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Microbial Production of C_{13}-Norisoprenoids and Other Aroma Compounds via Carotenoid Cleavage

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Carotenoids are important precursors of a variety of compounds: the C_{20}-retinoids, the C_{15}-phytohormones, and the C_9- to C_{13}-aromas. Among the last type, C_{13}-carotenoid-derived compounds (norterpenoids/norisoprenoids) such as ionones and damascones, constitute an essential aroma note in tea, grapes, roses, tobacco, and wine. Extraction of carotenoid-derived aroma compounds from plant sources is not economically realistic or considerably expensive. The biotechnological production of aroma compounds represents a feasible alternative and offers the production of enantiomerically pure molecules which can be labeled as "natural." To date, research in the production of ionones or the C_{10}-compound, safranal, has mainly been focused on plant dioxygenases that cleave carotenoids in the positions between carbons 9 and 10 (9′-10′) or 7 and 8 (7′-8′), respectively. Although relatively little is known about the microbial conversion of carotenoids into compounds with aroma due to the well known advantages of manipulating microorganisms, the aim of this work is to review the current state of the research in microbial production of norisoprenoids and other aroma compounds derived from carotenoid cleavage.

Keywords Carotenoid Cleavage; Carotenoid-Derived Aroma Compounds; C_{13}-Norisoprenoids

INTRODUCTION

"Odor" refers to any perception (biological, physical, and physiological) caused by molecules called odorants, which are principally organic compounds. To discern and identify the compounds responsible for particular odors constitute one of the principal aims in aroma research (Steinhart et al. 2000). Since ancient times, flavors and fragrances have been deeply appreciated and later on, studied by mankind. Obtaining mixtures of desired aromas was mainly accomplished, at an artisan level, by maceration, extraction, or distillation either from plant or animal sources. However, it was until the 19th century with the isolation of benzaldehyde and cinnamaldehyde, the production of coumarin, and the structure determination and the chemical synthesis of vanillin, when the flavoring industry really began (Figure 1).

The ability to synthesize compounds that reproduce the aroma or flavor characteristic of natural products gave birth to a completely new branch of the chemical industry (Armstrong et al. 1993), and during the past years it has experienced a rapid growth. In 1996 its worldwide size was estimated in USD 9.7 billion (Somogyi 1996); for 2001 its share corresponded to USD 12.2 billion, and by 2005 it was calculated in USD 16.0 billion (Leffingwell and Associates 2006). The structure of the industry of flavors and fragrances is quite complex due to the lack of homogeneity; nevertheless, it can be grouped in three branches: essential oils and natural extracts, chemical compounds with aroma, and formulated fragrances (Somogyi 1996). Up to now, food still remains as the largest market for this industry with beverages, bakery, dairy products, meat, and confectionery as the main sectors. Furthermore, aromas are widely applied in soaps, detergents, cosmetics, perfumery, and house-cleaning items, as well as new targets such as candles and aromatherapy products.

BIO-MANIA

According to the American (CFR 1990) and European (EC 1988) legislations, the term "natural flavor" is applied to the substance (essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate) derived from vegetal, animal, or microbial sources (spice, fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf, or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products) by physical, microbiological, or enzymatic processes, whose significant function in food is flavoring rather than nutritional. Lately, everything labeled as "natural" and very recently, as "organic," possesses an unfounded notion associated to good health. In contrast, anything containing the words "artificial,"

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This review is dedicated to Prof. Barbarín Arreguín-Lozano, Mexican pioneer in terpenoid research.

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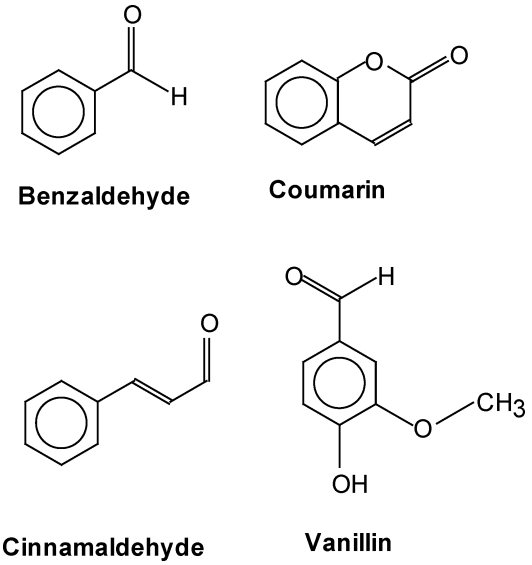


FIG. 1. Chemical structure of the base molecules for the aroma industry.

"chemical," or "synthetic" is conceived as negative or even harmful. Due to the consumer's preference and acceptance, a product sold in the market as natural is considerably more expensive than the same one obtained by chemical synthesis. A strikingly surprising example of this situation is given by vanillin, a chemical considered as the most important flavoring compound in terms of amount and value. Natural vanillin extracted from pods is estimated between USD 1000 and 4000 kg^{-1} while its synthetic counterpart costs around USD 10 to 15 kg^{-1}. Therefore, this "bio-" mania, defined as the perception of natural as better, constitutes an important driving force in the development of flavors and fragrances.

BIOTECHNOLOGICAL AROMA PRODUCTION

Although still in practice, obtaining natural products via extraction from plants represents an incredibly arduous task. Among the inconveniences encountered in this method are the strong dependence on agriculture and all the factors surrounding it (climate, seasons, social, and political situation), the diminishing supplies of raw material, the expenses in order to achieve higher yields, and the variability in the amount and quality of the final product (Welsh et al. 1989). As described above, in accordance to U.S. and Europe legislations, biotechnology represents a very attractive alternative for the production of flavors and fragrances that can be considered as natural. In addition, compared to chemical synthesis, the biotechnological approach offers the resolution of enantiomeric mixtures as they occur in nature, thus eliminating subsequent separation steps (Berger et al. 2002) and improving the perception of scent that could be affected by chirality. For example, of eight isomeric forms, only L-menthol confers the desired peppermint note (Figure 2). As Abate et al. (2004) emphasized, the need of enantiopure compounds in the fragrance field is covered by enzymatic catalysis, in particular, with the use of lipase-mediated reactions.

The biotechnological production of aromas can be accomplished via biocatalysis, either by whole cell (microbial or plants) or enzymatic methods. The difference between them might be their application, i.e., whole cells for complex targets and product mixtures, whereas enzymes for single step transformations (Krings and Berger 1998). In crude extracts or purified forms, enzymes such as lipases, esterases, proteases, nucleases, and glycosidases have been extensively employed in various food industry processes, as well as in the production of specific flavor chemicals. Over the use of enzymes, whole cell methods principally have the advantages of higher stability and for not requiring cofactors nor the necessity of regenerating them. Contrary to what is assumed, biocatalysis research and applications have been focused more in the food science and technology fields rather than in the pharmaceutical. Reports dealing with biocatalytical generation of flavors and fragrances have been extensively published (for an excellent and comprehensive

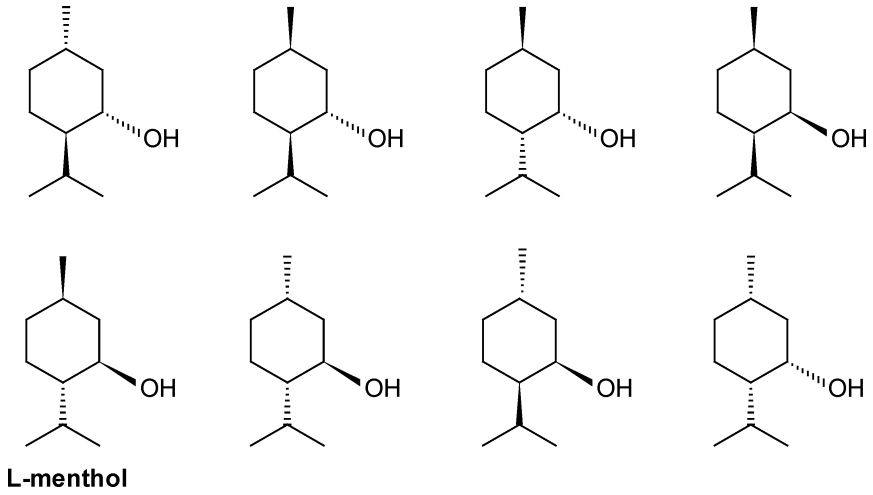


FIG. 2. Chirality in the monoterpene menthol molecule.

overview consult Berger 1995; Rabenhorst 2000; Berger et al. 2002; Cheetham 2004). Furthermore, reviews concerning this topic have focused on traditional aromas, particularly vanillin, benzaldehyde, alcohols, carboxylic acids, esters, lactones (γ-decalactone), 2-phenylethanol, green notes, terpenes (monoterpenes), and with less extent their terpenoid derivatives (Krings and Berger 1998; Vandamme and Soetaert 2002; Schrader et al. 2004; Serra et al. 2005; Longo and Sanromán 2006). Also from a general point of view, Aguedo et al. (2004) described the approaches of different researchers for the production of fragrances from lipids; nevertheless, their work included the important group of carotenoid-derived aroma products obtained by different means.

Compared to plant cells, microorganisms are easily culturable. Rapid growth rates, physiological control, manipulation of fermentation parameters and genes, and the possibility for scaling up make microbial cells excellent candidates as overproducers. Although the use of microbial processes for the production of traditional flavors in fermented foods and beverages has historically accompanied mankind, it was until the 1920s when the role of microorganisms was pointed out (Omelianski 1923). The options offered by the microbiological approach include synthesis *de novo* and bioconversions/biotransformations (Krings and Berger 1998). *De novo* synthesis involves the catabolism of macromolecules (carbohydrates, fats, and proteins) to breakdown products, and then, the production of more complex flavoring molecules derived from the secondary metabolism of microorganisms. On the other hand, the microbial capacity to modify or convert a chemical compound into a structurally related one is known as bioconversion (more than one step) or biotransformation (one step process).

ISOPRENOIDS

Isoprenoids, terpenes, or terpenoids, depending on the author, are the most structural diverse and abundant compounds in nature, primarily constituting part of essential oils in plants. Their building block is the hydrocarbon isoprene, $CH_2 = C(CH_3) - CH = CH_2$, and based on the number of units, they are classified in hemiterpenoids (1), monoterpenoids (2), sesquiterpenoids (3), diterpenoids (4), triterpenoids (6), tetraterpenoids (8), and polyterpenoids ($n > 8$) (Table 1) (Connolly and Hill 1991; Harborne 2001; Nes and Zhou 2001).

Isoprenoids are formed by consecutive condensations of C_5-isoprene units, specifically, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) (Figure 3). Depending on the type of organism, the biosynthesis of the precursor units (IPP and DMAPP) can follow two routes: the mevalonate (eukaryotes, archaebacteria, and cytosols of higher plants) and the nonmevalonate pathways (eubacteria, green algae, and plastids of higher plants) (Kuzuyama 2002). The overall biological role of isoprenoids is still to be elucidated; nevertheless, their basic functions in stabilizing membranes, enzymatic regulation, protein targeting, and respiration are now known.

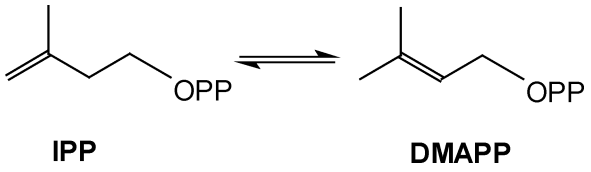


FIG. 3. Isoprenoid precursors.

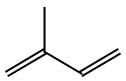
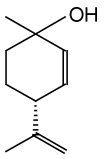
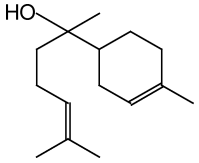
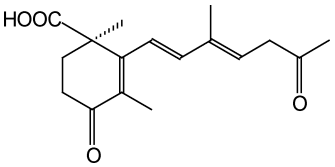
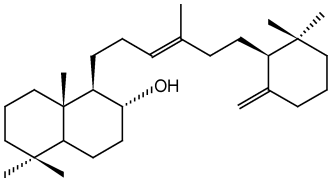
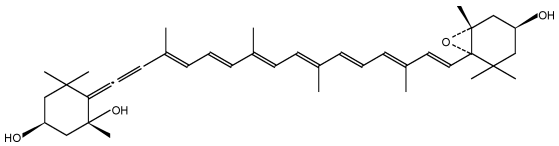
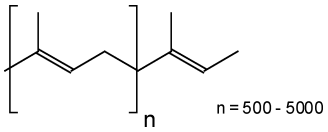
In plants, where the structure and function of isoprenoids have mostly diversified, they act as attractants (aromas and colors) for insects involved in pollination, expellants (aromas and flavors) for insects or certain animals, antimicrobial agents, especially against fungi, accessorial pigments (carotenoids) for photosynthesis, and growth factors. For many years plants represented the principal source of isoprenoids. The increasing demand of these compounds and the scarceness of raw materials make this option uneconomically feasible. Isoprenoids can also be isolated from microbes and marine organisms; however, the yields are very low. This situation has driven the search for new alternatives. As described above, microorganisms represent an excellent option, and a lot of research has been achieved in this area (Maury et al. 2005), being the most recent, the engineering of microbial cells to produce the desired levels of terpenoids or novel ones (Mijts and Schmidt-Dannert 2003; Chang and Keasling 2006). Furthermore, once obtained, isoprenoids can be biosynthetically modified by losing carbon atoms or by adding carbons or other functional groups. As de Carvalho and da Fonseca (2006) mentioned, terpenes and the products derived by biotransforming them have earned increasing attention due to their attractive uses as flavors and fragrances, as well as their very recent applications in pharmaceutics, therapeutics, plague control, and substitutes for friendlier technologies.

CAROTENOIDS: THE COLORFUL ISOPRENOIDS

Among the numerous group of compounds encompassed by isoprenoids, the lipophilic C_{40}-tetraterpenoids, known as carotenoids, are the most widely distributed natural pigments, ranging from bright yellow to deep red. These molecules are composed of a backbone of eight isoprenes, arranged in two C_{20} units that are condensed head to head, forming a long conjugated chain of double bonds, especially in the central portion. However, variants to this classic arrangement, exhibiting shorter C_{30} or longer C_{45} or C_{50} backbones have been found in bacteria. Generalities of these compounds can be found in reports by Goodwin (1986), Karnaukhov (1990), Britton (1995), and Olson and Krinski (1995). With respect to the molecule ends, they can be described as acyclic, monocyclic, or bicyclic. Up to now, about 600 different occurring structures have been described, with three forms as the basic ones: lycopene, α-, and β-carotene (Figure 4).

Although a new classification, based on novel compounds, already exists, carotenoids have been traditionally divided in two major classes: carotenes (hydrocarbons) and xanthophylls

TABLE 1
Classification, number of carbons, examples, and chemical structures of different type of terpenoids

Classification	Carbon atoms	Compound name	Chemical Structure
Hemiterpenoids	5	Isoprene	
Monoterpenoids	10	*p*-menthadienol	
Sesquiterpenoids	15	$\alpha-$bisabolol	
Diterpenoids	20	Trisporic acid	
Triterpenoids	30	Ambrein	
Tetraterpenoids	40	Neoxanthin	
Polyterpenoids	>40	Rubber	

(oxygenated carotenoids). As described above, carotenoids are formed from the isoprenoid pathway, and its biosynthesis only occurs in bacteria, fungi, algae, and plants. Animals are unable to synthesize them so they obtain these molecules via ingestion. Depending on the organism, carotenoid anabolism has been extensively studied (Armstrong 1994; Johnson and Schroeder 1996; Armstrong 1997; Britton 1998; Sandmann 2001; Bhosale and Bernstein 2005; Römer and Fraser 2005), and even though all share the same initial pathway (geranylgeranyl pyrophosphate and phytoene synthases) (Figure 5), their subsequent reactions are quite specific. For instance, desaturation of phytoene results in the production of lycopene. However, depending on the cyclase involved in the next steps, different cyclic carotenoids are formed.

Carotenoids exhibit different and essential biological roles. Shortly, their functions are involved in photosynthesis, membrane stability, development (hormone-like), adaptation, protection (photo and antioxidants), nutrition, and recently studied, disease prevention or treatment. Additionally, as mentioned by Lee and Schmidt-Dannert (2002) carotenoids can be commercially focused in the food industry as colorants, animal feed supplements, or nutraceuticals, and also in cosmetics and pharmaceuticals, thereby, increasing the efforts towards their production in useful quantities.

CAROTENOID CLEAVAGE AND APOCAROTENOIDS

By means of oxidative cleavage (involving molecular oxygen) carotenoids can be precursors of different compounds

FIG. 4. Molecular structures of lycopene (A), α- (B), and β-carotene (C) with their carbon numeration.

(initially aldehydes or ketones at the cleaving site), termed as apocarotenoids. The cleaving mechanism first established was the conversion of β-carotene (provitamin A) to retinal (vitamin A) (Moore 1930). Since then, due to the crucial functions exerted by apocarotenoids in living systems, in addition to the possible commercial applications in flavoring and perfumery, extensive studies in various fields have taken place. Carotenoid breakdown can be symmetric or asymmetric, and once formed, these compounds can be substrate for further cleavage. Therefore, rather than being formed from smaller molecules, the big

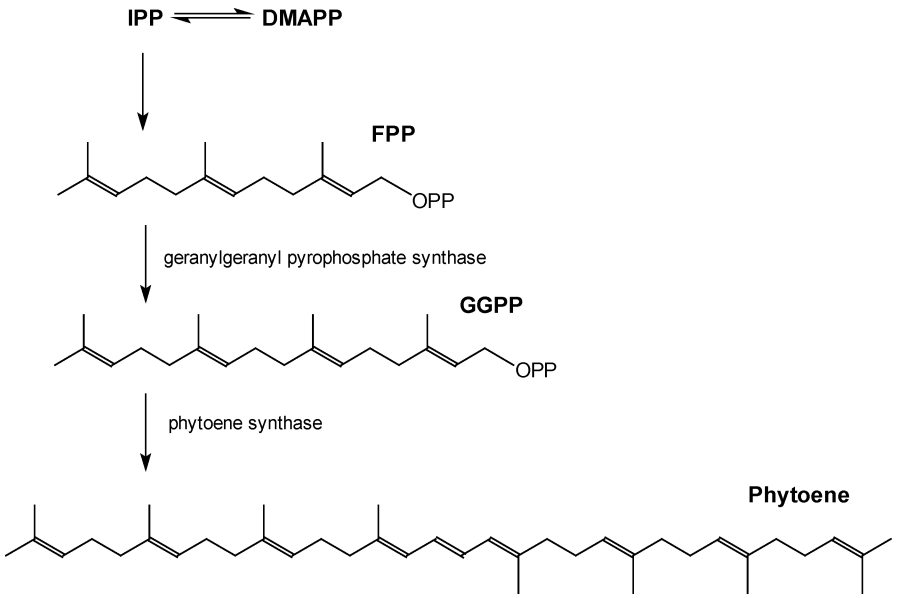


FIG. 5. Common pathway for carotenoid biosynthesis.

majority of apocarotenoids are derived from larger isoprenoids (carotenoids) (Figure 6), with the exception of the plant hormone, abscisic acid (ABA), which can be synthesized from farnesyl diphosphate in fungi (Inomata et al. 2004a, 2004b).

A very complete insight on carotenoid cleavage aspects has recently been described by Kloer and Schulz (2006). Although apocarotenoid generation can be achieved via non-enzymatic or enzymatic mechanisms, carotenoid cleavage oxygenases (CCO) are commonly responsible for it. The first cloned and isolated enzyme of this family was a VP14 recombinant protein from maize (*Zea mays*), involved in the formation of ABA. The cleavage of 9-cis-epoxy-carotenoids took place at the

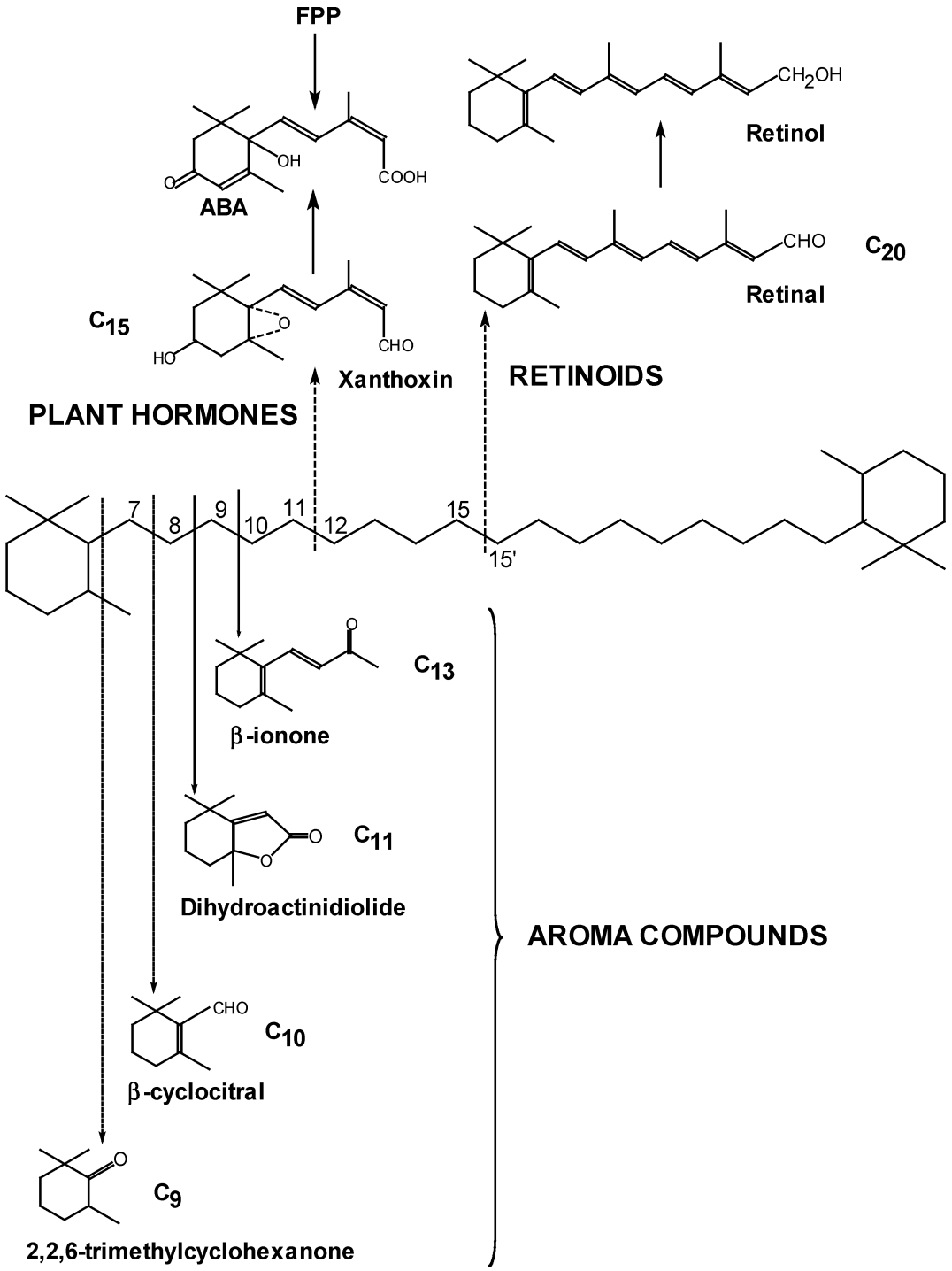


FIG. 6. Carotenoid cleavage products and the alternative biosynthetic route for ABA.

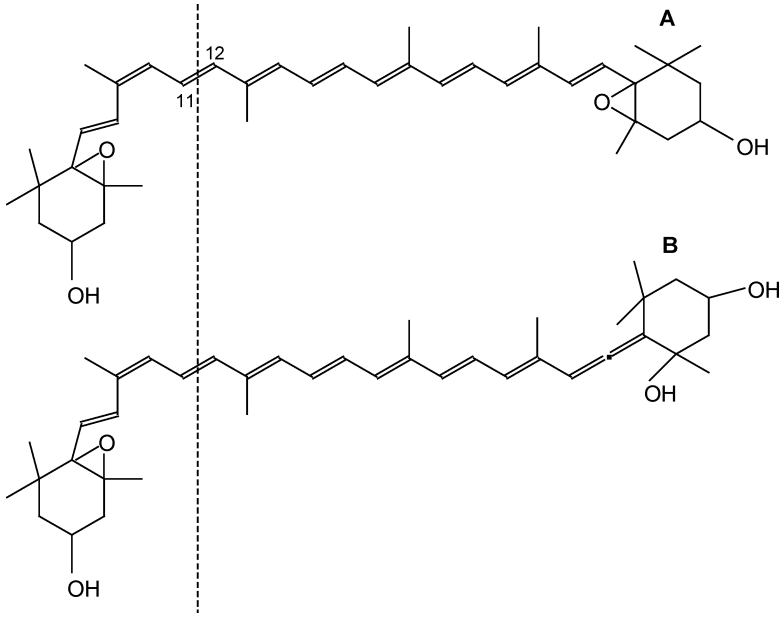


FIG. 7. Two examples of nine-cis-epoxy-carotenoids: 9-cis-violaxanthin (A) and 9-cis-neoxanthin (B), their chemical structure, and the position of cleavage by NCED.

C_{11}-C_{12} position (Figure 7), resulting in C_{25}-aldehydes and its C_{15}-precursor, xanthoxin (Schwartz et al. 1997). From then on, research concerning enzymes involved in carotenoid metabolism (catabolism) has been done, and reviews dealing with it, in plants (Bouvier et al. 2005; Auldridge et al. 2006), in animals (von Lintig et al. 2005), and from an evolutionary point of view (Moise et al. 2005) have been published. Enzymes belonging to the NCED (nine-cis epoxy-carotenoid dioxygenases) subfamily, described above, were isolated from other plant sources.

Another subclass of this type of enzymes, discovered also in *Arabidopsis thaliana* by Schwartz et al. (2001), was the carotenoid cleavage dioxygenases (CCD). This dioxygenase was responsible for the mostly occurring symmetrical cleavage at the C_9-C_{10}/$C_{9'}C_{10'}$, resulting in a C_{14}-dialdehyde and two C_{13}-products. On the other hand, studies on the synthesis of retinoids have mostly taken place in the animal kingdom, where β-carotene can be cleaved symmetrically between C_{15}-$C_{15'}$ (Figure 6) by β-carotene cleavage oxygenases (BCO1), yielding two molecules of retinal (von Lintig and Vogt 2000; Wyss et al. 2000), or asymmetrically (BCO2) in vertebrates, resulting in β-apo-10′-carotenal (Figure 8) and the C_{13}-compound, β-ionone (Kiefer et al. 2001).

CAROTENOID-DERIVED AROMAS: NORISOPRENOIDS AND OTHER COMPOUNDS

Due to their historical and commercial significance, low threshold values, and characteristic aroma notes, volatiles derived from carotenoid breakdown are highly appreciated in flavoring. Depending on the carotenoid precursor and, the breakdown position (Figure 6), a great diversity of C_9- to C_{13}-apocarotenoid compounds (Enzell 1985) can be generated (Table 2). It is now known that the cleavage in the 9-10 position is thermodynamically favorable; therefore, C_{13}-products, known as norisoprenoids, represent the most common and widespread group.

Winterhalter and Rouseff (2002) compiled a very complete work on carotenoid-derived aroma compounds, encompassing a general overview, generalities and analytical characteristics, biogenesis, biotechnological production, thermal formation, and occurrence, predominantly in plant tissues (leaves, fruits, flowers, and vegetables) or from other sources (beverages and spices). In the introductory chapter, they proposed a mechanism for the conversion of carotenoids into aromas, comprising three general steps: (1) an oxidative cleavage, (2) an enzymatic transformation, and (3) acid catalyzed conversions. Particularly for α- or β-carotene, the final product, α- or β-ionone,

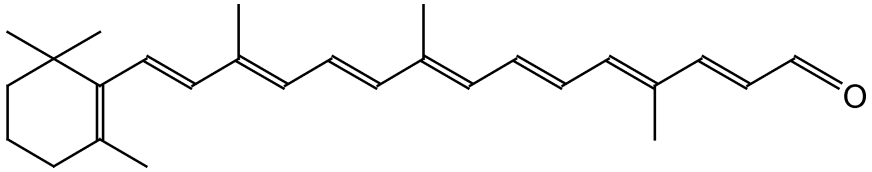
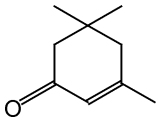
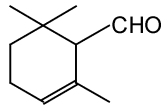
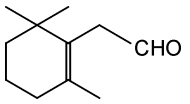
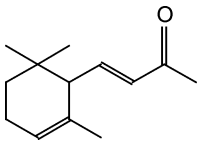
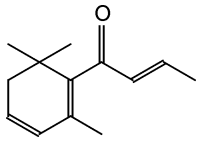
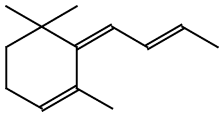
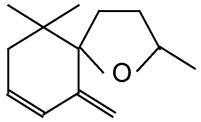
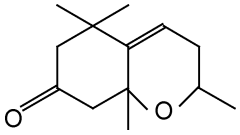
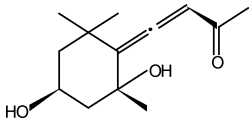


FIG. 8. Chemical structure of β-apo-10′-carotenal.

TABLE 2
Number of carbon atoms, cleavage position, examples, and chemical structure of C_9-to C_{13}-norisoprenoids

Carbon number	Cleavage position	Compound name	Structure
C_9	6–7	Isophorone	
C_{10}	7–8	$\alpha-$cyclocitral	
C_{11}	8–9	$\beta-$homocyclocitral	
C_{13}	9–10	Ionones (α-ionone)	
		Damascones (β-damascenone)	
		Megastigmanes (megastigma-4,6,8-triene)	
		Others: Vitispirane	
		Oxoedulan	
		Grasshopper ketone	

respectively, is obtained after the first step. On the other side, the primary breakdown product of neoxanthin, the grasshopper ketone undergoes a series of reactions for the generation of β-damascenone, the best known damascone for its rose-like note.

In the attempt to find the odorous principle of violets, the first synthesis of norisoprenoids was composed of a mixture of isomeric ionones. Almost 40 years later, the floral smelling (violet-like) compound, β-ionone, with an odor threshold of 0.007 ppb (in water), was isolated from a natural source

(*Boronia megastigma*). Since then, studies regarding the isolation and identification of further volatile compounds have been conducted. Regarding its importance in industrial applications and as starting materials for other natural products (vitamin A, carotenoids, edulan derivatives, and theaspiranes) ionones, mostly β-ionone, have drawn particular attention. Therefore, their preparation, even from a chemical point of view, and properties have been subject of study (Brenna et al. 2002). Finally, the research on carotenoid-derived aroma compounds during the past years has focused on non-volatile constituents due to the fact that in plant tissues they are predominantly found as bound forms, i.e., glycosylated. Apart from norisoprenoids, the aglycone moiety found in glycosidic aroma precursors can be monoterpenes and benzene derivatives, thus converting glycosidases or other hydrolases from plant or microbial sources as an ideal means for volatile release (Sarry and Günata 2004). Within the microbiological choice, the hydrolysis concerning the winemaking process has been the most studied. As an example, the ability to release aromas from a glycosidic extract of Muscat grape juice of different wine and non-wine yeasts strains was evaluated by Fernández-González et al. (2003). *Hanseniaspora uvarum* (wine yeast) and *Candida molischiana* (non-wine yeast) yielded the highest concentrations and varieties of volatiles.

Direct breakdown for the production of carotenoid-derived aroma compounds involve two methods: non-enzymatic and enzymatic. The first one comprise physicochemical treatments, such as photo-oxygenation, auto-oxidation, and thermal degradation. Within the enzymatic option, in addition to CCD, which were introduced in the former section, alternative enzymes (lipoxygenases, xanthine oxidase, phenoloxidases, and peroxidases) involving the co-oxidation of carotenoids, i.e., β-carotene (Bosser and Belin 1994; Wu et al. 1999; Waché et al. 2002, 2006) can be employed. Buonaurio and Servili (1999) reported on the activity enhancement, caused by a bacterial infection, of a lipoxygenase (LOX) from pepper (*Capsicum annuum* L.), responsible for the generation of volatile compounds. Leaf inoculation was accomplished with water as a control and two *Xanthomonas campestris* pv. v*esicatoria* strains (virulent and avirulent to pepper). For the avirulent infection, a considerable increase was observed in levels of volatiles derived from the LOX pathway, such as (*E*,*E*)-2,4-hexadienal, 1-hexanol, 3-hexen-1-ol, 2,4-hexadienal, and 2,4-eptadienal. In particular, after 12 h of inoculation, α- and β-ionone, derived from LOX degrading action over carotenoids, exhibited an 8.4 and 10.6 fold increase, respectively, compared to the control levels. A hypothetical explanation for the occurrence of the norisoprenoids might be the plant response towards the bacterial infection, as it is known that they can act as antibacterial agents (Anzaldi et al. 1999). Therefore, this research constitutes a very novel study, involving the association between a plant and a bacteria, in which the pathogenic interaction results in the production of volatiles derived from LOX action.

CAROTENOID-DERIVED AROMA COMPOUNDS PRODUCTION BY PLANT DIOXYGENASES

To date, research concerning the enzymatic generation of carotenoid-derived aroma compounds has mostly been achieved in plants. Characterization of the enzymes responsible for cleaving β-carotene to β-ionone as the major product was done in quince (*Cydonia oblonga*) (Fleischmann et al. 2002) and star fruit (*Averrhoa carambola*) (Fleischmann et al. 2003). In both cases, degradation of the carotenoid was only detected in the extracts obtained from ripened fruit. On the other hand, based on the homology to *A. thaliana* genes encoding dioxygenases from *A. thaliana* (Schwartz et al. 2001), other CCDs from saffron crocus (*Crocus sativus*) (Bouvier et al. 2003), tomato (*Lycopersicon esculentum*) (Simkin et al. 2004a), petunia (*Petunia hybrida*) (Simkin et al. 2004b), grapes (*Vitis vinicola*) (Mathieu et al. 2005), and melon (*Cucumis melo*) (Ibdah et al. 2006) have been successfully cloned and expressed. After the sequence analysis, homology between them was above the 80%, and depending on the carotenoid substrate, two different C_{13}-norisoprenoids and one C_{14}-dialdehyde were generated. Particularly for the case of *Crocus sativus*, although a dioxygenase (CsCCD) cleaving at the 9-10 (9′-10′) double bonds was identified, another dioxygenase (CsZCD), responsible for the 7-8 (7′-8′) breakdown of zeaxanthin was also found. When studying plant signaling in *A. thaliana* mutants, a carotenoid cleavage dioxygenase (CCD7), involved in the regulation of shoot branching, was discovered (Booker et al. 2004). Afterwards, it was found that this enzyme (AtCCD7) cleaves β-carotene in a different manner (Schwartz et al. 2004). Although the cleavage at the 9-10 position also took place, the obtained products were β-ionone and a C_{27}-apocarotenal, indicating an asymmetrical breakdown. This last product (10′-apo-β-carotenal) is sequentially cleaved by a second dioxygenase (AtCCD8), rendering a 13-apo-β-carotenone (C_{18}) and a C_9-dialdehyde (Figure 9). Very interestingly, Lewinsohn et al. (2005) studied the similarity between tomato and watermelon in the carotenoid content of mutants, evidenced by different flesh pigmentation patterns. Sets of volatiles (monoterpenes and norisoprenoids) derived from the pigments, varied according to the occurrence and/or the proportion of the carotenoids present in the wild-type and the mutant species.

CAROTENOID-DERIVED AROMA COMPOUNDS PRODUCTION BY MICROBIAL OXYGENASES

A 7,8 (7′,8′)-Cleaving Oxygenase from *Microcystis* PCC 7806

Aroma compounds derived from carotenoid breakdown and isolated from microorganisms were reported in the cyanobacterium *Microcystis aeruginosa*, with β-cyclocitral as the major compound liberated (Jüttner 1976). Even though plant dioxygenases have been predominantly reported in literature, cyanobacteria represent a good source of carotenoid cleaving oxygenases.

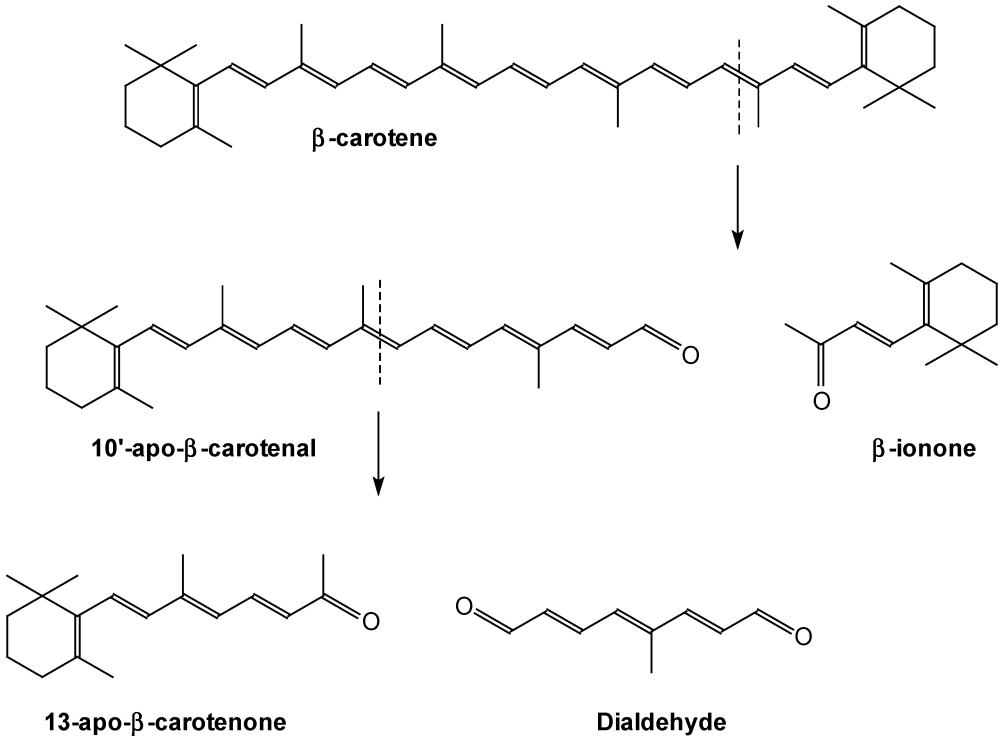


FIG. 9. Asymmetrical cleavage of β-carotene by AtCCD7 and its subsequent breakdown by AtCCD8 (Schwartz et al. 2004).

A membrane bound microbial oxygenase, termed β-carotene oxygenase, was first described by Jüttner and Höflacher (1985) in the genus *Microcystis*, particularly *Microcystis* PCC 7806. When comparing native versus activated cells, obtained after freezing the cyanobacterial pellet, a marked change in the pigment composition, associated with β-cyclocitral occurrence, was detected. A decrease in β-carotene and zeaxanthin levels was accompanied with the apparition of crocetindial. The compounds generated were characterized and the stoichiometric relationship was calculated. Per molecule of carotenoid, one molecule of crocetindial and two molecules of β-cyclocitral from β-carotene (or two molecules of hydroxy-β-cyclocitral from zeaxanthin) were obtained, suggesting a symmetrical cleavage at the 7,8 and 7′,8′ positions (Figure 10). No decrease was observed for other pigments like myxoxanthophyll and echinenone, possibly due to differences in their chemical structure or their location in the cell, not allowing the contact with the enzyme. When focusing on the oxygenase, no reaction could proceed under strict anaerobic conditions, pointing out dioxygen as the oxidant in the degradation of the carotene, which in turn was incorporated into the β-cyclocitral molecule. No β-carotene co-oxidation attributed to fatty acids appeared to be

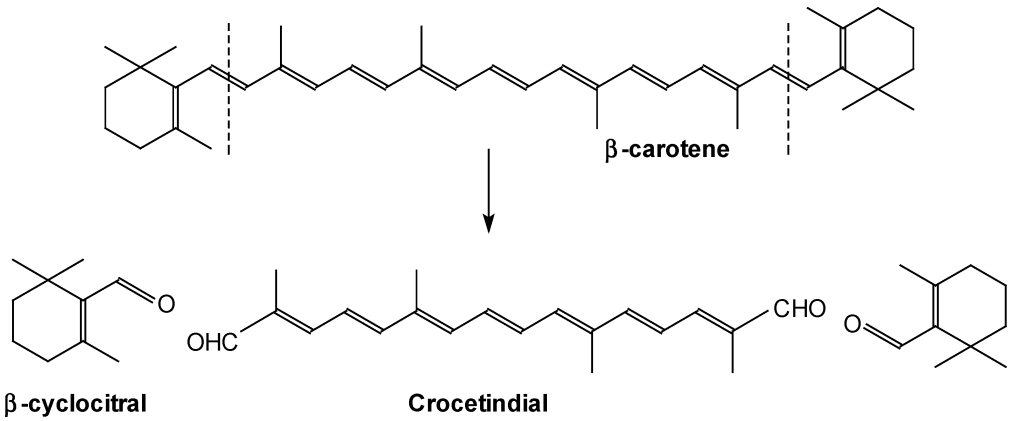


FIG. 10. Symmetrical cleavage of β-carotene by a membrane bound dioxygenase from the cyanobacteria *Microcystis* PCC 7806 (Jüttner and Höflacher 1985).

involved in the reaction mechanism, and iron seemed to play a crucial role as part of the enzyme.

Compounds with Aroma from *Calothrix* and *Plectonema* Cultures

Further studies showed that, in spite of producing unpleasant odors, cyanobacteria might also be an excellent source of norisoprenoids. The origin, whether bio- or non biogenic, of water off-flavor components, was determined in axenic cultures of *Calothrix* (*C. parientina* PCC 6303 and *Calothrix.* sp. PCC 7507) and *Plectonema* (*P. notatum* and *Plectonema* sp. PCC 7410) (Höckelmann and Jüttner 2004). Both genus are commonly found in cyanobacterial biofilms of streams and littoral lake zones. In addition to aliphatic alcohols, ketones, and long chain hydrocarbons, isoprenoids represented a large group of odor compounds, from which all except limonene appeared to have a biogenic origin, measured by ^{13}C-labelling. C_{13}- (β-ionone and dihydro-β-ionone), C_{11}- (dihydroactinidiolide), C_{10}- (β-cyclocitral), and C_9-compounds (2,6,6-trimethylcyclohexanone and 2-hydroxy-2,6,6- trimethylcyclohexanone) were detected in *C. parientina* and the two strains of *Plectonema*. β-cyclocitral, was only present in *Plectonema*, which together with dihydro-β-ionone, suggested further transformation, i.e., reduction reactions, of their respective preceding molecules, β-cyclogeraniol and β-ionone. Additionally to *C. parietina* and *Plectoderma*, *Phormidium* sp. cultured under monoxenic conditions, proved to be a new producer of β-ionone derivatives, such as dihydro-β-ionol, tetrahydroionone, and 4-oxo-β-ionone (Höckelmann and Jüttner 2005). Based on *Microcystis* (Jüttner and Höflacher 1985), in all cases, oxygenases seemed responsible for carotenoid degradation and the generation of their breakdown products.

Three Oxygenases from *Nostoc sp.* PCC 7120 with Different Cleaving Activities

Recently, Marasco et al. (2006) found predicted gene homologs of eukaryotic CCDs to be present in different cyanobacteria of the genus *Nostoc*, *Prochlorococcus*, *Synechocystis*, and *Synechococcus*. After sequence alignments, in some cases, very low identity was observed between cyanobacterial CCDs, in contrast to the higher homology degrees shared with the plant control dioxygenase from *A. thaliana* (AtCCD1) (Schwartz et al. 2001). Cloning and expression of the cleaving dioxygenases genes were carried out in *Escherichia coli* (Marasco et al. 2006). These cells were previously engineered to co-express different sets of genes involved in carotenoid biosynthesis; thus, different types of C_{40}-carotenoids (β-carotene, zeaxanthin, torulene, and lycopene) and a novel linear C_{30}-carotenoid (diapocarotenedial) were tested. The highest loss of color in the *E. coli* cells was observed for AtCCD1, probably implying that the carotenoid substrates employed for the microbial enzymes were not their natural ones or an affection in their accessibility might be responsible for these results. However, among the cyanobacterial dioxygenases, NSC1 and NSC2 from *Nostoc sp*. PCC 7120 and SYO from *Synechococcus elongates* PCC 7942 exhibited the highest bleaching activity.

The three enzymes from *Nostoc sp*. PCC 7120 were chosen for further characterization (Marasco et al. 2006), even though β-carotene levels in cells expressing NSC3 were quite similar to those quantified in the negative control (empty vector) cell. For *in vitro* assays, it was necessary to improve the protein solubility, purify the enzymes, and set the appropriate conditions for the measurement. Histidine tagging at the N-terminus resulted in the loss of the cleaving activity. The characterization of the cleavage for each dioxygenase was performed, rendering the following results. NSC1 was able to cleave β-carotene as well as β-apo-8′-carotenal (Figure 11) at the 9,10 (9′,10′) and 9,10 double bonds, respectively. In both cases, the volatile C_{13}-norisoprenoid, β-ionone, was identified by GC/MS (gas chromatography coupled to mass spectrometry). Additional products of the apocarotenoid suggested the occurrence, with less extent, of the breakdown at the 7,8 position. A 9,10 cleavage was also observed for NSC3 with a certain particularity. Although no breakdown of the carotenoids tested *in vivo* was detected, after the *in vitro* assay, this enzyme exhibited cleaving activity of β-apo-8′-carotenal. Therefore, NSC3 constituted the first case of a dioxygenase cleaving apocarotenoids at the 9,10 position.

For NSC2, trans-retinal was detected either with β-carotene or β-apo-8′-carotenal (Marasco et al. 2006). These results suggested the occurrence of the breakdown at the 15,15′ position, similar to the one performed by the firstly reported dioxygenase (Diox1) from *Synechocystis sp*. PCC 6803 (Ruch et al. 2005) or SYC2, as called by Marasco et al. (2006). However, the latter enzyme was only able to cleave apocarotenoids (β-apocarotenals, (3R)-3-OH-β-apo-carotenals, and apo-lycopenals), with different velocities and values of Km calculated depending on the substrate (Ruch et al. 2005). The structure of this enzyme, later called SynACO (*Synechocystis* apo-carotenoid oxygenase), was solved at a resolution of 2.4 Å, exhibiting a cavity that might act as a bottleneck by probably hindering the

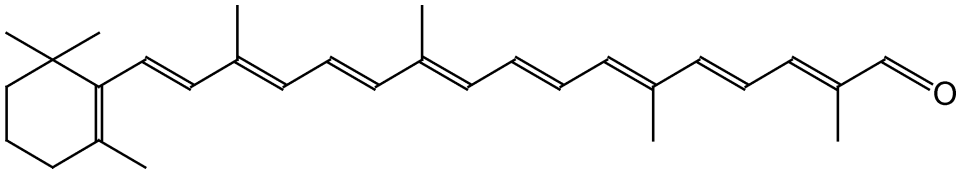


FIG. 11. Chemical structure of β-apo-8′-carotenal.

β-ionone end-group and positioning correctly the substrate for retinal formation (Kloer et al. 2005). At the same time and independently to the research group of Schmidt-Dannert (Marasco et al. 2006), Scherzinger and coworkers (2006) also focused on *Nostoc sp*. PCC 7120, mainly to the oxygenase responsible for retinal formation, NosACO (Nostoc apo-carotenoid oxygenase). The latter enzyme was able to cleave different β-apo-carotenals, (3R)-3-OH-β-apo-carotenals, apo-lycopenals, and the corresponding alcohols, depending on the substrate chain length. Finally, based on the crystal structure of SynACO (Kloer et al. 2005), an homology model was generated for NosACO, indicating that both enzymes are membrane proteins (Scherzinger et al. 2006).

According to the report by Marasco et al. (2006), where NSC1, NSC2, and NSC3 were identified, the genome of this cyanobacterium contains three members of the carotenoid oxygenase family (NosACO, NosDiox2, and NosDiox3) (Scherzinger et al. 2006). Nevertheless, in the study performed by Scherzinger and collaborators (2006), no breakdown of β-carotene was observed *in vivo*, nor cleavage of this carotene or other major carotenoid compounds (echinenone and myxoxanthophyll) was found *in vitro*. Even though the findings on the substrate cleaved by NSC2 (Marasco et al. 2006) and NosACO (Scherzinger et al. 2006) might apparently seem contradictory, the first group measured a very low breakdown activity on full-length carotenoids, i.e., β-carotene. Studies concerning cyanobacteria are expected to be accomplished, as this field offers an exploitable option for the identification, isolation, characterization, and production of (apo)carotenoid cleavage oxygenases.

CAROTENOID-DERIVED AROMA COMPOUNDS PRODUCTION BY MICROBIAL CELLS

Bioconversion of Lutein to Tobacco Aroma Compounds

The bioconversion process of lutein to products with aroma, carried out by a microbial mixture, was reported by Sánchez-Contreras et al. (2000). With the purpose of screening microorganisms able to degrade lutein, the carotenoid was added to a chemically defined solid medium as an oily extract. This extract, mainly composed of the xanthophyll (89% lutein), was obtained from the dehydration process of marigold flower (*Tagetes erecta*), representing as well the microbiological source for the screening. Therefore, due to their ability to grow on lutein as the only carbon source, our research group isolated nineteen microbial colonies. Primarily, pigment degradation was visualized by the appearance of colorless halos around the colony. When grown in submerged cultures, only two out of the nineteen colonies were capable for producing volatile compounds. Regarding its better efficiency for degrading lutein and its unique ability for generating a strong tobacco aroma, one of them was selected for further study. After purification, it was found that the microbial colony was comprised by two organisms, a bacterium and a yeast in a 100:1 proportion (Sánchez-Contreras et al. 2000), later identified as *Paenibacillus amylolyticus* and *Trichosporon asahii*, respectively (Rodríguez-Bustamante et al. 2005).

A 60% of lutein degradation was quantified in the presence of both microorganisms; whereas, when cultured separately, lower values were measured (Sánchez-Contreras et al. 2000; Maldonado-Robledo et al. 2003). At 96 h of the process, a considerable decrease in the absorption profile of organic extracts from the culture medium, corresponding to lutein (430, 445, and 460 nm) was observed. On the other side, an increasing peak at 270 nm, which might be correlated to the compounds responsible for the tobacco aroma, was registered in the absorption spectra (Sánchez-Contreras et al. 2000). Burton and Kasperbauer (1985) observed the decrease in chlorophyll and carotenoid (neoxanthin, violaxanthin, lutein, and β-carotene) content of tobacco leaves during senescence and curing, so the breakdown of lutein was further studied. In order to improve the degradation, different agitation conditions (0, 60, and 100 rpm) were tested, finding that the intermediate stirring allowed a 95% of carotenoid breakdown and a better yield volatile formation (Sánchez-Contreras et al. 2000). Using this agitation speed, a complete fermentation kinetics was carried out, following the evolution of microbial growth, consumption of lutein, and generation of products with aroma. A negligible carotenoid degradation took place in those conditions, and no volatile production was registered in the control without inoculum, thus eliminating the fermentation conditions as responsible for lutein conversion. However, compounds with a strong tobacco aroma were synthesized from lutein only when both microorganisms were present in the culture.

Analysis of the volatiles by GC/MS at three different times (0, 36, and 96 h) of the process pointed out that at 36 h, β-ionone comprised the only lutein derived product, while no α-ionone was detected. At the final time of the process, the following products were identified in different percentages: β-ionone (9.4%) and its hydrogenated derivatives: 7,8-dihydro-β-ionol (84.2%), 7,8-dihydro-β-ionone (3.5%), and 3-hydroxy-β-ionone (2.9%) (Sánchez-Contreras et al. 2000). Wahlberg (2002) focused on norisoprenoids from tobacco and pointed out β-ionone, α-ionone, 3-hydroxy-β-ionone, and 3-hydroxy-5,6-epoxy-β-ionone as the identified primary cleavage products. The fact of not detecting 3-hydroxy-β-ionone at the initial time of the bioconversion process of lutein might indicate that this compound is first dehydroxylated before cleavage (Sánchez-Contreras et al. 2000). On the other side, the two steps transformation of β-ionone to 7,8-dihydro-β-ionone and 7,8-dihydro-β-ionol by immobilized cells of *Nicotiana tabacum* were reported by Tang and Suga (1994). Hypothetically, a similar ionone conversion scheme might be followed from β-ionone, suggesting the probable existence of two reductases. When zeaxanthin, a xanthophyll similar to lutein but with two β-rings, was employed, the products generated were doubled (Sánchez-Contreras et al. 2000). These results suggested that an asymmetrical lutein breakdown took place and that the

cleaving system exhibited preference for cyclic β-bonds. Consequently, the bioconversion route of lutein to tobacco aroma compounds, constituted by four steps (dehydroxylation, β-ionone synthesis via carotenoid cleavage, and two consecutive transformations of the ionone into its reduced derivatives) was proposed (Figure 12). No explanation on the post-occurrence of 3-hydroxy-β-ionone was given.

The synthesis of ionone derivatives from α- or β-ionone as starting materials has been achieved using microorganisms such as *Aspergillus niger* (Mikami et al. 1981; Yamazaki et al. 1988; Larroche et al. 1995; Grivel and Larroche 2001), *Lasidiplodia theobromae* (Krasnobajew and Helmiger 1982), and *Streptomyces* strains (Lutz-Wahl et al. 1988). Likewise to ionones, Schoch et al. (1991) described the microbial conversion of α-damascone to its oxygenated derivatives by *Botrytis cinerea*, a fungal genus used to carry out different types of biotransformations (Aleu and González Collado 2001). Nevertheless, the study with lutein constituted the first report on coupled microbial action responsible for the generation of norisoprenoids from a carotenoid substrate (Sánchez-Contreras et al. 2000).

In order to determine the role exerted by each microorganism in the bioconversion pathway,*T. asahii* and *P. amylolyticus* were grown independently (Maldonado-Robledo et al. 2003). As a reference, the microbial mixture was also employed in the experiments, agreeing with the work by Sánchez-Contreras et al. (2000); the tobacco aroma was only produced when the two microbial cells were used together. *T. asahii* cultures supplemented with lutein showed β-ionone formation, meanwhile no reduced derivatives of this compound were detected. In contrast to the mixture, where β-ionone concentration decreased after 24 h of the process, the levels of the ionone obtained from *T. asahii* alone, remained constant after 48 h. *P. amylolyticus* cells grown in the presence of the xanthophyll did not produce any volatiles at all. On the other hand, when studying the ability of each microorganism to produce the tobacco aroma from β-ionone, only in the mixed, as well as in the bacterial cultures, the hydrogenated derivatives of the C_{13}-norisoprenoid were found. As mentioned above, in addition to their antibacterial properties (Anzaldi et al. 1999), ionones exert an antifungal action, presumably by inhibiting the respiratory enzymes (Larroche et al. 1995), so its effect over the microbial growth was evaluated.

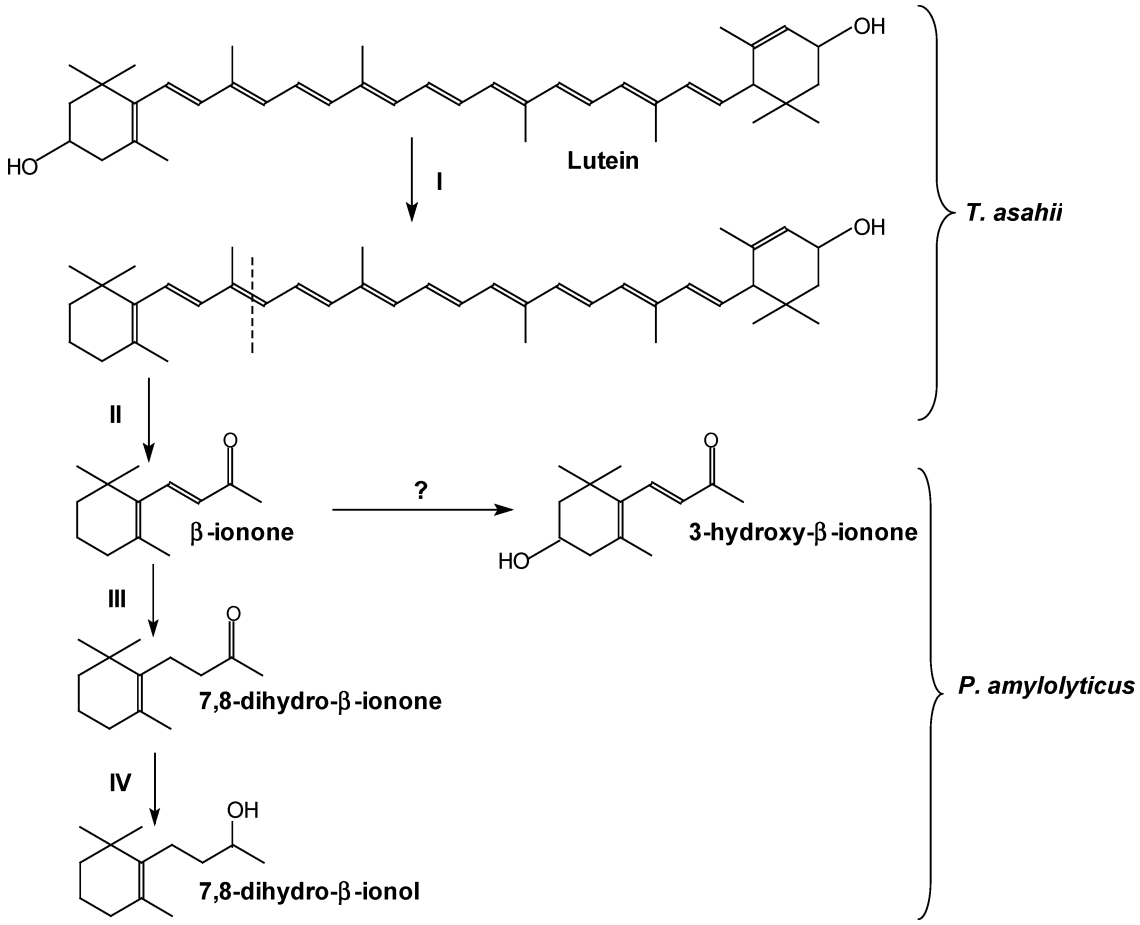


FIG. 12. Bioconversion scheme of lutein to tobacco aroma compounds, including the specific role of the microorganisms involved in it (Sánchez-Contreras et al. 2000; Maldonado-Robledo et al. 2003).

Although both microorganism were susceptible to the ionone, the yeast exhibited more sensitivity to lower concentrations of this compound.

Subsequent experiments showed that the bacterium was able to incorporate β-ionone, transform it into the aroma compounds, and secrete them to the culture medium (Maldonado-Robledo et al. 2003). These actions resulted in better microbial growth and in the detoxification of the culture medium (Rodríguez-Bustamante et al. 2006). Therefore, it was concluded that the bioconversion pathway of lutein could be divided in two parts, with respect to the role played by each microorganism (Figure 12). The first part of the process, performed by *T. asahii*, comprised the carotenoid cleavage to render β-ionone, while *P. amylolyticus* is responsible for assimilating and reducing this compound to yield the products with a tobacco note. Apparently more beneficial to the fungus, a microbial association of this type, which is involved in the synthesis of carotenoid-derived aromas, was firstly described by Maldonado-Robledo et al. (2003).

Research on this process is still going on. Studies focused on maximizing the aroma production and designing a recovery system of the synthesized volatiles, i.e. β-ionone, have been recently published (Rodríguez-Bustamante et al. 2005, 2006). By following a classical design of experiments methodology, the effect of different components of the culture medium was evaluated, and an almost 10-fold increase in the titer was attained. Surprisingly, in spite of its low amount, compared to other nutrients, a negative and significant estimate was calculated for glucose (Rodríguez-Bustamante et al. 2005). Therefore, an inverse relationship has been observed between the sugar concentration and the production of the aroma compounds (Rodríguez-Bustamante et al., in preparation). On the other side, after increasing the agitation rate in *T. asahii* cultures, a decrease in the production of β-ionone was observed, as described earlier by Sánchez-Contreras and collaborators (2000) for volatiles generated from mixed cultures. Therefore, by taking advantage of the ionone volatility, a headspace method was employed, reaching a 98% recovery of this compound with the aid of an adsorbent (Rodríguez-Bustamante et al. 2006). Thus, the use of a mesoporous silicate material allowed a 4.5-fold higher recovery than the obtained with a commercial adsorbent (silica gel), even though the surface areas were equaled. Currently, we are investigating the enzymes involved in the bioconversion route. For this purpose some of them were purified, sequenced and then will be cloned and overexpressed.

Fungal Cleavage of β-Carotene to Flavor Compounds

The screening of more than 50 yeasts and filamentous fungi, known for being involved in terpene reactions (synthesis or biotransformations), was performed by Zorn and coworkers (2003a). Discrimination between degrading and non-degrading organisms was also accomplished by a visual method, in which β-carotene containing agar plates were inoculated with approximately 1 cm^2 of agar pieces covered with mycelium. Depending on the organism (yeast or filamentous fungus), after one or two weeks, carotenoid bleaching was only observed in ten of the strains tested, exhibiting, as defined by the authors, strong (S) or weak (W) discoloration (see Table 3).

TABLE 3
Screening of the β-carotene degrading fungi (Zorn et al. 2003a)

Organism	Fungus type	Carotene bleaching
Cryptococcus laurentii	Yeast	W[1]
Cyathus pallidus	Basidiomycete	S[2]
Ganoderma applanatum	Basidiomycete	S
Hypomyces odoratus	Ascomycete	S
Ischnoderma benzoinum	Basidiomycete	S
Kuehneromyces mutabilis	Basidiomycete	S
Marasmius scorodonius	Basidiomycete	S
Phaffia rhodozyma	Yeast	W
Trametes suaveolens	Basidiomycete	S
Trametes versicolor	Basidiomycete	S

[1]W (weak).
[2]S (strong).

Positive carotenoid bleaching fungi were further studied. Low yields of dihydroactinidiolide as the sole product were measured in submerged cultures of *G. applanatum*, *H. odoratus*, *K. mutabilis*, and *T. suaveolens*. Inoculum-free medium and cultures with no addition of β-carotene, where no volatiles were detected, served as negative controls.

As pointed out by the pigment-fading test, it was thought that the enzymes responsible for the carotenoid degradation were extracellular; therefore, the possibility of using mycelium-free medium instead was contemplated. β-Ionone, β-cyclocitral, dihydroactinidiolide, and 2-hydroxy-2,6,6-trimethylcyclohexanone were found in the culture supernatants obtained from three basidiomycetes, with the ionone as the major compound in all cases. *I. benzoinum*, *M. scorodonius*, and *T. versicolor*, exhibited a β-carotene degradation values of 95, 93, and 98%, respectively, and even though *C. pallidus* was able to break down 85% of the carotene, no aroma compounds were detected. Ultrafiltration of the supernatants revealed the involvement of enzymatic activity in the cleaving process, as no carotenoid degradation was found in the permeates, contrary to the observed with the retentates. This is the first time that medium free of mycelium with secreted fungal enzymes was employed for converting β-carotene into aroma compounds, primarily β-ionone.

Finally, the cleavage activity of *M. scorodonius* was partially characterized by a photometric method, monitoring the decrease in the absorbance of β-carotene with respect to time. A rapid degradation profile, in which degradation of the 40% of the initially added carotenoid after 60 min, was observed. Control blanks, using water instead of the extracellular proteins, exhibited practically no breakdown even after 120 min. Maximum activity took place at 27°C and a pH value of 5.0.

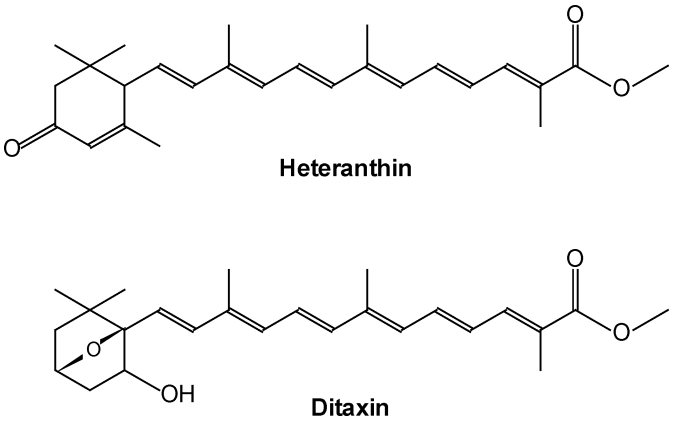


FIG. 13. Chemical structure of the apocarotenoids heteranthin and ditaxin (Méndez-Robles et al. 2006).

Generation of Aroma Compounds from a Newly Discovered Apocarotenoid

Ditaxis heterantha is a native plant of the Euphorbiaceae family that grows in the semiarid regions of Mexico. As the seed endosperm possesses an intense yellow coloration, its total pigment was extracted for chemical characterization. After purification it was found that the pigment could be separated into seven fractions, exhibiting three maximal peaks in their absorbance spectrum, a typical property of carotenoids. From these fractions, two new apocarotenoids, F4 and F5, later named heteranthin and ditaxin (Figure 13), respectively, were in major proportion (Méndez-Robles et al. 2004, 2006).

Based on the methodology previously described by Sánchez-Contreras et al. (2000), nine microbial colonies, grown on lutein as their only carbon source and able to degrade it, were isolated from *T. erecta* (Del Toro-Sánchez et al. 2006). When their capability to cleave the total pigment from *D. heterantha* seeds and its two main fractions was tested, only one of them was able to degrade the pigment and heteranthin. A herbal aroma was formed in both cases, while practically no volatiles where detected from cultures supplemented with ditaxin. Microscopic observations of the colony showed a yeast morphology, and molecular methods enabled the identification of the yeast as *Saccharomyces cerevisiae* by database comparison of the DNA region for the 5.8S rRNA gene (Del Toro-Sánchez et al. 2006).

Better pigment and apocarotenoid degradation, as well as aroma production, occurred at an agitation rate of 60 rpm. *S. cerevisiae* cells were cultured supplemented with either the total pigment or heteranthin, monitoring both substrates degradation, aroma generation, glucose consumption, and growth kinetics. The maximum aroma production was attained at 60 h of the process, with heteranthin yielding 15% less volatile compounds. In both cases the C_{10} compounds, safranal and 3-hydroxy-β-cyclocitral, represented the principal constituents in the following proportions, respectively: 26.9 and 10.8% for the total pigment, and 35.6 and 15.9% for F4. A possible degradation scheme of heteranthin to safranal was proposed (Figure 14), in which 3-hydroxy-β-cyclocitral appeared to be the precursor of this flavor compound. However, the primary cleavage product derived from heteranthin was not identified and therefore, the initial reaction remained unknown (Del Toro-Sánchez et al. 2006). The occurrence of C_{13}-norisoprenoids, i.e., ionones, was also observed, indicating that in addition to the cleavage at position 7,8 a breakdown at the 9,10 double bond also took place. 3-oxo-α-ionone, 3-oxo-7,8-dihydro-α-ionone, and 3-oxo-α-ionol, probably responsible for the floral aroma perceived, were detected in higher amounts in cultures supplemented with F4 rather than

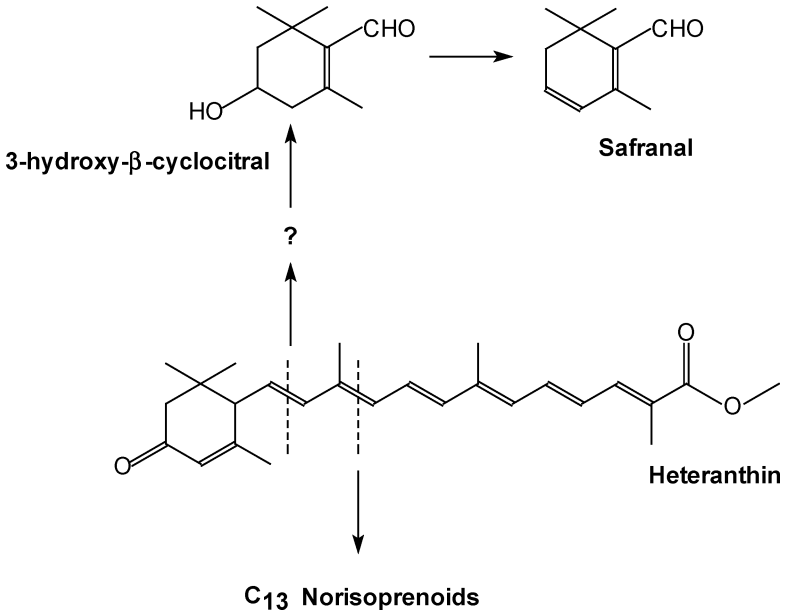


FIG. 14. Bioconversion scheme of heteranthin to safranal (Del Toro-Sánchez et al. 2006).

with the total pigment. Other heteranthin-derived volatiles detected in minor quantities were the C_9-compounds: isophorone and 4-oxo-isophorone.

To obtain more information on the bioconversion process, the cleaving activity was further studied. By a color fading test, using agarose plates stained with the pigment or the apocarotenoid, halos were only observed with intracellular extracts derived from cultures grown in the presence of either substrate. No degradation took place with extracellular protein. *In vitro* analysis showed that the cleavage was due to enzymatic activity, as no significant values were measured in the negative controls, where no protein extract was added. After 60 min, a degradation of 60 and 42% of the total pigment and heteranthin was respectively registered, suggesting the possible existence of one or more intracellular oxidases involved in the process (Del Toro-Sánchez et al., in preparation).

Although its major application relies in its desirable property of transmitting color and flavor to food, saffron has been used as textile dye, beauty product, and medicinal agent. This spice, derived from *C. sativus* flower, particularly the dried stigmas, is a traditional ingredient still employed in European cuisine (Spanish, French, Italian, and with less extent, German) (Winterhalter and Straubinger 2000). The aim to identify the key components of saffron flavor (taste, aroma, and color) has driven research into knowing its chemical composition. Therefore, during the past decades extensive studies on the synthesis, nature, and stability have been performed to its volatile and non-volatile constituents.

Regarding the volatile compounds, as safranal represents the major component of the essential oil content (60–70%), for many years it was thought to be responsible for the characteristic aroma (Cadwallader 2002). Further analysis have shown the contribution of more than 100 compounds, from which a part of them is derived from glycoside precursors. However in contrast to what other authors affirm, Carmona et al. (2007) concluded that the number of volatiles in saffron is not as high as believed and the aroma profile depended of its geographical origin. The generation of aroma compounds from saffron is still unclear, although a pathway for the formation of safranal was proposed by Pfander and Schurtenberger (1982). In this process, zeaxanthin is symmetrically cleaved at the 7,8 (7′, 8′) positions by the dioxygenase described above (CsZCD) (Bouvier et al. 2003), resulting in the formation of picrocin and crocetin. By the action of glycosidases or during thermal treatment or cooking, the first compound might be converted into safranal (Figure 15). Very interestingly, Carmona et al. (2006) described the generation of this volatile by carotenoid thermal degradation, following a different route. As they proposed, crocetin or its ester derivatives might play a crucial role.

Even when carotenoid breakdown at the 7,8 double bond has been described in cyanobacteria (see above), the research performed by Del Toro-Sánchez and coworkers (2006) constituted the first report on microbial (apo)carotenoid cleavage resulting in safranal production. On the other side, a food flavoring application might be contemplated, as *D. heterantha* seeds are more economic than saffron, so far, the most expensive spice in the

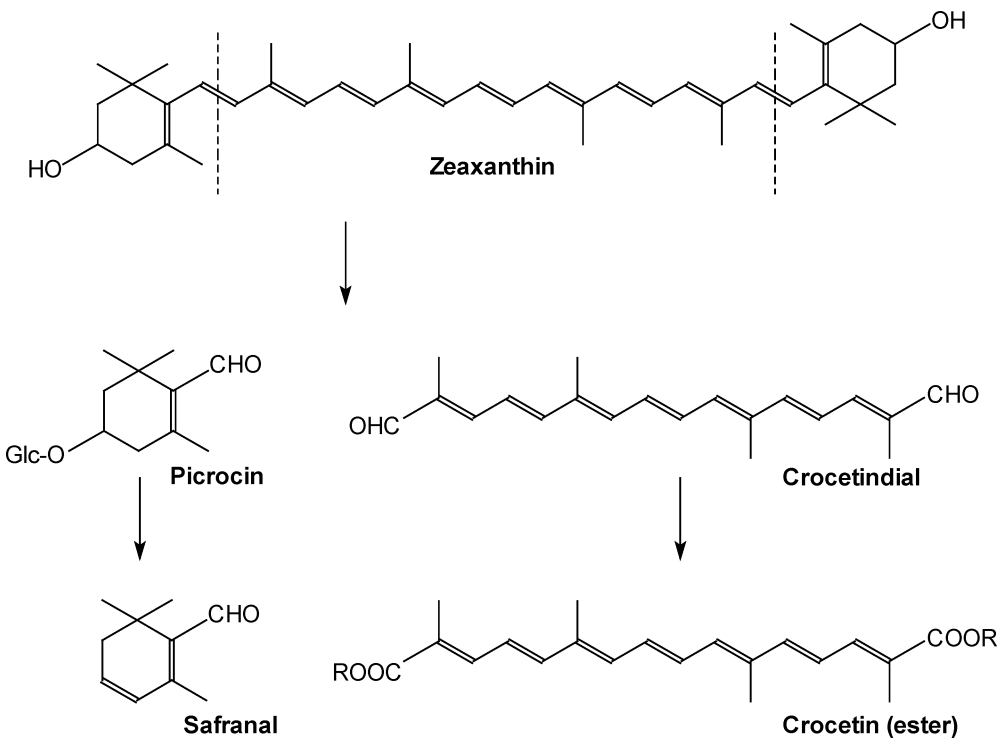


FIG. 15. The widespread pathway for safranal formation from zeaxanthin.

market. A novel possibility is opened in this area, which most probably might be further studied and later exploited in the ongoing years.

CAROTENOID-DERIVED AROMA COMPOUNDS PRODUCTION BY OTHER CLEAVING MICROBIAL ENZYMES: A PEROXIDASE FROM *LEPISTA IRINA*

Zorn et al. (2003b) isolated and characterized on a molecular level, an enzyme, from the edible mushroom *L. irina*, able to degrade β-carotene. After a few days, carotenoid bleaching was observed around the mycelium grown on agar plates supplemented with the carotene. With a similar approach to their previous experiments (Zorn et al. 2003a), the supernatant obtained from submerged cultures of *L. irina* was mixed with solubilized β-carotene, finding almost a complete degradation after 14 h of incubation. As described above, the same volatiles were detected, with β-ionone as the major products; however, the occurrence of β-apo-10′-carotenal was revealed by an HPLC method developed for the identification of non-volatile compounds. A rapid reaction profile was observed by a kinetic assay, in which a carotenoid degradation of 90% is reached within 30 min, and the maximal amount of β-ionone produced is attained after 60 min.

Concentrated supernatants were analyzed by isoelectric focusing gel chromatography with immobilized pH gradient, coupled to a zymographic test. An agarose layer stained with β-carotene was used to cover the focused gel, and after incubation, a distinctive bleaching band was detected. After comparison with reference proteins, the isoelectric point of the enzyme responsible for carotenoid breakdown was calculated, exhibiting a value of 3.75. Next, a purification protocol for the protein of interest was established, following three separation techniques (hydrophobic interaction, anion exchange, and size exclusion chromatographies). The maximum degradation was found at the tenth day of culture, at 34°C and pH values between 3.5 and 4.0. A yield of 63% and a purification factor of 43 were attained at the end of the process. The typical absorption spectra for heme proteins was observed for the purified enzyme.

Gel electrophoresis analysis (SDS-PAGE and IEF) showed the presence of two proteins: a main band with a molecular weight of 50.5 kDa and pI of 3.75, and a second faint band of 45 kDa and pI of 3.50. However, subsequent characterization exhibited identical N-terminal sequences for both proteins, probably suggesting the occurrence of isoenzymes. Further sequence information of the main protein allowed the construction of a cDNA library, screened by means of polymerase chain reaction (PCR). A 1.28 kbp cDNA was obtained and sequenced, showing great similarity to reported peroxidase from the white rot fungus *Pleurotus eryngii* (Ruiz-Dueñas et al. 1999). The cloned fragment contained an open reading frame of 1083 nucleotides, which encoded a polypeptide of 361 residues with a 30 aa signal peptide. In contrast to the molecular weight estimated by SDS-PAGE, a 34.6 kDa mass was calculated, attributing this considerable difference to a high degree of glycosylation (Zorn et al. 2003b).

Based on the sequence analysis, it was concluded that the enzyme responsible for β-carotene cleavage at the double bond between C_9-C_{10} belonged to a novel class of peroxidases. Even though the presence of H_2O_2 is necessary for peroxidase activity, the purified enzyme was able of breaking down the carotenoid in the absence of peroxides. Enzymes from white rot fungi known for their degrading action over a wide variety of compounds have been described in literature; nevertheless, the work by Zorn and collaborators (2003b) represented the first report on an extracellular microbial peroxidase capable of carotenoid cleavage. Future research conducted by this group is expected in this area, as the activity observed from *M. scorodonius* might follow a similar mechanism.

FUTURE PROSPECTS: NOT ONLY AROMAS

As stated above, flavors and fragrances have been appreciated by mankind for centuries. Traditionally, essential oils were extracted from natural substrates, making this a very tedious and laborious task. In general, the production of aromas by microbial methods offers new advantages. Novel approaches are still to come, and for the case of carotenoid-derived aroma compounds, research in various fields might certainly take place, as new substrates, enzymes and genes from different organisms (animals, plants, and microorganisms) are being discovered. A gene encoding a carotenoid oxygenase (*carX*) was identified in the ascomycete *Fusarium fujikuroi* (Thewes et al. 2005), and very recently, Prado-Cabrero et al. (2007) reported on the synthesis of retinal by the gene product. Car X expressed in engineered *E. coli* cells exhibited cleavage activity at the 15,15′ double bond of β-carotene but not of lycopene. Additionally, *in vitro* tests showed the ability of this oxygenase to synthesize retinal from β-carotene, as wells as from torulene, γ-carotene, and β-apo-8-carotenal (Prado-Cabrero et al. 2007).

Little is known about the function exerted by carotenoid breakdown products in living systems, with the exception of retinoids and the so-called plant hormones, both still to be studied. With the exception of the mechanism proposed by Zorn et al. (2003b) for the *L. irina* peroxidase, the oxidative cleaving reactions by oxygenases from different taxa and their regulation have not been elucidated yet, so further research is awaiting.

Besides their applications in flavoring, innovative studies have been conducted in the pharmacological sector. It has been shown that a diet rich in fruits and vegetables, considered as isoprenoid reservoirs, renders a protective effect against diverse kinds of cancer (Singletary 2000). A chemopreventive and inhibitory effect exerted by β-ionone has been described in cancer types such as melanoma (He et al. 1997; Jung et al. 1998), gastric adenocarcinoma (Liu et al. 2004), and hepatocarcinogenesis (de Moura Espíndola et al. 2005). In all cases, the inhibition of mevalonate synthesis, possibly representing the impairing of the malignant cells is assumed.

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