007), demonstrated that β-ionone can be synthesized in yeast by metabolic engineering.

More recently, two studies have reported the de novo synthesis of β-ionone in *S. cerevisiae* (Fig. 4). Beekwilder et al. (2014) employed polycistronic expression of crtYB/crtI/crtE, the biosynthetic genes of β-carotene from *Xanthophyllomyces dendrorhous*. These enzymes allow β-carotene synthesis from farnesyl diphosphate (FPP), an intermediate of the ergosterol pathway in yeast. The addition of the CCD1 gene from raspberry allowed the generation of the first yeast strain producing β-ionone from glucose. López et al. (2015) constructed an alternative platform for the synthesis of β-ionone through the use of integrative and episomal constructs. This strategy includes the overexpression of a truncated version of HMG1 encoding 3-hydroxy-3-methyl-glutaryl-CoA reductase, an enzyme responsible for the synthesis of mevalonate, a key intermediate in the FPP generation. The synthesis of GGPP from FPP was achieved by overexpression of endogenous BTS1 gene. CrtYB and the CrtI genes promoted the transformation of two GGPPs into β-carotene, whereas the CCD1 of *Petunia hybrida* catalyzed the oxidative cleavage of carotene to β-ionone. All these modifications were carried out in an FPP-overproducing *S. cerevisiae* strain (Scalcinati et al. 2012). A β-ionone titer of 5 mg/L (1 mg/g of biomass) was achieved after 50 h fermentation in 1.5 L bioreactors.

Biocatalytic methods for preparing β-damascenone from carotenes have not been reported yet. This could be due to the complexity of the proposed biosynthetic pathway, which includes both enzyme-catalyzed steps, as well as subsequent, putative acid-catalyzed transformations of precursors, that can be facilitated by plant product processing conditions, such as low pH and high temperature. Indeed, it is widely accepted that allene triol is a key intermediate that undergoes a series of non-enzymatic reactions to give β-damascenone (Sefton et al. 2011). This compound derives from the enzymatic or purely chemical reduction of the grasshopper ketone, which in turn is obtained from the oxidative cleavage of neoxanthin, which can be catalyzed by CCD1 (Schwartz et al. 2001; Huang et al. 2009a). This opens a new possibility for the de novo whole cell production of these key intermediates and/or their glycosylated derivatives, which can be subsequently transformed into β-damascenone by heat and pH treatments. Nevertheless, the existence of enzymes that can catalyze the final reactions of the synthesis of this compound cannot be discarded. The picture is similar for damascones. Indeed, a biosynthetic pathway for β-damascone has been postulated, analog of the β-damascenone pathway, but from deoxyneoxanthin as a precursor (Isoe et al. 1973). Unfortunately, this carotenoid has not been found in nature yet. An alternative proposed pathway for β-damascone formation in tobacco is the reduction of β-ionone to β-ionol, subsequent oxidation to an allenic diol and final rearrangement (Wahlberg 2001). These transformations probably occur in curing and aging processes of tobacco leaves. Similarly, the natural origin of dihydroionones is also unclear, but possible pathways include the conversion from ionones by reductases or direct cleavage of 7,8-dihydrocarotenes.

Comparison between chemical and biotechnological synthesis of ionones

The chemical synthesis of ionones is already optimized for industrial use. On the other hand, the development of biotechnological methods for apocarotenoid synthesis is relatively recent and, to the best of our knowledge, has been conducted only at a laboratory scale. Nevertheless, significant differences between both strategies are evident, indicating that they might apply to different product categories. In particular, the biotechnological synthesis is rapidly emerging as an environmental friendly and sustainable method to produce natural flavors.

Processes

The β-ionone production strategy of BASF prompted the implementation of a citral plant in 2004, with a production capacity of 40,000 metric tons per year (Jäkel and Paciello 2010). The synthesis of this intermediate is carried out in a

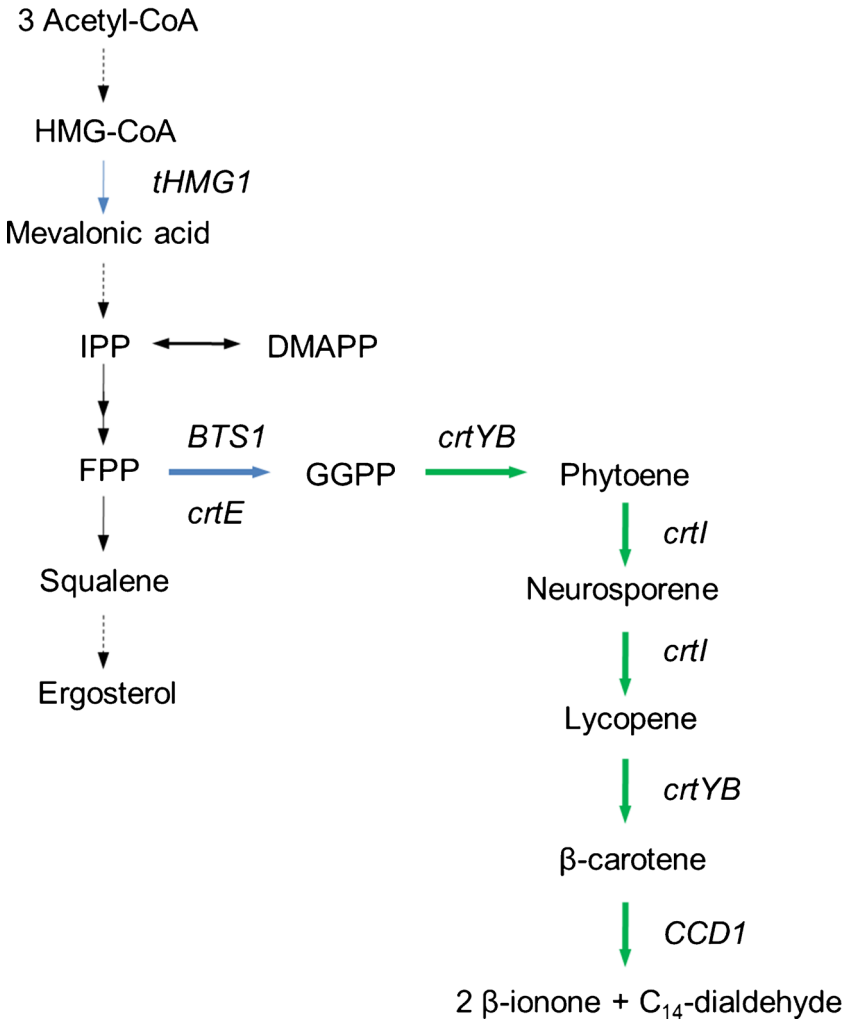

Fig. 4 De novo synthesis of β-ionone in metabolically engineered *S. cerevisiae* (Beekwilder et al. 2014; López et al. 2015). The expressed genes of the metabolically engineered steps are shown in *italics*. *Blue arrows* indicate optimized native reactions, and *green arrows* show heterologous reactions

complex, multi-step continuous process, that uses catalysts and demands high temperature conditions, which requires heating up to 360 °C. Additionally, intermediate purification steps through distillation are required (Wegner et al. 2008). Citral purification from the final mixture can be done by fractional distillation (Sasser 1992).

In a continuous and separate process, citral is condensed with acetone in order to obtain pseudoionone at temperatures above 100 °C. Resulting pseudoionone is purified by distillation (Dobler et al. 2006). Finally, the cyclization of pseudoionone is highly exothermic, so it is necessary to include organic solvents in the reaction, with considerable cooling capacity. Post-reaction, the organic phase is distilled for purification of the ionones.

The yield of these industrial syntheses of ionones is not reported, but it is presumed to be high. For example, the yield for pseudoionone cyclization is close to 90 % (Steiner et al. 1995; Rheude et al. 2001). Companies specialized in flavor production probably initiate the synthesis from citral and acetone, which makes the process considerably more expensive.

Biosynthetic processes are much simpler than those required for chemical production. The synthesis process only requires enzymatic reactors or bioreactors with soft temperature control, substrate feeding, and oxygen control. For the de novo synthesis in *S. cerevisiae*, two phase fermentations have been used, employing 10 % *v/v* dodecane for in situ ionone extraction (internal removal), which maximizes the recovery of β-ionone and reduces the toxic effects of this molecule on yeast cells (Beekwilder et al. 2014; López et al. 2015). There is an urgent need for the development of downstream methods to purify ionones from dodecane or other solvents.

Product labeling

The increasing demand for natural products in the food industry is unquestionable. This includes flavoring substances, where consumers are increasingly demanding the replacement of synthetic for natural ingredients. However, considering the low concentrations of ionones in natural sources, commercial extracts from natural sources are not economically viable.

Thus, a biotechnological system is a promising alternative for the sustainable production of "natural" ionones. Most global bodies follow the regulatory frameworks of the USA or the European Union (EU) to define an ingredient as natural. Both regulations have similarities, but the Food and Drug Administration (FDA, USA) focuses preferentially on the starting material rather than the process; whereas the European Food Safety Authority (EFSA, EU) pays more attention to the process, accepting a limited list of procedures, but with a vaguer definition for the starting material. Both regulations allow enzymatic catalysis and fermentation to produce the flavor with a natural claim. Hence, both strategies, i.e., enzymatic and whole cell biocatalysis, allow ionones to be labeled as natural. However, for this to be possible, it is necessary to use only natural raw materials. For enzymatic biocatalysis and whole cell bioconversion, carotenes can be extracted from vegetable sources, like carrots (Cavalcanti et al. 2013); and for the de novo synthesis, it is imperative to employ a carbon source that could be labeled as natural, as well as the rest of the culture medium components. However, although the flavors produced through metabolically engineered microbes can legally be defined as natural, they are being increasingly questioned, as consumers have a different perception of what is natural. Additionally, the regulatory definitions have not been updated and have yet to consider processes via genetically engineered microorganisms (Waltz 2015).

Currently, some US distributors offer α- and β-ionones labeled as natural. These products are mainly produced in China and are chemically synthesized from citral and acetone. This inconsistency results from the fact that the process employs natural starting materials, i.e., citral extracted from lemongrass or *Litsea cubeba* and acetone derived from fermentation, together with natural pH adjusting materials (Müller 2007). This is sufficient to comply with the US requirements for natural flavoring ingredients. However, ionones obtained by this process do not qualify as natural in the EU. Thus, there is currently no β-ionone flavor that could comply with the EU regulation to be sold as natural. Moreover, biotechnological synthesis is foreseen as the only alternative for natural β-ionone in this market. Another difference between US and EU regulations is reflected in the case of α-ionone, whose natural enantiomer is the (R). In the EU, the labeled substance must be chemically identical to the one naturally found in nature, including their isomer distribution. Therefore, only the enantiomerically pure (R)-α-ionone qualifies as natural in the EU; while in the US a racemic mixture is sufficient. The latter represents an attractive opportunity for the biotechnological synthesis of (R)-α-ionone.

Environmental impact and sustainability

Modern processes for the chemical synthesis of ionones have included several environmentally friendly practices, allowing much of the unconsumed reactants to be recycled. Residual mineral acids and bases in this process result in corrosion and toxicity problems (Díez et al. 2009), thus require to be neutralized before disposal. Nevertheless, these chemical synthetic methods are based on petrochemical consumption and have limited sustainability, which also involves the environmental effects of the exploitation of these resources. On the contrary, biotechnological processes are sustainable because they use renewable materials and lower amounts of energy (Gavrilescu and Chisti 2005). Biotechnological synthesis of ionones is not the exception, because the process uses raw materials derived from vegetables and microbial resources. Energetic costs will depend on final volumetric productivity and product concentration. Table 2 summarizes the comparison of chemical and de novo biotechnological syntheses of ionones.

Future directions for de novo synthesis of β-ionone

Current yield, titer, and volumetric productivity of de novo synthesis of β-ionone are too low to allow industrial synthesis. However, optimization of the biosynthetic pathway with synthetic biology tools, as well as of the fermentation process, should enable a significant improvement of these parameters. Particularly relevant is to increase the product titer, because it is critical for downstream processing operational costs (Carlquist et al. 2015). At the laboratory level, it will probably be necessary to reach a minimum titer of 1 g/L of β-ionone in batch fermentations to begin considering scaling-up to pilot plant level. This involves an increase of 200 times with respect to the highest product concentration reported (López et al. 2015). However, product titer can be increased further. Artemisinic acid, a precursor of the antimalarial drug artemisinin, is an example of the successful optimization of the de novo synthesis of terpenoids in *S. cerevisiae*. Paddon et al. (2013) achieved 25 g/L of artemisinic acid, and Westfall et al. (2012) reached 40 g/L of its precursor amorphadiene in fed-batch fermentations. This is a 220-fold and 9000-fold increase in production, respectively, with respect to batch fermentations with first reported strains (Ro et al. 2006).

Several metabolic engineering strategies could allow for the significant increase in β-ionone yields. One of the most direct alternatives is the optimization of the biosynthetic pathway of β-carotene. Meanwhile, a successful strategy was recently reported to enhance the production of β-carotene by adaptive laboratory evolution (Reyes et al. 2014). The flux through the carotenoid pathway could be further enhanced by increasing the limited cytosolic pool of acetyl-CoA, introducing heterologous acetyl-CoA biosynthetic enzymes, for example, cytosolic localized pyruvate dehydrogenase (Lian

Table 2 Comparison of chemical and de novo biotechnological syntheses of α- and β-ionones

Properties	Chemical synthesis (BASF and related methods)	Biotechnological synthesis in *Saccharomyces cerevisiae*
Main starting materials	Petrochemicals (BASF from isobutene and formaldehyde, others producers from citral and acetone)	Glucose or other carbohydrates
Complexity of synthesis process	High. 3 separate processes, each one with several steps (BASF)	Low. Bioreactor fermentation
Downstream processes	Purification of ionones from solvent phase by distillation	Purification of ionones from solvent phase. Distillation also can be utilized
Regioselectivity (isomeric purity of α- or β-ionone)	~90–96 %	~100 %. The resulting isomer depends on the precursor carotene
Enantioselectivity for (R)-α-ionone synthesis	No. Requires additional processes, or more laborious and costly synthesis strategies	Potentially yes. Experimental confirmation is necessary
Can the products be labeled as natural flavors?	No[a]	Yes, in both US and EU regulations
Sustainability	Low. Raw materials are non-renewable resources	High. Raw materials are renewable resources derived from vegetables
Main applications of the products	Synthetic fragrances and flavors. Intermediate for the synthesis of other compounds such as vitamin A	Natural flavor (potentially)

[a] Some US distributors offer natural ionones, obtained by chemical synthesis from natural citral, acetone, and pH regulators. EU regulations do not consider these products as natural

et al. 2014). Downstream of the biosynthetic pathway, the oxidative cleavage of β-carotene could be increased by using CCDs with higher activity than those currently employed. The low solubility of β-carotene in water is a major bottleneck for de novo β-ionone synthesis. This hydrophobicity promotes β-carotene localization preferentially in the core of lipid membranes (Gruszecki and Strza 2005). Another possibility is the accumulation of β-carotene in cytoplasmic droplets that has already been observed in the red yeast *Sporobolomyces roseus* (Davoli and Weber 2002). Despite the fact that carotenoids have a recognized role in oxidative damage protection of biological membranes, high-level production of β-carotene in *S. cerevisiae* leads to membrane stress and induces a pleiotropic drug resistance response (Verwaal et al. 2010). This suggests that β-carotene accumulation may cause a perturbation of membrane structure and dynamics and restrict the final yield. Future studies are necessary to determine the targets and strategies to overcome this limitation. One alternative could be carotenoid-binding proteins (Bhosale and Bernstein 2007) that can buffer β-carotene localization. However, the substrate accessibility of CCDs could decrease, and the high-energy cost associated with extra protein synthesis could be detrimental. An alternative approach benefits from the fact that the structural model of maize CCD1 shows a putative hydrophobic patch for membrane binding (Messing et al. 2010) that might be necessary to access membrane-embedded substrates. Accordingly, CCDs can be modified by protein engineering to achieve better accessibility. These modifications can be primarily designed from the structure of some enzymes of the CCD family that have been already crystallized (Sui et al. 2013).

Furthermore, it will only be possible to achieve a high industrial titer of β-ionone and other apocarotenoids by minimizing the growth inhibitory effect of this apocarotenoid. Indeed, as most terpenoids, β-ionone shows strong biostatic and biocidal capacities even at low concentration (10 mg/L of this compound inhibits 20 % growth rate of *S. cerevisiae*, and 50 mg/L completely stops growth; unpublished results). For this purpose, several in situ product removal strategies (ISPR) could be tested. Internal (inside the bioreactor) product removal using two-liquid phase extraction is the only ISPR method that has been used in de novo synthesis of β-ionone. An external loop is an alternative suitable configuration of two-liquid phase product recovery (Schewe et al. 2015). Organophilic pervaporation is one of the most promising ISPR methods for the recovery of β-ionone. This technique enables product recovery without use of solvents, and it has been successfully employed for the in vitro biocatalytic synthesis of β-ionone (Nacke et al. 2012). However, due to the high hydrophobicity of β-ionone and its possible accumulation in cell membranes, the non-use of solvents could reduce β-ionone transfer from inside the cell to the extracellular medium in de novo synthesis. A complementary strategy to avoid a β-ionone toxic effect is to obtain more resistant strains by evolutionary engineering (Winkler and Kao 2014).

Finally, intracellular glycosylation of some C_{13}-apocarotenoids by cloning glycosyltransferase genes could increase solubility and reduce the toxicity of these molecules. This strategy has been successfully used in the synthesis of vanillin in *S. cerevisiae* (Brochado et al. 2010). Although ionones cannot be glycosylated due to the absence of a hydroxyl group, this approach could be of interest for future de

novo synthesis of some hydroxylated C_{13}-apocarotenoids such as β-ionol or β-damascenone precursors.

Conclusions

The experience accumulated over the past few decades in the industry for the chemical synthesis of C_{13}-apocarotenoids has led to robust and optimized processes, with consequent low production costs. On the other hand, biotechnology is a recent developing and promising technology that has gained much attention recently because of its versatility, specificity, and sustainability but has yet to be further optimized. In this regard, in terms of profitability, the actual biotechnological methods can hardly compete with the chemical ones, if the former enter in the same commercial category of synthetic products, at least in the short term. However, as C_{13}-apocarotenoids obtained by biotechnology can be considered as natural in both the US and EU regulations, the niche of natural flavors has the greatest potential for these fermentative products. This is also sustained by the fact that the prices of natural flavors are significantly higher than those prepared by chemical synthesis (e.g., natural β-ionone is 10 times more expensive) However, long-term applications could be more diverse due to the undeniable need for more environmentally sustainable technologies.

Among the possible biotechnological methods available, de novo synthesis in metabolically engineered microorganisms probably has the greatest potential due to low cost of starting materials. The successful synthesis of β-ionone in *S. cerevisiae* represents the starting point for future biotechnological syntheses of other C_{13}-apocarotenoids (such as α-ionone and β-damascenone). Whether chemical synthesis will continue to dominate the industry or if biotech alternatives will gain traction is an ongoing discussion, which remains to be defined.

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Compliance with ethical standards

Conflict of interest Vicente F. Cataldo declares that he has no conflict of interest. Javiera López declares that she has no conflict of interest. Martín Cárcamo declares that he has no conflict of interest. Eduardo Agosin declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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